MODIFICATIONS OF NK-92MI CELL LINE THROUGH THIRD GENERATION LENTIVIRAL VECTOR

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MODIFICATIONS OF NK-92MI CELL LINE THROUGH THIRD GENERATION LENTIVIRAL VECTOR

by

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LIST OF SYMBOLS

%	Percentage
*	Asterisk
+	Plus
=	Equal to
±	More or less
×	Multiply
×g	Times gravity
÷	Divide
⩽	Less than or equal to
°C	Celsius
µg	Microgram
µL	Microliter
µm	Micrometre
bp	Base pair
g	Gram
L	Litre
M	Molar
mL	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
™	Trademark
V	Volt

ƶ	Zeta
α	Alpha
β	Beta
γ	Gamma
ε	Epsilon

LIST OF ABBREVIATIONS

ADAM17	A-disintegrin and metalloproteinase-17
ADCC	Antibody dependent cellular cytotoxicity
AML	Acute myeloid leukemia
ASCT-2	Sodium dependent neutral amino acid transporter-2
BiKE	Bispecific killer engager
$CaCl_2$	Calcium chloride
CAR	Chimeric antigen receptor
CAR-NK	Chimeric antigen receptor bearing natural killer
cDNA	Complementary DNA
CIK	Cytokine-induced killer cell
CO_2	Carbon dioxide
CRS	Cytokine release syndrome
dH_2O	Distilled water
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC2	Extracellular domain 2
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
FACS	Fluorescence assisted cell sorting
FasL	Fas ligand
FBS	Foetal bovine serum
Fc	Crystallisable fragment
FSC	Forward scatter

GFP	Green fluorescence protein
GvHD	Graft versus host disease
HeBSS	HEPES buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HSC	Hematopoietic cell
HSCT	Hematopoietic stem cell
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iPSC	Induced pluripotent stem cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor G1
LDL-R	Low density lipoprotein-receptor
LGL	Large granular lymphocyte
LIR	Leukocyte inhibitory receptor
LLT1	Lectin-like transcript
LTR	Long terminal repeat

mAb	Monoclonal antibody
MAC	Membrane attack complex
MFI	Median fluorescence intensity
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MMP25	Membrane type-6 matrix metalloproteinase
MOI	Multiplicity of infection
MPSIIIA	Mucopolysaccharidosis type IIIA
mRNA	Messenger ribonucleic acid
MV	Measles virus
NCBI	National Center for Biotechnology Information
NCR	Natural cytotoxicity receptor (NCR)
NIH	National Institutes of Health
NK	Natural killer
NKG2D	Natural killer group protein 2 family member D
NS	Netherton syndrome
OD	Optical density
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PI	Phosphatidylinositol
PMN	Polymorphonuclear neutrophil

ppt	Polypurine tract
PQC	Performance quality control
RCV	Replication competent virus
RE	Restriction enzyme
RNA	Ribonucleic acid
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev-responsive element
RSV	Rous sarcoma virus
SD	Standard deviation
SFFV	Spleen forming focus virus
SIGLEC7	Sialic-acid-binding immunoglobulin-like lectin
SIV	Simian immunodeficiency virus
SNV	Single nucleotide variation
SSC	Side scatter
TIR	Terminal inverted repeat
TNF-α	Tumour necrosis factor-α
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis inducing ligand
TriKE	Trispecific killer engager
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
VSV-G	Vesicular stomatitis virus glycoprotein G
WPRE	Wooden hepatitis virus post-transcriptional regulatory element

αMEM	Alpha minimum essential medium

MODIFIKASI SEL NK-92MI MELALUI VEKTOR LENTIVIRAL GENERASI KETIGA

ABSTRAK

Sel pembunuh semulajadi (NK) ialah limfosit sitotoksik yang memainkan peranan penting dalam imuniti semula jadi dan adaptif. Kajian berkaitan mekanisme dan interaksi sel dihamparkan oleh proses pengekstrakan sel primer yang sukar dan ia memaparkan fenotip yang tidak konsisten disebabkan oleh variasi genetik. Sel line NK seperti NK-92MI boleh digunakan sebagai pengganti tetapi ia kekurangan ciri-ciri seperti reseptor-reseptor CD16 bagi ADCC. Pengubahsuaian genetik melalui vektor lentiviral generasi ketiga mempunyai profil keselamatan yang lebih baik. Sistem lentiviral RRL telah diuji dalam pelbagai jenis sel, tetapi sel NK masih belum diuji. Dalam kajian ini, sel line NK-92MI telah ditransduksi dengan lentivirus yang membawa gen GFP intraselular atau reseptor CD16, transduksi juga dilakukan dengan penambahan polibren. Keputusan kajian menunjukkan ekspresi GFP pada purata 3.49 % tanpa polibren dan purata ini meningkat kepada 7.18 % apabila polibren ditambah. Ekspresi ekstraselular CD16 berpurata pada 1.03 % tanpa polibren dan meningkat kepada purata 3.12 % apabila polibren ditambah. Penambahan polibren telah meningkatkan ekspresi sebanyak dua kali ganda. Kajian ini menunjukkan kebolehlaksanaan menggunakan vektor lentiviral RRL generasi ketiga untuk mengubahsuai sel NK. Pengoptimasian lanjut diperlukan untuk meningkatkan ekspresi transgen sebelum sel ini boleh digunakan bagi ujian *in vitro*, seperti assay ADCC. Keupayaan untuk mengubahsuai genetik sel line NK membolehi penyesuaian sel NK pada masa hadapan dan menyediakan sumber sel yang konsisten bagi kajian sel NK.

