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Harnessing NK cells for cancer immunotherapy: immune checkpoint receptors and chimeric antigen receptors

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Running Title: Boosting NK cells based on ICRs and CARs

Keywords: natural killer cells, immune escape, immune checkpoint receptors, chimeric antigen receptors, cancer immunotherapy

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ABSTRACT

Natural killer (NK) cells, key antitumor effectors of the innate immune system, are endowed with the unique ability to spontaneously eliminate cells undergoing a neoplastic transformation. Given their broad reactivity against diverse types of cancer and close association with cancer prognosis, NK cells have gained considerable attention as a promising therapeutic target for cancer immunotherapy. NK cell-based therapies have demonstrated favorable clinical efficacies in several hematological malignancies but limited success in solid tumors, thus highlighting the need to develop new therapeutic strategies to restore and optimize anti-tumor activity while preventing tumor immune escape. The current therapeutic modalities yielding encouraging results in clinical trials include the blockade of immune checkpoint receptors to overcome the immune-evasion mechanism used by tumors and the incorporation of tumor-directed chimeric antigen receptors to enhance NK cell anti-tumor specificity and activity. These observations, together with recent advances in the understanding of NK cell activation within the tumor microenvironment, will facilitate the optimal design of NK cell-based therapy against a broad range of cancers and, more desirably, refractory cancers.

INTRODUCTION

Immune checkpoint receptors, such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), have recently emerged as molecular targets for cancer immunotherapy (1). Even before the investigation of the importance of such receptors in T cells (2, 3), the foundation of NK cell immunology was established by determining the quintessential roles of killer cell immunoglobulin-like receptors (KIRs), the inhibitory receptors in human NK cells. In 1986, Kärre *et al*. proposed the "missing-self" hypothesis (4). NK cells kill major histocompatibility complex (MHC) class I-deficient tumor cells but fail to kill MHC class I-expressing tumor cells. The recognition of "missing-self" MHC class I is mediated by KIRs in humans and Ly49s in mice. Later, S. Kim *et al*. suggested that "licensing" of NK cells is also mediated by the interaction between Ly49s and MHC class I (5). Licensing is a host MHC class I-dependent functional maturation process. Only NK cells that are licensed by self-MHC class I molecules during development are fully functional. Licensing also occurs in human NK cells (6). Thus, immune checkpoint receptors are fundamental for determining NK cell functionality. Nonetheless, NK cells express multiple immune checkpoint receptors, including natural killer group 2A (NKG2A), CTLA-4, PD-1, T cell immunoglobulin mucin 3 (TIM-3), and T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT), which have been explored as promising therapeutic targets to enhance the specificity and activity of NK cells against a broad range of cancers.

Another promising cancer treatment modality that has raised considerable interest is the incorporation of tumor-directed chimeric antigen receptors (CARs) in immune effector cells. The clinical success of Kymriah® and Yescarta®, two CAR-T cell therapies targeting hematologic malignancies, is sure to promote the growth of CAR-T cell therapies in clinical

trials, thereby treating a range of cancers. Nevertheless, the limitations of CAR-T cell therapies, in terms of off-the-shelf utility, safety, and target antigen escape, necessitate alternatives. With an array of innate receptors responding to cellular transformation, NK cells can efficiently kill a range of tumor cells without MHC restriction, thereby complementing MHC-restricted tumor lysis by cytotoxic T cells. With radical differences in tumor cell recognition, cytokine production profile, and *in vivo* persistence, NK-CAR cell therapies are viewed as an attractive alternative or complement to CAR-T cell therapies, as they potentially overcome several clinical challenges presented by CAR-T cell therapies. In this review, we summarize recent advances in NK cell-based cancer immunotherapy with a focus on immune checkpoint receptors, some of which are unique to NK cells and CAR-NK cells.

IMMUNE CHECKPOINT RECEPTORS

KIR, LIR, and CD94/NKG2A

Inhibitory KIRs, *i.e.* 2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 3DL2, and 3DL3, have long cytoplasmic tails comprising two ITIMs (7). Inhibitory KIRs recognize human leukocyte antigen (HLA)-A, B, or C (Figure 1). The main roles of KIRs in NK cells are described above. They have highly polymorphic immunoglobulin (Ig) domains specific to HLA molecules (8). Multiple myeloma (MM) cells upregulate MHC class I molecules, thus blocking inhibitory KIRs could enhance the antitumor effect of NK cells in MM. Treatment with anti-KIR2D antibody (Ab) (Lirilumab; IPH2102) has been proven safe without mediating toxicity and autoimmunity in patients with MM and acute myeloid leukemia (AML) (9, 10). Anti-KIR Ab treatment enhanced *ex vivo* NK cytotoxicity in patients with MM, but the Phase II clinical trial of lirilumab in MM resulted in failure due to lack of efficacy and presumably a loss of KIR2D expression in NK cells (11). Anti-KIR Abs have

been tested alone or in combination with other therapeutics, including lenalidomide, anti-CD20 Ab (rituximab), and immune checkpoint blockades in various hematological disorders, including MM, lymphoma, and myelodysplastic syndromes (12-15). The latest addition to this group is lacutamab (IPH4102), a first-in-class anti-KIR3DL2 Ab. It has been demonstrated to be safe, and 36% of patients with relapsed/refractory cutaneous T cell lymphoma responded to it in a Phase I trial (16). Apart from immune checkpoint blockade, pre-treatment with IL-12/15/18 reduces the expression of KIRs in NK cells and enhances NK cytotoxicity against tumor cells (17), suggesting that *ex vivo* expanded NK cells could be potent anti-tumor therapeutics by themselves or as CAR bearers. Clinical trials using immune checkpoint blockade are summarized in Table 1.

Among leukocyte immunoglobulin-like receptors (LIRs), LIR-1, also known as LIR subfamily B member 1 (LIRB-1), immunoglobulin-like transcript 2 (ILT2), and CD85j, recognizes HLA-G, a non-classical MHC class I molecule. LIR-1 contains ITIM motifs to recruit phosphatases, such as SHP-1 (18). HLA-G is expressed in various tumors and is often associated with reduced NK function or progressive tumors (19). Soluble HLA-G (sHLA-G) also plays a role in mediating regulatory function in some tumors, such as thyroid and colorectal cancers (20, 21). Blocking LIR-1 alone did not enhance the cytotoxicity of NK cells against MM cells (22), but a dual blockade of LIR-1 and NKG2A increased the cytotoxicity of KIR^- NK cells against acute leukemic cells *in vitro* (23). NKG2A is well known as an HLA-E receptor but has recently been suggested as an HLA-G receptor (24). However, the action mechanisms for the dual blockade may require further investigation.

CD94/NKG2A is a heterodimeric inhibitory receptor related to C-type lectins, recognizing another non-classical MHC class I molecule, HLA-E. ITIMs are phosphorylated upon receptor engagement and recruit tyrosine phosphatases SHP-1 and SHP-2 (25, 26).

