Immunology & Cell Biology 2023; **101**: 705–726
www.wileyonlinelibrary.com/journal/icb

REVIEW

Advances in natural killer cell therapies for breast cancer

Seyedeh Zahra Fotook Kiaei[1], Alireza Nouralishahi[2], Mohammad Ghasemirad[3], Maryam Barkhordar[4], Sasan Ghaffari[5], Hadis Kheradjoo[6], Mahshid Saleh[7], Saman Mohammadzadehsaliani[8,a] & Zahra Molaeipour[9,a]

1 Department of Pulmonary and Critical Care, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran
2 Eye Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
3 Department of Periodontics, Faculty of Dentistry, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
4 Hematology, Oncology and Stem Cell Transplantation Research Center (HORCSCT), Tehran University of Medical Sciences, Tehran, Iran
5 Department of Immunology, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, USA
6 Laboratory Department, Buraimi Hospital, Buraimi, Oman
7 Wisconsin National Primate Research Center, University of Wisconsin Graduate School, Madison, WI, USA
8 Ophthalmology Department, Buraimi Hospital, Buraimi, Oman
9 Hematology Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Keywords
Adoptive cell therapy, breast cancer, CAR-NK cells, NK cells

Correspondence
Zahra Molaeipour, Hematology Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
E-mail: zmolaeipour@gmail.com

Saman Mohammadzadehsaliani, Ophthalmology Department, Buraimi Hospital, Buraimi, Oman.
E-mail: dr_saliani@yahoo.com

[a]Equal contributors.

Received 21 March 2023;
Revised 28 April and 5 May 2023;
Accepted 6 May 2023

doi: 10.1111/imcb.12658

Immunology & Cell Biology 2023; **101**: 705–726

Abstract

Breast cancer (BC) is the most common cause of cancer death in women. According to the American Cancer Society's yearly cancer statistics, BC constituted almost 15% of all the newly diagnosed cancer cases in 2022 for both sexes. Metastatic disease occurs in 30% of patients with BC. The currently available treatments fail to cure metastatic BC, and the average survival time for patients with metastatic BC is approximately 2 years. Developing a treatment method that terminates cancer stem cells without harming healthy cells is the primary objective of novel therapeutics. Adoptive cell therapy is a branch of cancer immunotherapy that utilizes the immune cells to attack cancer cells. Natural killer (NK) cells are an essential component of innate immunity and are critical in destroying tumor cells without prior stimulation with antigens. With the advent of chimeric antigen receptors (CARs), the autologous or allogeneic use of NK/CAR–NK cell therapy has raised new hopes for treating patients with cancer. Here, we describe recent developments in NK and CAR–NK cell immunotherapy, including the biology and function of NK cells, clinical trials, different sources of NK cells and their future perspectives on BC.

INTRODUCTION

According to the Global cancer burden reported in 2020, 2.26 million women were diagnosed with breast cancer (BC), with the American Cancer Society recently estimating BC to account for almost 32% of all cancers among women in 2023 in the USA,[1] making it the most common type of cancer in women. Among women, BC is the second leading cause of cancer death, accounting for 15% of cancer mortalities.[2] The treatment approach for BC can be stratified based on the status of three hormone receptors. The majority (65%) of BCs are progesterone receptor–positive, and about 80% of BCs are estrogen receptor positive.[3,4] If BC cells are positive for either or both of these receptors, they are hormone receptor–positive and hormone therapy would be a viable treatment modality. If not, BC cells are hormone receptor negative with poor prognosis. Human epidermal growth

factor receptor 2 (HER2) is the third receptor that is present in about 20% of BCs.[3] The upside is that HER2^{+} BC cells are also responsive to treatment similar to hormone receptor–positive BC. In about 10–20% of patients with BC, tumor cells are categorized as being negative for both hormone receptors and HER2. With the absence of focused therapeutic alternatives, this disease, known as triple-negative BC, is well regarded as having the worst prognosis.[5,6] It is necessary to make numerous treatment decisions for patients with BC in accordance with the tumor's morphology; grade; metastases; size and the expression of estrogen receptors, HER2 and progesterone receptors.[7–9] The usual treatment for estrogen receptor–positive/progesterone receptor–positive BC involves blocking these receptors with hormone therapy drugs such as tamoxifen or preventing hormone production altogether with aromatase inhibitors such as anastrozole and letrozole.[4,10]

A growing understanding of cancer immunology has led to the development of immunotherapy—the use of immune cells directly or by manipulation of their activity—as a therapeutic approach.[11] By activating immune responses, immunotherapies are designed to initiate an active or passive antitumor response against cancer.[12,13] A famous branch of cancer immunotherapy is adoptive cell therapy, in which immune cells are reprogrammed to attack tumor cells with greater precision than conventional chemo/radiation therapy and surgery. To date, several cancer immunotherapies have been employed in clinical practice including adoptive cell therapy of natural killer (NK) cells and NK T cells.[14–17] NK cells are cytotoxic innate lymphocytes capable of lysing malignant or virally contaminated cells.[18] Cancerous cells can be indirectly eliminated by NK cells *via* influencing immune system cells.[11] To distinguish between normal and malignant cells, NK cells have developed a variety of inherent strategies. It is noteworthy that the destruction of tumor cells by NK cell is independent of major histocompatibility complex I (MHC) molecules and antibodies.[19] The expression of MHC-I on the surface of tumor cells is commonly reduced or deleted to avoid detection by tumor-invading cytotoxic T lymphocytes.[19] The absence of MHC-I expression is detectable by NK cell inhibitory receptors. Even if the diseased cell expresses sufficient MHC-I to inhibit NK cell cytotoxicity, NK cells can circumvent this by detecting stress-triggered self-ligands.[20] The influence of NK cells on the function of multiple immune cells, including dendritic cells, macrophages, T cells, and B cells, confirms their immunomodulatory effects. These immune cells interact with one another to create a variety of cytokines, growth factors, and chemokines.[21,22] In this review paper, we discuss the anticancer benefits of NK cells in BC and the several sources of NK cells used in clinical trials.

NATURAL KILLER CELLS' ACTIVATION MECHANISM

The innate immune system acts rapidly and nonspecifically to prevent the spread of foreign pathogens. Complement activation, cytotoxic molecule release and immune cell stimulation are all ways to achieve innate immunity.[23] NK cells are large, granular lymphocytes that play extremely important roles in the innate immune system. They defend the body against viruses, parasites, bacteria and perhaps most significantly, tumor cells.[21] NK cells are identified by CD56 expression and the lack of CD3, which is a T-cell marker.[24] They can be further divided based on CD56 intensity and CD16 (FcγRIIIA) expression. The two major subsets are CD56$^{dim/bright}$ and CD16$^{dim/bright}$ NK cells, each with distinct functions. NK cells fight tumors through direct cytotoxicity or the release of proinflammatory cytokines or cytolytic granules. Almost 90% of NK cells are CD56^{dim}.[25] Cell-mediated apoptosis or the release of cytotoxic molecules, such as perforins and granzymes, are the main means of killing target cells. Another NK cell–mediated cytotoxic response is antibody-dependent cell-mediated cytotoxicity (ADCC) induction, in which CD16 binds to the crystallizable fragment (Fc) site of target cell–attached immunoglobulin G to trigger cell lysis. Activating the TNF-related apoptosis-inducing ligand or Fas death receptors, which induce classical apoptosis, is another alternative. CD56^{bright} is an immune regulator generally involved in cytokine/chemokine production, including tumor necrosis factor-α and interferon (IFN)-γ, which helps bridge innate and adaptive immunity.[26–28]

In addition to providing meaningful clinical responses for patients, ADCC might be a useful mechanism for therapeutic antibodies. As compared with affinity- and valency-optimized NK cell engagers, monoclonal antibodies are not sufficient to fully leverage ADCC potential.[29] An array of bispecific, trispecific and multispecific NK cell–engaging constructs is being investigated. These include trispecific killer engager and bispecific killer engager molecules that engage CD16 in NK cells.[30,31] NK cell–mediated responses have been enhanced and prolonged by these novel engagers through targeting several different activating NK cell receptors.[15,32] The trispecific molecule is generated by the interaction between the interleukin-15 (IL-15) cytokine and the two antibody domains.[33] trispecific killer engager molecules function as cytokine signals to promote NK cell growth while also inducing targeted NK cell–mediated death of tumor targets.[34] Trispecific killer engagers improve

NK cell–mediated killing, activation and expansion by creating an antigen-specific synapse, which overcomes the nonspecific processes of normal NK cell cytotoxicity.[35] The outstanding efficacy and substantial antitumor effects of HER2–trispecific killer engager especially in ovarian cancer were demonstrated by Vallera *et al.*[35]

Inhibitory and activating receptors present on NK cells, which are the "nuts and bolts" of NK cell function, strongly influence their function.[36] MHC-I molecules determine whether a cell is killed or spared by NK cells. Normal, healthy cells present "self" antigens to NK cells *via* MHC-I molecules, which are recognized by killer immunoglobulin–like receptors (KIRs) and inhibit NK cell activation. Leukocyte-like immunoglobulin receptors, KIRs and type C receptors are also NK cell inhibitory receptors.[37,38] Tumor cells or virally infected cells that express low or no MHC-I receptors inhibit KIR activity on NK cells. Stress-induced cells show a reduction in MHC-I expression and an increase in molecules that further activate NK cells. While the lack of MHC-I alone to block KIR-mediated NK cell inhibition is insufficient to stimulate NK cells, the presence of additional cytotoxicity receptors can stimulate them as a second signal (Figure 1).[39] The inhibitory KIR receptors regulate NK cell activities by interacting with particular self-HLA class I ligands, a process called licensing or education in the NK cells' maturation program.[20,40] The license gives NK cells the capacity to find, identify and kill stressed target cells that have lost HLA class I molecules as a result of viral infection or tumor transformation. Lack of inhibitory KIR–HLA connections causes hyporesponsive or anergic NK cells.[41–43] Two groups of patients with BC and controls exhibited similar distributions of four inhibitory KIR–HLA class I ligand combinations, suggesting that patients may generate functionally active NK cells equivalent to controls. The functional activity of mature NK cells can be reset by changing HLA environments in tumor tissue with reduced HLA class I expression. This is a mechanism tumors acquire to resist adaptive immune responses.[44,45] However, NK licensing is not completely permanent.[44] Ashouri *et al.*[46] analyzed KIR and HLA polymorphisms in 162 patients with BC and 278 healthy controls. According to their findings, an immunotherapeutic approach for BC may involve using autologous activated NK cell clones with specific KIR–HLA compositions that favor antitumor activity.

The natural cytotoxicity receptors NKp30, NKp46 and NKp44, as well as CD16 and natural killer group 2

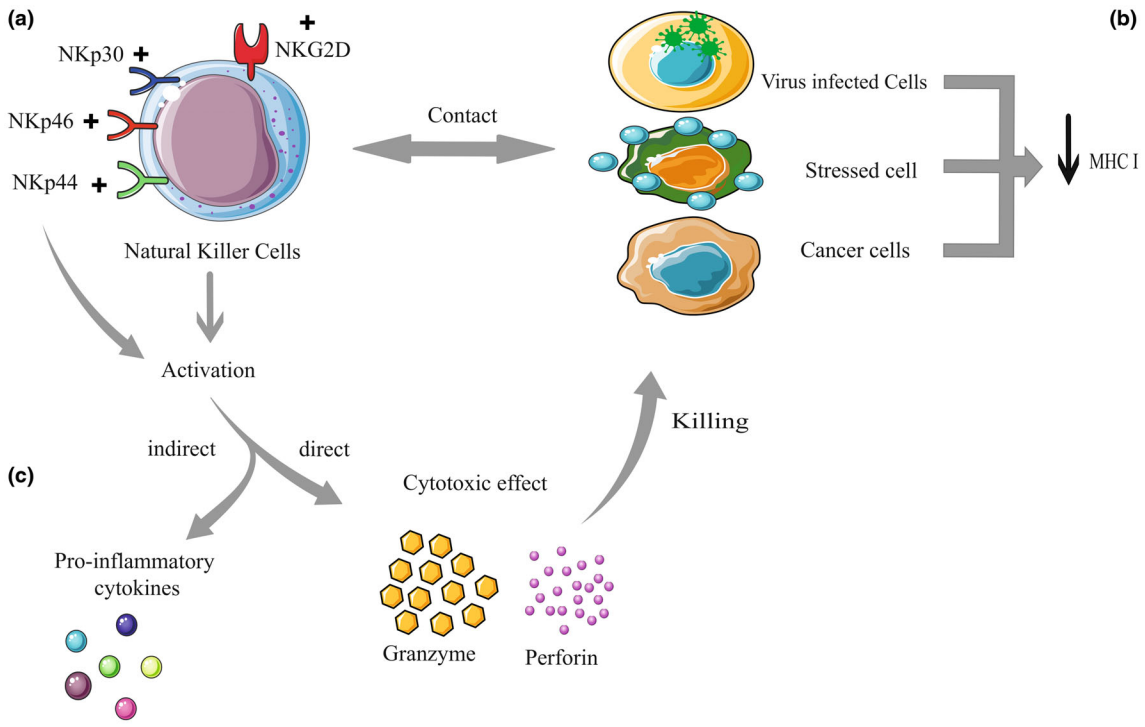

Figure 1. Natural killer (NK) cell activation process. **(a)** NK cells' activation signals go through the natural cytotoxicity receptors NKp30, NKp46 and NKp44, as well as CD16 and natural killer group 2 member D (NKG2D), which are the main NK cell activating receptors. **(b)** Virus-infected cells, stressed cells and cancer cells, which express low or no major histocompatibility complex I (MHC-I) receptor, trigger NK cells. **(c)** NK cells exert their antitumor function through direct cytotoxicity or the release of proinflammatory cytokines or cytolytic granules.

