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REVIEW

NK cell-based immunotherapy for malignant diseases

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Natural killer (NK) cells play critical roles in host immunity against cancer. In response, cancers develop mechanisms to escape NK cell attack or induce defective NK cells. Current NK cell-based cancer immunotherapy aims to overcome NK cell paralysis using several approaches. One approach uses expanded allogeneic NK cells, which are not inhibited by self histocompatibility antigens like autologous NK cells, for adoptive cellular immunotherapy. Another adoptive transfer approach uses stable allogeneic NK cell lines, which is more practical for quality control and large-scale production. A third approach is genetic modification of fresh NK cells or NK cell lines to highly express cytokines, Fc receptors and/or chimeric tumor-antigen receptors. Therapeutic NK cells can be derived from various sources, including peripheral or cord blood cells, stem cells or even induced pluripotent stem cells (iPSCs), and a variety of stimulators can be used for large-scale production in laboratories or good manufacturing practice (GMP) facilities, including soluble growth factors, immobilized molecules or antibodies, and other cellular activators. A list of NK cell therapies to treat several types of cancer in clinical trials is reviewed here. Several different approaches to NK-based immunotherapy, such as tissue-specific NK cells, killer receptor-oriented NK cells and chemically treated NK cells, are discussed. A few new techniques or strategies to monitor NK cell therapy by non-invasive imaging, predetermine the efficiency of NK cell therapy by *in vivo* experiments and evaluate NK cell therapy approaches in clinical trials are also introduced.
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INTRODUCTION

Surgery, chemotherapeutic agents and ionizing radiation have been used for decades as primary strategies to eliminate the tumors in patients; however, the development of resistance to drugs or radiation led to a significant incidence of tumor relapse. Therefore, investigating effective strategies to eliminate these resistant tumor cells is urgently needed. The importance of immune system in malignant diseases has been demonstrated by recent major scientific advances.

Both innate and adaptive immune cells actively prevent neoplastic development in a process called 'cancer immunosurveillance'. Innate immune cells, including monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells, mediate immediate, short-lived responses by releasing cytokines that directly lyse tumor cells or capture debris from dead tumor cells. Adaptive immune cells, including T and B cells, mediate long-lived, antigen-specific responses and effective memory.[1] Despite these immune responses, malignant cells can develop mechanisms to evade immunosurveillance. Some tumors protect themselves by establishing an immune-privileged environment. For example, they can produce immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β) to suppress the adaptive antitumor immune response, or skew the immune response toward a Th2 response with significantly less antitumor capacity.[2–4] Some tumors alter their expressions of IL-6, IL-10, vascular epithelial growth factor or granulocyte monocyte-colony stimulating factor (GM-CSF), impairing DC functions *via* inactivation or suppressing maturation.[5] In some cases, induced regulatory T cells suppress tumor-specific $CD4^+$ and $CD8^+$ T-cell responses.[6] Tumor cells also minimally express or shed tumor-associated antigens, shed the ligands of NK cell-activating receptor such as the NKG2D ligands UL16-binding protein 2, major histocompatibility complex (MHC) class I chain-related molecules A and B molecules (MICA/MICB) or alter MHC-I and costimulatory molecule expression to evade the immune responses.[7–9] Malignant cells may also actively eliminate immune cells by activation-induced cell death or Fas ligand (FasL) expression.[10,11] In addition, primary cancer treatments like chemotherapy and ionizing radiation can compromise antitumor immune responses by their immunosuppressive side effects.

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Tumor cells can be eliminated when immune responses are adequate; when they are not, tumor growth and immunosurveillance enter into a dynamic balance until tumor cells evade immunosurveillance, at which point neoplasms appear clinically as a consequence. Therapies designed to induce either a potent passive or active antitumor response against malignancies by harnessing the power of the immune system, known as tumor immunotherapy, is an appealing alternative strategy to control tumor growth. Until now, the cancer immunotherapy field has covered a vast array of therapeutic agents, including cytokines, monoclonal antibodies, vaccines, adoptive cell transfers (T, NK and NKT) and Toll-like receptor (TLR) agonists.[1,12,13] Adoptive NK cell transfer in particular has held great promise for over three decades. With progress in the NK cell biology field and in understanding NK function, developing NK cells to be a powerful cancer immunotherapy tool has been achieved in recent years. In this article, we will review recent advances in NK cell-based cancer immunotherapy, focusing on potential approaches and large-scale NK cell expansion for clinical practice, as well as on the clinical trials and future perspectives to enhance the efficacy of NK cells.

CONCEPTION OF NK CELLS

NK cells were first identified in 1975 as a unique lymphocyte subset that are larger in size than T and B lymphocytes and contain distinctive cytoplasmic granules.[14,15] After more than 30 years, our understanding of NK cell biology and function lends important insights into their role in immunosurveillance. It has been known that NK cells develop in bone marrow (BM) from common lymphoid progenitor cells;[16] however, NK cell precursors have still not been clearly characterized in humans.[17] After development, NK cells distribute widely throughout lymphoid and non-lymphoid tissues, including BM, lymph nodes (LN), spleen, peripheral blood, lung and liver.[18]

NK cells, defined as $CD3^{-}CD56^{+}$ lymphocytes, are distinguished as $CD56^{bright}$ and $CD56^{dim}$ subsets. Approximately 90% of peripheral blood and spleen NK cells belong to the $CD56^{dim}CD16^{+}$ subset with marked cytotoxic function upon interacting with target cells.[19,20] In contrast, most NK cells in lymph nodes and tonsils belong to the $CD56^{bright}CD16^{-}$ subset and exhibit predominantly immune regulation properties by producing cytokines such as interferon (IFN)-γ in response to IL-12, IL-15 and IL-18 stimulation.[19,21]

NK cells rapidly kill certain target cells without prior immunization or MHC restriction, whose activation is dependent on the balance between inhibitory and activating signals from invariant receptors.[22–24] The activating receptors include the cytotoxicity receptors (NCRs) (NKp46, NKp30 and NKp44), C-type lectin receptors (CD94/NKG2C, NKG2D, NKG2E/H and NKG2F) and killer cell immunoglobulin-like receptors (KIRs) (KIR-2DS and KIR-3DS), while the inhibitory receptors include C-type lectin receptors (CD94/NKG2A/B) and KIRs (KIR-2DL and KIR-3DL). Since some structural families contain both activating and inhibitory receptors, trying to understand how NK cell activity is regulated is often complicated.[25] At steady state, the inhibitory receptors (KIRs and CD94/NKG2A/B), which bind to various MHC-I molecules present on almost all cell types, inhibit NK cell activation and prevent NK cell-mediated killing. Under stress conditions, cells downregulate MHC-I expression, causing NK cells to lose inhibitory signaling and be activated in a process called 'missing-self recognition'. Additionally, the non-MHC self molecules Clr-b (mouse), LLT-1 (human) and CD48 (mouse) recognized by the inhibitory receptors NKR-P1B, NKR-P1A and 2B4, respectively, also perform this function.[26,27] In contrast to the self-expressed inhibitory receptor ligands, NK cell-activating receptors can recognize either pathogen-encoded molecules that are not expressed by the host, called 'non-self recognition', or self-expressed proteins that are upregulated by transformed or infected cells, called 'stress-induced self recognition'. For example, mouse Ly49H recognizes cytomegalovirus-encoded m157, and NKG2D recognizes the self proteins human UL16-binding proteins and MICA/MICB.[28,29] NK cells identify their targets by recognizing a set of receptors on target cells in an NK-target cell zipper formation; this results in the integration of multiple activating and inhibitory signals, the outcome of which depends on the nature of the interacting cells.[26] IFNs or DC/macrophage-derived cytokines, such as type I IFN, IL-12, IL-18 and IL-15, enhance the activation or promote the maturation of NK cells, which can also augment NK cell cytolytic activity against tumor cells.[30–32] Cytotoxic activity of NK cells can increase approximately 20–200 fold after exposure to IFN-α/β or IL-12. Despite these known innate immune cell functions, accumulating evidence in both mice and humans demonstrates that NK cells are educated and selected during development, possess receptors with antigen specificity, undergo clonal expansion during infection and can generate long-lived memory cells.[33,34]

After over 30 years of researching NK cells, evidence supports that they play critical roles in the early control of viral infection, in hematopoietic stem cell (HSC) transplantation (improved grafting, graft-*vs.*-host disease and graft-*vs.*-tumor), in tumor immunosurveillance and in reproduction (uterine spiral artery remodeling). The roles of NK cells in controlling organ transplantation, parasitic and HIV infections, autoimmunity and asthma have also been suggested, but remain to be explored further.[26] In particular, therapeutic strategies harnessing the power of NK cells to target multiple malignancies have been designed.

