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Natural Killer Cell-Based Immunotherapy for Cancer: Advances and Prospects

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ABSTRACT

Natural killer (NK) cells are key innate immune cells that provide the first line of defense against viral infection and cancer. Although NK cells can discriminate between "self" and "non-self," recognize abnormal cells, and eliminate transformed cells and malignancies in real time, tumors develop several strategies to escape from NK cell attack. These strategies include upregulating ligands for the inhibitory receptors of NK cells and producing soluble molecules or immunosuppressive factors. Various types of NK cells are currently being applied in clinical trials, including autologous or allogeneic NK cells, umbilical cord blood (UCB) or induced pluripotent stem cell (iPSC)-derived NK cells, memory-like NK cells, and NK cell line NK-92 cells, for the treatment of different types of tumors. Chimeric antigen receptors (CARs)-NK cells have recently shown great potential due to their redirect specificity and effective antitumor activity. In this review, we summarize the mechanisms of tumor escape from NK cell recognition, the current status and advanced progress of NK cell-based immunotherapy, ways of enhancing the antitumor capacity of NK cells *in vivo*, and major challenges for clinical practice in this field.

1. Introduction

Natural killer (NK) cells are a subset of major components of the innate immunity that provide the first line of defense against invading pathogens and malignancies. Once activated, NK cells can not only rapidly lyse malignant cells or viral-infected cells without prior sensitization and independently of major histocompatibility (MHC) restriction, but also act as regulatory cells by secreting several cytokines to initiate and broaden adaptive immune responses against tumor or infected cells [1,2]. The effector function of NK cells is tightly governed by a balance between inhibitory and stimulating receptors. NK cells distinguish normal tissues through the engagement of killer cell immunoglobulin-like receptors (KIRs) with MHC class I molecules [3,4], which results in the transmission of strong inhibitory signals to block NK cell activation. Once viral-infected cells or malignant transformed cells lose their MHC class I molecules, these activating NK cell receptors, such as NKG2D, NKp30, NKp46, and NKp44, can recognize the stress-induced ligands expressed on target cells, which thus provide positive signals for NK cell killing of the targets [5–7].

NK cells are usually considered to be one of the components of the innate immune cells and to recognize antigens without specificity. It is notable, however, that some researchers have found that NK cells may have features of adaptive immune cells, such as memory, in certain situations. A unique group of tissue-resident $CD49a^+DX5^-$ NK cells located in mouse liver has been found to have memory features during skin-contact inflammation and influenza virus infection [8–11]. More recently, a $CD49a^+$ NK cell subset—a likely human counterpart of mouse $CD49a^+DX5^-$ liver-resident NK cells—was found to have features of adaptive immune cells in the human liver [12]. These $CD49a^+$ NK cells can be induced from peripheral blood (PB) NK cells by stimulating with interleukin-2 (IL-2), IL-12, and IL-15 *in vitro*; they have comparable phenotypic and functional features to hepatic $CD49a^+$ NK cells, such as high expression of interferon-γ (IFN-γ) and NKG2C [13]. In addition, cytomegalovirus (CMV) and combinations of cytokines can induce NK cell memory responses. A murine Ly49H NK cell subset has been found to exert a memory response and be responsible for viral clearance during mouse CMV infection [14]. Similarly, human cytomegalovirus (HCMV)-induced memory NK cells with a response to CMV rechallenge have been identified in

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recipients who have acute HCMV infection received hematopoietic cell transplantation. The phenotypes of these CMV-specific memory NK cells are NKG2C$^+$, KIR$^+$, CD57$^+$, and NKG2A$^-$. These memory NK cells persist for a long time *in vivo* during the first year after allogeneic transplantation and are potent producers of IFN-γ during acute infection and upon secondary challenge [15,16]. In addition, brief pre-activation with IL-12, IL-15, and IL-18 induces human NK cell differentiation into memory-like NK cells with expression of CD94, NKG2A, and NKG2C, but a lack of CD57 and KIRs. These cytokine-induced memory-like NK cells show increased IFN-γ secretion after restimulation [17]. It is important to note that these findings on adaptive NK cells hold great promise for application in immunotherapy for cancers or infective diseases. In a phase I clinical trial on the adoptive transference of cytokine-induced memory-like NK cells, the NK cells showed robust antitumor capacity against acute myeloid leukemia (AML) [18].