MODIFICATIONS OF NK-92MI CELL LINE THROUGH THIRD GENERATION LENTIVIRAL VECTOR

ABSTRACT

Natural killer (NK) cells are cytotoxic lymphocytes that play important roles in both our innate and adaptive immunities. Studies of NK cell mechanisms and interactions were hampered by the laborious extraction process for primary cells, and inconsistent phenotypes due to genetic variations. NK cell line such as the NK-92MI can be used as substitutes but they lack features such as CD16 receptors, disabling their ADCC function. Thus, cell genetic modifications will be required. Compared to other techniques, third generation lentiviral vectors have a superior safety profile. The RRL lentiviral system was tested within clinical settings for a variety of cell types, but NK cells have not been tested. In this study, NK-92MI cell line was transduced with RRL lentivirus carrying the genes for either intracellular GFP or surface CD16 receptor, with the addition of polybrene polycation. Results showed that GFP expression averaged at 3.49 % without polybrene and averaged at 7.18 % when polybrene was added. Extracellular CD16 expression averaged at 1.03 % without polybrene and increased to an average of 3.12 % with polybrene. The addition of polybrene had significantly improved expression levels in NK-92MI cells and nearly doubled the percentages of transgene expression. This study demonstrated the feasibility of using the third generation RRL lentiviral vector for NK cell transduction. Further optimisations will be required to improve transgene expression levels before the CD16-positive NK-92MI cells can be used for *in vitro* testing, such as ADCC assays. The ability to modify NK cell lines may pave the way for future NK cell customisations and provide a consistent source of cells for NK cell studies.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Natural killer or NK cells are granular lymphocytes that play an important role in both the innate and adaptive immune systems. They are involved in the immune response towards virally infected cells and immune surveillance for abnormal cells such as tumours (Iannello et al., 2008). Human NK cells are found mainly in peripheral blood, but are also present in bone marrow, liver, uterus, spleen, lungs, lymphoid tissues, and the thymus (Yu et al., 2013). Circulating NK cells such as those in peripheral blood survey the human body for pathogen-infected and malignant cells. NK cells can recognize these targets and be directly cytotoxic against them without highly specific receptors. They could also detect for ligands such as the absence of major histocompatibility complex class I (MHC-I) on the surface of target cells and subsequently kill those cells (Sun & Lanier, 2011; Terunuma et al., 2008).

A wide range of activating and inhibitory receptors are present on the NK cell surface that control the activation state and subsequent functions of NK cells. Activation receptors include the major activating receptor CD16, natural cytotoxicity receptors (NKp46, NKp44), C-type receptors (NKG2C, NKG2D) and Ig-like receptors (2B4). The inhibitory receptors include killer immunoglobulin-like receptor (KIR), Ig-like receptors (CD158), C-type lectin receptors (NKG2A) and leukocyte inhibitory receptors (LIR1, LAIR-1) (Mandal & Viswanathan, 2015). The balance between activating and inhibiting signals transmitted within the cell will dictate the activation state and regulate the development of its cytotoxic capacity

(Kärre et al., 1986). When NK cells are activated, they release perforins that form pores within the target cell membrane, similar to the membrane attack complex (MAC) formed by the immune complement system. NK cells can also release granzymes and other cytokines that enter target cells through pores, initiating the caspase cascade pathway and cell apoptosis(Trapani, 1995). This ability is important for effective killing of virally infected cells as killing through cell lysis would release the virions from the cells, whereby apoptosis would destroy not only the host cell but the virus infecting it (Iannello et al., 2008).

NK cell lines are important substitutes for primary cells in current studies that decipher cell mechanisms and their use in immunotherapy. NK cell lines were established from patients with malignant leukemia or lymphoma, such as the NK-92 cell line which was derived from a patient with non-Hodgkin's lymphoma. NK cell lines commonly used for study include NK-92, YTS and NKL to name a few (Shin et al., 2020). YTS and NKL cell lines showed inconsistent cytolytic activity against conventional NK cell targets, while NK-92 cells are the most efficient effectors against them in comparison (Gunesch et al., 2019). NK-92 cultures showed consistent and reproducible antitumour cytotoxicity, making them suitable substitutes of primary NK cells for study but they are dependent on the application of exogenous IL-2 for propagation (Gong et al., 1994; Klingemann et al., 2016; Suck et al., 2016). IL-2 replenishes the granular stock of NK cells leading to enhanced perforin and granzyme-mediated lysis of target cells (Bhat & Watzl, 2007). However, high doses of IL-2 were shown to be toxic within the clinical settings (Wrangle et al., 2018). In addition to that, NK-92 cells do not express the CD16 receptors required for NK cell-mediated antibody dependent cellular cytotoxicity (ADCC) (Jochems et al., 2016). NK-92MI cell line is an IL-2-independent variant,

generated by the transfection of parental NK-92 cells with human IL-2 complementary DNA (cDNA) using particle-mediated gene transfer but they lack receptors such as CD16 and inhibitory KIRs (Tam et al., 1999).

To fully exploit the potential of NK cell lines, genetic modification methods are essential because cell lines lack certain features, such as activating and inhibitory receptors compared to primary NK cells. In some cases, cancer cells down-regulate ligands specific to NK cell receptors as an evasion mechanism, further impeding the use of NK cell lines for therapeutics. However, NK cell-based immunotherapies were reported with promising anti-tumour activity in several clinical studies involving patients with a variety of malignancies (Shin et al., 2020). Modifications can be made to overcome these problems and improve the use of NK cell lines. The advent of genetically modified NK cell lines could enable the manufacturing of "off-the-shelf" cell products, for various therapeutic purposes such as adoptive cell transfer therapy and chimeric antigen receptor-NK (CAR-NK) cell immunotherapy. This could be done through multiple approaches, such as silencing inhibitory NK cell receptors to enhance cytotoxicity capacity and redirecting killing by introducing activating receptors or chimeric antigen receptors (Carlsten & Childs, 2015).