NK CELL-MEDIATED ANTITUMOR MECHANISMS

NK cells originally described as large granular lymphocytes, exhibited natural cytotoxicity against certain tumor cells in the absence of preimmunization or stimulation.[35–37] $CD56^{dim}$ NK cells, which make up the majority of circulating cells, are the most potent cytotoxic NK cells against tumor cells. Evidence gathered from a mouse xenograft tumor model testing functionally deficient NK cells or antibody-mediated NK cell depletion supports that NK cells can eradicate tumor cells.[38–41] An 11-year follow-up study in patients indicated that low NK-like cytotoxicity was associated with increased cancer

risk.[42] High levels of tumor infiltrating NK cells (TINKs) are associated with a favorable tumor outcome in patients with colorectal carcinoma, gastric carcinoma and squamous cell lung cancer, suggesting that NK-cell infiltration into tumor tissues represents a positive prognostic marker.[43–45] As described above, NK-cell recognition of tumor cells by inhibitory and activating receptors is complex, and the three recognition models—'missing-self', 'non-self' and 'stress-induced self'—might be used to sense missing- or altered-self cells. Activated NK cells are thus in a position to directly or indirectly exert their antitumor activity to control tumor growth and prevent the rapid dissemination of metastatic tumors by 'immunosurveillance' mechanisms (Figure 1).

Direct tumor clearance by NK-mediated cytotoxicity

Upon cellular transformation, surface MHC-I expression on tumor cells is often reduced or lost to evade recognition by antitumor T cells. In parallel, cellular stress and DNA damage lead to upregulated expression of ligands on tumor cells for NK cell-activating receptors. Human tumor cells that have lost self MHC-I expression or bear 'altered-self' stress-inducible proteins are ideal NK cell targets, as NK cells are activated by initially recognizing certain 'stress' or 'danger' signals.[46] The 'missing-self' model of tumor cell recognition by NK cells was first demonstrated by observing that MHC-I-deficient syngeneic tumor cells were selectively rejected by NK cells; additionally, NK cell inhibitory receptors were shown to detect this absence of MHC-I expression.[47–49] NK cells can also kill certain MHC-I-sufficient tumor cells by detecting stress-induced self ligands through their activating receptors. Broad MICA/B expression has been detected on epithelial tumors, melanoma, hepatic carcinoma and some hematopoetic malignancies, representing a counter-measure by the immune system to combat tumor development.[31] NK cell-mediated cytotoxicity is also important against tumor initiation and metastasis *in vivo*.[50–52]

NK cells directly kill target tumor cells through several mechanisms: (i) by releasing cytoplasmic granules containing

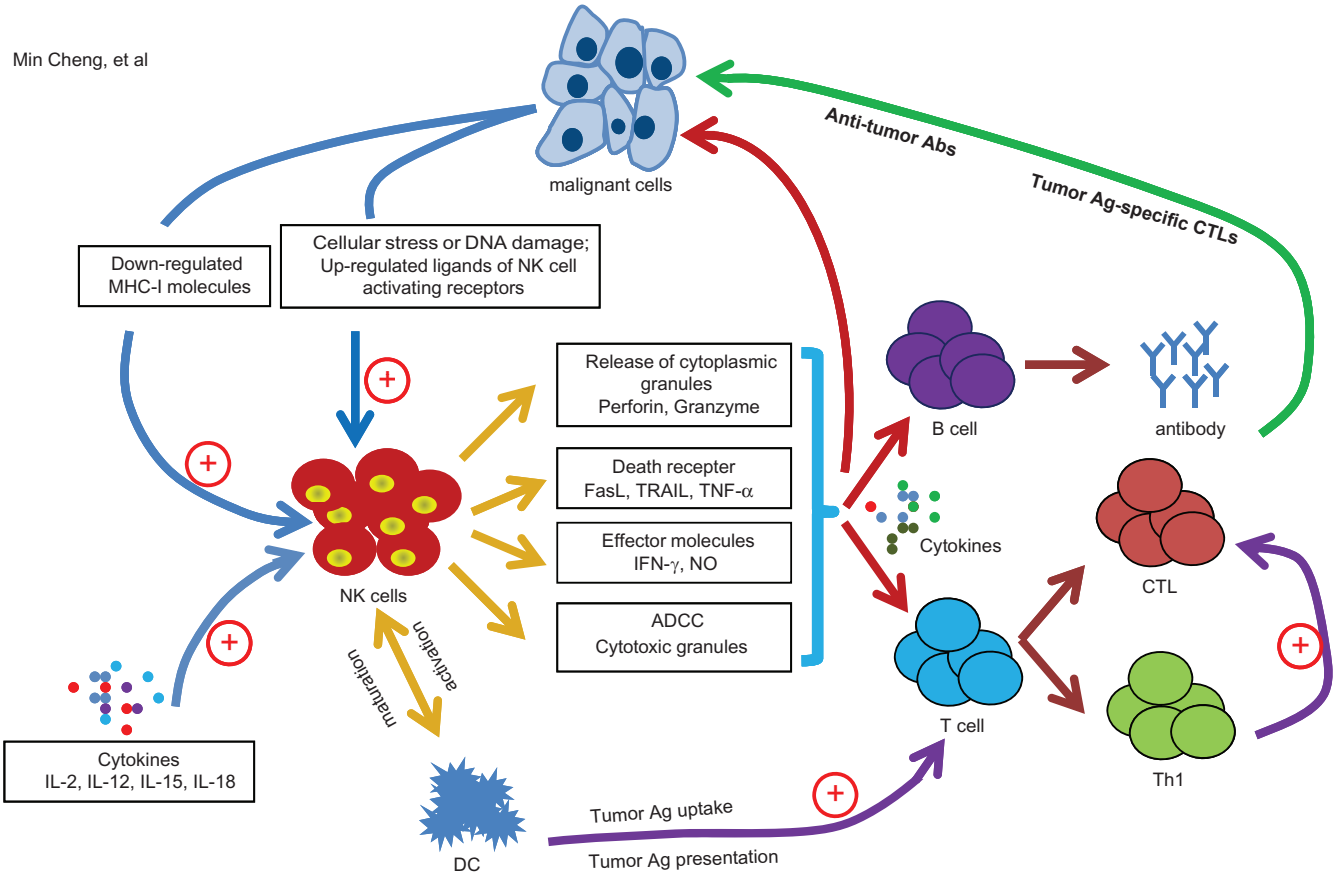

Figure 1 NK cells in tumor immunosurveillance. The diagram shows the potential roles of NK cells in tumor immunosurveillance. NK cells initially recognize the tumor cells *via* stress or danger signals. Activated NK cells directly kill target tumor cells through at least four mechanisms: cytoplasmic granule release, death receptor-induced apoptosis, effector molecule production or ADCC. Additionally, NK cells act as regulatory cells when reciprocally interact with DCs to improve their antigen uptake and presentation, facilitating the generation of antigen-specific CTL responses. Also, by producing cytokines such as IFN-γ, activated NK cells induce $CD8^+$ T cells to become CTLs. Activated NK cells can also promote differentiation of $CD4^+$ T cells toward a Th1 response and promote CTL differentiation. Cytokines produced by NK cells might also regulate antitumor Ab production by B cells. Ab, antibody; ADCC, antibody-dependent cellular cytotoxicity; CTL, cytotoxic T lymphocyte; DC, dendritic cell; IFN, interferon; NK, natural killer.

perforin and granzymes that leads to tumor-cell apoptosis by caspase-dependent and -independent pathways.[53,54] Cytotoxic granules reorient towards the tumor cell soon after NK–tumor cell interaction and are released into the intercellular space in a calcium-dependent manner; granzymes are allowed entry into tumor cells by perforin-induced membrane perforations, leading to apoptosis; (ii) by death receptor-mediated apoptosis. Some NK cells express tumor-necrosis factor (TNF) family members, such as FasL or TNF-related apoptosis-inducing ligand (TRAIL), which can induce tumor-cell apoptosis by interacting with their respective receptors, Fas and TRAIL receptor (TRAILR), on tumor cells.[55–59] TNF-α produced by activated NK cells can also induce tumor-cell apoptosis;[60] (iii) by secreting various effector molecules, such as IFN-γ, that exert antitumor functions in various ways, including restricting tumor angiogenesis and stimulating adaptive immunity.[61,62] Cytokine activation or exposure to tumor cells is also associated with nitric oxide (NO) production, where NK cells kill target tumor cells by NO signaling;[63,64] (iv) through antibody-dependent cellular cytotoxicity (ADCC) by expressing CD16 to destroy tumor cells.[40] The antitumor activity of NK cells can be further enhanced by cytokine stimulation, such as by IL-2, IL-12, IL-18, IL-15 or those that induce IFN production.[40,65–70]

Indirect NK-mediated antitumor immunity

NK cells act as regulatory cells when reciprocally interact with DCs, macrophages, T cells and endothelial cells by producing various cytokines (IFN-γ, TNF-α and IL-10), as well as chemokines and growth factors.[26,71] By producing IFN-γ, activated NK cells induce $CD8^+$ T cells to become cytotoxic T lymphocytes (CTLs), and also help to differentiate $CD4^+$ T cells toward a Th1 response to promote CTL differentiation.[72,73] NK cell-derived cytokines might also regulate antitumor antibody (Ab) production by B cells.[40] In addition, cancer cells killed by NK cells could provide tumor antigens for DCs, inducing them to mature and present antigen.[74] By lysing surrounding DCs that have phagocytosed and processed foreign antigens, activated NK cells also could provide additional antigenic cellular debris for other DCs. Thus, activated NK cells promote antitumor immunity by regulating DC activation and maturation,[75] as these DCs can facilitate the generation of antigen-specific CTL responses through their ability to cross-present tumor-specific antigens (derived from NK cell-mediated tumor lysis) to $CD8^+$ T cells.[76,77]

NK CELLS IN TUMOR IMMUNOTHERAPY

During tumor progression, tumor cells develop several mechanisms to either escape from NK-cell recognition and attack or to induce defective NK cells. These include losing expression of adhesion molecules, costimulatory ligands or ligands for activating receptors, upregulating MHC class I, soluble MIC, FasL or NO expression, secreting immunosuppressive factors such as IL-10, TGF-β and indoleam ine 2,3-d ioxygense (IDO) and resisting Fas- or perforin-mediated apoptosis.[31,78,79] In cancer patients, NK cell abnormalities have been observed, including decreased cytotoxicity, defective expression of activating receptors or intracellular signaling molecules, overexpression of inhibitory receptors, defective proliferation, decreased numbers in peripheral blood and in tumor infiltrate, and defective cytokine production.[60] Given that NK cells play critical roles in the first-line of defense against malignancies by direct and indirect mechanisms, the therapeutic use of NK cells in human cancer immunotherapy has been proposed and followed in a clinical context (Table 1).