SHP-1 mediates dephosphorylation of Vav1 (27). In addition, Crk phosphorylation contributes to the inhibition of NK cells through NKG2A-HLA-E interaction (28). ITIM-based inhibition appears to be dominant over activation in NK cells against normal cells. Recruitment of SHP-1 by MHC-I-specific ITIM-bearing receptors inhibited signaling at a proximal step, such that most downstream signals were prevented (29). HLA-E is overexpressed in human colorectal cancers with poor prognosis (30). Ovarian and cervical cancer cells express HLA-E that limits $NKG2A^{+}$ cytotoxic T cells, thereby resulting in less infiltration of NK cells in HLA-E-expressing gynecological cancer (31). In addition, $NKG2A^{-}NKG2C^{+}KIR^{+}CD56^{dim}$ NK cells are suggested as memory-like NK cells in patients with human cytomegalovirus infection (32). An anti-NKG2A Ab (monalizumab; IPH2201) ameliorates NK cell dysfunction in chronic lymphocytic leukemia (33). Monalizumab is currently under clinical investigation as a single agent in ovarian cancer or in combination with cetuximab (anti-EGFR Ab) and durvalumab (anti-PD-L1 Ab) for advanced stage solid cancers (34, 35). Interim results of a Phase II trial of monalizumab and cetuximab in previously treated squamous cell head and neck cancer showed a 31% objective response rate, where monalizumab improved anti-tumor immunity of T and NK cells (36). A combination of monalizumab and durvalumab demonstrated clinical efficacy and manageable toxicity in a Phase I trial of heavily pretreated metastatic microsatellite colorectal cancer (19). However, NKG2A blockade reportedly works through CD8 T cells rather than NK cells in mouse models that are set to block NKG2A/Qa-1b interaction using HPV16 E6 and E7-expressing tumors (37). Taken together, NKG2A blockade appears to be a promising immune-oncological therapeutic that promotes T and/or NK cell activation. Notably, NKG2A can recognize HLA-G as well (24), thereby suggesting the previously unexpected benefit of NKG2A blockade in tumor immunity.

CTLA-4 and PD-1

CTLA-4 plays a pivotal role in T cell expansion, whereas PD-1 is a central regulator of T cell effector function. CD80 (B7-1) and CD86 (B7-2) are the common ligands for the costimulatory receptor CD28 as well as the co-inhibitory receptor CTLA-4. However, CTLA-4 binds to ligands with greater affinity than CD28. Despite the absence of inhibitory ITIM, CTLA-4 inhibits the activation of Akt but not PI3K via activating the serine/threonine phosphatase PP2A (38). Engagement of CTLA-4 with CD80 leads to the reduction in IFN-γ production by mouse activated NK cells against mature dendritic cells (39). In head and neck cancer, CTLA-4 is upregulated on Treg cells that suppress NK cell anti-tumor cytotoxicity (40). In melanoma, anti-CTLA-4 treatment leads to Fc receptor-mediated selective depletion of Treg cells (41, 42). Moreover, clinical outcome of CTLA-4 therapy in melanoma is associated with the increased population of mature circulating $CD3^{-}CD56^{dim}CD16^{+}$ NK cells (43). Thus, anti-CTLA-4 therapy may enhance anti-tumor cytotoxicity of NK cells in both a direct and indirect manner such as depletion of $CTLA\text{-}4^{+}$ Treg cells. Triple immunotherapy with anti-CTLA4 antibodies, monophosphoryl-lipid-A, and indolamine-dioxygenase-1 inhibitor has been reported to enhance NK cell counts and the $CD3^{+}CD4^{+}$/Treg and $CD3^{+}CD8^{+}$/Treg ratios, in addition to the reduction in tumor mass, in a murine melanoma model (44). Combination therapies could provide additional benefits, although the B7/CTLA-4 axis may not play a key role in NK cell activation (45, 46).

PD-1 has one ITIM and one immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic domain. Specifically, the ITSM tyrosine (Y248) of PD-1 is known to recruit phosphatase SHP-2, which is mandatory for PD-1-mediated inhibition of the PI3K/Akt pathway (47). The cognate ligands for PD-1 are PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-1

expression is found on $CD56^{dim}NKG2A^{-}KIR^{+}CD57^{+}$ mature NK cells, but not on $CD56^{bright}$ NK cells (48). In ovarian cancer and Kaposi sarcoma, PD-1 expression is elevated on NK cells and associated with impaired NK cell function (49, 50). PD-1^{+} NK cells are considered to be functionally exhausted (32). Blockade of PD-1 enhances cytotoxicity of NK cells against autologous MM cells (51). In Hodgkin lymphoma and diffuse large B-cell lymphoma, PD-1 blocking also reverses the suppression of PD-1^{+} NK cells mediated by tumor-associated macrophage-like monocytes (52). In mice, tumor-infiltrated NK cells express PD-1, which suppresses NK cytotoxicity (53). PD-1/PD-L1 blockade, PD-1/PD-L1 genetic deficiency, or NK cell depletion prevents lung metastasis in a B16 melanoma model and tumor growth in a murine model using CT26 colon tumor cells and a breast cancer orthotopic model using 4T1 cells *in vivo* (53). However, activated human primary NK cells efficiently killed colorectal cancer cells in organoid culture independently of PD-L1 expression (54), and blockade of PD-L1 failed to increase cytotoxicity of human liver-associated NK cells against hepatocellular carcinoma cell lines *in vitro* (55). In line with these results, PD-1 is expressed only minimally in mouse and human NK cells in various infections and tumor models (56). Nevertheless, PD-1 expression in murine NK cells can be induced in the spleen and liver by glucocorticoid (57), and anti-PD-1 blockade can induce cytokine production, such as IFN-γ, which may boost NK cells indirectly (34).

Four ongoing clinical trials are evaluating the combined effect of infused NK cells and anti-CTLA-4, PD-1, or PD-L1. They are induced pluripotent stem cell (iPSC)-derived NK cells combined with nivolumab or pembrolizumab (NCT03841110), cytokine-induced memory-like NK cells and ipilimumab (NCT04290546), unmodified allogeneic NK cells and pembrolizumab (NCT03937895), and autologous NK cells combined with avelumab or pembrolizumab (NCT03941262).

