Chapter 4

Natural Killer Cell-Based Therapies

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Abstract

NK[1] cells are the main component of the innate immune system with the potential to function in immune surveillance and cytotoxicity against infected and malignant cells. Unlike B and T lymphocytes, NK cells can identify and eliminate abnormal cells without the necessity of prior sensitization. It is estimated that NK cells constitute about 5 to 15% of peripheral blood lymphocytes and are specifically immunophenotyped by surface markers such as CD56. In terms of activity, the balance between activating and inhibitory signals is critical in NK cytotoxic and immune-regulatory functions. In the last few years, the application of NK cells, as a promising tool for cancer therapy, has been extended in the clinical setting. Using various approaches, including NK cell engagers, monoclonal antibodies, and cytokine-based treatments, their cytotoxicity is enhanced.

[1]. Natural Killer cells

Novel approaches such as immune stimulants, CAR[1]-engineered NK cells, and adoptive transfer of NK cells that have grown *ex vivo* are beneficial in the targeted recognition of tumor cells. Besides, NK cells and their products, such as EVs[2], exhibit putative anti-tumor impacts in preclinical research, opening new avenues for NK cell-based treatments. In this chapter, recent progress and implications regarding the application of NK cells have been discussed.

Keywords: NK cells; Cell-based therapies; Anti-tumor activities.

1. Introduction

NK cells are a vital element of the immunological system, offering potent cytotoxicity in opposition to infection and malignant cells [1]. As key players in innate immunity, these cells show an essential role in cancer immune surveillance by targeting and eliminating aberrant or stressed cells without prior sensitization [2]. As a common belief, NK cells are putative cells in destroying CSCs[3], which often exhibit resistance to conventional therapies [3]. In this regard, immune cell-based therapies with the application of the body's immune system components have emerged as a significant treatment modality to increase the survival rate of cancer patients [4]. Recent breakthroughs in antibody and cell-based immune therapies have changed the conventional protocols with a focus on the administration of NK cells. To accomplish better therapeutic outcomes, the stimulation of NK cell cytotoxicity is a key strategy in cancer immunotherapy in combination with other medications [5]. To this end, NK cell engagers, monoclonal antibodies, and cytokine-based treatments are the center of attention [6]. The use of immune stimulants, including

[1]. Chimeric antigen receptor
[2]. Extracellular vesicles
[3]. Cancer stem cells

antibodies and cytokines, as well as the adoption of NK cells that have been grown ex *vivo,* can also be helpful [7]. Very recently, the development of CAR-engineered NK cells has increased the tumoricidal properties of these cells by targeted recognition of tumor cells [8]. Along with these approaches, NK cell EVs have anti-tumoral functions in preclinical research, opening new avenues for NK cell-based therapies [9]. As the study advances, optimizing NK cell-based strategies continues to have a lot of potential to enhance the results of cancer treatment.

2. General features of NK cells

NK cells, an essential part of the innate immunological system, are actively involved in timely and rapid reactions to infected and tumor cells [10]. These cells were first defined as a new lymphocyte population in mice between 1973 and 1982. From 1983 to 1992, NK cells were phenotypically characterized. In 1993–2002, the existence of inhibitory and activating receptors with relevant ligands was described. The progress in the identification of the NK cell memory hypothesis occurred from 2003–2012. Finally, from 2013 to the present time, new technologies have accelerated the precise identification of NK cell types and bioactivity [11]. Unlike T and B lymphocytes, NK cells can find and eliminate aberrant cells without prior sensitization [1]. Their inevitable role in immune surveillance and cytotoxicity has made them attractive target cells for immunotherapy, in particular cancer treatment [12]. In terms of morphological features, human NK cells are large-sized cells with granularity and comprise about 5 to 15% of circulating lymphocytes [13]. These cells can distribute across both non-lymphoid and lymphoid tissues, including the uterine, hepatic, and splenic tissues, and blood [14]. The paracrine activity of NK cells is prominent and usually follows with the production of several cytokines, like

IFN-γ[1], to stimulate the immune system cells and drive inflammation under pathological conditions [15]. NK cells encompass a heterogeneous population belonging to ILCs[2] with three distinct families (ILC1, ILC2, and ILC3). The highest fraction of NK cells is listed as group 1 innate lymphoid cells, which reside within peripheral tissues [16, 17]

2.1. NK origin and development

It is thought that BM[3] is the main site for the production of NK cells. Inside the BM, $CD34^+$ HSCs[4] undergo multi-stage orientation to generate different immune cells [18]. Along with NK cells, BM assists in the production of various progenitors and precursors with the ability to differentiate and mature into different lineages [19]. This phenomenon is commonly monitored by dynamic changes in surface marker expression, transcription factor expression, and functional capabilities [17]. In this regard, NK cell differentiation includes three consequential steps as follows: commitment to the lymphoid lineage, common NK/innate lymphoid cell lineage, and NK cell lineage. In short, HSCs differentiate into CLPs[5] to produce multiple immune lineages, including T, B, and NK/ILC progenitors [20]. Pre-pro NK progenitors, a subset within the CLP population, are Lin^- (negative for major immune lineage markers) but can express IL7R-α[6] ($CD127^+$), marking their transition into the lymphoid lineage [17]. Within this early progenitor population, a subset known as pre-NKPs[7] starts to up-regulate the CD122 (IL-2Rβ)[8], leading to their commitment to the NK cell lineage and further progression to NKPs[9]. It is thought that the

1. Interferon gamma
2. Innate lymphoid cells
3. Bone marrow
4. Hematopoietic stem cells
5. Common lymphoid progenitors
6. IL-7 receptor alpha
7. Pre-NK cell precursors
8. IL-2 receptor β chain
9. NK progenitors

