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Review Article

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NK Cell-Based Immunotherapies in Cancer

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ABSTRACT

With the development of technologies that can transform immune cells into therapeutic modalities, immunotherapy has remarkably changed the current paradigm of cancer treatment in recent years. NK cells are components of the innate immune system that act as key regulators and exhibit a potent tumor cytolytic function. Unlike T cells, NK cells exhibit tumor cytotoxicity by recognizing non-self, without deliberate immunization or activation. Currently, researchers have developed various approaches to improve the number and anti-tumor function of NK cells. These approaches include the use of cytokines and Abs to stimulate the efficacy of NK cell function, adoptive transfer of autologous or allogeneic *ex vivo* expanded NK cells, establishment of homogeneous NK cell lines using the NK cells of patients with cancer or healthy donors, derivation of NK cells from induced pluripotent stem cells (iPSCs), and modification of NK cells with cutting-edge genetic engineering technologies to generate chimeric Ag receptor (CAR)-NK cells. Such NK cell-based immunotherapies are currently reported as being promising anti-tumor strategies that have shown enhanced functional specificity in several clinical trials investigating malignant tumors. Here, we summarize the recent advances in NK cell-based cancer immunotherapies that have focused on providing improved function through the use of the latest genetic engineering technologies. We also discuss the different types of NK cells developed for cancer immunotherapy and present the clinical trials being conducted to test their safety and efficacy.

Keywords: NK cells; Cancer; Immunotherapy; Tumor microenvironment

INTRODUCTION

Due to the severe and/or obvious side effects of the available cancer drugs and radio therapeutic approaches, a much attention is being paid to cancer immunotherapy, which activates the immune system of patients with cancer. Among the different types of immune cells, NK cells represent innate immune cells that are CD3 negative and CD56 positive; they play important roles in cancer immune surveillance. Constituting approximately 5%–15% of the circulating lymphocytes in humans, NK cells can be classified into subpopulations based on their maturation status and functional characteristics. Unlike T cells or other

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Conflict of interest

The authors have no financial conflicts of interests.

Abbreviations

ADCC, Ab-dependent cellular cytotoxicity; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CAR, chimeric Ag receptor; CIK, cytokine-induced killer; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; CRS, cytokine release syndrome; EBV, Epstein-Barr virus; EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid cell lines; EGFR, epidermal growth factor receptor; ETC, endogenous T cell; FDA, Food and Drug Administration; FLT3, Fms-related receptor tyrosine kinase 3; GvHD, graft-versus-host disease; haNK, off-the-shelf CD16-targeted natural killer cells; HIF, hypoxia-inducible factor; HSCT, hematopoietic stem cell transplantation; iPSC, induced pluripotent stem cell; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, killer-cell Ig-like receptor; LGL, large granular lymphocyte; MDSC, myeloid-derived suppressor cell; NCT, National Clinical Trial; NKG2A, NK group protein 2 family member A; NKG2D, NK group protein 2 family member D; NSCLC, non-small cell lung cancer; PBL, peripheral blood mononuclear lymphocyte; RCC, renal cell carcinoma; SCF, stem cell factor; scFv, single-chain variable fragment; TCGF, T cell growth factor; TME, tumor microenvironment; TRAIL, TNF-related apoptosis-inducing ligand; UCSD, University of California, San Diego

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adaptive immune cells, NK cells exhibit high cytotoxicity against tumors and virus-infected cells without prior sensitization. Activated NK cells can also act as regulatory cells by secreting various cytokines to trigger and expand the adaptive immune responses against targeted tumor cells (1). Various NK cell sources are currently being used for adoptive cancer immunotherapy; they include autologous NK cells, allogeneic NK cells, NK cell lines from peripheral blood and stem cells, and genetically engineered NK cells (2).

The function of NK cells is tightly regulated by the balance between activating and inhibitory receptors. Inhibitory signals blocking NK cell activation generally result from the interaction between killer cell Ig-like receptors (KIRs) and MHC class I (3). Activating NK cell receptors, such as NK group protein 2 family member D (NKG2D), NKp30, NKp46, NKp44, and DNAM-1, allow for the recognition of the stress-induced ligands expressed on tumor cells, and prompt NK cells to kill tumor target cells through the release of cytotoxic granules containing perforin and granzyme B (4,5). NK cells also mediate Ab-dependent cellular cytotoxicity (ADCC), an immune mechanism through which Fc receptor-bearing NK cells can recognize and kill Ab-coated target cells expressing tumor-derived Ags on their surface. NK cells express the low-affinity Fc activating receptor CD16, which is composed of FcγRIIIa (CD16a) and FcγRIIIb (CD16b) (6). Upon binding to the Fc portion of IgG, the cross-linking of Fc receptor with the surface of NK cells induces phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by cellular Src kinases, resulting in cytotoxic granule release and lysis of the target cell via the predominant perforin/granzyme cell death pathway.

NK cells are also crucial to rebuffing undesirable cellular targets, which include tumors, virally infected cells, and allogeneic bone marrow through their ability to differentiate MHC-1 molecules between normal and abnormal non-self-cells (2). When MHC-I molecules that are present on target cells interact with NK cell inhibitory receptors, NK cells are unable to eliminate the target cells (7,8). A lack of MHC-I on target cells, however, leads to NK cell activation through a mechanism called 'missing-self recognition', which allows NK cells to dispose of the target cells (9). For example, due to the lack or downregulated expression of MHC on bacteria, viruses, and impaired or infected cells, NK cell activity, which is usually suppressed by inhibitory receptors on target cells, is unhindered and NK cells are more prone to attack these target cells (10). Thus, the MHC-dependent inhibitory signal is essential during the effector phase for NK cell tolerance.

MHC-I molecules can be categorized as classical MHC-Ia or non-classical MHC-Ib. NK cell receptors that interact with MHC-Ia consist of the human KIR family receptors and the murine Ly49 family of inhibitory receptors. MHC-Ib molecules, such as HLA-E in humans and Qa1 in mice, are recognized by receptors such as NKG2/CD94 in both humans and mice (11,12). KIRs are Ig superfamily receptors that are involved in NK cell inhibition and activation as well as missing-self recognition (10). Long-tailed KIR receptors have one or two immunoreceptor tyrosine-based inhibition motifs (ITIMs) that support inhibitory signaling. Since a considerable percentage of the human population does not have activating KIRs, these receptors are not as clinically critical for NK cell activation (13).

C-type lectin receptors can substitute the lack of activating KIRs (13). Ly49 and NKG2 family receptors are both type II C-type lectin-like receptors. Murine Ly49-family receptors include both inhibitory and activating receptors that are characterized by the existence or lack of ITIM domains in the cytoplasmic tail (14,15). Ly49 inhibitory receptors are capable of

inhibiting a missing self-response by binding to MHC-I. NKG2 family members contain 2 ITIMs in its cytoplasmic tail (16,17). NKG2A/CD94 is an inhibitory receptor that plays a significant role in hindering NK cell-mediated killing by ligating to HLA-E (18). Like inhibitory KIRs, NKG2A/CD94 also moderates missing self-recognition to aid NK cell self-tolerance. NKG2A/CD94 contributes to the migration of NK cells as well, which augments target cell binding and the probability of cell elimination (19). When Ly49 inhibitory receptors and NKG2A/CD94 receptors bind to MHC-I, the interaction leads to the phosphorylation of tyrosine residue in the ITIM domain, which then leads to recruitment of the Src-homology 2 domain-containing protein tyrosine phosphatases, such as SHP-1, which then generates inhibitory signals (20).

Based on the actions and abilities of NK cells, these cells attract significant attention, and their use shows a great promise in the field of cancer immunotherapy. In this review, we focus on the current status and recent advances in NK cell-based cancer immunotherapy, including autologous and allogeneic NK cells, NK cell lines, and human induced pluripotent stem cell (iPSC)-derived and chimeric Ag receptor (CAR)-NK cells, along with the clinical trials being carried out in this field.

CURRENT STATUS OF CANCER IMMUNOTHERAPY

Traditional methods of cancer treatment include surgery, chemotherapy, and radiotherapy. Despite the significant efficacy of conventional therapies with respect to eliminating primary tumors, tumor recurrence remains a common issue. Therefore, alternative strategies are required to solve these problems.

