

# The emerging role of off-the-shelf engineered natural killer cells in targeted cancer immunotherapy

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**Natural killer (NK) cells are innate lymphocytes that recognize and clear infected and transformed cells. The importance of NK cells in tumor surveillance underlies the development of NK cell therapy as cancer treatment. The NK-92 cell line has been successfully modified to express high-affinity CD16 receptor for antibody-dependent cellular cytotoxicity and/or chimeric antigen receptors (CARs) that can recognize antigens expressed on tumor cells and mediate NK cell activation. Since there is no need for human leukocyte antigen matching or prior exposure to the tumor antigens, NK-92 provides an opportunity for the development of next-generation off-the-shelf cell therapy platforms. CAR-engineered NK-92 cells have demonstrated robust antitumor activity in *in vitro* and *in vivo* preclinical studies, propelling the clinical development of CAR NK-92 cells. Preliminary phase 1 data indicate that CAR NK-92 can be safely administered in the clinic. In this review, we provide an overview of recent advances in the research and clinical application of this novel cell immunotherapy.**

## INTRODUCTION

Natural killer (NK) cells are innate effector lymphocytes that play an important role in clearing infected and transformed cells. In humans, NK cells are typically defined as CD3<sup>-</sup>CD56<sup>+</sup> cells and represent 5%–20% of the circulating lymphocytes. Human NK cells can be subclassified into immature NK cells (CD56<sup>bright</sup>CD16<sup>dim</sup>), which are potent inflammatory cytokine producers that play a role in immunomodulation, and mature NK cells (CD56<sup>dim</sup>CD16<sup>bright</sup>), which mediate the cytolytic function of the human NK cells. In mice, NK cells are phenotypically CD3<sup>-</sup> cells that express NK1.1, NCR1, and/or CD49b, depending on mouse background.<sup>1</sup>

In contrast to T cells and B cells, NK cell recognition is not dependent on antigen specificity and does not require prior sensitization. Instead, the activity of NK cells is controlled by the integration of signals from various NK cell surface inhibitory and activating receptors. Most of the inhibitory receptors bind major histocompatibility class (MHC) class I molecules and can detect missing self-markers on target cells that downregulated MHC class I to evade T cells. In humans, examples of inhibitory NK receptors include killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs), and

CD94-NKG2A receptors. Non-human leukocyte antigen (HLA)-specific inhibitory receptors such as PD-1, TIGIT, and CD96 can also be upregulated on the NK cell surface in pathological conditions to limit exacerbated immune responses during viral infection; conversely, they may enable tumor escape. Activating receptors discriminate between healthy cells and abnormal cells by detecting pathogen or cell stress-induced cell ligands. Examples of NK cell activating receptors are Fc gamma receptor (FcγR)IIA (also known as CD16), NKG2D, and members of the natural cytotoxicity receptors (NCRs) such as NKp46, NKp30, and NKp44. Except for CD16, activating receptors must be simultaneously crosslinked in pairs or combinations to elicit synergistic activation signals for NK cell function.<sup>2–4</sup>

NK cells can directly kill infected and transformed cells through several processes. One NK cytotoxic response involves the targeted release of cytolytic granules that contain perforin and granzymes. NK cells also mediate killing of target cells via engagement of death receptors. Death ligands on NK cells, such as Fas ligand (FasL) and TRAIL, bind to their cognate receptors on target cells and result in apoptosis of target cells.<sup>1,5</sup> NK cells are also able to induce antibody-dependent cell-mediated cytotoxicity (ADCC) through the CD16 receptors that detect target cells that are opsonized with immunoglobulin (Ig)G antibodies. The ADCC function of NK cells is considered to contribute to the clinical activity of cancer therapeutic antibodies.<sup>6,7</sup> In addition to direct killing, NK cells are also potent producers of cytokines such as interferon-γ (IFNγ), tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as chemokines (e.g., CCL3, CCL4, CCL5, XCL1, and CXCL8) that can modulate the function of other immune mediators.<sup>1</sup>

The ability of NK cells to differentiate normal cells from those that have undergone malignant transformation makes them a key player in tumor immunosurveillance. Tumor-infiltrating NK cells correlate with

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improved patient prognosis and survival in colorectal carcinoma,<sup>8</sup> gastric carcinoma,<sup>9</sup> pulmonary adenocarcinoma,<sup>10</sup> and squamous cell lung carcinoma.<sup>11</sup> In renal cell carcinoma, NK cell infiltration of the lung metastases is associated with improved survival.<sup>12</sup> Inversely, an 11-year follow-up prospective cohort study from Japan suggested that low NK cell cytotoxicity is associated with increased cancer risk.<sup>13</sup> Low NK activity was also correlated with cancer recurrence following surgical tumor resection of colorectal cancer<sup>14</sup> and incidence of metastasis in head and neck<sup>15</sup> and pharyngeal<sup>16</sup> cancer.

