CHAPTER II

LITERATURE REVIEW

2.1 NK cell-based immunotherapy

Growing evidences in the field of adoptive immunotherapy have shed light on the impact of Natural killer cells (NK cells) in cancer treatment. In principle, NK cell-based immunotherapy is a type of cancer treatment that use NK cells to fight against cancer. The reason was that NK cells, named by their inheritance killing ability, are cytotoxic lymphocyte endowed with array of germline-encoded surface receptors that recognize and trigger cytotoxic response to virally-infected, stressed, or cancerous cells in the absence of prior sensitization or antigen presentation (Shaver *et al.*, 2021). Role in immunosurveillance of NK cells is mediated by the net balance between signals from activating receptors (e.g., natural killer group 2 member D (NKG2D), DNAX Accessory Molecule-1 (DNAM-1), and the natural cytotoxicity receptors NKp30, NKp44, and NKp46) and inhibitory receptors (e.g., killer immunoglobulin-like receptors (KIR) and NK group 2 member A (NKG2A)) (Hu *et al.*, 2019). To date, more than one tumor recognition models of NK cells have been proposed, figure 2.1: missing-self, induced-self, and antibody-dependent cell-mediated cytotoxicity (ADCC) model (Morvan *et al.*, 2016). When the activating signals overcome inhibitory signals (a process known as “licensing”), NK cells destroy target cells through an array of killing machineries, figure 2.2. Directly, NK cells lyse target cells through the release of lytic granules containing perforin and granzymes, expression of death ligands (death receptor pathway), and activation of the antibody-dependent cell-mediated cytotoxicity (ADCC) cascade. Indirectly, NK cells mediate their killing activities by secreting inflammatory cytokines (e.g., interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α)) and chemokines (e.g., interleukin-8 (IL-8), CCL5, and XCL1) to recruit and activate the adaptive immune response (Barnes *et al.*, 2021). This important linkage between innate (NK cells) and adaptive immune responses (e.g., T cells, B cells, dendritic cells) provides a complete framework for

establishing the cancer-immunity cycle, ensuring efficient control of cancer growth (Ramírez-Labrada *et al.*, 2022).

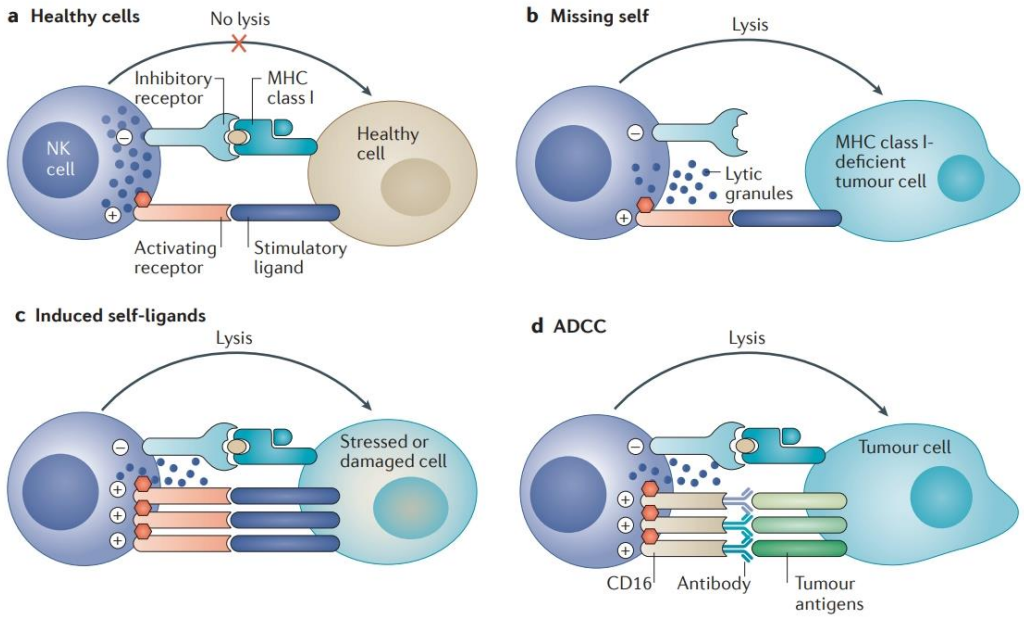

Figure 2.1 Tumor recognition models of NK cells. A) In healthy cells, prevention of self-lytic attack governs by inhibitory signals from inhibitory receptors that turn off cytotoxic responses of NK cells when recognize major histocompatibility complex (MHC) class I molecules on the surface of healthy normal cells. B) Missing self-recognition model; low or absent expression of inhibitory ligands (MHC class I molecules) on virally infected cells or transformed cells turn on NK cell cytotoxic responses. C) Induced self-recognition model; the expression of activating receptor ligands (e.g., MICA, MICB, UL16-binding protein, hemagglutinin of influenza virus (Vogler et al., 2022)) on damaged or stress cells led to the activation of NK cell cytotoxicity. D) ADCC model; the binding between FcγRIII receptor (CD16) of NK cells surface and antigen-specific antibodies initiate ADCC cascade. Reference picture from Morvan & Lanier, 2016.

