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Chapter 5

Clinical Applications of Natural Killer Cells

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Abstract

Natural killer (NK) cells are an essential component of the innate immune system, and they play a crucial role in immunity against malignancies. Recent advances in our understanding of NK cell biology have paved the way for new therapeutic strategies based on NK cells for the treatment of various cancers. In this section, we will focus on NK cell immunotherapy, including the enhancement of antibody-dependent cellular cytotoxicity, the manipulation of receptor-mediated activation, inclusion criteria based on killer cell immunoglobulin-like receptor (KIR) ligand mismatches, and adoptive immunotherapy with *ex vivo* expanded chimeric antigen receptor (CAR)-engineered or engager-modified NK cells. In contrast to T lymphocytes, donor NK cells do not attack any recipient tissues based on allogeneic human leukocyte antigens (HLAs), suggesting that NK-mediated antitumor effects may be achieved without the risk of graft-versus-host disease (GvHD). Despite reports of clinical efficacy, the application of NK cell immunotherapy is limited. Developing strategies for manipulating NK cell products, host factors, and tumor targets are thus current subjects of diligent study. Research into the biology of NK cells has indicated that NK cell immunotherapy has the potential to become the forefront of cancer immunotherapy in the coming years.

Keywords: NK cell, KIRs, immunotherapy, HSC transplantation, genetic modification

1. Introduction

Natural killer (NK) cells have been used in clinical studies in order to treat various malignancies. Missing-self is one of the mechanisms of the NK cell response that works by the detection of the loss of autologous major histocompatibility complex (MHC) class I expression.

We will mention four elements of NK cells in this chapter: (1) mechanisms of NK cells; (2) activation of NK cells; (3) inclusion criteria based on KIR ligand mismatches; and (4) genetic modifications *ex vivo*.

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NK cells, which are thought to have emerged much later than B cells and T cells based on the evolutionary convergence of variable receptors, rarely cause autoimmune diseases. We will introduce the applicability of the NK cell to cancer treatment.

2. Activation of NK cells

NK cell-based immunotherapy has been explored for decades. Several animal experiments indicated the potential efficacy of NK cells in cancer treatment [1–3]. However, *ex vivo* NK cell expansion techniques have been insufficient regarding the numbers of cells, purity and antitumor activity to use in clinical settings. NK cells comprise only a minor population (i.e., 5–15% of peripheral lymphocytes), and only a small number of NK cells is isolated after a typical apheresis procedure. For example, approx. $77.8 \pm 14.4 \times 10^9$/L (range 62.7–$95.9 \times 10^9$/L) of leukapheretic products are the result of a single apheresis of peripheral blood in a normal adult human with a mean percentage of lymphocytes of 59.8% ± 6.1 (range 53.9–66.4%), and subsequently $5–10 \times 10^8$ NK cells can be obtained [4]. On the other hand, at least 2×10^7/kg or 6×10^6–6.5×10^9/body NK cells are required for each effective injection with multiple administrations [5, 6]. In addition, the reported engraftment period of NK cells was 2–189 days (median 10 days), which showed no correlation with the number of NK cells administered [7]. The NK cell infusion should thus be repeated in order to maintain a sufficient number of NK cells meeting clinical requirements, but this would be a burden on patients.

Scientists have been working to develop various methods for proliferating NK cells *ex vivo* with high cytotoxicity and high purity. Several studies used an anti-CD3 antibody (clone: OKT3) in the first few days of culture for the activation of autologous T cells to help the NK cell expansion, subsequently producing high numbers of undesirable T cells or NKT cells in the final product [8–10]. However, particularly in haploidentical NK cell transplantation, T cells must be excluded prior to the infusion in order to prevent graft versus host disease (GvHD). Other studies removed $CD3^+$ cells by magnetic beads with or without CD56-positive selection at the beginning of culture [11–13].

To acquire highly purified and expanded NK cells, an initial efficient depletion (<1%) of $CD3^+$ cells and a relatively long-term culture (over 12 days) seem to be essential [12, 13]. Because only a minor fraction of circulating NK cells is reactive to target cells (tumor cells) *in vitro*, primary NK cells show insufficient cytotoxicity [14]. Various types of stimulation have thus been reported to enable NK cells to achieve their full effector potential, such as interleukin (IL)-15 produced by dendritic cells (DCs) [15] or macrophages [16], IL-2 [17], IL-12 [18], IL-18 [19] and IL-21 [20]. Currently, the additional cytokines used in the cultivation of NK cells include IL-2, IL-15, and IL-21. IL-2, IL-15, and IL-21 share the receptor subunits IL-2/15Rβ and common γ chain [21] on the NK cell surface and have a synergetic effect. Use of IL-2 combined with IL-15 for cultivation leads to good viability and good proliferation of NK cells [22]. IL-2 is also important for NK cell infiltration and killing, and IL-15 is important for both NK cell maturation and survival [21]. IL-2 and IL-15 induce the expression of KIRs and activating receptors (NKG2D and NKp44) on NK cell surface [23]. IL-21 modifies the expression of killer

cell immunoglobulin-like receptors (KIRs) and NKp44 by reducing expression of DAP-12, subsequently promote cell maturation, the ability of killing and survival [22, 23]. Several experiments used feeder cells to provide essential stimulation for NK cell cultivation through cytokine production or cell-to-cell contact [20, 23, 24]. The various cytokines and feeder cells used in some clinical trials are mentioned in a later section of this article.

2.1. Killer cell immunoglobulin-like receptors (KIRs)

NK cells express KIRs, most of which are inhibitory (partially activating) receptors that recognized MHC class I molecules. In the 1980s, KIRs were first described as explaining the NK cell-mediated rejection of allogeneic bone marrow transplants from a homozygous donor to a hemizygous host in lymphoma and in F1-hybrid anti-parental resistance in a rodent model [25]. In these situations, the graft fails to express at least one MHC class I allele of the host, and NK cells are highly capable of identifying the difference, thus causing rejection. This can be explained by the concept that NK cells have inhibitory receptors and begin to attack target cells if they do not express ligands that interact with the specific inhibitory receptors. This phenomenon was termed the "missing-self hypothesis", which is now accepted as one of the complex target recognition mechanisms of NK cells.

2.1.1. Regulation of NK cell activity

The activation of NK cells is regulated by various receptors including KIRs, CD94–NKG2 family, leukocyte immunoglobulin-like receptors (LILRs), natural cytotoxicity receptors (NCRs), and FcγRIIIa (CD16) [26–28]. Among these receptors, KIRs, CD94/NKG2A heterodimers, and LILRs belong to the large family of inhibitory receptors of MHC class I, mediating NK cell function by signaling through intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) [29]. Each NK cell has a threshold of activation through a balance of total stimulation between inhibitory and activating signals [30] (**Figure 1**). In other words, NK cells selectively kill target cells that down-regulate MHC class I molecules and/or up-regulate other activating ligands [28] such as MHC class I chain (MIC)-related antigens MICA, MICB and UL-16 binding protein (ULBP). MICA, MICB, ULBPs are ligands of NKG2D homodimer, which belong to C-type lectin receptor NKG2 family expressed on the surface of NK cells and $CD8^+$ T-lymphocytes. NKG2A/CD94 and NKG2B/CD94 heterodimers transmit inhibitory signals, while NKG2C/CD94, NKG2E/CD94, NKG2H/CD94 heterodimers and NKG2D homodimer are activating receptors.

2.1.2. Genetics of KIRs

The KIR gene family includes 14 loci (KIR2DL1, KIR2DL2/3, KIR2DL4, KIR2DL5A/B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S1, KIR3DL2, KIR3DL3 and two pseudogenes, KIR2DP1 and KIR3DP1) [31]as shown in **Table 1**. These loci are located on chromosome 19q13.4, which is known as the leukocyte receptor cluster (LRC); each haplotype has 9–15 KIR genes in a row [32]. Different NK cells within individuals each express a subset of the available KIR repertoire, leading to an allelic polymorphism of KIRs. Based on studies of KIR genotype variation, two major KIR haplotype groups termed the groups 'A' and 'B' are defined [33].

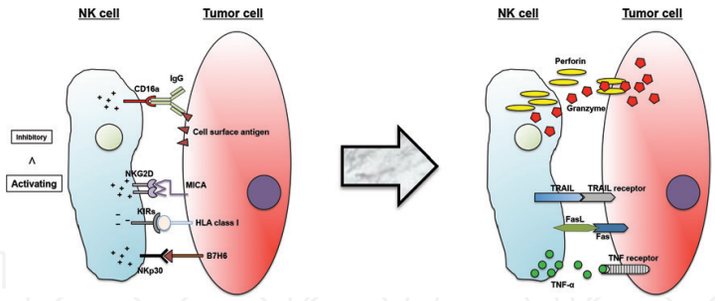

Figure 1. The mechanisms of cytotoxicity by NK cells.