Immune cell-based immunotherapy—and chimeric antigen receptor (CAR)-T cells in particular—has recently become a breakthrough in cancer therapy [19,20]. More specifically, acute lymphoblastic leukemia (ALL) clinical complete response rates were as high as 70%–90% in patients who accepted infusions of CD19-targeting CAR-T cells [21]. In fact, NK cell-based immunotherapy has several advantages over T cell- or CAR-T cell-based therapy. Phase I and II clinical trials have shown that the adoptive transfer of allogeneic NK cells is safe and well-tolerated, and will not lead to graft-versus-host disease (GVHD) [22–24]. The source of T cells is usually restricted to autologous cells, but NK cells can be prepared from autologous or allogeneic PB, obtained from bone marrow or umbilical cord blood (UCB), or induced from human embryonic stem cells or pluripotent stem cells; furthermore, the NK-92 cell line can be used directly. In addition, many drugs are designed to arm NK cells and achieve good therapeutic effects. For example, a superagonist complex of IL-15 and IL-15Rα named ALT-803 can bind to the surface IL-15 receptor and activate NK cells [25,26], thus promoting NK cell proliferation and cytotoxicity against hematologic malignancies and solid tumors, including multiple myeloma, ovarian cancer, bladder cancer, and breast and colon carcinomas [27–30]. In a phase I clinical study, 19% of evaluable patients with hematologic cancers were observed to have responses after administration with ALT-803, including one complete remission lasting seven months [27]. Thus, NK cell-based tumor immunotherapy has shown great promise. In this review, we provide a summary of the mechanisms of tumor escape from NK cell recognition, the current status and advanced progress of NK cell-based immunotherapy, strategies to enhance the efficacy of NK cells *in vivo*, and major challenges for clinical practice in this field.

2. Mechanisms of tumor escape from NK cell immunosurveillance

Although NK cells are major components of the innate anticancer immunity, tumors develop various strategies to evade NK cell attack or to impair the activity and function of NK cells. These strategies have a strong impact on the efficacy of NK cell therapy. NK cell dysfunction has been reported in various hematologic malignancies and solid tumors [6,31–33]. For example, under immune stress, tumor cells often upregulate the expression of ligands for the inhibitory NK receptors, such as human leukocyte antigen-G (HLA-G), which is a ligand for KIR2DL4, immunoglobulin-like transcript 2 (ILT2), and ILT4, in order to evade NK cell-mediated cytotoxicity [34]. Ectopic HLA-G expression has been found to correlate with poor prognosis in tumor patients, which implies that this molecule plays a role in tumor immune escape [35]. HLA-G inhibits the proliferation and cytotoxicity of NK cells and reduces the production of IFN-γ and tumor necrosis factor-α (TNF-α) through engagement with ILT2 [36]. It has been reported that HLA-G expressed on Ewing sarcomas suppresses the activity of GD2-specific CAR-expressing NK cells [37]. The blocking of HLA-G on tumor cells in patients with chronic lymphocytic leukemia (CLL) increased the tumor's susceptibility to NK cell-mediated cytotoxicity [38].

Another important mechanism of tumor escape from NK cell surveillance involves the soluble molecules or ligands produced by tumor cells. IL-2 is required for the proliferation and activation of NK cells. However, soluble IL-2Rα produced by tumors can bind to IL-2 and prevent it from binding to membrane IL-2R on the surfaces of NK cells, resulting in insensitivity to exogenous IL-2 administration and impairment of NK activity [39]. Impaired NK cell function has also been reported to correlate with elevated serum levels of soluble ligands, such as BAG6 for NKp30, and MHC class I-related chain A (MICA) and ULBP1-3 for NGK2D [32,40]. Soluble ligands shed from tumor cells prevent specialized NK cells from recognizing the ligands on the membrane of the tumor cells [41–43]. Tumor cells shed the ligands from the cell membrane through the activity of protein disulfide isomerase ERp5, disintegrins, and the metalloproteinases ADAM10 and ADAM17 [42,44]. Ferrari de Andrade et al. [45] recently designed antibodies directed against the site of proteolytic cleavage of MICA and MHC class I-related chain B (MICB), and confirmed that these antibodies effectively prevent the shedding of MICA and MICB from the surfaces of tumor cells, thus reactivating the antitumor immunity of NK cells.

The major mechanisms of tumor escape are associated with the tumor microenvironment (TME), which consists of immunosuppressive cells (e.g., regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs)), soluble factors, suppressive molecules expressed on tumor cells or antigen-presenting cells, and the extracellular matrix. The immunosuppressive microenvironment not only promotes tumor growth and migration, but also helps the tumor cells evade the surveillance of the host immunity and resists immunotherapy [46–49]. Tumor cells secrete various immunosuppressive factors, such as transforming growth factor-β (TGF-β), IL-10, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2), which suppress NK cell antitumor activity [50]. Several groups have demonstrated that tumor-cell-derived IDO and PGE2 sharply suppress the cytotoxicity and cytokine production of NK cells [51,52]. Several suppressive cells such as Tregs, MDSCs, and M2-macrophages can impair the cytotoxicity of intratumoral NK cells by secreting IL-10 and TGF-β [53,54]. Tumor cells, antigen-presenting cells, and stromal cells in the TME express high levels of inhibitory molecules, such as programmed-death ligand 1 (PD-L1), to prevent NK cell activation through ligation with their respective inhibitory receptors on NK cells, thus leading to NK cell dysfunction or even exhaustion [6,55–57]. Therefore, tumor-infiltrating NK cells usually exhibit an exhausted state and are prone to apoptosis; they are also characterized by decreased expression of activating receptors, upregulated inhibitory receptors (e.g., NKG2A, the co-inhibitory receptor T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), and the T cell immunoglobulin and mucin-domain containing-3 (Tim-3)), and low secretion of IFN-γ and TNF-α [33,55,58]. Blockading these inhibitory checkpoint receptors can restore NK cells from exhaustion and significantly improve the therapeutic efficacy of immunotherapy [59].

