

Challenges of NK cell-based immunotherapy in the new era

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Abstract Natural killer cells (NKs) have a great potential for cancer immunotherapy because they can rapidly and directly kill transformed cells in the absence of antigen presensitization. Various cellular sources, including peripheral blood mononuclear cells (PBMCs), stem cells, and NK cell lines, have been used for producing NK cells. In particular, NK cells that expanded from allogeneic PBMCs exhibit better efficacy than those that did not. However, considering the safety, activities, and reliability of the cell products, researchers must develop an optimal protocol for producing NK cells from PBMCs in the manufacture setting and clinical therapeutic regimen. In this review, the challenges on NK cell-based therapeutic approaches and clinical outcomes are discussed.

Keywords natural killer cells; immunotherapy; adoptive transfer; genetic modification; immune checkpoint inhibitor

Introduction

Natural killer (NK) cells are the direct killers of tumor cells [1,2]. NK cells recognize target cells through two classes of receptors: killer immunoglobulin-like receptors (KIRs) and killer activation receptors (KARs). KIRs recognize “self” molecules, pair with human leukocyte antigen (HLA) class-I molecules, and transmit inhibitory signals to maintain tolerance to NK cells. KARs pair with damage-associated proteins to recognize “abnormal” molecules on target cells and transmit activation signals. In tumor cells, HLA molecules are often reduced or absent, and damage-associated proteins are upregulated, reducing inhibitory signals through KIRs and promoting activation through KARs on NK cells [3]. Such recognition models between KIRs and HLA molecules are referred to as “missing-self” recognition, and KARs and damage-associated proteins are involved in “stress-induced” recognition [4]. Nevertheless, the triggering of the cytotoxicity of NK cells involves a balance between inhibitory and activation signals [4–6]. Activated NK cells can kill tumor cells directly by (1) secreting granules that contain perforin and granzymes and (2) ligation through Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand. Moreover, acti-

vated NK cells can secrete several cytokines and chemokines that can regulate innate and adaptive immune cells to achieve indirect cytotoxic activities (Fig. 1) [7,8].

Other well-known killer cells are cytotoxic T lymphocytes (CTLs) and natural killer T (NKT) cells. These cells recognize target cells through their antigen-specific T cell receptors (TCRs) and transmit activation signals. TCRs in CTLs or NKT cells pair with specific antigens on target cells presented by major histocompatibility complex (MHC) class-I molecules [9] or CD1d molecules [10,11]. These distinct recognition and activation mechanisms between T cells and NK cells may result in diverse actions and outcomes in cancer immunotherapy.

In this review, the contribution and challenges of NK cell-based immunotherapy are discussed.

Approaches of NK cell-based immunotherapy

Adoptive cell transfer (ACT)

ACT has been extensively applied in anticancer therapy in clinical trials [12]. In NK cells, the greater the alloreactivity between donor and recipient is, the better the antitumor efficacy is [13–16]. This relationship is consistent with the mechanisms of NK-cell recognition, whereby autologous NK cells are potentially inhibited by self MHC class-I molecules on cancer cells. Based on the genotypes of KIR

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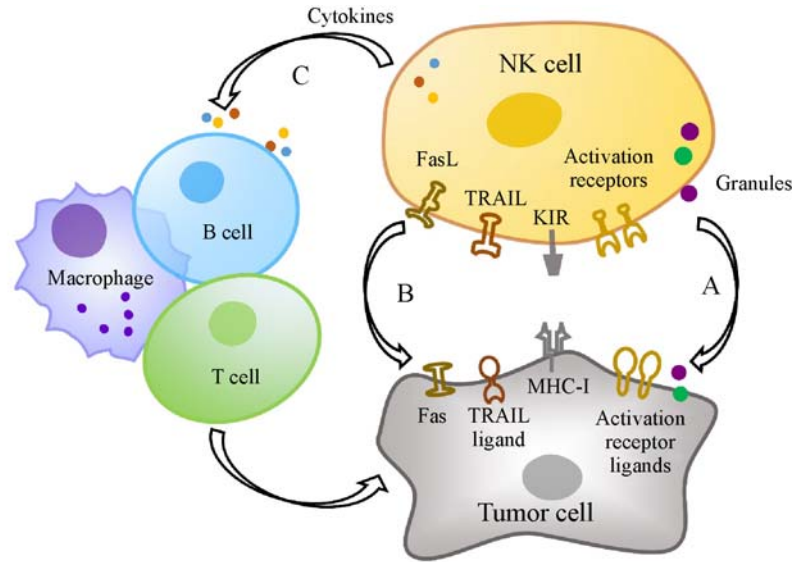


