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CAR-NK cells for gastrointestinal cancer immunotherapy: from bench to bedside

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Abstract

Background Gastrointestinal (GI) cancers represent a significant health burden worldwide. Their incidence continues to increase, and their management remains a clinical challenge. Chimeric antigen receptor (CAR) natural killer (NK) cells have emerged as a promising alternative to CAR-T cells for immunotherapy of GI cancers. Notably, CAR-NK cells offer several advantages, including reduced risk of graft-versus-host disease, lower cytokine release syndrome, and the ability to target cancer cells through both CAR-dependent and natural cytotoxic mechanisms.

Main body This review comprehensively discusses the development and applications of CAR-NK cells in the treatment of GI cancers. We explored various sources of NK cells, CAR design strategies, and the current state of CAR-NK cell therapy for GI cancers, highlighting recent preclinical and clinical trials. Additionally, we addressed existing challenges and propose potential strategies to enhance the efficacy and safety of CAR-NK cell therapy.

Conclusions Our findings highlight the potential of CAR-NK cells to revolutionize GI cancer treatment and pave the way for future clinical applications.

Keywords CAR-NK cells, Gastrointestinal cancers, Immunotherapy, Combination therapy, Tumor microenvironment

Background

Gastrointestinal (GI) cancers represent a significant health burden worldwide. Their incidence continues to increase, and their management remains a clinical challenge [1]. These cancers are often diagnosed at advanced stages, and their complex biology, coupled with a dense stroma and immunosuppressive microenvironment, complicates treatment [2]. Conventional therapies are limited by their non-specific nature and the high toxicity associated with systemic treatments, leading to poor prognosis and high mortality rates in patients with GI cancers [3].

Immunotherapy has rapidly emerged as an exciting and promising avenue of cancer treatment in recent years [4, 5]. Among these, Chimeric antigen receptor (CAR) T cell therapy has shown remarkable success in the treatment of hematological malignancies, offering hope to many patients [6]. Notably, CAR-T cells are engineered to express receptors that target specific antigens on cancer cells, thereby directing the immune system to attack these cells with precision [6]. However, CAR-T cell therapy faces significant hurdles in the treatment of solid tumors, including GI cancers [7]. Challenges such as on-target off-tumor toxicity, cytokine release syndrome (CRS), and immunosuppressive tumor microenvironment (TME)

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limit the effectiveness and safety of CAR-T cells in these cancers [8].

CAR-natural killer (NK) cells are a promising alternative to CAR-T cells, offering several advantages that could address the limitations of CAR-T therapy [9]. Notably, NK cells, a type of lymphocytes in the innate immune system, possess natural cytotoxic abilities to target and kill cancer cells without prior sensitization. They are less likely to cause severe side effects such as CRS and can be sourced from allogeneic donors without causing graft-versus-host disease (GVHD), allowing for the development of "off-the-shelf" therapies [10]. The potential advantages of CAR-NK cells over CAR-T cells include the following. First, unlike T cells, they are associated with a reduced risk of GVHD because NK cells do not require a perfect match between the donor and recipient. This makes NK cells an attractive option for allogeneic cell therapy. Second, NK cells produce lower levels of pro-inflammatory cytokines than T cells, which reduces the risk of severe CRS, a potentially life-threatening complication of CAR-T cell therapy. Third, NK cells recognize and kill cells with downregulated MHC class I molecules, a common mechanism by which cancer cells evade T cell detection. Finally, CAR-NK cells have demonstrated safety and potential efficacy in early-phase clinical trials, showing promise for the treatment of solid tumors, including GI cancers [11].

This review aims to provide a comprehensive overview of the development and application of CAR-NK cells in the treatment of GI cancers. We discuss the sources and generation of NK cells, design of CAR for NK cells, and current state of CAR-NK cell therapy in GI cancers, including recent clinical trials and preclinical studies. Additionally, we address the existing limitations of CAR-NK cell therapy and propose potential strategies to overcome these challenges. By exploring these aspects, we hope to highlight the potential of CAR-NK cells to revolutionize the treatment of GI cancers and pave the way for future clinical applications.

Generation of CAR-NK cells

Sources of NK cells

It is essential to understand that while present, they have a lower risk than T cells in causing GVHD [12–14], making them suitable for creating "off-the-shelf" allogeneic cell therapy products. These products in contrast can be prepared in large amounts and stocked so that when a number of patients need them they can be easily administered. Currently, the clinically compliant NK cells can be generated at large quantities from many sources such as NK 92 cell line, peripheral blood mononuclear cells (PBMCs), umbilical cord blood (UCB), CD34+hematopoietic progenitor cells (HPCs) and induced pluripotent stem cells (iPSCs). (Fig. 1).

Latest clinical trials employ CAR-NK cells primarily use the NK92 cell line because of the NK cells that replicate in vitro, and are less prone to the effects of cyclical freezing and thawing [15]. Using NK-92, an established NK cell line, as a replacement for patient- or donor-derived NK cells, a sufficient number of NK cells can be obtained to circumvent the problem of limited NK cell numbers for CAR-NK cell production. In addition, NK-92 cells are highly cytotoxic, have a short cell doubling time, lack inhibitory receptors, and are easier to genetically modify than other cell lines [16, 17]. However, NK-92 cells, being a tumor cell line, have inherent limitations, such as potential tumorigenicity, lack of CD16 and NKp44 expression, and diminished in vivo expansion potential due to the lethal irradiation required before infusion [18]. Therefore, modifying the NK-92 cells to address these limitations is crucial. To date, several advancements have been made with NK-92 cells. For example, NK-92 cells have been modified to generate cell lines expressing IL-2, such as NK-92ci and NK-92 mi cells [17, 19]. To overcome the lack of CD16 receptors, NK-92 cells were transduced with plasmids carrying high-affinity CD16 and IL-2, resulting in the generation of NK-92 cells expressing high-affinity Fc receptors [17, 19].

PBMCs are one of the primary sources of primary human NK cells often employed in clinical research. Furthermore, NK cells can be efficiently isolated from the PBMCs of healthy donors using specific isolation kits. Once isolated, these NK cells can be activated and expanded using specialized media containing cytokines, they have massive potential to be applied for preclinical studies as for GMP grade complex clinical trial production [20]. CAR-NK cells derived from PBMCs, some of which encode a multitude of activating receptors, can be safely given without the necessity of applying irradiation, allowing them to expand within the patient's body. Typically, these PBMC-derived NK cells (PB NK cells) are predominantly CD56dimCD16+cells, which are known for their mature phenotype, high cytotoxicity, and limited capacity for proliferation [21]. Since there is no GVHD, PB NK cells can be harvested from either MUD or HLA disparate unrelated donors [13, 22, 23], that means a diverse donors base and the refining of the end product. The same method can also be used for isolation of NK cells from UCB. UCB banks give the opportunity of choosing the donors with the specific HLA types and NK receptor patterns. However, the limited volume of a single UCB unit results in a smaller yield of NK cells, which poses a challenge for clinical scalability. Additionally, The NK cells isolated from UCB are usually of less mature subtype and display lower cytotoxic activity compared to PB NK cells. These UCB NK cells have reduced levels of adhesion molecules, CD16, killer immunoglobulin-like receptors (KIRs), perforin, and granzyme B, while