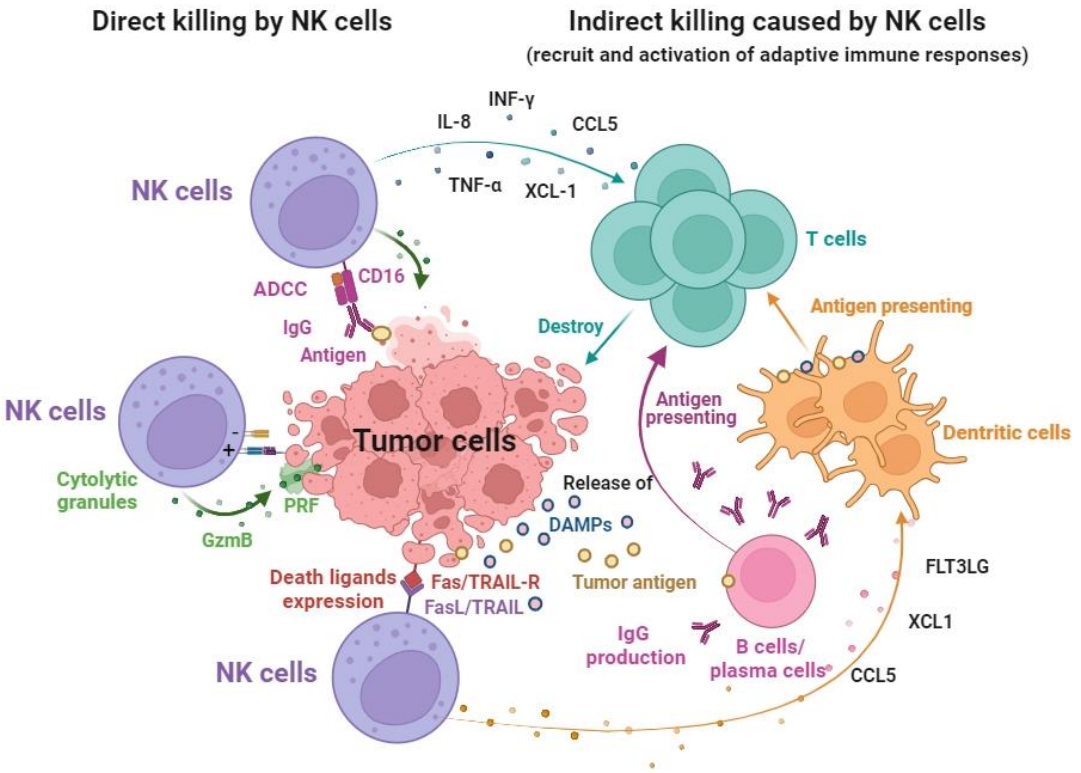

Figure 2.2 Mechanisms of NK cells cytotoxicity. After licensing, NK cells mediate cancer killing through both direct (left) and indirect (right) pathways. In the direct pathway, the elimination of target cells involves various mechanisms, such as synthesizing and releasing perforin (PRF; induces pore formation) and granzymes (GzmB; initiates apoptosis cleavage of the intracellular substrates), triggering cancer cell apoptosis through the expression of death ligands (Fas ligand; FasL and Tumor Necrosis Factor-related apoptosis-inducing ligand; TRAIL), and activating the ADCC cascade by expressing FcγRIII (CD16) to recognize target cells coated with antibodies (IgG). In the indirect pathway, NK cells recruit players from the adaptive immune system to mediate additional cancer elimination by secreting diverse types of immunomodulatory cytokines (e.g., interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and chemokines (e.g., interleukin-8 (IL-8), CCL5, and XCL1). After recognizing and engulfing Danger-Associated Molecular Patterns (DAMPs) and tumor antigens, dendritic cells subsequently present processed antigen fragments to

activate T cell responses. Upon stimulation by tumor antigens, B cells contribute to cancer elimination by performing dual functions: acting as antigen-presenting cells and producing antibodies (IgG) (Prokopeva *et al.*, 2024). Modified picture from Barnes *et al*., 2021. Created with BioRender.com.

The feasibility and safety of NK cells in cancer immunotherapy have been studied for several decades. In 1999, a pioneering study presented by Ruggeri and colleague demonstrated that donor NK cells exerted graft versus leukemia effects on recipient's target cells in acute myeloid leukemia patients underwent haploidentical hematopoietic stem cell transplantation (HSCT) (Ruggeri *et al.*, 1999). Since then, similar findings have been reported in different kinds of malignancy: acute myeloid leukemia, chronic myeloid leukemia, myelodysplastic syndrome, multiple myeloma, B cell non-Hodgkin lymphoma, neuroblastoma, renal cell carcinoma, melanoma, ovarian cancer, and breast cancer (reviewed in Shimasaki *et al.*, 2020).

2.2 Umbilical cord blood as a source of NK cells for cancer treatment

To observe a clinical benefit, sufficient numbers of NK cells with intact cytotoxic function are required. Several studies demonstrated that NK cells can be expanded from various cell sources; peripheral blood mononuclear cells (PBMCs), umbilical cord blood (CB), induced pluripotent stem cells (iPSCs), and NK cell lines (reviewed in Kundu *et al.*, 2021). Among all the options, considerable evidences suggested that CB are an attractive source of NK cells. Advantages of using CB cell source include, but not limited to, (i) CB contain substantial numbers of HSCs which possess the ability to generate all types of blood cells (Till *et al.*, 1961), (ii) aside from HSCs, CB contains a higher percentage of NK cells than peripheral blood; 15-20% versus 10-15%, respectively (Fang *et al.*, 2019), (iii) CB banking is currently available worldwide, (iv) CB units obtained from accredited cord blood bank were screened to be free form known specific infectious pathogens (Armitage, 2016), (v) fewer T cells exist along with lower risk of GvHD (Zhao *et al.*, 2020).

To exploit the therapeutic properties of umbilical cord blood-derived NK cells (CBNK cells) in cancer, completed and ongoing clinical trials have been or are being

undertaken (Table 2.1). Moreover, it was worth mentioning that the CBNK cells are currently commercially available as preserved living medicine. oNKord®, invented by Glycostem, is the first-generation of off-the-shelf cryopreserved CBNK cells product that received an orphan drug designation for AML from the United States food and drug administration (FDA) and European medicines agency (EMA) (Veluchamy, 2020). Results from phase 1 trial demonstrated that 4 out of 10 treated AML patients had a temporary disease-free period up to 60 months after infusion (Dolstra *et al.*, 2017). To further evaluate its safety and efficacy, this product is now move to phase I/IIa trial, refer to NCT04632316, in 2020.

Table 2.1 Cord blood-derived NK cells (CBNK cells) in clinical trials.