The field of cancer immunotherapy is rapidly growing and is becoming an attractive strategy for the use against severe malignant tumors. This includes mAbs such as immune checkpoint blockades, cancer vaccines, and adoptive cell therapies utilizing endogenous and engineered T cells, and NK cells (21,22). Among these, Ab-targeted therapy becomes the standard treatment for several malignant cancers. To date, immune checkpoint blockade has proved to be the most successful approach for use in the clinic (23). Immune checkpoint therapy comprises blocking Abs that obstruct proteins that inhibit T cell activation, thus allowing cytotoxic T cells to target tumors (24). Clinical trials using Abs against PD-1/PD-L1 have demonstrated durable response in melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), and Hodgkin's lymphoma (25). In addition, patients with melanoma have shown reliable clinical results with anti-CTLA-4 treatment (26). These convincing clinical results have enabled the Ab against CTLA-4 (ipilimumab) and 2 different Abs against PD-1 (pembrolizumab and nivolumab) to achieve Food and Drug Administration (FDA) approval in 2011 and 2014 for melanoma treatment (26).

In case of cancer vaccines, although preclinical studies have shown promising results over the past years, therapeutic cancer vaccination in humans has demonstrated only moderate success (27); only one cancer vaccine, sipuleucel-T (Provenge, Dendreon) for the treatment of asymptomatic or minimally symptomatic metastatic, castration-resistant prostate cancer, has been approved by the FDA until now (28).

The low-frequency endogenous T cells (ETCs) reactive to tumor Ags from the peripheral blood of patients can be isolated and expanded *ex vivo* for adoptive transfer for the treatment

of melanoma and other solid tumors. Once tumor Ag has been ensured, ETC is immediately available for clinical trials. This characteristic of ETC is useful to develop personalized Ag-specific T-cell therapy for solid tumor patients, including colorectal, pancreatic, and ovarian cancers. Clinical trials with MART-1 and gp100-specific $CD8^+$ T cells resulted in moderate clinical improvements in 8 of 10 metastatic melanoma patients (29).

For patients with refractory or relapsed acute lymphoblastic leukemia (ALL) who were treated with engineered CD8 T cells retrovirally transduced with anti-CD19 CAR constructs, over 90% remission rate was achieved (30). Despite its success, the safety of CAR-T therapy is still in question due to the toxicity reported in some studies (31,32). Other challenges to the use of CAR-T cell therapy in the mainstream include the exploration of target Ags that are not expressed in healthy tissues and overcome the tumor immunosuppressive microenvironment. In addition, adoptive immunotherapy with NK cells has shown great potential for treating malignant solid tumors (33). Unlike CAR-T cells, they do not need to be patient-specific, which makes them better applicable for use in cancer treatment. Several applications of NK cells in cancer immunotherapy will be discussed in this review.

The recent development of cancer immunotherapy, such as CAR-T cells, NK cell adoptive immunotherapy, and checkpoint inhibitors, provides wide treatment options for individual patient. Therefore, improved complete response (CR) and overall survival in advanced cancer patients have become more conceivable. In addition to these immunotherapeutics, personalized combination therapy specifically tailored to match the genetic and epigenetic characteristics of each patient proved to be a promising approach to boost the effect of cancer therapy.

NK CELL THERAPY: AN ALTERNATIVE TO CAR-T CELL THERAPY

First described in the 1970s, NK cells have been a promising tool in the field of adoptive immunotherapy (34). They have the ability to target and destroy tumor cells without prior sensitization, via activation of NK cell-activating receptors against ligands present on tumor target cells. The function of NK cells is defined by the balance between the inhibitory receptors (killer inhibitory receptors and NK group protein 2 family member A [NKG2A] and killer cell lectin-like receptor subfamily G member 1) and the activating receptors (natural cytotoxicity receptors, NKp30, NKp44, NKp46, NKG2D) (34). Under the normal condition, inhibitory KIRs bind to the HLA-I and inhibit the tumor-killing activity of NK cells. However, upon encountering tumor cells, NK cell activation is triggered by binding NK activation receptors with their respective ligands expressed on target tumor cells (35). NK cells eliminate target cells by various mechanisms, such as releasing perforin and granzyme, ADCC, and mediating cytotoxicity by apoptotic pathways including TNF or FAS ligands (36-38).

Several clinical studies have reported NK cell-based immunotherapy to be a promising treatment for cancer. In patients with cancer, NK cell function is generally inhibited due to the reduced expression of NK cell-activating receptors, thus impairing their tumor-killing activity. In this regard, adoptive immunotherapy with NK cells has emerged as a promising solution against a number of malignancies (39). One of the well-known methods of NK cell-based adoptive immunotherapy involves *ex vivo* expansion and activation. This method has been developed to increase both the number and antitumor activity of NK cells to overcome immunosuppression that is commonly observed in solid tumors.

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Several approaches have been developed to generate NK cells for adoptive immunotherapy. One of these approaches involves using cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-21, to culture and expand NK cells (40). These cytokines can upregulate the expression of activating receptors present on NK cells, thereby enhancing the anti-tumor activity of NK cells against the cells that express the respective receptor ligands. Co-culturing NK cells with growth-inactivated feeder cells may also be used to enhance NK cell proliferation and activation. Culturing NK cells *ex vivo* has shown to condition NK cells to target tumors that are resistant to the function of NK cells (41).

LIMITATIONS OF CAR-T CELL THERAPY

Currently, the most prominent form of immunotherapy uses CAR-T cells. Several pharmaceutical companies, including Gilead (Kite Pharma, Los Angeles, CA, USA), Novartis (Basel, Switzerland), and Juno Therapeutics (Seattle, WA, USA), have lined up several CAR-T cells in their pipelines. CAR-T cells are produced using autologous T cells from patients through genetic engineering to express a CAR for tumor-specific or tumor-associated Ags. Genetically expressed CAR sequences enable these modified T cells to kill tumor cells of corresponding patients via HLA-independent matter. Although CAR-T treatment represents a powerful and promising therapy for patients with cancer, CAR-T cells are associated with a high level of toxicity, potentially resulting in severe life-threatening conditions such as the cytokine release syndrome (CRS) (42).

CD19 CAR-T therapies have shown high remission rates in patients with ALL and B-cell lymphomas, but durable remissions have not been reported. Approximately 40% of patients showing disease remission at 1 month with CD19 CAR-T eventually relapse within 1 year of treatment, possibly due to the low rate of *in vivo* persistence and Ag loss (43). In addition, the use of CAR-T cells for the treatment of solid tumor has the limitation due to the complex structures of solid tumors such as extracellular matrix and inhibitory tumor microenvironment (43). These structures limit the contact of CAR-T cells to solid tumors (44).

Compared to the disadvantages of CAR-T cells, the significant advantages of NK cells for patients with cancer include their ability to be used as “off-the-shelf” treatments, as NK cells are not required to be specific to a particular patient. This advantage enables their use in the clinic, in combination with other drugs for cancer treatment. In this review, we discuss the key types of NK cell-based cancer immunotherapy (**Fig. 1**) considering the characteristics and advantages of NK cells.

AUTOLOGOUS AND ALLOGENEIC NK CELL THERAPY

NK cell therapy can be split into autologous and allogeneic cell therapy. Autologous cell therapy implies collection of the cells from a patient before being expanded, processed, and activated. These cells are then refused back into the initial patient. Cells in allogeneic NK cell therapy are not collected from the patient who undergoes treatment, but from various donors before being processed and infused into patients. Although autologous NK cell expansion and activation for adoptive cancer immunotherapy has been reproducibly shown *in vitro*, they have limited function against the autologous tumors in the clinic. The Rosenberg group has shown that the adoptive transfer of autologous NK cells does not demonstrate significant