Inhibitory	Ligands	Ligand missing	Notes
KIR2DL1	HLA-C2 group (Cw2, C*0307,Cw4, Cw5, Cw6, C*0707, C*0709, C*1204, C*1205, Cw15, C*1602, Cw17, Cw18)	HLA-C1/C1	
KIR2DL2	HLA-C1 group (Cw1, Cw3, Cw7, Cw8, Cw12, Cw13, Cw14, C*1507, C*1601/4)	HLA-C2/C2(except for C*02, *05) and HLA-B46, B73 negative	
	HLA-B46, B73		
	HLA-C2(C*02, C*05)(weak interaction)		
KIR2DL3	HLA-C1 group (Cw1, Cw3, Cw7, Cw8, Cw12, Cw13, Cw14, C*1507, C*1601/4)	HLA-C2/C2 and HLA-B46, B73 negative	
	HLA-B46, B73		
KIR3DL1	HLA-Bw4 epitope(including HLA-A23, A24, A32)	HLA-Bw6/Bw6 and HLA-A23/24/32 negative	Expression level: *01502, *020 > *001, *007 >*004
KIR3DL2	HLA-A03, A11(+ EBNA peptide),HLA-B27 dimer	HLA-A03, A11 negative, free of EBV	*001 is homo-dimer A03/A11 could not promote NK cell Licensing
KIR2DL5B	Unknown		
KIR2DL5T	Unknown		
Activating	Ligands	Ligand missing	Notes
KIR2DS4	HLA-A*1102,(A*1101, C*0304, C*0501)	HLA-A*1102 negative	
KIR2DS1	HLA-C2	HLA-C1/C1	
KIR2DS2	Unknown(HLA-C1?)		
KIR3DS1	Unknown(HLA-B*2705?)		
KIR2DS3/5	Unknown		
KIR2DL4	HLA-G		
Others	Ligands	Ligand missing	Notes
KIR2DP1	–	–	Pseudogene
KIR3DP1	-	-	Pseudogene

Table 1. Human KIRs.

Each haplotype is separated into two regions: the centromeric half (Cen) and the telomeric half (Tel). Cen and Tel motifs can be divided into Cen-A, Cen-B and Tel-A, Tel-B by the KIR genes they contain. Haplotype A is a combination of Cen-A and Tel-A, which consists of mainly inhibitory KIRs (KIR3DL3, 2DL3, 2DL1, 3DL1, 2DS4, 3DL2 and two pseudogenes). Other combinations are termed Haplotype B (such as Cen-A and Tel-B or Cen-B and Tel-B), composed of a large variation of genes characterized by the presence of more activating KIRs. All individuals can be categorized according to their haplotype: A/A, which is homozygous for group A haplotypes, or B/x, which contains either one (A/B) or two (B/B homozygotes) group B haplotypes[34]. As a consequence of this genetic variation, several studies report that donor-derived NK cells can mediate the graft-versus-leukemia (GVL) effect or even the graft-versus-tumor (GVT) effect after allogeneic hematopoietic cell transplantation (HCT) [34–36]. These are the results of a KIR-ligand mismatch, the details of which are described later in this article.

2.1.3. KIRs subtypes

KIRs are type I transmembrane glycoproteins expressed on the surface of NK cells, composed of two (2D) or three (3D) extracellular Ig-like domains and a cytoplasmic short (activating) or long (inhibitory) tail [31]. The length of the intracytoplasmic part determines the function; for example, receptors with long cytoplasmic tails with one or two ITIMs that bind to phosphatase SHP-1, 2 allow the transduction of inhibitory signals through its dephosphorylation. In contrast, receptors with short cytoplasmic tails possess a positively charged residue (lysine) in the transmembrane domain that enables it to associate with adaptor proteins including DAP12 and process the immunoreceptor tyrosine-based activation motifs (ITAMs) [29]. There is one exception: KIR2DL4, with a long cytoplasmic tail, binds to the activation motif of FcεRIγ and thus seems to transmit the activating signal [31].

MHC class I molecules are well-known ligands for KIRs, but HLA-C molecules, in particular, are the main ligands contributing greatly to NK cell activity. Polymorphisms in amino acids at positions 77 and 80 of HLA-C show specificity for its target KIRs. Group 1 HLA-C ligands (C1) include allele-encoded molecules with serine and asparagine (Ser77 and Asn80), whereas group 2 ligands (C2) are characterized by allele-encoded molecules with asparagine and lysine (Asn77 and Lys80). C1 epitopes bind specifically to KIR2DL2/3, and C2 epitopes are ligands for KIR2DL1 [37]. However, it was shown that KIR2DL2/3 might also bind to certain HLA-C2 epitopes (C*0501, C*0202, C*0401) and some HLA-B epitopes (HLA-B*4601, B*7301) with very low affinity [38].

Among the inhibitory KIRs, KIR2DL1 results in a stronger inhibition compared to the KIR 2DL2/3 [38]. The third inhibitory KIR is KIR3DL1, which binds to HLA-Bw4 epitopes and a subset of HLA-A epitopes (A*23, A*24, and A*32). All HLA-B have either the Bw4 or Bw6 epitope, but only the Bw4 epitope is a ligand for KIRs [39]. KIR3DL2 is a framework gene, and it recognizes HLA-A*03 and HLA-A*11 with a low level of inhibition [40]. Although haplotype A includes only a single activating KIR (2DL4), at least two to five activating KIR genes (2DS1, 2DS2, 2DS3, 2DS5 and 3DS1) are subject to haplotype B. However, KIR2DS1 alone is now confirmed to have matched ligand HLA-C2 with lower avidity compared to KIR2DL1 [41].

2.2. Death Ligands

NK cells express members of the tumor necrosis factor (TNF) superfamily, the so-called death ligands. The cognate of the receptor on target cells by these ligands on NK cells — which include TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL) and TNF-like weak inducer of apoptosis (TWEAK) — results in classical caspase-dependent apoptosis [42, 43]. In the TRAIL/TRAIL receptor system, at least five receptors have been identified and two of them, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), contain cytoplasmic death domains and are able to transduce an apoptotic signal [44]. Other TRAIL receptors — TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) and a soluble receptor called osteoprotegerin (OPG: TRAIL-R5) — lack a death domain, but they are dedicated as decoy receptors to regulate TRAIL-mediated cell death [45]. In target cell expressing TRAIL receptors, the ligation of TRAIL can lead to the activation of caspase-8 and subsequently caspase-3 to induce apoptosis [46, 47]. TRAIL is up-regulated after the stimulation of interferon-gamma (IFN-γ) *in vitro* [48, 49] and also *in vivo*, which demonstrates that TRAIL is required for the IFN-γ-mediated prevention of tumors [50].

FasL is expressed on activated NK cells and cytotoxic T lymphocytes (CTLs) [42]. Although it is known that FasL is expressed on NK cells at a low level, significant amounts are stored intracellularly [51]. An activating signal of rodent NK1.1 up-regulated the expression of FasL [52]. After FasL has bound to Fas, the Fas associated with two specific proteins, Fas-associated death domain (FADD) and caspase-8, to form the death-inducing signal complex (DISC). Fas is expressed on various tissues, but the molecule is downregulated in cancers during its progression [53]. NK cells are capable of directly inducing Fas expression on tumor cells via IFN-γ secretion, and NK cells show cytotoxicity to tumor cells expressing Fas [45].

2.3. Perforin and granzyme

Cytolytic killing using perforin and granzyme is a major mechanism in the elimination of infected cells and tumor cells by NK cells. NK cells contain cytoplasmic granules including perforin (a membrane-disrupting protein) and granzyme (a family of serine proteases). Once NK cells recognize target cells, they form an immunological synapse, and the secretory granules fuse with the presynaptic membrane and release perforin and granzyme into the synaptic cleft. Released perforin provides transmembrane pores on the target cell and enables granzyme to diffuse into the cell. Granzyme then initiates the apoptosis of the target cells, and the NK cells detach from the dying cells and can interact with other target cells to accomplish serial killing [54]. The release of such granules stored in the NK cells is dependent on the polarization of both microtubules and actin filaments in the cytoskeleton. The increase of intracellular calcium concentration triggered by a positive balance of activating and inhibitory signals initiate the rapid move of microtubule-organizing center (MTOC) in the cytoplasm towards the target cell, and then cytotoxic granules migrate along the MTOC [54]. Granules fuse with the presynaptic membrane, subsequently, the lytic granules can be released at the NK cell-target cell interface[55] (**Figure 2**).