TIM-3, CEACAM1, TIGIT, and LAG-3

TIM-3, whose cognate ligands are galectin-9 (Gal-9), phosphatidylserine, high mobility group box 1 (HMGB1), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1), does not have a classical signaling motif, but five conserved tyrosine residues (58). In particular, phosphorylated Tyr256 and Tyr263 are required for the Gal-9-mediated BAT3 release from TIM-3 and inhibitory signaling (59). Gal-9 and HMGB1 can be soluble as well as membrane-bound. TIM-3 is regarded as a marker for mature NK cells and TIM-3^+PD-1^+ NK cells are considered to be functionally exhausted (60). The expression of TIM-3 is elevated on peripheral NK cells in patients with advanced gastric cancer (61) and lung adenocarcinoma (62). It is also upregulated on the tumor-infiltrated NK cells in over 70% of patients with gastrointestinal stromal tumors (63). Interestingly, PD-1 expression is not found on the TIM-3^+ tumor-infiltrated NK cells. Anti-TIM3 treatment rescues TIM-3^+ exhausted NK cells from patients with advanced melanoma (64). Further, TIM-3 expression levels are correlated with the stage of the disease. Several anti-TIM-3 Abs are to be tested in Phase I or II clinical trials: TSR-022 by Tesaro, LY3321367 by Eli Lilly, MGB453 by Novartis, Sym023 by Symphogen, and BGB-A425 by Beigene (65, 66). The antibodies are often applied in combination with anti-PD-1 or anti-LAG-3 Abs in advanced solid tumors or AML. The studies are still recruiting patients, and results will be available in a few years. However, caution is warranted as the blockade of TIM-3 leads to the reduction in NK cell-mediated cytolysis of pancreatic cancer cell lines (67). Moreover, Ab-mediated Gal-9 blocking leads to a decrease in IFN-γ production in NK cells in response to primary AML blasts (68), thereby complicating the outcomes of TIM-3 blocking.

Interestingly, CEACAM1 is also expressed in NK cells and interacts with CEACAM5 (69). Recently, NEO-201, a monoclonal antibody (mAb) specific to the CEACAM family was demonstrated to enhance NK cytotoxicity against various human tumor cells through CEACAM5 on tumor cells and CEACAM1 on NK cells *in vitro* (70) and *in vivo* (71). CEACAM1-expressing NK cells produce IFN-γ by IL-12/18 in a mouse hepatitis virus infection model and the engagement of CEACAM1 was demonstrated to decrease the production IFN-γ (72). CEACAM1 on activated NK cells inhibits NKG2D-mediated cytolytic function and signaling (73), suggesting that CEACAM1 is an immune checkpoint receptor in NK cells, as well as a cognate ligand to TIM-3. Human CEACAM1 protein has a signaling cytoplasmic domain comprising either a long ITIM-containing domain or a short domain devoid of ITIMs (74). CEACAM1 could have 12 alternatively spliced isoforms that lead to the generation of proteins with potentially different functions.

TIGIT and CD96 are inhibitory receptors that compete with DNAM-1 (CD226), an activating receptor, for CD155 (PVR), and CD112 (Nectin-2). CD155 is highly expressed in many types of tumor cells. TIGIT and CD96 contain the ITIM motif. TIGIT contains an ITT-like motif in addition to an ITIM motif in the cytoplasmic tail, where phosphorylation of ITT-like motif upon ligand binding plays a critical role in inhibitory signaling via the recruitment of SHIP1. Engagement of TIGIT with CD155 induces its phosphorylation through Fyn and Lck and recruits SHIP1 in T cells (58). High TIGIT expression is associated with the exhaustion of tumor-infiltrated NK cells in patients with colorectal cancer (75). The blockade of TIGIT prevents NK cell exhaustion and elicit potent anti-tumor immunity in mice (75). Combined blocking of TIGIT and PD-1 showed significant tumor clearance in mice (76). TIGIT and PD-1 are often co-expressed in tumor-infiltrated NK cells (76), but only TIGIT is associated with NK cell exhaustion (75). As PVR expression is associated with unfavorable

prognosis in many solid tumors, such as colon, breast, lung, and pancreatic cancers, the "PVR-TIGIT axis" has been suggested as a novel target in immune checkpoint therapy (77). Notably, tiragolumab, an anti-TIGIT Ab developed by Genentech, is already being evaluated in two independent Phase-III clinical trials for small cell lung cancer and non-small cell lung cancer with atezolizumab, an anti-PD-L1 Ab (66), and chemotherapy. There are two other anti-TIGIT Abs, MTIG7192A and AB154, in Phase I or II trials for various solid tumors. The role of CD96 is relatively less elucidated in NK cells.

Lymphocyte activation gene-3 (LAG-3) is structurally similar to CD4 and binds to MHC class II molecules with a higher affinity than CD4. Fibrinogen-like protein 1 (FGL1) is a recently identified ligand for LAG-3 (78). LAG-3 transduces two independent inhibitory signals through the FXXL motif in the membrane-proximal region and the C-terminal EX repeat (79); the motifs are unique among the known inhibitory receptors. It is expressed on activated NK cells, and chronic stimulation of $NKG2C^{+}$ NK cells can induce high expression of LAG-3 (80). A soluble form of LAG-3-Ig fusion protein, IMP321 induces human NK cells to produce IFN-γ and TNF-α *in vitro* (81). In human trials, IMP321 induced NK cell activation as monotherapy in advanced renal cell carcinoma (82) and combination with paclitaxel in metastatic breast cancer (83). FGL1 blockade also potentiated anti-tumor T cell responses in mice (78), but that of NK cells is not yet known.

CD47, CD73, and SIGLEC FAMILY PROTEINS

In this section, we introduce some of the emerging immune checkpoint molecules in NK cell biology. CD47 is an integrin-associated protein with a short cytoplasmic domain, interacting with thrombospondin-1 (TSP-1) and signal regulatory protein α (SIRPα), an inhibitory transmembrane protein. CD47 regulates NK cell homeostasis and immune responses to

lymphocytic choriomeningitis virus infection (84) and NK cell recruitment and activation in the tumor microenvironment in mice (85). CD47 is quite ubiquitously expressed. Elevated CD47 expression is associated with reduced survival in some cancers. Cord blood cell-derived $CD16^+$ NK cells respond well to anti-CD47 Ab-treated T and B-ALL cell lines with an approximately 10% increase in cytotoxicity (86). CD47 blockade with trastuzumab (anti-HER-2 mAb) augmented anti-tumor efficacy, but the effect appears to be due to increased phagocytosis, rather than ADCC (87).

CD73, ecto-5′-nucleotidase, is probably the latest addition to immune checkpoint molecules in NK cells. The expression of CD73 is virtually absent in circulating human and mouse NK cells in healthy individuals, but tumor-infiltrated NK cells express substantial CD73 (88). It defines regulatory NK cells in the tumor environment in patients with breast cancer and sarcoma (89). $CD73^+$ NK cells in the tumor microenvironment express LAG-3, VISTA, PD-1, and PD-L1. NK cells transport CD73 upon engagement of 4-1BB on tumor cells, to express IL-10 via STAT3 activation (89). CD73 is suggested as a correlative factor of patient survival and NK cell infiltration in glioblastoma (90) and mediates immunometabolic dysfunction of NK cells under hypoxic conditions in solid tumors (91). Targeting CD73 has also been shown to suppress tumorigenesis. A first-in-class therapeutic anti-CD73 mAb, MEDI9447, is currently being evaluated in Phase I clinical trials in cancer patients (88).