Non-genetically modified cells					
NCT number	**Cancer types**	**NK cells platform**	**Cell number**	**Phase**	**Status**
NCT01914263	ZR751, MCF7, HepG2, SMMC-7721, Hela, A375, DU145, H1299 and A549	Cytokine induced CBNK cells, cytokine induced PBNK cells	$8x10^9$ cells, single dose	I	Unknown
NCT03019640	MCL, B-cell NHL	Expanded CBNK cells	ND	II	Completed
NCT02955550	MM	Expanded CBNK cells	ND	I	Completed
NCT01729091	MM	Expanded CBNK cells	ND	II	Active, not recruiting
NCT02781467	AML	Expanded CBNK cells	ND	I	Terminated
NCT02280525	CLL	Expanded CBNK cells	1×10^7 cells/kg.	I	Completed
NCT02727803	CML, ALL, MDS, Leukemia, MM	Unexpanded CB	ND	II	Recruiting
NCT01464359	AML	T-cell depleted CB (double unit CB transplants)	ND	II	Terminated
NCT02781467	AML	Expanded CBNK cells (named PNK-007)	ND	I	Terminated
NCT04632316	AML	Expanded CBNK cells (named oNKord®)	ND	I/II	Recruiting

Table 2.1 (Cont).

Genetically modified cells					
NCT number	**Cancer types**	**NK cells platform**	**Cell number**	**Phase**	**Status**
NCT04347616	AML	Expanded CBNK cells combination with subcutaneous IL-2	1.0-3.0 x 10^9 cells	I/II	Recruiting
NCT03420963	Relapsed or refractory solid tumors	Expanded CBNK	ND	I	Recruiting
NCT05110742	T-cell malignances, MCL, CLL	CD5-IL15 CAR-CBNK cells	1 x 10^7 cells, 1 x 10^8 cells, 1 x 10^9 cells, 1 x 10^{10} cells	I/II	Not yet recruiting
NCT05092451	B cell lymphoma, MDS, AML	CAR.70/IL15-transduced CB-NK	ND	I/II	Recruiting
NCT04796675	ALL, CLL, NHL	CD19 CAR-CBNK cells	1 x 10^5 cells, 1 x 10^6 cells, 1 x 10^7 cells	I	Recruiting
NCT03056339	B cell lymphoma, ALL, CLL, NHL	CD19-CD28-zeta-2A-iCasp9-IL15- CAR-CBNK cells	ND	I/II	Completed
NCT04991870	Glioblastoma	TGF-betaR2 -/NR3C1 - CBNK cells	ND	I	Recruiting
NCT05842707	B-cell NHL	DualCAR-NK19/70	ND	I/II	Recruiting
NCT05667155	B-cell NHL	CB dualCAR-NK19/70	2×10^6 cells/kg, 4×10^6cells/kg, 8×10^6 cells/kg	I	Recruiting
NCT05472558	B-cell NHL	CB anti-CD19 CAR-NK	2×10^6 cells/kg, 3×10^6 cells/kg, 4×10^6 cells/kg	I	Recruiting
NCT06083883	Synovial cell sarcoma, myxoid/round cell liposarcoma	NY-ESO-1 TCR/IL-15 NK	ND	I/Ib	Not Yet Recruiting

Table 2.1 (Cont).

Genetically modified cells					
NCT number	**Cancer types**	**NK cells platform**	**Cell number**	**Phase**	**Status**
NCT06066424	Solid tumor (breast)	TROP2-CAR-NK	ND	I	Recruiting
NCT06066359	MM	NY-ESO-1 TCR/IL-15 NK	ND	I/II	Recruiting
NCT03579927	B-cell NHL	CAR CD19-CD28-zeta-2A-iCasp9-IL15-transduced CBNK	ND	I/II	Withdrawn
NCT05703854	RCC, mesothelioma, osteosarcoma	(CAR).70/interleukin (IL)15-transduced CBNK	ND	I/II	Recruiting
NCT05922930	Ovarian cancer, mesonephric-like adenocarcinoma, pancreatic cancer	TROP2- CAR-NK	ND	I/II	Recruiting

Data from ClinicalTrial.gov, accessed on 8/2/2024, search term: cord blood-derived NK cells, cancer, and oNKord. Abbreviation: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukemia; HCC, hepatocellular carcinoma; MCL, mantle cell lymphoma; MDS, myelodysplastic syndromes; MM, multiple myeloma; ND, not determined; NHL, non-hodgkin's lymphoma; RCC, renal cell carcinoma.

2.3 CBNK cells expansion platform

Different platforms have been proposed to generate clinically relevant CBNK cell dose for adoptive cell therapy. Either artificial antigen-presenting cells (aAPCs or feeder cells)-based techniques or cytokines-based techniques have been reported.

2.3.1 Feeder cell-based techniques

In 2013, Shah and colleague have shown that cord blood derived mononuclear cells (CB-MNCs) can be expanded and can give rise to a mean of 1,848-fold and 2,389-fold expansion of NK cells (with > 95% NK cell purity) from fresh and cryopreserved CB, respectively (Shah *et al.*, 2013). In this study, the expansion took

place in GP500 bioreactor in the presenting of 100 IU/ml IL2 and K562-based aAPCs expressing membrane bound (mb) IL-21 “Clone9.mbIL21” at 2:1 aAPCs:cell ratio. Immunomagnetically CD3-depletion were performed on day 7 and the expansion were last for a total of 14 days. In 2015, Vasu *et al.* were demonstrated that a median of 6,092 x 10^6 $CD3^-CD56^+$ NK cells (range between 165–20,947 x 10^6 cells) can be obtained from only 1 ml of post-thaw CB unit after 35 days of expansion (Vasu *et al.*, 2015). In this study, the authors developed a selective access to cryopreserved samples device and employed the use of irradiated Epstein-Barr virus-transformed human B-cell lymphoblastoid cell lines, at 20:1 aAPCs:cell ratio, to stimulate and to expand 1 x 10^6 CD3-depleted total nuclear cells obtained from thawed CB in the medium containing 500 IU/ml IL2. A further study to evaluate the potential use of aAPCs to expand CBNK cells was published in by Ayello *et al.*, 2017 . In their study, they used irradiated genetically modified K562 cells expressing the NK-stimulatory molecules 4-1BB ligand and interleukin 15 (also known as K562-mbIL15-41BBL cell line) to co-cultured with CB-MNCs, at 1:1 aAPCs:cell ratio, in RPMI-1640 medium containing 10 IU/ml IL2. After 7 days of expansion, they demonstrated that the percentage of $CD3^-CD56^+$ NK cells significantly increased from 3.9% (at day 0) to 71.7±3.9%, 42.6±5.9%, and 9.7±2.4% in culture conditions contained K562-mbIL15-41BBL cells, wide-type K562 cells, and media alone, respectively. In addition, the same study also showed that the *in vitro* cytotoxic activity against B-cell non-Hodgkin lymphoma (B-NHL) of K562-mbIL15-41BBL-expanded CBNK cells was significantly higher than wide-type K562-expanded CBNK cells (40±3.1 vs. 18±2.1, respectively). Although a remarkable potential to expand CBNK cells has been reported from all of these studies, the use of tumor cell derived aAPCs/feeder cells rise a safety concern on final product purity (Halme *et al.*, 2006).