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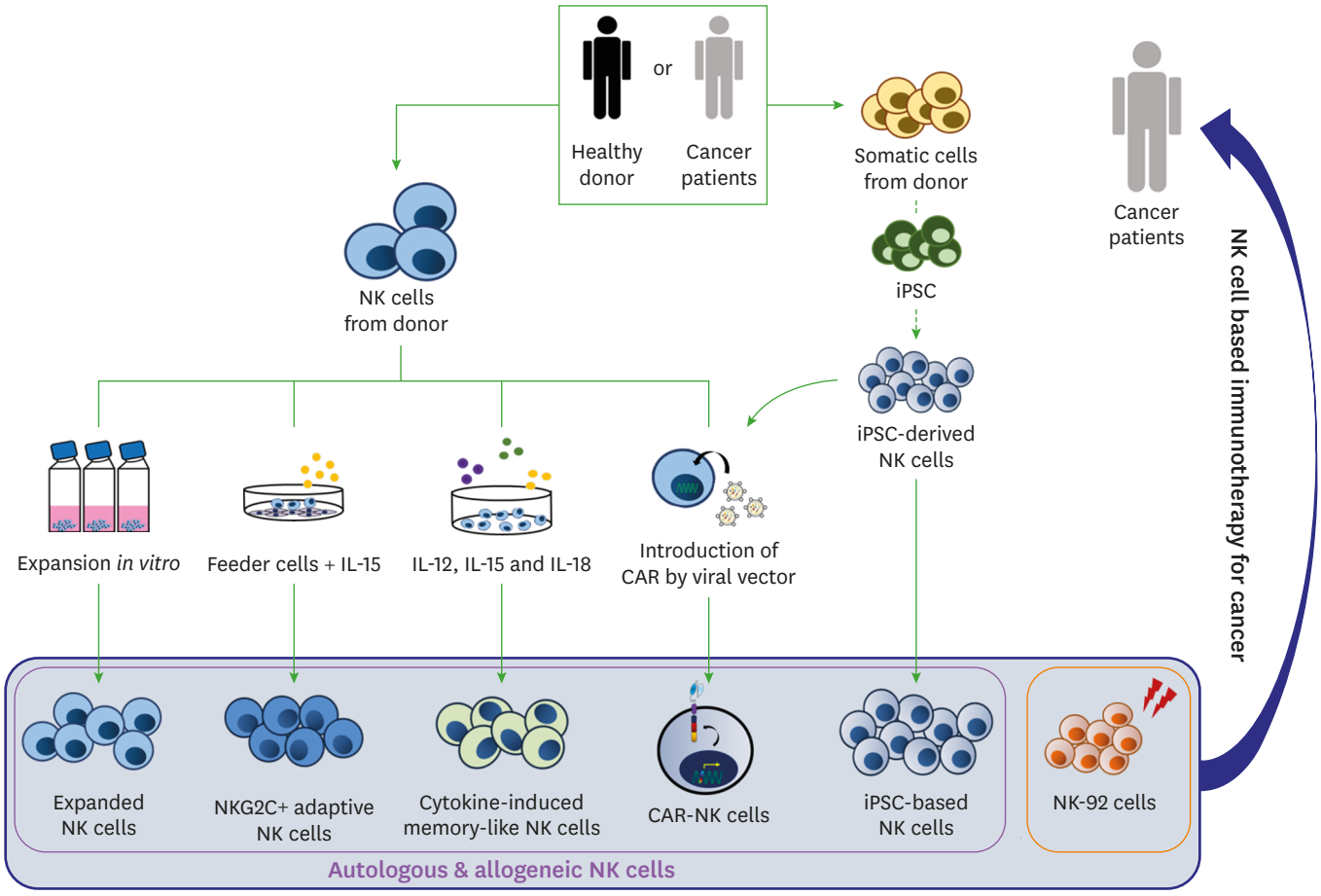

Figure 1. Methods of NK cell-based immunotherapy for cancer. Types of NK cells used for cancer immunotherapy. Allogeneic NK cells and autologous NK cells, manipulated with cytokines (IL-12, IL-15, and IL-18) and CAR, resulted in various types of NK cells for cancer immunotherapy. Somatic cells from the donor could be used as functional iPSC-based NK cells. Irradiated NK-92 cell line also served as an important resource for NK cell-based cancer therapy.

clinical results in patients with melanoma and renal cell carcinoma (RCC) (45). Although the adoptively transferred *ex vivo* IL-2 activated autologous NK cells survived for a long period of time, their clinical activity was minimal as well (45). This limitation may be explained partly by the inhibition of NK cell-mediated cell killing by the recognition of the "self" class I MHC on tumor cells by the inhibitory KIR.

Another reason underlying this limitation could be the difficulty of ensuring the pure and enhanced expansion of autologous NK cells from patients with cancer. In addition, autologous NK cells are vulnerable to the presence of resistant tumor cells, and show a tumor-targeting issue; therefore, tumor cells resistant to autologous NK cells can survive and grow (46). These significant disadvantages limit the use of autologous NK cells for cancer immunotherapy. Selected clinical trials with autologous NK cells, based on these features, are presented in **Table 1** (47).

With the discovery of inhibitory KIR that decrease NK cell-mediated killing of self-MHC-I expressing tumor cells, researchers have begun to explore the possibility of using allogeneic NK cells for cancer treatment, considering their enhanced anti-tumor activity (48). The functional activity of autologous NK cells is limited in patients with cancer, primarily due to the KIR ligand match (49). However, the alloreactivity of NK cells promoted by the mismatch

Table 1. Selected clinical trials with autologous NK cells

NCT number	Title	Status	Conditions	Interventions	Phase	Start date	Locations
NCT02481934	Clinical Trial of Expanded and Activated Autologous NK Cells to Treat Multiple Myeloma	Completed : Started participant 5 : Not completed 2 (death) : Completed 3 (not specified)	· Multiple myeloma	· Procedure: activated and expanded NK cells infusion · Drug: lenalidomide · Drug: bortezomib	Phase 1	March 2013	· Hospital Universitario 12 de Octubre, Madrid, Spain
NCT02185781	Phase I Study of Adoptive Immunotherapy With Enriched and Expanded Autologous Natural Killer (NK) Cells for Patients With Ph+ Acute Lymphoblastic Leukemia (ALL)	Recruiting	· ALL · Complete hematologic remission · Persistent/ recurrent minimal residual disease	· Other: autologous NK cells infusions	Phase 1	November 2014	· ISS/AIFA, Roma, Italy · Ospedale S. Eugenio, Roma, Italy · Università Cattolica del Sacro Cuore - Policlinico A. Gemelli, Roma, Italy · Università degli Studi "Sapienza" - Dip Biotecnologie Cellulari ed Ematologia - Divisione di Ematologia, Roma, Italy · Università degli Studi - Policlinico di Tor Vergata, Roma, Italy
NCT00720785	Natural Killer Cells and Bortezomib to Treat Cancer	Recruiting	· Chronic myeloid leukemia · Pancreatic Cancer · Colon/rectal cancer · Multiple myeloma · NSCLC	· Drug: NK cells + CliniMACs CD3 and CD56 systems · Biological: NK cells	Phase 1	August 1, 2008	· National Institutes of Health Clinical Center, 9000 Rockville Pike, Bethesda, MD, United States
NCT00328861	Natural Killer Cells Plus IL-2 Following Chemotherapy to Treat Advanced Melanoma or Kidney Cancer	Completed	· Metastatic melanoma · Metastatic kidney cancer	· Drug: NK lymphocytes · Biological: IL-2 · Drug: cyclophosphamide · Drug: fludarabine	Phase 2	May 2006	· National Cancer Institute (NCI), Bethesda, MD, United States
NCT03941262	Autologous Natural Killer Cells in Participants With Pathologically Confirmed Cancer	Recruiting	· Malignant neoplasm	· Drug: autologous NK cells	Phase 1	July 15, 2019	· Sarcoma Oncology Research Center, LLC, Santa Monica, CA, United States
NCT03894579	Autologous Natural Killer Cells in Subjects With Moderate to Severe Psoriasis	Recruiting	· Moderate to severe plaque psoriasis	· Biological: study · Product: SNK01	Phase 1	July 24, 2019	· Hospital Angeles, Tijuana, BC, Mexico
NCT03958097	A Pilot Study of NK Cell Combined With PD-1 Antibody as Second Line Therapy for Advanced Driver Mutation Negative Non-small Cell Lung Cancer	Recruiting	· NSCLC	· Combination product: NK cell and PD-1 Ab	Phase 2	May 17, 2019	· First Hospital of Jilin University, Changchun, Jilin, China
NCT01884688	UARK 2013-05 A Study of Autologous Expanded Natural Killer Cell Therapy for Asymptomatic Multiple Myeloma	Completed : Started participant 3 : Not completed 2 (screen failure) : Completed 1 (not specified)	· Asymptomatic multiple myeloma	· Drug: expanded NK cell infusion	Phase 2	April 2013	· University of Arkansas for Medical Science, Little Rock, AR, United States

(continued to the next page)