In an actively developing area of investigation, harnessing NK cells is proving to be an attractive strategy to target cancer, either by activating endogenous NK cells or adoptive cell transfer. The clinical application of NK cells in the cancer setting was first explored in the 1980s when high infusions of interleukin (IL)-2 and lymphokine-activated killer (LAK) cells were tested in renal cell carcinoma and melanoma.<sup>17</sup> LAK cells were generated from freshly isolated peripheral blood mononuclear cells (PBMCs) that were cultured with IL-2, which activates NK cytotoxicity and supports NK and T cell proliferation. The NK cell population was primarily responsible for the anti-tumor cytotoxicity of LAK cells;<sup>18</sup> however, due to the minimal benefit of LAK cells and the toxicities linked to high-dose IL-2, LAK cell-based therapies were not developed further.<sup>17,19</sup>

Since then, our understanding of NK biology has advanced, resulting in the development of improved NK cell therapies with a better safety profile and efficacy. Furthermore, the clinical success of chimeric antigen receptor (CAR)-engineered T cells in hematological malignancies propelled the development of CAR-engineered NK cells.<sup>19</sup> NK cells for adoptive cell therapy can be derived or generated from peripheral blood, umbilical cord blood, and hematopoietic stem cells/induced pluripotent stem cells. The advantages and limitations of isolating, expanding, and engineering NK cells from these sources were reviewed in Daher et al.<sup>20</sup> NK cells can also be sourced from immortalized NK cell lines such as NKG, KHYG-1, NK-YS, YT, YTS, NK3.3, NKL, and NK-92.<sup>21</sup> All of these cell lines proliferate easily in culture and can potentially provide a steady supply of off-the-shelf “pure” NK cells; however, only NK-92 has demonstrated consistent cytotoxic activity against cancer targets.<sup>22</sup> Compared to peripheral blood NK cells, NK-92 can easily be manipulated to express receptors or ligands that enhance tumor targeting.<sup>22</sup> In this review, we highlight recent developments in CAR-engineered NK-92 cells as off-the-shelf targeted cancer immunotherapy. Other advances in NK-based immunotherapy were expertly reviewed in Franks et al.<sup>21</sup>

## NK-92 CELLS

NK-92 is an established NK cell line derived from the peripheral blood of a 50-year-old male non-Hodgkin's lymphoma patient.<sup>23</sup> NK-92 cells express CD56<sup>bright</sup>, CD2, CD7, CD11a, CD28, CD45, and CD54 and are negative for the cell surface markers CD3, CD4, CD8, CD16, CD1, CD5, CD10, CD14, CD19, CD20, CD23, CD34, and HLA-DR.<sup>24</sup> Despite being CD56<sup>bright</sup>CD16<sup>-</sup>, which conventionally characterizes cytokine-producing NK cells,<sup>1</sup> NK-92 cells express high levels of granzyme B and perforin, making them highly cytotoxic.<sup>23,24</sup>

NK-92 cells express a relatively large number of activating receptors and very few inhibitory receptors. NKG2D, NKp30, NKp46, and 2B4 have all been detected on NK-92 cells and are important in the function of the cell line.<sup>24</sup> Target cells that express the NKG2D ligands MHC class I chain-related genes (MIC)A/B are susceptible to NK-92 killing, while those that do not are resistant to NK-92-mediated lysis.<sup>25</sup> NK-92 cells express the inhibitory receptors CD94/NKG2A and LIR-1 but lack most of the receptors in the KIR family, except for KIR2DL4.<sup>24</sup> KIR2DL4 is an unusual KIR that has an inhibitory potential due to the immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic tail, but it functionally activates NK cytotoxicity.<sup>26</sup> The presence of a variety of activating receptors and the relative absence of inhibitory receptors contribute to the superior cytotoxicity of NK-92 cells.

The ability to expand NK-92 cells easily and reproducibly in good manufacturing practice (GMP) conditions allows for the development of off-the-shelf cell therapy products using this cell line as a backbone.<sup>27</sup> NK-92 cells can be continuously grown in culture with a doubling time of 24–36 h.<sup>27</sup> NK-92 cell growth is dependent on IL-2, and withdrawal of IL-2 causes a decline in cytotoxicity after 24 h.<sup>23,28</sup> IL-7 can support short-term proliferation of NK-92 but cannot support sustained growth of the cells.<sup>23</sup> IL-15 was also reported to augment NK-92 cell proliferation and activity.<sup>29,30</sup>

NK-92 cells are effective against a broad spectrum of tumor targets. Early studies demonstrated the cytolytic activity of NK-92 against malignant cells of hematologic origin *in vitro*.<sup>23,28,31</sup> Preclinical studies in severe combined immunodeficient (SCID) mice have shown the antitumor activity of NK-92 in T acute lymphoblastic leukemia (T-ALL),<sup>31</sup> acute myelogenous leukemia (AML),<sup>31</sup> myeloma,<sup>32</sup> and melanoma<sup>33</sup> xenograft models. Meanwhile, targeting of non-malignant allogeneic cells, such as hematopoietic cells derived from normal donors, was not reported.<sup>31</sup> Furthermore, NK-92 cells were not tumorigenic in immunocompromised SCID mice.<sup>22</sup> These studies demonstrated that in the preclinical setting, NK-92 has antitumor activity with minimal side effects.