It is important to design appropriate strategies to overcome the mechanisms of tumor immune escape—and particularly to overcome the immunosuppressive microenvironment—in order to obtain ideal therapeutic efficacy for NK cell-based antitumor immunotherapy.

3. Current status of NK cell-based tumor immunotherapy

3.1. Peripheral blood-derived autologous or allogeneic NK cells for tumor immunotherapy

Initial attempts to treat tumors by means of adoptive immunotherapy involved transferring IL-2-activated autologous NK cells derived from PB [60], as these have been shown to reduce the growth of pulmonary metastases in murine models. However, only a limited antitumor effect was observed when this method was used clinically [60,61]. The main cause of this limited effect was that the self-HLA molecules on tumor cells match with the KIRs on autologous NK cells, resulting in the suppression of NK cell activity. Later, KIR ligand-mismatched allogeneic or haploidentical NK cells were adopted in order to overcome this suppression due to the "missing-self" recognition of tumor cells [62]. An infusion of allogeneic NK cells reduced host rejection of the transplant while eliminating leukemia relapse, thus ensuring both efficacy and safety.

Several clinical trials have shown that NK cell infusions are well-tolerated without GVHD, cytokine release syndrome (CRS), or neurotoxicity [24,63–71]; however, the therapeutic effects vary enormously in different types of cancers. Objective clinical responses vary from 25% to 100% for hematologic malignancies, such as refractory non-Hodgkin's lymphoma (NHL), myelodysplastic syndromes (MDS), and AML. A recent clinical trial showed that all the evaluable patients (5/+5 evaluable) with high-risk MDS and AML achieved objective responses after receiving infusions of haploidentical NK cells [65]. The efficacy of allogeneic NK cell infusions has also been evaluated, often in combination with antibody treatment, in solid tumors. For example, in a phase I clinical trial for the treatment of patients with advanced gastric or colorectal cancers, adoptive NK cell therapy was given in combination with trastuzumab or cetuximab chemotherapy. Four of the six evaluable patients were stable in disease progression. It is important to note that combination therapy significantly promoted IFN-γ production and decreased the number of Tregs in the periphery, thus resulting in a reduction of tumor size in three of these four stable patients [68]. Another phase I clinical trial, in which allogeneic NK cell infusion was combined with cetuximab for the therapy of liver metastasis from gastrointestinal cancer, showed objective clinical responses in three out of nine patients [70]. The common problem of poor response in these trials was associated with the limited donor NK cell lifespan *in vivo* (usually two weeks) and the increase in Tregs related to IL-2 administration. Two clinical trials (NCT00274846 and NCT01106950) used IL-2-diphtheria fusion protein to eliminate Tregs during the infusion of haploidentical NK cells for the therapy of AML. The results showed that therapy with IL-2-diphtheria fusion protein improved the rates of complete remission and disease-free survival, which were associated with the effective Treg depletion, donor NK cell expansion, and higher serum IL-15 levels [72]. The therapeutic efficacy may be further improved through the administration of exogenous IL-15 but not IL-2, because IL-15 does not promote the survival and expansion of Tregs [73,74].

3.2. NK cells derived from umbilical cord blood or induced pluripotent stem cells for tumor immunotherapy

Aside from NK cells from PB, UCB and induced pluripotent stem cells (iPSCs) are currently used as sources of functional NK cells by means of co-culturing with supportive feeder cells or through stimulation alone with combinations of cytokines. The prominent advantages of UCB or iPSCs, such as the wide range of available sources and ease of clinical-grade expansion under good manufacturing practice (GMP) standards, are expected to allow UCB- or iPSC-derived NK cells to become an "off-the-shelf" product for cancer immunotherapy.