member D (NKG2D), are the main NK cell activating receptors. Other molecules, such as DNAX accessory molecule 1 (DNAM-1), 2B4 and NKp80, increase NK cell activity.[47] In contrast to NKp46 and NKp30, which are expressed on all NK cells, only NKp44 is specifically expressed on IL-2–activated NK cells.[48] These receptors directly activate NK cells' cytotoxicity and cytokine production.[49] NK cells frequently express NKG2D and DNAM-1 (CD226). MHC-I homologs MICA/B and ULBP (cytomegalovirus UL16-binding protein) are two examples of NKG2D ligands in humans.[50] In response to the recognition of these ligands, NKG2D receptors are activated by the adapter protein DNAX-activating protein 10 (DAP10).[51] CD112 and CD155, which are abnormally expressed in cancer cells, are detected by DNAM-1.[52] In the event of low NKG2D ligand levels, DNAM-1 ligand expression by cancer cells becomes responsible for NK cell–mediated death.[53]

BREAST CANCER AND NATURAL KILLER CELLS

As NK cells are innate cells, unlike T cells, they can eliminate tumor cells without prior antigen sensitivity or clonal proliferation. Through their antitumor activity, NK cells are essential in monitoring cancer immunity.[54,55] According to studies, NK cell elimination has been linked to an increased risk of cancer. When the expression of MHC-I molecules on target cells is reduced, NK cells become antitumor in function.[56–58] Furthermore, tumor cells with positive regulation of stress-induced molecules such as NKG2D are more likely to be killed by NK cells.[59] NKG2D is one of the most prominent activating receptors on NK cells whose ligands (NKG2DL) are almost exclusively expressed on tumor or virus-infected cells.[60] In addition, NK cells have been shown to boost T-cell penetration, thereby triggering immunological responses by releasing chemokines and cytokines.[61,62] Antimetastatic action is also a feature of NK cells, and they are likely to eliminate circulating tumor cells.[62,63] In a study on patients with BC, the expression of active receptors (NKG2D, CD16, DNAM-1 and NKp30) on NK cells decreased while that of the inhibitory receptor (NKG2A) increased, and this malfunction of NK cells directly impaired NK cell cytotoxicity.[64] The precise process through which innate immune system members, specifically NK cells, interact with BC cells remains complex and obscure. Several substances secreted by BC cells in the tumor microenvironment (TME) are thought to have a role in this complicated physiological response to tumor cells. The TME is thought to be involved in several processes, including tumor growth, progression and metastasis. Most crucially, the TME inhibits the immune system's anticancer activities, resulting in immune suppression and tumor development.[65,66] In the TME, suppressive cytokines are present that counteract the protective functions of NK cells. Tumor growth factor (TGF-β), released by regulatory T cells (Tregs), tumor cells and other stromal cells, is one of the protumorigenic cytokines secreted in TME. TGF-β directly and indirectly inhibits NK cell activity. It lowers IFNγ production as well as NKG2D and NKp30 surface expression. In addition, TGF-β attaches to TGFBR1 (TGF-β receptor type 1) and TGFBR2 (TGF-β receptor type 2) subunits that transduce the message of phosphorylated SMAD2 and SMAD3 proteins and interact with SMAD4, forming a heterotrimeric transcriptional structure. SMAD proteins are the critical signal transducers for TGF-β superfamily receptors. TGF-β activates NK cells and converts them to NK–group 1 innate lymphoid cell, the intermediate cell types, which are weaker cytolytic cells by default compared with NK cells. This results in less effective cancer surveillance and ultimately paves the path for cancer evasion.[67] In addition, TGF-β promotes metabolic dysfunction of circulating NK cells in individuals with metastatic BC. To enhance NK cell–based immunotherapies, blocking TGF and/or glycoprotein A repetitions predominant can restore NK cell metabolism and function.[68] Glycoprotein A repetitions predominant is a transmembrane receptor mostly found on the surface of Tregs and platelets which regulates the activation and bioavailability of TGF-β1 and is implicated in NK cell dysfunction.

IL-6 and IL-10 are other immunosuppressive cytokines.[69,70] IL-6 disrupts NK cell function by activating the signal transducer and activator of transcription 3 (STAT3) signaling pathway, leading to a decrease in natural cytotoxicity receptor expression.[70] IL-10 promotes tumor cell proliferation and metastasis through immunosuppression. IL-10–mediated immunosuppression is caused by the production of IL-12, IL-1, tumor necrosis factor and chemokines, as well as the downregulation of CD80 and CD86 molecules. It has been reported that IL-10 can both promote and prevent tumor growth.[71] It appears that IL-10's ability to modulate the immune response is influenced by both the TME and the amount of IL-10 receptors on immune cells.[72] TME is characterized by metabolic dysregulation that results in NK cell dysfunction. For instance, lactate levels rise in the TME that suppresses the proliferation of cytotoxic T cells and NK cells and lowers their cytokine output.[73] TME-mediated acidification in hepatic metastasis has also been reported to lead to NK cell apoptosis.[74] The survival and effective performance of NK cells are limited because of low pH, low nutrient concentrations and hypoxia in the TME, and NK cells

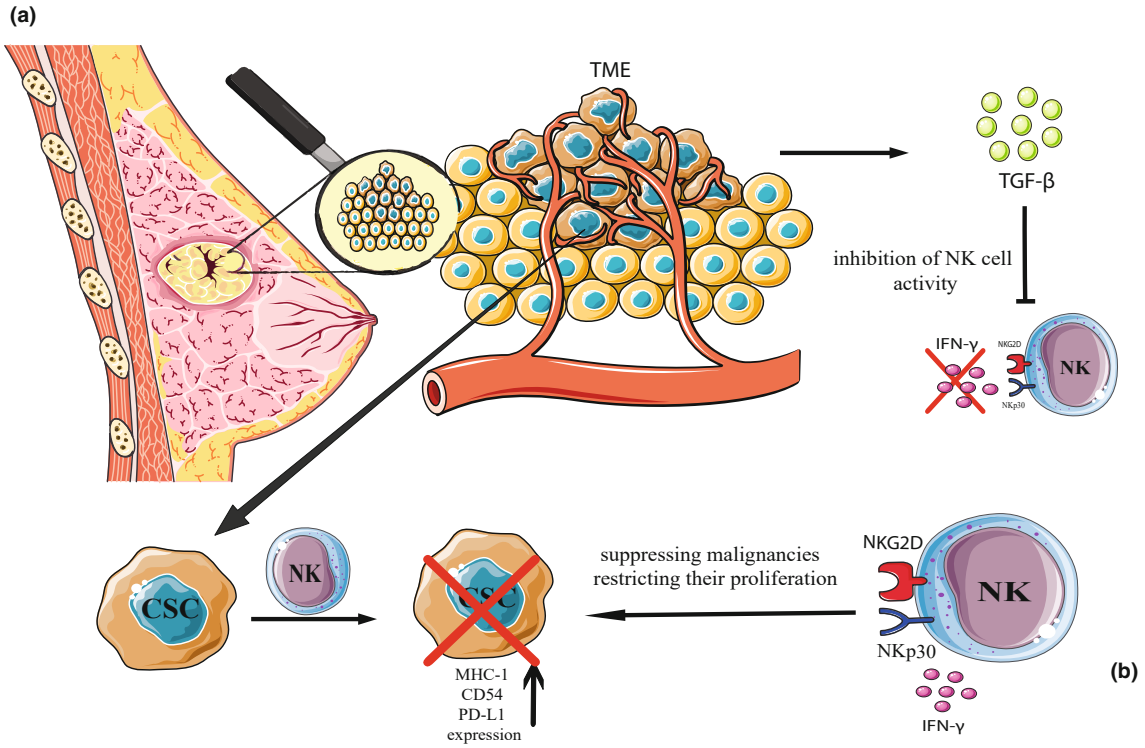

Figure 2. Interactions of natural killer (NK) cells in breast cancer with cancer stem cells (CSCs) and the tumor microenvironment (TME). Suppressive cytokines such as transforming growth factor (TGF)-β in the TME negate the protective functions of NK cells. **(a)** TGF-β lowers interferon alpha (IFNα) production as well as the surface expression of natural killer group 2 member D (NKG2D) and NKp30. **(b)** NK cells differentiate CSCs by boosting major histocompatibility complex I (MHC-I), CD54 and programmed death-ligand 1 (PD-L1) expression, inhibiting tumor development and metastasis.

have less penetration and lower toxicity in solid tumors as a result.[74] Abnormal angiogenesis in solid tumors causes hypoxic conditions that reduce cytokine production and activated receptor expression and lead to NK cell degranulation. All of these factors reduce NK cell toxicity. Therefore, immunotherapy can target hypoxic conditions to improve the function of NK cells.[75] Their effect on cancer stem cells (CSCs) demonstrates NK cells' ability to target tumor cells. CSCs are undifferentiated cells that are key to tumor formation. They are defined by low constitutive CD54 and programmed cell death protein 1 (PD-1) expression levels and high CD44 expression.[76] These characteristics improve CSC susceptibility to NK cell attack and increase chemotherapy resistance.[76] NK cells differentiate CSCs by boosting MHC-I, CD54 and programmed death-ligand 1 (PD-L1) expression and inhibit tumor development and metastasis.[76] This demonstrates the critical role of NK cells in suppressing malignancies and restricting their proliferation. In comparison, other studies indicated that CSCs in BCs are resistant to NK cell activity, implying that additional research is necessary to determine the interactions between NK cells and BC stem cells (Figure 2).[76] There is little information available regarding cancer cells' ability to reprogram NK cells and their transformation into a premetastatic condition.[77] Chan *et al.*[78] showed that NK cells respond to K14-positive cancer cell accumulations in the lungs. The basal epithelial marker K14 indicates the presence of highly migrating cells in cancer and their growth potential.[79] Some studies have been conducted regarding the expression and necessity of K14 in highly metastatic BC cells that result in systemic diffusion, cluster invasion and colonization of distant organs.[80,81] Invading cells with positive K14 markers escape immune surveillance by not expressing MHC-I molecules.[82] This indicates that these invading cells respond to NK cell targeting.[77]

NK CELL–BASED THERAPY FOR BREAST CANCER

A multitude of research published in the past few years has demonstrated that NK cells are potentially beneficial in treating malignancies.[83] Numerous pitfalls regarding