Fig. 1 Killing mechanisms of NK cell against tumor cells. Upon the formation of immunological synapse between activated NK cell and tumor cell, multiple killing mechanisms can be triggered, including direct killing of the tumor cell by the (A) release of granules containing perforin and granzymes and (B) induction of apoptosis through the ligation of Fas-FasL or TRAIL-TRAIL ligand, and indirect killing through (C) the secretion of factors that recruit and promote the activation of other inflammatory cells that indirectly kill a target cell.

and KIR ligands from donors and recipients, five donor-selection models have been developed and validated in clinical trials [17,18]. However, considering the feasibility and efficiency of selection of NK-cell donors, we should expand and evaluate the criteria for donor selection.

Two distinct basic protocols, with or without feeder cells, have been developed for the expansion of NK cells *ex vivo*. Both protocols can be used for expanding NK cells by over a thousandfold [19–22], remarkably improve NK-cell expansion to a greater extent than cytokine-induced killer (CIK) protocols, and lead to a new era of NK-cell therapy [23,24]. However, feeder cells derived from leukemia lines (e.g., K562) present safety consideration and require further purification and additional measures for quality control (Table 1). By contrast, feeder-free protocols

employ clinical-grade or US Food and Drug Administration-approved cytokines, stimuli, and antibodies with serum-free culture medium, and they can reach approximately 70% of NK cell purity from peripheral blood mononuclear cells (PBMCs) (Table 1). However, the number and purity of expanded NK cells from different donors remarkably vary, as observed in feeder-free protocols, which still need improvement. The purity and phenotype of the NK cells produced by different systems also differ [25,26]. These differences can directly influence the dose of the effector cells used for transfer and clinical efficacy. Overall, standardized measurements for the production and quality control of NK cells are lacking, possibly causing substantial variations in therapeutic outcomes.

Table 1 Manufacture of NK cells

Starting material	Protocol features	NK cell purity	Expansion fold	Properties	References
NK92	Cytokine	N/A	N/A	Additional irradiate step before use	[81,103]
CB-MNC	Allogeneic feeder cells	72%–95%	35–2389	–	[104,105]
Stem cell	Cytokines and antibodies	≥ 70%	1000–2100	Lack <i>in vivo</i> “education”	[106–111]
PBMC CD3 depleted or/and CD56 enriched	Cytokine combination	75%–99%	3–131	Additional purification	[79,112–115]
	Allogeneic feeder cells plus cytokine or/and anti body	≥ 90%	16–3637	step, low expansion rate	[116–120]
PBMC	Cytokines, antibodies, or/ and other stimulators	≥ 70%	140–5712	Simple protocols of expansion, low purity	[19,20,121]
	Feeder cells	66%–99%	20–14 116		[21,22,122,123]

CB-MNC, cord blood mononuclear cells; PBMC, peripheral blood mononuclear cell.

