



REVIEW

Technical advances in NK cell-based cellular immunotherapy

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ABSTRACT

Natural killer (NK) cells represent a promising future for tumor immunotherapy because of their unique biological functions and characteristics. This review focuses on technical advances in NK cell-based cellular immunotherapy and summarizes the developments of recent years in cell sources, genetic modification, manufacturing systems, clinical programs, and outcomes. Future prospects and challenges in NK cell immunotherapy are also discussed, including off-the-shelf NK cell exploitation, automatic and closed manufacturing systems, cryopreservation, and therapies involving regulatory checkpoints.

KEYWORDS

Natural killer cell; immunotherapy; adoptive cell transfer; genetic modification

Introduction

Natural killer (NK) cell-based immunotherapies are an attractive approach for treating malignancies because of their characteristic recognition and killing mechanisms¹⁻³. Despite increasing interest in NK cell immunotherapy for cancer, some challenges have emerged. Owing to the low frequency of NK cells in peripheral blood (PB; 10%–15%) and cord blood (15%–20%)^{4,5}, expansion is necessary to obtain them in clinical quantities. Feeder cell-based NK cell expansion systems can be used to obtain highly purified and quantitative products⁶; however, the use of a feeder cell line may result in unpredictable risks. Although feeder-free NK cell expansion systems are relatively simple, the expansion fold is limited⁷. Moreover, the transduction efficiency of NK cells varies, ranging from 25% to 50% in NK cell lines⁸, compared with less than 20% in primary NK cells and 6%–96% in *ex vivo* expanded NK cells⁹. The quantity of NK cells in a manufactured batch is not sufficient for multiple-dose adoptive transfer; thus, it is appropriate to cryopreserve NK cells for clinical use. However, NK cells are more

sensitive than T cells to the freeze and thaw process, and the effects of cryopreservation on their cytotoxicity remain controversial^{10,11}. Nevertheless, recent technical advances, including successful generation of stem-cell-derived NK cells, the development of an IL-15 super-agonist that can efficiently activate NK cells without side effects, and impressive clinical results from combination therapy and immune checkpoint inhibitors, have provided great encouragement and an indication of future trends in the development of NK cell immunotherapy.

Development of NK cell manufacturing

Sources of NK cells

Allogeneic or autologous PB cells have traditionally been the major sources of NK cells for immunotherapy. However, this cell population is donor dependent and heterogeneous, and the efficiency of expansion systems varies. Recently, CD34⁺ stem cells from sources such as cord blood and induced pluripotent stem cells (iPSCs) have been used to generate NK cells^{12,13}. Various protocols involving xenogeneic stromal feeder cell lines¹⁴ or a spin-embryoid body have been used to induce iPSC differentiation^{12,15}, producing more than 1,000-fold expansion of NK cells with purity of $\geq 90\%$. More importantly, by screening single iPSC clones, this approach

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provides a genetically defined, homogeneous NK cell population that can be genetically modified and expanded on a large scale to produce multiple doses. Therefore, stem-cell-derived NK cells represent a possible means of achieving “off-the-shelf” production, genetic modification, and defined and stable supplementation for NK cell generation.

Optimization of CARs for NK cells

Chimeric antigen receptor (CAR) autologous T cells have shown promising clinical outcomes against hematopoietic malignancies. NK cells have been explored as candidates for CAR engineering, enabling them to be directed to specific targets¹⁶. In recent years, several researchers have focused on the optimization of CAR constructs, including the extracellular antigen recognition domain and intracellular costimulatory signaling domain. Previously, CARs were designed to recognize tumor cells using the extracellular part; more recently, the targeting of CARs has focused on suppressor cells in the tumor microenvironment. NK cells engineered with a CAR that recognizes myeloid-derived suppressor cells (MDSCs) with overexpression of molecular NKG2D ligands can efficiently kill intra-tumoral MDSCs. This is a viable way to relieve immunosuppression and support other forms of immunotherapy¹⁷. Currently, most intracellular signaling domains of CARs are CD3- ζ chains incorporated with costimulatory signaling domains such as CD28. However, CD28 is not naturally expressed in NK cells, so the function of the CD28 signaling domain in NK cells is not clearly defined. Therefore, CAR constructs in NK cells suited to costimulatory signaling domains are needed. Kaufman's group reported that CAR constructs in NK cells typically expressing costimulatory signaling domains NKG2D-2B4 ζ showed greater capacity to induce NK cell cytotoxicity against targets. Notably, T cells engineered with T-CAR showed better activity than those engineered with NK-CAR¹². Optimization of CAR intracellular costimulatory signaling domains is needed, in order to find CARs suited to NK cells and T cells, respectively.