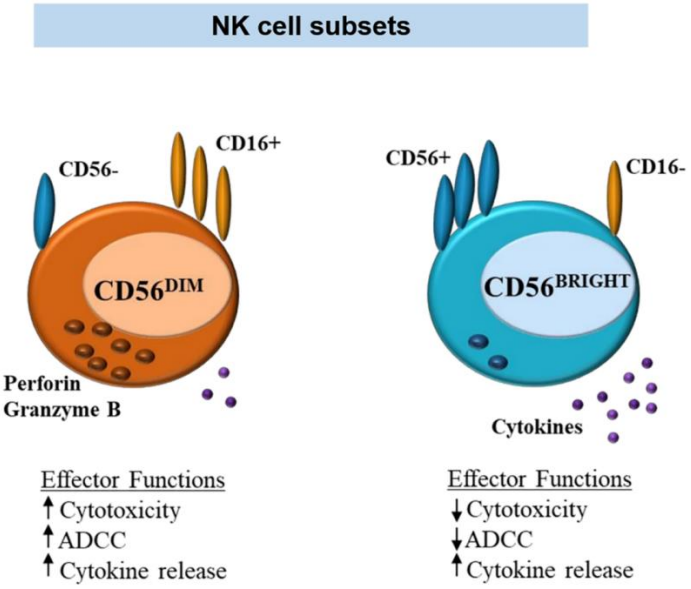
expression of CD122 is a critical phase in NK cell differentiation, and this receptor causes NK cells to respond to IL-15. Inside the BM, IL-15 is produced by stromal cells to control NK cell viability, differentiation, and development [19]. NKPs can further progress to immature iNK[1] cells via the activating receptors expression such as NKG2D[2], CD161, CD337 (NKp30), CD335 (NKp46), and CD314 (NKG2D) [21]. The procedure continues with the emergence of $CD56^{bright}$ NK cells with concomitant expression of NKp30, NKp46, NKG2D, CD161, and CD56. In the later step, $CD56^{bright}$ NK cells mature into $CD56^{dim}$ NK cells, with simultaneous expression of CD16 and down-regulation of CD56 [21].

2.2. NK cell subsets

Mature and functional NK cells constitute up to 20% of peripheral blood lymphocytes. However, their number is less in the BM and splenic tissue (5–10%) and rare in lymphatic tissues (below 1%) [22]. NK cells are devoid of the CD3ε signal transduction domain or the TCR[3], unlike T cells. Instead, NK cells can express CD56, and lymphocyte subsets are characterized with a general $CD3^{-}/CD56^{+}$ profile [23]. Of note, NK cells encompass a diverse population based on surface receptor CD56 and CD16 (FcγRIII)[4] expression. In the clinical setting and pre-clinical studies, CD56 is the most commonly used marker for NK cell identification [24]. However, NK cells can be classified into two separate subsets according to CD56 expression ($CD56^{bright}$ and $CD56^{dim}$ NK cells) [25]. These NK cell subsets exhibit different cytolytic activity, cytokine production, and homing capabilities [24]. iNK cells can initially mature into a $CD56^{+/+}$ ($CD56^{bright}$) subset, which consists of ~5% of total

1. Immature NK cells
2. Natural killer group 2 member D
3. T cell receptor
4. Fc gamma receptor III

NK cells, while the $CD56^{+/-}$ subset ($CD56^{dim}$) population is more than 90% [26]. The majority of $CD56^{bright}$ cells are present in lymphoid organs with few lytic granules (such as granzymes and perforin) [27]. However, $CD56^{bright}$ NK cells can produce and release high levels of cytokines like IFN-γ and TNF-α[1] [26]. Upon stimulation, these cells rapidly enter proliferation and gradually acquire cytolytic activity. In contrast, $CD56^{dim}$ NK cells harbor KIRs[2] and show strong cytotoxicity. Taken together, $CD56^{bright}$ NK cells exhibit regulatory functions while $CD56^{dim}$ NK cells have prominent cytotoxic functions [17, 25, 28]. Additionally, $CD16^{+}$ NK cells[3] can attach to antibodies and exert ADCC[4] activity. The relationship between CD16 and the Fc region of antibodies triggers NK cells to destroy antibody-coated target cells, such as cancer cells (**Figure 1**) [29, 30].



[1]. Tumor necrosis factor alpha
[2]. Killer cell immunoglobulin-like receptors
[3]. CD16 a receptor for the Fc portion of antibodies
[4]. Antibody-dependent cellular cytotoxicity

Figure 1. Different effector functions are displayed by CD56^DIM and CD56^BRIGHT subsets of NK cells. Reproduced with permission. [31]. International Journal of Molecular Sciences. 2020.

2.3. NK cell activation and inhibition

The bioactivity of NK cells is tightly controlled by a balance between activating and inhibitory receptors [32]. The activation of receptors by ligands leads to the engagement of distinct intracellular signaling chains. In the next steps, these effectors are phosphorylated by Src family protein kinases to stimulate several intracellular signaling transduction pathways, such as calcium flux [33]. The increase of intracellular calcium content results in exocytosis, gene transcription, production of cytokines, and NK cytotoxicity [34]. Conversely, inhibitory receptors, such as KIRs, show a vital role in NK cell regulation after the recognition of HLA[1] (A, B, and C) substances on the target cells [35]. Based on their intracellular domains, KIRs can send both activating and inhibiting signals [32]. Inhibitory KIRs use ITIMs[2] to transmit their signal. Upon activation, ITIM domains provoke several phosphatases that dephosphorylate key signaling effectors, resulting in the inhibition of NK cell activity [36]. The interaction of HLA Class I molecules with KIRs transmits inhibiting signals and blunts NK cell activity to ensure self-MHC-expressing cells are not mistakenly recognized [37]. This mechanism, known also as "missing-self" recognition, potentiates NK cells to detect and eradicate abnormal cells [38]. During NK cell maturation, KIRs are produced to monitor MHC I levels and maintain NK cell inactivity under normal conditions [39]. However, infected or abnormal cells with less MHC I content or upregulated stress-induced ligands switch the balance toward activation [39]. Along with the