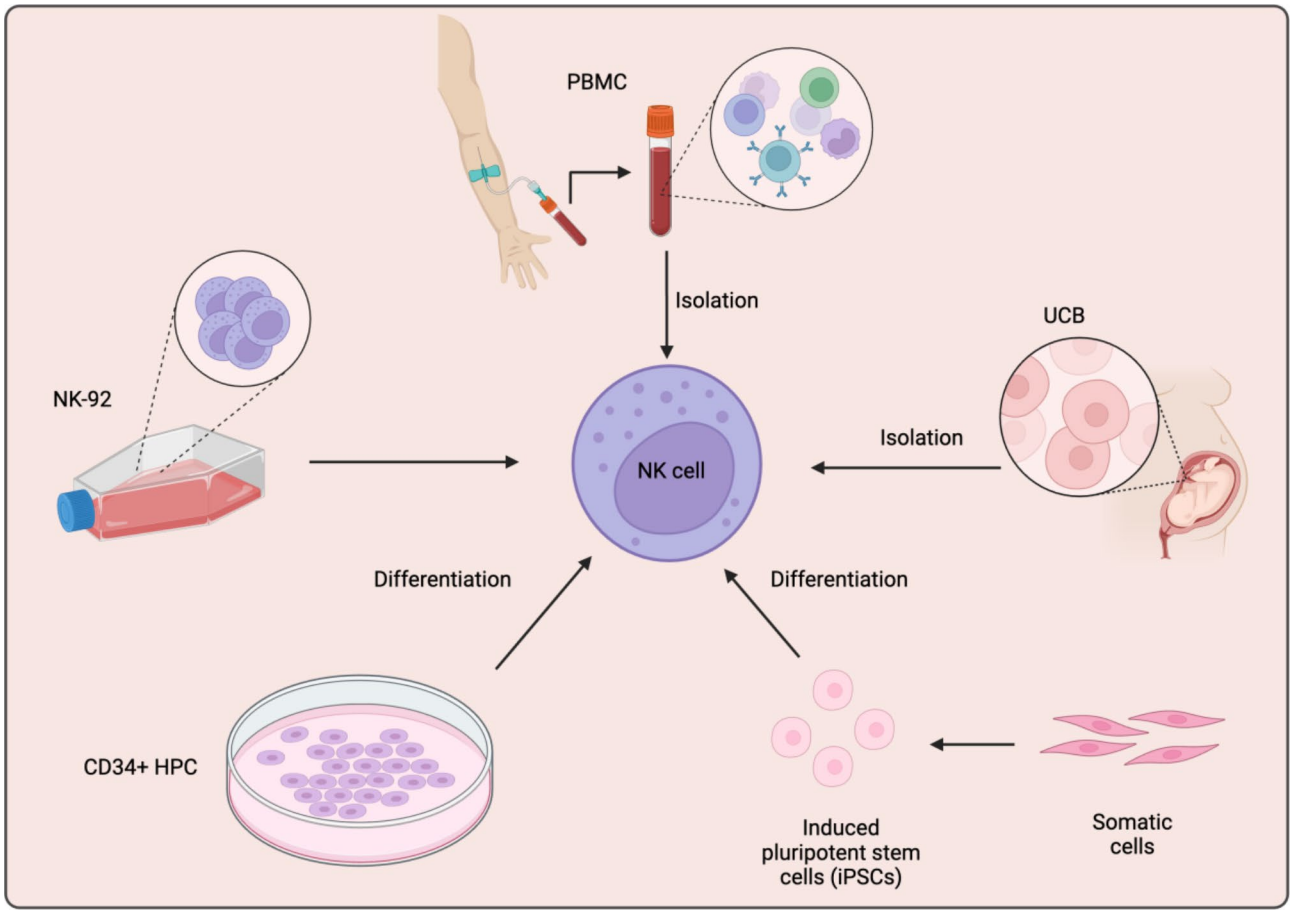

Fig. 1 Schematic diagram of the sources of NK cells

Producing more inhibitory molecules such as NKG2A [24]. The variation of the source of PBMC and UCB derived CAR-NK cells makes the standardization of these products for clinical use challenging [25].

Other strategies for the production of a vast number of mature NK cells for the clinical use include their generation from CD34+HPCs through their differentiation. These CD34+HPCs can be harvested from BM, ESCs, MPB and UCB but the last one is the most common source. These are further propagated and cultured into mature NK cells by applying a particular set of cytokines to the culture Dish [26]. The obtained CD56+CD3−NK cells are similar to PB NK cells in the expression of activating receptors and efficient killing of leukemia cells in both SCID-reconstituted mice and co-culture systems [27]. Recently, iPSCs have been checked as a potential source for CAR-NK cells based on their capability to self-renew indefinitely [28]. Another advantage over that the differentiated NK cells has is that the iPSCs can be transduced to stably express a CAR at a higher efficiency. Then, using these iPSCs generated by CAR-engineering, the cells can be cultured in a mixture of stem cell factor, VEGF, and BMP4 to develop into hematopoietic progenitor cells. Here, the progenitor cells are cultured with IL-3, IL-15, IL-7, SCF and FLT3L in the culture media for complete differentiation towards CAR-NK cells [29]. This process allows for the production of a large number of homogeneous CAR-NK cells from a single engineered iPSC, suitable for clinical use. Despite their advantages, NK cells derived from iPSCs, similar to those from UCB, often display an immature phenotype. They exhibit lower expression of KIRs and CD16 but higher expression of inhibitory receptors, such as NKG2A compared to PB NK cells [30]. However, iPSC-derived NK cells expressing NK-tailored CARs, instead of the traditional CAR directed to T cells, the course molecules have demonstrated a high tumor-killing capacity in vitro and in vivo. This makes iPSCs a precious and limitless resource for "off the shelf" CAR-NK cell products, providing a scalable solution for clinical applications [29].

CAR design for NK cells

The original idea of CARs was designed for T cells in order to improve their strength and functionality toward the target cells [31]. These engineered proteins consist of three essential components: an extracellular domain,

a transmembrane region, and an intracellular signaling domain [32, 33]. The extracellular domain, typically a single-chain variable fragment (scFv) derived from antibodies, facilitates antigen recognition. Recent advancements have also explored the use of single variable domains on heavy chains (VHH), which are known for their small size and high affinity, for this purpose [34, 35]. Some of these are as follows; the scFv or VHH that affix to the target antigen, the hinge region that connects the transmembrane and the transmembrane region that provides an attachment point of the CAR molecule to the cell membrane [10].

Intracellularly, CAR incorporates signaling domains derived from T cell or other activation receptors, which are crucial for initiating downstream signaling pathways that activate CAR-modified effector cells upon antigen encounter [36, 37]. Traditionally classified into three generations, CARs evolve from first-generation designs with a single CD3ζ signaling domain, which often require additional costimulatory signals for robust cytotoxic responses [38, 39]. Second-generation CARs address this by integrating a single costimulatory domain, such as CD28, CD137 (4-1BB), or CD134 (OX40), alongside CD3ζ. Third-generation CARs further enhance activation and persistence by incorporating two costimulatory domains fused to CD3ζ, although superiority over second-generation CARs remains inconclusive [40]. Advancements beyond third-generation CARs have led to fourth-generation CARs, leveraging synthetic biology to enhance functionality and safety [41–43]. These include the integration of cytokine genes for autocrine activation and inducible caspase 9 system as safety switches to manage effector cell toxicity [44].