Currently, the NK cell line, PB-NK, and stem-cell-derived NK cells can all be engineered with CARs. However, the efficiency of CAR gene transfer is lower in PB-NK, ranging from 10% to 60%, compared with the NK cell line and stem-cell-derived NK cells, which have efficiencies of up to 90%¹⁸. Moreover, which type of CAR-NK cell provides the greatest benefit is still the subject of research. The latest report shows that CAR-NK-92 cells have stronger cytotoxic activities compared with CAR-engineered PB-derived NK cells from healthy donors *in vivo*¹⁹. However, NK cell lines must be

irradiated before infusion, resulting in reduced cytotoxicity and migration. Overall, CAR-NK strategies require further study.

The extracellular part of CARs is usually a single-chain variable fragment, designed to rely on the antibody. This extracellular part enables the cells to recognize specific antigens only expressed on the target cell surface. However, this recognition model represents a bottleneck in CAR-engineering of cells. Recently, the T cell receptor (TCR), which can recognize antigenic peptides, has been used to establish a TCR-NK cell. The transgenic NK-92 cell line expressing the CD3 signaling complex acquired a T-cell-like profile and showed pMHC-specific cytotoxic function²⁰. TCR-NK cells can recognize all cellular proteins, enabling them to break through the bottleneck and expanding the range of potential applications.

Expansion systems for NK cells

Stem-cell-derived NK cells generation systems usually have two stages: the hematopoietic progenitor differentiation stage and the NK cell differentiation/expansion stage. Most early studies used two murine stromal cell lines, S17 or M210 in stage 1 and EL08-1D2 or AFT024 in stage 2²¹. However, the heterogeneity of these cell lines limited the clinical use of NK cells derived from such systems. A feeder-free system for differentiation and expansion of NK cells was established recently. This led to the development of a spin-dependent hematopoietic progenitor enrichment system that did not require sorting and could produce about 10 times more progenitor cells compared with a feeder-dependent sorting system. These two systems yielded a 1–2-log expansion of stem-cell-derived CD45⁺CD56⁺ NK cells in about 4 weeks¹⁵.

Most of the protocols for PB-derived NK cell expansion fall into two categories: feeder-cell systems and feeder-free systems. Several type of cells, including EBV-transformed lymphoblastoids and genetically modified HEK293 or K562 cell lines, are used as feeder cells^{22,23}. Genetically modified K562 is used most often. Expression of membrane-bound IL-21 in K562 feeder cells has significantly improved the expansion efficiency to 40,000-fold and the NK cell purity to 95% for 2–3-week cultures²⁴. However, there are safety concerns regarding the clinical application of cancer cell-derived feeder cells, although the feeder cells are lethally irradiated before use. Recently, irradiated autologous PBMCs combined with anti-CD16 antibodies have been used as feeder cells, resulting in a greater than 5,000-fold expansion rate²³. Furthermore, collected cell lysis particles from feeder cells have been used instead of whole cells for NK cell

expansion *in vitro* and *in vivo*; this may become an effective alternative to feeder-cell systems²⁵. On the other hand, feeder-free systems based on cytokines and antibodies have been developed to expand NK cells, but their expansion efficiencies range from hundreds- to thousands-fold, and the purity of the resulting NK cells ranges from 30% to 90%. Thus, the major focus for feeder-free NK cell expansion protocols is improving their reliability and efficiency. An IL-15 super-agonist complex, ALT-803, has shown NK cell-specific proliferation and cytotoxicity stimulate activities than its nature form IL-15²⁶. The development of these new artificial stimulators could improve feeder-free NK cell expansion systems and facilitate the production of NK cells of clinical grade in appropriate quantities.

Subsets of NK cells exert different cytotoxicities²⁷. CD56^{bright} NK cells harbor superior antitumor function compared with CD56^{dim} cells^{28,29} in general, although a subset defined as CD56^{dim}FE-1H10⁻ exhibited higher cytotoxicity than their CD56^{dim}FE-1H10⁺ counterparts³⁰. In addition, NK cell subsets with memory or memory-like profiles exhibited robust responses to tumor cells³¹⁻³³. These NK cell subsets could be exploited to improve NK cell-based immunotherapy, although strategies for their expansion require further study.