Autologous NK cells

Early studies aimed to improve the antitumor activity of NK cells through activating endogenous NK cells and promoting their proliferation in patients. One major strategy was systemic administration of cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21 and type I IFNs.[80–84] Upon cytokine stimulation, NK cells first become lymphokine-activated killer (LAK) cells and exhibit greater cytotoxicity against malignant targets, with upregulated effector molecules, such as adhesion molecules, NKp44, perforin, granzymes, FasL and TRAIL, as well as with enhanced proliferation and cytokine production; however, only limited antitumor activity of LAK cells was observed in cancer patients.[85] A poor clinical outcome was also observed, when LAK cells were adoptively transferred into cancer patients in combination with high IL-2 doses, as patients experienced severe life-threatening toxic side effects such as vascular leak syndrome. High IL-2 doses also promote expansion of regulatory T cells that directly inhibit NK-cell functions and induce activation-induced cell death of NK cells.[86,87] Another approach using autologous LAK cells in combination with daily administration of low IL-2 doses also resulted in limited clinical success.[88] More success was attained when adoptively transferring IL-2-activated LAK cells rather than administering IL-2 systemically.[89–91] Combining IL-2 and IFN-α with GM-CSF has been shown to be effective, providing a solid basis for using IL-2 to stimulate antitumor activity from endogenous NK cells.[60]

Other NK cell activators, such as IL-12, IL-15, IL-18 and IL-21, have been successfully tested in preclinical cancer models as part of various vaccination strategies.[92,93] In the presence of IL-15 and hydrocortisone (HC), autologous NK cells can be activated and expanded *in vitro*, and these cells are effective *in vivo* in a lung metastasis mouse model which accumulated in lung tissue and were retained in the tumor-bearing site for more than 3 days. Extremely high IL-15 doses were necessary to observe any meaningful antitumor effects *in vivo*. IL-15 was only effective at augmenting NK cell-mediated cytotoxity when presented *in trans*, as soluble IL-15 at physiological concentrations was not effective. Thus, strategies favoring IL-15 trans-presentation to augment NK-mediated immunosurveillance have been proposed.[94]

There are two important limitations for using cytokines for cancer treatment: toxicity of systemic cytokine administration and cytokine-induced NK-cell apoptosis.[95] Adoptively transferring *ex vivo* expanded and activated autologous NK cells has been evaluated clinically for cancer immunotherapy and was found to greatly improve clinical responses without any

Table 1 NK cells in tumor immunotherapy

	Administration	*Stimulation*	*Effector*	*Clinical trial*	*Limitation*	*References*
Autologous NK cells	Stimulation with cytokines *in vivo*; Adoptive transfer after activation/expansion *ex vivo*	Cytokines: IL-2, IL-12, IL-15, IL-18, IL-21, type I IFN Antibody: KIR Ab	Upregulated adhesion molecules NKp44, perforin, granzymes, FasL, and TRAIL; Enhanced proliferation ability and cytokine production.	Limited activity; metastatic RCC, malignant glioma and breast cancer	Toxicity of systemic cytokine administration; cytokine-activated NK cell apoptosis; suppressed by recognition of self-MHC molecules	[80–100]
Allogeneic NK cells	Adoptive transfer after *ex vivo* activation/ expansion; Infusion of unstimulated donor NK cells	IL-15/hydrocortisone; soluble factors, immobilized molecules, cellular activators	Greater tumor killing activity	Safe with minimal toxicity; successful for cancer immunotherapy, including metastatic melanoma, renal cell carcinoma, Hodgkin's disease and poor-prognosis AML; advanced non-small cell lung cancer	Rejection by a patient's immune system	[101–105]
NK cells *via* antibody-dependent cell-mediated cytotoxicity	Systemic administration	Tumor-specific monoclonal antibodies; Altered antibody including class switching, humanization, point mutations; Co-administering cytokines (IL-12, IL-2 and IL-21), TLR agonists (CpG), or agonist antibodies (anti-4-1BB); Antibodies with linked cytokines (Immunocytokines)	Higher cytotoxicity to Ab-coated target cells	CD20-specific mAb (rituximab) in non-Hodgkin's lymphoma patients; HER-2-specific mAb (Trastuzumab/ Herceptin) in patients with metastatic breast and gastric carcinomas; Humanized anti-GD2 mAb in melanoma, osteosarcoma, and soft-tissue sarcoma patients	-	[106–124]
NK cell lines	Adoptive transfer after *ex vivo* expansion	Expanded *in vitro* as necessary	High cytotoxicity to tumor cells; cytokine production	Safe and successful antitumor effects NK92: advanced malignant melanoma and renal cell carcinoma	Rejection by a patient's immune system	[125–139]
Genetic modification of NK cells	Adoptive transfer after genetic modification	Cytokine transgene; Overexpression of activating receptors by genetic modification; Silencing of inhibitory receptor expression by RNA interference; Retargeting NK cells by using a chimeric receptor	Increased tumor-cell killing efficiency; Stronger intracellular signals for activating NK cell cytotoxicity	Successful antitumor effects; IL-2–NK-92; IL-15–NK-92; IL-15–NKL; SCF–NK-92; Anti-HER-2/neu–CD3ξ, anti-CEA–CD3ξ; anti-CD33–D3ξ; anti-CD19–CD3ξ; anti-CD20–CD3ξ	Limited specificity of NK cells *via* cytokine transgene	[140–150]

Abbreviations: AML, acute myeloid leukemia; FasL, Fas ligand; IFN, interferon; KIR, killer cell immunoglobulin-like receptors; MHC, major histocompatibility complex; NK, natural killer; RCC, renal cell carcinoma; SCF, stem cell factor; TLR, Toll-like receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

obvious adverse side effects in metastatic renal cell carcinoma (RCC), malignant glioma and breast cancer patients.[96–98] However, these autologous NK cells could not yet exhibit their full cytotoxic capacity *in vivo* and were not consistently effective in cancer patients;[99,100] this may be due to MHC class I expression in cancer patients that suppress autologous NK cells *in vivo*. Moreover, endogenous NK and LAK cells might be insufficiently cytotoxic to combat advanced tumor cells.[40] Therefore, finding ways to overcome autologous NK-cell inhibition by self-HLA molecules is needed to effectively direct autologous NK cells to kill tumor cells. Blocking NK cell expressed inhibitory receptors specific for MHC-I by using anti-KIR Abs can increase NK-cell cytotoxicity against tumor cells, which is currently being tested in a phase I clinical trial in human patients with acute myeloid leukemia (AML).

Allogeneic NK cells

Alloreactive NK cells with KIR mismatch demonstrate greater tumor-killing activity and the ability to better control AML relapse.[101,102] Based on the effectiveness of NK-cell alloreactivity in this and other studies, specific criteria for selecting mismatched donors has been established. This convincing clinical evidence also strongly supports a therapeutic role for allogeneic NK cells in controlling human malignancies. Indeed, strategies using adoptively transferred human-mismatched (haploidentical) allogeneic NK cells have been more successful for cancer immunotherapy, including against leukemia and solid cancers, and have been shown to be a safe therapy with minimal toxicity. They can also expand in patients with various malignancies, including metastatic melanoma, renal cell carcinoma, Hodgkin's disease and poor-prognosis AML.[103] Adoptive transfer of allogeneic NK cells that were activated and expanded with IL-15/HC *in vitro* has been demonstrated to be safe and potentially effective in a phase I clinical trial when used in combination with standard chemotherapy in patients with advanced non-small cell lung cancer.[104] A disadvantage to this approach is that using KIR mismatched allogeneic NK cells eventually led to immune-mediated rejection due to MHC mismatch.[105]