Genetic modification of NK cells

Genetic modification of cells, such as chimeric antigen receptor (CAR)-NK cells and armed-NK cells, enables the specific targeting or augmented cytotoxicity of NK cells [27,28]. This technique has been successfully implemented in T cells. Approximately two-thirds of clinical trials are targeting hematologic malignancies involving antigens, such as CD19, CD20, CD123, CD22, and B cell maturation antigen [29,30]. The most frequently targeted antigen is CD19. Clinical trials with CD19-CAR-T cells have revealed an objective response rate (ORR) of > 60%, and other reported complete remission (CR) rates have reached > 85% [30]. The reported side effects and toxicities include neurologic toxicity, cytokine release syndrome, tumor lysis syndrome, immunogenicity, and on-target, off-tumor recognition [30]. To some extent, the long-lasting and production of pro-inflammatory cytokines from CAR-T cells *in vivo* is the cause of severe side effects [31]. NK cells have been considered better candidates for CARs because their short lifespans last for nearly 2 week *in vivo* and they mainly produce interferon (IFN)- γ [32,33]. Moreover, CAR-NK cell activation not only depends on CARs but also is compromised by integrated signals from inhibitory and activating receptors. These multiple signals on CAR-NK cells can prevent on-target, off-tumor effects, but this system can also have unfavorable consequences. GD2-CAR-NK cells failed to eliminate GD2-expressing Ewing sarcomas in a preclinical study when a xenograft model is used mainly because GD2-CAR cells upregulate immunosuppressive ligand HLA-G in cancer cells. HLA-G interact with KIR on NK cells and transduce inhibitory signals [34]. CAR-NK cells targeting well-known targets, such as CD19, CD20, SLAMP7, and EpCAM, have been tested *in vivo* with in-mouse models or *in vitro*. However, only few of these targets have proceeded to clinical trials, including CD19-CAR-NK cells and CD33-CAR-NK cells (NCT0194479, NCT00995137), and the results have not yet been reported [35]. Appropriate design of the CAR structure and transfection of the expression vectors into NK cells are challenging steps in the development of CAR-NK cells. These steps will be discussed in detail in the section, “Challenges in NK cell immunotherapy.”

Checkpoint inhibitor

Checkpoint inhibitors targeting inhibitory receptors can augment immune-cell function, thereby effectively suppressing tumor cells [36–38]. Inhibitory KIRs expressed in NK cells play key roles in maintaining tolerance to NK cells. The KIR-blocking antibody IPH2101 is under a phase-II clinical trial for multiple myeloma treatment but responded minimally in preliminary trials possibly due to

the trogocytosis of KIR2DL1/L2/L3 molecules from the surface of NK cells by neutrophils and eosinophils induced by IPH2101. This effect may be reversed by optimizing the clinical scheme and combination therapy [39]. Furthermore, several blocking antibodies, such as the anti-NKG2A blocking antibody monalizumab and the anti-Tim-3 blocking antibody MBG453 [40–42], target immune checkpoints on NK and T cells and can reverse NK-cell dysfunction in preclinical studies. However, the safety and efficacy of these inhibitors or combination therapies require further investigation.

Cytokines and immunomodulatory drugs

Several cytokines and immunomodulatory drugs, such as the common γ -chain family of cytokines (interleukin (IL)-2, IL-7, IL-15, and IL-21), thalidomide, and pomalidomide, boost NK-cell cytotoxicity [43,44]. However, the targets of these cytokines and drugs vary [45]. Optimizing and improving the side effects of these modulatory factors are the key steps toward their clinical application [46,47].

Contribution of NK cells to cellular immunotherapy

Several cell types, including antigen-sensitized dendritic cells (DC), tumor-infiltrating lymphocytes (TIL), CIK cells, and NK cells, have been used for ACT immunotherapy [48–50]. Immunotherapy using NK cells is a pan-specific ACT immunotherapy that does not rely on the recognition of HLA-mediated tumor antigen [51]. Autologous and allogeneic NK cells can be both used in ACT immunotherapy because their safety and tolerability have been proven [52–54]. Indeed, allogeneic NK cells exhibit better anti-tumor efficacy than autologous NK cells [15,16,55]. Various sources of NK cells, including peripheral blood-derived NK (PBNK) cells, CD34⁺ stem cell-derived NK cells, and NK cell lines, such as NK-92, have been used for ACT immunotherapy [56]. PBNK cells are the most widely used in clinical trials because they are safe, can be collected conveniently, and can strongly kill tumor cells. However, because of the low number of PBNK cells in blood, they require to be expanded *ex vivo*. Unlike T cells, donor NK cells last approximately 2–3 weeks *in vivo* [57,58]. The short lifespan of NK cells reduces the risk of unpredictable long-term side effects. Moreover, NK cell transfer cannot cause serious or uncontrollable graft-versus-host disease (GVHD) or toxicity [12,59,60]. NK cells have been used for treating hematopoietic malignancies (e.g., leukemia, lymphoma, and multiple myeloma) and solid tumors (e.g., melanoma, ovarian cancer, lung cancer, colorectal cancer, and glioblastoma) [53,61] (Table 2).