[1]. Human leukocyte antigen
[2]. Immunoreceptor tyrosine-based inhibitory motifs

suppression of inhibitory signaling, NK cell cytotoxicity is induced, and these cells efficiently target and eradicate abnormal or diseased cells (**Figure 2**) [40]. As a common belief, KIRs are key regulators in controlling human NK cell growth and effector function. To date, 17 KIRs have been identified, and these receptors are classified into three different groups based on their function. For example, inhibitory KIRs (KIR2DL1-4, KIR2DL5, KIR3DL1-3), activating KIRs (KIR2DS1-5, KIR3DS1), and dual-function KIRs (KIR2DL4) [41, 42]. The family of KIR genes exhibits significant diversity because of potent variations in genes between haplotypes, while the possibility of extensive allelic polymorphism is also high [42]. The two general categories of KIR haplotypes are A and B, and four genes, including KIR3DL3, KIR3DP1, KIR2DL4, and KIR3DL2, are shared by these haplotypes. However, they differ in their additional KIR gene content [43]. Four genes (KIR2DL3, KIR2DL1, KIR3DL1, and KIR2DS4) are fixed in Group A haplotypes and predominantly encode inhibitory receptors (KIR3DL1, KIR3DL2, KIR2DL1, and KIR2DL3) to the potential to recognize particular ligands of HLA class I: C2, C1, Bw4, and A3/A11, respectively [44, 45]. Notably, KIR2DS4 is the only activating receptor in this group [46]. Group B haplotypes exhibit greater genetic variability, containing one or more of seven additional genes (KIR2DL5, KIR2DL1, KIR2DS2-3-5, and KIR3DS1) [47]. Unlike group A, group B haplotypes can express multiple stimulating KIRs such as KIR3DS1 and KIR2DS1, 2, 3, and 5, contributing to a more dynamic and responsive NK cell repertoire [39, 48, 49].

NK cells harbor an inhibitory receptor named NKG2A/CD94 with the regulatory activity to control NK cell-target cell interaction by binding to the non-classical HLA Class I molecule and the HLA-E [50]. As a key immune checkpoint, the attachment of NKG2A to CD94 and recruitment of HLA-E

contribute to the generation of inhibitory signals to NK cells [51]. Such activity has been indicated in tumor immunological escape and various immune-related conditions, such as transplant rejection, inflammatory diseases, autoimmune disorders, and viral infections [49, 52]. NKp46, NKp30, and NKp44 are examples of NCRs[1] in NK cells that stimulate cytokine production and cytotoxic activity [53]. These receptors aid NK cells in recognizing and killing viral, bacterial-infected, and cancer cells. NKp30 and NKp46 are found in all NK cells, while NKp44 is expressed when NK cells are exposed to IL-2 [53]. Additionally, NKG2D is a receptor activation of NK cells that detects ligands caused by stress, such as MICA, MICB[2], and six ULBP1-6[3] on the stressed, infected, or transformed cells. NKG2D engagement enhances NK cell cytotoxicity, promoting the elimination of abnormal cells [54]. Emerging data have indicated that the PD-1/PD-L1[4] signaling pathway acts as an immune checkpoint and inhibits NK cell function [55]. The attachment of NK cell PD-1 to target cell PD-L1 suppresses NK cell activation via the inhibition of the PI3K/AKT signaling pathway. This regulation plays a role in immune evasion in tumor microenvironments and chronic infections. Interestingly, the inhibition of PD-1/PD-L1 signaling improves cytokine production capacity, degranulation, and viability in NK cells, leading to significant tumoricidal and anti-viral properties [56, 57].

2.4. NK cell function

NK cells are involved in the immune system with two primary functions, including cytotoxicity and immune modulation [27]. These cells can destroy aberrant cells by releasing granzymes and perforin without any prior

[1]. Natural cytotoxicity receptors
[2]. MHC class I chain-related protein A and B
[3]. UL16-binding proteins
[4]. Programmed cell death ligand 1

activation [58]. Upon stimulation through KARs[1], NK cells release ligands that cause death, including TNF-α, FasL[2], and TRAIL[3], and induce apoptotic changes in target cells [59]. The discharge of cytokines like CCL3, CCL4, CCL5, IL-10, and IFN-γ by NK cells facilitates close interaction between the immune system's innate and adaptive responses, resulting in immune modulation (**Figure 2**) [60, 61].

2.4.1. Cytotoxic function

The phenomenon of NK cell cytotoxicity occurs in four separate steps. First, an immunological synapse is generated between the NK cells and the target cells, followed by actin cytoskeleton remodeling [62]. Next, MTOC[4] and secretory lysosomes are guided toward the lytic synapse. In the next step, secretory lysosomes are physically connected to the NK cell's plasma membrane before merging with the target cell's plasma membrane [63]. Along with these changes, the production of cytotoxic chemicals like perforin and granzymes is stimulated, leading to NK cell degranulation [64]. During this step, LAMP-1 (CD107a)[5] and LAMP-2 (CD107b) are up-regulated on the NK cell surface [65]. The existence of LAMP-1 is thought to be a reliable indicator of the cytolytic function in NK cells [66]. Upon release, perforin is attached to target cells, and its polymerization generates pores that enable the entry of granzymes [67]. Granzymes are serine proteases with different types (A, B, H, K, and M) and are found commonly in cytotoxic lymphocytes [68]. Each granzyme has unique substrate specificities and promotes apoptosis in a caspase-dependent or -independent manner [69]. Inside the cells, granzymes are stored in cytotoxic granules to prevent possible damage to host cells [69].