Among sialic acid-binding immunoglobulin-like lectins (Siglecs), Siglec7 and Siglec9 are expressed in NK cells. Sialic acids, cognate ligands of Siglecs are 9-carbon-backbone monosaccharides, which are the glycan residues of glycoproteins and glycolipids. The cytoplasmic domains of Siglec7 and Siglec9 contain an ITIM and an ITIM-like motif (92). Siglec7 and Siglec9 share structural similarity and functionality but have different roles in virus infection and tumors. Siglec7 is expressed on mature or more cytotoxic NK cells and

can reduce NK cytotoxicity. Sialic acid-containing glycan has been reported to protect tumor cells from NK cells through Siglec7 (93). Hypersialylated tumor cells can bind to Siglec9, and Siglec9^{+} NK cells express higher levels of KIRs and LIR-1. Siglec7 interacts with gangliosides, while Siglec9 interacts with mucins (92). Importantly, the desialylation of tumor cells by neuraminidases enhanced NK cytotoxicity and cytokine production (94), thereby implying novel therapeutic approaches. Siglec3 (CD33) is just recently identified as an inhibitory receptor on NK cells (95). Siglec3 inhibits cytotoxicity triggered by NKG2D via Vav1 dephosphorylation, but not by NKp46 (95).

CHIMERIC ANTIGEN RECEPTORS

The potential advantage of CAR-NK cells over CAR-T cells

The majority of CAR-T cell therapies, including Kymriah® and Yescarta®, use autologous T cells collected from cancer patients. However, the use of autologous T cells has well-known disadvantages, including a complex manufacturing process and a low quantity of patient cells (96). Furthermore, T cell dysfunction can occur in patients who have received previous treatment with chemotherapy and/or certain other medications (97). To overcome these limitations, gene-editing tools to knockout T-cell receptors (TCRs) and human leukocyte antigen (HLA) have been employed for the generation of allogeneic CAR-T cells. These tools apply to non-HLA matched patients by reducing the potential risk of graft versus host disease (GVHD) (98-100). However, highly gene-edited cells come with unknown risks.

CAR-NK cell therapies traverse several of the limitations of CAR-T cell therapies. First, NK cells can recognize and kill tumors without HLA matching or prior antigen-sensitization (101). The transfer of allogeneic NK cells has even been shown to mediate graft-versus-tumor (GvT) responses without GvHD (102). Subsequently, NK cells can be obtained

from several different sources including peripheral blood, umbilical cord blood, embryonic stem cells, and induced pluripotent stem cells (iPSCs) (103-105).

In addition, NK cell lines such as NK-92 can be utilized as allogeneic off-the-shelf CAR-expressing cell products. In contrast to primary NK cells, CAR-expressing NK-92 cells can be manufactured from a functionally and molecularly characterized single-cell clone under good manufacturing practice-compliant conditions (106). The CRISPR-Cas9 genome editing technology may allow for site-specific integration of the CAR, thereby mitigating the risk of any dysfunction in the NK-92-CAR cells. Additionally, NK-92 cells require irradiation before infusion into patients to avoid potential malignant expansion. Nonetheless, repeated infusion of irradiated CAR-NK92 cells can maintain the efficacy of CAR-NK therapy depending on the dose and frequency.

A major safety concern with CAR T-cell therapy is cytokine release syndrome (CRS). Aberrant activation of CAR T cells can lead to massive production of inflammatory cytokines including IL-6 (107). Several clinical trials have demonstrated that the adoptive transfer therapy of allogeneic CAR-NK cells does not cause severe side effects (108). CAR-NK cells may be potentially safer than CAR-T cells because of their shorter lifespan after infusion (109). There exists little clinical evidence for the comparison of the side effects of CAR-T and CAR-NK cells (110). However, according to the clinical trial of CAR-NK cell therapy at the University of Texas MD Anderson Cancer Center, the treatment of CAR-NK cells derived from cord blood showed complete remission in 7 of the 11 patients (4 with non-Hodgkin's lymphoma and 3 with chronic lymphocytic leukemia) without CRS, neurotoxicity, or GvHD, which are all potential side effects of CAR-T therapy (111).

Antigen escape is a major obstacle for effective CAR-T therapy. The immune pressure by CAR-T cells results in the outgrowth of antigen loss variants. In hematologic

malignancies, CD19 loss after CAR-T therapy drives relapses (112, 113). CAR-NK cells could show effective antitumor activity against target antigen-negative tumors by endogenous activating receptors such as NKG2D, Nkp30, Nkp44, Nkp46, or DNAM-1 that are involved in tumor immune surveillance (114, 115). In addition, cytotoxic response via activating receptors including CD16 (FcγRIII) mediating antibody-dependent cellular cytotoxicity (ADCC) can synergistically enhance the antitumor activity of CAR-NK cells (116).

Enhancing the anti-tumor activity of CAR-NK cells

CARs comprise a signal peptide, a single-chain variable fragment (scFv), hinge region, transmembrane region, and intracellular domains. The composition of conventional CARs is as follows: a CD4, CD8, or IgG hinge; a CD3ζ, CD4, CD8, or CD28 transmembrane domain; a 4-1BB or CD28 costimulatory domain; and a CD3ζ activation domain (117, 118). Herein, we describe the current efforts to optimize the clinical outcome of CAR-NK therapies for each domain.

(1) Antigen recognition domain

The single-chain variable fragment (scFv) is an extracellular component of a CAR and confers antigen specificity. scFv is derived from the variable regions of the heavy chain and light chain of antibodies. Since the main function of scFv is antigen binding, the proper design of scFv against tumor antigens is required for better clinical outcomes. Typically, therapeutic antibodies with high-affinity binding to a specific antigen are preferred for predicted responses with minimal side effects. Although designing an scFv for a CAR is dependent on mAbs specific to the tumor-associated antigen, optimizing CAR scFv sequences for the best clinical outcome is still under discussion. Chmielewski *et al*. generated T cells expressing chimeric TCR pools against human epidermal growth factor receptor 2

(ERBB2, HER2) with a Kd ranging from 3.2 × 10-7 to 1.5 × 10-11 M. Researchers demonstrated that a higher affinity receptor did not correlate with the potency of T cell activation. Moreover, T cells with high-affinity receptors could induce an immune response to normal tissue expressing low antigen levels (119). Morgan *et al*. reported a clinical case study on the administration of CAR-T cells-derived from the FDA-approved therapeutic mAb, trastuzumab (Herceptin), that targets HER2, a tumor-associated antigen used to treat metastatic colon cancer. The patient presented side effects resulting from a dramatic pulmonary infiltration of CAR-T cells by the recognition of low expression levels of HER2 in normal lung epithelial cells and died 5 days after administration (120). Phase I clinical trial of NK-92/5.28.z (CAR-NK-92 cells targeting HER2 with trastuzumab scFv) is presently ongoing in patients with recurrent HER2-positive glioblastoma (CAR2BRAIN, NCT03383978, clinicaltrials.gov). So far, no remarkable adverse effects have been observed, but more detailed clinical signs should be confirmed in dose-escalating studies (121).

