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CAR-NK Cell: A New Paradigm in Tumor Immunotherapy

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The tumor microenvironment (TME) is greatly multifaceted and immune escape is an imperative attribute of tumors fostering tumor progression and metastasis. Based on reports, the restricted achievement attained by T cell immunotherapy reflects the prominence of emerging other innovative immunotherapeutics, in particular, natural killer (NK) cells-based treatments. Human NK cells act as the foremost innate immune effector cells against tumors and are vastly heterogeneous in the TME. Currently, there exists a rapidly evolving interest in the progress of chimeric antigen receptor (CAR)-engineered NK cells for tumor immunotherapy. CAR-NK cells superiorities over CAR-T cells in terms of better safety (e.g., absence or minimal cytokine release syndrome (CRS) and graft-versus-host disease (GVHD), engaging various mechanisms for stimulating cytotoxic function, and high feasibility for 'off-the-shelf' manufacturing. These effector cells could be modified to target various antigens, improve proliferation and persistence *in vivo*, upturn infiltration into tumors, and defeat resistant TME, which in turn, result in a desired anti-tumor response. More importantly, CAR-NK cells represent antigen receptors against tumor-associated antigens (TAAs), thereby redirecting the effector NK cells and supporting tumor-related immunosurveillance. In the current review, we focus on recent progress in the therapeutic competence of CAR-NK cells in solid tumors and offer a concise summary of the present hurdles affecting therapeutic outcomes of CAR-NK cell-based tumor immunotherapies.

Keywords: chimeric antigen receptor, natural killer cells, solid tumors, immunotherapy, tumor-associated antigens

INTRODUCTION

Currently, natural killer (NK) cell-based immunotherapy has become a promising and advanced scientific research topic in the context of cancer immunotherapy, either solid tumors or hematological malignancies (1). NK cells are innate lymphocytes holding a spectrum of functional aptitudes, comprising anti-cancer, anti-viral, and anti-graft-versus-host disease (GVHD) functions (2). They act as the foremost effector cells against tumor in innate immunity and are greatly heterogeneous in the microenvironment. Today, some restrictions such as the failure of T cells to identify and kill HLA-I negative tumor cells hinder their clinical efficacy (3); new strategies for cancer immunotherapy are emphasizing NK cells.

For the first time, NK cells were recognized in the 1970s as an exclusive lymphocyte subclass capable to identify and rapidly kill abnormal cells in the absence of prior sensitization or detection of specific tumor antigens, enabling shrinkage of the tumor (4). A few years later, it was shown that NK cells could lyse an MHC class I negative lymphoma cell line, while the original MHC class I positive cells were resistant to lysis. This delivered the proof of a hypothesis citing that NK cells are capable of sensing the lack of "self" MHC class-I molecules on cancerous cells, which is known as "missing self-hypothesis" (5). Later, this premise was supported following the discovery of inhibitory (6) and activating NK receptors (7). Based on the literature, the chief NK inhibitory receptors are the killer Ig-like receptors (KIRs) which identify allotypic determinants mutual by groups of HLA class-I alleles (8), and CD94/NKG2A heterodimer (9) which recognizes the non-classical HLA-E molecule. The activating NK cell receptors include a variety of non-HLA-specific receptors and co-receptors capable to elicit NK cell stimulation *via* straight interaction with ligands overexpressed or expressed *de novo* on malignant cells (10, 11).

Triggered NK cells can kill cancerous cells by direct cell cytotoxicity and/or generation of pro-inflammatory cytokines. In addition to the releases of perforin and granzymes for tumor cell elimination, NK cells exert antibody-dependent cellular cytotoxicity (ADCC) by the membrane receptor CD16 or apoptotic axis intermediated by Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) (**Figure 1**) (12, 13). Furthermore, modulation of anti-tumor immune responses by NK cells leads to secretion of cytokines and chemokines including interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (14). Although NK cells can identify and eliminate tumor cells; malignant cells continue to advance their mechanisms to avoid identification by NK cells or limit NK cells activities. Tumor cell immune evasion is thought to mainly rely on the generation of immunosuppressive cytokines or chemokines, ranging from IL-10 and transforming growth factor-beta (TGF-β) to the soluble IL-2 receptor (sCD25), CXCL9, and CXCL10 (15–17). As well, transformed cells can attenuate the expression of tumor-associated antigens (TAAs) (18) and also raise the expression of MHC class I-related molecules (19) to obstruct NK cells activation.

Recent observations have indicated that engineering NK cells to express a chimeric antigen receptor (CAR) can defeat immune evasion (20). In addition to an array of strategies such as CAR T cells, checkpoint inhibitors, antibodies, antibody–drug conjugates, and tumor vaccinations, the progress of "off-the-shelf" CAR-modified NK cells is considered as an emerging and rapidly evolving approach in the advancement of potent anti-cancer immunotherapeutic products (21). These CAR-modified cells express antigen receptors toward TAAs, which redirect their effector functions and improve tumor-specific immunosurveillance (20). A large number of preclinical studies have been executed, and some clinical trials are being carried out to address the clinical efficacy of CAR-NK cells in human tumors. Herein, we will discuss CAR-NK cell's therapeutic potential for treating solid tumors, focusing on *in vivo* researches, and also will deliver a brief overview of existing challenges in the context of CAR-NK cell-based cancer immunotherapy.

NK CELLS IN THE TUMOR MICROENVIRONMENT

The importance of NK cells in cancer is not limited to only hematological malignancies. Recent reports indicate that NK cells contribute to the modification of extravascular tumor growth, and to the primary steps of oncogenesis. Recent investigations have revealed that cancerous cells could progress more rapidly in spontaneous leukemia and prostate cancer models wherein the NK cells were exhausted, compared to those with normal NK cell activity (22). Correspondingly, fully advanced tumors from NK-cell-deficient rodents could present ligands for NKG2D, while tumors from NK-competent rodents did not show these ligands, thus signifying that tumors evolving in these rodents had been modulated by NK cells (22). In this regard, the prognostic importance of NK cells in patients suffering from colorectal carcinomas was first evidenced by Coca et al. (23) showing that patients with lower NK infiltration experienced shorter survival rates compared with those with widespread infiltration. Meanwhile, some studies proposed that the rate of tumor-infiltrating NK cells performed as an influential factor to determine the survival of patients with squamous cell lung cancer (24). However, Vaquero et al. study didn't support the existence of an association between the rate of NK-cell infiltration inside resected brain metastases and the period free of intracranial disease in patients with lung adenocarcinoma (25). Nevertheless, the number of cancer types in which a correlation between intratumoral NK-cell levels and prognosis has been established and is progressively rising. Indeed, regardless of recruitment into the solid tumors, NK cells can functionally affect the host–tumor relationship. Sun et al. found that NK cells density in the blood and tumor tissues of hepatocellular carcinoma (HCC) patients could be certainly associated with survival and prognosis. On the other hand, a cluster of NK cells-related genes in HCC tissues is related with sustained survival, therefore implying that NK cells and HCC