NK cell metabolism are being addressed in studies to improve their survival and antitumor potential in the TME of solid tumors, including BC. Injection of ILs, such as IL-2 or IL-15, improves NK cell survival.[84] According to a previous study, cord blood NK cells transduced with a retroviral vector that produces IL-15 significantly increased their function.[85] These strategies are thought to enhance the clinical delivery of this medicine and help overcome severe limitations associated with current chimeric antigen receptor (CAR)-T-cell therapies.[86] Numerous research groups have recently demonstrated that infusing mouse and human NK cells preactivated with an IL-15/12/18 cocktail increases and enhances sustained anticancer activities both *in vitro* and *in vivo*.[87–89] In addition to these cytokines, inhibiting GSK3 kinase with CHIR99021 (an aminopyrimidine derivative) stimulates the *ex vivo* maturation of human peripheral blood (PB) NK cells and enhances their antitumor capabilities *in vitro* and *in vivo*. Human NK cells expanded with IL-15 with GSK3 inhibition expressed significantly higher CD57 cell maturity markers and transcription factors associated with the final stages of NK cell maturation, such as zinc finger E-box-binding homeobox 2 (ZEB2), T-box expressed in T cells (T-bet) and B lymphocyte-induced maturation protein-1 (BLIMP-1) than those expanded with IL-15 alone. When expanded human NK cells were combined with Herceptin, an anti-HER2 antibody, they demonstrated increased ADCC and tumor control in a mouse ovarian cancer xenograft transplantation model.[90] Recent investigations have revealed an increase in the percentage of NK cells in women with HER2/neu$^-$ tumors (the most prevalent subtype), but no change in the percentage of NK cells in patients with HER2/neu$^+$ tumors was reported.[91] Chemokines released by lymphocytes attract circulating NK cells to cancerous areas, and the effect of these cells begins with an imbalance of signals sent by surface receptors.[92,93] In normal cells, ligands for NK cell activation receptors are poorly expressed. However, aberrant cell proliferation in cancer cells contributes to DNA replication stress and genomic instability. This results in the production of DNAM-1 and NKG2D ligands in stressed cells.[94] The expression of NKG2D and DNAM-1 receptors on NK cells also affects their ability to lyse leukemia, myelodysplastic syndrome, multiple myeloma and ovarian cancer cells.[53,95,96] To avoid detection by CD8$^+$ T cells, cancer cells decrease MHC-I molecule production, which stimulates NK cells and activates them.[97,98] NK cells produce and release perforins and granzymes immediately in the synaptic cleft, initiating apoptosis, whereas ligand-mediated apoptosis occurs later.[97] Although NK cells have weak penetration and cytotoxic capacity in the TME, they are essential for carcinogenesis suppression. As cancer development is connected to NK cell dysfunction, increasing NK cell activity is critical for anticancer immunity.[49]

Activation of NK cells after injection into the body is difficult, despite their high numbers.[98] In addition, for NK cell therapy to fulfill its therapeutic potential, both a sufficient number of and high-quality NK cells are required. According to Sawasdee *et al.*,[99] the use of doxorubicin in combination with NK cells increases NK cell activity. They also investigated how doxorubicin therapy modulated FasR protein (CD95) expression and the ability of NK cells to kill cancer cells.[99] This study showed that doxorubicin could be utilized as an adjuvant therapy with other cellular immunotherapies to boost immune cell function and combat rapidly proliferating cancer cells and their robust microenvironment. In addition, FasR/FasL signaling is crucial for immune cell functionality. The development and application of combination chemoimmunotherapy and immunomodulation for the treatment of BC are supported by other studies.[99]

PRECLINICAL AND CLINICAL STUDIES ON THE APPLICATIONS OF NK CELLS IN CANCER TREATMENT

NK cell dysfunction and reduction of its cytotoxic molecules lead to tumor growth and metastasis.[100–102] Studies have shown that large numbers of tumor-infiltrating NK cells are associated with a better prognosis in solid cancers, including breast, lung, head, neck, liver and colon cancers.[103,104] Recent studies have reported that an increase in activating receptors in NK cells is associated with improved outcomes in patients with prostate cancer and BC.[105,106] The effectiveness of NK cells in solid tumors remains limited, despite their success in hematologic malignancies. This may be related to the solid tumor's suppressive microenvironment, which is associated with impaired detection, penetration, activation and cytotoxic functions of NK cells.[107] CAR-modified NK cells can potentially overcome this. In contrast to CAR-T cells, CAR–NK cells do not proliferate in response to external stimuli.[108]

To date, the selective transfer of autologous NK cells has been explored in a variety of solid tumors with limited clinical benefit. This may be explained by the host immune system being suppressed, as the inhibitory receptors on autologous NK cells match molecules on the tumor cell surface.[109,110] In many clinical studies, NK cells were activated in the *ex vivo* environment, which reduced CD16 expression and rendered them unsuitable for ADCC (Table 1).[111] Therefore, Tian and colleagues[112] showed that Herceptin decreases CD16 expression on NK

Table 1. Current clinical trials in NK cell therapy on breast cancer.

Number	Title and sponsor	Trial ID	Location	Design	Primary outcome	Recruitment status	Phase
1.	ACE1702 in subjects with advanced or metastatic HER2-expressing solid tumors Sponsor: Acepodia Biotech Inc.	NCT04319757	Texas, USA Chicago, Illinois, USA	A phase I, open-label, dose-escalation study of ACE1702 cell immunotherapy in patients with advanced or metastatic HER2-expressing solid tumors (n = 36).	• Adverse events, including DLTs and serious adverse events • Phase Ib/II starting dose for ACE1702	Recruiting July 28, 2021	I
2.	FT500 as monotherapy and in combination with immune checkpoint inhibitors in subjects with advanced solid tumors Sponsor: Fate Therapeutics	NCT03841110	Texas, California, Minnesota, New Jersey, USA	An open-label, nonrandomized trial; FT500 as monotherapy and in combination with immune checkpoint inhibitors in patients with advanced solid tumors (n = 37).	• The incidence of patients with DLTs within each dose-level cohort	Recruiting October 25, 2021	I
3.	Supervised exercise to promote infiltration of NK-cells into the tumor Sponsor: VU University Medical Center	NCT04704856	The Netherlands	An open-label, randomized, supervised exercise to promote infiltration of NK cells into the tumor (n = 20).	• Participation rate • Successful examined biopsies rate	Recruiting January 12, 2021	Not applicable
4.	FATE-NK100 as monotherapy and in combination with monoclonal antibody in subjects with advanced solid tumors Sponsor: Fate Therapeutics	NCT03319459	Texas, California, Minnesota, Ohio, USA	A single-dose, open-label, dose-escalation study. FATE–NK100 as monotherapy and in combination with monoclonal antibody in patients with advanced solid tumors (n = 44).	• Incidence of DLTs	Completed November 22, 2021	I
5.	Clinical study on anti-tumor effect induced by activated primary natural killer (NK) cells Sponsor: Xuanwu Hospital, Beijing	NCT03634501	Beijing, China	An open-label, single-group clinical study on the antitumor effect induced by activated primary NK cells.	• Incidence of toxicity induced by NK infusion	Recruiting January 11, 2019	I II
6.	CAR-pNK cell immunotherapy in MUC1-positive relapsed or refractory solid tumor Sponsor: PersonGen BioTherapeutics (Suzhou) Co., Ltd.	NCT02839954	Jiangsu, China	An open-label, single-group clinical study evaluating the efficacy and safety of chimeric antigen receptor–modified pNK cells in MUC1-positive advanced refractory or relapsed solid tumor (n = 10).	• AEs attributed to the administration of the anti-MUC1 CAR–pNK cells	Unknown December 6, 2016	I II

(*Continued*)

Table 1. Continued.

Number	Title and sponsor	Trial ID	Location	Design	Primary outcome	Recruitment status	Phase
7.	NK cell infusions with trastuzumab for patients with $HER2^+$ breast and gastric cancer Sponsor: National University Hospital, Singapore	NCT02030561	Singapore, Singapore	An open-label, single-group clinical study; a phase I/II study of expanded, activated, autologous NK cell infusions with trastuzumab for patients with $HER2^+$ breast and gastric cancer (n = 29).	• Number of participants with serious and nonserious adverse events • Duration of tumor response measure • Time-to-event outcome measure	Unknown June 22, 2016	I II
8.	Allogeneic NK cells for ovarian, fallopian tube, peritoneal and metastatic breast cancer Sponsor: Masonic Cancer Center, University of Minnesota	NCT01105650	Minnesota, USA	An open-label, single-group clinical study, lympho-depleting chemotherapy and T-cell suppression followed by allogeneic NK cells and IL-2 in patients with recurrent ovarian, fallopian tube, primary peritoneal cancer and advanced metastatic breast cancer (MT2009-30; n = 13).	• Response rate	Completed December 28, 2017	II
9.	A pilot surveillance study to monitor NK cells and circulating tumor cells in women with previously treated non-metastatic triple negative breast cancer and women with previously treated non-metastatic breast cancer with a confirmed BRCA mutation. Sponsor: Cynvenio Biosystems	NCT02639832	California, USA	Observational; the LiquidBiopsy device will test for cells with tumor cell markers in blood. The genetic sequence of DNA-recovered samples will be studied. Using NK Vue researchers will test for NK cell activity (n = 210).	• Presence of cell-free tumor DNA and/or circulating tumor cells from a blood sample • NK cell activity levels	Unknown August 8, 2016	—
10.	Immunotherapy combined with capecitabine *versus* capecitabine monotherapy in advanced breast cancer Sponsor: The First People's Hospital of Changzhou	NCT02491697	China	An open-label, randomized, randomized controlled trial comparing dendritic cells cocultured with cytokine-induced killer cells immunotherapy combined with capecitabine *versus* capecitabine monotherapy in advanced breast cancer (n = 400).	• Overall survival	Not yet recruiting February 23, 2016	II

(*Continued*)

Table 1. Continued.

Number	Title and sponsor	Trial ID	Location	Design	Primary outcome	Recruitment status	Phase
11.	A study of combinations of D-CIK immunotherapy and anti-PD-1 in refractory solid tumors Sponsors: Sun Yat-sen University	NCT02886897	Guangdong, China	An open-label, single-group clinical study; a phase II clinical trial to investigate the safety, clinical activity and toxicity of combinations of D-CIK and anti –PD-1 antibody in patients with treatment-refractory solid tumors (n = 60).	• Progression-free survival	Unknown September 1, 2016	I II
12.	QUILT-3.067: NANT triple negative breast cancer (TNBC) vaccine: molecularly informed integrated immunotherapy in subjects with TNBC who have progressed on or after standard-of-care therapy. Sponsor: ImmunityBio, Inc.	NCT03387085	California, USA	This was a phase Ib/2 study to evaluate the safety and efficacy of metronomic combination therapy in patients with TNBC who have progressed on or after previous SoC chemotherapy. Phase II will be based on Simon's two-stage optimal design.	• Incidence of treatment-emergent AEs and SAEs, graded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03 • Objective response rate by RECIST	Active, not recruiting March 29, 2022	I II
13.	A study of Sacituzumab with chemoimmunotherapy to treat advanced TNBC after prior therapies Sponsor: ImmunityBio, Inc.	NCT04927884	California, USA	This is a phase Ib/2 open-label study to evaluate the safety and efficacy of sacituzumab govitecan-hziy in combination with chemoimmunotherapy (cyclophosphamide, N-803 and PD-L1 t-haNK) in patients with triple-negative breast cancer (TNBC) after at least two prior treatments for metastatic disease.	• Phase Ib: maximum tolerated dose or highest tolerated dose. Determine the maximum tolerated dose or highest tolerated dose and designate a recommended phase II dose • Phase Ib: safety profile of sacituzumab plus chemoimmunotherapy. Incidence of DLTs, treatment-emergent AEs and SAEs • Phase II: objective response rate per RECIST version 1.1. Phase II primary endpoint	Active, not recruiting March 31, 2022	I II

(*Continued*)

Table 1. Continued.