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[1]. Killer activating receptors
[2]. Fas ligand
[3]. TNF-related apoptosis-inducing ligand
[4]. Microtubule-organizing center
[5]. Lysosomal-associated membrane proteins-1

Among different granzymes, types A and B are the most prevalent and have been extensively studied for their critical role in eliminating malignant and transformed cells [70]. As aforementioned, perforin induces the formation of pores in target cells and facilitates the entry of granzymes, accelerating apoptosis [71]. Ultrastructural studies have revealed the interaction of perforin with the target cell membrane by using its calcium-binding C2 domain. This domain can oligomerize and generate pores through the MACPF[1] domain [72]. Irrespective of granzyme- and perforin-dependent cytotoxicity, NK cells can also mediate target cell elimination by apoptosis after the activation of the death receptor [72]. The expression of FasL, TNF-α, and TRAIL by NK cells helps these cells efficiently bind to cognate receptors on target cells [67]. For example, FasL interacts with the CD95/Fas receptor, while TRAIL attaches to TRAIL-R1 and -R2 on target cells [73, 74]. Upon attachment, receptors undergo conformational changes and provoke adaptor proteins, followed by apoptotic death via a caspase-dependent manner [1, 59, 67].

2.4.2. Cytokine production by NK cells

NK cells play critical roles in immune regulation with the production and release of important cytokines, including IFN-γ and TNF-α [75]. The $CD56^{bright}/CD16^{-}$ NK cells can produce abundant cytokine levels with significant immunoregulatory function [76]. The release of cytokines by these cells potentiates the host defense system against tumorigenic cells and various intracellular infections [77]. IFN-γ production promotes both innate and adaptive immune activation by recruiting immune cells to the site of infections, upregulating MHC molecules to promote Th1 differentiation, and helping T cells in the recognition processes [78]. Additionally, NK cells release TNF-α and other pro-inflammatory

[1]. Membrane attack complex-perforin

cytokines, as well as IL-10, which has immunomodulatory properties [75]. It is believed that the cytokine release is controlled by the same activation pathways that trigger NK cell-mediated cytotoxicity [79]. To be specific, cytokines are produced non-directionally and are transported via a different channel, whereas lytic granules containing perforin are secreted directionally at the immunological synapse [80]. The paracrine activity of NK cells is triggered in the presence of cytokines like IL-1, -2, -12, and -15. TNF-α and IFN-γ, once produced, can cause tumor cell death and stimulate immune cell activation [79]. TNF-α *per se* boosts IFN-γ production via the TNFR2 signaling axis, and IFN-γ makes cytotoxic NK cells more motile and cytotoxic, amplifying immune responses [1, 64].

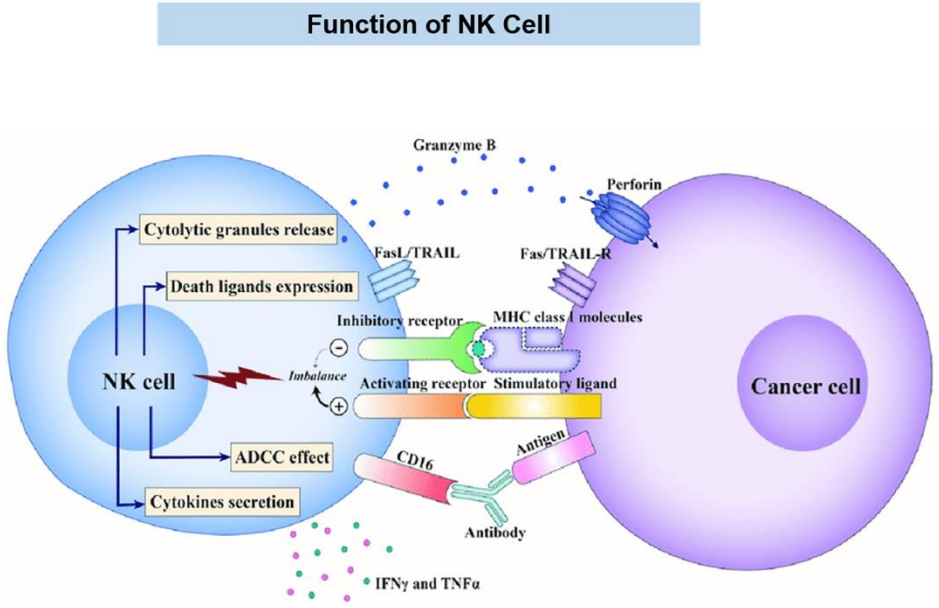

Figure 2. NK cells play a key role in anti-cancer immunity by balancing activating and inhibitory receptor signals. Cancer cells enhance NK activation by upregulating stimulatory ligands (e.g., NKG2D) and downregulating MHC class I molecules. Once activated, NK cells release cytolytic granules (perforin and granzymes) and express death ligands (FasL and TRAIL) to induce apoptosis in cancer cells. Additionally, NK cells mediate ADCC

through CD16 and secrete cytokines like IFN-γ and TNFα to enhance immune responses. Reproduced with permission. [81]. Cancers (Basel). 2021.

3. Possible sources for isolation of NK cells

Primary NK cells can be produced or isolated from several sources, such as iPSCs[1], PB[2], and UCB[3], as discussed here [82]. PB is one possible source for the isolation of NK cells for different therapeutic purposes [83]. It is possible to manufacture NK cells from autologous (patient-derived) or allogeneic (donor-derived) sources [84]. However, the limited quantity of NK cells isolated from PB is the main challenges that necessitate large-scale expansion in *in vitro* conditions [85]. Besides, autologous NK cells may exhibit compromised functionality due to patient conditions or conventional treatments. Thus, these changes make allogeneic NK cells preferred cells for clinical application [86]. Despite several limitations, PB NK cells are developed and do not require differentiation induction in *in vitro* settings [87]. Of course, it should not be forgotten that their gene transduction capacity is comparatively limited, and any manipulations can result in decreased cytotoxicity and telomere shortening *in vitro* [88, 89]. Furthermore, storage temperature stress due to cryopreservation adversely influences their viability and function [90]. In general, NK cell therapy requires the reinfusion of 10^6–10^8 cells per kilogram of body weight, and a low number of PB NK cells complicates large-scale *in vitro* culture. Despite these limitations, PB NK cells remain valuable due to their maturity, which eliminates the necessity of a prolonged period of differentiation [91, 92]. UCB is also a reliable biological source for the isolation of NK cells. In this regard, two main strategies are