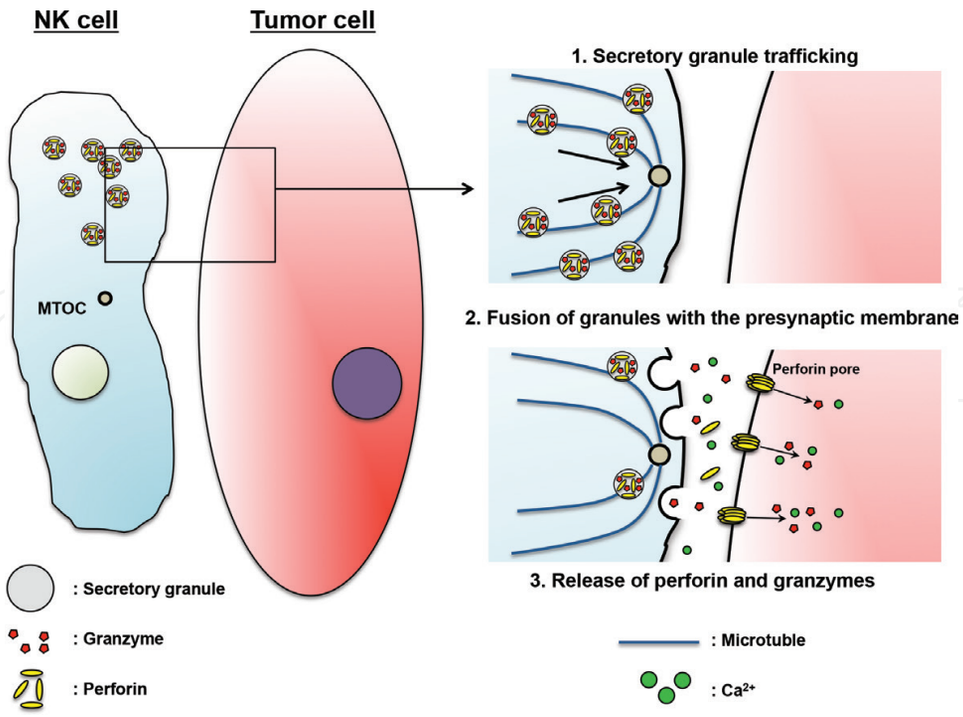

Figure 2. Interaction of an NK cell with a target cell.

3. NK cells for clinical use

3.1. Sources of NK cells

There are several options for sources of NK cell therapy, including peripheral blood mononuclear cells (PBMCs), umbilical cord blood (UCB), bone marrow (BM), cell lines, human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs).

3.1.1. Peripheral blood mononuclear cells (PBMCs)

PBMCs are the most common source of NK cells, and PBMCs can be collected by apheresis or a specific gravity centrifugal method (e.g., Ficoll separation). However, the percentage of NK cells in PBMCs is low (5–20%), and because there is a limit on the number of cells that can be recovered from a donor by lymphocyte apheresis, it is not possible that a sufficient number of NK cells for killing the target can remain in the recipient [7]. That is, in order to stay in the recipient's body until the target cells are killed, it is necessary to collect peripheral blood frequently, which is a heavy burden on the patient.

Numerous methods for amplification cultures of NK cells have been reported. Stem cell mobilization is a process whereby stem cells ($CD34^+$cells) are stimulated out of the bone marrow space (e.g., the hip bones and the chest bone) into the bloodstream, and granulocyte-colony stimulating factor (G-CSF) is widely used as a drug for harvesting peripheral blood stem cells from patients or healthy people. Another drug, Plerixafor [56], a CXCR4

antagonist approved in 2008, has an excellent mobilization effect in combination with G-CSF [57, 58].

An increase in a number of CD34-positive cells harvested by mobilization reduces the number of apheresis sessions required for cell therapy, which may reduce the burden on patients. When allogeneic hematopoietic stem cells are used, GVHD in the acute phase, which is thought to be caused by T-cell contamination, exists as a problem to be overcome. There is also a report that the induction of myeloid-derived suppressor cells (MDSCs) by the administration of G-CSF reduces the frequency of GVHD. It is important to note that MDSCs suppress T-cell function through an arginine depletion by arginase-1, the production of reactive oxygen species, and the induction of Treg cells [59, 60].

3.1.2. Bone marrow (BM)

Bone marrow aspiration removes a small amount of bone marrow fluid through a needle put into a bone under general anesthesia. Compared to apheresis, it is very rarely used as a starting material because of its high invasiveness to donors.

3.1.3. Umbilical cord blood (CB)

Hematopoietic reconstitution for Fanconi anemia treatment using cord blood was first performed in 1988 by Gluckman et al. [61]. Since then it has been widely accepted as a source of hematopoietic stem cells when autologous blood is not recommended or readily available. Cord blood (CB) can be donated to public CB banks for use by any patient worldwide for whom it is stored for potential autologous or family use. The majority of CB products used today are for hematopoietic stem cell transplantation and are accessed from public banks.

Cord blood presents no harm to the donor at the time of collection, and the frozen storage of collected samples is possible. In addition, cord blood of various blood types is classified and preserved in umbilical cord blood banks. When umbilical cord blood is used as the source of therapeutic NK cells, it is highly tissue-compatible according to the patient's blood type and accompanying transplantation. It is easy to select blood type-specific umbilical cord blood, with little side effects. In addition, it is possible to minimize the risk of GVHD from the absence of T cells [62–64]. As with PBMCs, a growth culture is essential because the amount of NK cells that can be obtained from a single CB sample is not an amount that can expect to provide a therapeutic effect.

3.1.4. Embryonic stem cells and induced pluripotent stem cells (ES/iPS)

Another possible source of NK cells is human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). A differentiation/expansion culture from hESCs and iPSCs to NK cells is a regimen requiring more sophisticated technology compared to the PBMCs and UCB described above. The differentiation process of human iPSCs is divided into three stages of "maintenance amplification", "structure construction", and "differentiation induction". The culturing step is to generate $CD34^+$ hematopoietic progenitor cells from hESCs and iPSCs and then differentiate these cells into NK cells.

A preclinical demonstration that NK cells can be separated from the sources of these pluripotent stem cells has been described [65, 66]. However, that demonstration enabled efficient proliferation by using mouse stromal cells as feeder cells, and the involvement of heterologous cells may limit clinical application. As with UCB, advances in the development of safe, effective and standardized clinical-grade manufacturing protocols will provide opportunities to develop ready-made personalization and immunogenic cell therapy.

3.1.5. Cell lines

The cell lines that have been derived from NK cells are NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, and IMC-1 cells [67], and several research groups are exploring the possibilities of using these cell lines for therapeutic applications. The primary advantage of an NK cell line is that it is "ready to use", and it is possible to establish comprehensive standardization and characterization of the cell source by using the master cell bank. The cell therapy product is thus considered to be an attractive merit in manufacturing. Moreover, a more homogeneous population is obtained compared to that from peripheral blood, and its homogeneous character is another advantage when performing a genetic modification operation.

NK cell line has been applied to genetic modification technology for expressing intracellular IL-2 for the forced expression of CD16, natural cytotoxicity receptor (NCR), chimeric antigen receptor (CAR) and NK cell activation [68, 69]. The most widely clinically used cell line is the NK-92 cell line, which is cytotoxic to a wide range of malignant cells [70, 71]. NK-92 cells express the receptor but hardly express KIR, NKp44 or CD16. NantKWest (Culver City, CA, USA) conducts clinical trials using NK-92 cells (Neukoplast™) and has completed a Phase 1 study (U. S. National Clinical Trial [NCT] #00900809 and NCT #00990717). Moreover, the company has begun phase 1 and phase 2 trials of haNK (high-affinity NK cells) [72] engineered to express CD16 (NCT #02465957 and NCT #03027128). In addition, as another attempt, the development of CAR-TNK expressing CD7 or CD33 has been advanced (NCT #02944162 and NCT #02742727).

3.2. Manufacturing method

The number of NK cells contained in a collectable amount of UCB or peripheral blood is not enough to achieve a clinical therapeutic effect. A long-term culture method is necessary to overcome this problem. For starting material, T cells and/or B cells are removed with magnetic beads to increase the purity of NK cells. This is also to prevent the proliferation of T cells caused by IL-2 during the culture period of NK cells and to avoid lower purity of the final product [8, 73].