ADCC

NK cells express only the activating type IIIA Fc receptor (FcRγIIIa; CD16a) on their surface, which enables NK cells to recognize Ab-coated target cells and trigger NK cell-mediated ADCC, resulting in rapid NK-cell activation and degranulation.[1,106] Strong evidence supporting an important role for ADCC comes from anti-CD20 (Rituxumab)-treated non-Hodgkin's lymphoma (NHL) patients as well as from anti-HER2 (Trastuzumab/Herceptin)-treated metastatic breast and gastric carcinoma patients.[107,108] Several modifications to alter antibody structure, including class switching, humanization and point mutations to reduce complement activation, have been generated to increase NK-cell ADCC function while reducing antibody-induced toxicity.[108,109] Humanized anti-GD2 mAb can stimulate NK cell effectors and simultaneously reduce some toxicity associated with anti-GD2 therapy.[110] A CD19-specific mAb with increased FcγRIIIA-binding affinity significantly increased NK cell-mediated ADCC, thus efficiently clearing malignant B cells in cynomolgus macaques *in vivo*.[111,112]

The effect of ADCC can be potentiated by coadministering cytokines, TLR agonists or agonist antibodies that activate NK cell receptors. IL-12 increased NK-cell responses to HER2-expressing breast tumor cells when used in combination with Herceptin.[113,114] IL-2 also increases ADCC activity against tumor cells for LAK cells.[115,116] IL-21 promotes the differentiation of $CD56^{dim}CD16^{+}$ NK-cell subset, which can potentially direct ADCC.[117,118] Combining the TLR agonist CpG with Rituximab increased antitumor NK cell ADCC in a mouse model.[119] Activated NK cells by an agonistic antibody to the activating 4-1BB receptor completely regressed subcutaneous murine lymphoma tumors during Rituximab treatment.[120] Antibodies containing cytokines linked to their Fc terminus, called immunocytokines (ICs), may wield certain advantages over traditional mAbs.[121] ICs enhance synapse formation between the mAb-coated tumor cell and the NK cell by enhancing both Fc and cytokine receptor binding. In several preclinical models, ICs demonstrated much greater antitumor effect than equivalent amounts of naked mAb infused with equivalent cytokine amounts.[108,122] Treating with Rituximab combined with an antibody that blocks inhibitory self-recognition or an immunomodulatory agent (Lenalidomide) that upregulates NK-cell activation markers enhanced NK cell-mediated cell lysis.[123,124]

NK cell lines

Using NK cell lines as the source for therapeutic allogeneic NK cells may be potentially beneficial, as the lack of KIR ligand(s) (recognizing HLA) in the recipient induces NK-cell function.[125] Seven established malignant NK cell lines, including NK-92, YT, NKL, HANK-1, KHYG-1, NK-YS and NKG, have been previously reviewed by us.[126] Among them, NK-92, KHYG-1, NKL and NKG have been well documented for their antitumor activity, while the other three cell lines YT, NK-YS and HANK-1 are useful for studying the biological characteristics of EBV-associated lymphoma/leukemia.[127–130] NK-92 cells have been demonstrated to be a safe and potentially beneficial therapy with successful antitumor effects, receiving FDA approval for testing in patients with advanced malignant melanoma and renal cell carcinoma.[46,131–133] NK-92 is currently the only NK cell line that has entered clinical trials and can serve as a platform for studying NK cell-based tumor immunotherapy in the future.

The KHYG-1 cell line is the first human NK leukemia derived from a patient expressing an aberrant p53 gene.[130] KHYG-1 cells exhibited greater cytoxicity than NK-92 cells.[134] Irradiated KHYG-1 cells with inhibited proliferation does not diminish their enhanced cytolytic activity against tumor targets, suggesting that KHYG-1 cells may be a feasible anticancer immunotherapeutic agent.[135] NKL cells showed natural killing ability, ADCC and proliferative responses very similar to $CD16^{-}CD56^{dim}$ NK cells, indicating that they represent a cell

line that has likely retained most of the original NK cell characteristics.[136] NKL cells have a different antitumor spectrum and are more cytotoxic to some human cancer cells compared with NK-92 cells.[137] NKL has potential use in adoptive immunotherapy against tumors.[138,139] NKG cells, established in China, was firstly demonstrated to be a promising new human NK cell line candidate for clinical cancer immunotherapy in a xenograft mouse model.[127]

A distinct advantage of using permanent NK cell lines is that they can easily be maintained *in vitro* and expanded to large numbers under good manufacturing practice (GMP) conditions for immunotherapy. Most importantly, their antitumor activities can be further enhanced.[131] Thus, adoptively transferring established NK cell lines with broad antitumor activity represents an alternative strategy that is more practical for quality control and large-scale production for use in clinical trials.

Genetic modification of NK cells

As summarized in Table 1, expressing cytokine transgenes, overexpressing activating receptors, silencing inhibitory receptors or retargeting NK cells *via* chimeric receptors might be effective genetic manipulation approaches to modulate and enhance NK–tumor cell interaction.

The cytokine gene transfer approach induces NK cell proliferation and increases survival capacity, further enhancing their activation. By using NK cell lines, modifying genes such as IL-2, IL-12, IL-15 and stem cell factor (SCF) have been demonstrated to restore their cytotoxic capacity as well as increase their proliferative rate, survival ability and *in vivo* antitumor activity.[140–145] However, the specificity of NK cells is still limited. The approach focuses on retargeting NK cells to tumor cells by gene transfer of chimeric tumor-antigen specific receptors, such as by fusing a single chain variable fragment receptor (Fv) specific for a certain tumor-associated antigen to intracellular signaling machinery (i.e., a CD3 ζ chain). Indeed, chimeric receptors against HER2/neu, carcinoembryonic antigen (CEA) and CD33 in NK cell lines showed increased specific cytotoxicity both *in vitro* and *in vivo*.[146–148] Additionally, the NK-92 cell line modified to contain a chimeric Ag receptor consisting of a CD20-specific scFv Ab fragment exhibited significantly enhanced cytotoxicity against $CD20^+$ target cells as compared with the control.[149] Moreover, NK cells transduced with a chimeric receptor specific for CD19 dramatically enhanced the cytotoxicity against $CD19^+$ malignant B cells.[150]

EXPANDING NK CELLS FOR CLINICAL PRACTICE

For NK cell immunotherapy, obtaining a sufficient number of functional NK cells is critical in clinical protocols. Therefore, the number, purity and state of NK cell proliferation and activation are considered as the key factors.[151] In Table 2, the purification/expansion of clinical-grade NK cells developed in recent years is summarized. They can be produced from cord blood, bone marrow, peripheral blood and embryonic stem cells. Overall, the summarized methods suggest that long-term *ex vivo* expansion of NK cells may present a clinical benefit, but not the short-term activation which is not sufficient for augmenting the functions of NK cells.[152]

Cord blood-derived NK cells

Umbilical cord blood (UCB) is an excellent source of HSCs, which produce a multitude of therapeutic cells, including NK cells. HSCs can be harvested from UCB for clinical applications by using the CliniMACS system to select $CD34^+$ cells.[153–155] Enriching $CD34^+$ cells from thawed UCB was optimized by using the EloHAES separation method within the CliniMACS system. The $CD34^+$ cell-derived NK cells were generated in static cell culture bags or in an automated bioreactor in order to produce clinical-grade NK cells in a closed environment. Large-scale production of highly active and functional NK cells was obtained by this method for a phase I dose-finding trial in elderly AML patients.[155] Another extremely efficient cytokine-based culture system for expanding $CD34^+$ cell-derived NK cells was also reported; this method could be used for both fresh and frozen $CD34^+$ UCB cells. These UCB-derived $CD56^+$ NK cells uniformly expressed high NKG2D levels and natural cytotoxicity receptors, efficiently targeted myeloid leukemia and melanoma cell lines, and lysed primary leukemia cells at low NK/target ratios.[154] $CD34^+$ cells expanded *ex vivo* in optimized serum-free medium provide a promising cell source with significantly higher NK-cell differentiation as well as enhanced IFN-γ secretion and cytotoxic ability compared with the freshly isolated $CD34^+$ cells.[153] UCB-derived $CD56^+$ cells separated by an anti-CD56 mAb and immunomagnetic beads could be expanded in an *ex vivo* culture system in the presence of irradiated autologous lymphocytes and various IL-2 concentrations while maintaining their antileukemic abilities.[156] However, prolonged exposure of purified NK cells to cytokines *in vitro* might induce cell exhaustion, rendering the NK cells unable to effectively kill tumor cells after infusion into the recipient.[152]

Stem cell/induced pluripotent stem cell (iPSC)-derived NK cells

Human NK cells can be differentiated from BM-derived $CD34^+$ hematopoietic progenitor cells cultured in certain conditions: IL-2 plus an allogeneic feeder cell layer; IL-2 plus other hematopoietic growth factors, such as c-kit ligand; IL-15; or in a marrow stroma-dependent long-term culture system.[157,158] $CD34^+$ cells derived from human embryonic stem cells (hESCs) enrich for hematopoietic colony-forming cells, which is similar to $CD34^+$ selection from primary hematopoietic tissues, such as BM and UCB, suggesting that hESCs might be a suitable novel cell source for therapy.[159] Studies on hESCs and iPSCs indicate that using hESC- and iPSCs-derived hematopoietic products for diverse clinical therapies is a reasonable expectation for the future.[159–161] Recently, efficient generation of functional NK cells from hESCs by a two-step culture method has been reported. These NK cells possess the ability to lyse tumor cells by direct cell-mediated cytotoxicity and ADCC, and exhibit a mature NK cell phenotype, including KIR and CD94/NKG2A expression as well as high expression

