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NK cell-based cancer immunotherapy: from basic biology to clinical application

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Natural killer (NK) cells, which recognize and kill target cells independent of antigen specificity and major histocompatibility complex (MHC) matching, play pivotal roles in immune defence against tumors. However, tumor cells often acquire the ability to escape NK cell-mediated immune surveillance. Thus, understanding mechanisms underlying regulation of NK cell phenotype and function within the tumor environment is instrumental for designing new approaches to improve the current cell-based immunotherapy. In this review, we elaborate the main biological features and molecular mechanisms of NK cells that pertain to regulation of NK cell-mediated anti-tumor activity. We further overview current clinical approaches regarding NK cell-based cancer therapy, including cytokine infusion, adoptive transfer of autologous or allogeneic NK cells, applications of chimeric antigen receptor (CAR)-expressing NK cells and adoptive transfer of memory-like NK cells. With these promising clinical outcomes and fuller understanding the basic questions raised in this review, we foresee that NK cell-based approaches may hold great potential for future cancer immunotherapy.

NK cell, cancer, cytokine infusion, adoptive transfer, immunotherapy

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Cancer is a major public health problem all over the world. In China, it is currently the second leading cause of death after heart disease, and is expected to be the foremost cause of death in the next few years. Traditional treatments for cancer include surgery, chemotherapy, and radiation therapy depending on the location of the tumor, the malignant degrees of carcinoma, and the stages of cancer development [1,2]. However, the arising resistance to drugs or radiation leads to a significant ratio of tumor recurrence. Therefore, it is crucial to develop new therapeutic strategies to eradicate drug-resistant tumor cells. Cancer immunotherapy that aims to elicit or amplify the patient's own immune response to eliminate tumor cells has recently emerged as one of such promising approaches.

The host immune response is composed of two interconnected arms: innate immunity and adaptive immunity. The innate immune system, also known as the nonspecific immune system, utilizes the germline-encoded molecular and cellular mechanisms to provide the first line of defense through immediate responses to foreign antigens. In contrast, the adaptive immune system, also known as the ac-

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quired immune system, is composed of highly specialized cellular subsets that recognize and eliminate the foreign invaders via their antigen-specific receptors, and generates immunological memory after an initial encounter to a specific pathogen, leading to a more intensive response to the same pathogen. NK cells have traditionally been recognized as innate lymphoid cells due to their rapid response to target cells without prior sensitization. Unlike T cells, NK cells are always switched on and can recognize target cells independent of antigen specificity and MHC matching, which allows anti-tumor effects without eliciting strong graft-versus-host disease (GvHD) responses. Moreover, NK cell based therapy will potentially be cheaper, more scalable and have a lower latency period from treatment decision to receipt of therapy compared to T-cell based competitors [3,4]. Therefore, approaches that utilize NK cells for cancer immunotherapy are gaining more and more attention worldwide. For example, in China, researchers attempt to suppress the recurrence of Hepatocellular carcinoma (HCC) after liver transplantation using NK Cells from sibship (ClinicalTrials.gov. NCT02399735); in France, scientists have initiated a trial to infuse haploidentical NK cells into patients bearing acute myeloid leukemia (AML) (NCT01947322); in Germany, NK cell-based adoptive immunotherapy is currently used for the treatment of patients with non-small cell lung cancer (NSCLC) (NCT02118415); in the USA, infusion of cytokine-induced memory-like NK cells into patients with AML or myeloid dysplastic syndromes (MDS) has already obtained a certain extent of success (NCT01898793); and umbilical cord blood NK cell transfer has been introduced as part of the therapy for patients with chronic lymphocytic leukemia (CLL) (NCT02280525). More clinical studies are summarized in table 1.

The present review summarizes the concepts and rationale for the clinical application of NK cells in cancer immunotherapy. We further overview strategies that can optimize NK cell cytotoxicity and alloreactivity for the improvement of therapeutic efficacy in the clinical practice.

1 NK cell biology

1.1 NK cell phenotype

In the mid-1970s, NK cells were originally identified by Kiessling as a sub-population of lymphocytes in mice that are larger in size than T and B lymphocytes and have the capacity to kill tumor cells without prior sensitization [5–7]. Since then, major advances in the understanding of NK cell biology have provided significant insights into its role in controlling various tumors and infections [8]. It is widely known that conventional NK cells originate from common lymphoid progenitor cells and further differentiate into immature/mature NK cells in bone marrow (BM) that are then distributed in peripheral lymphoid and non-lymphoid organs and tissues, including BM, lymph nodes (LN), spleen, peripheral blood, placenta, lung, liver and peritoneal cavity [9–12]. Resting NK cells that account for 10%–15% of peripheral blood lymphocytes circulate in the blood. After responding to a large array of cytokines and chemokines, they are capable of extravasation and recruitment into distinct inflamed or malignant tissues [13]. Human mature NK cells can be subdivided into two distinct major subpopulations based on cluster of differentiation 56 (CD56) expression levels, i.e. low CD56-expressing ($CD56^{dim}$) and high CD56-expressing population ($CD56^{bright}$). $CD56^{dim}$ subsets predominately reside in peripheral blood (90%–95%) and inflammatory sites, which exhibit a high cytotoxic potential after encountering target cells [14–16]. In contrast, approximately 90% NK cells in secondary lymphoid tissues belong to the $CD56^{bright}$ population that primarily exerts immunoregulatory function by producing abundant cytokines or chemokines [16,17]. Although murine NK cells do not express the CD56 receptor, the developmental status, homing properties and functional attributes of murine NK cells can be represented by four subsets according to the expression of CD11b and CD27: $CD11b^{lo}CD27^{lo}$, $CD11b^{lo}CD27^{hi}$, $CD11b^{hi}CD27^{hi}$, $CD11b^{hi}CD27^{lo}$ [18]. Further studies on human NK subsets defined by CD11b and CD27 markers have also revealed four populations with distinct maturation stages, tissue distribution patterns and functional properties [19,20]. Moreover, these studies have showed that the $CD56^{bright}$ population remains heterogenous. Thus, further understanding of distinct properties of these NK subsets at the steady state or under inflammatory/tumor settings may be instrumental for designing clinical applications of NK cells.