At present, large-scale clinical-grade expansion techniques for UCB- and iPSC-derived NK cells have been well established. A high log-scale *ex vivo* NK cell expansion method from $CD34^+$UCB has been established and can be used as a clinical-grade protocol for adoptive immunotherapy. This method permits greater than 15 000-fold expansion efficiency with 100% purity. $CD34^+$ hematopoietic progenitor cells from UCB were expanded with a novel clinical-grade medium supplemented with a combination of cytokines; this process generated functional $CD56^+$ NK cells with NK cell receptor expression and the ability to efficiently lyse tumor cells, including leukemia and solid tumors [75]. The iPSC-derived NK cells can be expanded 100–1000 fold through a two-stage culture system with a combination of cytokines (including IL-3, IL-7, IL-15, SCF, and Flt3L) without exogenous stromal cells [76]. The antitumor activity of UCB- or iPSC-derived NK cells has been evaluated on various types of tumors, including hematological and solid cancers [76–84]. However, NK cells expanded from UCB or iPSCs have poorer or similar antitumor functions compared with PB mononuclear cell (PBMC)-derived NK cells [76–81]. Moreover, UCB- and iPSC-derived NK cells express low levels of KIRs; this raises concerns about NK cell education, which is the process by which NK cells are endowed with diverse effector capacities while remaining tolerant to self-HLA [85–88]. Educated NK cells usually have lower thresholds for activation or inhibitory signaling, whereas uneducated NK cells are hyporesponsive to activation or inhibition. In contrast, a recent study shows that uneducated NK cells from UCB have superior cytotoxicity against HLA-expressing cervical tumor cell lines compared with educated NK cells from PBMCs [84]. Thus, it remains to be answered whether NK cell education is essential for immunotherapy against cancers.

Five clinical studies are presently focusing on UCB-derived NK cells for the therapy of relapsed or refractory hematological and solid tumors. Four studies (NCT01729091, NCT03019640, NCT02280525, and NCT03539406) are currently recruiting patients. One trial (NCT00354172) has finished its research. This clinical research study aimed to find the highest tolerable dose of NK cells that can be given with chemotherapy to patients with CLL, and to learn whether the addition of NK cells is effective in treating this disease. The clinical results showed that two of the fifteen patients with hematologic cancers were disease-free and alive for six months after treatment with UCB-derived NK cell infusions.

3.3. NK cell-line-based tumor immunotherapy

There are several established NK cell lines, including NK-92, NKL, YT, NK-YS, and NKG [89–91]. Among these, NK-92 cells display a robust and broad-spectrum cytotoxicity against malignant cells, and are the only cell line that has achieved US Food and Drug Administration (FDA) approval for clinical trials [92]. NK-92 cells have several advantages in clinical practice. They are easily expanded under GMP conditions compared with allogeneic or UCB-derived NK cells, which leads to lower costs for patients. NK-92 cells can be efficiently manipulated with viral or non-viral vectors to enhance their targeting, homing, and killing activity. Clinical trial results have confirmed the safety of infusion with NK-92 cells, even at a dose of 1×10^{10} cells·m^{-2} [93–95]. The efficacy of NK-92 cell infusion has been assessed in patients with melanoma, sarcoma, colorectal cancer, renal carcinoma, lung cancer, and AML. It seems that patients with lung cancer (three out of four) or renal cell cancer (five out of eleven) have better clinical responses than others in terms of achieving a mixed response or stable disease [93,94]. A recent phase I clinical trial using IL-2-activated NK-92 cells demonstrated their safety and feasibility, along with transient activity in three out of seven patients with

refractory and relapsed AML [95]. However, due to the limited persistence of these NK cells *in vivo*, the efficacy of NK-92-based therapy remains limited, even with repeated infusions. In addition, NK-92 cells must be irradiated before infusion in order to completely abrogate the proliferation, due to their origin from an NHL patient [96]. However, this irradiation process severely impairs their persistence *in vivo*, such that they are usually undetectable in circulation within several days [94]. Although more frequent cell infusions may be a simple approach to improve the persistence, this solution raises concern because it is likely to evoke a humoral immune response against the HLA antigens expressed on NK-92 cells. Therefore, several groups are attempting to redirect the specificity and improve the clinical results by means of genetic modifications, such as CAR-expressing NK-92 cells.

3.4. Potential immunotherapeutic benefits of memory-like NK cells

Although they are a main component of the innate immunity, recent evidence indicates that NK cells display memory-like adaptive features. Three types of NK cells with features of memory-like immune response have been reported: cytokine-induced memory-like NK cells, CMV-specific NK cells, and hepatic liver-resident NK cells [97]. The common features of memory-like NK cells are: expression of a higher level of NKG2C; long-term *in vivo* proliferation and persistence; and increased IFN-γ secretion after restimulation with antigens or cytokines, or when coming into contact with tumor cells. The three types differ in that cytokine-induced memory-like NK cells and CMV-specific NK cells exert stronger cytotoxicity against targets, whereas hepatic liver-resident NK cells display poor degranulation [12]. Although a great deal of progress has been achieved in NK cell immunotherapy, clinical efficacy is still limited due to the short lifespan and low cytotoxicity of NK cells *in vivo*. The memory features and the long-term *in vivo* proliferation and persistence of memory-like NK cells make them a possible breakthrough as a novel source of NK cell-based immunotherapy against tumors.