[1]. Induced pluripotent stem cells
[2]. Peripheral blood
[3]. Umbilical cord blood

applied to prepare NK cells from UCB for therapeutic purposes [93]. NK cells can be directly purified from UCB samples or developed by the differentiation of HSCs into NK cells [94]. It is estimated that only 10% of each UCB unit is required to harvest over 10^9 NK cells during two weeks after *in vitro* expansion [95]. Notably, 10^9 NK cells are enough for one therapy cycle. Interestingly, UCB NK cells exhibit limited inhibitory function with less expression of receptors. Therefore, the isolated cells are not fully differentiated, which in turn can increase the risk of carcinogenesis with allogeneic transplants. Despite these limitations, UCB NK cells possess suitable BM homing capacity, making them a valuable option for NK cell-based therapies [85, 93]. The development of NK cells from iPSCs takes about 3 to 5 weeks [96]. It is believed that this approach diminishes donor-recipient heterogeneity compared to allogenic cell sources. Despite these benefits, iPSC-derived NK cells face multiple difficulties, such as the possibility of malignant transformation and tumorigenesis after being transplantation [92]. Besides, their immunogenic entity would stimulate unexpected immune responses like cytokine release syndrome [97-99]. The reduced expression of CD16 in iPSC NK cells can impair ADCC [97]. Moreover, systemic administration of cytokines for NK cell activation is clinically not applicable due to high costs and potential adverse effects [100]. Highly active NK-92 cells are another NK source for therapeutic purposes [101]. Compared to PB NKs, NK-92 cells possess several advantages. These cells can be easily transduced and expanded without T lymphocyte contamination and the possibility of GVHD[1], making cost-effective and standardized production of NK cells [102-104]. NK-92 cells are devoid of KIRs, which increase the cytotoxic properties [90]. Since NK-92 cells are highly proliferative, their dynamic growth can be regulated by modalities such as radiation before transplantation [105]. In addition, these

[1]. Graft-versus-host disease

cells have genomic instability because of carrying the Epstein–Barr virus [106]. The low density of CD16 in NK-92 cells makes them non-suitable cells for ADCC and reduces regenerative potential in combined therapies [107]. However, NK-92 cells have gained FDA[1] approval for clinical application and are an alternative in NK cell-based therapies. Taken together, the strong intrinsic cytotoxicity of NK-92 cells against tumor cells makes them a promising cell tool in cancer therapy (**Figure 3**) [28, 108, 109].

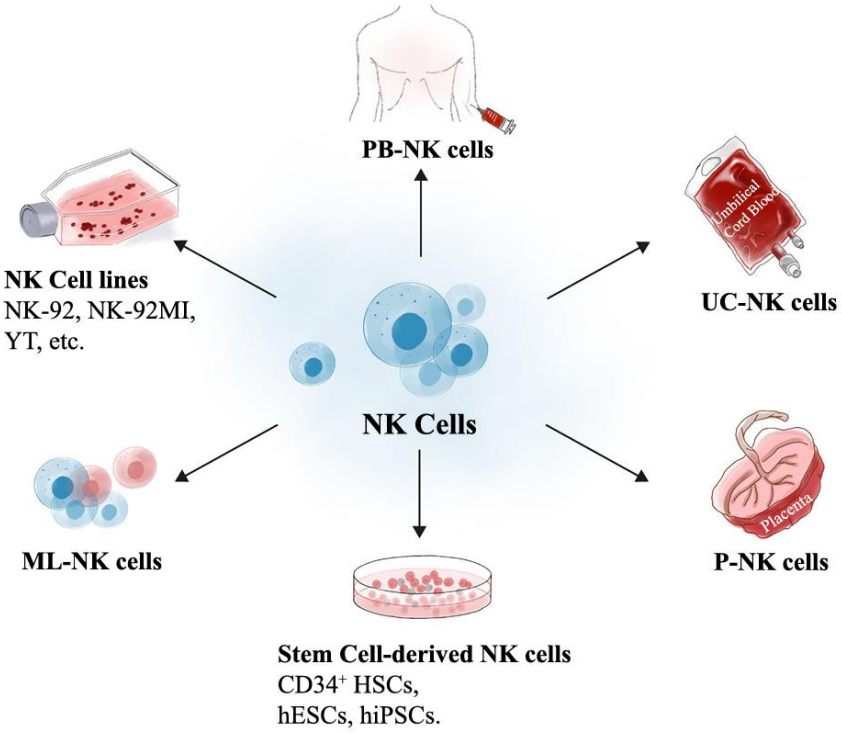

Figure 3. The main sources of NK cells for isolation of memory-like NK (ML-NK) cells, Placental blood-derived NK (P-NK) cells, Umbilical cord blood-derived NK (UC-NK) cells, and Peripheral blood-derived NK (PB-NK) cells. Reproduced with permission. [110]. Medical Review. 2023.