Table 1. (Continued) Selected clinical trials with autologous NK cells

NCT number	Title	Status	Conditions	Interventions	Phase	Start date	Locations
NCT02507154	Reactivating NK Cells in Treating Refractory Head and Neck Cancer	Recruiting	· Nasopharyngeal cancer · Head and neck squamous cell carcinoma	· Drug: cetuximab + NK cells	Phase 1 Phase 2	July 2015	· National University Hospital, Singapore, Singapore
NCT02030561	NK Cell Infusions With Trastuzumab for Patients With HER2+ Breast and Gastric Cancer	Unknown status	· Breast cancer · Gastric cancer	· Drug: trastuzumab + NK cells	Phase 1 Phase 2	January 2014	· National University Hospital, Singapore, Singapore · National University Hospital, Singapore, Singapore
NCT02805829	Combination Trastuzumab With Expanded Natural Killer Cells for Treating HER2-positive Gastric Cancer	Not yet recruiting	· Gastric cancer	· Drug: trastuzumab + NK cells	Phase 1 Phase 2	January 2017	· Xuzhou Medical University, Xuzhou, Jiangsu, China
NCT02734524	A Clinical Research of NK Cell Infusion Combined With Chemotherapy in the Treatment of Non-small Cell Lung Cancer	Recruiting	· NSCLC	· Biological: NK cells · Drug: Taxol · Drug: carboplatin	Phase 2	March 2016	· Southwest Hospital of Third Millitary Medical University, Chongqing, Chongqing, China
NCT03329664	Autologous Killer Cell Therapy in Colon Cancer Patients	Not yet recruiting	· Colon cancer stage II/III	· Biological: CIK cell · Other: chemotherapy and/or radiation therapy	Phase 1 Phase 2	April 2019	· Cell-based Therapies Research Center, Digestive Disease Research Institute, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
NCT02271711	Expanded Natural Killer Cell Infusion in Treating Younger Patients With Recurrent/Refractory Brain Tumors	Recruiting	· Recurrent childhood medulloblastoma · Recurrent ependymoma · Recurrent medulloblastoma	· Other: laboratory biomarker analysis · Biological: NK cell therapy	Phase 1	March 17, 2015	· MD Anderson Cancer Center, Houston, TX, United States
NCT03662477	Effect of NK Cell Immunotherapy on Advanced Lung Adenocarcinoma Adenocarcinoma With EGFR Mutation	Recruiting	· NK cell mediated immunity	· Biological: NK cells	Early Phase 1	January 1, 2018	· Shenzhen Luohu Hospital, Shenzhen, Guangdong, China
NCT03410368	NK Cell-based Immunotherapy as Maintenance Therapy for Small-Cell Lung Cancer	Recruiting	· Small cell lung cancer	· Biological: NK cells	Phase 2	April 1, 2018	· The First Hospital of Jilin University, Changchun, Jilin, China

EGFR, epidermal growth factor receptor.

between KIR receptors and their ligands expressed on the surface of target tumor cells in hematopoietic stem cell transplants (HSCT) has been demonstrated to induce potent anti-tumor activity and to limit graft-versus-host disease (GvHD) (50,51). Unlike T cells, NK cells are not related to GvHD since activated NK cells are not able to attack host Ag-presenting cells, and they are not able to proliferate in patients with cancer. Therefore, toxic effects resulting from the infusion of allogeneic donor NK cells are minimized.

Alloreactive NK cells can be used either as a type of HSCT or as adoptive immunotherapy. In an allogeneic HSCT settings, T cells from the donor are the main cause of GvHD. In comparison to T-cells, NK cells result in limited incidence of GvHD and have thus been used in T cell-depleted HSCT (52). HSCT is a well-known method for the treatment of hematological cancers, and NK cells are the first lymphocytes to be reconstituted after allogeneic HSCT (53).

In allogeneic NK cell adoptive therapy, NK cells can be obtained from umbilical cord blood, clonal cell lines (including NK92), and products of lymphapheresis from allogeneic donor PBMCs (54-56). While treatments with autologous NK cells have not shown persistent anti-tumor activity, researchers have demonstrated the improved efficacy of allogeneic NK cells co-cultured with feeder cells and cytokines, such as IL-12, IL-15, and IL-18, for treating hematological and solid cancers (57). Several methods have now been established to choose NK cell donors based on the genotype of their KIR and KIR ligands (58).

Despite the efficacy of allogeneic NK cells in hematological malignancies, the function of NK cells is often impaired in solid tumors. The high degree of heterogeneity of solid tumors causes complications in the progression of these NK cells, and conditions and components of the tumor microenvironment such as hypoxia and TGF-β promote the downregulation of activating NK cell receptors (59). Various approaches have been established for improving the effect of adoptively transferred NK cells into solid tumors, such as genetic engineering to confer trafficking to solid tumors as well as combination therapy with other targeted drugs, including checkpoint inhibitors, cytokines, Abs, and immunomodulatory agents (60,61). Selected clinical trials that have used allogeneic NK cells are presented in **Table 2** (47).

NK CELL LINES

NK cell lines that are sequestered from patients are important in understanding the underlying mechanisms and advancing immunotherapy. NK-92, YT, NK-YS, NKL, and NK3.3 are the most commonly used NK cell lines. These NK cell lines were established from expanded clones of patients with malignant leukemia/lymphoma, with the exception of NK3.3 that originated from the blood of a healthy donor (62). Each cell line has unique characteristics, which must be accounted for when creating an ideal environment to maintain and grow them. The proliferation of these cells can be easily compared to the expansion of PBMCs or stem cell-derived NK cells *ex vivo*. Due to this observation, these patient-isolated NK cell lines are excellent models for research on human NK cell immunotherapy (63,64).

The NK-92 cell line is generated from the peripheral blood of a patient with large granular lymphocyte (LGL) non-Hodgkin's lymphoma (65). As NK-92 is derived from a human tumor, it must be irradiated prior to infusion for safety reasons; this treatment eventually results in limiting its therapeutic efficacy (66). However, this cell line still displays a high degree of toxicity against various malignant cells (67). This cell line is dependent on recombinant IL-2 and the cells die within 72 hours due to the lack of the cytokine (65). NK-92 is positive for CD56, CD2, CD7, CD11a, CD28, and CD45, and negative for CD16, NKp44, and NKp46 (47). The lack of CD16, however, results in an impaired ADCC (65,68,69). The NK-92 cell line is the only U.S. FDA-approved cell line for use in clinical trials (70). Researchers are trying to enhance the specificity and efficacy of NK-92 cells through genetic manipulations, such as those used for creating CAR-NK92 cells. CAR-engineered NK-92 cells have several advantages over primary NK cells (71). Overall, the NK-92 cell line is unlimited, uniform, well-characterized, reproducible, and homogeneous. This homogeneity of NK-92 cells results in a more consistent and effective transduction efficiency as compared to those in primary NK cells.

The YT cell line is derived from the pericardial effusion of a patient with acute lymphoma and thymoma with an inducible receptor for T cell growth factor (TCGF) and IL-2. These cells do not require conditioned media to be maintained and can be grown in RPMI-1640 media

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Table 2. Selected clinical trials with allogeneic NK cells

NCT number	Title	Status	Conditions	Interventions	Phase	Start date	Locations
NCT03420963	*Ex-Vivo* Expanded Allogeneic NK Cells For The Treatment Of Pediatric Solid Tumors	Recruiting	· Non-myeloablative TCR alpha/beta depleted haploidentical hematopoietic stem cell transplantation · Recurrent lip and oral cavity carcinoma · Recurrent malignant endocrine neoplasm · And 26 more	· Biological: cord blood-derived expanded allogeneic NK cells · Drug: cyclophosphamide · Drug: etoposide	Phase 1	August 31, 2018	· MD Anderson Cancer Center, Houston, TX, United States
NCT02854839	A Study of MG4101 (Allogeneic Natural Killer Cell) for Intermediate-stage of Hepatocellular Carcinoma	Unknown status	· Hepatocellular carcinoma	· Biological: MG4101	Phase 2	September 2016	· Seoul National University Hospital, Seoul, Republic of Korea · Seoul Asan Medical center, Seoul, Republic of Korea · Samsung Medical Center, Seoul, Republic of Korea
NCT03937895	Allogeneic NK Cell ("SMT-NK") in Combination With Pembrolizumab in Advanced Biliary Tract Cancer	Not yet recruiting	· Biliary tract cancer	· Biological: 'SMT-NK' Inj (allogeneic NK cell) · Drug: pembrolizumab injection [Keytruda]	Phase 1 Phase 2	June 3, 2019	· Gachon University Gil Medical Center, Incheon, Republic of Korea · Severance Hospital, Seoul, Republic of Korea · Gangnam Severance Hospital, Seoul, Republic of Korea
NCT02650648	Humanized Anti-GD2 Antibody Hu3F8 and Allogeneic Natural Killer Cells for High-Risk Neuroblastoma	Recruiting	· Neuroblastoma · High-risk	· Drug: cyclophosphamide · Biological: NK cells · Biological: hu3F8 · Drug: rIL-2	Phase 2	January 2016	· Memorial Sloan Kettering Cancer Center, New York, NY, United States
NCT03539406	Intraperitoneal Infusion of ex Vivocultured Allogeneic NK Cells in Recurrent Ovarian Carcinoma Patients	Not yet recruiting	· Recurrent ovarian carcinoma · Recurrent fallopian tube carcinoma · Recurrent primary peritoneal carcinoma	· Biological: UCB-NK cells · Drug: chemotherapy	Phase 1	April 2019	No record
NCT03019640	Umbilical Cord Blood NK Cells, Rituximab, High-Dose Chemotherapy, and Stem Cell Transplant in Treating Patients With Recurrent or Refractory BCell Non-Hodgkin's Lymphoma	Recruiting	· Mantle cell lymphoma · Recurrent diffuse large B-cell lymphoma · Recurrent follicular lymphoma · Recurrent indolent adult non-Hodgkin lymphoma · Refractory diffuse large B-cell lymphoma · Refractory follicular lymphoma · Refractory indolent adult non-Hodgkin lymphoma	· Procedure: Autologous hematopoietic stem cell transplantation · Drug: carmustine · Biological: cord blood-derived expanded allogeneic NK cells · Drug: cytarabine · Drug: etoposide · Biological: filgrastim · Drug: lenalidomide · Drug: melphalan · Biological: rituximab	Phase 2	October 10, 2017	· MD Anderson Cancer Center, Houston, TX, United States
NCT02809092	Interleukin-21 (IL-21)-Expanded Natural Killer Cells for Induction of Acute Myeloid Leukemia	Recruiting	· AML	· Biological: NK cells + chemotherapy starting	Phase 1 Phase 2	April 1, 2017	· Centro Terapia e Tecnologia Celular, Porto Alegre, Rio Grande Do Sul, Brazil
NCT03669172	Effectiveness of Donor IL-15-stimulated NK Cells Post Transplant Infusion in in Acute Leukemia	Recruiting	· Acute leukemia	· Biological: donor IL-15 stimulated NK cells infusion	Phase 1 Phase 2	September 20, 2017	· Hospital General Universitario Gregorio Marañón, Madrid, Spain