Other studies attempting NK cell transfection determined that cell lines are less susceptible to genetic modification, mostly yielding low or short transgene expression and unfavourable effects on viability. The main methods of genetic manipulation include transfection via electroporation and retroviral-based transduction (Carlsten & Childs, 2015). For example, NK-92 cells were modified by plasmid-based transfection to express endogenous IL-2 and CD16 with high affinity 158V receptors, but the expression was only transient in nature (Jochems et al.,

2016). A recent study compared DNA electroporation and messenger RNA (mRNA) electroporation strategies for NK cell transfection. Although the DNA electroporation method resulted in lower efficiency, it led to improved persistence of the transfected gene up to 15 days (Ingegnere et al., 2019). The alternative method using retroviral vectors, requires the host cells to be actively dividing, which is an impediment to achieve complete transfection in NK cells (Guven et al., 2005; Kellner et al., 2016). Although retroviral transduction was reported to not affect the characteristics and functions of NK cells, cell viability following transduction was rarely reported (Carlsten & Childs, 2015).

In contrast to that, lentiviral vectors can incorporate transgene into the genome of non-dividing cells and sustain gene modifications without altering the phenotype or functional properties of NK cells. Previous studies have shown varying levels of efficiencies when lentiviral transduction was performed on NK cell lines (Boissel et al., 2013; Savan et al., 2010). In some cases, multiple rounds of lentiviral transduction were required (Micucci et al., 2006; Sahm et al., 2012). Multiple rounds of transduction could potentially increase the number of cells transduced, but it can also lead to unwanted mortality due to toxicity and multiple transduction events per cell (Denning et al., 2013). Excess transduction events increase the chances of insertional mutagenesis, stemming from gene dysregulation at the site of integration and the insertion of non-physiologic promoters (Schlimgen et al., 2016). Similarly to retroviral vectors, viability of NK cells after lentiviral transduction was rarely reported. More recently, a third-generation lentiviral vector, here termed the RRL lentiviral vector, has been extensively studied and approved for use in clinical settings, such as transduced acute myeloid leukemia (AML) blast and lentiviral fibroblast gene therapy (Chan et al., 2005; Di et al., 2013; Ingram et al., 2009; Lwin

et al., 2019). The RRL lentivirus was pseudotyped with vesicular stomatitis virus glycoprotein G (VSV-G) and constructed with various elements to improve transduction (Nordin et al., 2016). Its use for transduction has been shown in a variety of cell types, including hematopoietic and keratinocyte stell cells (Ellison et al., 2019). These findings highlighted the potential of this third-generation lentiviral vector for the modification of NK cell lines, such as NK-92MI, which could lead to further developments in NK cell-based technologies and benefits immunotherapy.

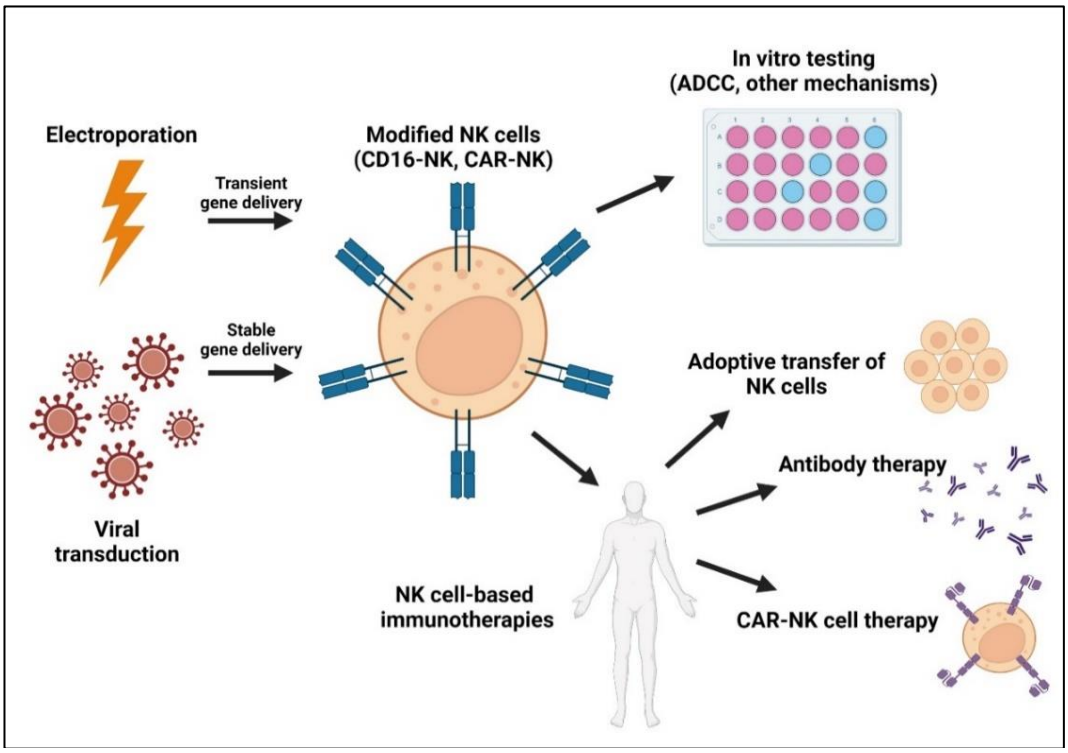

Figure 1.1 Brief summary of NK cell modification methods and the potential uses of modified NK cells.

1.2 Problem statement

NK cell lines have potent cytotoxic activity, they can act as a platform for investigation into NK cell-elicited immune responses and antibody functionality. Previously, the use of primary NK cells in various studies was impeded by the requirement for repeated extractions of human peripheral blood. Multiple extractions were required to obtain a sufficient number of functionally active NK cells for study, since NK cells comprised only about 10 % of circulating blood lymphocytes (Langers et al., 2012; Robertson & Ritz, 1990). This was further complicated by the variability of NK cells between different individuals, which leads to inconsistent results when testing cell functions. Alternate sources, such as NK cell lines (NK-92 and NK-92MI) are now available, providing consistent characteristics and mitigating the costly extraction process (Myers & Miller, 2021). However, these cells were found to lack certain elements compared to primary cells, such as activating the CD16 receptor and inhibitory KIRs. To expand their functionality, an efficient gene delivery technique to modify these cells is needed, as NK cell lines were notoriously less susceptible to genetic modifications (Guven et al., 2005; Imai et al., 2005). The feasibility of lentiviral vector use for stable transgene expression was established in different cell types (Kararoudi et al., 2020), supporting its potential use for modifying NK-92MI cell line.