2.3.2 Cytokines-based techniques

Cytokines-based approaches to generate large numbers of functionally competent CBNK cells have been described since 2010. Xing and team showed that an average 92-fold (range between 39–112-fold) expansion of CBNK cells could be obtained from $CD3^-CD56^+$ sorted CB-MNC cells after 14 days of culture in RPMI-1640 medium containing 10% human serum and 500 IU/ml IL2 (Xing *et al.*, 2010). In the same year, Spanholtz *et al*. published a two-step cytokine-based protocol that could

efficiently generated more than 10,000 x 10^6 CBNK cells from freshly selected $CD34^+$ CB-MNC cells (Spanholtz *et al.*, 2010). In this study, $CD34^+$ CB-MNC cells were first immunomagnetically sorted and expanded for 14 days in medium containing 10% human serum and cytokine cocktails: 10 pg/ml GM-CSF, 250 pg/ml G-CSF, 50 pg/ml LIF, 200 pg/ml MIP-Iα, 50 pg/ml IL6, 27 ng/ml SCF, 25 ng/ml FLT-3L, 25 ng/ml TPO, 20 ng/ml IL15, and 25 ng/ml IL7. These cells were then differentiated and further expanded for another 21 days in NK cell differentiation medium containing 10% human serum and cytokines: 10 pg/ml GM-CSF, 250 pg/ml G-CSF, 50 pg/ml LIF, 200 pg/ml MIP-Iα, 50 pg/ml IL6, 20 ng/ml IL7, 22 ng/ml SCF, 1,000 IU/ml IL2, and 20 ng/ml IL15. Next, in 2012, Tanaka *et al.* reported that proliferation of CBNK cells was achieved by supplementation of 10 ng/ml IL15, 5 ng/ml IL2, 10 – 1,000 ng/ml anti-CD3 monoclonal antibody (clone OKT3), 0.02 - 0.1 ng/ml tacrolimus and 5 – 10 IU/ml dalteparin sodium to the medium. (Tanaka *et al.*, 2012). In this study, the authors highlighting the beneficial effects of tacrolimus and dalteparin sodium as a T cell anti-proliferation and NK cell stimulation agent, respectively. After 3 weeks of expansion, about 40 x 10^6 CBNK cells with the maximum of 72.8% NK purity were obtained. In addition, Ma *et al*. found that a combination of high concentration of IL2 (2,000 IU/ml) and 5 μM zoledronate could possibly induce a greater expansion of CBNK cells (average 1,286-fold) from CB-MNCs (Ma *et al.*, 2018). Without cell sorting, this method yields up to 80.46% NK cell purity after 21 days of culture. Last but not least, Mu *et al.* employed the use of immunopotentiator extracted from group A streptococcus to reinforce CBNK cell proliferation (Mu *et al.*, 2019). In this study, CB-MNCs were first stimulated for 3 days in AIM-V medium supplemented with 2,000 IU/ml IL2, 0.01 KE/ml group A streptococcus, and 5 μM zoledronate. Then, the activated cells were expanded in the same medium containing only 2,000 IU/ml IL2 for 21 days. In the end of the process, this method could produce up to an average of 15,900 x 10^6 CBNK cells with more than 90% NK cell purity.

2.4 Approches to improve CBNK cell expansion

2.4.1 Notch signaling activator

During NK cell development, NK cell follow a carefully orchestrated journey starting from $CD34^+$ HSCs to common lymphoid progenitors and subsequently

to NK cell progenitors (Bi *et al.*, 2020). The pleiotropic functions of NOTCH signaling pathway have long been reported to plays an essential role in various physiological processes include cell proliferation, cell fate determination, and cell death (Wang, 2011). Activation of Notch signaling combined with NK associated cytokine milieu have been reported to have a significant role in *in vivo* NK cell development (Bachanova *et al.*, 2009; Rolink *et al.*, 2006). Under feeder-free expansion system and in the presence of Notch ligand delta 4, IL7, SCF, and FLT-3L, human cord blood $CD34^+$ cells could effectively differentiate into functional NK cells (Haraguchi *et al.*, 2009). A significant therapeutic value of small molecules that modulate Notch pathway has been reviewed by Sail *et al.*, 2012 . Azelaic acid, a nine-carbon dicarboxylic acid NOTCH signaling activator, have been found to promote the proliferation of PBNK cells (Dongdong *et al.*, 2019). Under a concentration of 10 μM/ml azelaic acid plus 100 IU/ml IL2, $CD3^-CD56^+$ sorted PBNK cells could proliferate nearly 1.5-fold after 24 hrs. of stimulation. Beyond PBNK cell proliferation, the aforementioned study highlighted that an additional advantage of using small molecules is a conformational stability of the substance. Among a larger collection of a newly synthesized small molecule drug, Yhhu-3792 is a 2-phenylamino-quinazoline-based compound (figure 2.3) which potentially activates the NOTCH signaling pathway. In mouse neural stem cell model, Yhhu-3792 treatment led to an increase in the diameter and number of neurospheres compared with the untreated group (Lu *et al.*, 2018). Based on the positive effect on promoting the self-renewal of neural stem cells, Yhhu-3792 is currently of great interest for its potential additional clinical applications.