(continued to the next page)

Table 2. (Continued) Selected clinical trials with allogeneic NK cells

NCT number	Title	Status	Conditions	Interventions	Phase	Start date	Locations
NCT03300492	Expanded Natural Killer Cells Following Haploidentical HSCT for AML/MDS	Recruiting	· AML · Myelodysplastic syndromes	· Other: NK-DLI	Phase 1 Phase 2	November 12, 2018	· University Hospital Basel, Basel, Switzerland
NCT02853903	Comparison of Autogenic and Allogenic NK Immunotherapy on the Outcome of Recurrent Solid Tumors	Unknown status	· Malignant solid tumour	· Biological: NK immunotherapy	Phase 2	July 2016	· Fuda Cancer Institute of Fuda Cancer Hospital, Guangzhou, Guangdong, China
NCT02727803	Personalized NK Cell Therapy After Chemotherapy and Cord Blood Transplant in Treating Patients With Myelodysplastic Syndrome, Leukemia, Lymphoma or Multiple Myeloma	Recruiting	· Accelerated Phase Chronic Myelogenous Leukemia, BCR-ABL1 Positive · Acute Biphenotypic Leukemia · ALL · ALL in remission · AML with myelodysplasia-related changes · AML with variant MLL translocations · And 20 more	· Biological: allogeneic NK cell line NK-92 · Biological: anti-thymocyte glowbulin · Drug: busulfan · Drug: clofarabine · Drug: cyclophosphamide · Drug: fludarabine phosphate · Other: laboratory biomarker analysis · Drug: melphalan · Biological: rituximab · Radiation: total-body irradiation · Procedure: umbilical cord blood transplantation	Phase 2	May 19, 2016	· MD Anderson Cancer Center, Houston, TX, United States
NCT01795378	Safety and Efficacy Study of Donor Natural Killer Cells Given After Haploidentical Hematopoietic Cell Transplantation	Completed : No results posted	· Acute myelogenous leukemia · ALL	· Biological: donor natural killer cell infusion	Phase 1 Phase 2	February 2013	· Asan Medical Center, Seoul, Republic of Korea
NCT00569283	Donor Natural Killer Cell Infusion in Preventing Relapse or Graft Failure in Patients Who Have Undergone Donor Bone Marrow Transplant	Completed : No results posted	· Cancer	· Biological: therapeutic allogeneic lymphocytes	Phase 1	May 2007	· Korea Research Institute of Bioscience and Biotechnology, Dajeon, Republic of Korea · Asan Medical Center - University of Ulsan College of Medicine, Seoul, Republic of Korea
NCT01898793	Cytokine-induced Memorylike NK Cells in Patients With Acute Myeloid Leukemia (AML) or Myelodysplastic Syndrome (MDS)	Recruiting	· AML	· Drug: fludarabine · Drug: cyclophosphamide · Procedure: leukapheresis · Biological: cytokine-induced killer cells · Biological: IL-2 · Drug: ALT-803 · Procedure: peripheral blood for correlative studies · Procedure: bone marrow for correlative studies	Phase 1 Phase 2	August 11, 2014	· Washington University School of Medicine, Saint Louis, Missouri, United States

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supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Unlike many NK cell lines, YT cells proliferate continuously *in vitro* in an IL-2–independent manner (72). Pre-treatment of these cells with IL-2 did not generate an increased cytolytic activity. This could be beneficial in NK cell adoptive transfer treatments where, oftentimes, high doses of IL-2 are needed to activate the cells, whether they are autologous or allogeneic NK cells (73). YT cells are negative for CD2, CD3, and CD16, and are IL-2-independent. They exhibit irregular cell size and nuclei and exert cytolytic effect against K562, MOLT-4, and HPB-ALL.

The NK-YS cell line is established from a patient with leukemic-state nasal angiocentric NK cell lymphoma and systemic skin infiltration (74). This cell line is generated through co-culturing leukemic cells from the patient with a mouse stromal cell line (SPY3-2) with recombinant human interleukin 2 (rhIL-2). NK-YS cells express CD2, CD5, CD7, and CD56, but are negative for CD3, CD16, and CD57. The NK-YS cells preserved toxicity against K562 and Jurkat cells and show a type-II latent infection of Epstein-Barr virus (EBV) (74).

The NKL cell line is established from the peripheral blood of a patient with LGL-leukemia (75). This cell line is IL-2 dependent and expresses CD2, CD6, CD11a, CD27, CD29, CD38, CD43, CD58, CD94, and CD95. In conditions of prolonged *in vitro* culture, the cell surface expression of CD16, CD56, and CD57 is rapidly decreased (75,76). These cells have diverse tumor-killing activities and exhibit high and low specific killing activity against NK-sensitive target cells, K562 and 721.221, respectively (75-78).

NK3.3 is a normal NK-derived cell line that originates from a mixed lymphocyte culture isolated from the peripheral blood of a healthy donor (79). This cell line is generated by mixing the peripheral blood mononuclear lymphocytes (PBL) from heparinized blood from a responder cell donor and a stimulator cell donor. The responder PBL is incubated with an equal amount of irradiated stimulator PBL in upright flasks for 6 days to generate a mixed lymphocyte culture. To maintain this cell line, the cells must be grown with IL-2 conditioned media to continue proliferation (62). These cells are IL-2-dependent, and express CD2, CD11a, CD38, CD45, CD16, and CD56 (62,80). NK3.3 cells exhibit strong cytolytic activity against NK-susceptible target cells, such as K562 and MOLT-4 (62,79).

Other than NK cell lines described above, we summarized patents introducing the methods of generating the NK cell lines in **Table 3** (81-84). EP3138905A1 provides a method for expanding human donor-derived NK cells with IL-21, IL-2, IL-15, and B cell-derived EBV-transformed lymphoblastoid cell lines (EBV-LCL) (81). This method resulted in a 1×10^{11}-fold expansion of NK cells in 7 wk (81). EP2539442A1 introduces a method for the generation and expansion of cytokine-induced killer (CIK) cells and NK cells from human peripheral blood cells in the presence of IL-15, IL-7 in combination with IL-2, stem cell factor (SCF), and Fms-related receptor tyrosine kinase 3 (FLT3) (82). This method showed 15- to 48-fold NK cell expansion within 3 weeks and significantly high toxicity against K562 target cells with the effector:target ratio 20:1 (82). WO2017017184 showed the methods of NK cell modification to produce increased cytotoxic phenotype (83). CD96, CD328, and TNF-related apoptosis-inducing ligand (TRAIL) ligand of KHYG-1 and NK92 cells were modified and showed increased toxicity against K562 or MM1.2 target cells (83). ZNK® cells have been developed by Tella Inc. (Tokyo, Japan) and Kyushu University from human PBMC and cord blood cells (84). ZNK® cells show several hundred to 10,000-fold expansion, 10 times more perforin and Granzyme B release than that before culturing, and elimination of almost all cancer cells within 2 h (84).