Table 2 Expansion of NK cells *in vitro* for clinical practice*

	Starting material	*Initial cell number*	*Medium*	*Stimulators* Feeder cells*	*Culture instrument*	*Culture time/ acquired cell number*	*Fold proliferation*	*Purity*	*Cytotoxicity*	*Phenotype, cytokine production*	*References***
Cord blood-derived NK cells	$CD34^+$ cell from cord blood (CliniMACS)	(0.89–6.34) $\times 10^6$	Glycostem Basal Growth Medium +10% HS	SCF, IL-7, IL-15, IL-2, Flt3L, TPO, G-CSF, IL-6, LMWH	Vuelife™ bags, WAVE Bioreactor System 2/10, BIOSTATH CultiBag RM system	6 weeks (1.6–3.7)$\times 10^9$	1435–2657	>90%	K562 (>40%, 10 : 1)	$CD56^+$, $CD3^-$, $NKG2D^+$, $NCRs^+$, $CD161^+$, $CD314^+$, $CD244^+$	[155]
	$CD34^+$ cell from cord blood (CliniMACS)	(0.84–2.50) $\times 10^6$	Glycostem Basal Growth Medium	SCF, TPO, IL-7, Flt3L, IL-15, IL-2, G-CSF, GM-CSF, IL-6, LIF, MIP-1α	24-well tissue culture plates	14–35 days (1.9–7.8)$\times 10^9$	$\sim 10^4$ (freshly UCB); $\sim 10^3$ (frozen UCB)	>95%	K562, Lama, Kasumi, BLM, nd FM3 (>75%) KG1a (~30%) (18 h 1 : 1)	$CD56^+$, $CD3^-$, NKG2D+, $NCRs^+$, $CD107^+$, $2B4^+$, $CD161^+$, IFN-γ	[154]
Stem cell/ iPSC-derived NK cells	$CD34^+$ $CD45^+$ cells (H9 hESC line)	—	RPMI 1640+15% defined fetal bovine serum; DMEM/Ham F12+20% heat-inactivated human serum AB	IL-3, IL-15, IL-7, SCF and Flt3L; Feeder cells: M210-B4; AFT024	—	30–35 days	~100	>37.5%	K562, MCF7, PC3 (55%–80%), NTERA2, and U87 (20%–30%)	$CD56^+$, $CD45^+$, $CD16^+$, $CD94^+$, $NKG2D^+$, $NKp46^+$, $CD158a^+$, $CD158b^+$, IFN-γ	[159,162,163]
	BM $CD34^+$	—	Dulbecco's medium supplemented with 12.5% fetal calf serum; 12.5% horse serum	IL-2; Feeder cells: stromal cells from irradiated BMMNC	—	—	~690	75%	K562 (80%, 6.6 : 1)	$CD3^-$, $CD56^+$, $CD2^+$, $CD7^+$, $CD8^+$, $CD16^+$	[157]
PBMCs	$CD3-CD56^+$ cells from PBMCs (CliniMACS)	(0.40±0.16) $\times 10^8$	CellGro SCGM serum-free Medium, 5% AB human serum	IL-2, IL-15, anti-CD3 monoclonal antibody (MAb) OKT3	Baxter LifeCell culture bags	19 days (85.5±17.2) $\times 10^8$	268.3±66.8	100%	K562 (>60%, 10 : 1)	$CD3^-$, $CD56^+$, $NKG2D^+$, $NCRs^+$, DNAM-1	[166]

Table 2 Continue

Starting material	*Initial cell number*	*Medium*	*Stimulators* Feeder cells*	*Culture instrument*	*Culture time/ acquired cell number*	*Fold proliferation*	*Purity*	*Cytotoxicity*	*Phenotype, cytokine production*	*References***
CD3−CD56$^+$ cells from PBMCs	3.0×10^6	SCGM Medium and 10% fetal bovine serum	IL-2; Feeder cells: K562-mb15-41BBL	VueLife bag system	7 days	90.5 (33–141)	83.1% (72.9%–85.9%)	K562, HL-60, KG1, and U937 (>40%, 4 : 1)	CD3$^-$, CD56$^+$, NKG2D$^+$, NCRs$^+$	[165]
CD56$^+$ cells from PBMCs	(9.5–85.8) $\times10^6$	Alpha-MEM, 20% fetal bovine serum	IL-15, HC	—	20–23 days	23 (3.2–131.3)	97.9% (82.7%–99.6%)	K562, (23.2%, 7.0–54.7%, 1 : 1)	CD3$^-$, CD56$^+$, NKG2D$^+$, NCRs$^+$	[196]
CD56$^+$ cells from PBMCs (CliniMACS)	2.0×10^8	X-VIVO 20 10% heat inactivated human AB serum	IL-2; Feeder cells: EBV-TM-LCL cells	Flasks and bags	21 days 3×10^{10}	490±260	84.3%± 7.8%	RCC (27.6±9.3%, 1 : 1)	CD3$^-$, CD56$^+$, CD244$^+$, CD48$^+$, NKG2D$^+$ sFasL, IFN-γ, GM-CSF, TNF-α, MIP-1α, MIP-1β	[164]
PBMCs	2×10^6 NK cells	SCGM Medium and 10% fetal bovine serum	IL-2; Feeder cells: K562-mb15-41BBL	G-Rex100 flasks	8–10 days	209 (38–338)	61% (54%–70%)	K562, U266 and Raji (>40%, 5 : 1)	CD3$^-$, CD56$^+$	[197]
PBMCs	—	Serum-free medium and 10% heat-inactivated human plasma	rhIL-2; OK432; anti-CD16	Cell-culture bag	21 days	637–5712	78.9%± 11.6%	K562, Raji and Daudi (>20%, 3 : 1)	CD3$^-$, CD56$^+$, CD158a$^+$, CD158b1/b2$^+$, CD159a$^+$, CD69$^+$, NKp30$^+$, NKp44$^+$, NKp46$^+$, IFN-γ, TNF-α	[198]
PBMC	1.5×10^6	cRPMI	Il-2; Feeder cells: K562-mbIL15-41BBL cells	T-25 or T-75 culture flasks	14 days	165 (4–567)	45.6% (7.4%–76.4%)	K562, MCF-7, LNCaP, DU145, PC-3	CD3$^-$, CD56$^+$, NKG2D$^+$, NCRs$^+$	[199]
PBMCs	(4.6–9.7) $\times10^8$	CellGro SCGM serum-free medium 5% human serum	IL-2	Wave Bioreactor System 2/10	21 days (9.8×10^9)	Mean 77-fold	Mean 37.5%	K562 (>25%, 10 : 1)	CD3$^-$, CD56$^+$, CD244$^+$, CD11a$^+$, CD69$^+$, NKG2D$^+$, NCR$^+$	[200]

Table 2 Continue

	Starting material	*Initial cell number*	*Medium*	*Stimulators* Feeder cells*	*Culture instrument*	*Culture time/ acquired cell number*	*Fold proliferation*	*Purity*	*Cytotoxicity*	*Phenotype, cytokine production*	*References***
	CD3-depleted PBMCs	10^7 CD3− depleted cells	AIMV media 10% hu AB serum	IL-2; Feeder cells: OKT3-loaded autologous PBMC	Cell-culture bags	21 days (4.70±2.10) $\times10^{10}$	—	⩾93%	888 (82±12%, 10 : 1)	$CD3^-$, $CD56^+$, $CD16^+$, $NKG2D^+$	[176]
NK cell lines	NK-92	(2.5×10^5/mL) ×25 mL/bag	X-Vivo 10 serum-free media amino acids and 2.5% human AB plasma	IL-2 (500 IU/ml)	1 l Vuelife culture bag	15–17 days $>1\times10^9$/bag	>200	⩾80% (viability)	K562 (72%); Raji (58%) (10 : 1)	$CD3^-$, $CD56^+$, IL-6, IL-8, IL-10	[170,171]
		1×10^7/ bioreactor	Optimized clinical-grade media	IL-2 (100∼ 500 IU/ml)	Controlled stirred bioreactor	11–16 days $>10^{10}$/ bioreactor	>1000	>95% (viability)	Highly lytic to leukemia, lymphoma, malignant melanoma, prostate cancer, squamous cell carcinoma, breast cancer	Positive: CD56, CD2, CD7, C11a, CD28, CD45, CD54 Negative: CD1, CD3, CD4, CD8, CD14, CD16, CD20, CD23, CD34, HLA-DR	[131]
	NKG	(1×10^5/ml) ×200 ml/bag	α-MEM medium 10% fetal bovine serum +10% horse serum	IL-2 (100 IU/ml)	WAVE Bioreactor	12–14 days $>10^{10}$/bag	>1000	>90% (viability)	K562 (>50%), Ho-8910 (>60%), Daudi (>70%), LoVo (>35%) (10 : 1)	$CD56^+$, $CD16^-$, $CD27^-$, $CD3^-$, αβTCR−, $\gamma\delta TCR^-$, $CD4^-$, $CD8^-$, $CD19^-$, $CD161^-$, $CD45^+$, $CXCR4^+$, $CCR7^+$, $CXCR1^-$, $CX3CR1^-$; IFN-γ, TNF-α, IL-6, IL-10	[127]

Abbreviations: GM-CSF, granulocyte monocyte-colony stimulating factor; hESCs, human embryonic stem cells; IFN, interferon; iPSCs, induced pluripotent stem cells; NCR, natural cytotoxicity receptor; NK, natural killer; PBMC, peripheral blood mononuclear cell; SCF, stem cell factor; UCB, umbilical cord blood.