Human cytokine-induced memory-like NK cells are obtained through pre-activation of human PB-derived NK cells with various combinations of the cytokines IL-12, IL-15, and IL-18 [17,18,98,99]. These memory-like NK cells are currently being assessed in clinical studies, and a phase I clinical trial has been completed. After brief pre-activation and adoptive transfer, these NK cells displayed robust antitumor activity against AML in a xenograft mice model [18]. In the clinical study, the transferred memory-like NK cells proliferated and reached a peak from day 7 to day 14; furthermore, they expanded by an average of 419 fold, when comparing day 7 with the first day (day 1) after infusion [18]. It is important to note that the expanded memory-like NK cells displayed potent anti-leukemia function. Five out of nine AML patients transferred with memory-like NK cells achieved a clinical response, with four complete remissions [18]. Thus far, the efficacy of cytokine-induced memory-like NK cells has only been tested in AML patients. Other types of tumor—and solid tumors in particular—remain to be evaluated.

A memory NK cell response has also been found during HCMV infection. Foley et al. [15] have shown that in allogeneic hematopoietic transplant recipients, donor-derived NK cells demonstrate memory-like features, with increased frequencies of $NKG2C^+$ NK cells and higher production of IFN-γ when reactivated by CMV infection—features that play a significant role in reducing relapse. The activating receptor NKG2C, whose ligand is HLA-E, might serve as a marker for these memory-like NK cells. CMV-induced $NKG2C^+$ NK cells have been shown to display strong cytolytic activity against tumors strongly expressing HLA-E *in vitro* through increased degranulation and IFN-γ secretion [100,101]. Furthermore, an efficient method for the *ex vivo* expansion of $NKG2C^+$ NK cells has been established [102]. $NKG2C^+$ NK cells are preferentially *ex vivo* expanded from healthy donors through culturing with HLA-E-transfected 721.221 cells as feeder cells, and then stimulating with IL-15 for two weeks [101,102]. The *ex vivo* expanded $NKG2C^+$ NK cells exhibit differentiated phenotypes, including low expression of NKG2A, CD7, CD16, and siglec-7 and high expression of CD2, CD57, CD226, and granzyme B [102]. It is important to note that these expanded $NKG2C^+$ NK cells carry a single self-specific KIR, which allows them to completely overcome the HLA-C barriers and exert stronger cytotoxicity against mismatched tumor cells. Despite certain challenges that require solutions, such as the low expansion (2–4 fold after a 14-day expansion), the features of the specific expansion of a single KIR-expressing NK cell subset and the high potential cytolytic activity of $NKG2C^+$-adaptive NK cells are appealing, so these NK cells hold promise to become the next generation of NK cell-based cancer immunotherapy [16,103]. It is notable that the $NKG2C^+$-adaptive NK cells were engineered with a third-generation anti-CD19-CAR, and exhibited superior cytolytic capacity compared with all other NK subsets, thus confirming their feasibility and demonstrating the great promise of CAR-modified primary adaptive NK cells in cancer immunotherapy [104].

Phenotypic features of liver antigen-specific memory NK cells were first identified in mice [8]. The liver contains two distinct types of NK cells: liver-resident $CD49a^+DX5^-$ NK cells and conventional $CD49a^-DX5^+$ NK cells. Liver-resident $CD49a^+DX5^-$ NK cells have a memory potential with robust recall responses after the challenge in contact hypersensitivity models [8,105,106]. Later, a population of intrahepatic $CD49a^+$ NK cells was similarly identified with memory-like features in humans [12]. Human intrahepatic $CD49a^+$ NK cells express a high level of NKG2C and low levels of NKG2A, CD16, CD57, and perforin. Upon stimulation, $CD49a^+$ NK cells produce large amounts of inflammatory cytokines, but degranulate poorly. Recent studies have shown that IL-12 and IL-15 can stimulate the differentiation of PB NK cells into $CD49a^+CXCR6^+$ NK cells *in vitro* [13]. These $CD49a^+CXCR6^+$ NK cells express a high level of NKG2C and produce IFN-γ, with a similar phenotype and function as liver-resident $CD49a^+$ NK cells. Human intrahepatic $CD49a^+$ NK cells may become a promising NK cell candidate for the immunotherapy of liver carcinoma or related chronic liver diseases.