## Clinical trials with NK-92 cells

Several phase 1 clinical studies have been completed to establish the safety of administering NK-92 cells as allogeneic cell therapy in hematologic and solid cancers. Four clinical trials included patients with solid tumors such as renal cell cancer, lung cancer, melanoma,<sup>34,35</sup> and blood-related malignancies including AML, multiple myeloma (MM), and other relapsed/refractory hematological cancers.<sup>35–37</sup> In these studies, one treatment course consisted of two to three infusions of escalating doses of NK-92 cells given 24–48 h apart. The NK-92 cells were irradiated at 10 Gy prior to infusion to prevent proliferation *in vivo* and eliminate the tumorigenic potential.<sup>28</sup>

These clinical studies demonstrated that NK-92 cell infusion is generally well tolerated and is correlated with some clinical responses. In a phase 1 study performed in Chicago, 12 patients with renal cell cancer or metastatic melanoma were enrolled and received NK-92 cell dose

levels of  $1 \times 10^8$ ,  $3 \times 10^8$ ,  $1 \times 10^9$ , or  $3 \times 10^9$  cells/m<sup>2</sup>. Most of the NK-92 infusion-related toxicities were mild except for one grade 3 fever and one grade 4 hypoglycemic episode in the cohort that received the highest dose level. The melanoma patient exhibited a minor response during the study period, while one renal cell cancer patient had a mixed response. A minor response was defined as the regression of a target tumor lesion by 10%–30% without the formation of new lesions and without the progression of non-target lesions, while a mixed response was the regression of some lesions but simultaneous progression of others. One patient was alive with disease at 4 years post-treatment.<sup>34</sup> In the study performed in Frankfurt, no NK-92 cell infusion-related toxicities were observed even at the highest dose level tested, which was  $1 \times 10^{10}$  cells/m<sup>2</sup>. One patient, however, had to discontinue the second infusion due to back pain that was likely related to abdominal distension caused by the fluid bolus given before and during the infusion. Three of the four patients with advanced lung cancer had some antitumor response. Two of the lung cancer patients had metastatic lesions that disappeared after two infusions of NK-92, while one patient had stable disease for approximately 2 years.<sup>35</sup> In the QUILT-3.018 study (ClinicalTrials.gov: NCT00900809), seven patients with AML received a total of 20 infusions of NK-92 at  $1 \times 10^9$  or  $3 \times 10^9$  cells/m<sup>2</sup>. No patient experienced dose-limiting toxicities during infusion or within the 21 days of the post-infusion observation period. In addition, no grade 3–4 toxicities related to the infusion were observed. In one patient the blast percentage was reduced, and in two patients the blast percentage remained stable.<sup>36</sup> In another study, 12 patients with lymphoma or MM who relapsed after autologous hematopoietic cell transplantation (AHCT) for relapsed disease were enrolled and the highest NK-92 dose level tested was  $3 \times 10^9$  cells/m<sup>2</sup> (ClinicalTrials.gov: NCT00990717). Minor acute infusion-related toxicities were observed but no grade 3 or 4 infusion-related toxicities, no delayed toxicity, no graft-versus-host disease, and no cytokine release syndrome were noted. Complete response was achieved in one patient with Hodgkin's lymphoma (alive 10 years after therapy) and one patient with MM. A mixed response was observed in two patients, one with Hodgkin's lymphoma and one with diffuse large B cell lymphoma (DLBCL). One patient with chronic lymphocytic leukemia (CLL) had clinical improvement in the trial.<sup>37</sup> Several phase 1 and 2 trials are underway to evaluate the safety and efficacy of NK-92 in combination with other anticancer agents in stage II or IV Merkel cell carcinoma (ClinicalTrials.gov: NCT02465957), hematological cancers (ClinicalTrials.gov: NCT02727803), and pancreatic cancer (ClinicalTrials.gov: NCT03136406) (Table 1).

### HIGH-AFFINITY NK CELLS

The parental NK-92 cell line is dependent on exogenous IL-2 for cytotoxicity<sup>23,28</sup> and is devoid of the ADCC-mediating receptor CD16.<sup>6,7,24</sup> Hence, a novel NK-92 cell line, called high-affinity NK (haNK), has been engineered to express to high-affinity 158V FcγRIIIa (CD16) receptor as well as endoplasmic reticulum-retained IL-2.<sup>38,39</sup> Injection of haNK cells into athymic mice did not result in tumor formation, but to further ensure safety, haNK cells were irradiated to decrease the tumorigenic potential. Irradiated haNK cells continued to produce IL-2, IFNγ, and IL-8 for at least 48 h and main-

tained cytotoxicity against various cancer cell lines *in vitro*. haNK cells have high levels of granzyme and are more cytotoxic against tumor targets compared to healthy human donor NK cells.<sup>39</sup>

Several studies have shown that patients with the 158V/V FcγRIIIa (CD16) polymorphism experience a better antitumor response and increased survival upon treatment with monoclonal antibodies (mAbs) such as anti-CD20 rituximab (Rituxan) for lymphoma,<sup>40,41</sup> anti-ErbB2/HER2 trastuzumab (Herceptin) for breast cancer,<sup>42</sup> and anti-epithelial growth factor receptor (EGFR) cetuximab (Erbix) for colorectal cancer<sup>43,44</sup> and squamous cell head and neck cancer.<sup>45</sup> However, only ~10% of the population are homozygous for the high-affinity 158V allele.<sup>46</sup> In addition, the role of ADCC in cancer therapy remains controversial since ADCC has yet to be directly observed.<sup>47</sup> Nevertheless, the findings in the abovementioned retrospective studies provide a strong rationale for the combination treatment of haNK cells with tumor-targeting IgG1 antibodies. Improved haNK cell-mediated targeting of cervical, ovarian, breast, and lung cancer cell lines was observed in the presence of cetuximab, trastuzumab, or anti-HER2 pertuzumab (Perjeta) mAbs. Blocking the CD16 receptor diminished tumor cell lysis, indicating that ADCC plays a role in the antitumor activity of haNK cells in combination with tumor-associated antigen (TAA)-targeted antibodies.<sup>39</sup> Furthermore, in a CD38<sup>+</sup> NCI-H929 multiple myeloma xenograft model in NOD-*scid* IL2Rγ<sup>null</sup> (NSG) mice, combination treatment with haNK cells and anti-CD38 mAb daratumumab (Darzalex) resulted in improved survival compared to tumor-bearing mice that received haNK cells with isotype control.<sup>38</sup>