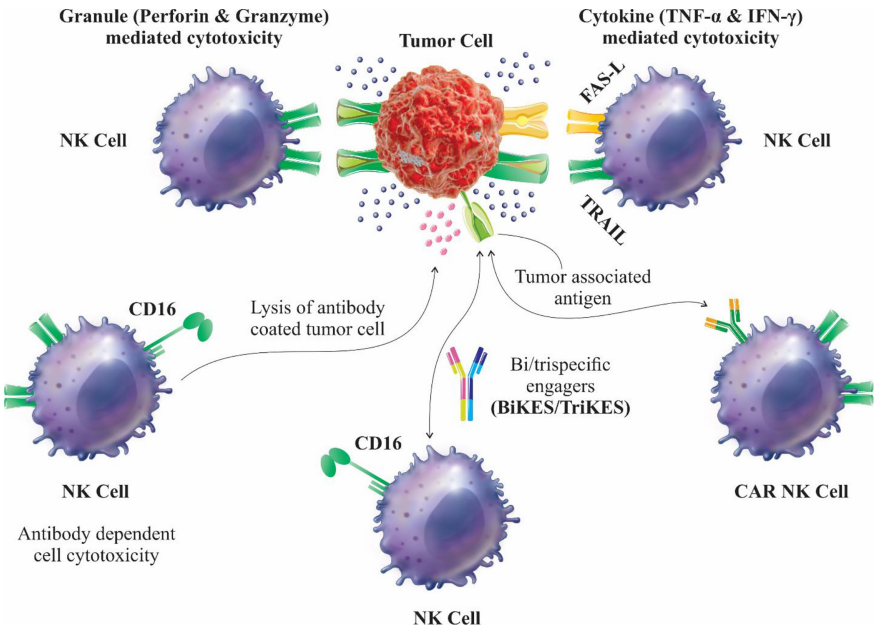

FIGURE 1 | Mechanisms of NK cell cytotoxicity against tumors. The Fc receptor CD16 is presented on NK cells following the identification of antibody-coated cells stimulates a signal to NK cells, enabling tumor cell eradication by direct lysis and cytokine generation. Despite the secretion of perforin and granzymes for tumor cell killing, NK cells elicit ADCC *via* the membrane receptor CD16, or apoptotic axis mediated through FASL and TRAIL. Furthermore, BiKEs and TRiKEs that induce NK cells toward one or more TAAs are the capable strategies for treating human solid tumors; on the other hand, CARs re-direct NK cells against tumor cells showing specific antigens, making key opportunities in the battle toward tumors. NKCs, Natural killer cells; BiKEs, Bispecific killer cell engagers; TRiKEs, Trispecific killer cell engagers; TAAs, Tumor-associated antigens; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; FasL, Fas ligand; ADCC, Antibody-dependent cellular cytotoxicity; CARs, Chimeric antigen receptors.

development are strongly associated (26). Moreover, it seems that any abnormalities in NK cells functions in patients with chronic Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections affect HCC development, and also depletion of NK cells that presents lesser cytotoxicity and compromised cytokine generation may serve as a prognosticator for HCC incidences (26). Molecular analysis displayed that impairment in NK cells-exerted cytotoxicity against cancer cells relies on unregulated signaling and expression of stem cell factor (SCF), c-myc, and signal transducer and activator of transcription 3 (STAT3) in NK cells (27). In STAT3 deficiency, NK cells progress typically and in normal frequencies, while show modifications in the kinetics of IFN-γ generation through direct bindings to IFN-γ promoter (28). In a variety of preclinical models of hematological malignancies, STAT3 deficiency in NK cells improves tumor surveillance, recommending that STAT3 inhibitors could trigger the NK cells-induced cytotoxicity against leukemia (28). Moreover, a study in 320 patients with stage II colon cancer suggested that the density of NK cells is linked with the lymph nodes (LNs) frequencies and is an independent predictive factor (29). Similarly, a study in 180 gastric cancer patients verified a significant association between the NK cells percentage and overall survival of enrolled participants (30). NK cells number was directly related to lymphocyte count and albumin but was conversely related to cancer antigen 125 (CA 125) and neutrophil–lymphocyte ratio. Remarkably, patients at early clinical stages had superior NK cell numbers over those at advanced clinical stages of gastric cancer (30).

CARS STRUCTURES AND FUNCTIONS

To date, most CAR-NK cell surveys use CAR constructs designed for CAR-T cells. Lately, new specific CAR constructs have been designed for NK cells, and diverse CAR constructs displayed variable influences on cytotoxicity and cytokine generation in NK cells (31).

Briefly, CAR is an engineered altered fusion protein based on the T cell receptor, encompassing an extracellular antigen identifying domain bonded to a diversity of intracellular signaling domains (32). The extracellular domain of CARs is typically an antibody single-chain variable fragment (scFv) detecting the specific antigen, which is regularly overexpressed on or is specific to cancer cells, and this detection is in the absence of presentation by major histocompatibility complex (MHC) molecules, similarly to an antibody (33). The intracellular domains commonly consist of CD28, 4-1BB, or OX40 for sustaining engineered-cell activation, and CD3ζ for cytotoxicity. While 4-1BB/CD28-comprising CARs that were firstly exploited in T cells could stimulate anti-cancer functions following use in NK cells; NK cells with 2B4 (CD244), a well-known NK-specific co-stimulatory domain, -comprising CAR

showed improved cytotoxic function, triggered rapid proliferation, augmented cytokine releases, and diminished apoptosis compared to NK cells bearing the typical 4-1BB comprising CAR (34). Besides, CARs containing signaling domain DAP12 showed more prominent anti-cancer potentials in primary NK cells or NK92 cell lines compared to CD3ζ containing CARs (35).

Genetically-altered T cells expressing a CAR can detect CAR-targeted antigen and thereby induce T cell activation, proliferation, cytokine secretion, and cytotoxicity toward tumor cells that express specific antigens (36). Accordingly, CAR-modified T cell therapy resulted in appreciated achievement for treating hematological malignancies, containing lymphoma, chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia (ALL) (37, 38). Especially, CD19-targeting CAR-T cells lead to complete response rate of 70 to 90% in patients suffering from ALL (39), while display usually non-significant clinical efficacy for solid tumors (36, 40). Due to the incidence of GVHD elicited by allogeneic T cells which impedes their clinical use, patient autologous cells are required for the construction of CAR-T cells (41). Alike with CAR-expressing T cells, CAR-NK cells show sustained tumor-specific targeting and cytotoxicity against cancer cells. Prominently, NK cells possess various superiorities over T cells in CAR-targeted immunotherapy, in particular, offering the chance of allogeneic NK cell application as GVHD rely usually on T cell, not NK cells. On the other hand, CAR-NK cells seem to be safer than CAR-T cells since they commonly do not induce cytokine storms (42, 43). In the next sections, we will explain in depth the differences between these two types of engineered cells and their potent advantages and disadvantages.

NK CELLS SOURCE AND TRANSDUCING PROCESS FOR CAR-NK GENERATION

To generate CAR-NK cells, NK cells have been firstly obtained from various sources and then transduced using varied vectors (**Figure 2**) (44). We will discuss various sources of NK cells and also evaluate transduction procedures applied typically to establish effector CAR-NK cells.

Source

NK cells are found in peripheral blood (PB) and umbilical cord blood (UCB) and also can be derived from stem cell sources, ranging from hematopoietic stem cells (HSCs) to human pluripotent stem cells (hiPSCs) (45, 46). The clinical scale expansion of NK cells enables the production of sufficient cells for immunotherapy. Further, allogenic NK cells can be utilized as effector cells because they are not responsible for GVHD but they improve graft-versus-leukemia (GVL) (47).