4. Strategies to enhance NK cell function

[1]. Food and Drug Administration

4.1. Cytokine-based stimulation

Cytokines are key tools for regulating the NK cell viability, expansion, and cytotoxicity behavior in immunotherapy [111]. Interleukins such as IL-2, -15, and -21 are involved in the activation of NK cells [111]. IL-2 is an early-stage cytokine for the activation of NK cells and approved by the FDA for cancer therapy [112]. This cytokine triggers NK cell growth and cytotoxicity via the expression of stimulating receptors and increasing cytotoxic molecule synthesis [113]. Previously, IL-2 has been used to generate LAK[1] cells consisting of immunological cells like NK, NKT, and T cells in early autologous cancer therapies [82]. Unfortunately, this cytokine can simultaneously activate Tregs[2] with the failure of a therapeutic strategy [82]. IL-15 shares receptor components with IL-2 (CD122 and CD132) but possesses an alpha chain, making it a superior co-therapeutic alternative [114]. Unlike IL-2, this cytokine does not activate Tregs and is safe for cancer therapy. IL-15 is necessary for the growth and survival, and cytotoxicity of NK cells, and exerts tumoricidal properties with less toxicity [6, 115]. The last cytokine, namely IL-21, acts as a two-edged sword in NK cell regulation [116]. At optimal doses, IL-21 promotes cytotoxic functions, ADCC, and IFN-γ production, while higher concentrations can induce apoptosis and limit NK cell viability [116]. Interestingly, IL-21 has synergistic effects when combined with IL-2 or IL-15, leading to enhanced NK cell growth and function (**Figure 4**) [82, 117].

4.2. NK cell engagers

The use of mAbs[3] has increased for the induction of NK cell responses [118]. These antibodies can block inhibitory receptors, stimulate NK cell

[1]. Lymphokine-activated killer
[2]. Regulatory T cells
[3]. Monoclonal antibodies

proliferation, and thereby facilitate ADCC [119]. A major challenge in NK cell therapy is the failure of the immune evasion strategies used by tumor cells [120]. Checkpoint-blocking antibodies counteract these mechanisms by targeting inhibitory ligands on tumor cells or inhibitory receptors on NK cells, thereby releasing the cytotoxicity of NK cells [121-123]. Both immunosuppressive cells and TME[1] present high levels of PD-L1, resulting in immune evasion [124, 125]. The use of mAbs targeting the PD-1/PD-L1 axis can reduce the suppression by enhancing the function of NK cells [126]. Anti-TIGIT, LAG-3, and TIM-3 antibodies improve NK cell cytotoxicity by preventing inhibitory signaling [127]. Moreover, Monalizumab increases NK-mediated tumor cell death by inhibiting the NKG2A-HLA-E interaction [128-130]. Several mAbs with the induction of ADCC in NK cells include Rituximab (targeting CD20, for B-cell malignancies). Trastuzumab (targeting HER2, for gastric and breast cancers) and Cetuximab (targeting EGFR, for colorectal and head & neck cancers). NK cells easily identify tumor cells coated with antibodies using CD16 (FcγRIII). The attachment of CD16 with mAbs triggers NK cell cytotoxicity [131-133]. However, the rapid shedding of surface CD16 by disintegrin and metalloproteinase 17 (ADAM17) leads to diminished NK cell function (**Figure 4**) [134-137].

4.3. CAR-NK cells

Engineered NK cells are a promising solution to circumvent several above-mentioned limitations and increase tumoricidal capabilities [138]. It is believed that CARs enhance the antitumor activity of immune cells by increasing tumor cell detection and attack with greater precision and accuracy [139]. CAR is a synthetic hybrid antigen receptor used to

[1]. Tumor microenvironment

increase target-specific recognition of TAAs[1] and the killing properties of immune cells [86]. Typically, CAR is composed of three major elements: an extracellular domain, a transmembrane area, and an intracellular signaling domain [140]. The extracellular domain has a scFv[2] that is obtained from tumor antibodies, enabling an appropriate antigen recognition [141]. Recently, VHH[3] with strong affinity and tiny size has been used for increasing CAR cell activity [142, 143]. The spacer area attaches the scFv or VHH to the transmembrane domain and has prominent flexibility to ensure suitable positioning of the receptor on the cell membrane [89]. The CAR molecule is directly attached to the cell membrane by the transmembrane domain to strengthen structural stability [86, 144, 145]. The intracellular signaling domain acts as an activator in CAR-expressing immune cells following antigen recognition [146]. This domain is derived from ITAMs[4] of TCRs or the cytoplasmic domains of other receptors. Upon antigen binding, phosphorylated ITAMs recall adapter molecules and stimulate downstream signaling cascades [147]. Regarding variations in their intracellular signaling components, CARs are classified into different generations, and each generation is designed to improve immune activation and therapeutic efficacy [109, 145].

Despite the existence of promising regenerative potential, CAR-T cell therapy has several bottlenecks [86]. A lake of appropriate TAA can lead to therapy resistance [148]. Besides, off-tumor" effects cause direct damage to healthy cells [144]. Serious adverse effects, such as CRS[5] and ICANS[6], increase the possibility of excessive immune activation [144]. The

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1. Tumor-associated antigens
2. Single-chain variable fragment
3. Single variable domains on heavy chains
4. Immunoreceptor tyrosine-based activation motifs
5. Cytokine release syndrome
6. Immune effector cell-associated neurotoxicity syndrome

homing and infiltration of CAR-T cells into the solid tumors' parenchyma is problematic, and due to the existence of various immunosuppressive signals, the activity of recruited cells can be altered [149]. A large number of manipulated cells are required for therapeutic purposes, which, *per se,* increases costs, production time, and detrimental effects in late-stage cancer patients [150]. In therapeutic protocols related to allogeneic CAR-T therapies, the occurrence of GVHD is a serious issue, and donor T cells may cause an immunological reaction against the recipient tissues [151]. To circumvent CAR-T treatment side effects, recent experiments are shifting towards the development of CAR-NK cells with a similar CAR structure. CAR-NK cells offer several advantages that make them a promising alternative [152, 153]. As aforementioned, NK cells can identify and destroy inflamed or malignant cells without previous antigen priming [100]. Unlike allogeneic CAR-T cells, GVHD cannot be caused by CAR-NK cells, making them a safer "off-the-shelf" therapy option [154]. The possibility of CRS and neurotoxicity is less in patients who received CAR-NK [155]. Unfortunately, the shorter lifespan of CAR-NK cells and antibody-based CAR ectodomain specificity restricts on-target capacity [156]. The recognition of TAA by CAR-T cells is based on single-chain antibodies, while CAR-NK cells identify target cells through multiple activating receptors (NKp46, NKp44, NKp30, CD226, and NKG2D) [157-159]. CAR-NK cells can be produced from various sources, including hESCs[1], NK-92 cell lines, UCB, PB, and iPSCs [155]. Currently, CAR-NK cells can be easily manipulated to express scFvs[2], ligands for HLA-independent antigen recognition, and various peptides [92]. Novel and sophisticated approaches, such as TCR-NK cell therapy, have been