### Clinical trials with haNK cells

A phase 1 3+3 dose escalation study, with a starting dose of  $2 \times 10^9$  haNK cells per infusion, has been designed to determine the safety of haNK cell infusion in patients with metastatic or locally advanced solid tumors (ClinicalTrials.gov: NCT03027128). This study has completed enrollment but has yet to post results. An ongoing phase 2 study aims to evaluate the therapeutic effect of haNK cells with anti-programmed death ligand 1 (PD-L1) avelumab (Bavencio) and the IL-15 superagonist N-803 in Merkel cell carcinoma patients who have progressed on or within 6 months of completing treatment with avelumab or anti-programmed cell death 1 (PD-1) pembrolizumab (Keytruda) by objective response rate (ORR) using response evaluation criteria in solid tumors version 1.1 (RECIST 1.1) (ClinicalTrials.gov: NCT03853317). Another ongoing phase 1b trial (ClinicalTrials.gov: NCT03387085) evaluates the safety and efficacy of haNK cell therapy in combination with immune checkpoint inhibition, IL-15 superagonist (N-803) administration, cancer vaccines, and chemoradiation in patients with refractory, metastatic, or unresectable triple-negative breast cancer (TNBC). Preliminary results indicate that the combination is safe and tolerable with a disease control rate of 78%, overall response rate of 67%, and complete response of 22%.<sup>48</sup> Similar phase 1/2 trials evaluate the safety and efficacy of haNK cells in combination with immunotherapy and chemoradiation in metastatic colorectal cancer (ClinicalTrials.gov: NCT03563157), squamous cell carcinoma (ClinicalTrials.gov: NCT03387111), and pancreatic cancer (ClinicalTrials.gov: NCT03329248) (Table 1).

**Table 1. Clinical studies with NK-92 and haNK cells**

Trial identifier: ClinicalTrials.gov:	Disease condition	NK cell	Combination agent	Phase	Status
NCT02465957	stage IIIB and IV Merkel cell carcinoma	NK-92		phase 2	active, not recruiting
NCT02727803	myelodysplastic syndrome, leukemia, lymphoma, MM	NK-92	biological: anti-thymocyte globulin, rituximab drug: busulfan, clofarabine, cyclophosphamide, fludarabine phosphate, melphalan radiation: total-body irradiation procedure: umbilical cord blood transplantation	phase 2	active, recruiting
NCT03136406	pancreatic cancer	NK-92	drug: cyclophosphamide, oxaliplatin, capecitabine, 5-fluorouracil, leucovorin, nab-paclitaxel biological: bevacizumab, avelumab, N-803, Ad-CEA, RAS-yeast	phase 1/2	active, not recruiting
NCT03387085	TNBC	haNK	drug: doxorubicin HCl, capecitabine, cisplatin, cyclophosphamide, 5-fluorouracil, leucovorin, nab-paclitaxel biological: N-803, Ad-CEA, Ad-brachyury, Ad-MUC1, RAS-yeast, CEA-yeast, brachyury-yeast, avelumab, bevacizumab procedure: SBRT	phase 1/2	unknown
NCT03563157	metastatic CRC	haNK	drug: doxorubicin HCl, capecitabine, cisplatin, cyclophosphamide, 5-fluorouracil, leucovorin, nab-paclitaxel biological: N-803, Ad-CEA, Ad-brachyury, Ad-MUC1, yeast-RAS, yeast-CEA, yeast-brachyury, avelumab, bevacizumab procedure: SBRT	phase 1/2	active, not recruiting
NCT03387111	squamous cell carcinoma	haNK	drug: doxorubicin HCl, capecitabine, cisplatin, cyclophosphamide, 5-fluorouracil, leucovorin, nab-paclitaxel biological: N-803, Ad-CEA, Ad-brachyury, Ad-MUC1, yeast-RAS, yeast-CEA, yeast-brachyury, avelumab, bevacizumab, necitumumab procedure: SBRT	phase 1/2	active, not recruiting
NCT03329248	pancreatic cancer	haNK	biological: N-803, Ad-CEA, RAS-yeast, avelumab, bevacizumab drug: capecitabine, cyclophosphamide, 5-fluorouracil, leucovorin, nab-paclitaxel, Lovaza, oxaliplatin procedure: SBRT	phase 1/2	active, not recruiting
NCT03853317	Merkel cell carcinoma	haNK	biological: avelumab, N-803	phase 2	active, recruiting

MM, multiple myeloma; Ad, adenovirus; CEA, carcinoembryonic antigen; RAS, rat sarcoma virus; TNBC, triple-negative breast cancer; haNK, high-affinity natural killer; SBRT, stereotactic body radiation therapy; CRC, colorectal cancer; MUC1, mucin 1.