The design of an scFv for CAR-NK therapies is still in its early stages. Because CAR-NK cells are generally more tolerant than CAR-T cells, we would select the one with the strongest affinity. Moreover, NK-92-CAR cells have to be irradiated before infusion, this process can decrease the potency of NK-92-CAR cells. Currently, the sequences for therapeutic mAbs are being adapted for scFv design, but we will consider all possible factors including biochemical (affinity), the transmission of activation signaling in the CAR-NK cells, compatibilities with the cytoplasmic domain of the CAR, and side effects observed in clinical trials, to obtain the best clinical outcome.

(2) Spacer, transmembrane, and costimulatory domains

Since NK cells express CD3z for transmitting activation signaling similarly to T cells (122), the intracellular domain of CD3z is generally used for CAR designs for NK cells. The first

generation of CARs generally contained only the CD3z domain for signal transduction. However, due to CD3z's weak potency, costimulatory domains (e.g., CD28 or 4-1BB, or both) were added to the cytoplasmic region of second/third generation CARs (123). A previous study showed that incorporation of DNAX-activation protein 12 (DAP12) instead of the CD3z activation domain allowed the lysis of KIR/HLA-matched prostate stem cell antigen (PSCA)-positive tumor cell lines and was considered suitable for CAR-NK therapy against solid tumors (124). The CD28 and 4-1BB costimulatory domains of third-generation CARs of iPSC-derived NK cells can be replaced with the combination of an NKG2D transmembrane and 2B4 cytoplasmic domain that can induce synergistic activation of NK cells with enhanced antitumor activity of iPSC-CAR NK cells (104). These studies highlighted that the selection of optimal transmembrane and cytoplasmic signaling domains of a CAR unique to NK cells can improve the antitumor activity of CAR-NK cells in a manner different from CAR-T cells.

In addition, distinct hinge domains alter the performance of CAR-T cell therapies. The extracellular spacer has been reported to affect the accessibility of a CAR to approach the target epitope and decides the cell-cell distance depending on the length (125). Moreover, the selection of an optimal hinge region contributes to CAR dimerization and performance. This may be due to structural interactions as well as the flexibility of the CAR (126, 127). Further, the incorporation of 4-1BB costimulatory domains has been demonstrated to ameliorate the exhaustion of T cells caused by CAR-mediated antigen-independent tonic signaling thereby leading to functional differences (128). The difference between CD28/CD3ζ and 4-1BB/CD3ζ is associated with kinetics and signal strength (129).

(3) Fourth-generation CARs

Fourth-generation CAR-T cells are engineered to secrete transgenic cytokines. Cytokine-

mediated “signal 3” is known to be important for T or NK cell activation and persistence. These CARs, armed with cytokines, were validated to enhance antitumor activities in several studies on T cells. The persistence of CAR-expressing effector cells is associated with improved clinical outcomes (127, 130). Adoptively transferred NK cells have limited persistence *in vivo*, thereby potentially limiting the efficacy of CAR-NK cells. Genetic engineering of CAR-NK cells to express IL-15, which is associated with increased proliferation and survival of NK cells, enhanced the persistence and efficacy of CAR-NK cells (103, 131). *Ex-vivo* NK cells cultured with IL-15 and a pharmacological inhibitor of the glycogen synthase kinase 3 (GSK3) enhanced CD57 upregulation, promoted late-stage maturation, and improved the antitumor activity of the NK cells (132).

Several interesting studies focusing on signal 3 were conducted in CAR-T cells. Kagoya *et al*. developed CARs with truncated IL-2Rbeta and STAT3 binding YXXQ motif in addition to the CD28-CD3z cytoplasmic domain, thereby resulting in enhanced antitumor activity (133). Chmielewski *et al*. developed TRUCKs (T cells redirected for antigen-unrestricted cytokine-initiated killing), an NFAT-based cytokine expression system to increase the cytotoxicity of CAR cells (134, 135). Hurton *et al*. engineered CARs with IL-15 fused to the IL-15Ralpha via a flexible linker (136). However, these mechanistic studies are based on T cells and the conventional CAR structure. The biological mechanisms behind CAR signaling when using different CAR constructs or different cell types such as NK cells are not well understood. It is hypothesized that the exploration of various CARs and activation pathways based on NK cells improves CAR-NK therapy by determining optimal constructs and methodological complications such as cell sources, *ex vivo* culture, infusion dose, and frequency. Next, we will describe current attempts conducted in industries for CAR-NK anti-cancer therapy employing various new technologies.

CAR-NK DEVELOPMENT IN INDUSTRY: CURRENT STATUS

Here, we summarize the current status of CAR-NK developments in the biotech and pharmaceutical industries (Figure 2). Bellicum Pharmaceuticals (Houston, TX) is developing the novel technology GoCAR™ NK cell therapy by introducing both rimiducid-inducible iMC (MyD88-CD40 dimerization) for NK cell activation and proliferation along with rapamycin-inducible iRC9 (Caspase-9) for safety into NK cells. Anti-CD123 and anti-HER2 CAR-NK cells with iMC and autocrine IL-15 showed enhanced persistence and anti-tumor activity in *in vivo* CD123+ AML and HER2+ solid tumor models, respectively (137). Bellicum has announced that GoCAR™ NK cells targeting BCMA are in preclinical development for treating multiple myeloma.

Fate Therapeutics Inc. is developing NKCAR-iPSC-NK cells that target the CD19 antigen, FT596, armed with a high-affinity, non-cleavable CD16 FcR and a novel IL-15 receptor fusion. The high-affinity CD16 receptor allowed the CAR-NK cells to overcome resistance induced by CD19 antigen loss in combination with rituximab (CD20 therapeutic mAb) in a Raji CD19-CD20+ lymphoma model [ASH 2018, 2019]. FT596 is now under Phase I clinical trial for patients with B cell lymphoma and chronic lymphocytic leukemia [NCT04245722].

Avectas and ONK therapeutics are developing a CAR-NK cell therapy by incorporating DR5 TRAIL variants to maximize cytotoxicity in various tumors including CD19 targeting B-cell lymphomas (patent US 10,034,925). The DR5 TRAIL variant showed a maximum of 1,000-fold or greater binding affinity compared to other variants, thereby resulting in TRAIL receptor-mediated apoptosis in target tumor cells.

GEMoaB Monoclonals GmbH is developing a universal CAR platform (Uni-CAR)

451 which uses a switchable turn-on/off mechanism by binding with cancer-specific targeting
452 modules (TM) (138). Uni-CAR NK-92 cells are redirected and activated by scFv- and IgG4-
453 based TM specific for tumor antigen GD2, thereby resulting in anti-tumor activity in GD2-
454 expressing solid tumor mouse models. *In vivo* pharmacokinetic analysis showed rapid
455 elimination of scFv-based TM with a half-life of 1.6 h.