While PB-NK cells can be easily obtained, low transduction efficiency along with poor expansion restrict their use (48). However, NK cells can be established in large numbers from hiPSC and are more permissive to engineering (49). Moreover, UCB-NK cells are more readily engineered because of their greater proliferative competence, as has been evidenced in the first available clinical trial of CAR-NK cells (50). Nonetheless, a potential difficulty is the comparatively immature nature of UCB-NK cells, leading to condensed cytotoxicity in comparison to PB-NK cells (51). Compared to PB-NK cells, UCB-NK cells express comparable levels of CD56, NCRs (NKp46 and NKp30) and NKG2D but a lower levels of CD16, adhesion molecules (e.g., CD2, CD11a, CD18, CD62L), KIRs, DNAM-1, NKG2C, IL-2R and CD57, and CD8 concomitant with a higher level of inhibitory receptor NKG2A (**Figure 3**) (52, 53). Although cell lines such as NK-92 are relatively easy to engineer; there are challenges related to the safety concerns, and the fact that they must be lethally irradiated before injection, impedes their persistence in the host consequently (43, 54). Currently, feeder cell lines are available to expand NK cells *ex vivo*. These MHC-negative cell lines, most importantly K562, are frequently engineered to generate IL-15 and IL-21 and are irradiated before use (55). Finally, NK cells can be generated from CD34-positive cells from the BM or UCB. These cells are usually similar to PB-NK cells and show functionality, the capability to eliminate leukemic cell lines and patient's tumor cells, and also generate cytokines following exposure to various stimuli *in vitro* and *in vivo*, while exhibiting low rates of inhibitory receptors (56).

Transduction

Transduction denotes introduction of genetic material using viral vectors, containing the retroviral and lentiviral-based vectors (57). Throughout the life-cycle of retroviruses, viral RNAs are reverse transcribed into double-stranded cDNA that subsequently integrate into the target cell's genome semi-randomly. Owing to this fact, this approach usually takes longer till the genes are expressed (58). Vectors constructed by these pathogens possess some benefits, which make it comparatively simple to form complex vectors and then insert them into the target cells. Normally, these vectors are up to 10 kb in size without experiencing substantial loss of titer throughout manufacture, permitting the introduction of up to 7–8 kb (59). Besides, these vector's integration support cell's prolonged alteration in the lack of antibiotic resistance markers, and then altered cells typically are upheld in the host during a long duration (60). Rendering reports, NK cell susceptibilities to external genetic material accompanying the demanding procedure of transduction usually leads to low rates of transduction along with high apoptosis, making the NK cells transduction efficiency lower than T-cells (59). It is because of the resistance to viral transduction exerted by the innate immune system directed by pattern recognition receptors identifying foreign genetic material (61). Given this problem, the use of PDK1 inhibitors such as BX795 is recommended as they negatively regulate the induction of signaling pathways elicited by RIG-I-like receptors or Toll-like receptor 3 (TLR3) (62), and eventually promote lentiviral transduction efficiency up to ~4 fold (63). However, to obtain sufficient transgene expression in this strategy, commonly a series of transduction is required.

Compared to gene expression using viral vectors, CAR expression *via* non-viral-based approaches is typically short-

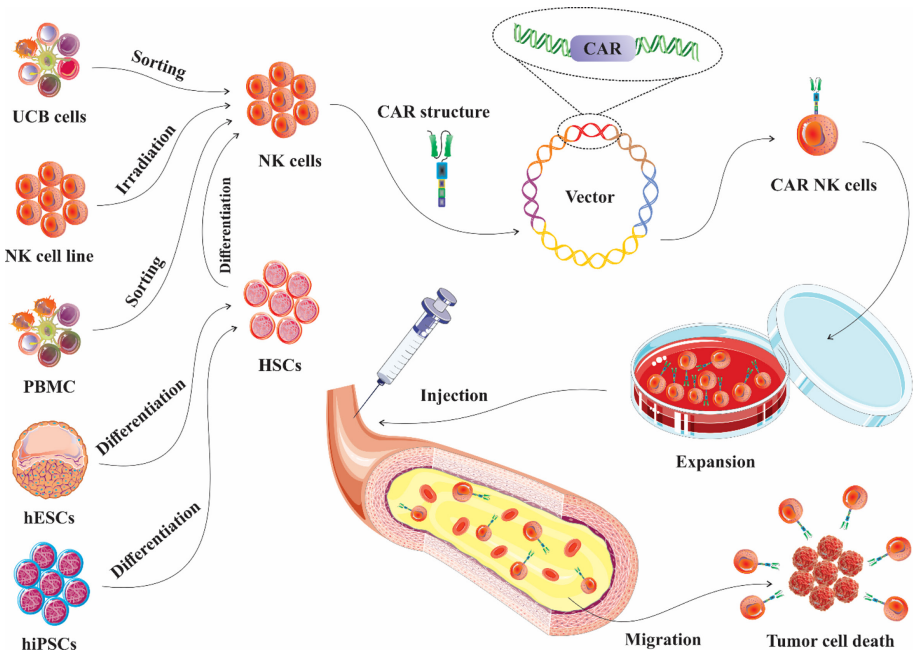

FIGURE 2 | Sources and generation procedure of CAR-NK cells. Isolated or established NK cells from various sources (e.g., PB, UCB, HSCs, hESCs, and hiPSCs) can be activated and genetically modified with CAR-expressing vectors (e.g., lentivirus or retrovirus) and then cultivated in NK cell-specific expansion media with cytokines for GMP-grade clinical use. UCB, Umbilical cord blood; PB, Peripheral blood; hiPSCs, Human induced pluripotent stem cell; hESC, Human embryonic stem cell; HSC, Hematopoietic stem cell; CAR-NK, Chimeric antigen receptor-natural killer cells; GMP, Good manufacturing practice.

lived, and lasts for a few days (64). Though, the permanent expression can be attained when the sequence is integrated through particular systems, such as transposon-based systems (64, 65). Moreover, nucleic acid integration is also carried out *via* electroporation, a well-known simple and cost-effective tactic, providing large-scale clinical applications (66, 67). A foremost drawback of electroporation is that permeabilization of cell membrane by electric pulses may lead to loss of a large number of cells due to formation of enduring membrane leakage (68).

In sum, the insertion of foreign genetic material and succeeding proliferation of NK cells is challenging, thus delaying the advances of feasible and reproducible GMP practices. Accordingly, selection of more appropriate and effective transfection approaches is an influential step for conduction of a successful clinical trial.