Number	Title and sponsor	Trial ID	Location	Design	Primary outcome	Recruitment status	Phase
14.	A study of allogenic NK cells in combination with trastuzumab and pertuzumab in adult patients with refractory metastatic Her2 positive breast cancer. NK-ACT-BC_2020 Sponsor: Vall d'Hebron Institute of Oncology	NCT05385705	Barcelona, Spain	A phase Ib study with a safety lead-in cohort and expansion phase of the safety, tolerability, biological effect and efficacy of allogenic NK cells in combination with trastuzumab and pertuzumab in adult patients with refractory metastatic HER2-positive breast cancer.	• Nature and frequency of AEs • Nature and frequency of SAEs • Treatment-limiting toxicity • Alterations in clinical laboratory test results • Alterations in electrocardiogram results • Alterations in vital sign measurements • Alterations in physical examination findings • Alterations in assessment of ECOG	Recruiting June 1, 2022	I

AE, adverse event; D-CIK, dendritic and cytokine-induced killer cell; DLT, dose-limiting toxicity; ECOG, Eastern Cooperative Oncology Group; HER2, human epidermal growth factor receptor 2; IL, interleukin; NK, natural killer; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; pNK, precursor natural killer; RECIST, Response Evaluation Criteria in Solid Tumors; SAE, severe adverse event; SoC, site of care; t-haNK, targeting high-affinity natural killer; TNBC, triple-negative breast cancer.

cells *via* monoclonal antibodies and used it in a patient with BC. They demonstrated that the relative response of a patient with progressive metastatic $HER2^+$ BC receiving Herceptin-treated NK cells (5.9×10^9, 3.9×10^9, 8.1×10^9 and 6.5×10^9) was associated with increased activity and proliferation of Herceptin-mediated NK cells *in vitro*.[112] In this pilot study, the authors reported that Herceptin increases the population of cytotoxic NK cells by interacting with them. By doing so, these cells migrate and cause cytotoxicity to $HER2^+$ cancer cells. This study demonstrated the therapeutic potential of Herceptin-treated NK cells in patients with BC who cannot tolerate Herceptin.[112] In another trial, Geller *et al.*[113] employed allogeneic NK cells to treat patients with recurrent ovarian and BC. The cells were delivered intravenously 2 days following the administration of fludarabine. On the day of the NK cell injection, patients started to receive subcutaneous IL-2 injections three times a week. They received a lymphatic drainage preparation regimen consisting of 525 mg m^{-2} fludarabine and 260 mg kg^{-2} cyclophosphamide, and seven patients received 200 cGy total body irradiation to enhance host immune suppression. The mean dosage of NK cells per kilogram was 2.16×10^7 cells.[113] In this study, the short persistence of donor NK cells was during the patients' lymphodepletion period under the preparation regimen. However, after 14 days, the host's T cells regenerated. This indicates that the factors induced by the TME may inhibit the proliferation of the donor NK cells.[113] The proliferation of NK cells transplanted to the patient may be limited by factors including effector T cells, myeloid-derived suppressor cells and recipient Tregs.[114–118] According to Geller and his colleagues,[113] half of the patients had significantly increased Treg proliferation. Hence, effective adaptive cell therapy requires Tregs depletion.

An 11-year prospective cohort study of healthy individuals discovered that poor NK cytotoxicity was related to a high risk of cancer development. Increased levels of tumor-infiltrating NK cells have been linked to favorable outcomes in patients with colorectal and gastric carcinomas, as well as lung squamous cell carcinoma, indicating that NK cell infiltration is an excellent prognostic factor.[119] Clinical investigations with autologous NK cells have revealed them to be a nontoxic but also relatively ineffective therapy, which may be related to NK cell suppression by self-MHC-I molecules. As a result, allogeneic therapy may offer a more effective treatment option. In 2002, it was demonstrated for the first time that alloreactive NK cells play a direct role in eliciting the antitumor effect of hematopoietic stem cell (HSC) transplantation.[120] NK cells promoted graft healing by supporting graft-*versus*-leukemia and

suppressing graft-*versus*-host disease (GvHD), mainly when a KIR ligand mismatch between the donor and host was found. It is suggested that GvHD reduction is achieved by the recipient's antigen-presenting cell lysis while the graft-*versus*-leukemia impact persists. These effects were then replicated in an animal model utilizing acute myelocytic leukemia (AML)-positive nonobese diabetic/severe combined immunodeficiency mice injected with alloreactive NK cells. The clearance of tumors demonstrated the importance of NK cells in maintaining graft-*versus*-leukemia effectiveness.[121]

Miller and colleagues[122] later established NK cell therapy in a clinical trial by injecting allogeneic NK cells into patients with metastatic melanoma, renal cell carcinoma, Hodgkin lymphoma and AML, plus subcutaneous IL-2. This study found that NK cells transplanted from a haploidentical donor can proliferate *in vivo*. *In vivo*, NK cells expand only in a high-dose preconditioning regimen (Hi-Cy/Flu). NK cell infusions were found to be feasible and safe, in addition to demonstrating complete remission in 5 of the 19 patients with poor prognoses.[122] Furthermore, the efficacy of haploidentical NK cell therapy in patients with refractory diseases was increased by host Tregs depletion using IL-2 diphtheria toxin fusion, which inhibits Tregs' immunosuppressive activity.[84] In addition to HSC transplantation, NK cell alloreactive potential might be used in different settings. Studies conducted on patients with glioma and malignant neuroblastoma, for instance, demonstrated that NK cell infusion was safe and somewhat effective.[123,124] Thus, individuals with different types of cancer can benefit from NK cell immunotherapy, as well as routine clinical trials, including pancreatic, lung, head and neck, breast and kidney carcinomas.[125] Therefore, activated NK cells can regulate tumor growth and prevent the rapid spread of metastatic cancers through immunological monitoring mechanisms. NK cells were isolated and grown in the laboratory using K562-mb15-41BBL cells and then reinjected intravenously into patients at doses of 1×10^6 cells kg^{-1}, 1×10^7 cells kg^{-1}, 5×10^7 cells kg^{-1} and 1×10^8 cells kg^{-1} in a study. NK cell therapy in combination with trastuzumab was well tolerated in these patients.[126] Although the number of NK cells did not increase in the patients, the phenotype of these cells changed significantly: for example, an increase in the CD56 marker and a decrease in the CD16 marker. It should be noted that autologous NK cells were expanded and activated, with no engineering changes to increase their toxicity.[126] Thus, allogeneic NK cells are not suppressed by self-MHC and may have stronger ADCC than autologous NK cells.[127]

In a study by Liang and colleagues,[128] the clinical results of allogeneic and autologous NK cell transplantation in patients with recurrent BC were compared. In this study, 36 patients with relapsed BC were transplanted with NK cells intravenously for 30 min on days 13–15. They showed that cell therapy using allogeneic NK cells has better clinical effectiveness than using autologous cells. It also significantly improved the patients' clinical outcome and immune system function, as well as reducing the number of circulating tumor cells.[128]

THE ADVANCES OF CHIMERIC ANTIGEN RECEPTOR NK CELL IMMUNOTHERAPY

Following the advent of CARs and the Food and Drug Administration approval of Kymriah and Yescarta CAR-T cells in 2017, adoptive cell therapy was revitalized.[129,130] CAR-T cells have shown great success by inducing complete remission and satisfactory overall survival in high percentages of patients with relapsed or refractory B-cell acute lymphoblastic leukemia[129,130] and chronic lymphocytic leukemia.[131,132] Investigating CAR–NK cell potential in oncotherapy was inevitable. CARs are synthetic receptors that recognize a certain antigen with their single-chain variable fragment (scFv) of the extracellular domain and transmit an activation signal to the immune cells upon which they are mounted *via* their intracellular signaling domain.[133,134] CAR itself is not cytotoxic but helps traffic the modified cells to the tumor site.[133,134] The scFv itself is composed of the heavy-chain variable region and the light-chain variable region of a monoclonal antibody that create the extracellular portion of CAR when attached to a hinge region. The transmembrane domain (e.g. NKG2D) fixes the construct to the cell, and the intracellular domain transmits the stimulatory signal to the immune cell. The latter consists of a costimulatory domain (e.g. 2B4, CD28, 4-1BB) and a signaling domain (e.g. CD3ζ, DAP10, DAP12). The costimulatory domain is crucial in activating the immune cell which, although not as optimized as CAR-T cells, is more potent when it is specifically NK tailored.[133,134] As mentioned, the lectin-like NKG2D receptor is instrumental in cytotoxic response, which it owes to the DAP10 signaling adaptor molecule.[60] DAP12 transmits the signal of NKG2C and NKp44 to downstream proteins of NK cells. This makes the fusion of the adaptor molecules with their corresponding receptor a viable strategy to produce potent CAR–NK constructs.[135] CAR–NK cells are defined in a sequential, physical order, for instance, HER2 scFv–(NKG2D)–2B4–DAP10. Unlike conventional T cells that require the presentation of antigen peptides by MHCs of an antigen-presenting cell, CAR-T cells are able to bypass the need for MHC molecules for target cell recognition. With NK cells'

similar inherent ability, CAR–NK cells can utilize two non-MHC-I–restricted recognition procedures.[135] After obtaining NK cells from any of the available sources, the CAR transgene is transferred to the cells either virally (retroviral or lentiviral) or nonvirally (DNA or messenger RNA electroporation). After confirming high-efficiency transduction *via* flow cytometry, the cells are expanded to large numbers and ready for infusion.

The MHC independence of NK cells, their established role in tumor suppression, their rapid response and their direct and indirect cytotoxicity prompted researchers to equip them with CARs for target therapy. Compared with CAR-T cells, CAR–NK cells are cheaper to produce and less time-consuming, can be derived from multiple sources and possess a range of cytotoxic abilities.[135] One of the main selling points of CAR–NK cells is their safety in relation to GvHD, neurotoxicity and cytokine storms.[136] Because of a lack of an endogenous T-cell receptor, NK cells are less likely to cause acute/chronic GvHD, a common problem with T-cell–based immunotherapies.[136] These features make investigating CAR–NK cells as an allogeneic cell therapy worthwhile.

The CAR aspect of CAR–NK cells redirects NK cells for a more directed approach. The target antigens are the same as CAR-T cells. CD19, for instance, is the most common target antigen for CAR-T and CAR–NK cells. HER2 (ErbB2) is a prime target for CAR–NK cell therapy. Before CARs became mainstream, Kruschinski *et al.*[137] engineered and retrovirally transduced "chimeric receptors" in primary NK cells that were functional against breast and ovarian cancer cell lines (HER2 scFv–CD28–CD3ζ). This was one of the first preclinical studies of CAR–NK cells in solid tumors, which was preceded by another study on CD19 B-acute lymphoblastic leukemia cell lines.[138] The BC preclinical studies that followed used either PB[137,139] or NK-92 cell lines[140–142] as a source. NK-92 cells are an enticing substitute for endogenous NK cells because of their high cytotoxicity against tumor cells, which they probably owe to a lack of MHC-responsive inhibitory receptors.[143] Anti-ErbB2 CAR NK-92 cells are cytolytic for ErbB2-expressing BC cells without prior sensitization, and their function and receptor expression are not affected by CAR expression. These cells suppress BC cells' growth *in vivo* as well as reducing lung metastasis and increasing IFNγ secretion.[140] Similarly, satisfactory results were achieved in another study where intravenously injected ErbB2-directed CAR NK-92 cells accumulated in ErbB2-positive breast carcinoma xenografts compared with unmodified NK cells.[141] These cells retained specific cytotoxicity against target cells after γ-irradiation as well. γ-Irradiation of cell products in phase I trials is a precautionary safety measure to prevent the engraftment and proliferation of the injected cells.[144] Epidermal growth factor receptor (HER1) is a potential target for triple-negative BC tumors because it is overexpressed in at least half of the patients.[145] The *in vitro* and *in vivo* cytotoxicity of epidermal growth factor receptor–directed CAR NK-92 cells[142] and PB-derived CAR–NK cells[139] toward triple-negative BC cells has been demonstrated. Furthermore, preclinical studies have demonstrated the feasibility of CAR–NK combination therapy for the treatment of BC. Chen *et al.*[142] showed how a combinational administration of anti–epidermal growth factor receptor CAR NK-92 cells and oncolytic herpes simplex virus 1 eradicated BC cells more efficiently *in vitro* and in an intracranial mouse model of BC brain metastasis. Interestingly, tumor cell lysis was more robust when the cancer cells were treated with CAR NK-92 cells first and oncolytic herpes simplex virus 1 second. Viruses aggregate and gradually lyse target cells which, in this case, could have reduced the surface area of tumor cells and hindered CAR NK-92 cells' function.