1.3 Research objectives

1.3.1 General objective

To design a gene delivery protocol using a third generation lentiviral vector to modify the NK-92MI cell line for *in vitro* studies.

1.3.2 Specific objectives

i. To construct lentiviral vectors containing the gene sequence for green fluorescence protein (GFP) or high affinity 158V allele CD16 receptor.

ii. To package lentiviral particles using HEK-293T cells and optimise the concentration process.

iii. To transduce NK-92MI cell line with the packaged lentivirus and investigate the expression of transgenes.

iv. To investigate the use of polybrene polycation for improving lentiviral transduction in NK-92MI cell line.

1.4 Significance of research

NK cell studies have been impeded by tedious and high-cost preparation methods. Customisation of NK-92MI cell lines through a third-generation lentiviral vector could provide a safe alternative for NK cell genetic modifications and pave the way for future *in vitro* studies. This approach could provide a reliable source of NK cells with the required features and consistent nature for various cell studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Killer (NK) cells

Natural killer or NK cells are large granular lymphocytes with potent cytotoxic and cytokine-producing effector functions. They secrete cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), boosting immune responses exerted by other immune cells such as macrophages and dendritic cells. NK cells are involved in immunosurveillance, circulating around our body for the detection and killing of tumour or virally infected cells (Vivier et al., 2008). They patrol our body for signs of infection or cancers, appearing throughout both lymphoid and non-lymphoid tissues. NK cells represent a small fraction of total lymphocytes, ranging from 2 % to 18 % of human peripheral blood lymphocytes (Grégoire et al., 2007).

NK cells can discriminate between healthy cells and target cells due to the myriad of activating and inhibitory receptors on the cell surface. Activation of NK cell functions are dictated by the balance of activating and inhibitory signals (Vivier et al., 2008). When the activating signals are dominant, NK cell enters the active state and are poised to carry out its cytotoxic functions. Activating NK cell receptors detect the presence of alert molecules such as stress-induced, non-self, and toll-like receptor ligands (Lanier, 2005). These target molecules are signs of unhealthy conditions and ‘mark’ target cells for NK cell killing. Inhibitory receptors in turn detect for constitutively expressed self-molecules on potential targets, such as detecting the absence of MHC-I molecules that may be lost during stressful conditions (Vivier et al., 2008). A lack of MHC-I on target cells can lead to NK cell

activation through missing-self recognition by inhibitory receptors, such as killer cell immunoglobulin-like receptor (KIR) and lectin like CD94-NKG2A heterodimers (Bix et al., 1991; Vivier et al., 2008). These interactions allowed for cytotoxicity against targets while ensuring tolerance to self. Maturing NK cells acquire cytotoxic capabilities by forming cytoplasmic lytic granules containing perforin and granzymes. Exocytosis of these granules at the immunological synapse allows NK cells to precisely direct the cytolytic process toward the target without affecting neighbouring cells (Freud et al., 2017; Quatrini et al., 2021).

NK cells interact with other components of the immune system such as T cells, dendritic cells and macrophages through cytokine secretion. Type-I interferon and interleukins such as IL-12, IL-15 and IL-18 are potent activators of NK cell function (Trinchieri, 1989; Vivier et al., 2008). Pre-activation of NK cells using a combination of IL-12, IL-15 and IL-18 enhanced NK effector response upon cytokine or activating receptor restimulation. IL-2 further promotes NK cell proliferation, cytotoxicity and cytokine secretions. NK cells in turn exert negative feedback on hyperactivated macrophages and reduce the risk of inflammatory disorders. Furthermore they kill immature dendritic cells, and this killing action leads to the cross presentation of target cell antigens on dendritic cells. Through the secretion of IFN-γ and tumour necrosis factor (TNF), NK cells promote dendritic cell maturation which in turn secretes IL-12 to further activate NK cells (Romee et al., 2016).

NK cells directly influence the adaptive immune response through their interactions with T and B cells. Secretion of IFN-γ by NK cells promote the priming of CD4 T helper type-I cells and they kill activated T cells with abnormal expression

of MHC-I molecules (Lu et al., 2007). NK cells can suppress autoreactive B lymphocytes and depletion of NK cells led to more severe autoimmunity (Takeda & Dennert, 1993). NK cells also contribute to immune defence against viral infections, where defects in NK cell activity such as IFN-γ production or cytotoxicity were shown to increase susceptibility to viral infections (Scalzo et al., 2007). This problem could stem from the evasion of viruses from NK cell targeting. Mechanisms of viral evasion from NK cell control include prevention of NK stimulation by down-regulating NKG2D ligands and the expression of decoy ligands, such as MHC-I homologs that inhibit NK cell activation by blocking activating receptors (Vidal & Lanier, 2006).