[1]. Human embryonic stem cells
[2]. Single-chain variable fragments

recently introduced in which NK cells are reinforced to express TCR complexes [160]. This technique combines the innate cytotoxicity of NK cells and the antigen specificity of TCR-based targeting and helps TCR-NK cells detect and present TAA by MHC-I [161]. Multi-specific NK cell treatment uses NK cells with the ability to leverage mAbs, bi-specific, or tri-specific antibodies for enhancing tumor cell recognition, and appropriate cell activation (**Figure 4**) [92, 162].

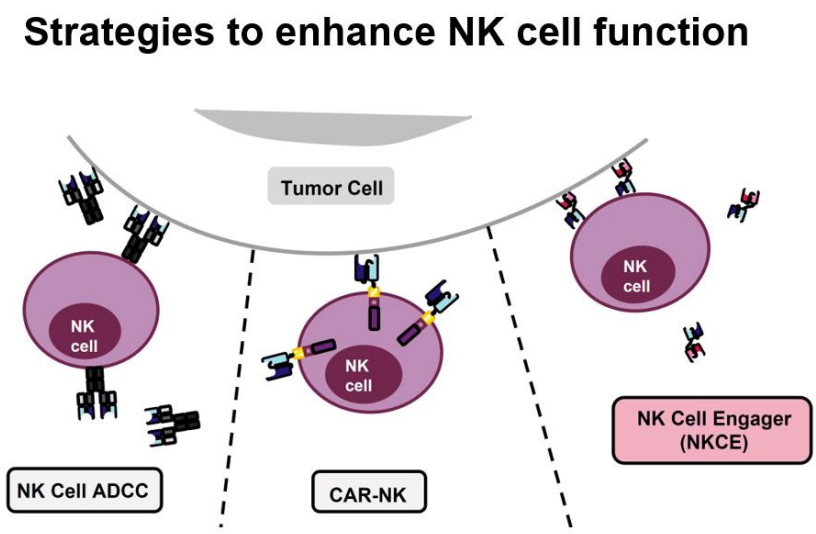

Figure 4. NK cell ADCC is triggered by CD16a on NK cells and guided by monoclonal antibodies .CAR-NK cells are genetically engineered to target tumor antigens, while NK cell engagers (NKCEs) link NK and tumor cells to activate NK cells. Adapted from [163].

4.4. NK EVs

EVs, nanosized intercellular communication tools, are involved in the regulation of immune response [164]. EVs harbor various bioactive substances like lipids, proteins, and carbs, and genetic materials such as miRNAs[1], etc., to alter the metabolic status of acceptor cells [165]. In

[1]. microRNAs

general, EVs are categorized into three basic groups based on their biogenesis and size as follows; a) Exos[1], with an average diameter between 30–200 nm, are produced by the endosomal system, b) MVs[2] range between 100 and 1000 nm, and are generated by evagination of plasma membrane, and c) apoptotic bodies are produced in apoptotic cells [166]. NK cells can release EVs for immune regulation and controlling the defense system [167]. Recently, NK-EVs[3] have been considered due to their potent tumoricidal properties [168]. NK-EV cytotoxic molecules like perforin, granzymes, FasL, and DNAM-1 induce apoptosis in melanoma, neuroblastoma, breast cancer, glioblastoma, leukemia, and other cancer types [169, 170]. Notably, resting and activated NK cell Exos harbor NK-specific markers such as CD56, NKp46, NKp30, NKp44, and NKG2D, which enhance their capacity to identify and eliminate tumor cells [171]. Interestingly, pre-treatment of NK cells with neuroblastoma cell Exos increases activating receptors and tumoricidal properties. Therefore, it is thought that tumor cell Exos educates and refine the NK cells against malignancies [67, 172, 173]. Recent advances in *ex vivo* NK cell expansion help researchers to provide large-scale NK-EV production for various biological purposes. These EVs are repertoires of NK cell proteins, such as perforin, granulysin, granzyme A, B, CD56, and FasL, which are comparable to parent NK cells in the elimination of tumor cells [172, 174]. Moreover, NK Exos are suitable bioshuttles for the transfer of cisplatin in boosting its anti-tumor effects, restoring NK cell activity, and reducing the immunosuppressive state (**Figure 5**) [169].