The recognition of NK cells as a viable alternative to CAR-targeted immunotherapy has gained traction [45]. Shared signaling components between T and NK cells, such as CD3ζ, CD28, and 4-1BB, allow adaptation of CAR designs originally for T cells to NK cells, showing effectiveness [46]. Recent studies exploring NK-specific signaling domains such as DNAX-activation protein (DAP)10 or DAP12 instead of CD3ζ have demonstrated superior cytotoxicity in PB-derived NK cells [47–50]. In a comparative study by Li et al. [29], evaluating CAR constructs in NK-92 cell lines and iPSC-derived NK cells, designs incorporating NK-specific transmembrane and costimulatory domains showed enhanced cytotoxicity and activation. These findings underscore the potential of optimizing CAR-NK cell designs to align with NK cell biology and improve anti-tumor efficacy.

Despite this progress, challenges persist in CAR-based therapies. Antigen escape, associated with poor outcomes, remains a significant concern [51]. Bispecific CARs, which are capable of targeting multiple antigens or incorporating dual target-recognition domains, aim to enhance tumor detection precision and reduce evasion [52]. Additionally, trogocytosis, where target cell molecules transfer to effector cells, poses risks of fratricide and dysfunction in CAR-T cells [53]. To address this, an inhibitory CAR (iCAR) targeting NK cell-specific inhibitory receptors has been introduced into NK cells. This iCAR emits a "don't kill me" signal, preventing NK cells from attacking each other. The combination of a tumor-targeting activating CAR or aCAR with the identification of self by iCAR has also been useful in preventing fratricide through the process of trogocytosis, thereby enhancing the activity and longevity of CAR-NK cells [54], (Fig. 2).

Application of CAR-NK cells in GI cancer treatment

Hepatocellular carcinoma (HCC)

Glypican-3 (GPC3) is a cell-surface heparan sulfate proteoglycan belonging to the glypican family. These proteoglycans play a crucial role in regulating cell growth and differentiation. GPC3 is highly expressed in several types of cancer, most notably hepatocellular carcinoma (HCC) [55, 56]. As a result, this specific protein is ideal for immunotherapy intervention since this particular protein is significantly changed in cancer tumors. I chose this article since Yu and colleagues developed GPC3-CAR-NK-92 cells using NK-92 cell line. These outcomes showed that GPC3-CAR-NK-92 cells effectively accumulated into GPC3 + HCC xenografts, restraining tumor cells' proliferation further promoting the apoptotic process [57]. Likewise, a study conducted in the present work used PB-NK cells to generate GPC3-CAR-NK cells [58]. More specifically, authors also found that individuals with HCC exhibit high serum sPD-L1, proving that the tumor influences the level of the receptor, which encumbers the activity of GPC3-targeted CAR-NK cells. To overcome this problem, it is necessary to use a high-affinity sPD-L1 variant (L3C7c-Fc); having incorporated it into GPC3-CAR-NK cells, the authors have found that L3C7c-Fc can effectively enhance the therapeutic activity of GPC3-CAR-NK cells by neutralizing the inhibitory effect of sPD-L1. This combine strategy shown a better prospects in enhancing the therapeutic efficacy for HCC patients. Additionally, the current study further optimized GPC3-CAR. A scFv was designed based on an existing humanized high-affinity GPC3 antigen [59]. Therefore, with the help of GT, the GPC3-O4-CAR, GPC3-CD8-CAR, and GPC3-ori-CAR genes were directly synthesized. The above synthesized CAR genes were cloned into the pLVX-IRES-Zsori1 lentiviral vectors to obtain pLVX-GPC3-O4-CAR, pLVX-GPC3-CD8-CAR and pLVX-GPC3-ori-CAR respectively [60]. Importantly, under similar transfection efficiencies, using the fluorescence microscopy, it was indicated that 293T cells transfect with GPC3-O4-CAR fluorescence intensity was