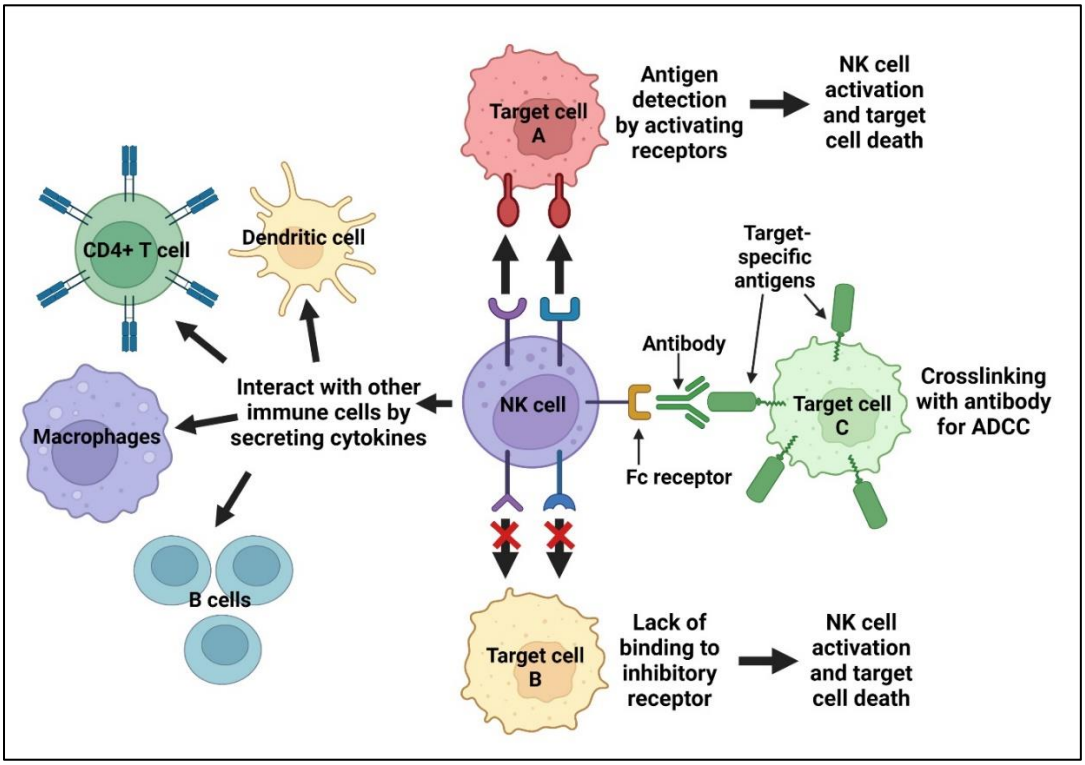

Figure 2.1 Overview of NK cell functions.

2.2 NK cell receptors

Human NK cells displayed an array of activating and inhibitory receptors that in turn dictates NK cell activity. Defining features for NK cells include the surface expression of CD56 and the loss of CD34 during cellular development. Developing NK cells express CD117 that is then down-regulated during an earlier maturation process into $CD56^{bright}$ NK cells, while the expression of CD57 marks a terminally differentiated NK cell (Freud et al., 2014; Lopez-Vergès et al., 2010). CD56 is the predominant phenotypic marker of NK cells, encoded by the *NCAM1* gene for neural cell adhesion molecule (Lanier, Chang, et al., 1991). $CD56^{positive}$ NK cell subsets can be divided into $CD56^{bright}$ and $CD56^{dim}$ NK cells. The $CD56^{dim}$ subset represented a more mature population of NK cells that is commonly associated with higher expression levels of CD16 and KIR. This subset was found to be more abundant in peripheral blood compared to the $CD56^{bright}$ subset (Angelo et al., 2015). Another distinction between these populations is their functional capacity, with the $CD56^{bright}CD16^{negative}$ subset abundantly secreting cytokines such as IFN-γ and TNF-α in response to stimulation but having inferior cytotoxic capacity and lower expression of lytic granules when compared to the $CD56^{dim}CD16^{positive}$ subset (Gunesch et al., 2019; Romagnani et al., 2007).

A transcriptional and phenotypic profiling study on NK cells across multiple tissues, showed that NK cells in blood, bone marrow, spleen and lung are predominantly of the more differentiated $CD56^{dim}CD16^{positive}$ subset (Dogra et al., 2020). Although the immature $CD56^{bright}$ subset was found at higher frequencies within secondary lymphoid tissues such as lymph nodes, tonsils, and gut tissue (Dogra et al., 2020; Michel et al., 2016). These findings suggested that tissue localization could drive further subset-specific transcriptional programs. It points to

anatomical compartmentalisations of NK cells where the lymphoid tissues and intestine represent precursor and immature NK cell reservoirs, while immunosurveillance and effector functions occur primarily within the blood, spleen and lungs.

During NK cell activation, phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the associated adaptor proteins are crucial for the relay of signals coming from activating receptors. This association was made possible by the presence of positively charge amino acid within the transmembrane domain of the activating receptor (Quatrini et al., 2021). Although inhibitory receptors display a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIM), where tyrosine phosphorylation will allow the recruitment of tyrosine phosphatases and allow the transmission of inhibitory signals (Rumpret et al., 2020). KIRs are immunoglobulin (Ig) superfamily receptors supporting ITIMs, however a considerable percentage of the human NK cell population does not have activating KIRs. Therefore, KIRs are not considered as clinically critical as other NK receptors for cell activation (Bashirova et al., 2006).

Major activating NK cell receptors include CD16 (FcγRIIIa), natural cytotoxicity receptors (NCR) and NK group protein 2 family member D (NKG2D) (A. Moretta et al., 2001). CD16 is responsible for binding to crystallisable fragment (Fc) regions of immunoglobulins and mediates ADCC by NK cells. Examples of natural cytotoxicity receptors are NKp46, NKp30 and NKp44 that are Ig-like transmembrane proteins. NKp46 and NKp30 are found on nearly all resting NK cells and are associated with CD3ƶ and FcεRIy. NKp30 plays an important role in the interaction between NK cells and dendritic cells, contributing to the maturation and

selection of dendritic cells with better antigen presentation capacities (L. Moretta et al., 2002). NKp44 is acquired upon cell activation and transmit their activating signals through KARAP/DAP12 adaptor molecules (A. Moretta et al., 2001).

NCR ligands enable NK cell activation when expressed on target surfaces but act to inhibit NK cell functions when released in the soluble form. NCR ligands encompass several hosts as well as pathogen-specific molecules. These molecules can exist as membrane-bound, extracellularly soluble glycoproteins, or nuclear proteins to be presented on target cell surfaces. NCRs influences both antitumour responses and infectious diseases, while their ligands are being studied as possible biomarkers in a myriad of pathological conditions (Barrow et al., 2019). NKG2D is expressed on both NK cells and cytotoxic T cells, it transduces activating signals through the adaptor protein DAP10. NKG2D binds to MICA/B and ULBP ligands that are commonly up-regulated in virally infected, stressed, as well as tumour cells (Bauer et al., 1999; Lanier, 2015). Similarly to NCRs, their ligands in soluble form were incriminated as key players within the tumour escape mechanism (Dhar & Wu, 2018).