In addition to the important cytokine IL-2, there are IL-15, which is necessary for both the maturation and survival of NK cells [21]. IL-2 and IL-15 share the same receptor component IL-2/15Rβ and a common γ chain, and they are used in a culture method without the use of feeder cells [74, 75]. IL-21 [76], a member of the IL-2 cytokine family, is a potent immunostimulatory cytokine that shows diverse regulatory effects on NK cells, T cells and B cells [77, 78] and also has the effect of enhancing rituximab-mediated antibody-dependent cell mediated cytotoxicity (ADCC) of mantle cell lymphoma [79]. In addition, there is a culture method

using feeder cells to efficiently expand NK cells *ex vivo*. As feeder cells, monocytes, irradiated PBMCs, the K562 cell line and a genetically modified cell line are used.

For example, there are systems using a co-culture with NK cells and monocytes [80], with CB $CD34^+$ cells and bone marrow stromal cells [81], or K562 cells transfected with IL-21 [82]. There is also a completely closed culture using Epstein-Barr virus-transformed lymphoblastoid cell lines [83]. Thus, the use of feeder cells is an important method for securing a number of cells that can be expected to have a therapeutic effect. However, because of the problem of infectious disease risk presented by the use of an allogeneic feeder, the regulatory hurdles in the manufacture of pharmaceutical products are high [84].

To overcome this problem, Yonemitsu et al. reported a method of culturing highly activated NK cells with ≥90% purity from PBMCs in a completely closed and feeder-free system under the good manufacturing practices (GMP) [13]. As another feeder-free culture method, Spanholtz and colleagues reported a culture method that achieved amplification efficiency and high purity of 10,000 times or more in 6 weeks from UCB $CD34^+$ hematopoietic stem cell (HSCs), using a closed system process based on GMP [85]. Knorr and colleagues reported differentiation induction from $CD34^+$ hematopoietic progenitor cells produced under feeder-free conditions to cytotoxic NK cells [86].

3.2.1. NK cell-based immunotherapy: autologous cells

Clinical trials using autologous NK cells have been performed targeting solid tumors such as colorectal cancer, non-small cell lung cancer, melanoma, kidney cancer and esophageal cancer [87–89]. In general, autologous NK cell therapy is safe without side effects such as GvHD [87], but its therapeutic effect is limited to some cancer types [74]. An activation culture with IL-2 and OKT3 or Hsp70 has been reported to be able to efficiently induce the proliferation of NK cells [74], in particular, the retronectin culture method of Sakamoto et al. showed a high amplification efficiency (about 4720-fold) [89].

3.2.2. NK cell-based immunotherapy: allogeneic cells

Allogeneic NK cell products are used for the treatment of malignant tumors such as leukemia, renal cell carcinoma, colorectal cancer, and lymphoma. The major risk of allogeneic NK cell transplantation is the onset of GvHD. Measures against GvHD include the use of immunosuppressive agents, injection of high-purity NK cells by CD3 depletion, and the selection of donors consistent with the host HLA [74, 75]. In the case of haploidentical donors and recipients, to avoid GvHD, it is necessary to strictly perform T-cell depletion. In many studies, $CD56^+$ is enriched after the removal of $CD3^+$ T cells [17, 90, 91]. In cases of an allogeneic type, HLA typing and confirmation of KIR by flow cytometry are carried out, particularly in order to select the optimum donor. For details on the selection criteria, please refer to a later section of this article.

3.2.3. Synergistic effect: antibody drugs

An antibody drug is, in short, a medicine that functions based on the specificity with which an antibody recognizes an antigen. The characteristics of antibody drugs are high specificity (low

toxicity) and high stability *in vivo*. The antibody binds only to the target antigen, and not to any other, which leads to the intended medicinal effect with only rare unexpected side effects. Antibodies are present at a stable level in the blood, and antibody medicines can also be detected at a stable level in the blood for a long period after administration, and they can exert their medicinal effects over a long term. More than 50 antibody drugs have been approved in Japan, the U.S. and Europe. Target diseases include cancer, rheumatoid arthritis and psoriasis, but most of the targets are cancers. One of the action mechanisms of antibody drugs used to treat cancer is ADCC, in which NK cell plays a central role. **Table 2** shows FDA-approved antibodies for ADCC to treat cancer.

ADCC mediated by NK cells begins with the recognition of antibodies bound to target cells. NK cells express two Fc receptors, CD16a (FcγRIIIa) [92] and CD32c (FcγRIIc) [93]. These Fc receptors recognize and bind to IgG1 and IgG3 and have a high affinity for IgG3 [94]. NK cells that recognize the antibody on the target cells transmit their signals intracellularly and kill the cells.

The signaling of human CD16 is mediated via FcεRIγ, CD3ζ, or FcεRIγ-CD3ζ heterodimer. These molecules contain an ITAM and are phosphorylated when the antibody binds to CD16 [42]. CD32c (FcγRIIc) contains an ITAM-like sequence in the cytoplasmic domain (which is

Drug name	Trade name	Type	Target	Cancers	Approved year
Rituximab	Rituxan MabThera	Chimeric IgG1κ	CD20	Non-Hodgkin)s B-cell lymphomas Chronic lymphocytic leukemia	1997
Trastuzumab	Herceptin	Humanized IgG1κ	HER2	Adenocarcinoma of the stomach or gastroesophageal junction Breast cancer	1998
Alemtuzumab	Campath	Humanized IgG1κ	CD52	B-cell chronic lymphocytic leukemia	2001
Cetuximab	Erbitux	Chimeric IgG1κ	EGFR	Colorectal cancer Head and neck cancer	2004
Ofatumumab	Arzerra	Human IgG1κ	CD20	Chronic lymphocytic leukemia	2009
Pertuzumab	Perjeta	Humanized IgG1κ	HER2	Breast cancer	2012
Obinutuzumab	Gazyva	Humanized IgG1κ	CD20	Chronic lymphocytic leukemia Follicular lymphoma	2013
Dinutuximab	Unituxin	Chimeric IgG1κ	GD2	Neuroblastoma	2015
Daratumumab	Darzalex	Humanized IgG1κ	CD38	Multiple myeloma	2015
Elotuzumab	Empliciti	Humanized IgG1κ	SLAMF7	Multiple myeloma	2015

Table 2. FDA-approved antibodies for ADCC to treat cancer.

similar to that of FcγRIIa), suggesting that it transmits a signal via SRC-SYK (the SRC family of kinases and spleen tyrosine kinase [SYK]) signaling pathways [95]. However, the expression of CD32c was less than half of that of NK cells [96, 97]. Many studies focusing on CD16 have thus been conducted.

Several genetic polymorphisms of CD16 exist. Among them, the amino acid at position 158 has been shown to be important for the strength of the affinity for antibodies. The affinity depends on whether the amino acid at position 158 is phenylalanine or valine, and the valine type (158 V) has a higher affinity for the Fc of IgG. A number of reports have indicated that differences in the affinity for antibodies are correlated with therapeutic effects, and many studies have analyzed the clinical responsiveness of this gene polymorphism and antibody therapy. Cartron et al. examined the effects of rituximab treatment for non-Hodgkin's lymphoma, and they reported a higher objective response rate in CD16 (158 V) homozygous patients compared to CD16 (158F) carrier patients [98]. Wang et al. analyzed the outcomes of rituximab treatment for Follicular Lymphoma and reported significantly more likely progression-free survival at 2 years in CD16 (158 V) homozygous patients compared to CD16 (158F) carrier patients, at 45 and 14%, respectively [99].

These results suggest that the affinity of CD16 for antibodies correlates with the therapeutic effect. Thus, focusing on CD16, the modification of NK cells has been attempted. Binyamin et al. reported that introducing CD16 (158 V) into NK-92 cells not expressing CD16 improved the cytotoxicity against B-cell lymphoma with rituximab [100]. Carlsten et al. reported high ADCC activity of cultured NK cells from healthy donors with CD16 (158F/F) and transduced CD16 (158 V) mRNA by electroporation against rituximab-coated $CD20^+$ B-cell lymphoma cells [101].

As described above, since NK cells mediate antibody-dependent cytotoxic activity via Fc receptors, compatibility with antibody drugs targeting ADCC is desirable. As a strategy to further augment the antitumor effect, a plausible strategy is to enhance the affinity between the Fc receptor and the antibody. Low-molecular-weight compounds that are able to inhibit the shedding of CD16 have been reported. It is known that CD16 is cleaved by a protease such as a disintegrin and a metalloprotease 17 (ADAM17) when cells are activated [102], and thus in order to exert more sustained and enhanced ADCC activity, a method of inhibiting the cleavage of CD16 on NK cells may be important. In fact, inhibitors of ADAM17 enhanced the activity of NK cells [102, 103]. Another method to inhibit the cleavage of CD16 is a genetic modification. The substitution of the serine residue at position 197 in CD16 by a proline prevents the cleavage of CD16 on NK cells [104]. It may be possible to promote the antitumor effect by using a CD16 mutant.