Table 2 Clinical outcome of NK cell-based immunotherapy

Source of NK cells	Patient characteristic	Clinical outcome	References
NK-92	Solid tumor (<i>n</i> = 31)	CR = 0, PR = 4/31, SD = 5/31	[81,124,125]
	Lymphoma (<i>n</i> = 3)	CR = 1/3, PR = 1/3, SD = 0	[81,124]
	Hematopoietic malignancy (<i>n</i> = 12)	CR = 1/12, PR = 1/12, SD = 2/12	[103,124]
CD34 ⁺ cell-derived NK cells	Hematopoietic malignancy, reached CR in previous therapy (<i>n</i> = 18)	DFS ≥ 12 months, 1-year OS 11/15, 2-year OS 4/15	[62,73,126]
	Hematopoietic malignancy (NK cell combination therapy) (<i>n</i> = 20)	CR = 9/20, PR = 9/20, SD = 0	[73,126]
Autologous PBNK	Solid tumor (<i>n</i> = 36)	CR = 0, PR = 1/36, SD = 10/36	[122,127,128]
	Hematopoietic malignancy (<i>n</i> = 9)	CR = 0, PR = 2/9, SD = 3/9	[129,130]
Allogeneic PBNK	Solid tumor (<i>n</i> = 58)	CR = 0, PR = 12/58, SD = 31/58	[78,79,128,131]
	Lymphoma (<i>n</i> = 6)	CR = 2/6, PR = 2/6, SD = 0	[55]
	Hematopoietic malignancy, reached CR in previous therapy (<i>n</i> = 16)	DFS ≥ 18 months, 1-year OS 13/16, 2-year OS 12/16	[63,64]
	Hematopoietic malignancy (<i>n</i> = 24)	CR = 10/24, PR = 1/24, SD = 0	[58,64,132]

PBNK, peripheral blood mononuclear cell-derived NK cells; CR, complete remission; PR, partial remission; SD, stable disease; DFS, disease-free survival; OS, overall survival.

Hematopoietic malignancies

Infusion of NK cells for hematopoietic malignancy treatment is used in two settings: stem-cell transplantation (SCT) and non-SCT.

Allogeneic NK cells control hematopoietic malignancies effectively because of the graft-versus-leukemia effect. In patients who have reached CR or morphologic CR, adoptive NK cell transfer can remarkably prolong disease-free progression. Of the 28 patients reported, 4 had CR lasting for about 1 year, 5 had CR lasting for about 1.5 years, and 10 had CR lasting for about 2 years [62–64]. Meanwhile, 24 out of 50 patients with active diseases reached CR [55, 58, 65–67]. In patients with relapsed or refractory acute myeloid leukemia (AML), the mean CR rate was approximately 40%, which was considerably higher than that obtained by conventional chemotherapy (10%) [53] (Table 2). Overall, such clinical trials demonstrated that allogeneic NK cell adoptive transfer, as immunotherapy for hematopoietic malignancies (especially AML), is efficacious and safe without severe GVHD or side effects.