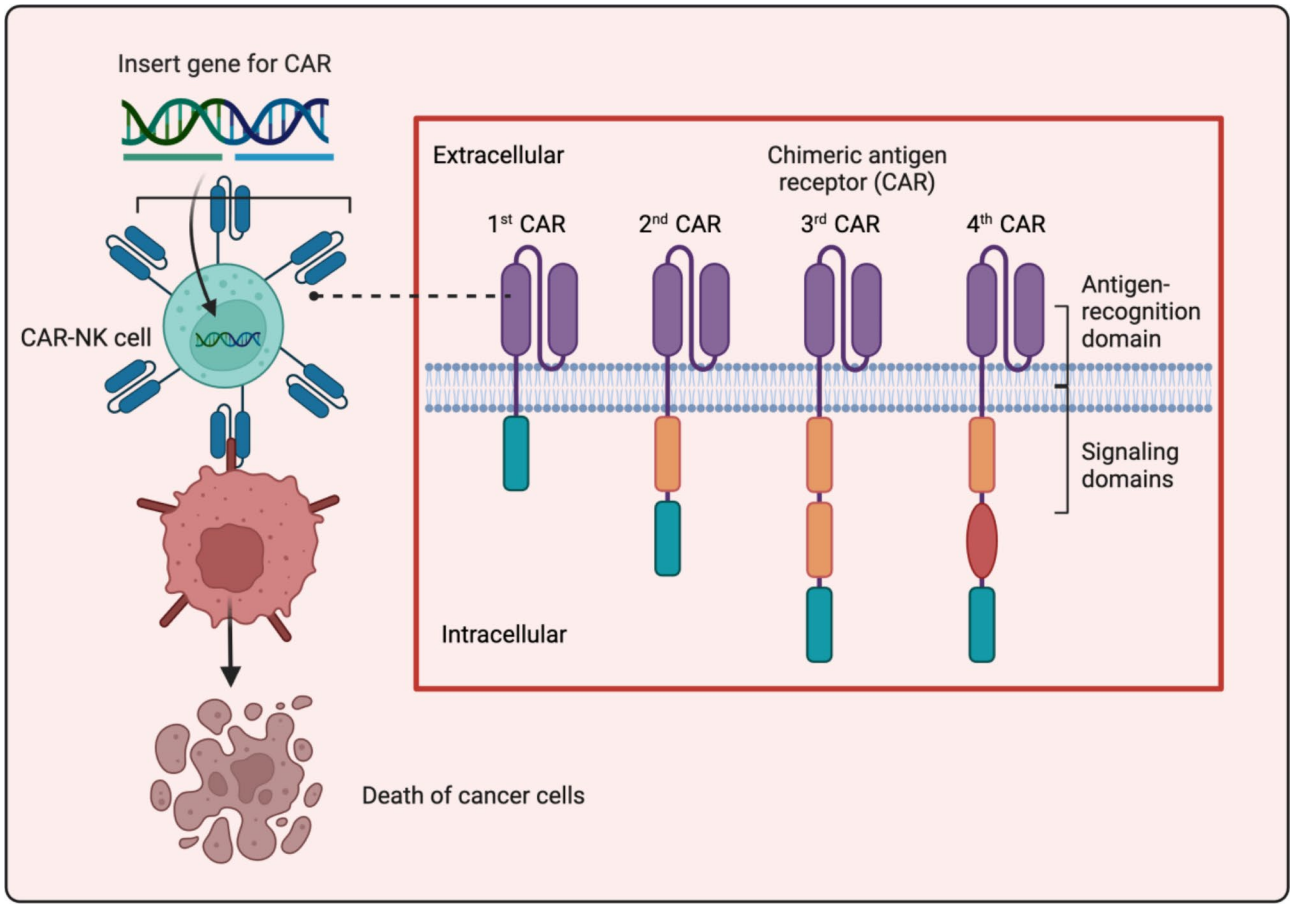

Fig. 2 Overview of the constructs of CAR-NK cells

higher photo, thus showing that the surface expression of GPC3-O4-CAR was higher than the other two constructs. In addition, cytotoxicity assays for the NK cells with the GPC3-O4-CAR construct indicated that the culture was more lethal, and cells secreted increased IFN-γ as compared to the other constructs [60]. This implies that the optimized structure increases binding and tumor cytotoxicity of GPC3-CAR-NK cells making it a better option in managing cancer.

c-MET is the protein product of the proto-oncogene MET which is present in the epidermal as well as endothelial cell, neurons, hepatocytes, and hematopoietic cells [61, 62]. Notably, c-MET has been considered as a novel oncogene in HCC, whose activity boost can promote, maintain or even worsen tumorigenic process and the disease progression [63]. Consequently, c-MET is one of the identified targets for applying immunotherapeutic CAR-T cell therapy for advanced HCC [64]. But CAR-T cells have some limitations in facing HCC through the absence of HCC specific tumor antigens and poor infiltration of CAR-T cells in to the tumor mass [65]. To overcome the limitations of c-MET-CAR-T cells in immunotherapy, scientists have tried to develop CAR-NK cells specific for c-MET. The in vitro cytotoxicity assays revealed that c-MET-CAR-NK cells are toxic to HepG2 cells with high endogenous c-MET expressing levels; thus, c-MET is possibly an effective target for CAR-NK immunotherapy for human liver cancer cells. However, this study did not evaluate the immunotherapeutic efficacy of c-MET-CAR-NK cells in vivo using animal models or organoid models, indicating the need for further investigation in future research [66].

Additionally, CAR-NK cells targeting CD147 have been constructed, and their role in HCC immunotherapy has been explored. As a transmembrane glycoprotein, CD147 participates in tumor progression and can serve as a prognostic marker for various cancers, including HCC [67, 68]. Importantly, Licartin, a radioactive drug known as [131]I-Metuximab (Metuximab Iodine-131), has been clinically proven to be effective in treating HCC [69]. Notably, CD147-CAR-NK cells have been shown to specifically kill CD147+HCC cells both in vitro and in vivo, including in PDX mouse models. Additionally, to mitigate the off-tumor toxicity of CD147-CAR, a synthetic Notch receptor "logic-gated" CD147-CAR targeting both GPC3 and CD147 has been developed. This approach aims to

minimize the potential toxicity in human CD147 transgenic mouse models [70]. This study proposes the possibility of combining anti-CD147 monoclonal antibodies with CD147-CAR-NK cells for HCC treatment. Future research should focus on constructing in vivo models to investigate whether this combined therapy improves therapeutic efficacy against HCC and to assess the safety profile of the treatment.

In addition, CAR-T cells targeting AFP [71], CD133 [72], NK group 2 member D (NKG2D) [73], and fibroblast activation protein [74] have been shown to exert specific anti-tumor immune effects against HCC. Future research should aim to develop CAR-NK cells targeting these antigens and explore whether they offer superior efficacy and safety compared with CAR-T cells for HCC treatment.

Colorectal cancer (CRC)

The CEA is a glycoprotein that is involved in the cell adhesion process. It can be secreted during the fetal period. In adults, its production is usually low, but elevated levels can be found in certain cancers, including colorectal cancer (CRC). CEA has already been utilized in the construction of CAR-T cells. In Phase I clinical trials, CEA-CAR-T cells significantly reduced tumor size in patients with CEA+CRC [75]. Similarly, CEA-CAR-NK cells, The target ligands obtained by electrotransferring NK-92 cells with an anti-CEA specific single-chain antibody fragment are able to selectively identify and destruct the CRC cells that derive from CEA [76]. However, the abundant release of soluble CEA from tumor cells can obstruct CAR recognition and migration through the TME [77]. To address this issue, Franzén and colleagues developed a next-generation CAR construct that specifically targets cell-associated CEA. The development of this concept involves the use of a checkpoint inhibitor and a chemokine receptor. These two factors can help improve the infiltration and homing of CAR-NK-92 cells into the TME, while preventing fratricidal effects [78]. Studies demonstrated that CEA-CAR-NK-92 cells possess potent cytotoxicity against CRC. Notably, the low cytotoxicity, specifically the low amount of deaths in non-cancerous cell lines, was used to further emphasize on the selectiveness of this kind of treatment. Moreover, this increase in homing effectiveness was because of the CCR4 migration receptor, which affixed to its target ligands – CCL17 and CCL22. Crucially, this CAR design did not induce significant trogocytosis-mediated fratricide [78]. Accordingly, it is suggested that the CEA-targeting CAR-NK cell therapy could provide a novel therapeutic option for CRC due to its advantageous features of accuracy and efficiency in the individualized treatment.

EpCAM (CD326) is a transmembrane glycoprotein that is highly expressed in 97.7% of colorectal adenocarcinoma patients [79]. When EpCAM is overexpressed, oncogenes are highly stimulated and cell multiplication is encouraged [80]. As molecular technology has advanced, EpCAM has emerged as a promising target for diagnosing and developing antibody-based immunotherapies for a variety of malignancies [81]. Therefore, Zhang and colleague created EpCAM specific CAR-NK cells. Experiments in this study proved that CAR-NK-92 cells could selectively adhere to EpCAM positive CRC cells and release cytokines by cytokine blotting including IFN-γ, perforin and granzyme B, exhibiting targeted cytotoxicity [82]. It is worth noting that the specificity of EpCAM-CAR-NK cells against CRC has been further evaluated in organoid models. The results provide additional support for the effective targeting of CRC by EpCAM-CAR-NK cells [83]. Recent studies suggest that beyond their direct anti-tumor effects, some TKIs have reported to alter the TME and increased immune pressure against tumor cells [84, 85]. Consequently, there is literature evidence that points at the exploration of the synergistic effects of TKIs alongside CRC's CAR-NK cells. Regorafenib has higher anti-cancer activity in combination with EpCAM-CAR-NK cells in animal models of human CRC., offering a novel strategy for CRC treatment [82].