4. Optimized selection of patients/donors

4.1. Clinical outcomes: on the KIR ligand mismatch model

Research reports focusing on the KIR of NK cells and the HLA of tumor cells for the purpose of treating leukemia have been drawing attention since the 2000's. In the report by Ruggeri et al.

published in *Science* in 2002, the cases in which HLA-ABC recognizable by NK cells is present in a donor but not in a recipient were defined as "KIR mismatch". It was speculated that in such cases, NK cells not receiving suppression signals from the donor HLA attack the cells of the recipient. When Ruggeri et al. started this research, it was not known that NK cells are a heterogeneous population expressing multiple inhibitory receptors, or that the specific antibodies that can be used for analysis are inadequate. Ruggeri and colleagues, therefore, examined only the HLA of donors and recipients without examining the KIR, and they analyzed patients divided into the two groups of HLA matched and mismatched transplantations. Although it may not have been an appropriate observation based on the current knowledge, at that time, the analysis was based on the following basic research data.

Ruggeri et al. examined whether CD56-positive NK cell clones collected from donors could attack leukemia cells of the recipient. They found that NK cell clones that attack leukemia cells exist, and their high frequency (≥2%) correlates well with the cases in which the donor's and recipient's KIR ligand (HLA) do not match in the GVH direction. Ruggeri et al. analyzed 35 acute lymphoblastic leukemia (ALL) and 57 acute myeloid leukemia (AML) patients who had allogeneic hematopoietic stem cell (HSC) transplantations at their institution. The result was a breakthrough result in 20 AML patients who were not KIR ligand mismatches in the GVH direction, with 0% relapse after transplantation [105]. This report was the subject of much attention, and then it was decided to conduct data analyses in medical institutions around the world seeking reproducibility of the results.

The analysis by Ruggeri et al. was by the method known as the "KIR ligand mismatch model", which examines only the donor and recipient HLA-ABC without checking the KIR. This method determines whether a KIR match or mismatch is determined. Researchers all over the world could thus easily use this method. However, most of the analysis results obtained in this way conflicted with the report by Ruggeri et al.

As an example, we will describe the analysis results obtained from the study of the Japan Marrow Donor Program (JMDP). In 2007, Morishima et al. reported the results of their analysis of 1790 patients who underwent an allogeneic bone marrow transplantation in accord with the KIR ligand mismatch model [106]. In conventional domestic allogeneic HSC transplantation, the criterion for donor selection is that the HLA-AB and -DR matched, and by this criterion 534 of the 1790 cases were HLA-C mismatched. As a result, the overall survival rate, the recurrence rate, and the incidence of GvHD were poor in the group in which the KIR ligand HLA-C was mismatched in the GVH direction.

The finding that allogeneic HSC transplantation has no merit in KIR mismatches is common in the report about cases from the U.S. National Marrow Donor Program (NMDP) and the European group for blood and marrow transplantation (EBMT) [107]. Comparing Ruggeri's 2002 report with Morishima's 2007 report, the biggest difference was the cell source for transplantations, the former being $CD34^+$ cell transplantation from haploidentical donors and the latter being conventional bone marrow transplantation. In addition, Ruggeri et al. used antithymocyte globulin (ATG) in all cases. A re-analysis of the registered JMDP cases reconfirmed that the transplantation performance of the HLA-C matched group was also good and that of the group with the HLA-C mismatch was poor [108].

Interestingly, the disadvantage of this HLA-C mismatch was not observed in the ATG administration group. In other words, it is suggested that not NK cell dysfunction but T cells induced by HLA-C mismatch had exacerbated the transplantation results. T cell-depleted grafting and ATG might avoid the T-cell response. There are certain reports that HLA-C mismatches are recognized by cytotoxic T lymphocytes (CTLs). There are two papers that reported a total of nine CTL clones from two patients who developed GvHD [109, 110]. Interestingly, all of the targets of the nine CTL clones were HLA-C, which was different from the HLA-C of the recipient. There is no doubt that the difference in HLA-C could be a target for CTL.

4.2. Receptor ligand model or missing ligand model

Although the KIR ligand mismatch model investigated only ligand (i.e., HLA) differences, eventually some researchers noted that donors' KIR should also be examined and analysed by different approaches were developed. In 2004, Leung et al. analyzed 36 children who received selective $CD34^+$ cell transplantation from haploidentical donors [111]. They first examined the presence or absence of inhibitory KIR in donor cells by flow cytometry (i.e., a phenotype assay). It was speculated that the recipient would have a KIR mismatch if the recipient did not have a ligand for KIR (suppressing type) of the donor. This is the "receptor-ligand model".

For example, when the donor has KIR2DL1 with HLA-C1/C1, when the recipient is HLA-C1/C1, it is considered a KIR match in the KIR ligand mismatch model, but it is a ligand mismatch of KIR2DL1 in the receptor-ligand model. This is a very frequent combination for Japanese (HLA-C1/C1 = 85%, $KIR2DL1^+$ = 99%). Conversely, if the donor does not have KIR2DL1 with HLA-C1/C2, if the recipient is HLA-C1/C1, it is considered a KIR mismatch in the KIR ligand mismatch model, but it is considered a KIR match in the receptor-ligand model. However, since there are few $KIR2DL1^-$ and HLA-C1/C2 among Japanese, this combination is extremely rare.

An analysis conducted to determine which model can predict recurrence more accurately revealed that the receptor-ligand model is superior [111]. Hsu et al. also reported their analysis based on the receptor-ligand model [112]. They investigated 178 cases of T cell-depleted transplantation and found that the positive rates of KIR2DL1, KIR2DL2/3, and KIR3DL1 were 93, 99, and 92%, respectively, in the donor gene, and that although there were 112 cases (63%) of the 178 patients who were expected to exhibit the GVL effect, there was a significant difference in the recurrence rate, and the disease-free survival rate/overall survival rate was also good in the AML and myelodysplastic syndrome (MDS) patients.

Hsu et al. presented a new idea in 2006 [113]. It is hard to examine the donor's KIR by genetic testing, but donors usually have genes of KIR2DL1, KIR2DL2/3, and KIR3DL1. Therefore, HLA-C1/C1 homozygous, HLA-C2/C2 homo and HLA-Bw6/Bw6 homo patients identified by examining only the recipient's HLA may experience the GVL effect from donor NK cells. This method is called the "missing ligand model". It was designed for transplantation performance analyses, not for donor selection. Hsu et al. analyzed 1770 patients who had allogeneic (unrelated) T-cell-depleted transplantation. A University of Minnesota study examined 2062

cases from the U.S. NMDP [114], and based on the idea that NK cells have a GVL effect, they excluded 568 Japanese subjects or analyzed only partial diseases with good prognosis. Nonetheless, that paper is very interesting as it shows the difference in the distribution of KIR ligand between Japanese and Westerners and the difference in the recurrence rate of disease [113]. We can see how Japanese are 'biased' toward HLA-C1.

Although few cases of GvHD in Japanese have been reported, the recurrence rate of HLA-C1/C1 patients is not much different from that of Westerners. In addition, among the Japanese, the recurrence rate is extremely small when the recipients are HLA-C2/C2 homozygous, which is a minority. However, caution is required in the interpretation of the results, as there are only three cases in which the recipient had HLA-C2/C2. Further detailed analysis is expected in the future.

4.3. Therapeutic effects of allogeneic transplantation of NK cells, and their limitations

In 2007, Ruggeri et al. reported the results of 122 cases of AML [115], which included 57 cases [105] and the other 55 cases. In the KIR ligand mismatch model, there were 51 cases of KIR mismatch in the GVH direction and 61 cases of matches, and the number of remission cases at the time of transplantation and the cases which were refractory to treatment were approximately 50% of the cases. The results showed that although the relapse rate was significantly lower in the cases of remission at the time of transplantation in the KIR mismatch in the GVH direction (3 vs. 47%, $p = 0.003$), in the cases in which the treatment was refractory, no treatment effect was observed (32 vs. 37%, p = NS).