1.2 NK cell-mediated recognition mechanisms

A current consensus is that NK cell cytotoxicity is modulated by an intricate balance between signals emanating from surface inhibitory receptors and those from activating receptors. NK cells are quiescent when encountering normal or healthy cells that express adequate MHC molecules for NK inhibitory receptors and no or few activating ligands. NK cell cytotoxicity is otherwise triggered when target "dangerous" cells exhibiting a reduced or altered MHC outfit along with the enough activating ligands expression [21]. Three recognition models have currently been proposed: "missing-self recognition", "stress induced-self recognition" and "non-self recognition". Under stressed conditions, such as cellular transformation, down-regulated MHC molecules expression by normal cells, resulting in NK cell activation in a process called "missing-self recognition". This model is based on the fact that NK cell activity is normally held in check by self-MHC molecules that interact with a large repertoire of inhibitory NK receptors, including the heterodimeric C-type lectin-like receptor (CTLR) called CD94/NKG2A (natural killer group protein 2 family member A) [22], the *NK*-gene complex (NKC)-encoded CTLR

Table 1 Clinical trials of NK cells in cancer immunotherapy

NCT Number	Age Groups	Patients	Phases	Conditions
NCT01944982	Child|Adult	10	Phase 1 |Phase 2	Relapsed/Refractory Pediatric T Cell Lymphoblastic Leukaemia and Lymphoma
NCT01795378	Child|Adult|Senior	85	Phase 1 |Phase 2	Acute Myelogenous Leukemia|Acute Lymphoblastic Leukemia
NCT02130869	Child|Adult	36	Phase 1	Neuroblastoma|Lymphoma|High-risk Tumor
NCT02280525	Adult|Senior	44	Phase 1	Leukemia
NCT01787474	Adult	30	Phase 1 |Phase 2	Leukemia
NCT01823198	Child|Adult	72	Phase 1 |Phase 2	Leukemia
NCT02271711	Child|Adult	24	Phase 1	Brain Cancer
NCT02074657	Child|Adult	10	Phase 2	Relapsed/Refractory Pediatric Acute Leukemia
NCT01884688	Adult|Senior	20	Phase 2	Asymptomatic Multiple Myeloma
NCT02316964	Adult|Senior	10		Adult Acute Myeloid Leukemia with 11q23 (MLL) abnormalities|Adult Acute Myeloid Leukemia with Del(5q)|Adult Acute Myeloid Leukemia with Inv(16)(p13;q22)|Adult Acute Myeloid Leukemia with t(15;17)(q22;q12)|Adult Acute Myeloid Leukemia with t(16;16)(p13;q22)|Adult Acute Myeloid Leukemia with t(8;21)(q22;q22)|Recurrent Adult Acute Myeloid Leukemia|Secondary Acute Myeloid Leukemia
NCT02100891	Child|Adult|Senior	20	Phase 2	Ewing Sarcoma|Neuroblastoma|Rhabdomyosarcoma
NCT01904136	Child|Adult	45	Phase 1 |Phase 2	Leukemia|Myeloproliferative Diseases
NCT02291198	Adult|Senior	1000		Natural Killer Cell Cytokine Production
NCT01974479	Child|Adult|Senior	20	Phase 2	B-cell Acute Lymphoblastic Leukemia
NCT02118285	Adult|Senior	20	Phase 1	Ovarian Cancer|Fallopian Tube Carcinoma|Primary Peritoneal Carcinoma
NCT02259348	Child|Adult	18	Phase 2	Acute Lymphoblastic Leukemia (ALL)|Acute Myeloid Leukemia (AML)|Myeloid Sarcoma|Chronic Myelogenous Leukemia (CML)|Juvenile Myelomonocytic Leukemia (JMML)|Myelodysplastic Syndrome (MDS)|Non-Hodgkin Lymphoma (NHL)
NCT02019628	Adult|Senior	20		Family Member
NCT02118415	Adult|Senior	90	Phase 2	NSCLC Stage IIIA/B
NCT02185781	Adult|Senior	6	Phase 1	Acute Lymphoblastic Leukemia|Complete Hematologic Remission (CHR)|Persistent/Recurrent Minimal Residual Disease (MRD)
NCT02409576	Child|Adult|Senior	20	Phase 1 |Phase 2	Ewing Sarcoma|Osteosarcoma|Rhabdomyosarcoma
NCT01857934	Child|Adult	42	Phase 2	Neuroblastoma
NCT02326727	Adult|Senior	30		Colonic Neoplasms
NCT02229266	Adult|Senior	56	Phase 2	Acute Myeloid Leukemia
NCT02477787	Adult|Senior	96	Phase 2	Acute Myelogenous Leukemia
NCT02123836	Child|Adult|Senior	20	Phase 1	Acute Leukemia|Myelodysplastic Syndrome
NCT01790269	Adult	40		Relapsing-Remitting Multiple Sclerosis
NCT01807611	Child|Adult	110	Phase 2	Leukemia|Lymphoma
NCT02395822	Adult|Senior	24	Phase 2	Acute Myelogenous Leukemia
NCT02399735	Adult	18	Phase 1	Hepatocellular Carcinoma
NCT01947322	Adult	11	Phase 1 |Phase 2	Acute Myeloid Leukemia
NCT02465957	Adult|Senior	24	Phase 2	Stage IIIB Merkel Cell Carcinoma|Stage IV Merkel Cell Carcinoma
NCT02370017	Adult|Senior	68	Phase 2	Non-small Cell Lung Cancer
NCT02301065	Child|Adult	100		Hematologic Malignancies
NCT01875601	Child|Adult	51	Phase 1	Solid Tumors|Brain Tumors|Sarcoma|Pediatric Cancers|Neuroblastoma
NCT02030561	Adult|Senior	29	Phase 1 |Phase 2	Breast Cancer|Gastric Cancer
NCT01898793	Adult|Senior	24	Phase 1	Leukemia, Myeloid, Acute
NCT01807468	Child|Adult	12	Phase 2	Neuroblastoma|Ewing Sarcoma|Rhabdomyosarcoma|Osteosarcoma|Soft Tissue Sarcoma
NCT02481934	Adult|Senior	5	Phase 1	Multiple Myeloma
NCT01853358	Adult|Senior	22	Phase 1	Hematological Malignancy
NCT01841294	Adult|Senior	50	Phase 4	Colorectal Cancer