Of these, the NKG2D present in the human NK cells, CD8+T cells and γδ T cells and NKT cells can bind to the eight NKG2D ligands (NKG2DLs) which when expressed can usually stimulate the activity of an immune cell [86]. Notably, NKG2DL is often expressed in cancers, making it a favorable therapeutic target for anti-cancer strategies [87, 88]. Xiao et al. developed NKG2D-CAR-NK cells and administered them in patients with metastatic CRC. This approach described clinical improvement in malignant ascites and tumor load reduction, which implies the therapeutic rationale for employing CAR-NK cell treatment in solid tumors [89].

Additionally, as a specific molecular biomarker of cancer stem cells, CD133 is considered a rational target for immunotherapy [90, 91]. More clinical trials have proven that immune therapy with CD133 CAR T cells is safe and they did not report any serious side effects [92, 93]. These findings provide impetus for investigating the therapeutic effects of anti-CD133 CAR-NK cells in CRC treatment. In a recent study, Wang and colleagues developed a fourth-generation CAR-NK cell targeting the CD133 antigen. To this CAR construct, an inducible TLR5 agonists CBLB502 (i502) expression cassette driven by the nuclear factor of activated T-cells (NFAT) –IL2 minimal promoter (i502-CAR133) [94]. Specific anti-tumor activity of the CAR133-i502-NK92 cells was also observed in the in vitro experiments as well as in the xenograft mouse models, with good tolerability. Notably,

CAR133-i502-NK92 cells produce CBLB502 protein, which sankering non-specific endogenous immune response [94]. This optimized CAR-NK cell approach not only specifically eliminates CD133+CRC cells but also indirectly eradicates CD133- CRC cells through a CBLB502-specific endogenous immune response, representing a promising new technology for treating antigenically diverse solid tumors. Interestingly, Yang et al. have derived from a fourth generation CD133-specific CAR-T cell, which expresses PD-1 blocking scFv. Designed to enhance CAR-T therapy efficacy, this strategy targets the cancer stem cell-associated antigen CD133 while simultaneously counteracting the immune suppression typically induced by PD-1 in the TME. Such a dual-action approach holds the potential to significantly improve therapeutic responses in treatments targeting solid tumors [72]. Indeed, applying this design to CAR-NK cells poses an intriguing possibility and represents one of the future challenges in the field. Integrating a PD-1 blocking scFv into CAR-NK cells could potentially enhance their efficacy by overcoming immune suppression mechanisms similar to those faced by CAR-T cells. This could broaden the therapeutic scope and improve outcomes in treating solid tumors, where the tumor microenvironment often impairs immune cell function. Exploring this approach will require careful consideration of the unique properties and activation pathways of NK cells compared to T cells.

Pancreatic cancer

Mesothelin (MSLN), a membrane glycoprotein that differentiates cells, is highly overexpressed on the surface of several tumor types, including pancreatic cancer cells [95]. MSLN expression has been linked to worsening tumor progression and increased chemoresistance, This receptor group is a promising target for developing CAR-T cell-based therapies [96]. To overcome the limitations of CAR-T cells, researchers are focusing on exploring the efficacy of CAR-NK cells targeting MSLN in tumor immunotherapy. Currently, Using CAR-NK cells specific to the MSLN antigen, very striking and selective anti-tumor effects have been established both in vitro and in subcutaneous and intraperitoneal xenotransplanted tumor models. Such cells have the ability to kill ovarian cancer cells and considerably increase the survival rates of mice with intraperitoneal tumors. As evidenced by this study, MSLN-targeted CAR-NK cells could be a viable approach in managing advanced ovarian cancer and most likely other MSLN positive malignancies [97]. Similarly, in GI cancers, The generated MSLN-CAR-NK cells derived from NK-92 cell line has been noticed to mediated the IFN-γ and granzyme B in vitro and specifically the lysing of the pancreatic cancer cell lines. This increased activity demonstrates the usefulness of MSLN-CAR-NK cells for selectively killing tumor cells in pancreatic and other GI cancers [98]. In addition to the cytotoxicity seen in vitro, MSLN-CAR-NK cells have also shown good tumor reduction in a pancreatic cancer mouse model in vivo. Even more significantly the co-treatment of these cells with the STING agonist cGAMP significantly restricted the tumor growth and significantly increased the survival of the mice. Regarding the above points, it indicates that enhancement of incompatible increase in the MSLN-CAR-NK-92 cells relating to the destruction of pancreatic cancer cells may be accomplished through the combination treatment [99]. Based on these findings, it introduces possibilities that the integration of STING agonist with CAR-NK cell may bring new form immunotherapy for pancreatic cancer.

The protein codes for a product within the axon guidance receptor group known as Robo [100], has also been said to work as a chemo attractant to T cells and as a tumor angiogenetic factor [101, 102]. Scientific publications have also indicated that Robo1 is up regulated in pancreatic cancer, suggesting its involvement in the progression and vascular development of the disease [103]. This makes Robo1 a potential target for therapeutic interventions in pancreatic cancer. Some researchers have demonstrated that Robo1-CAR-NK92 cells produce more cytokines such as IL-6, IL-10, TNF-α, and IFN-γ, compared to their parent NK-92 cells. Additionally, these engineered cells exhibit significantly enhanced specific lysis of target cells over that of the unmodified NK-92 cells [104]. In a study focused on innovative treatments, In a study, it was demonstrated that the use of a type of CAR-NK cell known as a robo1-specific CAR-NK cell boosted the effectiveness of 125I seed brachytherapy in treating human pancreatic cancer. This suggests that targeting Robo1 with CAR-NK cells could provide a potent adjunct therapy in the management of pancreatic cancer, particularly in combination with established treatment modalities like brachytherapy [105]. Future research on Robo1-CAR-NK cells should focus on conducting clinical trials to evaluate their safety and effectiveness in humans, exploring additional combination therapies, and understanding the mechanisms underlying their synergy with treatments like brachytherapy. The identification of biomarkers that would help in the identification of patients who would benefit most from drugs targeting Robo1 may help to make treatments more targeted. Additionally, extending these studies to other cancers where Robo1 is overexpressed could broaden the therapeutic impact of this promising approach.

Furthermore, through comprehensive bioinformatics and clinical pathology analysis, FRα and DR4/5 were statistically up regulated in patient derived pancreatic cancer cells. FRα and DR4/5 are biomolecules which are correlated to poor prognosis of the clinical situations;

therefore, they are possible therapeutic targets. Consequently, researchers have developed dual-targeted CAR-NK cells aimed at both FRα and DR4/5. Notably, these dual-targeted CAR-NK cells secrete more TRAIL (a ligand for DR4/5) and exhibit significantly higher cytotoxicity against pancreatic cancer cells compared to single-targeted CAR-NK cells [106]. This highlights their enhanced therapeutic potential and provides us with a new therapeutic strategy for constructing multi-targeted CAR-NK cells to improve treatment outcomes.