## CAR-ENGINEERED NK-92 AND haNK CELLS

### Advantages of CAR NK over CAR T cells

CAR T cells represent a cutting-edge immunotherapeutic approach that has revolutionized cancer treatment. Currently, the U.S. Food and Drug Administration (FDA) has approved four autologous CD19-directed CAR T cell therapies for the treatment of relapsed or refractory diffuse large B cell lymphoma, primary mediastinal B cell lymphoma, high-grade B cell lymphoma, and transformed follicular lymphoma,<sup>49</sup> as well as one autologous B cell maturation antigen

(BCMA)-directed CAR T cell therapy for the treatment of relapsed/refractory MM.<sup>50</sup> Despite the successes of CAR T cell therapy in the clinic, several challenges remain unaddressed. While CAR T cells have been effective in hematological cancers, they currently provide minimal therapeutic benefits in solid tumor settings.<sup>51</sup> Preparation of CAR T cells also requires an autologous source since allogeneic T cells cause graft-versus-host disease (GVHD).<sup>52</sup> Moreover, CAR T cell therapy may induce cytokine release syndrome and neurologic toxicities that can be life-threatening.<sup>53,54</sup>



NK cells provide a CAR-engineering platform that is safer and more advantageous compared to T cells.<sup>55</sup> With the absence of GVHD after allogeneic NK cell infusion<sup>34–37,56,57</sup> and the availability of immortalized NK cell lines such as NK-92,<sup>21</sup> CAR NK cells not only are a safer alternative but also one that potentially has a broader off-the-shelf clinical application not limited by individualized preparation.<sup>58</sup> Another safety advantage of CAR NK cells over CAR T cells is the type of cytokines that NK cells produce. The cytokine release syndrome induced by CAR T cells is associated with elevated levels of pro-inflammatory cytokines such as IL-6, TNF- $\alpha$ , and IL-1.<sup>54,55,59,60</sup> CAR NK-92 cells, alternatively, have a cytokine profile that is less likely to induce cytokine release syndrome. CAR NK-92 cells were reported to secrete high levels of IFN $\gamma$ , macrophage inflammatory protein (MIP)-1 $\alpha$  (CCL3), GM-CSF, and moderate levels of TNF- $\alpha$ .<sup>55,61</sup> Furthermore, persistent CAR T cells that can attack normal cells cause on-target/off-tumor effects. CD19-targeting CAR T cells can persist as memory CAR T cells and target normal B cells, which may lead to prolonged B cell deficiency.<sup>58,62</sup> In one colorectal cancer patient, HER2-targeting CAR T cells possibly recognized low levels of HER2/neu on lung epithelial cells, resulting in acute respiratory failure.<sup>63</sup> Meanwhile, irradiated parental NK-92 cells have a limited lifespan *in vivo* and do not develop memory, minimizing the risk of these side effects.<sup>28</sup> However, as a consequence, repeated and frequent infusions of irradiated NK-92 and NK-92-based cells would be required to maintain *in vivo* cell numbers.<sup>64</sup> Lastly, CAR T cells are mostly dependent on the artificial receptor for tumor targeting. The heterogenous nature of most tumors entails that a population of the malignant cells will not be recognized and attacked by the CAR T cells.<sup>65</sup> CAR NK-92 cells retain the expression of activating receptors, allowing these effector cells to detect even the tumor cells that do not express the CAR target.<sup>55</sup> In addition to natural cytotoxicity, CAR-engineered hANK cells may also have the potential to mediate ADCC in the presence of TAA-specific mAbs.<sup>38,39</sup>

Alternatively, the repeated infusion of an allogeneic cell product such as CAR NK-92 may trigger the patient's immune response and possibly limit the effect of NK-92 cell-based therapies. Hence, the development of humoral and T cell responses has to be monitored. In the phase 1 clinical trials, the formation of HLA antibodies against NK-92 cells occurred in less than half of the recipients.<sup>34,35</sup> Furthermore, mixed lymphocyte reactions using the patients' lymphocytes and irradiated NK-92 cells showed that NK-92 cells are only mild stimulators.<sup>22</sup> Whether the differences in the immune status of the patients and prior blood transfusion events contribute to the variability in host responses against NK-92 cell therapy is yet to be determined. Nevertheless, patients who develop HLA antibodies may have to avoid retreatment with NK-92 cell products beyond a 7-day window in order to avert an anamnestic response.<sup>34</sup>