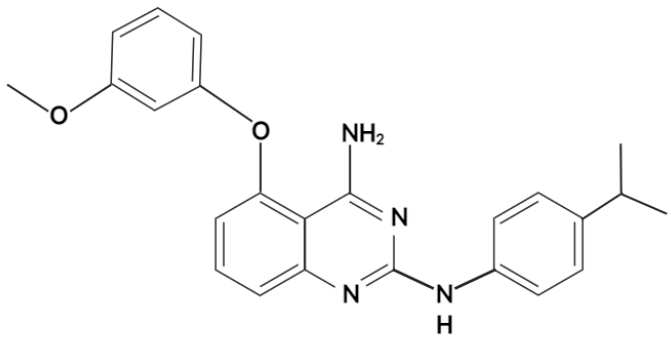

Figure 2.3 Structure of Yhhu-3792. Created with BioRender.com.

2.4.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major structural component of an outer membrane of most gram-negative bacteria envelope that owes an ability to stimulate the immune response (Bertani *et al.*, 2018). Typically, LPS consist of 3 structural domains named Lipid A, core oligosaccharide, and O antigen (figure 2.4). A line of evidence suggests that LPS is a potent activator of macrophages, T cells, and NK cells (Conti *et al.*, 1991; Mattern *et al.*, 1994; McAleer *et al.*, 2008). Study underpinning the role of LPS on PBNK cell proliferation has been published. In 2000, Goodier and Londei demonstrated that the percentage of $CD3^{-}CD56^{+}$ PBNK cells increased from 10.5±3.5% (range 3.8-16.7%) to 20.9±11.3% (range 9.1-48.4%) after stimulated with 1 μg/ml LPS for 9 days, before and after stimulation respectively (Goodier *et al.*, 2000). Later, it was discovered that LPS can directly promote IFN-γ production of PBNK cells (Kanevskiy *et al.*, 2013). Moreover, in the field of vaccine industry, synthetic LPS have long been pursued as a adjuvant of human vaccine (Zariri *et al.*, 2015).

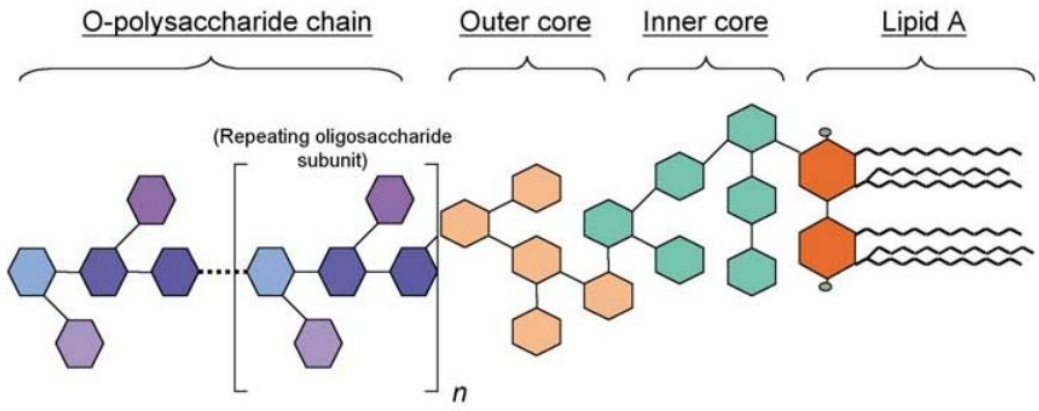

Figure 2.4 Structure of lipopolysaccharide. The classic LPS molecule consists of three main structural components: lipid A, core oligosaccharide, and O antigen. The immunogenic activity of LPS varies among different bacterial species, depending on the variation in lipid A and O antigen structure (Lin *et al.*, 2020). Reference picture from Erridge *et al.*, 2002.

2.5 Challenges in generating CBNK cells for adoptive cell transfer

2.5.1 Anti-cancer activity of CBNK cells

Although adoptive cell transfer (ACT) of *ex vivo* expanded CBNK cells is an attractive alternative source to PBNK cells, an important consideration when using CBNK cells is the functional activity of the cells. It has been reported that variations in the phenotype and function of NK cells exist among those derived or isolated from different sources: PBNK cells, CBNK cells (expanded from $CD34^+$ or $CD56^+$ sorted populations), and iPSC-derived cells, figure 2.5 (Goldenson *et al.*, 2020).