Previous studies have shown that the tumour microenvironment affects NK cell activity by exerting immune suppressive effects. Studies found dysfunctional NK cells within tumour samples, characterised by the downregulation of their main activating receptors for tumour recognition such as NCRs, DNAM-1 and NKG2D (Devillier et al., 2021). Some cancer cells developed the ability to release soluble NKG2D ligands, rendering these cells invisible towards NKG2D recognition (Molfetta et al., 2016). These soluble ligands also induced NKG2D downregulation, further impairing NK cell responses (Ferrari de Andrade et al., 2018). However a

separate study showed that within inflammatory autoimmune conditions, soluble NKG2D ligands could trigger NK cell activation without downregulating NKG2D expression. This could be due to high concentrations of pro-inflammatory cytokines, such as IL-15 and TNF-α (Groh et al., 2003).

NK cells have a wide repertoire of activating and inhibitory receptors, a few examples are listed in **Table 2.1**. NK cells interact with the environment differently through these receptors, while many receptors have yet to be studied extensively.

Table 2.1 NK cell receptors and their ligands

Receptor	Known ligand(s)	References
Activating receptor		
CD2 (T11)	CD15	(Warren et al., 1996)
CD16	Antibody Fc region	(Mandelboim et al., 1999)
CD96	CD155	(Fuchs et al., 2004)
CD226 (DNAM-1)	CD112, CD155	(Bottino et al., 2003)
CD244	CD48	(Brown et al., 1998)
NK group 2 member D (NKG2D)	MICA, MICB, ULBP1-5	(Eagle & Trowsdale, 2007)
NKp30	HLA-B associated transcript 3 (BAT3)	(von Strandmann et al., 2007)
NKp46	Influenza hemagglutinin	(Arnon et al., 2004; Mandelboim et al., 2001)
NKp44	Influenza hemagglutinin	(Arnon et al., 2004)
Inhibitory receptor		
CD66a	Carcinoembryonic antigen (CEA)	(Boulton & Gray-Owen, 2002)
CD94/NKG2A	HLA-E	(Braud et al., 1998; Lee et al., 1998)
CD161	Lectin-like transcript 1 (LLT1)	(Aldemir et al., 2005)
CD305	Collagen	(Meyaard, 2008)
Killer cell lectin-like receptor G1 (KLRG1)	E-cadherin, N-cadherin, R-cadherin	(Gründemann et al., 2006; Ito et al., 2006)
Sialic acid-binding immunoglobulin-like lectin (SIGLEC7)	Ganglioside GD3	(Nicoll et al., 2003)

2.3 CD16 receptor

FcγIII receptors, better known as CD16 receptors, are indispensable for mediating ADCC by immune cells such as NK cells and neutrophils. CD16 is a low affinity Fc activating receptor that exists in the isoform of CD16a or CD16b, which defers in cell type-specific expression and localisation strategy on the cell surface despite having nearly identical genomic sequences (Patel et al., 2019).

The CD16a surface receptor is encoded by the *FCGR3A* gene and acquired during the maturation of human NK cells. During the earlier maturation stages of NK cell development, maturing NK cell precursors do not display any expression of CD16 receptors. Later on during development, NK cell maturation is marked by the expression of transcription factors and acquisition of surface markers such as CD56, CD16 and CD57. As the $CD56^{bright}$ subset matures into the $CD56^{dim}$ subset, this progression was accompanied by higher expression levels of CD16 (Freud & Caligiuri, 2006; Scoville et al., 2017).

CD16 plays a pivotal role in NK cell activation and ADCC, and polymorphisms in the *FCGR3A* gene lead to differential binding affinity to antibodies. Polymorphism of phenylalanine (F) to valine (V) at amino acid residue 158 (rs396991, FcγRIII-F/V158 or FcγRIII-F/V176), leads to higher antibody binding affinity and increased activation of ADCC (Wu et al., 1997). This polymorphism occurs in the extracellular domain 2 (EC2) and affects ligand binding, the non-conservative T to G substitution at nucleotide 559 predicts the change in amino acid. The V/V homozygotes displayed higher binding affinity to the Fc region of the antibodies, subsequently leading to a higher level of NK cell activation (Koene et al., 1997; Wu et al., 1997).

Previous studies implicated strong associations between the lower binding phenotype (F/F) and several diseases (Wu et al., 1997), suggesting its potential role in predicting risk for certain diseases. Prior clinical studies demonstrated improved response and survival for individuals whose NK cells expressed CD16 homozygous V/V genotype, compared to heterozygous V/F or homozygous F/F genotypes (Bibeau et al., 2009; Musolino et al., 2008; Veeramani et al., 2011). Although there is no direct evidence of NK cell action in the enhanced clinical benefit, results remain compelling for indicating the potential use of these genetic variations to forecast treatment efficacy.

However, repeated activations through CD16 receptors decrease perforin secretion, which can be restored by subsequent activation through the NKG2D activating receptor instead. This decrease in perforin secretion was attributed to CD16 shedding triggered by cellular activation (Srpan et al., 2018). Albeit the drawback, CD16 shedding serves to elevate NK cell motility and facilitated NK cell detachment from target cells. Disassembly of this immune synapse allows NK cells to dissociate and move to other targets (Vanherberghen et al., 2013). Shedding of CD16 is beneficial by increasing serial engagement of target cells and also prevents excessive immune responses (Srpan et al., 2018). Previously, inhibition of target cell caspases to prevent target lysis had prolonged contact time with murine NK cells, leading to excessive cytokine secretions and hyperinflammation (Jenkins et al., 2015).

Down-regulation of CD16 in NK cells was caused by proteolytic cleavage of its extracellular portion by A-disintegrin and metalloproteinase-17 (ADAM17) or membrane type-6 matrix metalloproteinase (MMP25) (Peruzzi et al., 2013; Romee et

al., 2013). This CD16 shedding can be prevented by a single mutation (Ser197Pro), conferring shedding resistance and restoring initiation of ADCC. Cells expressing CD16 resistant to cleavage showed increased ADCC against lymphoma cells both *in vitro* and *in vivo* (Guo et al., 2019).