3.5. CAR-NK cells for tumor immunotherapy

Adoptive immunotherapy with CAR-T cells has had strikingly positive clinical results for the treatment of hematological malignancies [107,108]. The use of CD19-targeted CAR-T cells for the treatment of relapsed B-ALL and certain types of relapsed NHL has been approved by the FDA administration. However, a number of obstacles still remain that plague this clinical application. Infusions of CAR-T cells are often accompanied by side effects, such as CRS, neurotoxicity, and on-target/off-tumor effects [109].

CAR-T cells are usually prepared from T cells from the patients' autologous blood in order to prevent GVHD, thus making the preparation personalized, time-consuming, and costly. CAR-NK cells have several advantages that overcome these limitations [20,110]. CAR-NK cells can be prepared as an "off-the-shelf" product without the restriction of autologous cells due to their sufficient safety and low likelihood of triggering GVHD upon allogeneic infusion. NK cells usually secrete restricted levels of IFN-γ and GM-CSF, and do not secrete IL-1 and IL-6, which are the main cytokines to initiate CRS. Furthermore, CAR-NK cells retain their natural receptors, such as NKp46, NKp30, NKp44, NKG2D, and CD226, which recognize stress-induced ligands independent of CARs; as a result, they can decrease the likelihood of relapses related to a loss of CAR-targeting antigen.

Given the abovementioned advantages of NK cells, CAR-NK cells are now being explored for the therapy of both hematological and solid tumors, and have shown great promise. In preclinical studies, CAR-NK cells have displayed powerful antitumor efficacy in murine tumor-bearing models. For example, transferring wild-type epidermal growth factor receptor (EGFR) and EGFRvIII-targeting CAR-NK-92 cells significantly reduced the growth of glioblastoma and effectively prolonged the survival time of tumor-bearing mice, compared with the transference of unmodified NK-92 cells [111]. Therapy with glypian-3 (GPC3)-targeted CAR-NK-92 cells for hepatocellular carcinoma has also markedly reduced the tumor burden in an orthotopic xenograft model [112].

However, there are still several limitations affecting the successful clinical translation of CAR-NK cells. In contrast to T cells, arming CAR-NK cells is challenging due to the low transfection efficacy to PB-derived NK cells and the short persistence *in vivo* [110]. Many researchers are exploring methods to improve the efficacy of transfecting primary NK cells; however, no remarkable progress has yet been made. Shimasaki et al. [113] have used mRNA electroporation to transmit an anti-CD19-CAR into human primary NK cells. The expression level of the anti-CD19-CAR reached 40.3% in freshly purified NK cells and 61.3% in expanded NK cells, with a cell viability of 90%, at 24 h after electroporation. This method of modifying NK cells significantly enhanced the specific cytolysis of the NK cells against $CD19^+$ tumor cells *in vitro*. The tumor burden in mice after treatment markedly decreased, but relapse occurred during the later period due to the transient antitumor effects [113]. Thus far, two clinical trials that manufacture CAR-NK cells with mRNA electroporation are being carried out in order to assess the safety and feasibility of this therapy. One trial is targeting CD19 for the treatment B lymphoid malignancies (NCT01974479) sponsored by National University Health System in Singapore, and the other is using NKG2D ligand-targeting CAR-NK cells for the therapy of patients with metastatic solid tumors, sponsored by The Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) in 2018 (NCT03415100).

To overcome problems with the transient expression of CARs and the low transfection efficiency, and to obtain stable CAR-expressing NK cells, several groups have begun using NK-92 cells or UCB/iPSCs-derived NK cells as superior CAR drivers. CAR-NK-92 cells display robust activity against CAR-targeting tumors. Many tumor antigens are applied as targets in CAR-NK cell studies, including tumor antigens from hematological malignancies (e.g., CD19, CD20, CD33, CD138, and CS-1) [114–118] and solid tumors (e.g., HER2, erbB2, EpCAM, mesothelin, GD2, GPA7, GPC3, PSCA, EGFR, and EGFRvIII) [37,111,112,119–123]. Five clinical studies (NCT02892695, NCT02742727, NCT02944162, NCT03383978, and NCT02839954) at phases I and II are focusing on NK-92 cells that express CD19, CD7, and CD33-specific CAR for lymphoma and leukemia; HER2-specific CAR for glioblastoma; and MUC1-specific CAR for $MUC1^+$ relapsed or refractory solid tumors.