Clinical programs for NK cell-based cellular immunotherapy in cancer

NK cell adoptive transfer monotherapy

Owing to the pan-specific tumor cell recognition model of NK cells³⁴, adoptive transfer has been widely used in tumor immunotherapy clinical research. There are several clinical schemes for NK cell adoptive transfer. Some of the reported studies employed a single NK cell infusion scheme, with $1 - 10 \times 10^7$ NK cells/kg³⁵⁻³⁷, which was sufficient to evaluate safety and NK cell retention *in vivo*. However, considering that NK cells survive for about 2 weeks *in vivo*³⁸, a single infusion may not be sufficient to maximize the effect. Other studies employed an infusion scheme of $1 - 4 \times 10^7$ NK cells/kg three times weekly³⁹; this may guarantee the safety of each return, while increasing the dose of NK cells for total infusion. In addition, an infusion scheme of $1 - 4 \times 10^7$ NK cells/kg in 2 - 4 infusions once per week was employed⁴⁰. This infusion scheme may achieve the persistence and quantity of NK cells *in vivo* required for long-term therapeutic effect. However, this infusion scheme requires a weekly NK cells supply; considering the challenges of NK cells cryopreservation, the feasibility of this scheme is

uncertain. Both autologous and allogenic NK cells can be used in adoptive transfer cell therapy. However, the impaired development and function of patient-derived autologous NK cells and cell lines limit the clinical applications^{41,42}. Therefore, allogeneic NK cells have been employed in the majority of clinical trials of NK cell-based adoptive cell transfer⁴³. In addition to allogeneic PB-NKs, stem-cell-derived and iPSC-derived NK cells have been developed, and their tumor suppression effects have been confirmed in preclinical models^{13,44}. Clinical trials of stem-cell-derived and iPSC-derived NK cells against tumors are also ongoing (www.clinicaltrials.gov). Therefore, the optimization of the NK cells infusion scheme requires further study.

NK cell monotherapy has different clinical outcomes for different indications. Early clinical trials showed better clinical outcomes for allogeneic NK cell immunotherapy against hematopoietic malignancies, with complete and partial response rates of about 50%, compared with about 20% for NK cell immunotherapy against solid tumors^{38,45}. In recent years, the clinical scheme for NK cells immunotherapy has been optimized, and clinical outcomes have gradually improved (Table 1).

Although NK cell therapy has shown a promising tumor suppressive effect, the clinical outcomes of NK cell monotherapy are still limited. Hence, studies using NK cell therapy in combination with other therapies to maximize effectiveness have become mainstream.

Combinational therapy with NK cell adoptive transfer

In previous studies, NK cell therapy was usually combined with conventional cancer treatments such as chemotherapy or radiotherapy. Patients treated with 2 Gy whole-body irradiation followed by an infusion of 2×10^7 NK cells/kg 1 day later had significantly prolonged overall survival⁴⁶. Patients who received chemotherapy were subsequently infused with $1 - 3 \times 10^7$ NK cells/kg three times a week, leading to progression-free survival of about 4 months⁴⁷. For hematological malignancies, NK cell therapy was usually combined with hematopoietic stem cell transplantation (SCT); transferring 1×10^7 NK cells/kg 1 - 3 months after allo-SCT transplantation enhanced hematopoietic stem cell engraftment^{48,49}. However, several factors including dosage, intensity, and sequential order affect the outcomes of NK cells therapy. Therefore, increasing attention has focused on NK cell combination therapies in recent years (Table 1). Combined with chemotherapy, infusion of an increased dosage of 5×10^7 NK cells/kg prolonged the average disease-

Table 1 Clinical outcomes of NK cell immunotherapy (2017–2019)