Although preclinical studies are proof-of-concept for CAR–NK therapy, clinical studies are needed to first determine the safety and efficacy of this treatment and then to determine whether it would be worth investing in. With the exception of coronavirus disease-19 (NCT04324996), CAR–NK trials are exclusively focused on its application in patients with cancer. Looking into the registered clinical trials shows that the CAR–NK cancer therapy is still in its infancy. As of January 2023, we found 44 entries for CAR–NK in the clinicaltrials.gov database, 15 of which have focused on solid tumors and none on BC. Considering the TME of hematological malignancies, most novel therapies are prioritized for leukemia/lymphoma first. Except for one trial registered in 2009, three trials were registered in 2016 (same location), three in 2017, two in 2018 and seven in 2019, the rest were registered in the past 4 years, and the increasing number of trial registrations per year shows a trend of growing popularity. The status of 12 and 3 of the trials is unknown and withdrawn, respectively. The rest are still recruiting patients which is understandable given the recent date of most trials. Unsurprisingly, only one trial is in the second phase, and the rest are in phase I or phase I/II; 2 of the 44 trials are reported as completed (NCT05563545 and NCT00995137), but we found no published results, and 1 trial (NCT03415100) published its experiment on a xenograft mouse model.[146] The first-in-human trial (NCT02944162) of CAR–NK therapy was a safety test of administering three doses of CD33-directed irradiated CAR NK-92 cells every couple of days in three patients with relapsed or refractory AML after salvage chemotherapy.[147] The patients tolerated doses of up to 5×10^9 cells with minimal side effects,

GvHD or cytokine release syndrome, but one patient relapsed, one died and the third was unresponsive.[148] The next trial (NCT03415100) was unique in that the NK-specific DAP12 signaling domain was used instead of T cells' CD3ζ, and the NKG2D ligand–directed CAR–NK cells were sourced from PB NK cells of three patients with refractory colorectal cancer.[148] Two of the patients received autologous CAR–NK, and the other had haploidentical CAR–NK cells, and direct infusion of the cells into the malignant site greatly reduced tumor burden in all three patients. The most recent trial (NCT03056339) published its phase I/II results, and 7 of the 11 patients with $CD19^+$ relapsed or refractory hematological malignancies achieved complete remission after receiving cord blood–derived, partially matched CAR–NK cells.[147] GvHD, neurotoxicity and cytokine release syndrome were not seen in any of the patients. CAR–NK cells persisted for up to 12 months regardless of the infusion dose. Interestingly, responding patients had significantly higher CAR–NK expansion within the first 28 days than nonresponders. Apart from the unsatisfactory results of the first trial, which could be attributed to the aggressive nature of AML,[147] the published results of the trials show both the safety and efficacy of CAR–NK therapy. In all three trials, retroviral, lentiviral or messenger RNA transfer of CAR and local or systemic infusion of CAR had minimal side effects.[147–149] All three trials used different NK cell sources. This greatly supports the idea of off-the-shelf CAR–NK cell therapy, and with minimal side effects from mismatched donors, mass production of such cells could further reduce manufacturing costs. Although none of the trials enrolled patients with BC, with the positive results from solid tumors, and the progress in controlling BC reportedly stagnated,[150] employing CAR–NK therapy in breast tumors is a viable strategy.

Despite showing great promise, CAR–NK therapy suffers from some drawbacks as well. Indeed, CAR–NK cells have lower transduction rates, low *in vivo* persistence and are more susceptible to stress during *in vitro* production stages than CAR-T cells. As a result of the inherent, intracellular defense of NK cells against viruses, the transfer of the CAR gene into NK cells is a major obstacle. This results in subpar transduction efficacy.[151] One of the first *in vitro* studies to produce functional anti-CD19 CAR–NK cells used retroviruses for transduction.[138] Viral transduction of CAR provides a longer, more stable expression which offers persistent modified immune cells to prevent relapse. However, it might allow the population of CAR–NK cells to grow uncontrollably. This phenomenon is especially life-threatening in CAR-T cell therapies when T cells' overzealous cytokine production causes toxicities such as cytokine release syndrome and immune effector cell–associated neurotoxicity syndrome.[152] Although such side effects are negligible based on clinical studies, they should not be ignored. With messenger RNA transfection, a short-lived CAR–NK product can be produced, which allows for a controlled, safety test. However, larger cell doses must be injected in shorter intervals. The safety and tolerability of irradiated NK-92 cells were established long ago,[153,154] but their aggressiveness may also act as a double-edged sword. One flaw with using NK-92 cells is that since their parental cells are derived from malignant cells, they have to be irradiated in order not to overexpand in the host. The downside of irradiation is that it represses the cytotoxic capacity and proliferation of CAR NK-92 cells, causing their function and numbers to drop only days after infusion.[147] The short life of infused NK cells is exacerbated by the lack of autologous IL-2 secretion. Other sources of CAR–NK cells with moderate expansion, suicide genes introduction (e.g. inducible caspase 9[149])—previously demonstrated with CAR-T cells[155]—or transient CAR expression by using messenger RNA transfection should be studied to ascertain the optimal approach. While some interpret the fleeting CAR–NK presence in the patient as an advantage, the ever-present modified immune cells can inhibit any remaining tumor cells beyond the minimal residual disease to prevent relapse. A successful adoptive cell therapy is greatly dependent on the long-term persistence of the modified cells to eliminate any residual tumor cells and prevent relapse, a condition previously satisfied by long-lasting memory CAR-T cells that remain quiescent unless they are exposed to tumor antigens. As recently reported in an elegant study where two currently in-remission patients with chronic lymphocytic leukemia who had received CART19 therapy more than a decade ago, the presence of $CD4^+$ CAR-T cells with memory phenotypes was the main reason remission was sustained for more than 10 years.[156]

Future studies on the efficacy and cost-effectiveness of CAR–NK therapy, especially compared with its more mainstream cousin CAR-T cells, will judge its worth.

SOURCES OF NK CELLS FOR CLINICAL USE

NK cells mature and enter the bloodstream from the bone marrow, lymph nodes, the spleen, tonsils and the thymus.[157] A clinically relevant number of functional NK cells capable of surviving *in vivo* is required for NK cell immunotherapy to be effective. Numerous attempts have been made to produce higher numbers of NK cells from various sources. The cells can be isolated from cord blood or PB. However, because NK cells represent only

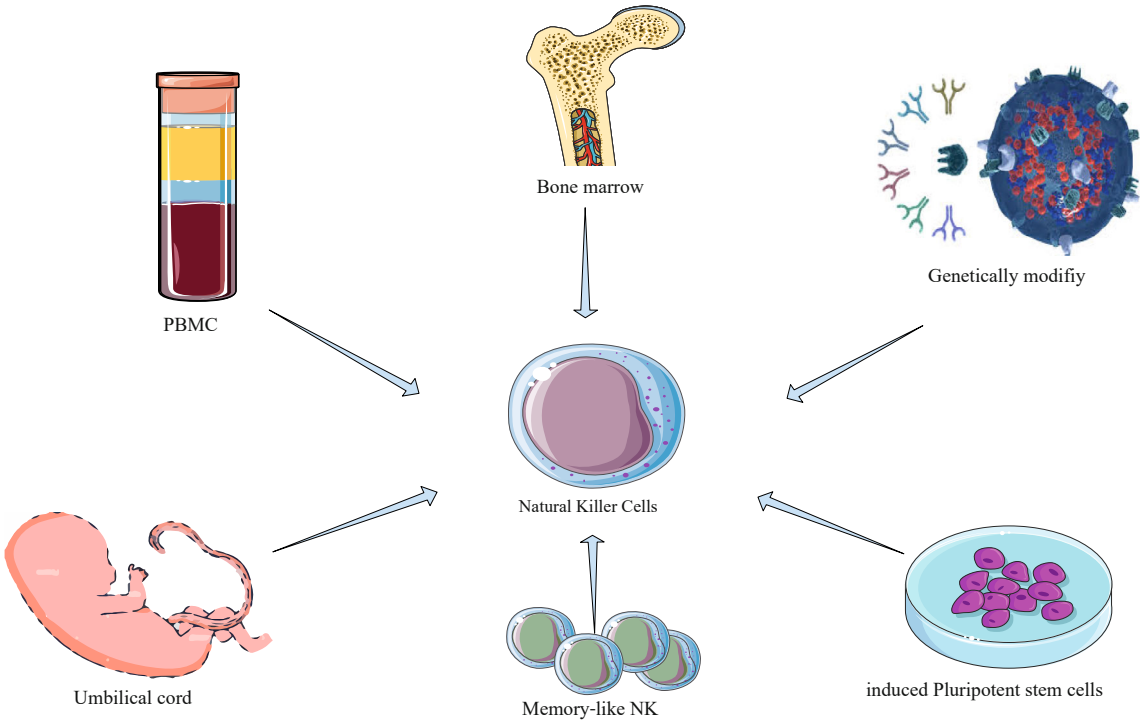

Figure 3. Allogeneic sources of natural killer (NK) cells, peripheral blood mononuclear cell (PBMC), bone marrow, genetically modified NK cells, induced pluripotent stem cells and umbilical cord. Numerous attempts have been made to produce higher numbers of NK cells from various sources. There are multiple strategies for raising cell count, including *in vitro* expansion of cells using various cytokine combinations with or without a nutritional layer, NK cell lines and derivation of NK cells from hematopoietic stem cells.

10% of circulating lymphocytes in the PB and 30% of lymphocytes in the cord blood,[158,159] the quantity of recovered cells is limited, and this method is not suitable for multiple injections. There are multiple strategies to raise cell count, including *in vitro* expansion of cells using various cytokine combinations with or without a nutritional layer, NK cell lines and the development of NK cells from HSCs (Figure 3).[125]

Umbilical cord blood–derived NK cells

Umbilical cord blood (UCB) is a rich source of HSCs, which differentiate into a variety of therapeutic cells, including NK cells. By selecting $CD34^{+}$ cells from cord blood, HSCs can be isolated using the CliniMACS method for therapeutic applications.[19] NK cells constitute the $CD3–CD56^{+}$ population of UCB cells and are categorized as immature NK cells ($CD56^{bright}$) or mature NK cells ($CD56^{dim}$). UCB has a high $CD56^{bright}$-to-$CD56^{dim}$ NK cell ratio than PB, according to studies.[158,160] UCB-derived NK cells are younger and more proliferative than PB-derived NK cells.[160] In addition, because of the low number of immature T cells in UCB, GvHD incidence is significantly diminished.[122,161] One study discovered that when UCB-derived $CD56^{bright}$ NK cells were treated with IL-12 and IL-18, they exhibited enhanced CD69 expression and produced significantly more IFNγ than PB-NK cells.[162] Fewer NK cells are generated from UCBs because of the UCB unit's modest volume.[157] UCB-derived NK cells had lower cytotoxicity against K562 cells than PB-derived NK cells because granzyme B in cord blood-NK cells is deficient.[163] According to published studies, UCB-derived NK cells express less CD16, DNAM-1, KIRs, IL-2R, NKG2C and granzyme B but more NKG2A, resulting in decreased effectiveness in killing target cells compared with PB-derived NK cells.[157,163] However, when these cells were activated with cytokines, their cytotoxicity was comparable to that of PB-derived NK cells.[160,164]

Peripheral blood–derived NK cells

PB-NK cells are predominantly $CD56^{dim}$ cells that are highly cytotoxic to target cells.[165,166] In comparison, roughly 2–10% of PB-NK cells are $CD56^{bright}$ with limited cytotoxicity, which interact with dendritic cells and T cells to participate directly in acquired immunity.[165,166] In general, NK cells are present in small

numbers in peripheral blood mononuclear cells. As a result, researchers have concentrated on optimizing NK cell expansion *in vivo* and *in vitro* under good manufacturing practice conditions for immunotherapy.[167,168] A phase I clinical trial evaluated the anticancer effects and safety of autologous peripheral blood mononuclear cell–derived NK cells in patients with advanced metastatic cancers and hematologic malignancies.[169] Large-scale, *ex vivo* generation of alloreactive NK cells suitable for multiple infusions has been described in patients with AML.[170]

Stem cells and induced pluripotent stem cells

Another approach for producing large numbers of functional cells is to differentiate NK cells *in vitro* from induced pluripotent stem cells or HSCs. Frozen bone marrow, human embryonic stem cells, cord blood stem cells and PB stem cells have been used to differentiate frozen HSCs into NK cells *in vitro*.[125] Human NK cells can be generated from bone marrow–derived $CD34^+$ hematopoietic progenitor cells cultured under specific conditions, such as IL-2 plus an allogeneic feeder cell layer or IL-2 plus additional growth factors such as IL-15 or c-kit ligand, or in a long-term culture system dependent on brain stroma.[171] In addition, human embryonic stem cell– and induced pluripotent stem cell–derived NK cells have several advantages over PB-derived NK cells, including successful genetic modification and increased *in vivo* survival. However, ethical concerns exist regarding collecting cells from embryos as young as 5–7 days old.[171]

Natural killer cell lines

NK cell lines are excellent sources of allogeneic therapeutic NK cells. Seven NK cell lines have been identified, including NK-YS, NK-92, NKL, HANK-1, KHYG-1, YT and NKG.[171] The anticancer activity of NK-92, NKL, KHYG-1 and NKG has been validated. The other three cell lines (YT, HANK-1 and NK-YS) are being used to investigate the biological aspects of leukemia/Epstein–Barr virus–related lymphomas. NK-92 cells are a safe and effective screening method to study patients with advanced malignancies, melanoma and renal cell carcinoma. The NK-92 cell line is currently the only NK cell line in clinical trials and may serve as a basis for future NK cell–based immunotherapies.[172] NK-92 cells are a convenient replacement for endogenous NK cells. Because of the lack of suppressive KIR receptors (except for KIR2DL4), these CD16-positive cells are more cytotoxic against a wide range of tumor cells and are unreactive toward normal cells.[143] The fact that they lack NK cells' antiviral defense mechanism and are consequently more transducible incentivizes their use over NK cells.