Is it true that NK cells exert a GVL effect? A direct answer to that question has been reported as a KIR-mismatch NK cell transplantation in recent years. Ten children with AML [7] and 13 adults with AML [116] underwent the transplantation of CD56-positive NK cells harvested from haploidentical donors. The collected NK cells were considered KIR mismatches, and in both studies, only NK cells were transplanted after pretreatment (fludarabine and cyclophosphamide). The number of transplanted NK cells was 29×10^6/kg for the children and 2.7×10^6/kg for the adults; the numbers of transplanted T cells were $<1 \times 10^3$/kg and $< 1 \times 10^5$/kg, respectively. After transplantation, 1–10 million units of IL-2 were administered every other day, 6 times.

As a result, transient engraftment of donor NK cells was observed in all cases. Donor-derived NK cells occupied 7% (1–30%) of the peripheral blood lymphocytes at the peak of day 14 in the children's report, and at day 28, donor-derived NK cells have been detected from three of 10 patients [7]. All of the child patients had AML in complete remission, and all cases did not recur. Among the adult patients, whose leukemia was worse, one of five patients with clear recurrence showed transient remission, and two patients with genetic recurrence AML were remitted [116]. From the above results, it was demonstrated that NK cells exert a sufficient antitumor effect if the residual tumor is relatively small. However, it is not yet clear whether the therapeutic effect is proportional to the number of NK cells administered. It should be noted that even though transplanted CD3-positive cells are limited to $\leq 1 \times 10^3$/kg, there was no case of GvHD onset in either group.

4.4. Selection of KIR in allogeneic hematopoietic stem cell transplant donors

The target diseases in which the therapeutic effect by NK cells is confirmed in analyses of various clinical tests are mostly limited to AML. Clinical trials using conventional NK cells often do not even target other diseases. There are reports that NK cells do not show a therapeutic effect against ALL because of lymphoid cells highly MHC class I, and therefore, the inhibitory signal is strong [117]. In addition, KIR ligand mismatch can lead to GvHD by CTLs as described above. In order to avoid this, ingenuity such as umbilical cord blood transplantation or the use of ATG may be necessary.

4.4.1. Donor selection based on inhibitory receptors

There is a reason to expect the GVL effect in donor NK cells not receiving a KIR signal from the recipient HLA. The main inhibitory receptors are KIR2DL1, KIR2DL2/3, KIR3DL1, and KIR3DL2. There is no evidence that the effects of these four inhibitory receptors are identical. Particularly with regard to KIR3DL2, there are reports that the KIR-positive NK cells are not even licensed, even if the recipient has HLA-A3 or A11 (KIR3DL2 ligands) [118]. Most NK cell transplantations have been expected to provide a therapeutic effect due to missing self of KIR2DL1 or KIR2DL2/3, and the number of transplantations in which a therapeutic effect by missing self of KIR3DL1 have been expected is very low. There is no transplantation in which the therapeutic effect by missing self of KIR3DL2 alone is expected.

For example, if the recipient is HLA-C1/C1 if the KIR ligand mismatch model is used, an HLA-C1/C2 or HLA-C2/C2 donor would be selected. Even if transplantation is assessed using the receptor-ligand model, the donor's KIR is often unknown. However, in Japanese, most (approx. 99%) donors KIR2DL1 can be considered positive. If so, in the receptor-ligand model, it seems that any donor could be chosen, but NK cells derived from an HLA-C1/C1 donor may show a lower GVL effect. When the recipient is HLA-Bw6/Bw6, it must first be confirmed that all of the HLA-A23, -24 and -32 are negative, and then an HLA-Bw4-positive donor should be selected. However, since 7% of Japanese are negative for KIR3DL1 gene, it must be confirmed that KIR3DL1 is positive for the donor by flow cytometry, or that the genotype is either KIR3DL1*01502 or KIR3DL1*020.

4.4.2. Donor selection based on haplotypes

It was reported that donors should be chosen for haplotype B when considering the activating receptors [34]. In that study, Cooley et al. analyzed 448 AML cases of NMDP, and they observed that the 3-year overall survival rate when the donor was haplotype BX was 31%, significantly higher than the rate of 20% when it was haplotype AA ($p < 0.01$). In addition, the recurrence decreases the most in cases that the donor had Cen-B/Cen-B, and it is reported that Tel-B also leads to a reduction of recurrence and improvement of prognosis [119].

Based on the data from three groups in the United States (Memphis, Sloan Kettering, Minnesota), Leung et al. advocated a donor selection algorithm for NK cells [120]; in the transplantation of T cells containing bone marrow or peripheral blood stem cells, donors should be HLA matches, and donors with KIR ligand mismatch should be avoided. Conversely, in

T cell-depleted transplantation or umbilical cord blood transplantation, a donor for a KIR ligand mismatch should be chosen. It is also recommended that a donor with KIR that can attack the recipient's HLA (possibly KIR haplotype B) be selected. In Japan as well, if KIR haplotype testing of the donor banks becomes possible in the future, or if the NK cell preparation is put to clinical use, it will be possible to test this algorithm described by Leung et al.

4.4.3. Donor selection based on activating receptor

KIR2DS1 is activated by HLA-C2. This means that if the recipient is positive for HLA-C2 and the donor is KIR2DS1-positive (Tel-B = haplotype BX), KIR2DS1-positive NK cells will be activated and will kill the recipient's tumor cells. In actual transplantation, when NK cells were cultured *in vitro* to examine the antitumor effect on leukemic cells of patients, KIR2DS1-positive NK cells killed tumor cells of recipients with HLA-C2 [121]. An analysis of the KIR gene of AML donors and recipients (1277 cases) from the U. S. NMDP and the Center for International blood and marrow transplant research (CIBMTR) provided large-scale proof using actual transplantations, and the report was published in *The New England Journal of Medicine* [122]. The analysis revealed that recurrence was significantly reduced when the donor had KIR2DS1 (26.5 vs. 32.5% (KIR2DS1-negative), $p = 0.02$). However, this effect was canceled when the donor was HLA-C2/C2. This is thought to be a disarming phenomenon, and it can be explained as follows: the HLA-C2/C2-derived KIR2DS1-positive clone is inactivated.

In that report [122], the proportion of donors with the KIR2DS1 gene was 33%. In the report by Yabe et al. [108], the KIR2DS1 gene-positive rate was 38%. Since Yabe et al. focused only on recipients of HLA-C2/C2, they did not analyze KIR2DS1-positive donors because the number of cases was too small. In addition, it was reported that when the donors had KIR3DS1, although a decrease in the recurrence rate was not observed, the mortality rate decreased slightly [122]. Yabe et al. also analyzed leukemia patients and reported that transplantation from KIR3DS1 donors reduced the rate of acute GvHD [123]. It was also reported that the likelihood of acute GvHD increases when the donor is haplotype AA [124, 125]. The mechanisms underlying KIR/HLA interactions remain unclear, but these reports may be a reference for donor selection.

4.5. Immune checkpoint inhibitors

4.5.1. Checkpoint of NK cells

Checkpoint inhibitors are an extremely promising approach among immunotherapies. Treatment with anti-CTLA4 or anti-PD-1 antibody restored the T-cell activity in cancer patients and resulted in tumor regression in several patients. A combination of both of the checkpoint inhibitors anti-CTLA4 and anti-PD-1 could further enhance therapeutic benefits [126, 127]. It has been shown that NK cells from patients with multiple myeloma and renal cancer express PD-1, the signal of which reduce the cytolytic activities of NK cells [128, 129]. Treatment using patient-derived PD-1^+ NK cells with the anti-PD-1 antibody (pidilizumab, CT-011) increased the NK cell-mediated killing of autologous cancer cells *in vitro* [128]. The therapeutic benefit of activating PD-1^+ NK cells in cancer patients is currently not well understood, and the major therapeutic effect is certainly due to the re-activation of exhausted T cells.

4.5.2. Combination with a checkpoint inhibitor: expansion of the therapeutic spectrum

A loss or down-regulation of HLA class I antigens in tumor cells has been frequently observed in a variety of human malignancies, and this represents an important cancer-immune escape mechanism [130–134]. Using a panel of monoclonal antibodies on tumor tissue sections, these loss or down-regulation has been found in 60–90% of tumors [135–140]. Early studies using immunohistological analyses of different tumors showed a very low frequency of allelic loss. However, with the arrival of other techniques, such as studies of microsatellites to detect the loss of heterozygosity (LOH) on chromosome 6, it has been shown that LOH (haplotype loss) is the most frequent alteration of HLA class I expression [139, 141–144]. This alteration is caused by various defects in the HLA genomic region (i.e., the short arm of chromosome 6, 6p21), including chromosomal dysfunction, mitotic recombination, and genetic conversion.