Although CAR modification could increase the cytolytic potential of NK-92 cells against targets, the persistence of NK-92 cells *in vivo* is still limited due to the irradiation occurs before administration. Liu et al. [124] have presented a novel method to generate CD19-specific CAR-NK cells from UCB-derived NK cells, and have claimed that their approach can improve the transfection efficiency and persistence of NK cells *in vivo*. NK cells were genetically modified with a retroviral vector containing the CD19-specific *CAR* gene and *IL-15* gene in order to drive the expansion and persistence of NK cells, and an inducible caspase-9 in order to eliminate the transduced cells when necessary. This modification, and particularly the introduction of IL-15, significantly improved the proliferation and persistence of NK cells *in vivo*, and augmented the antitumor activity compared with only CAR-transduced NK cells. It is notable that the CAR-NK cells were able to persist for at least 68 d post-infusion [124]. A clinical trial (NCT03056339) sponsored by the MD Anderson Cancer Center was approved in 2017 and aimed to evaluate whether infusions of UCB-derived CD19-specific CAR-NK cells would improve the disease in stem cell transplant patients with relapsed or refractory B lymphoid malignancies.

4. Challenges and perspectives

An increasing number of research studies and clinical trials have demonstrated the superiority of NK cells in clinical applications for cancer therapy. Various approaches are being employed to enhance the *in vivo* proliferation, persistence, and antitumor capacity of NK cells (Fig. 1). However, the efficacy of NK cells is still insufficient, especially for the treatment of solid tumors. Many challenges limit the efficacy of NK cell-based immunotherapy; particular challenges include the limited *in vivo* proliferation and persistence, and the immunosuppression of the TME. Different combinations of multiple strategies have been proposed to augment the antitumor efficacy of NK cells, prolong their survival and persistence *in vivo*, and restore NK functions from exhaustion in the TME. NK cells are a group of heterogeneous cells with different functional subsets, such as tissue-resident NK cells, memory-like NK cells, and single self-specific KIR-expressing NK cells. The selective expansion of a suitable NK cell subset and its application to an appropriate type of tumor are worth exploring. UCB-derived NK cells or memory-like NK cells have longer lifespans than PB-derived NK cells, and thus may be ideal sources for NK cell therapy. The introduction of a combination of cytokines such as IL-12, IL-15, and IL-21 may further enhance the *in vivo* proliferation and persistence of these NK cells. CAR is a powerful tool to improve the cytolytic activity of NK cells; however, only a few studies have designed CAR constructs based on NK cell features [120,125]. Thus, most CAR-NK cells share the main constructs of CARs with CAR-T cells, without considering the unique features of NK cells. A promising field of investigation is to design an optimized structure of CAR that is suitable for NK cells, and then transduce the CAR into memory-like NK cells or a specific NK cell subset. Kaufman and colleagues [126] recently designed and tested nine different NK cell-specific CAR constructs containing different NK cell-specific activating domains, and utilized human iPSCs to produce mesothelin-targeted CAR-NK cells. The NK-specific CAR-NK cells, and particularly the optimized NKG2D-2B4ζ-iPSC-NK cells, demonstrated markedly augmented cytolytic capacity, significantly inhibited tumor growth, and prolonged survival in an ovarian cancer xenograft model [126]. It is important to note that the NK cell-specific CAR-mediated signaling activity effectively improved the *in vivo* expansion and survival of the NK cells.

Furthermore, NK cells are usually induced in a state of dysfunction or exhaustion in the TME [6], which significantly impairs their persistence and cytotoxicity. Strategies that target the TME may improve the efficacy of NK and CAR-NK cell-based immunotherapy. Some studies have shown that blocking the PD-1 pathway could partially reverse the dysfunction and exhaustion of NK cells [127,128]. Levels of PD-1 were found to be specifically upregulated on tumor-infiltrating NK cells in various tumor models, and PD-1 engagement by $PD\text{-}L1^+$ tumor cells potently suppresses NK cell-mediated antitumor immune responses. Thus, the impaired antitumor activity of NK cells could be restored by a PD-1 and PD-L1 blockade [127]. It is notable that the TIGIT was found to be highly expressed on tumor-infiltrating NK cells and T cells, and that the level of TIGIT was positively correlated with tumor progression and the functional exhaustion of both T cells and NK cells, making TIGIT an important checkpoint receptor [59,129–131]. A blockade of TIGIT significantly reversed NK cell exhaustion and restored the cytotoxicity and cytokine secretion activity of both NK cells