CAR-NK CELLS SUPERIORITY OVER CAR-T CELLS

Regardless of the initial success of CAR-T cell therapy, specifically in hematological disorders, its large-scale clinical use is restricted through the individualized preparation and several unwanted effects, encompassing CRS, CNS-related toxicity, and also on-target/off-tumor effects (37). Given these problems, NK cells have been suggested to be superior CAR drivers than T cells. Especially, NK cells pose some benefits to T cells in the context of CAR-based cancer immunotherapy. In this regard, CAR-expressing NK cells seem to be safer than CAR-T cells in clinical application, and NK cell immunotherapy is considered a safe and feasible therapeutic approach, as shown by various clinical trials' outcomes (69). For instance, some phase I/II trials have indicated that allogeneic NK cell administration is well-tolerated and does not result in GVHD and other severe unwanted events (70–72), pointing that NK cells are general CAR drivers without any restriction to autologous cells. Moreover, on-target/off-tumor effects mediated by the persistence of CAR-T cells are chief side effects in CAR-T cell therapy. For example, CD19 CAR-T-cells boost intense and ongoing B-cell deficiency, which may depend on the cellular memory of T cells and activation of either normal mature or progenitor B cells (73). Conversely, the restricted lifespan of CAR-NK cells in the circulation supports limited on-target/off-tumor effects (43). Additionally, there are differences between cytokines established by NK cells and those generated by T cells. Stimulated NK cells typically produce IFN-γ and GM-CSF, while the cytokine storm exerted by CAR-T cells is mostly achieved by pro-inflammatory cytokines (e.g., TNF-α, IL-1 and IL-6) (74, 75). Despite eliminating cancerous cells by a CAR-specific mechanism which involves tumor-related antigen's identification through scFv, NK cells naturally eliminate malignant cells by detecting various ligands through a diversity of activating receptors, such as natural cytotoxicity receptors

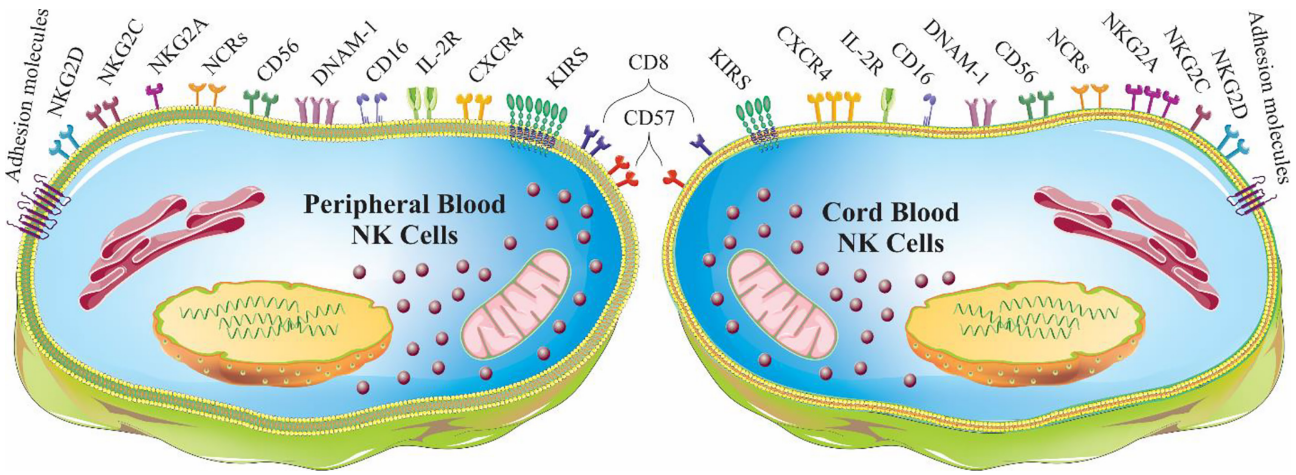

FIGURE 3 | Differences between UCB-NK cells and PB-NK cells phenotypic attribute. UCB-NK cells show lower expression levels of CD16, CD2, CD11a, CD18, CD62L, KIRs, DNAM-1, NKG2C, IL-2R, CD57, and CD8 along with higher expression levels of NKG2A and CXCR4 compared with PB-NK cells. UCB, Umbilical cord blood; PB, Peripheral blood; KIRs, Killer Ig-like receptors; DNAM-1, DNAX accessory molecule-1; NKG2C, NK cell group 2 isoform C; NKG2A, NK cell group 2 isoform A; CXCR4, C-X-C chemokine receptor type 4.

(NKp46, NKp44, and NKp30), NKG2D and DNAM-1 (CD226) (76). Indeed, these receptors typically identify stress-elicited ligands presented on cancer cells following initial exposure with immune cells or lasting treatment throughout tumor development. Also, NK cells facilitate the antibody-dependent cell-mediated cytotoxicity (ADCC) by FcγRIII (CD16) (77). CAR-T cells cannot eliminate malignant cells that are vastly heterogeneous (78); however, CAR-NK cells are capable of efficiently killing residual malignant cells that can modify their phenotypes following lasting treatment.

NK cells, as previously described, are found frequently in clinical samples and can be procured or generated from PB, UCB, hESCs, iPCSs, and even NK-92 cell lines. NK-92 cells deliver a homogeneous cell inhabitant and can be simply cultivated under reliable manufacturing practice standards for wider clinical use, allowing the "off-the-shelf" construction of CAR-NK-92 cells (2). Stimulated PB-NK cells present a broader series of activating receptors and can be infused without irradiation, permitting them to grow *in vivo* (79). NK cells derived from pluripotent stem cells, iPSCs and hESCs, merge the clinical benefits of PB-NK and NK-92 cells because they show a phenotype similar to PB-NK cells and are a homogeneous inhabitant. Notably, CARs are simply expressed in hESC- and/or iPSC-established NK cells *via* nonviral gene transfer strategies (80).

CAR NK CELLS IN SOLID TUMORS

As cited, CAR NK cells re-directed against cancer cells carrying particular antigens, make a chief prospect in the battle against cancer. Modified NK cells can be utilized as general CAR cells in the absence of any requirement for HLA matching or prior exposure to TAAs. Stimulating results from various studies have improved attentiveness in the ground of cancer immunotherapy due to competence of CAR-NK cells in manufacture of "off-the-shelf" anti-tumor immunotherapeutic products (**Tables 1** and **2**).

CAR NK in Neuroblastoma (NB)

Neuroblastoma (NB) is the most mutual extracranial tumor in children with a 5-year mortality rate of ~50% in the high-risk group (110). Though dinutuximab, a monoclonal antibody against ganglioside GD2, has revealed promising capacity to promote overall NB outcomes, it doesn't considerably improve the 5-year overall survival of high-risk patients (111). Currently, it has been suggested that frequency of NK cells may sponsor improved outcomes in NB. Indeed, NK cells hamper tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), eliminate neuroblasts and cancer stem cells (CSCs), and vigorously release cytokines to recruit supplementary immune effectors (112, 113).

NK cells bearing activating receptor NKG2D fused to the cytotoxic ζ-chain of the T-cell receptor (NKG2D.ζ) could target NKG2D ligands-overexpressing MDSCs within the TME (114). Significantly, NKG2D.ζ-NK cells could produce a variety of proinflammatory cytokines and chemokines in response to MDSCs at the tumor area and promote recruitment and anti-cancer activities of subsequently administrated CAR-T cells. *In vivo*, NKG2D.ζ-NK cells produced from patients suffering from NB could eradicate autologous intratumoral MDSCs which regularly hinder CAR-T activities (114). Moreover, Seidel and colleagues found that GD2-CAR-NK-92-scFv (ch14.18)-zeta, in addition to the exertion of remarkable cytotoxicity toward GD2-positive CHLA-20 NB cell line *in vitro*, could elicit a substantial anti-tumor response in a drug-resistant GD2-positive NB xenograft murine model (115). GD2-CAR-NK-92-scFv (ch14.18)-zeta meaningfully improved the median survival rate of the NB xenograft murine model to 52 days, while the median

survival rate of control groups was 30 days. Observations indicated that detection of GD2 by CAR is the principal mechanism contributed to NK-92-scFv (ch14.18)-zeta-achieved killing and is independent of activating NK cell receptor/ligand interactions (115). Also, GD2-NK-92-scFv (ch14.18)-zeta could provide effective detection and eradiation of GD2-positive NB cells that were resistant to parental NK-92. Intensely boosted cytotoxicity of the GD2-specific NK cells toward primary NB cells and GD2-expressing tumor cells of other origins, signify potential clinical application of the redirected NK cells (94).