named KLRG1 (killer cell lectin-like receptor subfamily G, member 1) [23] and members of killer-immunoglobulin like (KIR) family [24]. The numbers and subtypes of the KIR receptors are different in each individual [25]. The inhibitory KIRs that recognize human leukocyte antigen (HLA) class I proteins (HLA-A, -B, and -C) contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which can recruit and activate SH2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2, leading to inhibition of NK cells. Another ITIM-containing inhibitory receptor—human CD94/NKG2A heterodimers—binds to HLA-E, a non-classical HLA class I molecule, which also induces

inhibition of NK cells [26]. In addition, KLRG1 recognizes cadherin, which is often downregulated on epithelial tumor cells during tumor progression [27].

"Stress induced-self recognition" refers to that a number of NK activating receptors recognize self-proteins, which are upregulated in transformed or infected cells. The natural killer group protein 2 Family member D (NKG2D) activating receptor is constitutively expressed in all NK cells and recognizes UL16-binding proteins and the major histocompatibility complex class I chain-related genes A/B (MICA/MICB) in humans. Ligation of NKG2D and its ligands induces phosphorylation of the adapter protein DNAX-activating protein of 10 kD (DAP10), which contains a cytoplasmic tyrosine-isoleucine-asparagine- methionine (YINM) motif that upon phosphorylation leads to recruitment and activation of the p85 subunit of phosphatidylinositol-3-kinase (PI-3K), and subsequently induces NK cell activation [28,29]. Several members of activating KIR or NKG2 family, such as killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail (KIR2DS), killer cell immunoglobulin-like receptor, three domains, short cytoplasmic tail (KIR3DS), natural killer group protein 2 family member C (NKG2C) and natural killer group protein 2 family member E (NKG2E), contain the immunoreceptor tyrosine-based activation (ITAM)-bearing adaptor DAP12 that also exhibits immunostimulatory activity once the receptors are triggered [24,30,31]. In addition, DNAM-1, NKp80 and 2B4 (CD244) bind to ligands expressed by both normal and malignant cells, which induces NK cell activation under both circumstances [32–35].

"Non-self recognition" indicates that some NK activating receptors do not recognize endogenous ligands but interact with foreign pathogen-encoded molecules. For example, NKp46 and NKp44 recognize hemagglutinins derived from influenza and sendai viruses; NKp30 recognizes pp65 tegument protein from human cytomegalovirus and 2B4 recognizes Epstein-Barr virus-infected B cells [35–38]. These pairs of receptor-ligand interaction ultimately lead to NK cell activation.

1.3 NK cell function in innate and adaptive immunity

NK cells are initially regarded as cytotoxic lymphocytes that contribute to immune defense against infections and tumor transformation (Figure 1). NK cells are able to lyse target cells without prior sensitization through three major pathways: (i) Direct cytotoxicity by release of granules containing perforins and granzymes that induce apoptosis of target cells [39,40]. (ii) Lysis of targets through apoptotic pathways induced by ligation of tumor-necrosis factor (TNF) family members expressed by NK cells, such as FasL or TNF-related apoptosis-inducing ligand (TRAIL) [41–43].