NK cell adoptive transfer has been used extensively in combination with SCT for hematopoietic malignancies. SCT, combined with NK cells, improves two-year overall survival (OS) to 36% and reduces the risk of relapse, compared with SCT alone, which achieves a two-year OS of 15% [68–70]. The sequence of SCT and infusion of NK cells influence clinical outcomes. NK cell infusion performed before or within 2 weeks after SCT has a better clinical outcome than that performed 4 weeks after SCT [68–74], suggesting that NK cells contribute to the control of tumor metastasis, minimal residual disease, and tumor stem cells. Nevertheless, the optimal clinical procedures,

including timing, frequency, and dose of NK-cell infusion, may need more extensive studies.

Compared with NK cells, CIK cells are less effective against hematopoietic malignancies. Patients with hematopoietic malignancies receiving CIK treatments, the ORR was 30% (60/203), however the ORR was 58% with NK-cell treatment (Table 2) [75]. The reason for the low efficacy of CIK cells could be because > 70% of the CIK cells are T cells (mainly naive T cell). In addition, tumor-associated antigen-specific T cells, especially CAR-T cells, have become the central player and recently demonstrated exciting results against hematopoietic malignancies.

Although NK cell immunotherapy has demonstrated substantial clinical benefit against hematopoietic malignancies, the relapse rate still needs to be improved. NK cell therapy can be potentially used for combination therapy.

Solid tumors

NK cells have innate advantages in the treatment of certain solid tumors, such as melanoma and ovarian cancer. More than half of melanomas *in situ* have reduced or absent HLA expression, which is required in the mediation of CD8⁺ T cell recognition [76] but not in NK cells recognition. Therefore, NK cell therapy is an effective approach against these cancers. Moreover, IFN- γ is the major cytokine secreted by NK cells and has been shown to induce the permanent arrest of the growth of melanoma cells [77]. Ovarian cancer cells have high expression of MICA/B and ULBPs, which can activate NK cells through the active receptor NKG2D [51]. However, immunotherapies, which use NK cells for melanoma, ovarian cancer, and other solid tumors, can control disease progression but limitedly improve or abate disease [76,78–83]. The possible reasons

include (1) low infiltration rate of NK cells to the solid tumor; (2) inhibitory factors, such as transforming growth factor- β , in the tumor microenvironment; and (3) inhibition from tumor cells, such as residual HLA expression. Potential solutions for these crucial issues have been proposed, including enhancing the migration of NK cells to tumor sites, altering the tumor microenvironment through gene modification or clearance of immunosuppressive cells, and combining with a checkpoint inhibitor to activate NK cells. Recent studies have shown that IFN- γ can drive the fragility of T-regulatory cells to promote anti-tumor immunity, suggesting that NK cells, as an important source of IFN- γ , may substantially contribute for the immunotherapy of solid tumors [84].

Cancer stem cells (CSCs)

CSCs, which have low proliferation rate and asymmetrical growth, are resistant to conventional tumor therapy. The low expression of MHC class-I molecules, which mediate the recognition of CD8⁺ T cells to target cells, predicts the low cytolytic efficiency of CD8⁺ T cells to CSCs [85]. NK cells, which can kill “self-missing” tumor cells, preferentially target CSCs derived from colon tumors, melanomas, glioblastomas, pancreatic tumors, and breast tumors [85]. Furthermore, NK cells also play a key role in suppressing tumor metastasis formation with possible preferential targeting of CSCs [86,87]. Indeed, NK-cell therapy remarkably prolongs disease-free progression in hematopoietic malignancies [55,58,65–67], suggesting that NK cells may contribute in controlling the metastasis and recurrence of tumor cells by targeting CSCs preferentially. However, NK cells need further investigation and more evidence on their control of CSCs.

Combination therapy

Several studies have tested the combination of NK cells with traditional therapy and/or immunotherapy to improve clinical outcomes. Two strategies have been proposed.

First is the reduction of tumor burden and disruption of tumor stroma, thereby enhancing tumor cell sensitivity. Radiotherapy can upregulate the expression of stress-related ligands on tumor cells, thereby increasing their sensitivity to NK cells. Local radiation combined with NK cell adoptive transfer can remarkably prolong the survival of tumor-bearing mice [88]. An undergoing study have moved forward to phase-II clinical trials for the assessment of the effects of radiochemotherapy with NK cells on patients with non-small-cell lung cancer [89]. Several clinical trials combining NK cells and chemotherapy for solid tumor treatment are underway [61,90,91]. Chemotherapy has been shown to promote the sensitivity of tumor cells to NK cells and deplete immune cells to make room for infused NK cells [92,93].