CAR NK in Glioblastoma (GB)

Glioblastoma (GB) is the most invasive and most shared primary brain cancer identified in adults. It displays a poor prognosis, and existing treatment options are incapable of alleviating its clinical outcome, emphasizing the importance of developing innovative therapeutic strategies (116). Unfortunately, GB microenvironment can suppress immune cell activities *via* varied procedures, most importantly, recruitment of cell modulators. GB immunotherapy consists of diverse immune cells, including dendritic cells, cytotoxic T lymphocytes, and also NK cells (117, 118).

Molecular analysis has evidenced that epidermal growth factor receptor (EGFR) and its mutant form EGFRvIII are commonly overexpressed in GB, and immunotherapy based on EGFRvIII-specific vaccine has resulted in promising outcomes in GB clinical trials (119). Studies have shown that ErbB2 (also called human epidermal growth factor receptor 2 (HER2))-CAR-NK-92/5.28.z cells in contradiction of untargeted NK-92 cells could eliminate ErbB2-positive GB cells *in vitro* (101). Also, significant *in vivo* anti-cancer potential of modified NK cells was detected in orthotopic GB xenograft models in NSG mice, as evidenced by the improvement of median survival of transplanted models with ErbB2-CAR-NK-92/5.28.z cells to 200.5 days compared with 73 days in models treated with parental NK-92 cells (101). Importantly, another study suggested that EGFRvIII-CAR-NK-92 cells only could kill EGFRvIII-positive GB cells, whereas dual-specific NK cells showing EGFR inhibitor cetuximab-based CAR could trigger cytotoxic effects toward both EGFRvIII-positive and -negative

TABLE 1 | Overview of *in vitro* studies based on CAR-NK cell therapy for solid tumors.

Condition	Target Ag	Main results	Ref
Colorectal cancer	EpCAM	Recognition of EpCAM-positive colorectal cancer cells and the secretion of cytokines, such as IFN-γ, perforin, and granzyme B, and showing specific cytotoxicity by EpCAM-CAR-NK-92	(81)
Ovarian cancer	αFR	The elimination of αFR-positive ovarian cancer cells by αFR-CAR-NK-92 cells	(82)
Hepatocellular carcinoma	GPC3	Significant *in vitro* cytotoxicity and cytokine production by GPC3-CAR-NK-92 cells	(83)
liver cancer	c–MET	Remarkable cytotoxicity against HepG2 cells with high c–MET expression by c–MET–CAR–NK cells in comparison with the lung cancer cell line H1299 that demonstrate low rates of c–MET expression	(84)
Breast cancer	EGFR	EGFR-CAR-NK cell activation by TNBC cells resulted in cytotoxicity against these TNBC cells	(85)
Colorectal cancer	CEA	Targeting CEA-positive HCT116 cells and stimulating their elimination by CEA-CAR-NK cells	(86)
Gastric cancer	HER2	The killing of gastric cancer cells expressing HER2 mediated by the promotion of the cytokine releases by HER2-CAR-NK-92	(87)
Pancreatic cancer	Mesothelin	Successful engraftment of mesothelin-CAR-NK-92 cells along with interferon-γ and granzyme B secretion, and specific elimination of pancreatic cancer cell lines	
Breast cancer	HER2	Specific elimination of HER2-expressing tumor cells, and serial target cell killing by HER2-CAR-NK-92 cells	(88)
Glioblastoma	EGFRvIII	Specific elimination of EGFRvIII-positive glioblastoma cells by EGFRvIII-CAR-NK-92 cells	(89)
Lung cancer	NKG2D	Antitumor function against human lung cancer H1299 cells by NKG2D-CAR-NK cells	(90)
Various cancers	HER2	Antitumor function against HER2-positive tumor cells by HER2-CAR-NK-92 cells	(91)
Breast cancer	HER 2	Induction of elimination of HER2–expressing human breast cancer cell lines MDA-MB-453 and SKBr3 by HER2-CAR-NK-92 cells	(92)
Glioblastoma	EGFRvIII	Suppression of glioblastoma cell-growth upon induction of apoptosis by EGFRvIII-CAR-NK-92 cells	(93)
Neuroblastoma	GD2	Effective recognition and elimination of GD2 expressing neuroblastoma cells by GD2-CAR-NK-92 cells	(94)
Breast cancer	EpCAM	Promoted selective cytotoxicity against EpCAM-expressing breast carcinoma cells by EpCAM-CAR-NK-92 cells	(95)
Ovarian cancer	HER2	Specifically activation of HER2-CAR-NK cells following recognition of HER-2 positive tumor cells concomitant with high levels of cytokine release and degranulation	(96)
Glioma and Neuroblastoma	Robo1	The specific cytotoxicity of Robo1-CAR-NK-92 cells against glioma and neuroblastoma accompanied by secretion of a variety of cytokines including IL-6, IL-10, TNF-α and IFN-γ	(97)

EpCAM, Epithelial cell adhesion molecule; αFR, Folate receptor alpha; GPC3, Glypican 3; EGFR, Epidermal growth factor receptor; CEA, Carcinoembryonic antigen; HER2, Human epidermal growth factor receptor 2; NKG2D, Natural killer group 2 member D; Robo1, Roundabout homolog 1; TNBC, Triple-negative breast cancer.

TABLE 2 | Overview of *in vivo* studies based on CAR-NK cell therapy for solid tumors.