#### Generation of CAR NK-92 cells

CARs are based on the T cell receptor (TCR) and are composed of an extracellular antibody single-chain variable fragment (scFv) that recognizes specific surface antigens on the tumor, a hinge, a transmembrane domain, and an intracellular signaling domain.<sup>66,67</sup> Most CAR NK-92 studies utilize first-generation CARs, which contain a single

signaling domain composed of CD3 $\zeta$ , Fc $\epsilon$ R1 $\gamma$ , or DAP-12.<sup>61,68,69</sup> CAR-modified NK-92 cells were found to lyse tumor cells more effectively than CD16-engineered NK-92 cells acting through ADCC *in vitro*,<sup>64,70</sup> which indicates that even first-generation CARs are more potent than ADCC-mediated tumor killing. Second- and third-generation CARs employ one or two costimulatory domains in conjunction with CD3 $\zeta$  to improve cytotoxic activity. The co-activating proteins are usually based on the CD28 family (CD28 or ICOS), the TNF receptor family (4-1BB, OX40, or CD27), or the signaling lymphocytic activation molecule (SLAM)-related receptor family (2B4).<sup>67,71</sup> A study comparing ErbB2-targeted NK cells expressing CD3 $\zeta$  alone, CD28/CD3 $\zeta$ , or CD137/CD3 $\zeta$  found that the second-generation constructs displayed increased cytotoxicity compared to the first-generation CAR.<sup>72</sup>

Sustained CAR expression on NK cells requires stable gene transfer. The main gene modification methods utilized to generate CAR NK cells are viral transduction and transfection.<sup>58,71</sup> Retrovirus and lentivirus-based vectors have been widely applied in the production of CAR NK cells due to stable gene integration and high transduction rates, especially in blood-derived NK cells. Transduction levels of up to 60% were achieved in peripheral blood NK cells using retroviral vectors,<sup>73</sup> while a 73% transduction efficacy was achieved using lentiviral vectors in NK cells derived from cord blood.<sup>74</sup> Viral transduction, however, carries the risk of insertional mutations that can result in oncogenesis and other adverse events. Non-viral transfection methods with either naked plasmid DNA, transposase DNA-mediated integration, or mRNA by electroporation are inexpensive and low immunogenicity alternatives to viral transduction.<sup>58</sup> In fact, electroporation with mRNA was found to result in high transfection efficiencies in NK-92 cells. This method results only in transient gene transfer and, hence, short-term CAR expression.<sup>22,74,75</sup>

#### Preclinical studies with CAR NK-92 cells

The first CAR NK-92 reported was engineered using a first-generation CAR transgene that consisted of a ErbB2 (HER2/neu)-specific scFv, a CD8 hinge, and a CD3 $\zeta$  signaling domain that was delivered via a retroviral vector.<sup>76</sup> The value of targeting ErbB2 has been demonstrated by the positive preclinical and clinical observations with ErbB2-specific antibodies and CAR T cells.<sup>77,78</sup> ErbB2-targeting CAR NK-92 cells demonstrated specific cytotoxicity toward ErbB2-expressing breast, ovarian, and squamous cell carcinoma cells *in vitro*. *In vivo*, cell therapy with ErbB2-CAR-NK-92 suppressed the tumor growth of human-ErbB2<sup>+</sup> NIH 3T3 fibroblasts in CD-1 nude mice.<sup>76</sup> ErbB2-CAR-NK-92 cells were also demonstrated to migrate and accumulate in ErbB2<sup>+</sup> tumors, further illustrating the specificity of the modified cells.<sup>79–81</sup> Second-generation ErbB2-CAR-NK-92 cells that included the costimulatory CD28 domain in addition to CD3 $\zeta$  displayed increased cytotoxicity compared to the first-generation CAR<sup>72</sup> and exhibited potent antitumor activity in glioblastoma<sup>82</sup> and breast cancer models.<sup>83</sup>

Other CAR NK-92 cells have been developed against different TAA targets and have shown *in vivo* activity in preclinical tumor models.

CAR NK-92 cells specific for B cell differentiation antigens CD19 and CD20 in CLL, B cell ALL (B-ALL), and lymphoma,<sup>84–87</sup> CD138 and CS1 in MM,<sup>88,89</sup> and CD3, CD5, and CD7 in T-ALL<sup>90–93</sup> inhibited the tumor progression of these hematological cancers in corresponding xenograft models in NSG mice. CAR NK-92 cells for various surface antigens expressed by solid tumors have also been studied. Targeting EGFR/EGFRvIII in glioblastoma,<sup>94,95</sup> epithelial cell adhesion molecule (EPCAM) in colorectal and renal cell carcinoma,<sup>96,97</sup> GD2 in neuroblastoma,<sup>98</sup> GPC3 in hepatocellular carcinoma,<sup>99</sup> mesothelin in ovarian cancer,<sup>100</sup> and prostate-specific membrane antigen (PSMA) in prostate cancer<sup>101</sup> using CAR NK-92 cells decreased disease burden and/or prolonged the survival of tumor-bearing mice. In addition to TAAs, NK-92 cells engineered with scFv that recognizes TCR peptide epitopes complexed with HLA-A2, such as gp100/HLA-A2 in melanoma and WT1/HLA-A2 in leukemia, were shown to be effective in targeting the epitope-presenting tumors.<sup>102,103</sup> Immune checkpoint molecules, which are expressed by multiple tumor types and are essential in immune evasion,<sup>104</sup> represent another optimal target for CAR NK-92. For instance, B7-H3-targeted CAR NK-92 suppressed the tumor growth of non-small cell lung cancer and improved the survival of the tumor-bearing mice.<sup>105</sup> PD-L1 targeted haNK (PD-L1 t-haNK) cell therapy is highly cytotoxic against a broad spectrum of tumor cell lines *in vitro* and induces potent anti-cancer effects in murine xenograft models of TNBC, bladder cancer, and lung cancer.<sup>64,106</sup> Overall, these pre-clinical data demonstrate the potential therapeutic benefit of CAR NK-92 cell therapy and, therefore, provide a strong rationale for the application of this novel cell therapy in the clinic.