Second is the enhancement of cytolytic ability or tumor-cell targeting of NK cells. Federico *et al.* used anti-GD2 monoclonal antibody combined with chemotherapy and transferred haploidentical NK cells to treat recurrent or refractory neuroblastoma, resulting in a promising anti-tumor activity. Out of 11 patients, four had a CR, one had an excellent partial response, and three had a partial response; the one-year OS was 77%, and the median time to progression was 274 days [94]. Moreover, checkpoint inhibitors, such as the antibodies anti-KIR [95], anti-CD137 [96], and anti-TIGIT [97], have been exploited for the augmentation of NK cell functions. Bi- or tri-specific killer engagers that can enhance the targeting of NK cells to tumor cells and augment the cytotoxicity of NK cells have been intensively studied [98,99]. However, the outcomes of these combination therapies are diverse, and the role of these accelerants in combination with NK-cell therapy remains to be validated clinically [61,78,90,91]. These phenomena are probably caused by poor persistency and proliferation of NK cells *in vivo*. These conditions are expected to be improved by *ex vivo* activation and optimization of therapy regimen (e.g., dose, intensity, and the sequential order of the different treatments).

Challenges in NK cell immunotherapy

Preclinical and early clinical trials with NK-cell immunotherapies exhibit safety and encouraging clinical outcomes against hematologic malignancies. However, only a few of early-stage clinical trials on solid tumors have not reached any conclusion (Table 2). More studies on solid tumors are needed for the exploration of tumor types sensitive to NK cell immunotherapy.

Various cell types have been developed for NK cell generation for immunotherapy, including induced pluripotent stem cells, cord blood, peripheral blood NK cells, and NK-92 line. Although each cell type exerts some unique features, the potent cytotoxic response against different types of tumors remains to be elucidated. Moreover, developing off-the-shelf and strong cytotoxic NK cells is necessary in clinical applications.

NK and T cells use distinct tumor cell recognition mechanisms. They can be sensitive or synergistic to different tumor types. So, the clinical outcome of combination therapy with NK and T cells or other tumor therapy strategies need to be confirmed with large-scale clinical trials.

Allogenic NK cells from peripheral blood are safe and satisfactorily effective against tumors. Meanwhile, variations across individual donors for *in vitro* NK cell expansion rate and cytotoxicity are observed. Although predicted donor selection models, considering KIR-MHC I mismatches, has been proposed [17], and biomarkers influencing other NK cell properties and cytotoxicity, such

as matrices of KARs and KIRs, in both donor and recipient remain to be further elucidated.

CAR expression enables carrier cells to recognize antigens on tumor-cell surfaces without MHC restriction. Transfection efficiency for primary NK cells particularly remains the bottleneck. The transduction efficiency can reach approximately 70% in retroviruses in T cells [29], 15%–40% in NK cell lines [100], less than 20% in primary NK cells, and 6%–96% in *ex vivo* expanded NK cells [101]. Transiently inhibiting the antiviral defense signaling pathway leads to remarkably increased virus transduction efficiency, but it is not practical for the large-scale manufacture of CAR-NK cells [102]. Electroporation can substantially increase the transfection efficiency, but transient expression limits its CAR expression when the cell proliferated *in vivo* [101]. In summary, an efficient, reliable, and convenient transfection protocol is the bottleneck for developing gene-modified NK cells.

Conclusions

NK cells, as the only innate immune cells in lymphocytes, have unique advantages for tumor immunotherapy. The challenges in both optimized clinical schemes and techniques of NK cell generation remain to be developed. Optimal combinations with NK cells and other therapeutic methods, including T cell therapy could be synergistic to tumor immunotherapy.

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Compliance with ethics guidelines

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