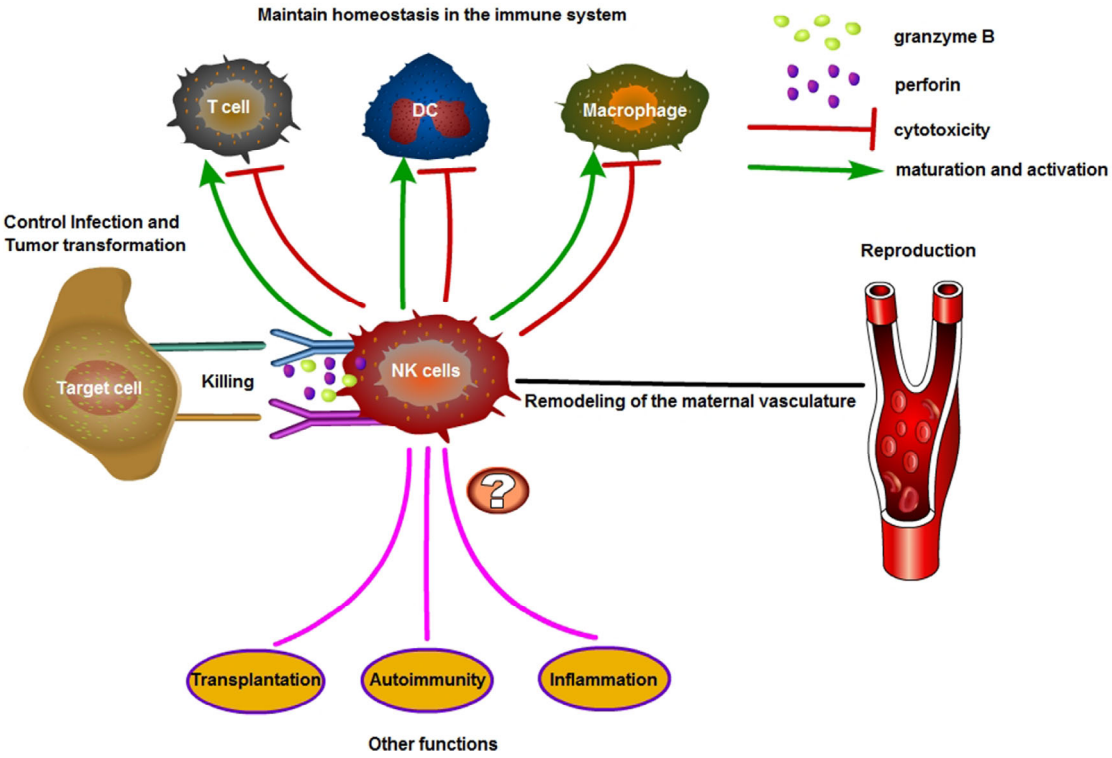

Figure 1 (color online) Functional activities of natural killer cells. The diagram presents the known and predicted roles of human NK cells in immune surveillance and regulation. NK cells can directly eliminate target cells to resolve infection and induce tumor regression. NK cells are also known to function as regulatory cells by inducing the activation, maturation or demise of other immune subsets, such as activated $CD4^+$ T cells, hyperactivated macrophages and DCs, which enables NK cells with the capacity to regulate immune homeostasis and autoimmune/inflammatory responses. In addition, NK cells may also regulate processes in reproduction and transplantation.

(iii) Lysis of targets through antibody-dependent cellular cytotoxicity (ADCC) by triggering the NK CD16 receptor, which binds to the IgG and antibody-coated targets [44].

NK cells are also known to function as regulatory cells that act as a link between the adaptive and innate immune responses and control immune homeostasis, inflammatory and autoimmune responses (Figure 1). They modulate other immune subsets by direct contact or releasing various cytokines/chemokines. NK cells have been demonstrated as the major source of IFN-γ *in vivo*, which is considered to be crucial in restricting tumor angiogenesis and shaping adaptive immunity [45–47]. IFN-γ from NK cells after stimulation with exogenous cytokines or through target cell recognition promotes the differentiation of $CD4^+$ T helper 1 subset (T_H1) and helps priming $CD8^+$ cytotoxic T-cell (CTL) response. However, NK cells can also exert negative regulatory effects on T_H cells, including T_H17 and follicular helper T cells (T_{FH}), in the context of autoimmunity [48,49]. Some NK subsets suppress T-cell responses by producing regulatory cytokines, including IL-5, IL-13, IL-10 or transforming growth factor Beta (TGF-β). NK cells also crosstalk with dendritic cells (DC) and macrophages and influence the overall immune responses [50,51]. For example, NK cells facilitate DC maturation and cytokine production, which further regulates antigen-specific T-cell responses; NK cells interact with macrophages and amplify the inflammatory response [50]. Epidemiologic reports and cellular analysis also indicate that NK cells may regulate other biological processes, including those in reproduction and transplantation (Figure 1). Future studies are required to better understand these NK cell-mediated regulatory activities.

Traditionally, NK cells are considered as innate lymphoid cells since they lack the ability to undergo somatic rearrangements that induce the expression of antigen-specific receptors. However, several recent studies demonstrate that NK cells display adaptive or "memory-like" properties both in human and mice, including long-term persistence, enhanced functional responsiveness and other phenomena that phenotypically and epigenetically distinct from their naive counterparts after pathogen infection or exposure to other stimulation. Human studies have demonstrated that infections with cytomegalovirus (CMV) [52–54], hantavirus [55], or chikungunya virus [56] result in an increased number of $NKG2C^+$ NK cells. Functionally, these $NKG2C^+$ NK cells produce more IFN-γ in response to target cells than their naive counterparts [52]. Furthermore, adoptively transferred $NKG2C^+$ NK cells from CMV-seropositive persons in recipients display enhanced effector function against a secondary CMV challenge compared to those in recipients who receive $NKG2C^+$ NK cells from CMV-seronegative individuals, suggesting that a prior sensitization of $NKG2C^+$ NK cells is necessary for the generation of memory-like responses [57].

1.4 Cross-talk between NK cells and other immune cells in cancer

The tumor initiation and progression are associated with aberrant regulation of multiple immune subsets, including T-cells, DCs and NK cells, under tumor-induced immunosuppressive conditions. A large population of tumor-infiltrating NK cells displays the immature $CD11b^{lo}CD27^{lo}$ phenotype [20]. Some of these NK cells upregulate inhibitory receptors, such as PD-1 and Tim-3, indicative of NK cell exhaustion [58]. The maturation arrest of NK cells and impaired NK cytotoxicity in patients bearing tumors are often attributed to the abnormal immune subsets that secrete various suppressive cytokines and/or enzymes. For example, tumor-associated accumulation of regulatory T-cells can produce TGF-β that downregulates the activating NKp30 and NKG2D receptors and impairs the tumouricidal activity of NK cells and the functional interactions between NK cells and DCs [59]. The bidirectional crosstalk between NK cells and DCs (as described in section 2.3) plays an important role in the induction of optimal CTL-mediated tumor-specific immune response against cancer, which has provided the rationale for the combined application of NK cells and DCs in the cancer immunotherapy. However, dysregulated NK cells in tumor microenviroment can also lead to accumulation of immature or tolerogenic DCs [60], which impairs anti-tumor CTL responses and aggravates the immunosuppression [59].