#### Clinical trials with CAR NK-92 cells

The first-in-human clinical trial using an off-the-shelf CAR NK-92 targeted CD33 in patients with relapse or refractory AML (ClinicalTrials.gov: NCT02944162).<sup>107</sup> This CD33 CAR NK-92 cell line was generated via lentiviral transfection and utilized a third-generation CAR involving costimulatory molecules CD28 and CD137. The highest dose administered was  $5 \times 10^9$  cells and even at this level, no grade 3–4 adverse events were observed. However, all three patients suffered from infusion-related fever that abated after a day or two. In a similar phase 1 study using CD33-targeted CAR T cells for treatment of relapsed or refractory AML (ClinicalTrials.gov: NCT03126864), grade 3 cytokine release syndrome and grade 2 neurotoxicity syndrome were observed in one of the three patients.<sup>108</sup> The CD33 CAR NK-92 cells were detected 1 week post-infusion in all three patients, suggesting that irradiated CAR NK-92 cells can persist, albeit in the short term, *in vivo*.<sup>107</sup> Although no obvious clinical efficacy was observed, this phase 1 study showed that CAR NK-92 cell therapy may be used safely in patients and may provide an off-the-shelf treatment option for AML and/or other cancers.<sup>107</sup> Other CAR NK-92 cell products being clinically tested for safety in hematological cancers target CD7 (ClinicalTrials.gov: NCT02742727) and CD19 (ClinicalTrials.gov: NCT02892695) in leukemia and lymphoma, and BCMA (ClinicalTrials.gov: NCT03940833) for MM (Table 2).

A phase 1 3+3 dose escalation trial evaluating the safety, maximum tolerated dose (MTD), and response to a second-generation CAR

NK-92 (NK-92/5.28.z) specific for ErbB2 in glioblastoma (ClinicalTrials.gov: NCT03383978) is underway (Table 2).<sup>109</sup> In this study, patients with recurrent or refractory ErbB2-positive glioblastoma are administered with irradiated CAR NK-92 cells intracranially during relapse surgery. As of 2019, administration of the first two dose levels ( $1 \times 10^7$  and  $3 \times 10^7$  cells) concluded and no dose-limiting toxicities were observed. The highest dose planned for this trial is  $1 \times 10^8$  cells. Once dose escalation is completed, a planned expansion cohort would include patients who will receive up to 12 additional injections of the ErbB2-CAR-NK-92 cells into the resection cavity through an implanted catheter and reservoir. Peripheral blood and cerebrospinal fluid will be collected and analyzed for soluble factors and cells to determine the effects of the CAR NK on endogenous immune cells over the course of therapy.<sup>109</sup>

Another TAA being used as a CAR NK-92 target is mucin 1 (MUC1), an aberrantly glycosylated transmembrane glycoprotein overexpressed in a variety of epithelial cancers.<sup>110</sup> A MUC1-CAR-NK-92 has been engineered by lentiviral gene transfer to express third-generation anti-MUC1 CAR with CD28/CD137 signaling moiety and has been shown to lyse MUC1<sup>+</sup> tumor cells *in vitro* and *in vivo*.<sup>111</sup> In a phase 1 clinical trial, 13 patients with MUC1<sup>+</sup> expressing lung cancer, pancreatic cancer, colon cancer or ovarian cancer were enrolled and received  $1 \times 10^9$  cells per infusion (ClinicalTrials.gov: NCT02839954; Table 2). No severe cytokine release syndrome and/or bone marrow suppression were reported, indicating that MUC1-CAR-NK-92 can be safely applied as therapy against different solid tumors. Furthermore, some minor clinical response was observed. Nine patients (69.2%) presented stable disease, and one patient had progressive disease.<sup>111</sup>

Based on preclinical data indicating the potency of PD-L1 t-haNK cells against a broad range of tumors,<sup>64,106</sup> PD-L1 t-haNK cells have entered a phase I clinical trial to evaluate the safety and preliminary efficacy of these CAR-modified high affinity NK cells in locally advanced solid tumors and metastatic cancer (ClinicalTrials.gov: NCT04050709). This study will determine the maximum tolerated dose and designate the recommended dose for future phase 2 studies. PD-L1 t-haNK cells are also being studied in combination with other anticancer agents in several phase 1/2 trials. In a phase 2 trial, the efficacy of PD-L1 t-haNK with immune checkpoint inhibitor and N-803 is being determined in patients with solid tumors that have progressed and/or relapsed after checkpoint inhibitor therapy (ClinicalTrials.gov: NCT03228667). The primary and secondary outcomes will address objective response rate, survival, and incidence of adverse events. A similar combination with PD-L1 t-haNK, N-803, and anti-PD-1 (pembrolizumab) will be used to treat patients with advanced-form gastric or head and neck cancer in an upcoming phase 2 trial (ClinicalTrials.gov: NCT04847466). In patients with pancreatic cancer, the comparative efficacy and overall safety of PD-L1 t-haNK is being evaluated in combination with chemoradiation and N-803 (ClinicalTrials.gov: NCT04390399). Based on the positive initial results observed with haNK cells in combination with N-803 and low-dose chemotherapy in refractory TNBC,<sup>48</sup> a phase 1B/2 study