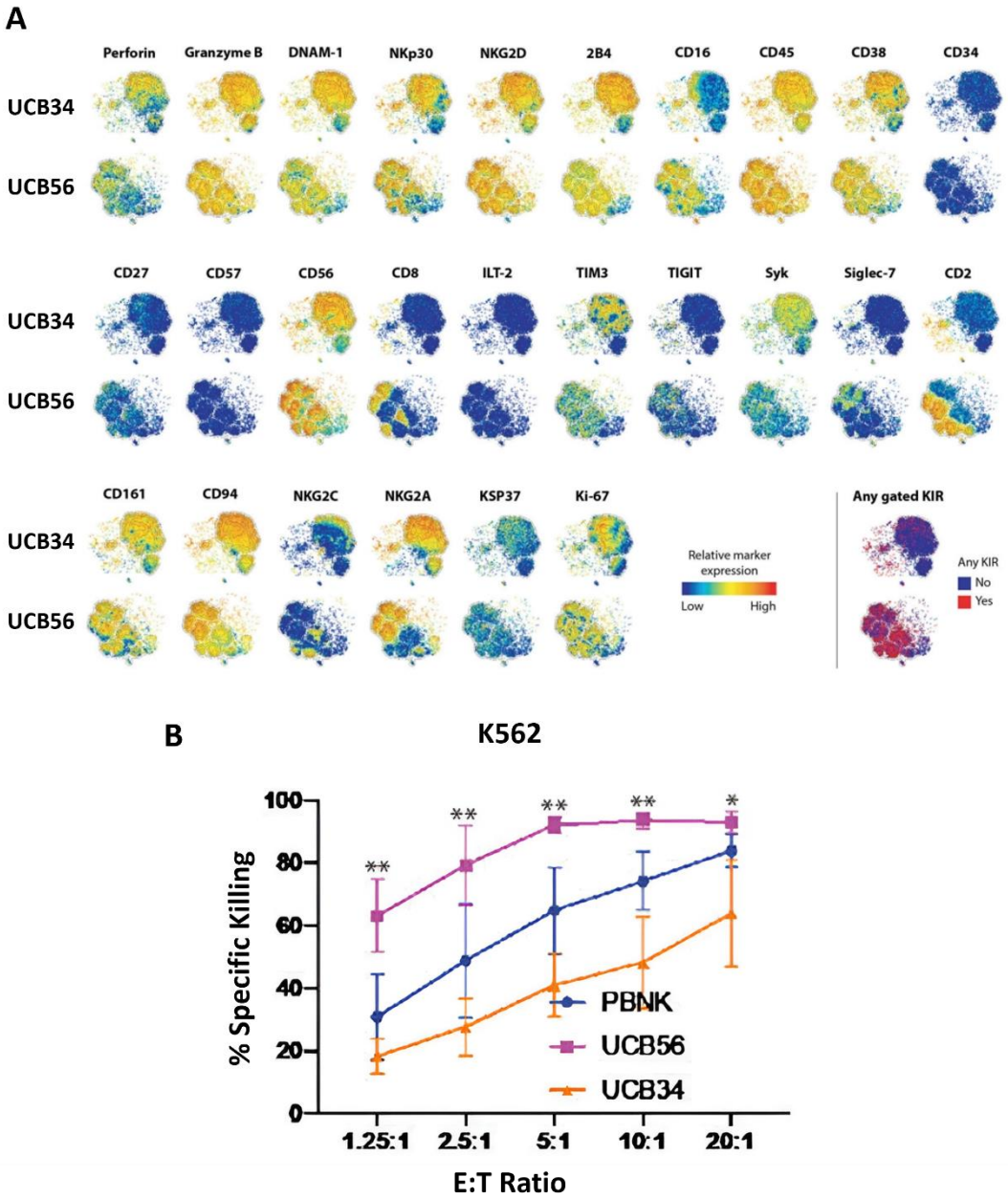

Figure 2.5 Distinct characteristics of NK cells. A) Representative t-distributed Stochastic Neighbor Embedding (t-SNE) plot from mass cytometry analysis showing the expression of NK cell surface markers in NK cells expanded from $CD34^+$ (UCB34) and $CD56^+$ (UCB56) populations obtained from the umbilical cord blood of the same donor. B) Analysis of NK cell killing activity using caspase-3/7 green apoptosis assay. UCB34-NK, UCB56-NK, and PBNK cells show differences in their cytolytic ability against K562 cells. Reference from Goldenson *et al*., 2020.

As shown in figure 2.5A, results from mass cytometry analysis reveal significant differences in the expression of CD16 and CD2 between NK cells expanded from CD34+ (UCB34) and CD56+ (UCB56) sorted populations, irrespective of their shared UCB origin (Goldenson *et al.*, 2020). Moreover, the same study also shows that UCB56 derived NK cells (UCB56-NK) displayed a more potent cytotoxicity compared to PBNK cells and UCB34 derived NK cells (UCB34-NK) (figure 2.5B). Inaddition, anti-cancer activity of the expanded CBNK cells varies between studies. In 2012, Luevano *et al*. demonstrated that expanded CBNK cells failed to lyse K562 cells and produced less IFN-γ than PBNK cells. (Luevano *et al.*, 2012). This lower activity could be explained by their immature phenotype, marked by the downregulation of key activating receptors (e.g. NKG2D, CD16, NKp30, NKp44 and NKp46) coupled with the upregulation of inhibitory receptors (e.g. NKG2A) (Shokouhifar *et al.*, 2021). Conversely, efficient expansion procedures for the production of functionally competent CBNK cells have been reported. For example, Kang *et al*. developed a 21-day feeder cell-based cultivation process, yielding a substantial number of highly cytotoxic CBNK cells that have the ability to kill a wide variety of tumor cell models (figure 2.6) (Kang *et al.*, 2013).

Taken together, all the studies described above indicate unique characteristics (both phenotype and function) of the expanded CBNK cells obtained from different approaches. Therefore, it would be indispensable to assess the anti-cancer activity of the expanded CBNK cells before transferring this technology into a clinical setting.

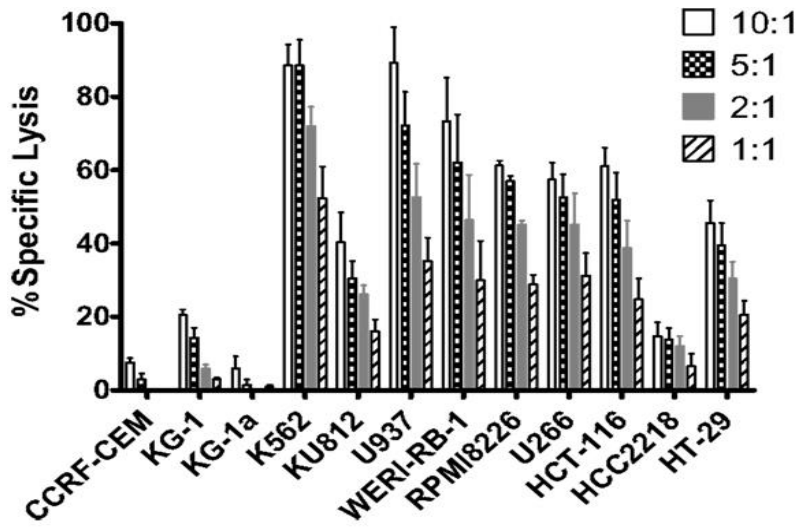

Figure 2.6 Anti-cancer activity of the expanded CBNK cells. Results of the lactate dehydrogenase assay demonstrated that expanded CBNK cells destroy target cancer in a dose-dependent manner. Reference picture from Kang *et al*., 2013.