1.5 Epigenetic regulation of NK cells

Epigenetic regulation refers to functionally relevant modifications to the genome that impact gene expression, cellular phenotypic and functional traits without inducing changes in the nucleotide sequence. Such modifications include DNA methylation; and acetylation, methylation and phosphorylation of the N-terminal tails of histone proteins; as well as the reverse processes of these modifications, which can induce changes in chromatin configuration on target gene loci. Cells utilize these mechanisms to integrate genetic programs with external or environmental signals in order to establish or maintain cellular identity. Although epigenetic regulation of the differentiation and function of T- and B-cells is well-characterized, less is understood regarding its role in NK cell development and function. Vijayalakshmi and his colleagues have recently observed that the histone H2A deubiquitinase Myb-Like, SWIRM and MPN domains 1 (MYSM1) is necessary for NK cell maturation but not for NK lineage specification and commitment [61]. Most recently, our group has discovered that the histone methyltranferase Ezh2 enhances NK development, in part through repression of NKG2D through the histone H3K27me3 modification on its regulatory locus (unpublished data). Many studies have also shown that epigenetic controls of genes encoding IFNγ, perforin and

granzymes are important for NK cell function [62].

Conclusive evidence has indicated that changes in chromatin scaffold in the precursors can be passed on to daughter cells, forming the basis for memory. Although epigenetic regulation has been well illustrated in memory T-cells [63], less is understood with regards to mechanisms underlying the formation of "memory-like" NK cells. Two independent studies have recently revealed that memory-like NK subsets in HCMV (Besides Human cytomegalovirus)-infected individuals display reduced expression of many signaling proteins and transcription factors, which are associated with hypermethylation of their gene promoters [64,65]. Further understanding of the epigenetic regulation of NK cell differentiation, memory formation and functional specialization may require generation of conditional mouse models that induce NK cell-specific deletion of crucial epigenetic modifiers. Genome-wide chromatin immunoprecipitation sequencing coupled with transcriptional profiling studies may help reveal the regulatory network that determines NK cell fate and functionality.

2 NK cell-based immunotherapy in malignancy

During the tumor development and progression, many malignant cells acquire the ability either to evade from NK-cell recognition or to impair NK cell function. Several general mechanisms are suggested including inaccessibility of NK cells to the tumor due to defective vascularization, deficient expression of adhesion molecules or activating receptors on NK cells, high expression of MHC class I on tumor cells, resistance to the Fas- or perforin-mediated apoptosis and secretion of immunosuppressive factors such as IL-10 or TGF-β by tumors [1,66]. Therefore, approaches to improve cytotoxicity or restore immune system are necessary to effectively enhance cancer immunotherapy. The success of chimeric antigen receptors (CARs) T cell therapy in cases of ALL and CLL has revitalized cell-based therapies as one of the promising approaches for the treatment of malignancy. In contrast to T cells, NK cell-mediated cytotoxicity does not require the antigen presentation by self-HLA. Thus, cancer cells can be recognized by allogeneic NK cells based on ''missing-self recognition" mechanism. Moreover, NK cells are known to contribute to graft-versus-tumor (GvT) effect but do not induce graft-versus-host disease (GvHD). Based on these unique properties of NK cells, the therapeutic use of autologous or allogeneic NK cells may provide a pivotal and clinical significance in human cancer immunotherapy (Figure 2).

2.1 Cytokine-induced NK cell activation

Tumorigenesis is a delicate process, which is under the stringent surveillance of NK cells as well as other immune