2.4 Antibody dependent cellular cytotoxicity (ADCC)

ADCC is an immune mechanism through which antigen specific antibodies act as a homing mechanism for NK cell-targeted killing. It occurs when NK cells bearing the Fc receptor (CD16) recognise and kill antibody-bound target cells that express surface tumour antigens. Cross-linking between Fc receptor and the antibody Fc portion induces phosphorylation of ITAMs by cellular Src kinase. Resulting in the release of cytotoxic granules such as perforin and granzymes, eventually inducing apoptosis of target cells via the Fas and tumour necrosis factor-related apoptosis inducing ligand (TRAIL) pathway (Chiossone et al., 2018; Legris et al., 2016; Patel et al., 2019; Shin et al., 2020). ADCC could be harnessed for therapeutic purposes through the use of NK cells and various retargeting mechanisms for effectors.

Interestingly, activating signals by CD16 receptor alone was capable of inducing NK cell-mediated ADCC. This was not possible for other activating receptors on NK cell, which required complementary binding with other receptors to enable degranulation (Long et al., 2013; Wang et al., 2015). Furthermore, clinical studies shown that higher binding affinity CD16 V/V genotype correlated with better survival and increased ADCC activity during antibody treatment (Siebert et al., 2016; Trotta et al., 2016). Similarly, the V/V genotype was also associated with

higher magnitudes of human immunodeficiency virus (HIV)-specific ADCC (Talathi et al., 2019).

Advents in antibody engineering technology introduced a myriad of antibody products capable of exploiting NK cell-mediated ADCC. Most studies focused on IgG because of their stability *in vivo* and ADCC inducing function. Many monoclonal antibodies (mAb) capable of inducing ADCC are now commercially available, including anti-CD20 Rituximab (Meyer et al., 2018), anti-CD38 Daratumumab (Vidal-Crespo et al., 2020), anti-epidermal growth factor receptor (EGFR) Cetuximab (Inoue et al., 2017; Nakamura et al., 2019; Trotta et al., 2016) and anti-human epidermal growth factor receptor 2 (HER2) Trastuzumab (Maadi et al., 2018) to name a few. Nevertheless, more specific antibodies are constantly being developed to combat new and discrete immunotherapy targets through ADCC. Recently, anti-CD123 mAb and disialoganglioside GD2 targeting antibodies were introduced and showed potent ADCC through NK cells (Modak et al., 2018; Xie et al., 2017). The combined use of different antibodies also showed additive effects that exceeded monotherapies, however, antibody dose-response curves from *in vitro* study showed that ADCC improvement is saturable, limited by the number of effector cells present for antibody binding (Tóth et al., 2016).

Induction of ADCC can be substantially affected by their binding interfaces, whereas alterations in amino acid compositions can disrupt or strengthen binding. Several strategies were proposed to augment binding, including modulating glycosylation patterns and removing fucose subunits in the N-glycan of antibody Fc region (Wang et al., 2015; Zahavi et al., 2018). Simultaneous core defucosylation and terminal galactosylation of IgG1 had enhanced NK cell-mediated ADCC

(Dekkers et al., 2017; Wada et al., 2019). Galactosylation of fucosylated Fc regions showed an increase of at least 1.5-fold in terms of CD16 binding affinity, potentially improving the induction of ADCC (Hajduk et al., 2020). Furthermore, defucosylated antibodies were proven to induce greater magnitudes of ADCC (Cao et al., 2020; Temming et al., 2019; Wirt et al., 2017).

Several other bispecific molecules are available that can simultaneously engage different antigens or distinct epitopes on the same antigen, further expanding the range of targets for NK cell-mediated ADCC (Huang et al., 2018; Qi et al., 2019). These products include bispecific antibodies, bispecific killer engagers (BiKE) and trispecific killer engagers (TriKE). Furthermore, bispecific antibodies with additional CD16 binding moieties could enhance ADCC by increasing the number of effector cells engaged at a given time (Ellwanger et al., 2019). BiKE constructs can be designed to carry scFv against CD16 and tumour antigen, while modified IL-15 crosslinkers can be incorporated into different BiKEs to create TriKE and improve ADCC mediated by NK cells (Schmohl et al., 2016, 2017; Vallera et al., 2020). The use of a TriKE molecule such as GTB-5550 induced greater cell degranulation, interferon release, and overall cell killing by NK cells (Khaw et al., 2022). While studies demonstrated the wide range of ADCC targets, newer engager molecules targeting ADCC will require stable NK cell platforms to unveil their interaction with NK cells.

2.5 NK cell lines

Reliable models for cell studies are required to dissect the fundamentals of NK cell mechanism that governs their functions, development and causes of dysregulation in various diseases. A plethora of sources are now available for both

NK cell study and use in adoptive cancer immunotherapy, such as haploidentical NK cells, umbilical cord blood NK cells, induced pluripotent stem cell (iPSC)-derived NK cells, cytokine-induced memory-like NK cells, chimeric antigen receptor NK cells and NK cell lines (Morvan & Lanier, 2016; Myers & Miller, 2021). In these cases, immortalised NK cell lines are attractive NK models due to their consistent features and ease of propagation (**Table 2.2**).

Table 2.2 List of available NK cell lines.