2.5.2 Manufacturing practice of CBNK cells

To fulfill the fast-paced development of NK cell-based immunotherapy, common challenges in the cell therapy industry include scaling up production and implementing quality controls and release criteria. Due to the nature of adoptive cell therapy (ACT) as a living treatment, its success depends on both the appropriate dosage and the integrity of the transferred cells (figure 2.7). In a clinical trial setting, the number of transferred NK cells ranges from 5×10^6 to 1×10^8 cells per kilogram of body weight (Heipertz et al., 2021). Given this range, a single dose of NK cell treatment for an adult weighing 70 kilograms is between 35×10^7 to 7×10^9 cells. Therefore, the commercial-scale production of NK cell products requires a large-scale expansion system that can handle liters, hundreds, or thousands of liters of culture working volume per batch. To reach this goal, bioreactors are now the favored choice in NK cellmanufacturing, facilitating efficient and scalable production processes.

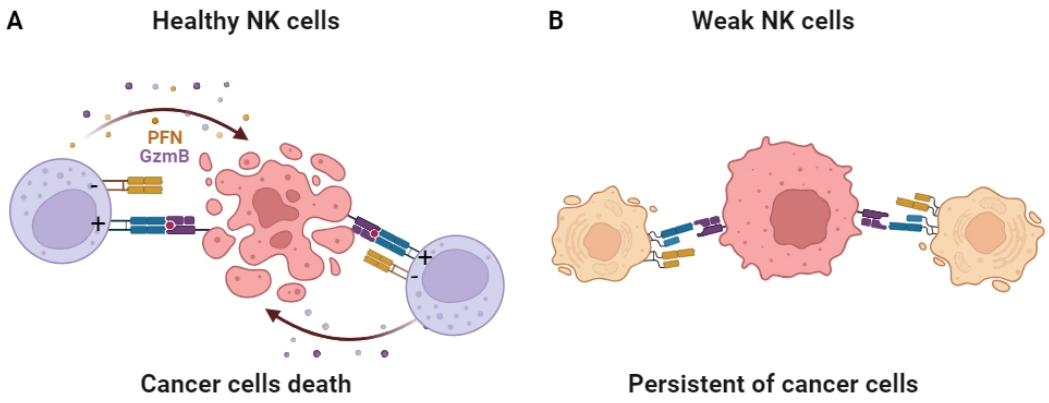

Figure 2.7 Efficiency of the living treatment. Healthy NK cells mediate effective cancer killing (A), while weak or nearly dying NK cells exert minimal to no response to cancer cells (B). Created with BioRender.com.

In addition, to comply with Good Manufacturing Practice (GMP) standards, it is advisable to choose a single-use, closed, and disposable cell production kit with an automatic system (Garcia-Aponte *et al.*, 2021). This helps avoid most technical complexities, such as handling multiple T-flasks or bags simultaneously, and minimizes the risk of errors and contamination. To date, automatic and semi-automatic manufacturing systems that have been reported to support NK cell production include CliniMACS Prodigy® from Miltenyi Biotec (Albinger *et al.*, 2024; Granzin *et al.*, 2015), Zellwerk's Z®RP platform from Zellwerk GmbH (Bröker *et al.*, 2019), and Xuri Cell Expansion System W25 from Cytiva (Veluchamy, 2020). The existence of batch-to-batch variation in NK cell products stems from various factors, including variations in the phenotypes of initially seeded cells, differences in cytokine and medium due to lot-to-lot inconsistency, and variations in process conditions such as dissolved oxygen, carbon dioxide, and pH. Therefore, it would be wiser to employ process analytical technology (e.g., raman spectroscopy, sequential injection capillary electrophoresis, optical biosensors, electrical impedance, etc.) to monitor and control the cell expansion process (Garcia-Aponte *et al.*, 2021). In line with this, real-time in-line monitoring, label-free, and contact-free techniques are more favorable to consider.

Like many types of medicinal products, quality controls and release criteria should be taken into great consideration. Since regulations and regulatory agencies can vary from country to country, the cell therapy industry should understand and follow the regulatory framework and legislation for cell therapy products in each country. The two biggest regions leading in this field are the European Union (EU) and the United States (US). In EU, cell therapy products were classified as "advanced therapy medicinal products" (ATMPs), while in US, NK cell products were classified as Cell and Gene Therapy Products (CGT) (Salazar-Fontana, 2022). The classification and definition of ATMPs and CGT products also differ. For instance, ATMPs are divided into somatic cell therapy medicinal products (SCTMP), tissue-engineered products (TEP), gene therapy medicinal products (GTMP), and combined ATMPs (cATMPs), while CGT products are simply categorized into two groups: gene therapy and cellular therapy products. EMA/CHMP/BWP/271475/2006 rev.1 and FDA-2008-D-0520 are specific guidance for cell therapy Industry on potency testing of ATMPs and CGT products, respectively (Iglesias-Lopez *et al.*, 2019). Overall, cardinal requirements of these guidelines include the identity, quality, purity, strength, and stability of the product.

Lastly, but most importantly, storing and distributing off-the-shelf NK cell products may inevitably require a cryopreservation process. In the cell therapy industry, cryopreservation is a useful technique that facilitates long-term storage of the living cells. Technically, cryopreservation provides extra time for many vital activities, including quality tests, donor and recipient matching (if needed), long-distance product delivery, and multiple treatments of the same batch. Unfortunately, the common problems of cryopreserved NK cells include cell loss caused by multiple washing step of freezing/thawing process, cell damage due to the toxicity of cryoprotectant agents (CPAs) (Lamers-Kok *et al.*, 2022), and a significant drop in cytotoxicity. Recently, results from the standard 2-dimensional (D) chromium release assay on fresh and cryopreserved (frozen-thawed) expanded NK cells demonstrated that the cytotoxicity of cryopreserved NK cells significantly declined when compared to fresh NK cells, figure 2.8A (Mark *et al.*, 2020). To investigate the underlying mechanisms of this decrease, the same study employs a 3-D collagen gel matrix coupled with time-lapse imaging and reveals that the loss in antitumor activity of the cryopreserved NK cells is caused by a reduction in motile NK cells, figure 2.8B. To

solve this problem, a variety of approaches have been reported, Table 2.2 (modified from Saultz *et al.*, 2023).