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Table 3. Generation of NK cell line from patents for future therapeutic aspect

Source of NK cells	Cytokines added	Feeder cells added	NK cell fold expansion	Genetic modifications	Cytotoxicity	Reference
Human donor-derived NK cells	IL-21 (0.1 and 1,000 ng/ml), IL-2 (1 and 5,000 U/ml), IL15 (0.1 and 1,000 ng/ml)	B cell-derived and EBV-LCL	1×10^{11}-fold expansion of NK cells in 7 wk	N/A	N/A	(81)
Human peripheral blood cells	IL-15 (40 ng/ml) and IL-7 (40 ng/ml), in combination with IL-2 (80 ng/ml), SCF (40 ng/ml), and FLT3 ligand (40 ng/ml)	N/A	$CD3^{-}CD56^{+}$ NK cells were expanded nearly 15 to 48-fold in 3 wk	N/A	· Cytotoxicity of effector: target ratio 20:1 was significantly higher than that of 10:l. against K562 target cell line	(82)
KHYG-1, NK92	N/A	N/A	N/A	CD96, CD328, TRAIL ligand	· Increased cytotoxicity in CD96 knockdown KHYG-1 cells against K562 target cells · Increased cytotoxicity in CD328 knockdown NK92 cells against K562 target cells · Increased cytotoxicity in TRAIL variant (TRAIL D269H/E195R) expressing KHYG-1 against MM1.S target cells · Synergistically enhanced cytotoxicity in CD96 knockdown and TRAIL variant expression simultaneously in KHYG-1 cells against both K562 and MM1.S target cells	(83)
Human PBMCs Human cord blood cells	N/A	N/A	PBMC: several hundred times Human cord blood cells: about 10,000 times	N/A	· Ten times more perforin and granzyme B release than before culturing · NK cells derived from PBMC can kill almost all cancer cells at a ratio of one NK cell for each cancer cell within 2 h	(84)

Although these NK cell lines are relatively easy to culture *ex vivo*, they need to be irradiated prior to clinical use owing to the risk of generating chromosomal abnormalities from the malignant transformation of the cell line . For this reason, *in vivo* persistence is limited, which leads to frequent injecion of NK cell lines into the patients to achieve promising clinical results. Furthermore, lack of NK activating receptors on some of the NK cell lines make them less cytolytic to tumor cells, hence require genetic incorportation of proper NK activating receptors, such as NKG2D and DNAM-1. However, NK cell lines retain characteristics similar to those of normal human NK cells and serve as an important tool in NK cell research, despite having different origins and phenotypes.

HUMAN IPSC-DERIVED NK CELLS

Human pluripotent stem cells, particularly the iPSCs, are considered to be the standard starter tool for the generation of immune cells, such as NK cells (85-87). NK cells generated from iPSCs are regarded as effective potentiators of tumor lysis, both *in vitro* and *in vivo*, as the receptor and gene expression profiles of these cells are similar to those of NK cells purified from peripheral blood or human umbilical cord blood (88,89). Moreover, human NK cells derived from iPSCs can be generated at a scale appropriate for clinical studies on cancer immunotherapy (**Fig. 2**) (90). Compared to T cells, primary NK cells are difficult to isolate, purify, and genetically modify, as they represent a heterogeneous mixture of cells that expand hardly (91,92,93). However, human iPSCs can be effectively differentiated into NK cells, without the risk of generating chromosomal abnormalities (89,94,95); this is in addition to other advantages arising from their being homogeneous and excluded from the donor variation (96). Another advantage of human iPSC-NK cells is that they can be genetically engineered with relative ease using viral vectors and CRISPR technologies (95).

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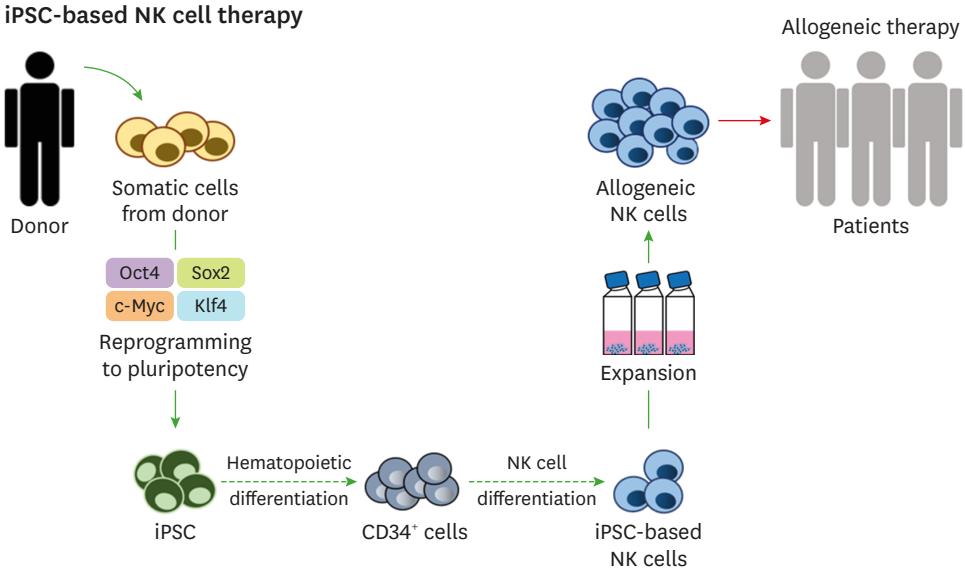

Figure 2. Schematic representation of human iPSC-based NK cell therapy. iPSCs are generated from somatic cells of the donor by reprogramming them using crucial various transcription factors, including Oct4, Sox2, c-Myc, and Klf4. The iPSCs are further differentiated into $CD34^+$ cells and NK cells and can be expanded for clinical use.

A number of studies have reported the development of mature NK cells from human iPSCs (86,97). Methods for the generation of NK cells from human iPSCs involve the creation and proliferation of $CD34^+$ hematopoietic precursor cells while retaining NK cell cytokine production (IL-15, IL-3, IL-7, SCF, and FLT3 ligand) and the use of a cell line expressing a membrane-bound IL-21 to boost their development, thereby scaling-up NK cell production to a clinical-level (40,85). Human iPSC-NK cells are reported to efficiently kill hematological malignancies, including acute myeloid leukemia (AML) and multiple myeloma, as well as solid cancer such as ovarian cancer by direct ADCC, and IFN-γ production (86,89). Furthermore, human iPSC-NK cells can kill leukemia and ovarian cancer cells efficiently, without further generating teratomas (89). Studies have demonstrated the role of KIRs in human iPSCs, and have provided a way to produce NK cells with customized KIR expression in patients with different HLA types (85). In 2018, Li et al. (98) reported the modification of iPSC-derived NK cells with CAR to increase the killing activity of NK cells against mesothelin-expressing tumors, both *in vitro* and *in vivo*. In this regard, CAR-iPSC-NK cells may provide an attractive option for CAR therapy, although the safety concerns and clinical effectiveness need to be resolved.

In February 2019, Fate Therapeutics and the University of California, San Diego (UCSD) undertook the first clinical trial for evaluating the effect of FT500 cell therapy (99). FT500 is an "off-the-shelf" and one master iPSC line-derived NK cell product. In this ongoing trial, FT500 is being tested for safety, along with patient responses to its different doses for the treatment of various tumors (**Table 4**) (47). If this trial is successful, a new era of "off-the-shelf" cancer immunotherapy might be introduced.

CAR-NK CELLS

In 2017, the U.S. FDA had approved the first CAR-T cell therapy (Tisagenlecleucel, marketed as Kymriah; Novartis) for the treatment of B-cell ALL in children and young adults (100).

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Table 4. Clinical Trial with human iPSC-derived NK cells

NCT number	Title	Status	Conditions	Phase	Sponsor/ collaborators	Dates	Locations
NCT03841110	FT500 as Monotherapy and in Combination With Immune Checkpoint Inhibitors in Subjects With Advanced Solid Tumors	Recruiting	· Advanced solid tumors · Lymphoma · Gastric cancer · Colorectal cancer · Head and neck cancer · Squamous cell carcinoma · EGFR positive solid tumor · HER2-positive breast cancer · Hepatocellular carcinoma · Small cell lung cancer · Renal cell carcinoma · Pancreas cancer · Melanoma · NSCLC · Urothelial carcinoma · Cervical cancer · Microsatellite instability · Merkel cell carcinoma	Phase 1	Fate therapeutics	Study start: February 15, 2019 Primary completion: March 2022 Study completion: June 2022 First posted: February 15, 2019 Results first posted: no results posted Last update posted: May 14, 2019	· UCSD Moores Cancer Center, San Diego, CA, United States · University of Minnesota Masonic Cancer Center, Minneapolis, MN, United States · MD Anderson Cancer Center, Houston, TX, United States

EGFR, epidermal growth factor receptor.