* *Ex vivo* expansion of NK cells for clinical immunotherapy under GMP conditions.

of a variety of effector molecules for natural cytotoxicity, such as FasL, TRAIL, NKp46, NKp44, NKG2D and CD16.[162] Furthermore, these hESC-derived NK cells are uniformly $CD94^{+}CD117^{low/-}$ and mediate an effective antitumor response in an *in vivo* xenogeneic mouse model, which was more effective as compared to UCB-derived NK cells.[163] Additionally, hESC- and iPSC-derived NK cells also provide advantages compared with PB-derived NK cells, including effective genetic modification and promoting survival *in vivo*.[161] The most promising future direction for these cells may be to engineer hESCs or iPSCs to express chimeric antigen receptors (CARs) for specific tumor antigens that are capable of directing CTLs to tumor sites; they can also be modified to express cloned T cell receptors for specific tumor antigens, which remains to be tested *in vivo*.[161] Indeed, producing NK cells from $CD34^{+}$ stem cells has become alluring, since stem cells can be isolated and frozen, and can overcome some obstacles presented by using purified NK cells.[152]

Peripheral blood mononuclear cells (PBMCs)

NK cells are normally present in low numbers in PBMCs. Thus, many researchers have focused on successfully expanding NK cells *ex vivo* under GMP conditions for clinical immunotherapy. As summarized in Table 2, Epstein–Barr virus-transformed lymphoblastoid cell lines, genetically modified K562 cells, or irradiated autologous cells were used as feeder cells to promote NK cell expansion from PBMCs.[164–167] The Campana group has developed a master cell bank of K562 feeder cells expressing a membrane-bound form of IL-15 (mbIL15) and 4-1BB ligand (K562-mb15-41BBL) under cGMP guidelines, and demonstrated that large-scale expansion and activation of human NK cells for clinical studies was feasible.[165] These NK cells demonstrated cytotoxic activity toward tumor cells even higher than observed in the initial small-scale experiments. Additionally, K562 feeder cells genetically modified to coexpress MICA, 4-1BB ligand and IL-15 (K562-MICA-4-1BBL-IL-15) showed potential for *ex vivo* NK cell expansion for clinical immunotherapy.[167] Compared with mbIL15, K562-based genetically engineered artificial antigen-presenting cells with membrane-bound IL-21 supported human NK cell proliferation with longer telomeres and less senescence, resulting in enhanced expansion and tumor killing.[168] Large-scale *in vitro*-expanded NK cells using irradiated Epstein–Barr virus-transformed lymphoblastoid cell lines feeder cells were also found to be more cytotoxic to tumor cells, with upregulated activating receptors and death receptor ligands as well as altered cytokine secretion profiles.

The safety and antitumor effects of autologous NK cells expanded from PBMCs were investigated in a phase I trial in patients with advanced metastatic tumors and hematological malignancies.[164] Large-scale *ex vivo* alloreactive NK cell expansion suitable for multiple donor lymphocyte infusions in AML was reported. This protocol involved that NK cells purified by CliniMACS were cultured in closed air-permeable culture bags with certified culture medium and other components, including human serum, IL-2, IL-15, anti-CD3 antibody and autologous irradiated feeder cells.[166] Furthermore, highly active human NK cells expanded in a large-scale, clinical-grade, feeder-free way were established using an automated bioreactor. Bulk PBMCs were directly cultured without feeder cells or any separation strategies, leading to an NK cell-enriched population that was distinct from either LAK or cytokine-induced killer cells. These expanded NK cells displayed significantly higher cytotoxic capacity and higher NKp44 expression than NK cells expanded in flasks.[169] As described earlier in the present review, allogeneic NK cells activated and expanded *in vitro* with IL-15/HC were safe and potentially effective in a phase I clinical trial when used in combination with chemotherapy in advanced non-small cell lung cancer patients.[104]

NK cell lines

Compared with autologous or allogeneic NK cells from PBMCs or stem cells, the large-scale expansion of NK cell lines under GMP conditions is easier and more available for clinical adoptive therapy. As shown in Table 2, individual samples of NK-92 cells or NKG cells from a master cell bank can be thawed and expanded in FDA-approved therapeutic-grade media supplemented with the required cytokines and freshly frozen plasma.[127,131,170,171] Substantial improvement in the purity and quantity of NK cells could be obtained by using an optimized cell culture medium and a controlled stirred bioreactor for NK-92 cells, or a WAVE bioreactor for NKG cells.

CLINICAL TRIALS OF NK CELL-MEDIATED TUMOR IMMUNOTHERAPY

Results from treating hematological malignancies demonstrated a critical role for NK cells in clinical immunotherapy, as alloreactive NK cells highlighted the graft-*vs.*-leukemia effect in AML patients.[172] The graft-*vs.*-tumor effect of alloreactive NK cells was also strengthened by mismatched IL-2-activated lymphocytes in patients with solid tumors or hematological malignancies.[173] As discussed above, autologous NK cells, allogeneic NK cells, NK cell lines and genetically modified NK cells were investigated for effectiveness as tumor immunotherapies. The clinical study designs evaluating the efficacy of these various NK cell-mediated tumor therapies are summarized in Table 3.

Adoptive transfer of autologous NK cells was shown not to have any clinical benefit for treating melanoma, RCC, lymphoma or breast cancer patients in several previously described clinical trials using *ex vivo*-generated LAK cells.[100,174] Based on these studies, a clinical study was initiated in metastatic cancer patients who were adoptively transferred with autologous, *in vitro*-activated NK cells. While reinfusion of activated autologous NK cells was found to be safe with no negative side effects in metastatic colorectal cancer, non-small cell lung cancer, metastatic melanoma or renal cell carcinoma patients, no significant clinical responses were observed.[175,176] Adoptively transferred NK cells persisted in the peripheral circulation for at least 1 week, but they expressed significantly lower levels of the key activating receptors, such as NKG2D, and showed weak ability to kill tumor cells. Additional studies in patients with

Table 3 Clinical trials of tumor immunotherapy by using NK cells

Source of NK cells	*Stage*	*Subjects*	*Treatment*	*Styles*	*Effects*	*Status*	*Country*	*Reference/ ClinicalTrials.gov Identifier*
Autologous NK cells (from PBMC)	Phase I	11 metastatic colorectal cancer; 1 NSCLC	$(0.001–0.3)\times10^9$ cells/dose; i.v. 1–4 doses/cycle, 1–6 cycle	Used alone	No toxicities	Completed	Germany	[175]
	Phase I	7 metastatic melanoma; 1 metastatic renal cell carcinoma	$(4.70\pm2.10)\times10^{10}$ cells; i.v.	Combined with chemotherapy	No toxicities	Completed	United States	[176]
	Phase I	Metastatic nasopharyngeal	i.v.	Used alone	—	Completed	Singapore	NCT00717184
	Phase I	Metastatic cancers; hematological malignancies	i.v.	Used alone	—	Recruiting participants	United States	NCT00720785
	Phase I	Breast cancer, glioma, hepatocellular cancer, squamous cell lung cancer, pancreatic cancer, colon cancer, prostate cancer	i.v.	Used alone	—	Suspended	United States	NCT00909558
Allogeneic NK cells (from PBMC)	Phase I	15 advanced NSCLC	$(0.2–29)\times10^6$ cells/kg/dose; i.v. 2–4 doses	Combined with chemotherapy	No side effects; clinically effective	Completed	Greece	[196]
	Phase I	Acute myeloid leukemia	i.v.	Combined with chemotherapy	—	Ongoing	United States	NCT00187096
	Phase I	Lymphoma; leukemia; stem cell transplantation	i.v.	Combined with Rituximab	—	Completed	United States	NCT00383994
	Phase I	Lymphoma	1×10^7 cells/kg; i.v.	Combined with SCT	No toxicities	Ongoing	United States	NCT00586690; NCT00586703; [178]
	Phase I	Non-B lineage hematological malignancies and solid tumors	i.v.	Combined with chemotherapy	—	Recruiting participants	United States	NCT00640796
	Phase I	Lymphoma; myeloma; leukemia	i.v.	Used alone	—	completed	United States	NCT00660166
	Phase I	ALL; CML; JMML; MDS; NHL	i.v.	Combined with chemotherapy and immunotherapy	—	Recruiting participants	United States	NCT00697671
	Phase I	13 acute myeloid leukemia	$(1–5)\times10^6$ cells/kg; i.v.	Combined with chemotherapy	No toxicity including GVHD	Unknown	Italy	NCT00799799; [177]
	Phase I	Neuroblastoma	i.v.	Combined with chemotherapy	—	Recruiting participants	United States	NCT00877110