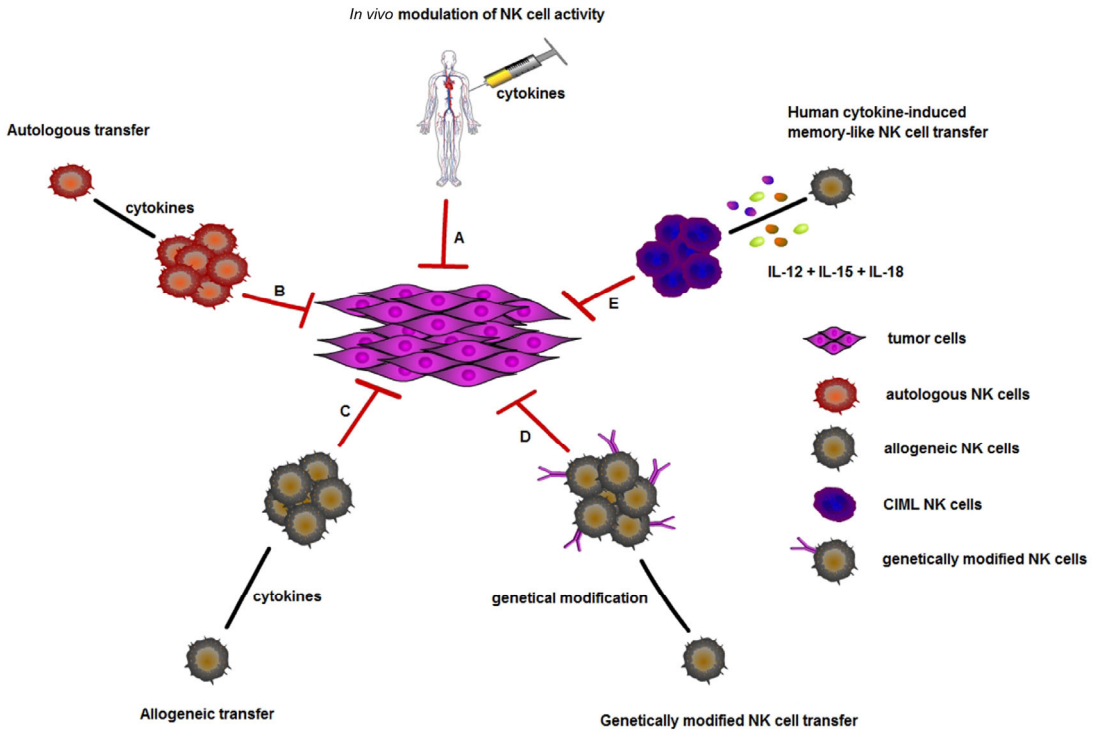

Figure 2 (color online) Schematic diagram for cancer immunotherapy by NK cells. The diagram shows an overview of current approaches to NK cell-based cancer immunotherapy. A, NK cell activity can be modulated *in vivo* by cytokine infusion. Autologous (B) or allogeneic (C) NK cells can be expanded or activated with cytokines *in vitro* prior to infusion into patients. D, NK cells can be genetically modified prior to infusion. E, Transfer of cytokine-induced memory-like (CIML) NK cells can provoke robust and effective anti-tumor responses.

subsets. During tumor progression, many cancer cells obtain the ability to escape immunosurveillance. In the early days, the therapeutic methods were focused on either improving the antitumor activity of NK cells, or promoting NK cell expansion. It is well-known that IL-2, a growth factor that induces proliferation and expansion of lymphocytes and plays key roles in immune responses, can activate NK cells and enhance NK cytotoxicity against malignant cells [67]. Many studies using animal models have demonstrated the effectiveness of IL-2 on NK cell activation and anti-tumor responses [68–81]. Robinson and Morstyn [82] has also reported that NK cells from lung cancer patients that fail to kill tumor cells, can regain the cytotoxicity against targets after activation by IL-2. Although the FDA has approved the IL-2-based therapy for renal cell carcinoma (RCC) in 1992, the clinical results are not satisfactory due to the limited anti-tumor activity of NK cells and excessive toxicity when high doses of cytokine are administered. Rosenberg et al. [83] has first reported that systemic administration of autologous lymphokine can activate killer cells and application of recombinant IL-2 (rIL-2) induces remission in patients with advanced cancer. This promising outcome has revealed a cytokine-based strategy for cancer therapy that has drawn wide attention, albeit with some observed side effects. A phase I/II trial reported that daily subcutaneous injection of IL-2 supplemented with two intravenous injections can generate peripheral blood mononuclear cells (PBMCs) with enhanced cytotoxicity against NK-resistant targets, but showed limited efficacy when compared with matched controls [84]. A similar study that uses regional arterial administration of lymphokine-activated killer cells (LAK) and low doses of rIL-2 also did not lead to satisfied clinical success [85].

Overall, data from the previous reports have showed some promising outcomes. However, activation of NK cells using high-dose IL-2 *in vivo* has significant but manageable side effects because of severe capillary leaky syndrome. Moreover, recipient regulatory T cells, which express high affinity IL-2 receptor α chain (CD25) and are more sensitive to low dose IL-2, can compete with $CD56^{bright}$ NK cells for cytokines and "space" and induce immune suppression. Thus, low-dose IL-2 treatment is not an optimum method for most candidates. Based on these studies, some groups combined IL-2 and other NK cell activators to improve the therapeutic efficacy and safety. Hellstrand et al. [86] administered IL-2 together with histamine to 22 AML patients, and showed a good clinical outcome. In addition, 21/22 complete remission (CR) patients could treat themselves with histamine at home throughout the trial. Other researchers combined IL-2 and IFN-α to induce a significant increase in peripheral blood NK cells [87–89]. Improved expansion and/or activation of various effector cells, including NK cells, were also reported after combined use of IL-2, IFN-α and GM-CSF [90]. In a different strategy from the above researchers, Bachanora et al. [91] utilized IL-2 diphtheria toxin (IL2DT), a recombinant cytotoxic fusion protein composed of the amino acid sequences of diphtheria toxin and truncated amino acid sequences of IL-2, to deplete Treg cells, and therefore improve donor NK cell expansion. In this study, they performed high-dose cyclophosphamide and fludarabine (Hi-Cy/Flu) lymphodepleting chemotherapy and adoptive transfer of allogeneic NK cells to treat refractory AML patients, and among these candidates, fifteen patients also received IL2DT. These patients exhibited improved complete remission rates at day 28 (53% vs 21%) and disease-free survival at 6 months (33% vs 5%). These data demonstrate that depletion of host Tregs, which exhibit sensitivity to low dose IL-2, is associated with improved *in vivo* donor NK-cell expansion and remission induction.

2.2 Adoptive transfer of autologous NK cells

Based on a series of investigations with successful experimental animal models, various researchers hold the view that adoptive transfer of autologous NK cells is safe and promising [81,92–94]. Ten metastatic RCC patients received a combination of high-dose IL-2 and lymphokine-activated natural killer (LANAK) cell infusions. Partially effective clinical outcomes were observed with four complete responses and two additional patients whose tumor mass was further reduced [95]. Ishikawa et al. [96] injected autologous NK cells together with IFN-β into 9 patients with recurrent malignant glioma and showed that NK cell therapy was safe and partially effective. However, Burns et al. [84] treated non-Hodgkin's lymphoma and RCC patients with *ex vivo* IL-2–activated autologous NK cells followed by daily subcutaneous IL-2 injection, which demonstrated no improvement in the disease status. Studies from other groups involved with metastatic breast cancer, colon cancer and lung cancer also failed to observe that transfer of autologous NK cells was consistently effective in patients [97–100]. After analyzing the clinical data mentioned above, Alici et al. [101] believed that failure to recover the NK cytotoxicity was due to insufficient NK cell activation. They further compared the effectiveness of long-term with short-term activation of NK cells from multiple myeloma (MM) patients and found that long-term activation can significantly enhance NK cytotoxicity against autologous tumor cells. However, Miller and his colleague conceived that many tumors expressing high levels of HLA class I receptor and/or low levels of ligands for activating receptors are largely resistant to the NK-cell-mediated lysis. Thus, they are currently focusing on therapies using allogenic NK cells or other strategies to prevent such NK cell resistance (described below).