The nature of the antigens that allow the immune system to distinguish cancer cells from non-cancer cells has long remained obscure. Recent technological innovations have made it possible to dissect the immune response to patient-specific neoantigens that arise as a consequence of tumor-specific mutations, and emerging data suggest that the recognition of such neoantigens is a major factor in the activity of clinical immunotherapies. These observations indicate that the neoantigen load may form a biomarker in cancer immunotherapy and provide an incentive for the development of novel therapeutic approaches that selectively enhance T-cell reactivity against this class of antigens.

If there is a neoantigen that can be a target of CTL and the patient has MHC on which the antigen is presented, and if the MHC is not lost from the tumor cells, treatments using CTL as an effector (e.g., checkpoint inhibitors) may be effective. NK cells that preferentially kill tumor cells whose expression of MHC has decreased by MHC non-restriction are expected to have a synergistic effect with a checkpoint inhibitor.

5. Future directions

5.1. Genetic modification–Gene transfer to NK cells

In order to genetically modify NK cells, efficient methods for gene transduction into NK cells are necessary. To date, such methods include viral transduction, electroporation, and nucleofection [145]. Gene transfer to not only NK cell lines such as NK-92, but also primary NK cells has been conducted. Although various gene transfer efficiencies have been described, their transduction efficiency into NK cells is generally not high. Mainly retroviral vectors and lentiviral vectors are used for gene transfer. Since a retrovirus vector cannot transduce genes into non-dividing cells, such a vector is suitable for use with NK cell lines such as NK-92 cells. When a retrovirus vector is used for primary NK cells, it is necessary to amplify the NK cells, and the transduction timing is important.

In contrast, lentiviral vectors are capable of gene transfer into both dividing and non-dividing cells. The lentiviral vectors RD114, 10A1, GALV, and VSV-G are used for the envelope of the

viral vector, and RD114 and VSV-G seem to be suitable. However, it has been reported that viral vectors are recognized by antiviral mechanisms such as intracellular pattern recognition receptors, and apoptosis is induced [146]. The introduction efficiency was therefore not high.

Efforts are underway to improve the transduction efficiency. For example, by using a cytokine combination (e.g., IL-2 + IL-15 or IL-2 + IL-21), the transduction efficiency into NK cells by VSV-G pseudotyped lentiviral vector was improved by approx. fivefold compared to single cytokine-stimulated NK cells [146]. In addition, inhibitors of intracellular antiviral responses were evaluated, and the results indicated that BX795 (an inhibitor of the TBK1/IKKe complex) improved the transduction efficiency by approx. 10-fold [146]. Guven et al. reported transduction into 75.4% of NK cells after 21 days of culture by a two-round transduction with the GALV-pseudotyped retroviral vector [147]. Further improvement of the transduction efficiency into NK cells by using viral vectors is desired.

In non-viral gene transfer, mainly electroporation has been studied. Electroporation is a method of physically pulling a minute hole in a cell membrane by applying an electric pulse to a cell suspension and sending the nucleic acids into the cells. The transfer of genes into NK cells using this method has been performed. High transfection efficiency and a high survival rate have been reported in both NK cell lines and primary NK cells. Much higher transfection efficiency was achieved using mRNA compared to using DNA [148–152]. Introduction by nucleofection has also been tried, but the efficiency was not high [153, 154].

In light of these reports, the transfection of mRNA by electroporation is considered to be an efficient method from the viewpoint of gene transfer into NK cells. Shimasaki and colleagues reported that NK cells transfected with mRNA encoding CD19-specific CAR (anti-CD19-4-1BB-CD3ζ) by electroporation showed enhanced cytotoxicity for tumors in animal models [154]. On the other hand, the transfection of mRNA can obtain transient gene expression. Further research is required to determine whether a treatment effect can be expected. When persistent gene expression is the goal, gene transfer by retroviral or lentiviral vectors is suitable.

It is necessary to select a suitable gene transfer method for the purpose of treatment. In any case, it is desirable to develop more efficient, simple, highly reproducible and clinically applicable gene transfer methods for NK cells.

5.2. Improved persistence of NK cells *in vivo*

As a strategy for the genetic modification of NK cells to improve their survival *in vivo*, methods for transducing cytokines such as IL-2 and IL-15 into NK cells have been reported, since it was demonstrated that a local administration of IL-2 resulted in enhanced functioning of NK cells [155, 156]. IL-15 has already been used in patients with metastatic melanoma and metastatic renal cell carcinoma, and it has been reported to induce the proliferation and clinical response of NK cells. Following these findings, attempts to introduce IL-2 and IL-15 genes into NK cells were reported. Nagashima et al. showed that the NK-92 cell line transduced with IL-2 genes with a retroviral vector propagated for >5 months irrespective of IL-2 and showed higher antitumor activity than the parental cell line *in vitro* and *in vivo* [157]. Imamura

et al. showed that the transfection of membrane-bound IL-15 into human PBMC-derived NK cells using a retroviral vector resulted in the cells' survival for 2 months without the addition of cytokines *in vitro*, and that the cytotoxicity of the transduced NK cells was enhanced; moreover, the antitumor activity was observed in a mouse model [158].

Sahm et al. transduced IL-15 gene into NK-92 cells and observed cell proliferation in the absence of cytokines. They also showed that the co-transduction of an EpCAM-specific chimeric antigen receptor and IL-15 into NK-92 cells enhanced the specific and efficient cytotoxic activity [159]. Jiang et al. reported high cytotoxic activity of a human NK cell line (NKL) transduced with IL-15 *in vitro* against human hepatocellular carcinoma; the transduced NKL suppressed tumor growth and prolonged survival in human hepatocellular carcinoma-transplanted model mice [160]. These reports suggested that NK cells transduced IL-2 or IL-15 can proliferate sustainably *in vitro* and *in vivo*, which resulted in improved antitumor activity.

5.3. Improvement of homing

It was reported that NK cells expressed different chemokine receptors depending on the activating state [161, 162]. Proper homing to the tumor tissue is an important factor in eliciting the antitumor activity of NK cells. Carlsten et al. showed that *ex vivo* expanded NK cells derived from PBMCs transfected with CCR7 mRNA using electroporation migrated significantly to CCL19, a ligand of CCR7 [163]. Sonamshi et al. reported that NK cells transferred CCR7 protein from feeder cells using trogocytosis, but not genetic manipulation, promoted the migration to CCL19 and CCL21 *in vitro*, and that the NK cells were transferred to lymph nodes in a mouse model [101]. There are few reports of genetic modification targeting homing receptors, but further progress in this field is expected.

5.4. Improvement of tumor-specific cytotoxic activity

As an approach to enhance specificity to tumor cells, a technique using a chimeric antigen receptor (CAR) should be mentioned. CAR is a chimeric protein composed of a single-chain antibody (the antigen-specific binding site) in which a light chain and a heavy chain derived from a monoclonal antibody recognize a tumor cell surface antigen, a transmembrane domain, and an intracellular signal domain.

In the first-generation CARs, the intracytoplasmic signal domain is composed of only the CD3ζchain. In addition to CD3ζ, the second-generation CARs have another T cell costimulatory signal domain (CD28, 4–1BB, OX40, etc.), and the activation signal is efficiently transmitted. In the third-generation CARs, two or more costimulatory signal domains are inserted. CAR-introduced T cells have already been used clinically, and excellent results have been obtained in some cases [164–167]. One of the problems with CAR-T cell therapy is a serious side effect. CAR-T cells attack not only target cells but also non-target normal cells. For example, CD-19 CAR-T cells kill not only tumor cells but also normal B cells, which cause B-cell deficiency [168]. In HER2-specific CAR therapy for colorectal cancer with lung metastasis, death due to a pulmonary complication accompanied by high cytokinemia has been reported [169].

NK cells are an attractive alternative to T cells, as NK cells have the following advantages. HLA matching is not necessary, and NK cells are used as allogenic cells. Their lifespan is limited, and they can be expected to be excluded from the body before severe side effects occur, after they kill cancer cells. The reported CAR-NK cells are summarized in **Table 3**. NK cells transduced with CD19-CAR or CD20-CAR have been used when targeting B-cell malignancies. Bossel et al. showed that NK-92 cells transfected with anti-CD19-CAR by using electroporation had high cytotoxic activity against a $CD19^+$ cell line and primary chronic lymphocytic leukemia (CLL) [150]. They also showed that tumor cells can be eliminated in xenograft mouse models by using NK-92 cells transduced with anti-CD19 CAR with lentiviral vectors [170]. Imai et al. also showed that primary NK cells transduced with anti-CD19–CD3ζ with a retroviral vector killed $CD19^+$ cells and that the cytotoxicity was improved by adding a signaling domain of 4–1BB to anti-CD19–CD3ζ [171].