456 NantKwest Inc. is developing t-haNK cells that target the PD-L1 in non-small cell lung
457 cancer. The t-haNK cells express an anti-PD-L1 CAR, a high-affinity CD16, and an
458 endoplasmic reticulum retained IL-2 (139). T-haNK is now under Phase I clinical trial for
459 patients with locally advanced or metastatic solid tumors (NCT04050709).

460 Nkarta Therapeutics Inc. is developing NKX-101: CAR-NK cells consisting of NKG2D
461 receptors in the extracellular domain, OX40-CD3z in the costimulatory domain, and
462 membrane-bound IL-15. The NKG2D receptor binds to eight NKG2D ligands that are
463 upregulated in a range of leukemic and solid tumors. CAR-NK cells targeting these ligands
464 showed antitumor activity in a murine model of osteosarcoma (140). NKX-101 showed *in*
465 *vitro* anti-tumor activity and increased cell delivery in an *in vivo* model of colorectal cancer
466 liver metastasis (ASCO 2020).

467 Takeda, under license from MD Anderson Cancer Center, is developing TAK-007: cord
468 blood-NK cells that are transduced to express CAR targeting CD19 with a CD28
469 costimulatory domain, IL-15, and an inducible caspase 9 suicide gene. In a recent small-scale
470 clinical trial, TAK-007 showed a response rate of 73% for patients with relapsed or refractory
471 CD19-positive cancers (111). Some additional CAR-NK cell therapies are under development
472 targeting several tumor-associated antigens including MUC1 for solid tumors (PersonGen
473 Biomedicine Co. Ltd), CD38 for AML (Celularity Inc), EGFRvIII for PD-L1 positive solid
474 tumors (PharmAbcine), CD7 for T cell leukemia (Gracell Biotechnology Ltd), GPC-3 for

hepatocellular carcinoma (Baylor College of Medicine, Kuur therapeutics), and CSPG4 for triple-negative breast cancer (Baylor College of Medicine). The ongoing clinical trials with NK-CARs are presented in Table 2.

CONCLUSIONS AND PERSPECTIVES

NK cells have the potential to kill a broad spectrum of tumor cells without mutational burden and neoantigen presentation. This property of MHC-unrestricted tumor lysis by NK cells without the risk of GVHD, which is unique among immune effector cells, has positioned NK cells as key components in the arsenal of cancer therapeutics. Recent studies on cancer therapies using NK cells have demonstrated favorable clinical efficacies in hematologic malignancies, but limited success in solid tumors. This may be attributed to the limited capacity of NK cells to infiltrate tumors, persist *in vivo*, or resist in the immunosuppressive tumor microenvironment. Thus, rational strategies for improving NK cell-based therapy have been developed to overcome the existing challenges. These include the blockade of immune checkpoint receptors to rescue dysfunctional NK cells and the incorporation of tumor-directed CAR to augment anti-tumor NK cell specificity and activity.

Since the early studies on KIRs, numerous immune checkpoint receptors revealed to be functionally quintessential in NK cell function. Immune checkpoint blockades that can stimulate NK cells have tremendous potential in cancer therapy. They could not only stimulate NK cells but also T cells in direct and indirect ways, particularly under the situation of blocking immune checkpoint receptors that are expressed on both the cell types. In addition, combination therapies with immune checkpoint blockade and chemotherapy or CAR-T/NK therapy could become a part of standard therapeutic regimens in the near future.

Despite challenges in the genetic manipulation of NK cells, CAR-NK cells have

499 received increasing attention as next-generation therapeutics against refractory malignancies
500 including solid tumors. In addition to the redirected specificity, they hold promise with "off-
501 the-shelf" clinical utility and low toxicity usually not causing immune-related adverse events.
502 Moreover, further engineering of CAR-NK cells with on-board cytokines (e.g., IL-15) that
503 leads to enhanced *in vivo* persistence and resistance to immunosuppression has paved the way
504 for new therapeutic options to improve clinical efficacy (103, 141). These observations, along
505 with several reliable protocols for GMP-grade large scale expansion of NK cells, will render
506 NK cell-based therapy a viable modality to treat refractory cancers, possibly in rational and
507 optimal combination with other therapies.
508

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513

514 **CONFLICTS OF INTEREST**

515 The authors declare no competing financial interests.
516

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FIGURE AND TABLE LEGENDS

Figure 1. Interactions between immune checkpoint receptors and their cognate ligands.

NK cells express multiple immune checkpoint receptors, which can interact with their cognate ligands on tumor cells as well as other immune cells, in particular, dendritic cells and Tregs. The red circles represent immune checkpoint receptors while the blue circles represent the ligands. SIGLEC7 and SIGLEC9 have common ligands that are sialic acids. The pink squares represent the classical ITIM motif and the light blue squares represent the ITSM motif, which have been implicated in mediating inhibitory signals. The light green squares represent the ITT-like motif. Cytoplasmic domains of other immune checkpoint receptors contain fewer known motifs (not marked as squares). CD73 is a nucleotidase, which does not have conventional inhibitory signaling domains. The black lines indicate receptor-ligand interactions.

Figure 2. Development of CAR-NK cell therapies in the industry.

(A) GoCAR-NK utilizes Rimiducid and Rapamycin for inducible activation and suicide signaling, respectively. (B) FT596, combined with Rituximab, can target both CD19 and CD20 with CD16 Fc receptor in B cell malignancy. IL-15 receptor fusion protein with a flexible IL-15 is introduced for further NK cell activation. (C) ONK therapeutics use TRAIL signaling for maximizing tumor cell apoptosis. A high-affinity variant of TRAIL is recognized by the DR5 TRAIL receptor, thereby resulting in activation of FADD-Caspase8 signaling in a target tumor cell. (D) t-haNK is a versatile platform targeting tumors with a combination of CAR and therapeutic antibody. In this figure, we illustrate an example of NSCLC which expresses a high level of PD-L1 and EGFR. ER-retained IL-2 also drives NK cell activation and persistence. (E) NKX-101 uses NKG2D-CAR for tumor antigen recognition, membrane-bound IL-15 for

932 further activation as well. NKG2D ligands (MIC and RAET1 families) are highly expressed in
933 various tumor cells.
934