Condition	Target Ag	Main results	Ref
Colorectal cancer	EpCAM	Suppression of colorectal cancer growth upon combination therapy with regorafenib and EpCAM-CAR-NK-92 cells in EpCAM-positive tumor xenografts model	(81)
Ovarian cancer	GPC3	The significant therapeutic effect resulted in prolonged survival of the mouse xenograft model of ovarian cancer upon injection of GPC3-CAR-iPSC-NK cells	(98)
Ovarian cancer	αFR	Inhibition of cancer cells growth in a mouse xenograft model of ovarian cancer leading to the knowingly extended survival of tumor-bearing mice by αFR-CAR-NK-92 cells	(82)
Hepatocellular carcinoma	GPC3	Significant anti-tumor activities of GPC3-CAR-NK-92 cells against hepatocellular carcinoma xenografts with both high and low GPC3 expression, as showed by reduced tumor proliferation, and boosted tumor apoptosis	(83)
Ovarian cancer	NKG2D	Significantly improved antitumor activities in mice carrying established peritoneal ovarian cancer xenografts by NKG2D-CAR-NK cells	(99)
Breast cancer	EGFR	Inhibition of breast tumors proliferation in mice models by EGFR-CAR-NK cell	(85)
Hepatocellular carcinoma	CD147	Stimulation of apoptosis by CD147-CAR-NK cells in a human CD147 transgenic mouse HCC model	(100)
Gastric cancer	HER2	Eliminating of small but not larger gastric tumor xenografts by HER2-CAR-NK-92 cells	(87)
Glioblastoma	HER2	Potent *in vivo* antitumor responses of HER2-CAR-NK-92 cells in orthotopic glioblastoma xenograft models in NSG mice	(101)
Pancreatic ductal adenocarcinoma	Robo1	Exerting anti-tumor effects on pancreatic cancer in an orthotopic nude mouse model by Robo1-CAR-NK-92 cells	(102)
Lung cancer	B7-H3	Inhibition of tumor growth in mouse xenografts of non-small cell lung cancer and promotion of survival of transplanted mice by B7-H3-CAR-NK-92 cells	(103)
Lung cancer	NKG2D	Eliciting of cytotoxicity against CD73-positive human lung cancer xenograft models by NKG2D-CAR-NK cells	(104)
Breast cancer Renal cancer	HER2	Specific lysis of tumor cells and anti-tumor functions exerted by HER2-CAR-NK-92 cells in orthotopic breast carcinoma xenografts, and decrease of lung metastasis in a renal cell carcinoma model by HER2-CAR-NK-92 cells	(88)
Glioblastoma	EGFRvIII	Complete tumor remission resulted in promoted survival by EGFRvIII-CAR-NK cells in the murine model	(105)
Breast cancer	HER2	Elimination of HER2-positive tumors, as showed by MRI analysis upon systemic injection of HER2-CAR-NK cells	(106)
Breast cancer	HER 2	Reduction in tumor volume and lung metastasis of nude mice bearing established MDA-MB-453 cells upon injection of HER2-CAR-NK-92 cells	(92)
Hepatocellular carcinoma	NKG2D	Inhibition of tumor growth in a hepatocellular carcinoma xenograft tumor model by NKG2D-CAR-NK-92 cells	(107)
Glioblastoma	EGFRvIII	Inhibition of tumor growth and promoted survival rate of the orthotopic glioblastoma xenograft mouse models following intracranial injection of EGFRvIII-CAR-NK-92 cells	(108)
Glioblastoma	HER2	Eliciting of endogenous antitumor immunity upon treatment with HER2-CAR-NK-92 cells in glioblastoma xenograft mouse models	(109)

EpCAM, Epithelial cell adhesion molecule; αFR, Folate receptor alpha; GPC3, Glypican 3; EGFR, Epidermal growth factor receptor; HER2, Human epidermal growth factor receptor 2; NKG2D, Natural killer group 2 member D; Robo1, Roundabout homolog 1; iPSCs, Induced pluripotent stem cells.

GB cells *in vitro* (89). *In vivo*, local application of dual-specific NK cells showed superiority over treatment with the corresponding monospecific CAR NK cells in xenografts GB models, supporting promoted survival without triggering fast immune escape as commonly detected following treatment with monospecific effectors (89). Moreover, further modification of EGFRvIII-specific NK cells with the chemokine receptor CXCR4 could provide particular chemotaxis to CXCL12/SDF-1α-producing U87-MG GB cells, and consequently improve survival of xenografts compared to treatment with NK cells expressing only EGFRvIII-specific CAR (105). Interestingly, EGFR-CAR-NK cells have shown more prominent cytolytic competence and IFN-γ secretion succeeding co-culture with GB cells or patient-derived GB stem cells, thus eliciting inhibition of tumor development leading to the promoted tumor-bearing mice survival in orthotopic GB xenograft models (108).

CAR NK in Liver Cancers

The great introduction of antigens makes the liver a significant immunological organ whose exclusive microenvironment forms both innate and adaptive immune reactions for supporting a precise balance between immune tolerance and immune activation. Deregulation of immune responses in the liver is responsible for the pathogenesis of various hepatic diseases, containing viral hepatitis, autoimmune disorders as well as tumors (120). The liver immune system includes varied innate effectors, including NK cells, NKT cells, gamma delta (γδ) T cells, and adaptive lymphocytes, such as αβ T cells and B cells. The probability of the modification of NK cell activities has recently been introduced as an innovative treatment option for liver disorders, as proved for infections and tumors (121).

Recently, glypican-3 (GPC3) has been described as a logical immunotherapeutic target for hepatocellular carcinoma (HCC). GPC3-specific NK-92/9.28.z cell treatment could lead to substantial *in vitro* cytotoxicity and cytokine generation against HCC cells (83). As well, modified NK-92/9.28.z cells were capable of induction of cytotoxicity in multiple HCC xenografts with either high or low GPC3 expression, but not GPC3-negative models. Potent infiltration of NK-92/9.28.z cells reduced the tumor development along with boosted tumor cell eradication in the GPC3-positive HCC xenografts, which suggested clinical efficacy of GPC3-specific NK-92/9.28.z cell in HCC (83). Moreover, evaluating the specificity and efficiency of c–MET–specific–NK cells against human c–MET–positive HepG2 revealed that c–MET–CAR–NK cells induced more specific cytotoxicity against HepG2 cells with high c–MET

expression than H1299 cells, a human lung cancer cell line with low c–MET expression, suggesting that c–MET could be a rational target for CAR–NK immunotherapy in liver cancer (84). In another study, T and NK cells transduced with a CD147-specific CAR could efficiently eliminate several HCC cell lines *in vitro* and HCC tumors in xenograft murine models. The use of logic-gated (log) GPC3-synNotch-inducible CD147-CAR for reducing on-target/off-tumor toxicity in HCC showed that LogCD147-CAR could selectively eliminate dual antigen (GPC3-positive CD147-positive), but not single antigen (GPC3-negative CD147-positive) positive HCC cells without any serious on-target/off-tumor toxicity in a human CD147 transgenic murine model (100). Besides, respecting the TGF-β capability to inhibit NK cell function, Wang and his coworkers genetically modified NK-92 cells to present a chimeric receptor with TGF-β type II receptor extracellular and transmembrane domains associated with intracellular domain of NKG2D, termed as TN chimeric receptor. *In vitro*, NK-92 cells expressing TN receptors displayed potent resistance against TGF-β-elicited inhibitory signaling, and triggered higher cytolytic competence and IFN-γ secretion toward tumor cells (107). More excitingly, NK-92 cells presenting TN receptors demonstrated a higher infiltration rate to tumors expressing TGF-β, and also reserved the differentiation of human naïve CD4-positive T cells to regulatory T cells. Also, NK-92-TN cell infusion resulted in suppressed tumor development in an HCC xenograft murine model, thereby implying that these chimeric receptors could be utilized to increase anti-cancer efficacy in NK cell adoptive therapy (107).

CAR NK in Breast Cancers

The growth of breast tumors is a complicated procedure comprising several cell types. HER2 has been introduced as a pivotal oncogene in breast cancer, which its activation is mediated mainly *via* gene amplification and re-arrangement (122–124). Overexpression of HER2 usually is detected in about 20% of primary breast cancers accompanying by poor prognosis and mainly promote CSCs proliferation through PTEN/Akt/mTORC1 axis (125).