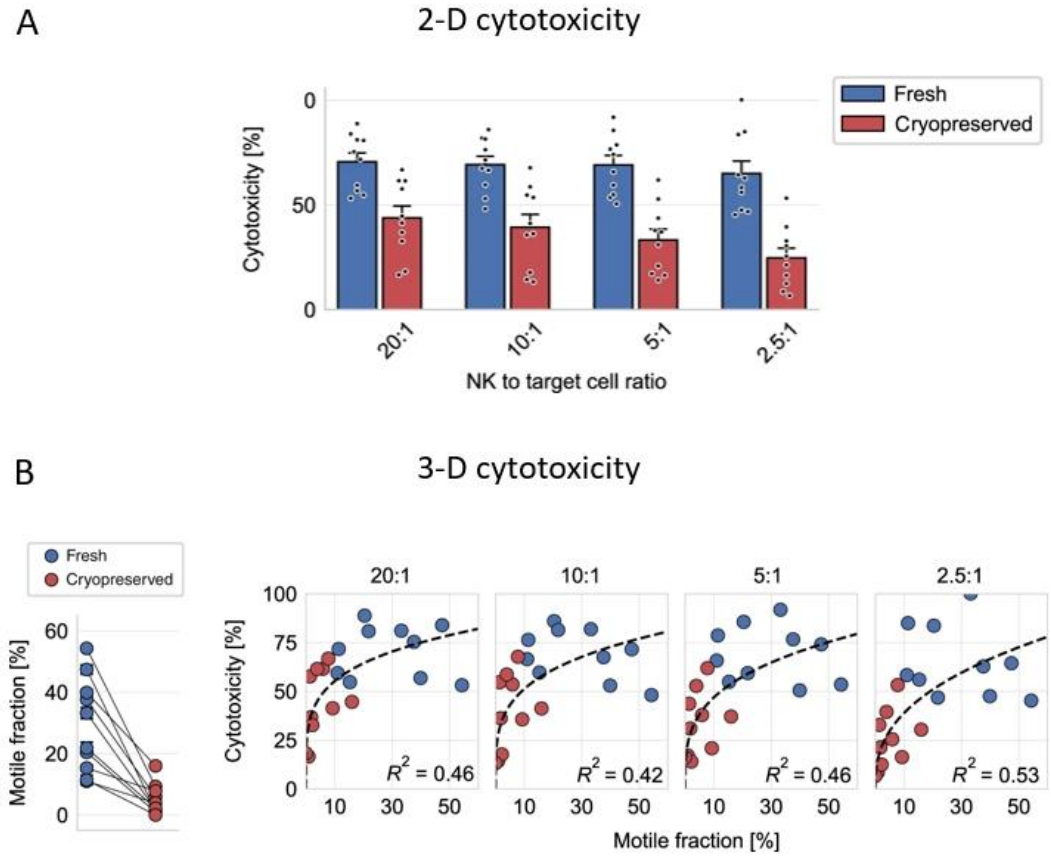

Figure 2.8 Effect of cryopreservation on NK cell cytotoxicity. A) In 2-D conditions, decreased cytotoxicity was observed on cryopreserved NK cells but not on fresh NK cells. B) Results of pairwise experiments on 3-D cell motility (left) and 3-D cell cytotoxicity (right) reveal that the fraction of motile NK cells decreased after cryopreservation, and this reduction is significantly related to lower cytotoxicity. For all tested NK-to-target cell ratios. Reference picture from Mark et al., 2020.

Table 2.2 Different approaches for a better outcome of NK cells post-thaw recovery.

No.	Cell concentration/cryomedia/container	Freezing/storage condition	Thawing /post-thaw recovery	Outcome	Reference
1.	**Cell density:** 1-5×10^6 cells/mL. **Cryomedia 1:** human AB serum + 10% DMSO **Cryomedia 2:** DMEM + 10% human AB serum + 10% DMSO **Container:** ND	**Freezing:** ND **Storage:** LN_2	**Thawing:** 1:10 dilution in media **Post-thaw:** ND	**Cell viability:** does not affected **Cell cytotoxicity:** does not affected	(Domogala *et al.*, 2016)
2	**Cell density:** 1×10^8 cells/mL. **Cryomedia:** RPMI 1640 + 20% albumin + 25% dextran-40 + 5% DMSO **Container:** ND	**Freezing:** ND **Storage:** LN_2	**Thawing:** 37°C water bath, slow diluted with RPMI1640 + 10% FBS **Post-thaw:** no recovery step (analyzed immediately)	**Cell viability:** ruduce from 95.9%±0.5% to 92.2%±0.8% **Cell cytotoxicity:** NS between fresh and cryopreserved NK cells.	(Min *et al.*, 2018)
3	**Cell density:** 1-2.5×10^7 cells/mL. **Cryomedia:** 50% RPMI1640 + 40% FBS, 10% DMSO **Container:** Cryotube vial	**Freezing:** by Mr. Frosty™ freezing container (-80°C, overnight) **Storage:** LN_2	**Thawing:** 37 °C water bath, dropwise 5-fold dilution in RPMI 1640 + 10% FBS + 1% antibiotic **Post-thaw:** recovery in RPMI 1640 + 10% FBS + 1% antibiotic +100 IU/mL IL2 (16 h, overnight thaw)	**Cell viability:** does not affected and can be recovered **Cell cytotoxicity:** thawed and rested (16 h) NK cells showed no decrease in cytotoxicity compared to fresh NK cells.	Oyer *et al.*, 2022

Abbreviation: CRF, Controlled Rate Freezer; DMSO, Dimethyl sulfoxide; FBS, fetal bovine serum; ND, not determined; NS, not significant different; RPMI 1640, Roswell Park Memorial Institute 1640 medium

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