It is a membrane-associated proteins encoded by the gene PSCA that functions in intracellular signaling and growth neoplasia; of high grade prostatic intraepithelial neoplasia [107, 108]. Interestingly, abnormal overexpression of PSCA has also been detected in the tumors of 60–80% of patients diagnosed with pancreatic cancer. Therefore, PSCA is a promising target for CAR-NK cell therapies aimed at treating pancreatic cancer and in other malignancies in which PSCA expression is prevalent, highlighting its potential utility beyond prostate cancer [109]. Teng et al. isolated NK cells from the UCB and engineered them to create PSCA-CAR-NK cells [110]. These cells demonstrated significant tumor-suppressive effects in vitro against PSCA-positive pancreatic cancer and substantially prolonged the survival of mice transplanted with human pancreatic cancer cells, with no observed signs of systemic toxicity. These PSCA-CAR-NK cells were produced using an optimized expansion platform and cryopreserved as "off-the-shelf" products, which aid in their clinical deployment and application [110]. This approach not only highlights the therapeutic potential of PSCA-CAR-NK cells but also underscores the feasibility of using these engineered cells in a clinical setting, offering a promising avenue for advancing immunotherapy for pancreatic cancer.

In agreement with the findings in CRC, NKG2D-CAR-NK cells also exhibited significant anti-tumor activity against pancreatic cancer, both in vitro and in vivo. Notably, further research has revealed that the GPR116 receptor suppresses NK cell function through the Gαq/HIF1α/NF-κB signaling pathway. Downregulation of the GPR116 receptor enhances the anti-tumor efficacy of NKG2D-CAR-NK cells against pancreatic cancer both in vitro and in vivo [111]. This insight provides a novel strategy for enhancing the therapeutic effectiveness of NKG2D-CAR-NK cells in pancreatic cancer treatment. Targeting the GPR116 pathway could potentially improve the immunosurveillance capabilities of these engineered cells, paving the way for more effective immunotherapeutic approaches to combat this challenging disease.

Gastric cancer (GC)

Due to the marked overexpression of the HER2 protein in a significant number of GC cells [112], scientists have engineered second-generation chimeric antigen receptor (CAR) NK-92 cells, known as HER2-CAR-NK cells. These cells specifically target and destroy HER2-positive GC cells and produce higher levels of cytokines [113]. These cells specifically target and destroy HER2-positive GC cells and produce higher levels of cytokines. While HER2-CAR-NK cells were highly effective in eliminating small tumor xenografts in vivo, their efficacy was reduced in larger solid tumors. To overcome this challenge, further research demonstrated that apatinib treatment enhances NK cell infiltration into larger tumors, thereby boosting the therapeutic effectiveness of HER2-CAR-NK cells [113]. This approach suggests a novel strategy for treating large or resistant tumors by combining pharmacotherapy with immunocellular therapy to improve overall treatment outcomes.

Like in pancreatic cancer, MSLN is also overexpressed in GC. To leverage this, researchers created MSLN-CAR-NK cells aimed at enhancing immunotherapy's effectiveness in gastric cancer patients [114]. In vitro studies confirmed that these cells selectively target and eliminate MSLN-positive GC cell lines, such as N87, MKN-28, and AGS, without affecting MSLN-negative cells like Huh-7. Moreover, MSLN-CAR-NK cells demonstrated efficacy against GC cells in both subcutaneous and intraperitoneal tumor models. Crucially, patient-derived xenografts treated with these cells exhibited substantial anti-tumor activity and extensive NK cell infiltration, indicating their promising therapeutic potential for GC treatment [114].

Additionally, CAR T cells redirected to target Claudin18.2 (CLDN18.2) [115], c-Met [116], folate receptor 1 [117], ICAM-1 [118], and PSCA [119] showed promising efficacy against GC in a preclinical study. Specifically, CLDN18.2-CAR-T cell therapy has progressed to Phase I clinical trials (NCT03874897) for GI cancers [120], with overall response and disease control rates reported at 38.8% and 91.8% among 98 patients. The median progression-free survival was 4.4 months, and overall survival was 8.8 months. However, 96.9% of patients experienced CRS [120]. Therefore, future research directions involve developing CAR-NK cells that target the aforementioned antigens and investigating their anti-tumor efficacy and safety in GC.

Esophageal squamous cell carcinoma (ESCC)

Liu et al. identified CD22, a well-known tumor surface marker in hematological malignancies that is also expressed in ESCC and potentially serves as a target for CAR-NK cell therapy. They developed CD22-CAR-NK cells and found that these cells exhibited in vitro inhibitory effects on ESCC cell lines [121]. However, no in vivo experiments have been conducted to analyze the anti-tumor capabilities of these cells and their safety in the body.

Additionally, CD276, a type I transmembrane glycoprotein, is widely overexpressed in various solid tumors, including ESCC [122, 123]. Lin et al. developed CD276-targeted CAR-NK cells derived from iPSCs and showed that these cells exhibited significant cytotoxicity in human ESCC cell lines, patient-specific organoid models of ESCC, and primary cultured ESCC cells. Moreover, in mouse tumor models, CD276-CAR-NK cells significantly prolonged the survival rate of mice [124]. These preclinical results highlight the potential of CD276-CAR-NK cells for ESCC treatment and underscore the need for further clinical research to explore their efficacy and safety in human trials Table 1.

Strategies to address the limitations of CAR-NK cells therapy

The results of the first large-scale trial of CAR-NK cells (NCT03056339) demonstrated their safety and robust clinical activity in patients with CD19+chronic lymphocytic leukemia and B-cell lymphoma [125]. As mentioned earlier, CAR-NK cells offer several advantages over CAR -T cells as cancer immunotherapy options. However, similar to CAR-T cell therapy, CAR-NK cell therapy also faces challenges, such as the loss of target antigens, tumor heterogeneity, and a hostile TME. Therefore, considering strategies that can maximize the efficacy of future CAR-based NK cell therapies is crucial.

Table 1 Application of CAR-NK cells in GI cancers treatment

Targets	Cancer	NK cell source	Ref
GPC3	HCC	NK-92	[57]
c-MET	HCC	PB-NK	[66]
CD147	HCC	NK-92	[70]
GPC3-O4	HCC	PB-NK	[60]
GPC3	HCC	PB-NK	[58]
CEA	CRC	NK-92	[76]
EpCAM	CRC	NK-92	[82]
NKG2D	CRC	PB-NK	[89]
EpCAM	CRC	NK-92	[83]
CD133	CRC	NK-92	[94]
CEA	CRC	NK-92	[78]
MSLN	Pancreatic cancer	NK-92	[98]
Robo1	Pancreatic cancer	NK-92	[105]
FRα and DR4/5	Pancreatic cancer	NK-92	[106]
PSCA	Pancreatic cancer	UCB-NK	[110]
MSLN	Pancreatic cancer	NK-92	[99]
NKG2D	Pancreatic cancer	NK-92	[111]
HER2	GC	NK-92	[113]
MSLN	GC	NK-92	[114]
CD22	ESCC	iPSC-NK	[121]
CD276	ESCC	iPSC-NK	[124]

Target antigens for CAR-NK cells

Notably, CAR-NK cells possess a unique ability to exert cytotoxic effects on cancer cells through both CAR-dependent pathways and natural mechanisms. Therefore, a CAR that triggers only a mild activation signal, such as a CAR lacking a costimulatory domain, can be engineered, allowing CAR-NK cells to primarily utilize their innate cytotoxic abilities, while minimizing CAR-induced direct cytotoxicity [126]. Furthermore, NK cells can be modified to carry a non-activating CAR that does not directly trigger cytotoxicity but instead enhances NK cell targeting, adhesion, and migration towards cancer cells [127]. This design leverages the natural cytotoxic pathways of NK cells, thereby reducing the risk of damage to normal cells that may also express the target antigen. Consequently, this approach enables targeting of a broader spectrum of antigens, including HER2, EGFR, and mesothelin.