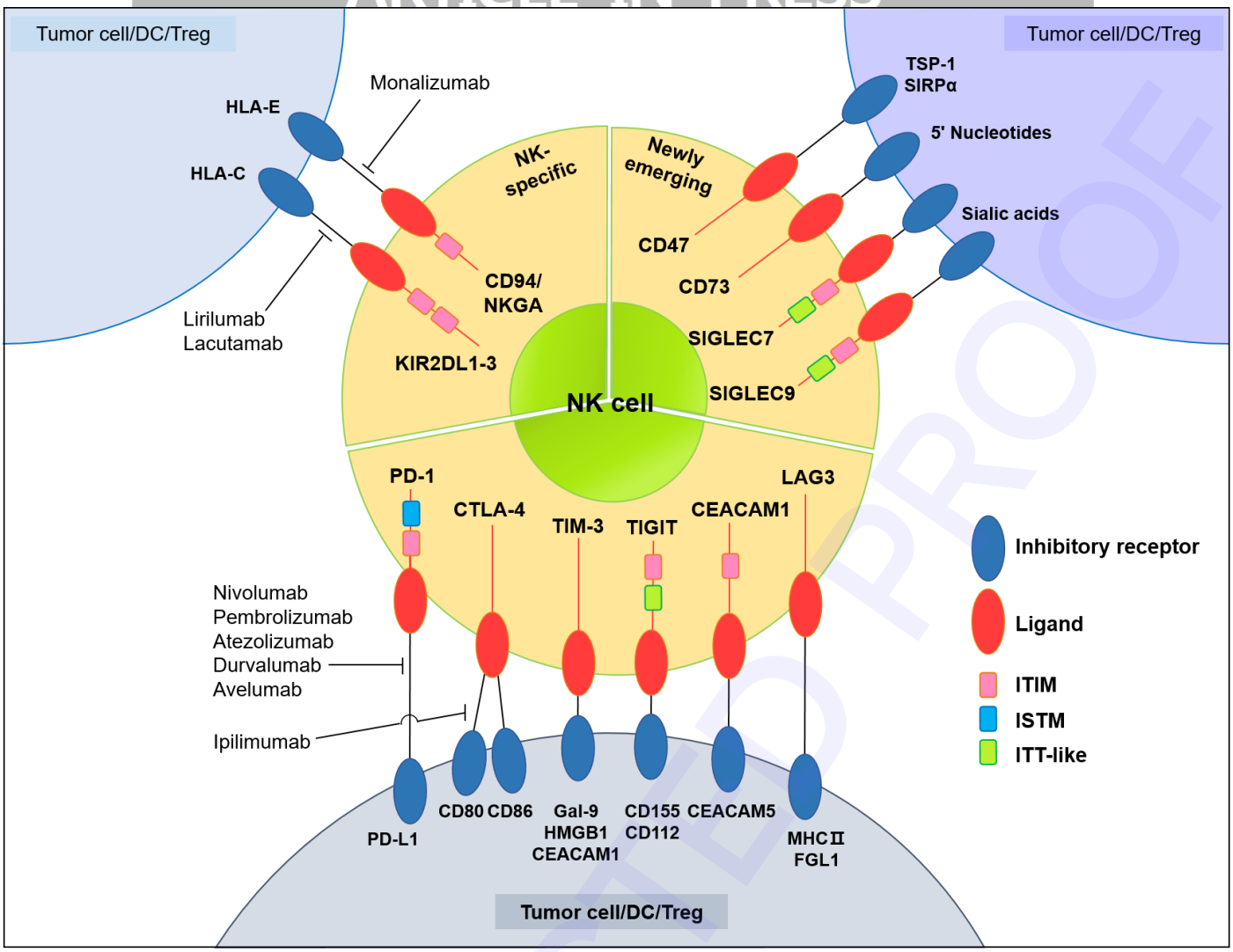

Fig. 1. Figure 1

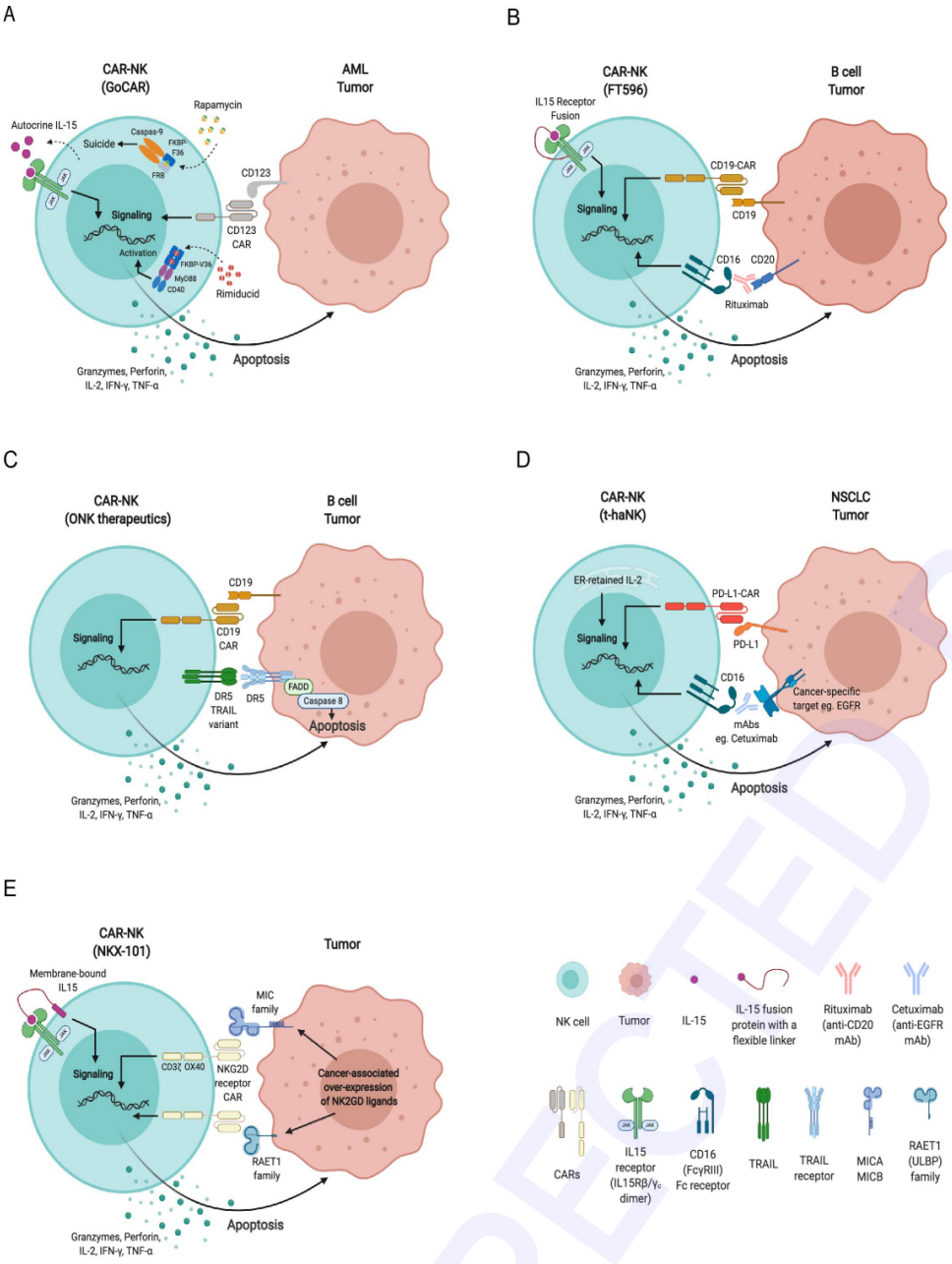

Fig. 2. Figure 2

Table 1. Current status of clinical trials based on immune checkpoint receptors.