After this, the second CAR-T cell therapy (Axicabtagene ciloleucel, marketed as Yescarta; Kite Pharma) was approved for the treatment of certain non-Hodgkin's lymphomas such as diffuse large B-cell lymphoma (101,102). Despite these outstanding clinical results, CAR-T cell therapy still has disadvantages that need to be overcome, such as CRS and neurotoxicity. CAR-T cells are generally composed of three domains, extracellular domain, transmembrane domain, and intracellular signaling domain (**Fig. 3**). Extracellular domain has a single-chain variable fragment (scFv) that recognizes tumor surface Ag. Various tumor Ag-binding domains have currently been designed and tested as CAR extracellular domains. Between the extracellular and transmembrane domains, there is a hinge region that is generally derived from CD8 or IgG4. The intracellular signaling domain is most important, as this region determines the functionality of CAR. Currently, CAR is in the fourth generation of its technical development (103). The first generation of CARs had a domain with a scFv that recognized cancer Ags, along with an immunoreceptor tyrosine-based activation motif (ITAM, generally CD3ζ) (104). However, the structure of these first generation CARs could not produce long-term cell proliferation signals to retain anti-cancer activity. In second and third generations of CARs co-stimulatory molecules, including CD28, CD134 (OX40), and CD137 (4-1BB) were introduced to increase tumor cytotoxicity and T cell proliferation (105). The fourth generation CAR cells consist of two co-stimulatory molecules (CD28, CD134, or CD137) and secrete cytokines to fully enhance the anti-cancer activity by activating the innate immune system (106,107).

The clinical success of CAR-T cells is being used to drive the development of CAR-NK cells. Similar to CAR-T cells, CAR-NK cells have a basic structure, including the extracellular, transmembrane, and intracellular signaling domains. To develop an intracellular signaling

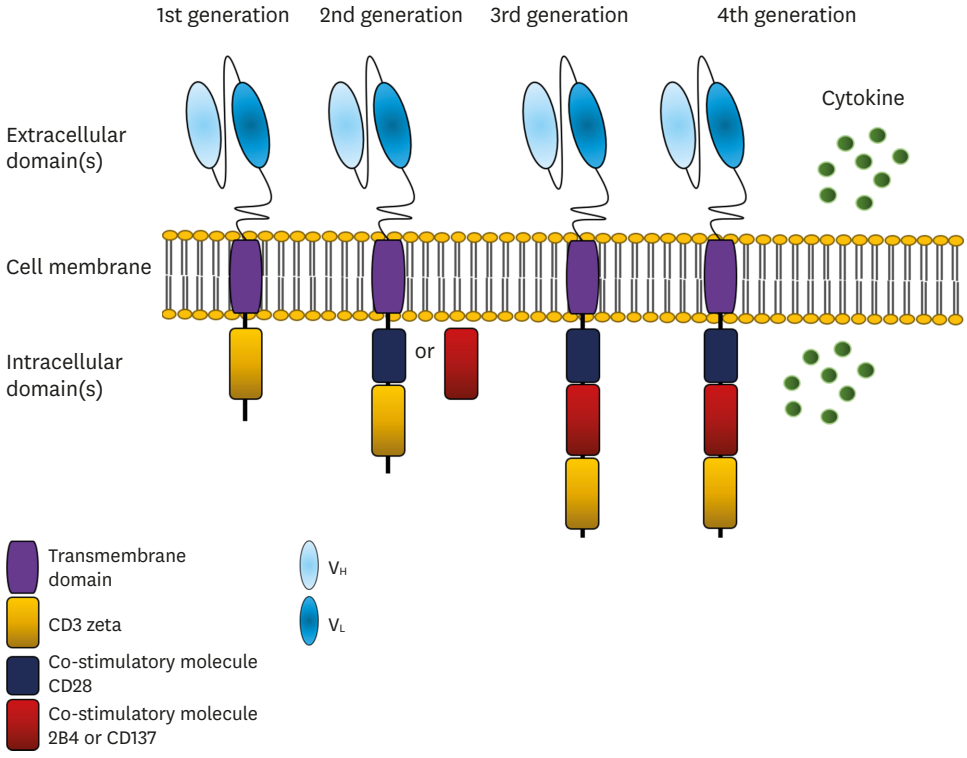

Figure 3. Structure of CAR-NK cells. Design of CAR-NK cells. Three domains constitute the central part of the CAR structure (extracellular domain[s], a transmembrane domain, and intracellular domain[s]). The scFv region of the extracellular domain is composed of heavy and light chains. Components of the intracellular domain include CD3 signaling domain and co-stimulatory domains, such as CD28, 2B4, and CD137.

motif, CAR-NK cells generally use CD3ζ as the first signal domain, followed by a co-stimulatory domain, such as CD28 or CD137 (4-1BB). Other co-stimulatory molecules, including NKG2D and CD244 (also known as 2B4), are also used by NK cells to promote cytotoxicity and secretion of cytokines by NK cells (108-112). Compared to CAR-T cells, CAR-NK cell therapy has advantages such as significantly reduced safety issues like on-target/off-tumor effects, GvHD, CRS and tumor lysis syndrome along with additional tumor-killing activity, such as ADCC (113,114). NK cells produce low amounts of IFN-γ and GM-CSF and do not secrete the central cytokines that promote CRS, such as IL-1 and IL-6. In addition, CAR-NK cells maintain their activating receptors, such as NKp30, NKp44, NKp46, NKG2D, and DNAM-1. Therefore, relapses may be reduced due to the loss of CAR-targeting Ag.

While CAR-NK cells cause reduced side effects, including the low occurrence of cytokine storms, further studies, including clinical trials, are required to thoroughly evaluate their safety. At present, there are on-going clinical trials registered on clinicaltrials.gov to test the safety and efficacy of CAR-NK cell therapy in both hematological and solid cancers (**Table 5**) (47).

IMMUNE CHECKPOINT THERAPY IN COMBINATION WITH NK CELLS

Immune checkpoint inhibitors are Abs blocking the PD-1:PD-L1 and CTLA-4. These molecules have shown significant effects for the treatment of NSCLC, BRAF wild-type melanoma, and metastatic RCC (115-117). In 2011, ipilimumab, a CTLA-4 blocking Ab, was

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Table 5. Clinical trials with CAR-NK cells

NCT number	Title	Status	Conditions	Interventions	Phase	Start date	Locations
NCT03941457	Clinical Research of ROBO1 Specific BiCAR-NK Cells on Patients With Pancreatic Cancer	Recruiting	· Pancreatic cancer	· Biological: BiCARNK cells (ROBO1 CAR-NK cells)	Phase 1 Phase 2	May 2019	· Department of Radiology, Shanghai Ruijin Hospital, Shanghai, China
NCT03940833	Clinical Research of Adoptive BCMA CAR-NK Cells on Relapse/Refractory MM	Recruiting	· Multiple myeloma	· Biological: BCMA CAR-NK 92 cells	Phase 1 Phase 2	May 2019	· Department of Hematology, Wuxi People's Hospital, Nanjing Medical University, Wuxi, Jiangsu, China
NCT03940820	Clinical Research of ROBO1 Specific CAR-NK Cells on Patients With Solid Tumors	Recruiting	· Solid tumor	· Biological: ROBO1 CAR-NK cells	Phase 1 Phase 2	May 2019	· Radiation Therapy Department, Suzhou Cancer Center, Suzhou Hospital Affiliated to Nanjing Medical University, Suzhou, Jiangsu, China
NCT03824964	Study of Anti-CD19/CD22 CAR-NK Cells in Relapsed and Refractory B Cell Lymphoma	Not yet recruiting	· Refractory B-cell lymphoma	· Biological: anti-CD19/CD22 CAR-NK cells	Early Phase 1	February 1, 2019	Unknown
NCT03692767	Study of Anti-CD22 CAR-NK Cells in Relapsed and Refractory B Cell Lymphoma	Not yet recruiting	· Refractory B-cell lymphoma	· Biological: anti-CD22 CAR-NK cells	Early Phase 1	March 2019	Unknown
NCT03692637	Study of Anti-Mesothelin CAR-NK Cells in Epithelial Ovarian Cancer	Not yet recruiting	· Epithelial ovarian cancer	· Biological: anti-mesothelin CAR-NK cells	Early Phase 1	March 2019	Unknown
NCT03690310	Study of Anti-CD19 CAR-NK Cells in Relapsed and Refractory B Cell Lymphoma	Not yet recruiting	· Refractory B-cell lymphoma	· Biological: anti-CD19 CAR-NK cells	Early Phase 1	March 2019	Unknown
NCT03415100	Pilot Study of NKG2D-Ligand Targeted CAR-NK Cells in Patients With Metastatic Solid Tumours	Recruiting	· Solid tumours	· Biological: CAR-NK cells targeting NKG2D ligands	Phase 1	January 2, 2018	· Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China
NCT03383978	Intracranial Injection of NK-92/5.28.z Cells in Patients With Recurrent HER2-positive Glioblastoma	Recruiting	· Glioblastoma	· Biological: NK-92/5.28.z	Phase 1	December 1, 2017	· Department of Neurosurgery, Johann W. Goethe University Hospital, Frankfurt, Germany · Senckenberg Institute of Neurooncology, Johann W. Goethe University Hospital, Frankfurt, Germany
NCT02944162	CAR-pNK Cell Immunotherapy for Relapsed/Refractory CD33^{+} AML	Unknown status	· Leukemia	· Biological: anti-CD33 CAR-NK cells	Phase 1 Phase 2	October 2016	· PersonGen BioTherapeutics (Suzhou) Co., Ltd., Suzhou, Jiangsu, China
NCT02892695	PCAR-119 Bridge Immunotherapy Prior to Stem Cell Transplant in Treating Patients With CD19 Positive Leukemia and Lymphoma	Recruiting	· Leukemia and lymphoma	· Biological: anti-CD19 CAR-NK cells	Phase 1 Phase 2	September 2016	· PersonGen BioTherapeutics (Suzhou) Co., Ltd., Suzhou, Jiangsu, China
NCT02839954	CAR-pNK Cell Immunotherapy in MUC1 Positive Relapsed or Refractory Solid Tumor	Unknown status	· Solid tumours	· Biological: anti-MUC1 CAR-pNK cells	Phase 1 Phase 2	July 2016	· PersonGen BioTherapeutics (Suzhou) Co., Ltd., Suzhou, Jiangsu, China
NCT02742727	CAR-pNK Cell Immunotherapy in CD7 Positive Leukemia and Lymphoma	Unknown status	· Leukemia and lymphoma	· Biological: anti-CD7 CAR-pNK cells	Phase 1 Phase 2	March 2016	· PersonGen BioTherapeutics (Suzhou) Co., Ltd., Suzhou, Jiangsu, China