Additionally, identifying suitable tumor-specific antigens that are predominantly expressed on GI cancer cells but not on healthy tissues is crucial. The ideal target should be highly expressed on cancer cells, with minimal or no expression on normal tissues. This specificity is essential for reducing the risk of off-target effects and associated toxicity [128, 129]. Moreover, the target antigen should ideally be involved in tumor growth or survival, providing a rationale for targeting it in therapy [130]. Given the heterogeneity of tumors, especially GI cancers, identifying a panel of antigens or developing multi-target CAR-NK cells may be beneficial to enhance efficacy and prevent tumor escape variants that may downregulate a single target antigen [131]. This approach could improve treatment outcomes and address the challenge of antigen loss during tumor evolution.

Tumor immunosuppressive microenvironment

The tumor microenvironment (TME) significantly hinders immune cell-mediated tumor destruction. Cancer-associated fibroblasts (CAFs), a key component of the tumor stroma, are well-known for promoting tumor progression [132]. Therefore, targeting CAFs offers a potential strategy to counteract the negative impacts of the TME. Sakemura and colleagues explored this by creating BCMA/CAF-CAR-T cells [133]. Studies with human multiple myeloma cells and mouse models revealed that this dual-targeting CAR can restore CAR-T cell function and enhance anti-tumor effectiveness [133]. Additionally, adjusting the expansion conditions of CAR-NK cells may alleviate the suppressive effects of the TME, as demonstrated in preliminary CAR-T cell studies [134].

The TME contains various immunosuppressive elements, including hypoxia, TGF-β, PGE-2, and extracellular metabolites like lactate and adenosine, which hinder immune cell activity [135]. Modifying NK cells to resist

these suppressive factors presents a promising approach. For example, reducing TGF-β receptor II expression on NK cells has shown to partly counteract TME's negative effects without compromising anti-leukemia activity [136]. Other explored methods include engineering CARs to express negative TGF-β receptors or combining them with TGF-β receptor inhibitors [137].

Tumor cells can evade immune clearance by upregulating ligands for immune cell inhibitory receptors, exploiting the immune system's negative feedback mechanisms. Strategies to overcome this include knocking down receptors like NKG2A on NK cells or combining therapy with specific inhibitory checkpoint antibodies to improve anti-tumor effects [138, 139]. Designing CAR structures targeting inhibitory checkpoints also shows promise [140], as demonstrated in initial studies for solid tumors [141].

Persistence of CAR-NK cells

NK cells, by nature, have a short lifespan and high turnover rate in vivo, which distinguishes them from longer-lived lymphocytes like T cells [142]. CAR-NK cells, therefore, tend to persist for only 1–2 weeks, limiting their efficacy without cytokine support. However, exogenous cytokines may trigger side effects and activate other immune cells, such as regulatory T cells, which could impair NK cell function [143]. Developing strategies to extend CAR-NK cells' longevity and functionality within a hostile tumor environment is crucial.

Advanced genetic engineering techniques allow the creation of NK cells that not only carry CARs but also additional therapeutic agents like cytokines, antibodies, and proteases to boost their growth, motility, and tumor penetration. For instance, IL-15 plays a vital role in NK cell development, survival, and activation. CAR-NK cells engineered to express IL-15 demonstrate enhanced persistence and increased anti-tumor activity in vivo [144]. Additionally, a recent study incorporated a short hairpin RNA cassette targeting PD-1 into a BCMA-CAR with an OX-40 costimulatory domain, showing reduced T cell exhaustion and a higher percentage of memory T cells in vitro compared to parental BCMA-CAR-T cells [145]. This genetically engineered CAR structure offers a promising new avenue for designing "armored" CAR-NK cells. Another innovative approach is targeting apoptotic signals in tumor cells or using gene-editing tools to remove pro-apoptotic genes in CAR-NK cells, potentially enhancing resistance to apoptotic signals in the TME [106]. These multifunctional CAR-NK cells, often referred to as "armored" CAR-NK cells or "NK cell pharmacies," provide a distinct and potentially safer method for locally modifying the TME, potentially minimizing or eliminating systemic side effects [146].

Combination of CAR-NK therapy with other therapeutic approaches

Combining CAR-NK cell therapy with conventional treatments such as chemotherapy or radiotherapy can create synergistic effects, increasing tumor antigen exposure and enhancing the sensitivity of cancer cells to immune-mediated killing. This multifaceted approach aims not only to improve tumor eradication but also to reduce recurrence risks and improve patient outcomes by attacking tumors from multiple angles [147]. Several studies have indicated that CAR-T/NK cells retain their cytotoxicity in the presence of cisplatin, and cisplatin can enhance their cytotoxic effects while showing strong anti-tumor activity [148, 149]. Recent findings have shown that CD44-targeted CAR-NK cells remain cytotoxic when combined with cisplatin, with the combination showing greater anti-tumor efficacy than sequential treatment [150]. Additionally, CAR-NK92 cells targeting CD133, when combined with cisplatin, exhibit the strongest anti-tumor effects against ovarian cancer, with cisplatin not impacting CAR-NK cells' cytotoxicity or viability [151]. Moreover, the synergistic effects of CAR-T/NK cells combined with radiotherapy have been validated for treating glioblastoma and pancreatic cancer, highlighting the ongoing critical role of chemotherapy in advancing CAR-NK cell therapy. Radiotherapy remains a key treatment modality for most cancers, whether curative or palliative. Emerging evidence suggests that radiotherapy, especially stereotactic body radiotherapy, can synergize with immunotherapy approaches, such as anti-PD-1 or anti-CTLA4 antibodies [152, 153]. Proposed mechanisms from preclinical studies include increased tumor antigen availability, release of immunostimulatory cytokines and danger signals, and disruption of the tumor-supporting stroma. These factors contribute to recruiting and activating antigen-presenting cells, which trigger specific tumor immune responses, leading to tumor regression at distant, non-irradiated sites, a phenomenon known as the "abscopal effect." [154]. Additionally, radiation-induced DNA damage can lead to the expression of NKG2D ligands on tumor cells, potentially enhancing NK cells' activation and cytotoxicity against tumor cells [155, 156]. Consequently, combining CAR-NK cell therapy with localized radiotherapy offers a promising alternative treatment strategy, particularly against solid tumors.