[1]. Exosomes
[2]. Microvesicles
[3]. NK cell-derived EVs

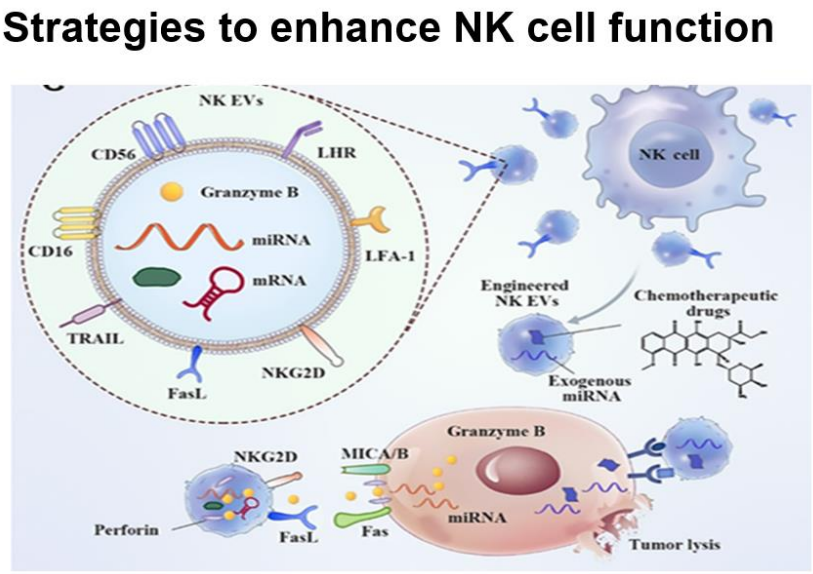

Figure 5. NK EVs interact with tumor cells, exert cytotoxic effects by releasing granzymes, perforin, and antimicrobial peptides, and ultimately induce tumor cell apoptosis. Furthermore, engineered NK EV-coated chemotherapeutic drugs have been developed to facilitate targeted drug delivery for chemotherapy. Adapted from [175].

4.5. Combination therapy

Combination therapy improves the anticancer efficacy of NK cells by disabling limitations in NK cell function [139]. This modality enhances tumor targeting and blunt immunosuppression within the tumor parenchyma, and increases tumor cell sensitivity to cytotoxic NK cells [139]. Current research has highlighted the tumoricidal properties of combination therapies using NK cells in conjunction with other conventional medications [176]. For instance, the simultaneous use of reoviruses and NK cells is an efficient method in bladder cancer treatment [177]. In an experiment conducted by Mazloumi et al, they found that combining NK cell therapy with concurrent telomerase inhibition significantly increases apoptosis in breast cancer cells [178]. In addition,

HCC[1] patients subjected to high-dose NK cell transplantation and four cycles of HAIC[2] using 5-fluorouracil and cisplatin showed better therapeutic outcomes [179].

5. NK cell therapy limitations

Despite the promising potential of NK cell treatment for cancer patients, several challenges limit the wide clinical success and application [180]. The viability of transplanted NK cells is less inside the body, which can blunt the efficiency of cell-based therapies in cancer patients over time [181]. Upon infusion, NK cells undergo apoptosis, or their retention time is not long. Approaches like genetic modifications or cytokine treatments can, in part, prolong the survival rate [182]. The response to NK cells against tumor cells varies between patients because of genetic traits in NK cell profiles and the heterogeneity of tumor cells [183]. It is also possible that tumor cells may express different levels of TAA or provoke mechanisms to escape NK cell recognition [38]. Inside the TME, low levels of target antigens or secretion of immunosuppressive substances like TGF-β[3] and IL-10 exhaust NK cells [184-186]. The penetration of NK cells into solid tumors is hindered, resulting in abnormal NK cell function [14]. Reverse patterns in TME inhibitory receptor ligands and activating receptor ligands can circumvent NK cell regulation [120]. Besides, the production of genetically induced NK cells from allogenic and autologous sources has some ethical concerns, GVHD, and the possibility of uncontrolled genetic changes should also be considered [187].

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[1]. Hepatocellular carcinoma
[2]. Hepatic arterial infusion chemotherapy
[3]. Transforming growth factor-β

6. Clinical Trial of NK cell therapy

Clinical trials of NK cell therapy are being conducted in multiple countries using various sources and across phases I and II, as shown in Table 1.

Table 1. clinical trial of natural killer cell therapy

Ref	Phase	Cancer Type	NK Cell Type	Trial Status	ClinicalTrials.gov Identifier
[188]	Phase I/II	B-cell Malignancies	Cord Blood-derived CAR NK Cells	Completed	NCT03056339
[189]	Phase I	solid tumors	iPSC-derived-NK cells	Ongoing	NCT03841110
[180]	Phase I	Biliary Tract Cancer	Allogeneic NK Cell	Completed	NCT03358849
[190]	Phase I	solid tumors	Expanded NK cell+ mAbs	Ongoing	NCT05069935
[191]	Phase I/II	Alzheimer's disease	autologous NK cells	Ongoing	NCT04678453
[192]	Phase I	Solid Tumors	Allogeneic NK Cell	Ongoing	NCT05027607
[193]	Phase I	Multiple Myeloma	iPSCs	Recruiting	NCT05182073
[186]	Phase I	B-cell acute lymphoblastic leukemia (B-ALL)	iPSC-derived	Recruiting	NCT04245722
[186]	Phase I	Acute myeloid leukemia (AML)	Cord Blood	Recruiting	NCT03940820
[186]	Phase I	AML and myelodysplastic syndromes (MDS)	Peripheral Blood	Recruiting	NCT04623944
[186]	Phase I	Relapsed/Refractory Solid Tumors	NK92 Cell Line	Recruiting	NCT05528341
[186]	Phase I	Metastatic Castration-Resistant Prostate Cancer	Human Primary NK Cells	Recruiting	NCT03692663
[186]	Phase I	Glioblastoma	NK92 Cell Line	Recruiting	NCT03383978
[186]	Phase I	Various Solid Tumors	Allogeneic NK Cells	Recruiting	NCT05395052

7. Conclusion

NK cells are emerging as a powerful tool in cancer immunotherapy due to their innate ability to target and eliminate tumor cells without prior sensitization. Recent advancements in NK cell engineering, such as CAR-NK cells, immune stimulants, and ex vivo expansion techniques, are enhancing their clinical potential. Additionally, NK cell-derived extracellular vesicles show promise in furthering anti-tumor effects. As research continues, NK cell-based therapies offer a promising approach to improving cancer treatment, with the potential to be integrated into clinical trials for more effective and targeted immunotherapy.

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