Checkpoint receptor	Ab/Drug	Combination drugs	Disease	Phase	Clinical trials identifier
KIRs	Anti-KIR (1-7F9, IPH2101)	Single	MM	Phase I	NCT00552396
		Single	MM, SMM	Phase II	NCT01248455
	Lirilumab (IPH2102, BMS-986015)	Lenalidomide	MM	Phase I	NCT01217203
		Nivolumab, Azacitidine	MDS	Phase II	NCT02599649
		Single	Gynecologic cancer	Phase I	NCT02459301
CD94/NKG2A	Monalizumab (IPH2201)	Durvalumab (MEDI4736)	Advanced solid tumors	Phase I/II	NCT02671435
		Cetuximab, Anti-PD-L1	Head and neck carcinoma	Phase I/II	NCT02643550
		Ibrutinib	CLL	Phase I/II	NCT02557516
CTLA-4	Ipilimumab (BMS-734016)	Single	Advanced melanoma	Phase I	NCT00920907
		Nivolumab	Advanced/metastatic melanoma	Phase II	NCT01783938
		Paclitaxel, Cisplatin, Carboplatin	NSCLC	Phase II	NCT01820754
PD-1	Pembrolizumab (MK-3475)	Single	Hepatocellular carcinoma	Phase II	NCT02658019
	Nivolumab	Ipilimumab	Advanced/metastatic melanoma	Phase II	NCT01783938
	Durvalumab (MEDI4736)	Tremelimumab	Metastatic pancreatic ductal adenocarcinoma	Phase II	NCT02558894
TIM-3	BGB-A425	Tislelizumab	Advanced or metastatic solid tumors	Phase I/II	NCT03744468
	MBG453	Decitabine, PDR001	AML and high risk MDS	Phase I	NCT03066648
TIGIT	MTIG7192A	Atezolizumab, Carboplatin, Cisplatin, Pemetrexed, Paclitaxel, Etoposide	Advanced/metastatic tumors	Phase I	NCT02794571
LAG-3	LAC-3-Ig (IMP321)	Single	Metastatic Breast Cancer	Phase I	NCT00349934
		Montanide ISA-51	Melanoma	Phase I/II	NCT01308294
CD47	IBI188	Single	Advanced malignancies	Phase I	NCT03763149
CD73	Oleclumab (MEDI9447)	Paclitaxel, Carboplatin, Durvalumab	Triple Negative Breast Cancer	Phase I/II	NCT03616886
	Ciforadenant (CPI-444)	Pembrolizumab	Advanced cancer	Phase I	NCT03454451
CD33 (Siglec 3)	Vadastuximab talirine (SGN-CD33A)	Azacitidine, Decitabine, Placebo	AML	Phase III	NCT02785900

MM: Multiple Myeloma; SMM: Smoldering Multiple Myeloma; AML: Acute Myeloid Leukemia; MDS: Myelodysplastic Syndromes; CLL: Chronic lymphocytic leukemia; NSCLC: Non-Small Cell Lung Cancer; MBC: Metastatic Breast Carcinoma

Table 2. Clinical trials with CAR-NK cells.

Clinical Trials.gov Identifier	Title or CAR-NK Strategy	Target Disease	Source of NK cell	Sponsor	Start Date	Status
NCT03692767	Anti-CD22 CAR NK	Relapsed and Refractory B Cell Lymphoma		Allife Medical Science and Technology Co., Ltd.	March 2019	Not yet recruiting
NCT03690310	Anti-CD19 CAR NK	Relapsed and Refractory B Cell Lymphoma		Allife Medical Science and Technology Co., Ltd.	March 2019	Not yet recruiting
NCT03692637	Anti-Mesothelin Car NK	Epithelial Ovarian Cancer		Allife Medical Science and Technology Co., Ltd.	March 2019	Not yet recruiting
NCT03415100	NKG2D-Ligand Targeted CAR-NK	Metastatic Solid Tumors		The Third Affiliated Hospital of Guangzhou Medical University	January 2, 2018	Unknown
NCT04324996	NKG2D-ACE2 CAR-NK	COVID-19	cord blood	Chongqing Public Health Medical Center	February 21, 2020	Recruiting
NCT03692663	Anti-PSMA CAR NK	Castration-Resistant Prostate Cancer		Allife Medical Science and Technology Co., Ltd.	December 2018	Not yet recruiting
NCT03940820	ROBO1 Specific CAR-NK	Solid Tumors		Asclepius Technology Company Group (Suzhou) Co., Ltd.	May 2019	Recruiting
NCT03940833	BCMA CAR-NK 92 cells	Relapse/Refractory Multiple Myeloma	NK-92 cell line	Asclepius Technology Company Group (Suzhou) Co., Ltd.	May 2019	Recruiting
NCT03824964	Anti-CD19/CD22 CAR NK	Relapsed and Refractory B Cell Lymphoma		Allife Medical Science and Technology Co., Ltd.	February 1, 2019	Not yet recruiting
NCT02944162	anti-CD33 CAR-NK	Relapsed/Refractory CD33+ AML	NK-92 cell line	PersonGen BioTherapeutics (Suzhou) Co., Ltd.	October 2016	Unknown
NCT02892695	PCAR-119 Bridge Immunotherapy before Stem Cell Transplant (anti-CD19 CAR-NK)	CD19 Positive Leukemia and Lymphoma	NK-92 cell line	PersonGen BioTherapeutics (Suzhou) Co., Ltd.	September 2016	Unknown
NCT03941457	ROBO1 Specific BiCAR-NK	Pancreatic Cancer		Asclepius Technology Company Group (Suzhou) Co., Ltd.	May 2019	Recruiting
NCT03931720	ROBO1 Specific BiCAR-NK/T	Malignant Tumor		Asclepius Technology Company Group (Suzhou) Co., Ltd.	May 2019	Recruiting
NCT03056339	Umbilical & Cord Blood (CB) Derived CAR-Engineered NK;	B Lymphoid Malignancies	cord blood	M.D. Anderson Cancer Center	June 21, 2017	Recruiting

	(iC9/CAR.19/IL15-Transduced CB-NK)					
NCT04245722	FT596 as a Monotherapy and in Combination with Anti-CD20 Monoclonal Antibodies	B Cell Lymphoma, Chronic Lymphocytic Leukemia	iPSC	Fate Therapeutics	March 19, 2020	Recruiting
NCT04050709	QUILT 3.064: PD-L1 t-haNK	Locally Advanced or Metastatic Solid Cancers	NK-92 cell line	NantKwest, Inc.	July 18, 2019	Active, not recruiting
NCT03383978	Intracranial Injection of NK-92/5.28.z (CAR2BRAIN)	Recurrent HER2-positive Glioblastoma	NK-92 cell line	Johann Wolfgang Goethe University Hospital	December 1, 2017	Recruiting