Immune checkpoint inhibitors (ICIs) such as anti-PD-1/PD-L1 [157] and anti-CTLA4 antibodies [158] have shown remarkable clinical results in various cancers. Notably, PD-1 pathway inhibition has enhanced CAR-T cell therapy's effectiveness [159–161]. However, systemic ICIs application can lead to increased immune-related adverse effects [162]. Recent advances include delivering anti-PD-1 antibodies or scFv directly to tumor sites using

engineered CAR-T cells, improving anti-tumor effects and reducing side effects compared to conventional systemic treatments [163, 164]. A recent study constructed CLDN6-CAR-NK cells and found that combining these cells with anti-PD-L1 synergistically enhanced CLDN6-targeted CAR-NK cells' anti-tumor efficacy, further supporting the potential of combining CAR-NK cells with PD-1/PD-L1 inhibitors for cancer therapy [165]. Additionally, CRISPR/Cas9 technology has been used to remove the PD-1 gene from CD19-targeted CAR-T cells, amplifying their efficacy against CD19-positive cancer cells [166]. Similarly, knocking down PD-1 in CAR-T cells reduced T-cell exhaustion and resulted in a higher percentage of memory T cells in vitro, thereby enhancing antitumor immunity [145]. These findings highlight the potential of PD-1 gene editing in improving CAR-T cell performance and offer a promising strategy for CAR-NK cell design, where similar approaches could enhance their antitumor capabilities. Activated NK cells express several T-cell immune checkpoint molecules, such as PD-1, CTLA-4, LAG3, and TIM3, which can suppress their tumor-fighting capabilities. Blocking these checkpoints can significantly enhance NK cell functions [167, 168]. Therefore, integrating CAR-NK cells with systemic or localized ICI therapies or incorporating gene-editing techniques to disrupt checkpoint expression represents a forward-looking strategy for enhancing CAR-NK cell therapy, particularly for treating solid tumors.

Additionally, sequential therapy using CAR-NK cells followed by CAR-T cells offers a potent and secure method to synergistically and continuously eradicate tumors, especially in patients with significant tumor loads. Readily available "off-the-shelf" CAR-NK cell products can be administered immediately to reduce tumor burden before CAR-T cell infusion, which typically takes 2–4 weeks to prepare. Some CAR-T cells may persist as memory T cells and maintain long-term anti-tumor activity. Therefore, sequential CAR-NK and CAR-T cell administration could facilitate swift and lasting tumor reduction. Furthermore, the initial tumor load reduction by CAR-NK therapy could decrease the likelihood of CRS and neurotoxicity in subsequent CAR-T cell therapy, thereby minimizing potential adverse effects [9].

Manufacturing and scalability

The production of CAR-NK cells must be efficient and scalable to meet clinical demands. Standardizing protocols for cell isolation, genetic modification, and expansion, while ensuring consistent quality and potency, are essential.

Safety concerns

Although NK cells are generally considered less toxic than T cells, a risk of CRS and other adverse effects still exists [125]. Therefore, developing strategies for monitoring and mitigating these risks is critical.

Regulatory challenges

Navigation of the regulatory landscape of novel cell therapies is complex. Ensuring compliance with regulatory requirements while advancing CAR-NK cell therapies through clinical trials is a challenge that must be overcome [169].

Clinical trial design

Designing reliable clinical trials to fully evaluate the safety and efficacy of CAR-NK cells in different patient populations and at various stages of GI cancer can be complex. Large-scale clinical trials are needed to explore the optimization of CAR-NK cell-based tumor immunotherapy, including administration schedules, dosage, duration, pharmacokinetics, interactions with endogenous immune cells, and underlying mechanisms of CAR-NK cell function [170].

Conclusion and future perspectives

Notably, CAR-NK cells are promising and versatile alternatives to CAR-T cells for the treatment of GI cancers. The unique properties of NK cells, such as their natural cytotoxicity, lower risk of GVHD, and reduced incidence of CRS, make them particularly suitable for developing "off-the-shelf" allogeneic cell therapies. Preclinical and early clinical studies have demonstrated significant antitumor activity of CAR-NK cells against various GI cancers, highlighting their potential to overcome some of the limitations associated with CAR-T cell therapies. Significant progress has been made in advancing CAR-NK cell therapy through two primary approaches: enhancing CAR structure and improving CAR-NK production. Enhancing the CAR structure involves innovating the CAR design to improve antigen recognition capabilities. On the production side, improvements include exploring diverse sources of NK cells, optimizing gene delivery systems, and increasing the efficiency of CAR transduction and transfection into NK cells.

Despite these advantages, several challenges must be addressed to fully realize the potential of CAR-NK cell therapy. A significant challenge is the TME, which can inhibit the activity and persistence of CAR-NK cells. Future strategies should focus on engineering CAR-NK cells to resist the suppressive effects of the TME and combining CAR-NK cell therapy with other treatments that modify the TME to enhance its efficacy. Antigen heterogeneity and the potential for antigen escape are other critical areas of research. The development of multi-specific or universal CAR platforms that can target multiple antigens may prevent tumor cells from evading immune detection. Additionally, enhancing the persistence and

proliferation of CAR-NK cells in vivo remains a key focus. Incorporating genes that promote NK cell survival, optimizing NK cell sources, and improving scalable production processes is essential to produce consistent and potent CAR-NK cell products.

Combining CAR-NK cell therapy with conventional treatments such as chemotherapy, radiotherapy, and ICIs can synergistically increase tumor cell death and overcome TME-induced immune suppression. Such combination therapies could potentially enhance the overall therapeutic efficacy of CAR-NK cells. To advance the clinical application of CAR-NK cells, regulatory strategies and well-designed clinical trials are necessary to assess their safety and efficacy against various GI cancers. Identifying predictive biomarkers for patient selection is crucial for tailoring treatments to individual patients and maximizing therapeutic outcomes.

Additionally, a recent head-to-head comparison study demonstrated that CAR-T cells outperformed CAR-NK cells in terms of CAR-mediated effector function. Therefore, future research may be needed to engineer CAR-NK cells so that they have enhanced anti-tumor activity, ultimately fulfilling the promise of effective "off-the-shelf" products [171].

Abbreviations

95% CI	95% Confidence interval
BCMA	B cell maturation antigen
CAFs	Cancer-associated fibroblasts
CAR	Chimeric antigen receptor
CEA	Carcinoembryonic antigen
CLDN18.2	Claudin18.2
CRC	Colorectal cancer
CRS	Cytokine release syndrome
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CLDN6	Claudin 6
DAP	DNAX-activation protein
DR4/5	Death receptors 4/5
ESCC	3.5 esophageal squamous cell carcinoma
FRα	Folate receptor alpha
GC	Gastric cancer
GI	Gastrointestinal
GPC3	Glypican-3
GVHD	Graft-versus-host disease
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
HPCs	Hematopoietic progenitor cells
iCAR	Inhibitory chimeric antigen receptor
ICIs	Immune checkpoint inhibitors
IFN-γ	Interferon gamma
iPSCs	Induced pluripotent stem cells
KIRs	Killer immunoglobulin-like receptors
L3C7c-Fc	High-affinity sPD-L1 variant
MHC	Major histocompatibility complex
MSLN	Mesothelin
NK cells	Natural killer cells
NKG2D	NK group 2 member D
NKG2DL	NKG2D ligands
PD-1	Programmed cell death protein 1
PBMCs	Peripheral blood mononuclear cells
PB NK cells	PBMC-derived NK cells
PSCA	Prostate stem cell antigen
SCF	Stem cell factor
scFv	Single-chain variable fragment
sPD-L1	Soluble programmed death ligand 1
STING	Stimulator of Interferon genes
TGF-β	Transforming growth factor beta
TKIs	Tyrosine kinase inhibitors
TME	Tumor microenvironment
UCB	Umbilical cord blood
VHH	Variable domains on heavy chains

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