BCMA, B-cell maturation Ag.

approved by the U.S. FDA for the treatment of melanoma (118). These checkpoint inhibitors have been reported to improve overall survival and the survival rate of many cancer patients and promote immune responses and tumor regression by reducing the immune suppressive mechanisms in many cancer patients.

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Although checkpoint blockade has shown significant responses, side effects including gastrointestinal and pulmonary toxicities and endocrine failure still remain to be a hurdle to overcome. Other limitations of immune checkpoint inhibitors are a low efficacy in cancers with the low mutational burden (119-121).

To improve the response rate to checkpoint inhibitors, clinical trials of checkpoint inhibitors in combination with NK cells, are now on-going. We have summarized these clinical studies in **Table 6** (47). NCT03853317 is a phase 2 study of combination therapy with an IL-15 superagonist (N-803), off-the-shelf CD16-targeted natural killer cells (haNK), and avelumab without cytotoxic chemotherapy in patients with Merkel cell carcinoma that has progressed on or after treatment with a checkpoint inhibitor. NCT03937895 is a phase 1/2a clinical trial to test the efficacy of allogeneic NK cells ("SMT-NK") in combination with pembrolizumab (Keytruda) for patients with gemcitabine-refractory biliary tract cancer. NCT04143711 is a phase I/II study to investigate the safety, tolerability, pharmacokinetics, biological, and clinical activity of DF1001 (new molecule that targets NK cell activation signals to specific receptors on cancer cells) in combination with pembrolizumab (Keytruda) in patients with locally advanced or metastatic solid tumors.

TUMOR MICROENVIRONMENT AND NK CELLS

Tumor microenvironment (TME), which is created when a tumor progresses and invades surrounding tissues, provides a protective environment around the tumor cells, immune cells, stromal cells, and extracellular matrix. General conditions of the TME include low oxygen concentration, modified metabolic status, acidic pH, Tregs, myeloid-derived suppressor cells (MDSC), and a series of immunosuppressive cytokines, including TGF-β, IL-10, and IL-6 produced by the tumor cells, Tregs, and MDSCs (122). These harsh immunosuppressive conditions are able to cause differentiation of regulatory immune cells and inhibition of immune cell activation and proliferation, and act as negative regulators of NK cell infiltration into solid tumors (123,124).

Microenvironmental hypoxia is a well-known TME of solid tumors and is involved in the up-regulation of hypoxia-inducible factor (HIF)-1α, altered gene transcriptional profiles (125), a metabolic shift to glycolysis (126), and loss of immune reactivity by inducing

Table 6. Clinical trials of checkpoint therapy in combination with NK cells

NCT number	Title	Status	Conditions	Interventions	Phase	Start date	Locations
NCT03853317	QUILT-3.063: A Study of N-803, haNK and Avelumab in Patients With Merkel Cell Carcinoma That Has Progressed After Checkpoint Therapy	Active, not recruiting	· Merkel cell carcinoma	· Biological: avelumab · Biological: N-803 · Biological: haNK™	Phase 2	March 8, 2019	· Chan Soon-Shiong Institute for Medicine, El Segundo, CA, United States
NCT03937895	Allogeneic NK Cell ("SMTNK") in Combination With Pembrolizumab in Advanced Biliary Tract Cancer	Not yet recruiting	· Biliary tract cancer	· Biological: 'SMTNK' Inj (allogeneic NK cell) · Drug: pembrolizumab injection [Keytruda]	Phase 1 Phase 2	June 3, 2019	· Gachon University Gil Medical Center, Incheon, Republic of Korea · Severance Hospital, Seoul, Republic of Korea · Gangnam Severance Hospital, Seoul, Republic of Korea
NCT04143711	Study of DF1001 in Patients With Advanced Solid Tumors	Recruiting	· Solid tumor, adult	· Drug: DF1001 · Drug: pembrolizumab	Phase 1 Phase 2	November 11, 2019	· MD Anderson Cancer Center, Houston, TX, United States

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immunosuppressive mechanisms (127). HIF-1α, a master regulator of hypoxic conditions, regulates key genes involved in cell proliferation, apoptosis, and metabolic pathways. HIF-1α, which is ubiquitinated or degraded in normoxic conditions, is generally expressed and stabilized in highly-glycolytic and hypoxic TME by interacting with HIF-1β in the nucleus (128). HIF-1α downregulates the expression of NK cell-activating receptors, such as NKp30, NKp44, and NKp46, NKG2D, granzyme B, and perforin (129). Hypoxic conditions in TME upregulates HIF-1α-dependent pro-angiogenic genes, such as VEGF and TGF-β, in NK cells (130). In addition, tumor-infiltrating NK cells present a $CD56^{bright}$ phenotype when they bind to PD-L1 in hypoxic TME (131).

Accumulated glucose metabolic products in TME affect the tumor-infiltrating NK cells by modifying the expression of PMK2, PGK1, GLUT1, and FAS in a HIF-1α-dependent manner (132). Metabolic end products, such as lactate, adenosine, and tryptophan, accumulated in TME, promote the immunoregulatory functions of tumor-infiltrated NK cells (122). Various pathways, including HIF-1α, myc, p53, PI3K/Akt, and mTOR, have been reported to be involved in these dynamic metabolic changes (133).

To improve persistence within a solid tumor microenvironment, adoptively transferred NK cells would need to avoid immunosuppressive factors to realize effective NK-cell-based therapies for solid tumors.

CONCLUSION

Despite the promising results of preclinical and clinical trials, cancer immunotherapy using NK cells still has several hurdles to overcome. Some of these obstacles include a lack of tumor trafficking, *in vivo* persistence, tumor cytotoxicity, and tumor immune escape. The lifespan of NK cells is also relatively short, and this causes a reduction in *in vivo* persistence and therapeutic efficacy of adoptively transferred NK cells. iPSC-derived or genetically engineered CAR-NK cells show promising pre-clinical results but are still in the early stages of development compared to CAR-T-based immunotherapy. The approaches to produce engineered NK cells, including optimal gene construct design and delivery methods, have not been fully established as well. Overcoming these challenges for clinical use in the next decades would greatly advance NK cell-based cancer therapeutics.

Another important limitation of NK cell immunotherapy is its low efficacy against solid tumors. This may due to the low ability of NK cells to traffic through the solid tumor tissues. The tumor microenvironment is another obstacle to the adoptive therapy of NK cells since tumor microenvironment interferes with NK cell activation by secreting immunosuppressive cytokines and facilitating differentiation into MDSC, Treg, M2 regulatory immune subtypes. Further in-depth understanding of NK cells within tumor microenviroment will greatly facilitate NK cell therapy to be effective in various solid tumor types.

As NK cells have natural anti-tumor toxicity, NK cell-based cancer immunotherapy can be used in a complementary or combinatory method with other anti-cancer agents. Although more research must still be conducted to establish the NK cell therapy as a legitimate form of therapy, NK cells possess the potential to become an 'off-the-shelf' product that may shift the current paradigm of cancer treatment modalities in the near future.

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