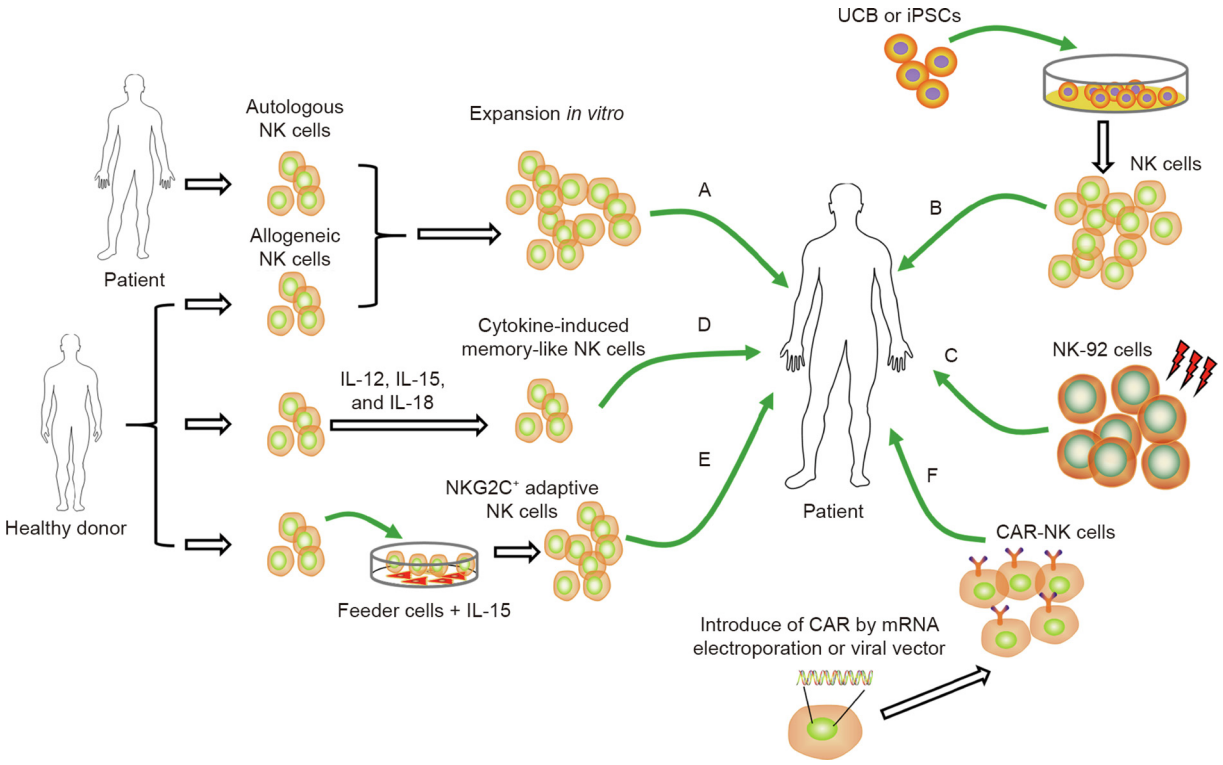

Fig. 1. Various sources of NK cells used for tumor immunotherapy. A: Autologous NK cells or allogeneic NK cells with mismatched KIRs from a healthy donor are purified from the PB and then activated with cytokines (IL-2 or IL-15) before administration into the patient. B: UCB and iPSCs are used as a source of functional NK cells by co-culturing with supportive feeder cells or by stimulation alone with a combination of cytokines. C: NK cell line NK-92 cell, irradiated with 1000 cGy prior to infusion, is another important source for NK cell therapy. D: Cytokine-induced memory-like NK cells are obtained through pre-activation of human PB-derived NK cells with a combination of cytokines such as IL-12, IL-15, and IL-18. E: NKG2C$^+$-adaptive NK cells are preferentially *ex vivo* expanded from healthy donors through culturing with HLA-E-transfected 721.221 cells as feeder cells and IL-15. F: NK cells can be genetically modified with CARs through mRNA electroporation or viral vectors in order to redirect specificity and enhance the antitumor efficacy of NK cell-based immunotherapy.

and T cells in different solid tumor models; it also finally prolonged the overall survival of tumor-bearing hosts [59]. Another co-inhibitory receptor, Tim-3, is also reported to be highly expressed on tumor-infiltrating NK cells from patients with different types of solid tumors, such as metastatic melanoma, hepatocellular carcinoma, mammary adenocarcinoma, and colon cancer. The level of Tim-3 on tumor-infiltrating NK cells was found to positively correlate with tumor progression and poor prognosis of patients with melanoma. However, a blockade of Tim-3 with antibody was found to reverse NK cell exhaustion and markedly restore the antitumor efficacy of NK cells [132,133]. Therefore, CAR-transduced NK cells combined with checkpoint inhibition (e.g., a blockade of PD-1, TIGIT, NKG2A, or Tim-3) might further increase antitumor immune responses to cancer. Similar strategies include combination therapy with the elimination or blockage of immunosuppression from MDSCs, Tregs, TAMs, TGF-β, and other immunosuppression in the TME.

In summary, the striking antitumor activity of NK cells is finally being translated into the clinic, where it has shown remarkable promise. Elaborately designed ideal strategies that can be combined with advanced techniques to improve the proliferation, persistence, and functions of NK cells and to overcome the bottlenecks in clinical translation will maximize the efficacy of adoptive NK cell immunotherapy.

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Compliance with ethics guidelines

Yuan Hu, Zhigang Tian, and Cai Zhang declare that they have no conflict of interest or financial conflicts to disclose.

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