Table 3 Continue

Source of NK cells	*Stage*	*Subjects*	*Treatment*	*Styles*	*Effects*	*Status*	*Country*	*Reference/ ClinicalTrials.gov Identifier*
	Phase I	Acute lymphoblastic leukemia	i.v	Used alone	—	Recruiting participants	United States	NCT00995137
	Phase I	Malignant lymphomas; solid tumors	Singe-dose infusion: Cohort1: 1×10^6 cells/kg Cohort2: 1×10^7 cells/kg; Repeated dose infusion: Cohort3: 1×10^6 cells/kg; Cohort4: 3×10^6 cells/kg; Cohort5: 1×10^7 cells/kg; Cohort6: 3×10^7 cells/kg; i.v	Used alone	—	Recruiting participants.	Korea	NCT01212341
	Phase I	Leukemia, lymphoma, neuroblastoma, sarcoma, desmoplastic small round cell tumor	3 dose levels (1×10^5, 1×10^6, and 1×10^7 cells/kg); i.v	Combined with chemotherapy	—	Recruiting participants.	United States	NCT01287104
	Phase I	Acute myeloid leukemia	Four cohorts of escalating doses receiving 0, 1, 10, or 20×10^6 NK cells/kg; i.v	Combined with chemotherapy and immunotherapy	—	Recruiting participants	United States	NCT01478074
	Phase I	Neuroblastoma	minimum of 0.1×10^6 cells/kg; maximum of 400×10^6 $CD45^+$ cells/kg, given once; i.v	Combined with chemotherapy and immunotherapy	—	Recruiting participants	United States	NCT01576692
	Phase I	Leukemia Chronic lymphocytic leukemia	$(3–7)\times10^8$ cells; however, if the dose of 3×10^8 cells is not achieved, all available NK cells will be infused; i.v.	Combined with chemotherapy	—	Not yet open for participant recruitment	United States	NCT01619761
	Phase I/ II	Acute myelogenous leukemia	$(2–3)\times10^7$ cells/kg; i.v.	Combined with chemotherapy	—	Ongoing participants	United States	NCT00303667

Table 3 Continue

Source of NK cells	*Stage*	*Subjects*	*Treatment*	*Styles*	*Effects*	*Status*	*Country*	*Reference/ ClinicalTrials.gov Identifier*
	Phase I/II	Leukemia Lymphoma	$(1.5–8)\times10^7$ NK cells/kg; i.v.	Combined with chemotherapy and immunotherapy	—	terminated	United States	NCT00625729
	Phase I/II	Brain and central nervous system tumors; chronic myeloproliferative disorders; leukemia; lymphoma; lymphoproliferative disorder; multiple myeloma and plasma cell neoplasm; myelodysplastic syndromes; myelodysplastic/ myeloproliferative neoplasms	i.v.	Combined with SCT	—	Recruiting participants	Korea	NCT00823524
	Phase I/II	Melanoma	i.v.	Combined with chemotherapy	—	Completed	Korea	NCT00846833
	Phase I/II	Multiple myeloma	3 dose levels (1.5×10^6 cells/kg, 1.5×10^7 cells/kg and 1×10^8 cells/kg), if safe, continuing with maximally 7 doses of 1×10^8 cells/kg; i.v.	Combined with chemotherapy and SCT	—	Recruiting participants	Switzerland	NCT01040026
	Phase I/II	Acute myeloid leukemia; advanced hematological malignancies; indication for allogeneic stem cell transplantation	1×10^7 cells/kg; i.v.	Combined with chemotherapy and radiation therapy and SCT	—	Recruiting participants	Germany	NCT01220544
	Phase I/II	Childhood solid tumor	i.v.	Used alone	—	Recruiting participants	Spain	NCT01337544
	Phase I/II	Acute myeloid leukemia; precursor cell lymphoblastic leukemia-lymphoma; myelodysplastic syndromes; lymphoma; neuroblastoma; rhabdomyosarcoma	$\geqslant1\times10^7$ NK cells/kg; i.v.	Combined with HLA-haploidentical HSCT	—	ongoing	Switzerland	NCT01386619

Table 3 Continue

Source of NK cells	*Stage*	*Subjects*	*Treatment*	*Styles*	*Effects*	*Status*	*Country*	*Reference/ ClinicalTrials.gov Identifier*
	Phase I/II	Acute myeloid leukemia	1×10^6 NK cells/kg or 3×10^6 cells/kg; i.v.	Used alone	—	Not yet open for participant recruitment	United States	NCT01520558
Allogeneic NK cells (from PBMC)	Phase II	14 ovarian cancer; 6 breast cancer	$(8.33–39.41)\times10^6$ NK cells/kg; i.v.	Combined with chemotherapy	PR (4 patients); SD (12 patients); PD (3 patients)	Completed	United States	[201]
	Phase II	Acute lymphoblastic leukemia; lymphoma, lymphoblastic	i.v.	Combined with chemotherapy and SCT	—	Recruiting participants	United States	NCT00186875
	Phase II	Metastatic melanoma; metastatic kidney cancer	i.v.	Combined with chemotherapy	—	Completed	United States	NCT00328861
	Phase II	Breast cancer	$(1.5–8.0)\times10^7$ NK cells/kg; i.v.	Combined with chemotherapy and radiation therapy	—	Terminated	United States	NCT00376805
	Phase II	Leukemia; myelodysplastic syndromes	i.v.	Combined with chemotherapy	—	Recruiting participants.	United States	NCT00526292
	Phase II	Fallopian tube cancer; ovarian cancer; peritoneal cavity cancer	$(1.5–8.0)\times10^7$ NK cells/kg; i.v.	Combined with chemotherapy and radiation therapy	—	Terminated.	United States	NCT00652899
	Phase II	Neuroblastoma	i.v.	Combined with chemotherapy and SCT	—	Terminated	United States	NCT00698009
	Phase II	Leukemia; pediatric cancer	i.v.	Combined with chemotherapy	—	Completed	United States	NCT00941928
	Phase II	Ovarian cancer; fallopian tube cancer; primary peritoneal cancer; breast cancer	8.0×10^7 cells/kg; i.v.	Combined with chemotherapy	—	Recruiting participants	United States	NCT01105650
	Phase II	Acute myelogenous leukemia	$\leqslant8.0\times10^7$ nucleated cells//kg; i.v.	combined with chemotherapy	—	Ongoing	United States	NCT01106950
	Phase II	Relapsed lymphoma; B cell non-Hodgkins lymphoma; refractory lymphoma; high risk chronic lymphocytic leukemia	$(1.5–8.0)\times10^7$ cells/kg; i.v.	Combined with chemotherapy	—	Recruiting participants	United States	NCT01181258
	Phase II	Acute myeloid leukemia; myelodysplastic syndrome	i.v.	Combined with SCT	—	Recruiting participants	United States	NCT01370213

Table 3 Continue

Source of NK cells	*Stage*	*Subjects*	*Treatment*	*Styles*	*Effects*	*Status*	*Country*	*Reference/ ClinicalTrials.gov Identifier*
	Phase II	Leukemia; chronic myelogenous leukemia	i.v.	Combined with chemotherapy	—	Recruiting participants	United States	NCT01390402
	Phase II	Myelodysplastic syndrome	i.v.	Combined with chemotherapy	—	Not yet open for participant recruitment	United States	NCT01593670
	Phase II	Acute myelogenous leukemia	i.v.	Combined with chemotherapy	—	Not yet open for participant recruitment	United States	NCT01639456
NK-92 cells	Phase I	11 advanced renal cell cancer; 1 melanoma	1×10^8 or 3×10^8 or 1×10^9 or 3×10^9 NK-92 cells/m^2 body surface; i.v. three doses (3 patients/ group)	Used alone	No severe hemodynamic or hematologic toxicities	—	United States	[171]
	Phase I	Acute myeloid leukemia	1×10^9 or 3×10^9 or 5×10^9 NK-92 cells/m^2 body surface; i.v. two doses	Used alone	Status: suspended	—	United States	NCT00900809
	Phase I	Leukemia; lymphoma; myeloma; Hodgkin's disease	1×10^9 or 3×10^9 or 5×10^9 NK-92 cells/m^2 body surface; i.v. on days 1, 3, and 5 of each cycle; 6 cycles monthly	Used alone	Status: suspended	—	Canada	NCT00990717
	Phase I/ II	4 sarcoma; 2 medulloblastoma; 1 PNET; 1 B-cell ALL	$(1–3)\times10^9$ NK-92 cells/ m^2 body surface; i.v. two doses	Used alone	Without any significant side effects; no conclusions as to the efficacy can be drawn		Germany	[131]
NK cells from UCB	Phase II	Leukemia; myelodysplastic syndromes	i.v.	Combined with chemotherapy and SCT	—	Terminated	United States	NCT00354172

Abbreviations: ALL: Acute Lymphoblastic Leukemia; CML: Chronic Myelogenous Leukemia; JMML: Juvenile Myelomonocytic Leukemia; MDS: Myelodysplastic Syndrome; NHL: Non-Hodgkin's Lymphoma; NK, natural killer; PBMC, peripheral blood mononuclear cell; UCB, umbilical cord blood.

lower tumor burden, or adoptive NK cell transfer coadministered with an mAb or cytokine, deserve evaluation.[175,176] The phase I clinical trials in breast cancer, glioma, squamous cell lung cancer, pancreatic cancer, hepatocellular cancer, colon cancer or prostate cancer patients have since been suspended (www.clincaltrial.gov).