NK cell type	Target antigen	Cancer	Co-stimulatory domain	Gene transfer method	Ref
NK-92 cell line	ErbB2 (HER-2)	Breast, ovarian carcinoma	CD3ζ	Retrovirus	[175]
NK-92 cell line	CD20	B-ALL, CLL	CD3ζ	Retrovirus	[170]
NK-92 cell line	CD19	B-ALL, CLL	CD3ζ	Electroporation (mRNA)	[151]
NK-92 cell line	EpCAM	Breast carcinoma	CD28/CD3ζ	Lentivirus	[160]
NK-92 cell line	HLA-A2 EBNA3C	EBV+ T-cell Lymphoma	CD28/CD3ζ	Retrovirus	[178]
NK-92 cell line	GD2	Neuroblastoma	CD3ζ	Retrovirus	[179]
NK-92 cell line	CD19/CD20	B-ALL, CLL	CD3ζ	Electroporation (plasmid DNA)	[180]
				Lentivirus	
NK-92 cell line	CD19/CD20	B-ALL, CLL	CD3ζ	Lentivirus	[68]
NK-92 cell line	CS1	Multiple myeloma.	CD28/CD3ζ	Lentivirus	[181]
NK-92 cell line	CD138	Multiple myeloma	CD3ζ	Lentivirus	[182]
NK-92 cell line	ErbB2 (HER-2)	Breast carcinoma	CD28/CD3	Electroporation (plasmid DNA)	[176]
NK-92 cell line	ErbB2 (HER-2)	Breast, ovarian and renal cell carcinoma	CD3ζ	Lentivirus	[183]
			CD28/CD3ζ		
			CD137/CD3ζ		
YTS cell line	PSCA	Prostate cancer	CD3ζ	Lentivirus	[184]
			DAP12 TM and signaling		
YT cell line	CD33	AML	CD3ζ	Electroporation (plasmid DNA)	[150]

NK cell type	Target antigen	Cancer	Co-stimulatory domain	Gene transfer method	Ref
YT cell line	CEA	Colon carcinoma	CD3ζ	Electroporation (plasmid DNA)	[148]
PBMC-NK	CD19	B-ALL, CLL	CD3ζ	Retrovirus	[171]
			DAP10		
			CD137/CD3ζ		
PBMC-NK	ErbB2 (HER-2)	Breast, ovarian and renal cell carcinoma	CD28/CD3ζ	Retrovirus	[174]
PBMC-NK	CD19/GD2		CD3ζ or 2B4 alone	Retrovirus	[185]
			2B4/CD3ζ		
			CD137/CD3ζ		
PBMC-NK	CD19	B-ALL, CLL	CD137/CD3ζ	Electroporation(mRNA)	[152]
PBMC-NK	CD20	B-ALL, CLL	CD137/CD3ζ	Electroporation(mRNA)	[186]
PBMC-NK	NKG2D ligands	wide range	CD3ζ (with DAP10)	Retrovirus	[177]
				Electroporation(mRNA)	

Table 3. CAR-NK cells.

The gene human epidermal growth factor receptor 2 (HER2) is overexpressed in many breast cancers and is correlated with disease progression [172, 173]. It is therefore considered one of the suitable targets of CAR. Kruschinski et al. transduced anti-HER2–CD28-CD3ζ into human primary NK cells using a retroviral vector, and the results demonstrated cytotoxicity to an $HER2^+$ cell line. This cytotoxicity was correlated with the HER2 expression level on target cells [174]. Uherek et al. reported that anti-HER2–CD3ζ-CAR retrovirally transduced NK-92 cells efficiently killed cell lines derived from ErbB2-positive breast carcinoma, ovarian carcinoma, and squamous cell carcinoma *in vitro* and *in vivo* [175]. In a study by Liu et al., the plasmid coding anti-HER2–CD28-CD3ζCAR was transfected into NK-92 cells by electroporation, and the cells specifically killed the ErbB2-expressing human breast cancer cell lines MDA-MB-453 and SKBr3. The adoptive transfer of NK-92 cells specifically reduced the tumor size and lung metastasis of nude mice transplanted with MDA-MB-453 cells and significantly prolonged the survival of these mice [176].

As shown in **Table 3**, CAR against various tumor-associated antigens (TAA) has also been evaluated in NK cells. Chang et al. reported a modification by CAR using NKG2D, one of the human NK cell activating receptors, instead of the antigen-binding site of the antibody. Since NKG2D can bind to eight types of ligands expressed in solid tumors and blood tumors, it can be applied to a broader range of tumor cells. Primary human NK cells were transduced with a retroviral vector, using constructs designed to combine the extracellular domain of NKG2D with CD3ζ and further to express DAP10 simultaneously. This approach showed strong cytotoxic activity against various tumor cell lines and showed no damage to normal cells. It also showed strong tumor growth suppression in a mouse model of osteosarcoma [177].

6. Conclusions

Because NK cells are difficult to culture and it is a challenge to transduce foreign genes into NK cells, research concerning NK cells has been delayed compared to research involving T cells. However, culture and gene transfer technologies for NK cells are now developing. As introduced here, genetically modified NK cells acquired enhanced antitumor functions. These NK cells are very intriguing and are expected to be revolutionary cellular medicines for the treatment of malignancies.

NK cells are heterogeneous populations that exhibit various maturation stages and different KIR expression patterns. Since this heterogeneity has not been completely elucidated, it is not easy to choose the appropriate subset of NK cells for cancer treatment. Although allogeneic NK cell therapy using a KIR mismatch shows a strong antitumor effect against several blood cancers, the mechanisms underlying the activation and maintenance of NK cells in cancer patients are not completely understood. Cancer patients usually undergo a variety

	Gamida Cell Lth.	Nant Kwest Inc.	Glycostem Therapeutics	GreenCross LabCell	Fate Therapeutics Inc.	GAIA BioMedicine Inc.
H.Q.	Israel	USA	Netherlands	Korea	USA	Japan
Auto/Allo	???	Allogeneic (Cell line)	Allogeneic	Allogeneic (Health donor)	Allogeneic	Allogeneic (Haploidentical)
Materials	PBMC/BM/CB	NK-92	CB CD34+ (CliniMACS)	PBMC	PBMC (CMV+ donnor)/iPS	PBMC(CliniMACS Prodigy)
Feeder cell	–	–	–	Irradiated (2000 rad) PBMC	–	–
Irradiation	–	Before administration	–	–	–	–
Purity	>97% (CD3−/CD56+)	–	>98.1 ± 0.88% (CD3−/CD56+)	>98.1 ± 0.88% (CD3−/CD56+)	>90.9 ± 2.2% (CD3−/CD56+)	>98.0% (CD3-/CD56+)
K562 killing	E:T=1:1, 40–50%	E:T=5:1 (4 hr), 50%	E:T=2:1 (4 hr), 40%	E:T=10:1 (4 hr), 70–80%	–	E:T=1:1 (2 hr), >80%
Handling	CPC	Wash, Irradiation, CPC	CPC	CPC	CPC	Completely closed system
Companion diagnosis	–	–	–	–	–	HLA/KIR selection
R & D Phase	Non-clinical(Phase I/II in 2017)	Phase II	Phase I	Phase II	Non-clinical(Phase I in 2017)	Non-clinical(Phase I in 2017)
Target disease	-	Merkel cell carcinoma	AML	Hepatocellular carcinoma	AML	NSCLC, Neuroblastoma

Table 4. Products of NK cells for clinical use.

of standard treatments before receiving immunotherapy, and it is important to understand the factors that influence NK cell activity in order to select the correct clinical setting for NK cell therapy. In this review, we have explained the combination with antibodies, the genetic modification technique, and the KIR mismatch pattern (which is the basis of patient selection) that has been tested to maximize the use of NK cells as a treatment for cancer. Products of NK cells for clinical use that have been developed worldwide are shown in **Table 4**.

Advances in the understanding of NK cells may also lead to the development of small-molecule inhibitors targeting intracellular signals. Because NK cells are difficult to handle, their development is delayed compared to several other immunotherapies, but it is highly likely that they will be established as innovative cell therapy in the near future.

Conflicts of interest

Y. Harada is a member of the Scientific Advisory Boards of GAIA BioMedicine Inc. K. Teraishi is a researcher from Ono Pharmaceutical Co., Ltd. H Ban is a researcher from GAIA BioMedicine Inc. Y. Yonemitsu is a member of the Board of Directors on Science and Medicine at GAIA BioMedicine Inc.

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