NK cell lines have the unique advantage of being easily stored and subsequently expanded to large numbers under good manufacturing practice conditions. Their anticancer activity could be further enhanced as well.[173] Kotzur *et al.*[143] showed that it is possible to produce NK-92 cells quicker, cheaper and easier while still maintaining their ability to cause cytotoxicity. This makes them even easier to use and supports the therapeutic and research utility of these cells. Thus, selective transfer of registered cell lines that exhibit broad antitumor activity provides more feasible quality control and bulk manufacturing technique in clinical trials.

Memory-like NK cells

The cytokine-induced memory-like (ML) NK cells that are activated with IL-12, IL-15 and IL-18 show robust antitumor responses and can successfully induce complete remissions in patients with leukemia.[174] The induction of remissions in patients with AML by ML NK cells with the predominant NKG2A checkpoint expression, which is phenotypically different from the *in vivo* conventional NK cells, has been deemed safe and efficient and is regarded as a new route to promote CAR–NK cell treatments.[88,175] As compared with conventional NK cells, ML NK cells respond more rapidly and effectively to a variety of triggers, including cancer cells.[88,176] Results from preclinical experiments suggest that human ML NK cells engineered to express 19-CARs (19-CAR-ML NK cells) have significantly improved responses to typically NK-resistant B-cell lymphoma malignancies *in vitro* and *in vivo*, offering a novel method for treating blood cancer through cell therapy.[174] *In vivo*, the developed ML CAR–NK cells showed higher activation receptors *versus* myeloid leukemia and longer survival without the usual KIR–KIR ligand interactions, which is noteworthy.[88,177] PB-derived ML-NKs with a shortened CD19–CAR transduction showed dramatically increased IFNγ production and degranulation, broader identification and targeted killing against NK-resistant lymphoma.[174]

ADVANTAGES AND DISADVANTAGES OF ALLOGENEIC AND AUTOLOGOUS NK CELL SOURCES

Autologous NK cells are a viable option because of their ease of obtaining, the absence of immunological suppression and minimal GvHD risk. However, increasing NK cell numbers in the PB by inhibiting self-HLA molecules does not produce the therapeutic

response expected in patients with hematologic malignancies, renal cell carcinoma and metastatic melanoma.[98,178,179] Unlike autologous NK cells, allogeneic NK cells are not restricted by a tumor's expression of HLA molecules. This significantly boosts their anticancer activity in the patient.[120,180] In addition, autologous NK cells' proliferation and functional state are limited compared with allogeneic NK cells, because these cells are frequently obtained from patients who have previously received aggressive treatment.[113] Unlike autologous cells, allogeneic NK cells have the advantage of sharing KIR–KIR mismatch ligands between host and donor cells; however, these therapies are associated with a significant risk of GvHD, which can result in severe tissue damage in the patient.[181] It is also difficult to evaluate the anticancer effects of injected autologous NK cells in patients. The reason for this is that it is difficult to distinguish between altered autologous NK cells and those transferred from circulating, unmodified NK cells.[182] To prevent the recipient from developing an immune response, nonmyeloablative chemotherapy regimens are administered before the transfer.[122]

CONCLUSION

Cell therapy is a relatively burgeoning approach for treating diseases such as cancer. NK cells play a crucial role in the body's defense against tumors, and a number of drugs can impair their activity in various ways. As NK cells cannot attack tumor cells in patients with cancer for various reasons, strategies to restore or replace NK cell cytotoxicity may be required for successful host defense against cancer. In order for NK cell–based immunotherapy to be effective, a significant number of active NK cells must be present. Moreover, the persistence of these cells in a patient's body contributes to the fight against cancerous cells. Despite its potent anticancer activity, NK cell therapy faces significant obstacles that limit its effectiveness. Nevertheless, NK and CAR–NK cell therapies have multiple advantages over other cell therapies, such as T-cell–based therapies, which make investigating them worthwhile. There is a lack of research on BC, but positive results from other solid tumors are encouraging. As a result, there is a need for further research on novel and realistic NK cell expansion and activation techniques in the laboratory to elicit the therapeutic benefits of these cells against tumor cells.

AUTHOR CONTRIBUTIONS

Seyedeh Zahra Fotook Kiaei: Writing – review and editing. **Alireza Nouralishahi:** Writing – review and editing. **Mohammad Ghasemirad:** Writing – review and editing. **Maryam Barkhordar:** Writing – review and editing. **Sasan Ghaffari:** Conceptualization; writing – review and editing. **Hadis Kheradjoo:** Writing – review and editing. **Mahshid Saleh:** Conceptualization; writing – review and editing. **Saman Mohammadzadehsaliani:** Writing – review and editing. **Zahra Molaeipour:** Conceptualization; writing – review and editing.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7–30.
2. Rao H-L, Chen J-W, Li M, *et al.* Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. *PLoS One* 2012; **7**: e30806.
3. Youness RA, Gad MZ. Long non-coding RNAs: functional regulatory players in breast cancer. *Noncoding RNA Res* 2019; **4**: 36–44.
4. Youness RA, Hafez HM, Khallaf E, Assal RA, Abdel Motaal A, Gad MZ. The long noncoding RNA sONE represses triple-negative breast cancer aggressiveness through inducing the expression of miR-34a, miR-15a, miR-16, and let-7a. *J Cell Physiol* 2019; **234**: 20286–20297.
5. Youness RA, Assal RA, Motaal AA, Gad MZ. A novel role of sONE/NOS3/NO signaling cascade in mediating hydrogen sulphide bilateral effects on triple negative breast cancer progression. *Nitric Oxide* 2018; **80**: 12–23.
6. Awad AR, Youness RA, Ibrahim M, *et al.* An acetylated derivative of vitexin halts MDA-MB-231 cellular progression and improves its immunogenic profile through tuning miR-20a-MICA/B axis. *Nat Prod Res* 2021; **35**: 3126–3130.
7. Sørlie T, Perou CM, Tibshirani R, *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; **98**: 10869–10874.
8. Perou CM, Sørlie T, Eisen MB, *et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–752.
9. Nielsen TO, Hsu FD, Jensen K, *et al.* Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 2004; **10**: 5367–5374.
10. Junttila TT, Parsons K, Olsson C, *et al.* Superior in vivo efficacy of afucosylated trastuzumab in the treatment of HER2-amplified breast cancer. *Cancer Res* 2010; **70**: 4481–4489.
11. Vishwasrao P, Hui SK, Smith DL, Khairnar V. Role of NK cells in cancer and immunotherapy. *Onco* 2021; **1**: 158–175.
12. Eggermont LJ, Paulis LE, Tel J, Figdor CG. Towards efficient cancer immunotherapy: advances in developing artificial antigen-presenting cells. *Trends Biotechnol* 2014; **32**: 456–465.

13. Martin JD, Cabral H, Stylianopoulos T, Jain RK. Improving cancer immunotherapy using nanomedicines: progress, opportunities and challenges. *Nat Rev Clin Oncol* 2020; **17**: 251–266.
14. Zhang C, Liu Y. Targeting NK cell checkpoint receptors or molecules for cancer immunotherapy. *Front Immunol* 2020; **11**: 1295.
15. Myers JA, Miller JS. Exploring the NK cell platform for cancer immunotherapy. *Nat Rev Clin Oncol* 2021; **18**: 85–100.
16. Wolf BJ, Choi JE, Exley MA. Novel approaches to exploiting invariant NKT cells in cancer immunotherapy. *Front Immunol* 2018; **9**: 384.
17. Bae E-A, Seo H, Kim I-K, Jeon I, Kang C-Y. Roles of NKT cells in cancer immunotherapy. *Arch Pharm Res* 2019; **42**: 543–548.
18. Watzl C. How to trigger a killer: modulation of natural killer cell reactivity on many levels. *Adv Immunol* 2014; **124**: 137–170.
19. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK cell-based immunotherapy for malignant diseases. *Cell Mol Immunol* 2013; **10**: 230–252.
20. Anfossi N, André P, Guia S, *et al*. Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 2006; **25**: 331–342.
21. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol* 2008; **9**: 503–510.
22. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev* 2006; **214**: 73–91.
23. Chaffey Na B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell. *Ann Bot* 2003; **91**: 401.
24. Lanier LL, Testi R, Bindl J, Phillips JH. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med* 1989; **169**: 2233–2238.
25. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001; **22**: 633–640.
26. Fauriat C, Long EO, Ljunggren H-G, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 2010; **115**: 2167–2176.
27. Freeman BE, Raué H-P, Hill AB, Slifka MK. Cytokine-mediated activation of NK cells during viral infection. *J Virol* 2015; **89**: 7922–7931.
28. Zhang Y, Huang B. The development and diversity of ILCs, NK cells and their relevance in health and diseases. *Regul Inflamm Signal Health Dis* 2017; **1024**: 225–244.
29. Pinto S, Pahl J, Schottelius A, Carter PJ, Koch J. Reimagining antibody-dependent cellular cytotoxicity in cancer: the potential of natural killer cell engagers. *Trends Immunol* 2022; **43**: 932–946.
30. Carter PJ, Lazar GA. Next generation antibody drugs: pursuit of the'high-hanging fruit'. *Nat Rev Drug Discov* 2018; **17**: 197–223.
31. Davis ZB, Vallera DA, Miller JS, Felices M. Natural killer cells unleashed: checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. *Semin Immunol* 2017; **31**: 64–75.
32. Demaria O, Gauthier L, Debroas G, Vivier E. Natural killer cell engagers in cancer immunotherapy: next generation of immuno-oncology treatments. *Eur J Immunol* 2021; **51**: 1934–1942.
33. Vallera DA, Felices M, McElmurry R, *et al*. IL15 Trispecific killer engagers (TriKE) make natural killer cells specific to $CD33^{+}$ targets while also inducing persistence, In vivo expansion, and enhanced FunctionImprovement of bispecific antibody by insertion of IL15. *Clin Cancer Res* 2016; **22**: 3440–3450.
34. Schmohl JU, Felices M, Todhunter D, Taras E, Miller JS, Vallera DA. Tetraspecific scFv construct provides NK cell mediated ADCC and self-sustaining stimuli via insertion of IL-15 as a cross-linker. *Oncotarget* 2016; **7**: 73830–73844.
35. Vallera DA, Oh F, Kodal B, *et al*. A HER2 tri-specific NK cell engager mediates efficient targeting of human ovarian cancer. *Cancer* 2021; **13**: 3994.
36. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 2008; **9**: 495–502.
37. Parham P. Influence of KIR diversity on human immunity. In: Gupta S, Paul WE, Steinman R, eds. Mechanisms of Lymphocyte Activation and Immune Regulation X: innate Immunity. Boston, MA: Springer; 2005:47–50.
38. Braud VM, Allan DS, O'Callaghan CA, *et al*. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998; **391**: 795–799.
39. Paul S, Lal G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. *Front Immunol* 2017; **8**: 1124.
40. Kim S, Sunwoo JB, Yang L, *et al*. HLA alleles determine differences in human natural killer cell responsiveness and potency. *Proc Natl Acad Sci USA* 2008; **105**: 3053–3058.
41. Chewning JH, Gudme CN, Hsu KC, Selvakumar A, Dupont B. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group *in vitro*. *J Immunol* 2007; **179**: 854–868.
42. Hayley M, Bourbigot S, Booth V. Self-association of an activating natural killer cell receptor, KIR2DS1. *PLoS One* 2011; **6**: e23052.
43. Sivori S, Carlomagno S, Falco M, Romeo E, Moretta L, Moretta A. Natural killer cells expressing the KIR2DS1-activating receptor efficiently kill T-cell blasts and dendritic cells: implications in haploidentical HSCT. *Blood* 2011; **117**: 4284–4292.
44. Madjd Z, Spendlove I, Pinder SE, Ellis IO, Durrant LG. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. *Int J Cancer* 2005; **117**: 248–255.
45. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T–cell recognition: molecular mechanisms and functional significance. *Adv Immunol* 1999; **74**: 181–273.