Indication	Source of NK cells	Therapeutic regimen	Outcome	Reference
Allogenic PB-NK	Hepatic carcinoma	20×10^6 NK cells/kg on day 1 to day 3, monthly, 1–6 infusions	3/16 PR 8/16 SD 5/16 PD PFS was 7.5 months (range, 2–12 months)	64
Allogenic PB-NK	Children with intermediate- or high-risk AML	Cyclophosphamide (day -7), fludarabine(days -6 through -2), and subcutaneous interleukin-2 (days -1, 1, 3, 5, 7, and 9), $3.6\text{--}62.2 \times 10^6$ NK cells/kg were infused on day 0, 4–5 infusions	8/21 relapse occurred between 186 and 629 days after NK cell infusion 3/21 died of the disease	65
Allogenic PB-NK	Liver metastases of gastrointestinal carcinoma	$3.1\text{--}12.1 \times 10^6$ NK cells/kg were infused on day 0, cetuximab intravenously first on day 7, weekly, 7 infusions	1/9 PR 2/9 SD 5/9 PD 1/9 Rd	66
Allogenic PB-NK	Children with recurrent/refractory neuroblastoma	hu14.18K322A, 40 mg/m ² /dose, days 2–5, 7 infusions GM-CSF, and IL2 with chemotherapy: cyclophosphamide/topotecan (courses 1,2), irinotecan/temozolomide (courses 3,4), and ifosfamide/carboplatin/etoposide (courses 5,6) $4.7\text{--}59.5 \times 10^6$ /kg NK cells were administered with courses 2, 4, and 6	4/13 CR 4/13 PR 5/13 SD Median time to progression was 274 days (range, 239–568 days) 10/13 patients survived 1 year	67
Allogenic PB-NK	High-risk neuroblastoma	NK cells at one of five dose levels ranging from 1 to 50×10^6 CD3 ⁺ CD56 ⁺ cells/kg	10/35 CR or PR 8/35 PD 17/35 no response	68
Allogenic PB-NK	AML CML MDS	Melphalan (day -7), fludarabine (days -7, -6, -5, and -4). 200 cGy total body irradiation (day -3), 1×10^5 to 1×10^8 /kg/dose NK cells (days -2, -7, and -28). Bone marrow graft was infused fresh on day 0	54% developed grade 1–2 aGVHD; a low incidence of viral complications was observed. 1/13 died of non-relapse mortality 1/13 relapsed 11/13 alive and in remission at last follow-up (median 14.7 months)	52
Autologous PB-NK	Gastric or colorectal cancer	IgG1 antibodies treated on weeks -3, 3, 6, 10. NK cells, 3 doses 0.5×10^9 cells/injection on week 0, 3 doses 1.0×10^9 cells/injection on week 3, 2.0×10^9 cells/injection on week 6	4/6 SD 2/6 PD	69
Autologous PB-NK	Locally advanced colon carcinoma	Pre-treatment with 5-fluorouracil or oxaliplatin, then patients were treated with chemotherapy combined with NK cells $2.4\text{--}4.0 \times 10^9$ /injection	Median PFS 23.5 months Prevented recurrence Prolonged survival with acceptable adverse effects Poorly differentiated carcinomas	50
CB-NK	MM	Lenalidomide on days -8 to -2, melphalan on day-7, 5×10^6 to 1×10^8 CB-NK cells/kg on day -5 and auto-HCT on day 0	8/10 CR Median follow-up of 21 months, 4/10 progressed or relapsed, 2/10 died	51

AML: acute myeloid leukemia; CML: chronic myeloid leukemia; CR: complete response; MDS: myelodysplastic syndrome; MM: multiple myeloma; PD: disease progression; PFS: progression-free survival; PR: partial response; Rd: dissociated response; SD: disease stabilization.

free survival time to 23.5 months⁵⁰. In addition, NK cell infusion 1 week before hematopoietic stem cell transplantation benefited the regeneration of hematopoietic stem cells⁵¹. Moreover, patients with hematopoietic malignancies treated with radiotherapy followed by hematopoietic stem cell transplantation and three courses of 1×10^8 NK cells/kg achieved 85% remission lasting for about 14.7 months⁵². Based on the regulation of other cells by NK

cells^{53,54}, NK cell therapy can be combined with novel immunotherapies. Copik's group reported that adoptive transfer NK cells could induce the expression of PD-L1 on tumor cells; thus, NK cell infusion combined with anti-PD-L1 treatment could significantly improve the NK cells' anti-tumor efficacy, with prolonged NK cell persistence and retention⁵⁵. However, this has only been shown in animal models, with no clinical trial outcomes reported as yet.

Recently, a case study showed that a combination of radiochemotherapy, NK cell adoptive transfer, and PD-1 inhibition could effectively induce long-term tumor control in a patient with stage IIIb non-small cell lung cancer⁵⁶. However, NK cell combination therapies require further study and clinical verification.