In vivo, infusion of NK-92-scFv (FRP5)-zeta cells presenting HER2-specific CAR resulted in the elimination of HER2-expressing human breast tumor cells (126). As well, investigation of the capacity of focused ultrasound (FUS) to deliver targeted NK-92 cells to the brain using a model of metastatic breast cancer verified FUS capabilities to augment the targeting of iron-loaded immune cell therapy of brain metastases, as shown by MRI test 16 h following treatment (126). Moreover, studies delivered proof of the concept that EGFR-CAR-NK cells could be applied for treating patients suffering from triple-negative breast cancer (TNBC) displaying heightened EGFR expression. EGFR-CAR-NK cells could stimulate potent cytotoxicity against TNBC cell lines, HS578T, MDA-MB-468 and MDA-MB-231 cells, with upregulated EGFR expression and selectively stimulated lysis of these cells *in vitro* (85). EGFR-CAR-NK cells co-cultured with TNBC cells showing promoted EGFR expression produced greater rates of IFN-γ, granzyme B and perforin compared to EGFR-CAR-NK cells in co-culture condition with the MCF7 cells, a non-TNBC cell line. Moreover, there was a consistency between levels of cytokine generation by modified NK cells and EGFR expression by experimental cell lines (85). Similarly, modified NK cells inhibited the tumor growth in breast cancer cell line-derived xenograft (CLDX) and patient-derived xenograft (PDX) murine models (85). Besides, genetically modified NK cells to identify a prominent surface antigen expressed in 50–85% of patients with TNBC, tissue factor (TF), demonstrated significant efficacy *in vivo* for the treatment of mouse models of orthotopic CDX and PDX. This was evidenced with a striking reduction in tumor weight between control and treatment groups without any significant change in mice body weight. *In vitro*, the analysis proposed that though TF-CAR-NK cells could kill TF-positive MDA-MB-231 cells, their efficacy could be improved when used in combination with TF-targeting therapeutic antibody-like immunoconjugates, such as L-ICON1 (127). As well, Sahm et al. found that effector NK-92 cells which co-expressed epithelial cell adhesion molecule (EpCAM), a type I transmembrane glycoprotein identified as a TAA—specific CAR and IL-15 could proliferate without exogenous cytokines *in vitro* and exhibited potent and specific cell-killing functions against EpCAM-expressing breast carcinoma cells those were resistant to unmodified NK cells-induced cytotoxicity (95). In another research, combinatorial treatment with EGFR-CAR-NK-92 cells and oncolytic herpes simplex virus (oHSV) elicited improved cytolytic functions and IFN-γ generation when co-cultured with breast cancer cell lines MDA-MB-231, MDA-MB-468, and MCF-7 in comparison to the mock-transduced NK-92 cells. As well, intratumoral injection of either EGFR-CAR-NK-92 cells or oHSV-1 abrogated tumor development, while combination of EGFR-CAR NK-92 cells with oHSV-1 led to more effective elimination of MDA-MB-231 tumor cells compared to monotherapies in MDA-MB-231 cell bearing mice (128).

CAR NK in Gastrointestinal (GI) Cancers

The particular activity of NK cell in gastrointestinal (GI) cancer was firstly revealed by a retrospective study with an 11-year follow-up showing that infiltration and cytotoxicity of NK cells have a tight association with cancer risk, thus suggesting an efficient role of NK cell in tumorigenesis (129). Then in colorectal cancer (CRC), through evaluating NK cells in the TME and peripheral blood, the lower frequencies of NK cell were concluded to be allied with a promoted risk of both cancer incidence and progress with poor prognosis (130).

Recent findings revealed that NKG2D-CAR adoptive NK cell therapy could increase the cytolytic activity of effector cells toward CRC cell lines *in vitro* and deliver therapeutic advantages to mice with CRC (131). Moreover, intraperitoneal infusion of NKG2D CAR mRNA-engineered NK cells to three participants with metastatic CRC verified its safety and efficacy in two of them. Respecting the results of Doppler ultrasound imaging, fast tumor deterioration was proven in the liver area, thereby pointing to a capable therapeutic competence of using RNA CAR-modified NK cells for treating metastatic CRC (131). On the other hand, HER2-CAR NK-92 cells significantly eliminated HER2-positive gastric cancer cells mediated by

advanced levels of cytokine generation *in vitro*. *In vivo*, effector cells could eradicate small tumor xenografts, while larger gastric tumors were not significantly affected by HER2-CAR NK-92 cells (87). Nevertheless, NK cells infiltration into large tumor xenografts and their therapeutic capacity were promoted following infusion in combination with apatinib, a tyrosine kinase inhibitor that exclusively suppresses the vascular endothelial growth factor receptor-2 (VEGFR2) (87). Further, despite the release of a variety of cytokine (e.g., IFN-γ, perforin and granzyme B) and cytotoxicity induced by EpCAM-specific CAR-NK-92 cells against EpCAM-positive CRC cells *in vitro*, synergistic influences of multi-kinase inhibitor regorafenib and modified CAR-NK-92 cells were supported in a murine model with human CRC xenografts. Accordingly, combination therapy with regorafenib and CAR-NK-92 cells showed superiority over monotherapy with CAR-NK-92 cells or regorafenib in terms of inhibition of CRC growth in xenografts (81). Other *in vitro* studies proposed that anti- carcinoembryonic antigen (CEA)-CAR NK-92MI cells selectively recognized and eliminated high CEA-expressing CRC tumor cell lines (LS174T); without any cytolytic effects on low CEA-expressing tumor cells (HCT116) (86). Interestingly, anti-CEA-CAR NK-92MI combination therapy with either histone deacetylase-inhibitor sodium butyrate (NaB) or the methylation-inhibitor 5-azacytidine (5-AZA) caused selective killing of HCT116, which imply the clinical importance of epidrugs for prompting CAR-NK cell therapeutic efficacy in human CRC (86).

CAR NK in Ovarian Cancers

Rendering findings, immunotherapy could be an effective therapy for ovarian cancer (OC), as OC is an immunogenic disorder with existence of T and NK cell infiltration in the TME. Remarkably, presence of tumor-infiltrating CD3-positive T cells directly associates with survival in OC patients (132). Additionally, CD103-positive tumor-infiltrating NK cells usually co-infiltrate with CD8-positive CD103-positive T cells, while the involvement of NK cells in promoting outcome is hard to evaluate (133). Furthermore, *ex vivo*-cultivated PB-NK cells of OC patients seem to be cytotoxic toward autologous primary OC cells (134).

Recently, *in vivo* study in OC xenograft models has revealed that NKG2D-CAR- iPSC-derived NK cells with 2B4 co-stimulatory domain and CD3ζ signaling domain could exert robust cytotoxicity against cancerous cells. Meanwhile, NKG2D-CAR-iPSC-NK cells displayed *in vivo* function similar to NKG2D-CAR-iPSC-T cells, while showing less toxicity (135). As well, GPC3-CAR-iPSC-NK cells could produce higher levels of IFN-γ against GPC3-expressing tumor cells *in vitro*, and also stimulated substantial therapeutic effect in OC murine models, as supported by prolonged survival of these models compared with the control group. Importantly, infusion of modified cells into immunodeficient mice caused no acute systemic toxicity or tumorigenicity (136). Besides, mesothelin (MSLN)-CAR NK92 cells selectively eliminated MSLN-positive OC cells (OVCAR-3 and SK-OV-3), rather than MSLN-negative cells (SK-HEP-1), *in vitro*. On the other hand, effector NK cells significantly killed OC cells in both subcutaneous and intraperitoneal tumor models resulted in improvement of survival of intraperitoneally tumor-bearing mice (137). Also, CD24-CAR-NK-92 cells presented great cytolytic functions towards CD24-positive OC cell lines (SKOV3, OVCAR3), but not CD24-negative cell lines (A2780, HEK-293T), suggesting for the first time that anti-CD24-CAR will be assessed in the future clinical trials as an interesting immunotherapeutic strategy against OC (138). The importance of CD24, as a small sialoglycoprotein commonly localized in lipid rafts through its glycosylphosphatidylinositol (GPI) anchor, in OC therapy relies on its influential role in development, invasion and metastasis of OC cells through targeting a variety of signaling axis, in particular, Akt and ERK pathways (139). Moreover, Klapdor et al. cited that CD33-CAR-NK-92 cells selectively eliminated CD33-positive OC cells *in vitro* mainly achieved by IFN-γ secretion. They also showed that NK cells retain their cytolytic competencies under cisplatin treatment and, prominently, successive treatment with cisplatin followed by CAR-NK cells resulted in the strongest killing effect, representing an encouraging strategy to prevent recurrent disease (140).