46. Ashouri E, Rajalingam K, Barani S, Farjadian S, Ghaderi A, Rajalingam R. Coexistence of inhibitory and activating killer-cell immunoglobulin-like receptors to the same cognate HLA-C2 and Bw4 ligands confer breast cancer risk. *Sci Rep* 2021; **11**: 1–11.
47. Cózar B, Greppi M, Carpentier S, Narni-Mancinelli E, Chiossone L, Vivier E. Tumor-infiltrating natural killer CellsTumor-infiltrating natural killer cells. *Cancer Discov* 2021; **11**: 34–44.
48. Vitale M, Bottino C, Sivori S, *et al*. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non–major histocompatibility complex–restricted tumor cell lysis. *J Exp Med* 1998; **187**: 2065–2072.
49. Du N, Guo F, Wang Y, Cui J. NK cell therapy: a rising star in cancer treatment. *Cancer* 2021; **13**: 4129.
50. Lanier LL. NKG2D receptor and its ligands in host defense. *Cancer Immunol Res* 2015; **3**: 575–582.
51. Wu J, Song Y, Bakker AB, *et al*. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 1999; **285**: 730–732.
52. Bottino C, Castriconi R, Pende D, *et al*. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med* 2003; **198**: 557–567.
53. El-Sherbiny YM, Meade JL, Holmes TD, *et al*. The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res* 2007; **67**: 8444–8449.
54. Paul S, Kulkarni N, Shilpi LG. Intratumoral natural killer cells show reduced effector and cytolytic properties and control the differentiation of effector Th1 cells. *Onco Targets Ther* 2016; **5**: e1235106.
55. Sconocchia G, Eppenberger S, Spagnoli GC, *et al*. NK cells and T cells cooperate during the clinical course of colorectal cancer. *Onco Targets Ther* 2014; **3**: e952197.
56. Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol* 2001; **13**: 459–463.
57. Orange JS. Natural killer cell deficiency. *J Allergy Clin Immunol* 2013; **132**: 515–525.
58. O'Sullivan T, Saddawi-Konefka R, Vermi W, *et al*. Cancer immunoediting by the innate immune system in the absence of adaptive immunity. *J Exp Med* 2012; **209**: 1869–1882.
59. Mandal A, Viswanathan C. Natural killer cells: In health and disease. *Hematol Oncol Stem Cell Ther* 2015; **8**: 47–55.
60. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol* 2013; **31**: 413–441.
61. Gooden M, Lampen M, Jordanova ES, *et al*. HLA-E expression by gynecological cancers restrains tumor-infiltrating $CD8^+$ T lymphocytes. *Proc Natl Acad Sci USA* 2011; **108**: 10656–10661.
62. Malmberg KJ, Carlsten M, Björklund A, Sohlberg E, Bryceson YT, Ljunggren HG. Natural killer cell-mediated immunosurveillance of human cancer. *Semin Immunol* 2017; **31**: 20–29.
63. López-Soto A, Gonzalez S, Smyth MJ, Galluzzi L. Control of metastasis by NK cells. *Cancer Cell* 2017; **32**: 135–154.
64. Mamessier E, Sylvain A, Thibult ML, *et al*. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *J Clin Invest* 2011; **121**: 3609–3622.
65. Poli A, Michel T, Thérésine M, Andrès E, Hentges F, Zimmer J. $CD56^{bright}$ natural killer (NK) cells: an important NK cell subset. *Immunology* 2009; **126**: 458–465.
66. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev* 2018; **32**: 1267–1284.
67. Gao Y, Souza-Fonseca-Guimaraes F, Bald T, *et al*. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nat Immunol* 2017; **18**: 1004–1015.
68. Slattery K, Woods E, Zaiatz-Bittencourt V, *et al*. TGFβ drives NK cell metabolic dysfunction in human metastatic breast cancer. *J Immunother Cancer* 2021; **9**: e002044.
69. Cai G, Kastelein RA, Hunter CA. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur J Immunol* 1999; **29**: 2658–2665.
70. Wu J, Gao FX, Wang C, *et al*. IL-6 and IL-8 secreted by tumour cells impair the function of NK cells via the STAT3 pathway in oesophageal squamous cell carcinoma. *J Exp Clin Cancer Res* 2019; **38**: 321.
71. Sheikhpour E, Noorbakhsh P, Foroughi E, Farahnak S, Nasiri R, Neamatzadeh H. A survey on the role of Interleukin-10 in breast cancer: a narrative. *Rep Biochem Mol Biol* 2018; **7**: 30–37.
72. Dennis KL, Blatner NR, Gounari F, Khazaie K. Current status of interleukin-10 and regulatory T-cells in cancer. *Curr Opin Oncol* 2013; **25**: 637–645.
73. Fischer K, Hoffmann P, Voelkl S, *et al*. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* 2007; **109**: 3812–3819.
74. Harmon C, Robinson MW, Hand F, *et al*. Lactate-mediated acidification of tumor microenvironment induces apoptosis of liver-resident NK cells in colorectal liver metastasis. *Cancer Immunol Res* 2019; **7**: 335–346.
75. Zhang W, Zhao Z, Li F. Natural killer cell dysfunction in cancer and new strategies to utilize NK cell potential for cancer immunotherapy. *Mol Immunol* 2022; **144**: 58–70.
76. Kaur K, Nanut MP, Ko MW, Safaie T, Kos J, Jewett A. Natural killer cells target and differentiate cancer stem-like cells/undifferentiated tumors: strategies to optimize their growth and expansion for effective cancer immunotherapy. *Curr Opin Immunol* 2018; **51**: 170–180.
77. Chan IS, Ewald AJ. The changing role of natural killer cells in cancer metastasis. *J Clin Invest* 2022; **132**: e143762.
78. Chan IS, Knútsdóttir H, Ramakrishnan G, *et al*. Cancer cells educate natural killer cells to a metastasis-promoting cell state. *J Cell Biol* 2020; **219**: e202001134.

79. Sun P, Yuan Y, Li A, Li B, Dai X. Cytokeratin expression during mouse embryonic and early postnatal mammary gland development. *Histochem Cell Biol* 2010; **133**: 213–221.
80. Cheung KJ, Gabrielson E, Werb Z, Ewald AJ. Collective invasion in breast cancer requires a conserved basal epithelial program. *Cell* 2013; **155**: 1639–1651.
81. Cheung KJ, Padmanaban V, Silvestri V, *et al*. Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. *Proc Natl Acad Sci USA* 2016; **113**: E854–E863.
82. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986; **319**: 675–678.
83. Shin MH, Kim J, Lim SA, Kim J, Kim SJ, Lee KM. NK cell-based immunotherapies in cancer. *Immune Netw* 2020; **20**: e14.
84. Bachanova V, Cooley S, Defor TE, *et al*. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood* 2014; **123**: 3855–3863.
85. Liu E, Tong Y, Dotti G, *et al*. Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. *Leukemia* 2018; **32**: 520–531.
86. Elaraby E, Malek AI, Abdullah HW, Elemam NM, Saber-Ayad M, Talaat IM. Natural killer cell dysfunction in obese patients with breast cancer: a review of a triad and its implications. *J Immunol Res* 2021; **2021**: 9972927.
87. Ni J, Miller M, Stojanovic A, Garbi N, Cerwenka A. Sustained effector function of IL-12/15/18-preactivated NK cells against established tumors. *J Exp Med* 2012; **209**: 2351–2365.
88. Romee R, Rosario M, Berrien-Elliott MM, *et al*. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med* 2016; **8**: 357ra123.
89. Leong JW, Chase JM, Romee R, *et al*. Preactivation with IL-12, IL-15, and IL-18 induces CD25 and a functional high-affinity IL-2 receptor on human cytokine-induced memory-like natural killer cells. *Biol Blood Marrow Transplant* 2014; **20**: 463–473.
90. Cichocki F, Valamehr B, Bjordahl R, *et al*. GSK3 inhibition drives maturation of NK cells and enhances their antitumor activity. *Cancer Res* 2017; **77**: 5664–5675.
91. Muraro E, Martorelli D, Turchet E, *et al*. A different immunologic profile characterizes patients with HER-2-overexpressing and HER-2-negative locally advanced breast cancer: implications for immune-based therapies. *Breast Cancer Res* 2011; **13**: R117.
92. Bald T, Krummel MF, Smyth MJ, Barry KC. The NK cell-cancer cycle: advances and new challenges in NK cell-based immunotherapies. *Nat Immunol* 2020; **21**: 835–847.
93. Huntington ND, Cursons J, Rautela J. The cancer-natural killer cell immunity cycle. *Nat Rev Cancer* 2020; **20**: 437–454.
94. Cerboni C, Fionda C, Soriani A, *et al*. The DNA damage response: a common pathway in the regulation of NKG2D and DNAM-1 ligand expression in normal, infected, and cancer cells. *Front Immunol* 2014; **4**: 508.
95. Carlsten M, Björkström NK, Norell H, *et al*. DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells. *Cancer Res* 2007; **67**: 1317–1325.
96. Carlsten M, Baumann BC, Simonsson M, *et al*. Reduced DNAM-1 expression on bone marrow NK cells associated with impaired killing of $CD34^{+}$ blasts in myelodysplastic syndrome. *Leukemia* 2010; **24**: 1607–1616.
97. Prager I, Liesche C, van Ooijen H, *et al*. NK cells switch from granzyme B to death receptor-mediated cytotoxicity during serial killing. *J Exp Med* 2019; **216**: 2113–2127.
98. Parkhurst MR, Riley JP, Dudley ME, Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. *Clin Cancer Res* 2011; **17**: 6287–6297.
99. Sawasdee N, Wattanapanitch M, Thongsin N, *et al*. Doxorubicin sensitizes breast cancer cells to natural killer cells in connection with increased Fas receptors. *Int J Mol Med* 2022; **49**: 40. https://doi.org/10.3892/ijmm.2022.5095.
100. Gorelik E, Wiltrout RH, Okumura K, Habu S, Herberman RB. Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. *Int J Cancer* 1982; **30**: 107–112.
101. Smyth MJ, Thia KY, Cretney E, *et al*. Perforin is a major contributor to NK cell control of tumor metastasis. *J Immunol* 1999; **162**: 6658–6662.
102. Takeda K, Hayakawa Y, Smyth MJ, *et al*. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* 2001; **7**: 94–100.
103. Zhang S, Liu W, Hu B, *et al*. Prognostic significance of tumor-infiltrating natural killer cells in solid tumors: a systematic review and meta-analysis. *Front Immunol* 2020; **11**: 1242.
104. Nersesian S, Schwartz SL, Grantham SR, *et al*. NK cell infiltration is associated with improved overall survival in solid cancers: a systematic review and meta-analysis. *Transl Oncol* 2021; **14**: 100930.
105. Ascierto ML, Idowu MO, Zhao Y, *et al*. Molecular signatures mostly associated with NK cells are predictive of relapse free survival in breast cancer patients. *J Transl Med* 2013; **11**: 145.
106. Pasero C, Gravis G, Granjeaud S, *et al*. Highly effective NK cells are associated with good prognosis in patients with metastatic prostate cancer. *Oncotarget* 2015; **6**: 14360–14373.
107. Nayyar G, Chu Y, Cairo MS. Overcoming resistance to natural killer cell based immunotherapies for solid tumors. *Front Oncol* 2019; **9**: 51.
108. Shimasaki N, Coustan-Smith E, Kamiya T, Campana D. Expanded and armed natural killer cells for cancer treatment. *Cytotherapy* 2016; **18**: 1422–1434.