Future prospects

Exploitation of off-the-shelf NK cells

Owing to the one-donor, one-patient strategy, the manufacture of clinical grade NK cells is a labor-intensive process. In addition, each batch of cell product may differ in yield, purity, and cell activity. In clinical applications, this requires a huge amount of work on product verification and a resulting lag-time to patient infusion. Although researchers have tried to introduce some criteria to predict or guarantee the success rate of cell production, it remains difficult to guarantee 100% cell infusion in less than 3–5 weeks. This may greatly limit the clinical applications of cell therapy. Therefore, there is a great need for an “off-the-shelf” product. Such a product should have sufficient cells for clinical use, and a clearly illustrated cell profile. Taking into account these quality and quantity criteria, monoclonal CD34⁺ stem cells, including iPSC-derived NK cells, may be suitable candidates. CD34⁺ stem cells are an unlimited NK cell source. NK cells derived from monoclonal CD34⁺ stem cells are homogeneous and can be clearly genetically defined. Kaufman’s group has established a manufacturing system for clinical grade iPSC-derived NK cells. Using genetic modification, they established a CAR-iPSC-NK cell line. A xenograft mouse model showed that CAR-iPSC-NK cell infusion could significantly inhibit tumor growth and prolong survival¹². This manufacturing strategy for monoclonal CD34⁺ stem-cell-derived NK cells may represent a feasible off-the-shelf cell product system, although this awaits confirmation through clinical research.

Manufacturing cell products

Currently, NK cells used for preclinical studies or clinical applications are usually cultured in T-flasks or bags. Handling these culture vessels is labor-intensive work and requires highly skilled experts. Using these culture vessels for every batch product leads to low levels of standardization. Thus, there is a great need to explore closed and automatic manufacturing systems, with the aim of achieving highly standardized cell manufacturing and reducing dependency

on specialized cell manufacturing centers. Miltenyi Biotech has developed a fully automated, large-scale NK cell expansion system, in which centrifugation, magnetic cell separation, and cell cultivation are performed in a closed system. NK cells produced by this automated system have similar yield and cell function to manually produced NK cells⁵⁷. Although this system was developed before 2015, its application is not universal. This is probably owing to the diversity of NK cell manufacturing processes with different initial cells, stimulators, and cell passaging principles. Currently, Boya Beijing, Novartis, and Kite Pharma are developing closed and automatic cell manufacturing systems. It is both challenging and necessary to develop an automated system suitable for diverse expansion systems.

NK cells cryopreservation

The condition of a patient, especially in cases of hematopoietic malignancy, may change at any time. It is difficult to guarantee that an NK cell product can be delivered to the patient at the appropriate time during the stable period of NK cell product quality, meaning that every newly started course of NK cell therapy needs the NK cells to be re-produced. Thus, there is a great need for NK cell cryopreservation technologies; these would increase the convenience of NK cell therapy in clinical applications, while reducing production costs and promoting the development of off-the-shelf products. However, current cryopreservation technology for NK cells has some limitations. The optimization of the cryoprotectant formula is ongoing, including the usage and proportion of autologous plasma, dimethyl sulfoxide (DMSO), and plasma substitution^{58,59}. A recent study showed that NK cells cryopreserved using freezing media containing RPMI1640, albumin, dextran, and DMSO retained about 90% viability and had no reduction in cytotoxicity after thawing. Moreover, viability and cytotoxicity were not significantly influenced after thawing when the cells were incubated in the presence of IL-2⁵⁹. This is a promising result for NK cell immunotherapy, although it requires verification.

Investigating the regulatory checkpoints of NK cell therapies to ensure safety and efficacy

Until now, regardless of whether autologous or allogeneic NK cell therapy was used, most clinical trials have shown better safety, less severe uncontrollable graft-versus-host disease (GVHD), and fewer severe adverse effects above grade 4. However, other cells in the final product used for NK cell

therapy represent a potential risk in clinical infusion; these include CD4⁺ T cells and B cells. At present, there are no criteria for the composition of NK cell therapy products. It is necessary to establish standards to define the proportion of NK cells and other immune cell subsets to avoid potential risks. NK cells can pan-specifically recognize malignant cells and have a broad-spectrum cytotoxicity to various malignant cells. However, clinical trial outcomes show that NK cell therapy has different effects on different tumors. Activation of NK cell function is determined by the integration of the received activating and inhibitory signals, so the activation trigger of NK cells is partially affected by the number and abundance of activating and inhibitory ligands expressed on the surface of target cells. In addition, various factors including cytokines and immunosuppressive cells in the tumor microenvironment affect NK cell activation and the clinical outcomes of NK cell therapy^{27,60-63}. Therefore, some regulatory checkpoints from the perspective of tumor cells and the tumor microenvironment will be beneficial to improve the outcomes of NK cell therapy. Establishing molecular and cell indices for NK cell therapy will also promote its clinical applications. The overall goal is to maximize the benefits of NK cell therapy while minimizing the risk.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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