2.3 Adoptive transfer of allogeneic NK cells

Previous clinical observations and basic science research

have greatly improved our understanding of functionality of NK cells within hosts bearing different MHC genotypes. Autologous NK cells are unable to kill host tumor cells due to inhibition by self-MHC or by tumor-induced immunosuppression. Functional responsiveness of mature NK cells can be reconfigured when they encounter an altered MHC environment [102], suggesting that donor NK cells can be "re-educated" by host HLA. These "re-educated" donor NK cells can acquire cytotoxicity against host tumor cells without causing GvHD. Quite a few cases using allogeneic T-cell-depleted hematopoietic cell transplantation from haploidentical donors in patients with AML have showed that NK cells can effectively control AML relapse and improve engraftment without causing GvHD [103–105], supporting that mature haploidentical NK cells alone may provide a pivotal role in achieving anti-tumor responses. Based on these important findings, a series of clinical trials showed convincing evidence that allogeneic NK cells display great therapeutic efficacy in controlling human malignancies. Koehl et al. [106] developed a protocol for clinical-use of highly enriched and IL-2-stimulated NK cells and infused these haploidentical NK cells to 3 pediatric leukemia patients. No GvHD response was observed and NK cell therapy was well tolerated. The similar efficacy was observed in trials by Passweg who infused haploidentical NK cells into 5 leukemia candidates after haploidentical hematopoietic stem cell transplantation (HSCT) [107]. Miller and his colleague performed a series of clinical trials using adoptively transferred haploidentical allogeneic NK cells to treat AML patients, including pediatric AML patients and older AML patients. They first reported that 5 out of 19 poor prognosis AML patients achieved complete remission [108]. They further reported that ten pediatric patients have a disease free survival rate of 100% at a median of 2 years follow-up after infused mismatched NK cells and high doses of IL-2 [109]. In addition, a clinical trial of older AML patients reported that 3 of 6 patients in CR remained disease-free at 34, 32, and 18 mon, respectively [110]. Besides hematopoietic-derived tumors, strategies using adoptively transferred human-mismatched (haploidentical) allogeneic NK cells also provide effectiveness on solid cancers, including HCC, NSCLC, colorectal cancer ovarian and breast cancer [21,108,111,112].

2.4 Applications of chimeric antigen receptor (CAR)-expressing NK cells

Chimeric antigen receptors (CARs) are engineered receptors that consist of an external antibody-derived targeting domain fused with one or more intracellular signaling domains. The external domain is responsible for targeting antigens either overexpressed or expressed uniquely on the surface of tumor cells, whereas the intracellular domain is designed to transmit the activating signal. When expressed by lymphocytes, CARs are able to enhance specific targeting and activation towards various malignancies. Based on the revolutionary effect of CAR-T cell therapy in cases of ALL and CLL, cell-based therapies are becoming increasingly promising for the treatment of cancer. The development of CAR-T cell-based therapies is accelerating the clinical emergence of tumor immunotherapy, but T cell-related transplantations must be restricted to autologous cells because T cells are major cause of graft-versus-host-disease (GvHD). In contrast, NK cells are not responsible for GvHD as they do not require HLA matching, and can be used as allogeneic effector cells [113], which makes NK-based therapies a viable alternative for adoptive immunotherapy. Transplanted allogeneic donor NK cells mediate reduction of GvHD by inhibiting activated, alloreactive T cells while retaining graft-versus-tumor (GvT) effects [110]. It has also been reported that the expression of CARs in NK cells may allow these cells to more effectively kill solid tumors that are often resistant to NK cell-mediated cytotoxicity [108,112]. As such, CAR-expressing NK cells have therefore gained clinical significance providing a targeted and allogeneic cell population that universally treats refractory malignancies.

To date, numerous pre-clinical investigations have used CAR-modified peripheral blood NK cells (PBNK) or NK-92 cells to direct against HER-2, CD244, CD19, CD20 and so on (reviewed in ref. [3,114]). Moreover, authorities have approved two clinical studies using CAR-expressing NK cells for the treatment of B-lineage ALL. One of the clinical studies (NCT00995137) is aimed to identify the maximum tolerance dose of genetically modified NK cells for patients with relapsed or refractory B-lineage ALL at St. Jude Children's Research Hospital. In this clinical study, allogeneic NK cells were first expanded by co-culture with irradiated K562 cells that were modified to express membrane bound IL-15 and 41BB ligand (K562-mb15-41BBL)-overexpression of these proteins promotes selective growth of NK cells. The *in vitro* expanded NK cells were then transduced with vectors encoding a signaling receptor that binds to CD19 which is only expressed on B-lineage ALL cells. A similar study (NCT01974479) performed by the National University Hospital in Singapore investigated the persistence and phenotype of redirected NK cells in participants with residual B-lineage ALL after chemotherapy. In that study, donor NK cells were activated and expanded by K562-mb15-41BBL cell line combined with IL-2 and then transduced with vectors encoding a signaling receptor targeting CD19.