109. Hu W, Wang G, Huang D, Sui M, Xu Y. Cancer immunotherapy based on natural killer cells: current Progress and new opportunities. *Front Immunol* 2019; **10**: 1205.
110. Koopmann J, Buckhaults P, Brown DA, *et al*. Serum macrophage inhibitory cytokine 1 as a marker of pancreatic and other periampullary cancers. *Clin Cancer Res* 2004; **10**: 2386–2392.
111. Basse PH, Whiteside TL, Chambers W, Herberman RB. Therapeutic activity of NK cells against tumors. *Int Rev Immunol* 2001; **20**: 439–501.
112. Tian X, Wei F, Wang L, *et al*. Herceptin enhances the antitumor effect of natural killer cells on breast cancer cells expressing human epidermal growth factor Receptor-2. *Front Immunol* 2017; **8**: 1426.
113. Geller MA, Cooley S, Judson PL, *et al*. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy* 2011; **13**: 98–107.
114. Watanabe S, Deguchi K, Zheng R, *et al*. Tumor-induced $CD11b^{+}gr\text{-}1^{+}$ myeloid cells suppress T cell sensitization in tumor-draining lymph nodes. *J Immunol* 2008; **181**: 3291–3300.
115. Talmadge JE. Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. *Clin Cancer Res* 2007; **13**: 5243–5248.
116. Pandolfi F, Cianci R, Lolli S, *et al*. Strategies to overcome obstacles to successful immunotherapy of melanoma. *Int J Immunopathol Pharmacol* 2008; **21**: 493–500.
117. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing $CD25^{+}CD4^{+}$ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 1999; **163**: 5211–5218.
118. Curiel TJ, Coukos G, Zou L, *et al*. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**: 942–949.
119. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* 2000; **356**: 1795–1799.
120. Ruggeri L, Capanni M, Urbani E, *et al*. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002; **295**: 2097–2100.
121. Ruggeri L, Mancusi A, Capanni M, Martelli MF, Velardi A. Exploitation of alloreactive NK cells in adoptive immunotherapy of cancer. *Curr Opin Immunol* 2005; **17**: 211–217.
122. Miller JS, Soignier Y, Panoskaltsis-Mortari A, *et al*. Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* 2005; **105**: 3051–3057.
123. Ishikawa E, Tsuboi K, Saijo K, *et al*. Autologous natural killer cell therapy for human recurrent malignant glioma. *Anticancer Res* 2004; **24**: 1861–1871.
124. Tarek N, Le Luduec JB, Gallagher MM, *et al*. Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *J Clin Invest* 2012; **122**: 3260–3270.
125. Domogala A, Madrigal JA, Saudemont A. Natural killer cell immunotherapy: from bench to bedside. *Front Immunol* 2015; **6**: 264.
126. Lee SC, Shimasaki N, Lim JSJ, *et al*. Phase I trial of expanded, activated autologous NK-cell infusions with trastuzumab in patients with HER2-positive cancers. *Clin Cancer Res* 2020; **26**: 4494–4502.
127. Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. *Nat Rev Drug Discov* 2020; **19**: 200–218.
128. Liang S, Xu K, Niu L, *et al*. Comparison of autogeneic and allogeneic natural killer cells immunotherapy on the clinical outcome of recurrent breast cancer. *Onco Targets Ther* 2017; **10**: 4273–4281.
129. Park JH, Rivière I, Gonen M, *et al*. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N Engl J Med* 2018; **378**: 449–459.
130. Maude SL, Laetsch TW, Buechner J, *et al*. Tisagenlecleucel in children and Young adults with B-cell lymphoblastic leukemia. *N Engl J Med* 2018; **378**: 439–448.
131. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* 2011; **365**: 725–733.
132. Turtle CJ, Hay KA, Hanafi LA, *et al*. Durable molecular remissions in chronic lymphocytic leukemia treated with CD19-specific chimeric antigen receptor-modified T cells after failure of ibrutinib. *J Clin Oncol* 2017; **35**: 3010–3020.
133. Li Y, Hermanson DL, Moriarity BS, Kaufman DS. Human iPSC-derived natural killer cells engineered with chimeric antigen receptors enhance anti-tumor activity. *Cell Stem Cell* 2018; **23**: 181–192.e185.
134. Müller N, Michen S, Tietze S, *et al*. Engineering NK cells modified with an EGFRvIII-specific chimeric antigen receptor to overexpress CXCR4 improves immunotherapy of CXCL12/SDF-1α-secreting glioblastoma. *J Immunother* 2015; **38**: 197–210.
135. Töpfer K, Cartellieri M, Michen S, *et al*. DAP12-based activating chimeric antigen receptor for NK cell tumor immunotherapy. *J Immunol* 2015; **194**: 3201–3212.
136. Sanber K, Savani B, Jain T. Graft-versus-host disease risk after chimeric antigen receptor T-cell therapy: the diametric opposition of T cells. *Br J Haematol* 2021; **195**: 660–668.
137. Kruschinski A, Moosmann A, Poschke I, *et al*. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proc Natl Acad Sci USA* 2008; **105**: 17481–17486.
138. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* 2005; **106**: 376–383.
139. Liu Y, Zhou Y, Huang KH, *et al*. Targeting epidermal growth factor-overexpressing triple-negative breast cancer by natural killer cells expressing a specific chimeric antigen receptor. *Cell Prolif* 2020; **53**: e12858.

140. Liu H, Yang B, Sun T, *et al*. Specific growth inhibition of ErbB2-expressing human breast cancer cells by genetically modified NK-92 cells. *Oncol Rep* 2015; **33**: 95–102.
141. Schönfeld K, Sahm C, Zhang C, *et al*. Selective inhibition of tumor growth by clonal NK cells expressing an ErbB2/HER2-specific chimeric antigen receptor. *Mol Ther* 2015; **23**: 330–338.
142. Chen X, Han J, Chu J, *et al*. A combinational therapy of EGFR-CAR NK cells and oncolytic herpes simplex virus 1 for breast cancer brain metastases. *Oncotarget* 2016; **7**: 27764–27777.
143. Kotzur R, Duev-Cohen A, Kol I, Reches A, Mandelboim O, Stein N. NK-92 cells retain vitality and functionality when grown in standard cell culture conditions. *PLoS One* 2022; **17**: e0264897.
144. Deacon DH, Hogan KT, Swanson EM, *et al*. The use of gamma-irradiation and ultraviolet-irradiation in the preparation of human melanoma cells for use in autologous whole-cell vaccines. *BMC Cancer* 2008; **8**: 360.
145. Hsu JL, Hung MC. The role of HER2, EGFR, and other receptor tyrosine kinases in breast cancer. *Cancer Metastasis Rev* 2016; **35**: 575–588.
146. Ng YY, Du Z, Zhang X, Chng WJ, Wang S. CXCR4 and anti-BCMA CAR co-modified natural killer cells suppress multiple myeloma progression in a xenograft mouse model. *Cancer Gene Ther* 2022; **29**: 475–483.
147. Tang X, Yang L, Li Z, *et al*. First-in-man clinical trial of CAR NK-92 cells: safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. *Am J Cancer Res* 2018; **8**: 1083–1089.
148. Xiao L, Cen D, Gan H, *et al*. Adoptive transfer of NKG2D CAR mRNA-engineered natural killer cells in colorectal cancer patients. *Mol Ther* 2019; **27**: 1114–1125.
149. Liu E, Marin D, Banerjee P, *et al*. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. *N Engl J Med* 2020; **382**: 545–553.
150. Giaquinto AN, Sung H, Miller KD, *et al*. Breast cancer statistics, 2022. *CA Cancer J Clin* 2022; **72**: 524–541.
151. Sutlu T, Nyström S, Gilljam M, Stellan B, Applequist SE, Alici E. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. *Hum Gene Ther* 2012; **23**: 1090–1100.
152. Lynn RC, Powell DJ Jr. Strain-dependent lethal toxicity in NKG2D ligand-targeted CAR T-cell therapy. *Mol Ther* 2015; **23**: 1559–1561.
153. Arai S, Meagher R, Swearingen M, *et al*. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. *Cytotherapy* 2008; **10**: 625–632.
154. Tonn T, Schwabe D, Klingemann HG, *et al*. Treatment of patients with advanced cancer with the natural killer cell line NK-92. *Cytotherapy* 2013; **15**: 1563–1570.
155. Hoyos V, Savoldo B, Quintarelli C, *et al*. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia* 2010; **24**: 1160–1170.
156. Melenhorst JJ, Chen GM, Wang M, *et al*. Decade-long leukaemia remissions with persistence of CD4(+) CAR T cells. *Nature* 2022; **602**: 503–509.
157. Hsu LJ, Liu CL, Kuo ML, Shen CN, Shen CR. An alternative cell therapy for cancers: induced pluripotent stem cell (iPSC)-derived natural killer cells. *Biomedicine* 2021; **9**: 1323.
158. Luevano M, Daryouzeh M, Alnabhan R, *et al*. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Hum Immunol* 2012; **73**: 248–257.
159. Kotylo PK, Baenzinger JC, Yoder MC, Engle WA, Bolinger CD. Rapid analysis of lymphocyte subsets in cord blood. *Am J Clin Pathol* 1990; **93**: 263–266.
160. Dalle JH, Menezes J, Wagner E, *et al*. Characterization of cord blood natural killer cells: implications for transplantation and neonatal infections. *Pediatr Res* 2005; **57**: 649–655.
161. Rubnitz JE, Inaba H, Ribeiro RC, *et al*. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *J Clin Oncol* 2010; **28**: 955–959.
162. Nomura A, Takada H, Jin CH, Tanaka T, Ohga S, Hara T. Functional analyses of cord blood natural killer cells and T cells: a distinctive interleukin-18 response. *Exp Hematol* 2001; **29**: 1169–1176.
163. Wang Y, Xu H, Zheng X, Wei H, Sun R, Tian Z. High expression of NKG2A/CD94 and low expression of granzyme B are associated with reduced cord blood NK cell activity. *Cell Mol Immunol* 2007; **4**: 377–382.
164. Verneris MR, Miller JS. The phenotypic and functional characteristics of umbilical cord blood and peripheral blood natural killer cells. *Br J Haematol* 2009; **147**: 185–191.
165. Malhotra A, Shanker A. NK cells: immune cross-talk and therapeutic implications. *Immunotherapy* 2011; **3**: 1143–1166.
166. Zhu H, Kaufman DS. Engineered human pluripotent stem cell-derived natural killer cells: the next frontier for cancer immunotherapy. *Blood Sci* 2019; **1**: 4–11.
167. Fujisaki H, Kakuda H, Shimasaki N, *et al*. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res* 2009; **69**: 4010–4017.
168. Denman CJ, Senyukov VV, Somanchi SS, *et al*. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One* 2012; **7**: e30264.
169. Berg M, Lundqvist A, McCoy P Jr, *et al*. Clinical-grade *ex vivo*-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. *Cytotherapy* 2009; **11**: 341–355.
170. Siegler U, Meyer-Monard S, Jörger S, *et al*. Good manufacturing practice-compliant cell sorting and large-scale expansion of single KIR-positive alloreactive human natural killer cells for multiple infusions to leukemia patients. *Cytotherapy* 2010; **12**: 750–763.

171. Cheng M, Zhang J, Jiang W, Chen Y, Tian Z. Natural killer cell lines in tumor immunotherapy. *Front Med* 2012; **6**: 56–66.
172. Klingemann H, Boissel L, Toneguzzo F. Natural killer cells for immunotherapy – advantages of the NK-92 cell line over blood NK cells. *Front Immunol* 2016; **7**: 91.
173. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. *J Hematother Stem Cell Res* 2001; **10**: 535–544.
174. Gang M, Marin ND, Wong P, *et al*. CAR-modified memory-like NK cells exhibit potent responses to NK-resistant lymphomas. *Blood* 2020; **136**: 2308–2318.
175. Berrien-Elliott MM, Cashen AF, Cubitt CC, *et al*. Multidimensional analyses of donor memory-like NK cells reveal new associations with response after adoptive immunotherapy for leukemia. *Cancer Discov* 2020; **10**: 1854–1871.
176. Romee R, Schneider SE, Leong JW, *et al*. Cytokine activation induces human memory-like NK cells. *Blood* 2012; **120**: 4751–4760.
177. Cooper MA, Elliott JM, Keyel PA, Yang L, Carrero JA, Yokoyama WM. Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci USA* 2009; **106**: 1915–1919.
178. Rosenberg SA, Lotze MT, Muul LM, *et al*. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med* 1985; **313**: 1485–1492.
179. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* 2008; **8**: 299–308.
180. Ruggeri L, Capanni M, Casucci M, *et al*. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 1999; **94**: 333–339.
181. Yoon SR, Kim TD, Choi I. Understanding of molecular mechanisms in natural killer cell therapy. *Exp Mol Med* 2015; **47**: e141.
182. Veluchamy JP, Kok N, van der Vliet HJ, Verheul HMW, de Gruijl TD, Spanholtz J. The rise of allogeneic natural killer cells as a platform for cancer immunotherapy: recent innovations and future developments. *Front Immunol* 2017; **8**: 631.