**Table 2. Clinical studies with CAR-expressing NK-92 and haNK cells**

Trial identifier: ClinicalTrials.gov:	Antigen target	Disease condition	NK cell	Combination agent	Phase	Status
NCT02944162	CD33	AML	NK-92		phase 1/ 2	unknown
NCT03940833	BCMA	MM	NK-92		phase 1/ 2	active, recruiting
NCT02742727	CD7	leukemia, lymphoma	NK-92		phase 1/ 2	unknown
NCT02892695	CD19	leukemia, lymphoma	NK-92		phase 1/ 2	unknown
NCT03383978	ErbB2	glioblastoma	NK-92		phase 2	active, recruiting
NCT02839954	MUC1	hepatocellular carcinoma, pancreatic carcinoma, glioma, gastric carcinoma, NSCLC, TNBC, CRC	NK-92		phase 1/ 2	unknown
NCT04050709	PD-L1	advanced solid tumor, metastatic tumor	haNK		phase 1	active, not recruiting
NCT03228667	PD-L1	NSCLC, SCLC, HNSCC, RCC, CRC, urothelial carcinoma, Merkel cell carcinoma, melanoma, gastric cancer, cervical cancer, hepatocellular carcinoma	haNK	biological: N-803, pembrolizumab, nivolumab, atezolizumab, avelumab, or durvalumab	phase 2	active, not recruiting
NCT04847466	PD-L1	GEJ cancer, advanced HNSCC	haNK	biological: N-803, pembrolizumab	phase 2	not yet recruiting
NCT04390399	PD-L1	pancreatic cancer	haNK	biological: N-803 drug: aldoxorubicin HCl, nab-paclitaxel, gemcitabine, cyclophosphamide, 5-fluorouracil, leucovorin, irinotecan liposome procedure: SBRT	phase 2	active, recruiting
NCT04927884	PD-L1	TNBC	haNK	biological: N-803 drug: sacituzumab govitecan-hziy, cyclophosphamide	phase 1/ 2	not yet recruiting

AML, acute myeloid leukemia; BCMA, B cell maturation antigen; MM, multiple myeloma; MUC1, mucin 1; NSCLC, non-small cell lung cancer; TNBC, triple-negative breast cancer; CRC, colorectal cancer; haNK, high-affinity natural killer; PD-L1, programmed death ligand 1; SCLC, small cell lung cancer; HNSCC, head and neck squamous cell carcinoma; RCC, renal cell carcinoma; GEJ, gastroesophageal junction.

has been planned to evaluate the efficacy of sacituzumab govitecan-hziy (Trodelvy) with PD-L1 t-haNK cell therapy, cyclophosphamide, and N-803 in patients with TNBC after at least two prior regimens for metastatic disease (ClinicalTrials.gov: NCT04927884). Trodelvy is a Trop-2-directed antibody and topoisomerase inhibitor drug conjugate that is approved by the FDA for the treatment of TNBC patients with metastatic disease who have received at least two prior therapies.<sup>112</sup> So far, no preliminary data have been posted on any of the PD-L1 t-haNK cell therapy studies (Table 2).

#### FUTURE PERSPECTIVES

Despite the tremendous potential of CAR NK-92 cells, several issues need to be addressed to inform better construction and application of this therapy. First, most of the CAR constructs being utilized in the generation of CAR NK-92 were designed for CAR T cells and may not be optimal for NK cells. For instance, NK cells do not naturally express CD28, and the function of this costimulatory molecule in CAR NK cells remains unclear.<sup>113</sup> Furthermore, in YTS cells, CAR containing DAP12 outperformed a CAR that included CD28/CD3ζ, suggesting that DAP12 may be a better signaling moiety for NK

cells.<sup>68</sup> Second, the effects of an immunosuppressive environment on CAR NK-92 cells need to be further elucidated. In addition, methods to interfere with or redirect these negative signals present a scheme to enhance the antitumor capabilities of CAR NK-92 cells. In fact, a CAR NK-92 has been engineered to convert the immunosuppressive signal induced by TGF-β into an activating signal.<sup>114</sup> Lastly, to increase the potency of CAR NK-92 cells especially against solid tumors, tumor infiltration must be promoted. Engineering chemokine receptors, such as CXCR4, into CAR NK-92 cells may enhance chemotaxis to the tumor.<sup>69</sup>

#### CONCLUSIONS

CAR NK-92 cells provide an off-the-shelf therapeutic platform that could be readily available and broadly applicable. Numerous preclinical studies suggest that CAR NK-92 cells have great promise as an effective cellular immunotherapy against a broad range of tumor types. The concluded NK-92 phase 1 studies and, importantly, initial reports on various CAR NK-92 cell therapies indicate that CAR NK-92 treatment strategy is safe for clinical use. Upcoming clinical data will illuminate the therapeutic potential of this CAR NK-92 and

expound on the feasibility of combining this cell therapy with other anticancer agents. Advances in CAR NK-92 technology guarantee that the field will evolve and progress, potentially resulting in improved clinical outcomes for cancer patients, especially those with limited treatment options.

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#### AUTHOR CONTRIBUTIONS

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#### DECLARATION OF INTERESTS

The authors declare no competing interests.

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