Due to the self-tolerance associated with autologous NK cells, adoptively transferred allogeneic NK cells were explored as an alternative. Highly purified NK cells from haploidentical KIR-ligand mismatched donors were effective in a cohort of elderly patients with high-risk AML (registered at www.clinicaltrial.gov as trial NCT00799799). Further studies are

highly warranted to specifically assess the role of NK cell therapy in post-remission management in adult AML patients.[177] Adoptive transfer in patients with other malignancies, including lymphoma, leukemia, non-B lineage hematological malignancies and solid tumors, has been proven safe and clinically effective.[104,178] Phase II clinical trials for PBMC-derived allogeneic NK cells in patients with hematological malignancies and solid tumors have been completed or are ongoing, as shown in Table 3. Another phase II study of allogeneic NK cell therapy in patients with recurrent ovarian and breast cancer indicated that adoptive NK cell transfer after lympho-depleting chemotherapy was associated with transient donor chimerism. Strategies to augment *in vivo* NK cell persistence and expansion might be required to reduce this chimeric effect; Treg reconstitution in the recipient, for example, can repress this chimerism.[179] Additionally, a phase II clinical trial using UCB-derived allogeneic NK cells has been performed in leukemia and myelodysplastic syndrome patients, but was terminated, and no conclusions can be drawn as to its efficacy.

As allogeneic NK cells, NK-92 cells, as the only NK cell line that has entered clinical trials, were demonstrated to be with no severe hemodynamic toxicities or significant tissue side effects in patients with advanced malignant melanoma and renal cell carcinoma in either Europe or the United States, suggesting that NK-92 cells might be an excellent candidate for adoptive cellular immunotherapy.[131,171]

FUTURE REMARKS

Application of tissue-specific NK cells in tumor immunotherapy

Strong evidence supports that NK cells are able to adapt to their microenvironment by expressing divergent phenotypic and functional features that are specific to each organ, such as liver, mucosal tissues, uterus, pancreas, joints, brain and peripheral blood. The various roles NK cells play in different organs are often complex and sometimes even paradoxical.[180] Immunotherapeutic approaches targeting NK cells should prove useful in inducing more effective immune responses to improve treatments. In 1985, the idea was put forth that organ-associated NK cell activity was a possible mechanism influencing the therapeutic effects of biological response modifier treatment.[67] Liver is the only organ with arterial and venous blood supply from the gut, which is a unique immunological organ with an overwhelming innate immune system. Indeed, the unique features of liver NK cells include higher TRAIL, perforin and granzyme expression, and the lack of Ly-49 inhibitory receptors. Augmented antitumor activity was observed in hepatic NK cells as compared to splenic NK cells; even tumor cells killed by hepatic NK cells are otherwise resistant to killing by splenic NK cells. The important role of NK cells in tumor immunosurveillance within the liver microenvironment makes tissue-specific NK cells an attractive target for immunotherapeutic approaches that aim to control tumor metastasis in the liver.[181]

NK cell receptor-mediated immunotherapy

NK cell-mediated tumor immunosurveillance was limited in AML patients due to the decreased expression of activating receptors on NK cells and/or the heterogenous expression of ligands on leukemic blasts.[182] Since the balance between inhibitory and activating receptors regulates NK cell activation, therapeutic strategies designed to target NK cell receptors may be able to potentiate NK cell activity in treating cancer. For example, reducing inhibitory KIR function by specifically blocking ligand recognition would be particularly effective in treating patients with HLA-expressing tumors that are resistant to NK cell-mediated lysis. A recent report by Binyamin *et al.*[124] demonstrated that antibody-mediated KIR blockade significantly augmented NK cell ADCC responses. However, blocking KIR alone did not significantly increase NK-mediated killing of autologous tumor cells in an *in vitro* study. A novel human anti-KIR receptor therapeutic antibody that blocks KIR2DL1-3 impressively augments NK-mediated tumor cell killing.[183] Study in a preclinical mouse model with all NK cells educated by a single transgenic inhibitory receptor, human KIR2DL3, by engaging its ligand, HLA-Cw3 indicated that anti-KIR mAb treatment induced HLA^{+} target cell lysis without breaking self-tolerance *in vivo*, and long-term anti-KIR mAb infusion did not abolish NK cell education or tumor cell recognition.[184] Therefore, blocking inhibitory receptors like KIR may be an effective way to enhance NK cell-mediated cytotoxicity toward tumors.[185]

Drugs affecting NK-cell function

In some instances, immunomodulatory drugs can directly or indirectly activate NK cells. As discussed above, cytokines or growth factors used in combination with NK cells have successfully treated several human cancers (Section on 'NK cell-mediated antitumor mechanisms'). In addition to these cytokines, broad activators of immune function—some of which also stimulate NK cells—are also implicated in antitumor immunity. In multiple myeloma (MM), thalidomide and immunomodulatory drugs trigger NK cell-mediated tumor-cell lysis by activating their cytotoxicity and ADCC functions. This partially explains the mechanism of action of these drugs and further supports their therapeutic use in MM.[186] Immunostimulatory DNA oligonucleotides containing CpG motifs (CpG ODNs) were reported to stimulate immune responses against primary human ALL cells *in vivo*, reducing systemic leukemia burden, controlling disease, and improving survival. Since NK-cell depletion significantly reduced the CpG ODN-mediated antileukemic activity *in vivo*, NK-cell activation was partially responsible for the enhanced antitumor activity.[187] Additionally, an essential role for NK cells was demonstrated during adjuvant intravesical bacillus Calmette–Guérin immunotherapy when used to treat superficial bladder cancer.[188]

Monitoring NK cell-based immunotherapy by non-invasive imaging

Evaluating the *in vivo* accumulation, distribution and quantity of transferred NK cells within tumor regions is clinically necessary to

monitor progress of NK cell-based immunotherapies after systemic administration. Using imaging techniques, monitoring NK cells in recipient patients can be performed non-invasively. Since the results can be obtained instantly and in real time, these images may serve as a surrogate readout for tumor response.[189,190] Radioisotope imaging techniques, such as positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), optical imaging and fluorescence and bioluminescent imaging, have been investigated as methods to track NK cells *in vivo*. In addition to these imaging methods currently used in the clinic, new handheld endoscopic and tomographic imaging systems are also being tested.[189]

Patients with renal cell carcinoma receiving NK cell immunotherapy were monitored by SPECT imaging. NK cells were labeled with a ^{111}In radiotracer during NK cell therapy, and SPECT images provided evidence for the activity, redistribution and tracer accumulation with high sensitivity, indicating an effective immunotherapeutic approach.[191] However, many limitations for PET/SPECT still exist, including high cost, low resolution (1–2 mm), radiation exposure, tracer decay and limited ability for follow-up studies (2–3 h for the ^{18}FDG radiotracer or 4–7 days for ^{111}In). The low resolution issue may be improved by integrating the PET/SPECT images with high-resolution CT images (200–400 μm), which is currently being investigated.[189] The optical and bioluminescent imaging methods can track transferred NK cells labeled with an exogenous fluorescent dye or those containing a fluorescent protein gene, such as green fluorescent protein or luciferase. OI offers an inexpensive, fast and radiation-free way to monitor NK cells *in vivo* with high sensitivity and low background noise, but with limited tissue penetration and low resolution (2–3 mm).[192,193] The MRI method can track NK cells labeled with iron-oxide nanoparticles that produce a strong negative enhancement on T2-weighted images. This method provides readily available clinical translation, high resolution (100 μm in plane), high soft-tissue contrast, no radiation exposure and longer signal persistence. In addition, several FDA-approved iron-oxide nanoparticles are suitable for clinical use, including ferumoxides, ferumoxytol and ferucarbotran. However, MRI cell-tracking techniques also require high costs, long scan times and limited sensitivity.[189] Among these approaches, MRI and PET will likely be at the center stage for whole-body applications for humans in the future; if used in tandem in a combined clinical MRI–PET scanner, these imaging methods can compliment each other by using MRI to localize the tracked cells and PET to measure viability and other functional parameters of the tracked cells.[190,194,195] Thus, using imaging techniques to monitor NK cells during immunotherapy can serve as surrogate readouts for NK cell tumor accumulation and tumor response; additionally, monitoring NK cell immunotherapy in real time can help to detect and avoid ineffective treatment in patients who are unknowingly resistant to NK cell therapy.

CONCLUSION

NK cell-based immunotherapy holds great promise for cancer treatment. However, only modest clinical success has been achieved thus far using NK cell-based therapies in cancer patients. Progress in the field of understanding NK cell biology and function is therefore needed to assist in developing novel approaches to effectively manipulate NK cells for the ultimate benefit of treating cancer patients.

COMPETING FINANCIAL INTERESTS

The authors have declared that no competing interests exist.

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