CAR NK in Other Cancers

There are evidences indicating that intratumoral and intraperitoneal delivery of GD2-specific CAR-NK cells could not eradicate GD2-expressing cells in Ewing sarcomas (EwS) xenografts possibly sustained by upregulation of the immunosuppressive ligand HLA-G by tumor cells (141). These finding signify that HLA-G is a candidate immune checkpoint in EwS involved in stimulating resistance to NK cell therapy (141). Conversely, ErbB2-CAR-NK cells established robust cytotoxicity against ErbB2-positive sarcoma cells in 3D tumor spheroids (142). Similarly, ErbB2-CAR-NK cells selectively activated and eliminated ErbB2-positive melanoma cells, as shown through high levels of cytokine generation and degranulation, in both *in vitro* and in recombination activating gene 2 (Rag2) knockout mice (96). Likewise, MSLN-specific CAR-NK-92MI cells selectively eliminated pancreatic cancer lines *in vitro* by secreting IFN-γ and granzyme B (143), and also Roundabout homolog 1 (Robo1)-specific CAR-NK cells demonstrated great cytotoxicity against pancreatic ductal adenocarcinoma (PDAC) in an orthotopic nude mouse model (102). It has been suggested that NK cells engineered to express a PD-L1 specific CAR could eradicate human and murine head and neck cancer cells at low effector-to-target ratios in a PD-L1-dependent manner (144). Furthermore, EGFR-CAR-NK-92 cells displayed synergistic therapeutic efficacy with cabozantinib toward human renal cell carcinoma (RCC) xenograft models as cabozantinib could improve EGFR and attenuate PD-L1 membrane surface expression in RCC cells. This data provided the proof of concept that combination therapy using chemotherapeutic agents and CAR-modified NK cells is an operational approach for treating solid tumors (145).

CHALLENGES OF CAR-NK CELLS THERAPIES IN HUMAN SOLID TUMORS

To date, several clinical trials have been designed and conducted based on CAR-NK cell therapy for human solid tumors (**Table 3**); however, the encouraging consequences displayed in CAR NK cell therapy of hematological malignancies have not yet

TABLE 3 | A brief overview of clinical trials in the context of the CAR NK cell-based therapy for human solid tumors registered in ClinicalTrails.gov (March 2021).

Diseases	Target Ag	Intervention Models	Participant Number	Study Phase	Study Location	Dose	Status	Masking	NCT number
Castration-resistant prostate cancer	PSMA	Sequential Assignment	9	Early 1	China	0.5–3 × 10^7/kg	Not yet recruiting	Open-Label	NCT03692663
Various cancers	Robo1	Single Group Assignment	20	1/2	China	N.A	Recruiting	Open-Label	NCT03940820
Ovarian cancer	Mesothelin	Single Group Assignment	30	Early 1	China	0.5–3 × 10^7/kg	Not yet recruiting	Open-Label	NCT03692637
Metastatic solid tumors	NKG2D	Single Group Assignment	30	1	China	N.A	Unknown	Open-Label	NCT03415100
Neuroblastoma	GD2	Single Group Assignment	0	1	USA	3 × 10^6–1 × 10^8/kg	Withdrawn*	Open-Label	NCT02439788

NKG2D, Natural killer group 2 member D; Robo1, Roundabout homolog 1; PSMA, Prostate-specific membrane antigen; N.A., Not available.
**Researcher's explanation: "Based on newly available preclinical data we changed the CAR construct to a more effective version and will now study that product on a different protocol".*

been shown in solid tumors due to tumor heterogeneity concomitant with the hostile TME (146). For instance, CD19 is vastly and homogenously presented on the surface of transformed B cells in a variety of hematological disorders, and CD19-specific effectors do not face large anatomical barricades in the blood before establishing communication with their targets. However, CAR-NK cells must transfer in the bloodstream and then migrate into the tissue to finally shape interaction with the tumor cells while combating several suppressive molecules in the TME (147). Nonetheless, it seems that local infusion, intra-peritoneal infusion, and FUS-guided delivery of CARs into tissues can defeat the anatomical hurdles faced by the CAR-NK cells in solid tumors (148). CAR NK cell therapy appears more favorable in breast, ovarian, and prostate cancers compared with other forms of solid tumors as they are simply and safely available and devastation of the normal tissue can be tolerated (88, 149). Besides, in solid tumors, TAAs commonly are presented by both cells in the tumor and also in pivotal organs, making it difficult to evade "on-target, off-tumor" effects (42). On the other hand, though *in vitro* cultivated NK cells show remarkable cytotoxicity toward transformed cells, they lose this capability following administration *in vivo* due to existence of immunosuppressive molecules such as TGF-β, IL-10, PD-1, or arginase, produced by neutrophils, macrophages and Tregs in the TME (148). Tregs and immunosuppressive MDSCs are vigorously infiltrated into the TME, wherein they shape a robust immunosuppressive environment encouraging tumor development (150). Currently, some reports evidenced that injection of TGF-β kinase inhibitors in combination with NK cells conserves the cytotoxic capacity and expression of activating NK receptors NKG2D and CD16 (151).

Also, transformed malignant cells escape immune surveillance by expression of checkpoint proteins averting immune responses. For instance, TIGIT moderates NK cell cytolytic activities by opposing CD226 (152), and also PD-1-positive NK cells demonstrate diminished proliferation and effector functions, while PD-L1-positive cells own improved effector activities (153). Accordingly, combining CARs with inhibitors of checkpoint proteins including PD-1, CTLA-4, LAG3 and TIGIT can support desired outcomes in human solid tumors (127).

CONCLUSION

Current developments in gene manipulation systems have permitted construction of novel CAR-NK cell products with effective anti-cancer influences, without toxicity against normal tissues. Manifold approaches such as CRISPR-based gene manipulation and introduction of novel genes to modify tumor microenvironment in CAR structure can result in noteworthy achievements in this regard. With the improving safety and sustaining cytotoxicity in preclinical reports and clinical studies accompanying advanced efforts to address existing hindrances, it seems that CAR-NK cell therapy can lead to auspicious therapeutic outcomes in the clinic. Taken together, we believe promoting CAR construction for ideal NK cell functions and cytotoxicity, boosting CAR-NK cell infiltration into TME, modifying these effector cells to defeat tumor inhibition and escape, generating CAR-NK cells with memory possessions *in vivo* for enduring tumor's surveillance, and also improving CAR-elicited selective killing are of paramount importance.

AUTHOR CONTRIBUTIONS

FM, HS, MM, AH, AA-A, AM, WA, YE, and MC drafted the main text, figures, and tables. MJ supervised the work and provided the comments and additional scientific information. FM and AH also reviewed and revised the text. All authors contributed to the article and approved the submitted version.

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