Although extensive pre-clinical studies have led to successful clinical phase I/II studies with CAR-T cells, different safety concerns persist for such long-lived highly-proliferative effector cells. These concerns include on-target/off-tumor effects, induction of GvHD, tumor lysis syndrome and cytotoxicity to normal tissues due to selectivity restrictions of the chosen target antigen [115–117]. To eliminate such side effect, various "suicide systems" have

been developed to improve the safety of cell therapy [118] by limiting the amount of time that CAR-engineered effector cells circulate in patients. To date, no such systems have been introduced for mature allogeneic CAR-engineered NK cells, as they are expected to induce anti-tumor effects and disappear after a few days [111]. Theoretically, however, such systems may be required in some NK cell-based therapies, as different subsets, such as umbilical cord NK cells, and immature NK cells can circulate for weeks to months, and as a result, may exhibit notable side effects if used in chimera-based therapies. The absolute necessity of a suicide switch in CAR-NK cell therapy still requires further investigation.

2.5 Adoptive transfer of memory-like NK cells

Immunological memory is a phenomenon that describes the elicitation of more robust reactions by immune cells to certain antigens that have been previously recognized. These cells, called memory cells, are phenotypically and epigenetically distinct from their naive counterparts and can be maintained in the human body for a few months, or even decades, to provide protection from the secondary invasion of the same pathogen. NK cells, which are classified as innate immune cells, have been considered incapable of undergoing somatic rearrangements to confer antigen specificity and forming memory. In contrast to this long-held paradigm, a subset of NK cells have recently been shown to be long-lived and mediate adaptive or ‘‘memory-like’’ responses to certain antigens in both mice and human, including viruses and haptens [119–122]. This subset of memory cells exhibits enhanced expansion and effector function following a secondary antigen challenge. Many people have been infected with HCMV (50%–100% depending on geographical location), and remain infected for life (9). Interestingly, many groups have reported that HCMV reactivation after allogeneic hematopoietic cell transplantation (allo-HCT) imposes a long lasting effect on relapses in patients with AML. Using multivariate analysis of time-dependent covariate functions for grades II to IV acute and chronic GvHD, Elmaagacli et al. [123] first confirmed that the reduced relapse risk is involved with early HCMV reactivation. Further analysis of a large cohort of patients receiving allo-HCT has identified that HCMV reactivation is associated with a reduced risk of early relapse in patients with AML but not in patients with ALL, MDS, lymphoma and chronic myeloid leukemia (CML) [124]. Recently, Shivaprasad et al. [125] has reported that the impact of HCMV reactivation on relapse after allo-HCT in AML patients is influenced by conditioning regimens. HCMV reactivation can significantly reduce the risk of relapse in patients who received myeloablative (MA) cohort but not in those with reduced intensity conditioning (RIC) regimens. HCMV reactivation probably induces expansion of $NKG2C^+$ memory-like NK cells, as described above (section 2.3). However, it remains unknown whether this subset of “memory-like” NK cells directly contributes to the reduced relapse in patients with AML. A better understanding of “memory-like” NK cell biology would be important for further clinical utility.

Besides HCMV-Induced “memory-like” NK cells, Yokoyama and colleagues have found that activation of NK cells overnight *in vitro* with IL-12, IL-18, and IL-15 also leads to generation of NK cells with memory-like properties in mice [126] as well as in human [127]. Based on a series of basic investigations and preclinical studies [126–131], a phase I trial (NCT01898793) at Washington University School of Medicine was conducted to study the side effects and best dose of activated NK cells in treating patients with relapses or refractory AML and MDS. In this study, patients first received chemotherapy to inhibit cancer cell growth before infused with cytokine-induced memory-like (CIML) NK cells to induce anti-tumor responses.

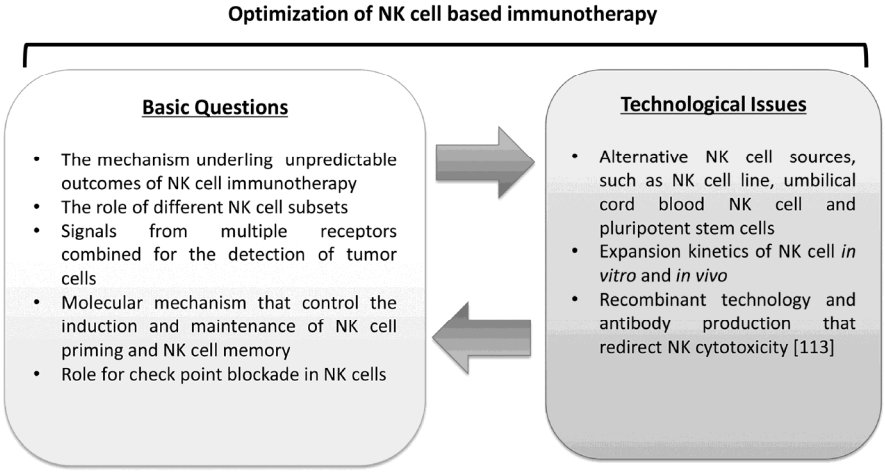

Figure 3 (color online) Future perspectives to optimize NK cell-based immunotherapy.

3 Outlook

Current efforts in immunotherapy have focused mainly on boosting T-cell responses to tumors. More effective approaches depend on mobilization of effector cells that belong to both the adaptive (T-cell) as well as the innate immune system to induce cooperative antitumor activity. The clinical studies reviewed the above provided ample evidence that NK cell-based cancer immunotherapy is both feasible and promising. However, there remain many questions and challenges in this field (Figure 3). For example, the out- comes of NK cell immunotherapy are still unpredictable, especially in solid tumors. Moreover, an improved study in the role of different subsets, receptor acquisition, memory- like responses and *in vitro*/*in vivo* expansion kinetics of NK cells will be pivotal for designing strategies to enhance the clinical efficacy. Despite the questions that are yet unanswered, it is reasonable to believe that NK cell-based immunotherapy may revolutionize the prospects for cancer therapy.

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