Cell line	Cytokine dependence	References
HANK1	IL-2 dependent	(Kagami et al., 1998)
KHYG-1	IL-2 dependent	(Yagita et al., 2000)
NK3.3	IL-2 dependent	(Kornbluth et al., 1982)
NK-92	IL-2 dependent	(Gong et al., 1994)
NKL	IL-2 dependent	(Robertson et al., 1996)
NK-YS	IL-2 dependent	(Tsuchiyama et al., 1998)
SNK-6	IL-2 dependent	(Nagata et al., 2001)
SNT-8	IL-2 dependent	(Nagata et al., 2001)
YT	IL-2 independent	(Yodoi et al., 1985)

As in the case of most NK cell lines, except the YT cell line, application of exogenous IL-2 was required for NK cell proliferation. IL-2 also replenishes the granular stocks of NK cells, leading to enhanced perforin and granzyme-mediated lysis of target cells (Bhat & Watzl, 2007). Due to their pro-inflammatory nature, recombinant IL-2 may cause overestimations of NK cell responses during *in vitro* testing. High doses of IL-2 were shown to be toxic in clinical settings (Wrangle et al., 2018), limiting the use of NK cell lines for therapeutics. Excessive IL-2 can also be toxic and cause health issues such as capillary leak syndrome, manifesting in various organs such as heart, lung and kidney (Schwartz et al., 2002). Thus, it may

be beneficial to generate NK cell lines that produce endogenous IL-2, just enough for cell propagation and maintenance.

Among the NK cell lines commonly used in cell studies are NK-92, YTS, NK3.3 and NKL cell lines. Genome-wide analysis and functional profiling found that these cell lines shared similarities in growth patterns and survival pathways, with differential expression of genes for cell development, survival and function. NK-92 cells displayed phenotype associated with the $CD56^{bright}$ subset, while YTS and NKL cells displayed phenotypes closer to $CD56^{dim}$ subset. Study identified NK-92 cell line as the most efficient effector at killing canonical NK targets, such as human leukocyte antigen (HLA)-null 721.221 cells, K-562 erythroleukemia cell line and Raji B cells. The lytic activity of NK-92 and YTS cells was comparable to that mediated by *ex vivo* NK cells, while other cell lines showed minimal lytic functions (Gunesch et al., 2019).

NK-92 cell line is an NK cell line derived from the peripheral blood of a male patient with large granular lymphocyte (LGL) non-Hodgkin's lymphoma and is dependent on exogenous IL-2 for propagation (Gong et al., 1994). This cell line is positive for most if not all NK cell surface markers such as CD56, CD2, CD7, CD11a, CD28 and CD45, but it lacks CD16 expression (Gong et al., 1994; Maki et al., 2001). NK-92 cells expressed a diverse activating receptor repertoire, including NKp30, NKp46, NKG2D, CD28 and 2B4 (Drexler & Matsuo, 2000; Maki et al., 2001). They also expressed Fas ligand (FasL), TRAIL and TNF-α, indicating their potential roles in lytic granule-independent pathways for killing. NK-92 lack or have extremely low levels of inhibitory KIR, allowing lysis of tumour cells expressing major histocompatibility complex (MHC) molecules (Jochems et al., 2016; Maki et

al., 2001). Moreover, further modifications have generated NK-92MI cell line that produce their own IL-2 and thereby enable proliferation without the addition of exogeneous IL-2 (Tam et al., 1999).

The YTS cell line was subcloned from YT-NK cell line, originated from the pericardial fluid of a male patient with acute lymphoblastic lymphoma (Yodoi et al., 1985; Yoneda et al., 1992). YTS cells express CD56, CD7, CD28 and CD45RO, while they are negative CD2 and CD16 (Yoneda et al., 1992). Unlike NK-92 cell line, YTS cells are independent of IL-2 for propagation, but they exhibited reduced cytolytic potential against common NK cell targets (Chen et al., 2006). NKL cell line was derived from the peripheral blood of a male patient suffering LGL leukaemia and also require IL-2 for propagation. These cells are positive for the surface markers CD2, CD6, CD11a, CD27, CD29 and CD94 (Robertson et al., 1996). This cell line was reported to have inconsistent lytic functions of common NK cell targets such as K-562 and 721.221 cells (Chen et al., 2007; Matsuo & Drexler, 2003).

NK3.3 is an example of non-malignant NK cell line that was generated by the *in vitro* cell cloning from the peripheral blood of a healthy donor. The cells were expanded in mixed lymphocyte culture and are dependent on IL-2 for propagation (Kornbluth et al., 1982; Mahle et al., 1989). NK3.3 cells are positive for CD2, CD11a, CD38, CD45, CD16 and CD56 (Kornbluth et al., 1982). Although these cells proved difficult to culture long term and their specific lysis activity plateaus after 1 hour in coculture with target cells (Mahle et al., 1989), they are therefore not a suitable model for studying the lysis capacities of NK cells.

Nowadays NK cell-based therapies are emerging as safe and effective alternatives for immunotherapy, thus increasing the repertoire of NK cell line use. Several cases of severe adverse events have been reported in conventional T cell therapy, such as severe to fatal cytokine release syndrome (CRS) and graft versus host disease (GvHD) (Acharya et al., 2019). Compared to T cell therapies, NK cell therapy provides a more manageable safety profile, limiting side effects from cell transplantation. Severe adverse effects are less likely to happen due to the limited *in vivo* persistence of NK cells (Veluchamy et al., 2017). NK cells have shown their potential in treating malignant solid tumours and in contrast to T cells, they do not need to be patient-specific which widens their application in cancer treatment (Sharma et al., 2017; Shin et al., 2020).

NK cell-based therapies and are not limited by the requirement for autologous cells, this paves the way for the development of "off-the-shelf" products based on NK cells. While autologous NK cells are limited by expansion efficiency, allogeneic NK cells such as NK cell lines and iPSC-derived NK cells can be expanded *ex vivo*, serving as stable alternatives for clinical application (Koehl et al., 2016; Shankar et al., 2020). Previously, the NK-92 cell line was tested in phase I clinical trials for various malignancies, showing minimal toxicity, but irradiation prior to infusion was required to prevent risk of graft (Arai et al., 2008; Klingemann et al., 2016; Tonn et al., 2013; Williams et al., 2017). Despite the allogeneic nature of NK-92 cells, the formation of anti-human leukocyte antigen (HLA) antibodies was observed in less than half of patients (Jochems et al., 2016). Other methods to enhance the therapeutic potential of NK cells include modification of effector NK cells, alteration of target cell antigens, use of cytokine-based agents and immune checkpoint inhibitors (Chin et al., 2021).

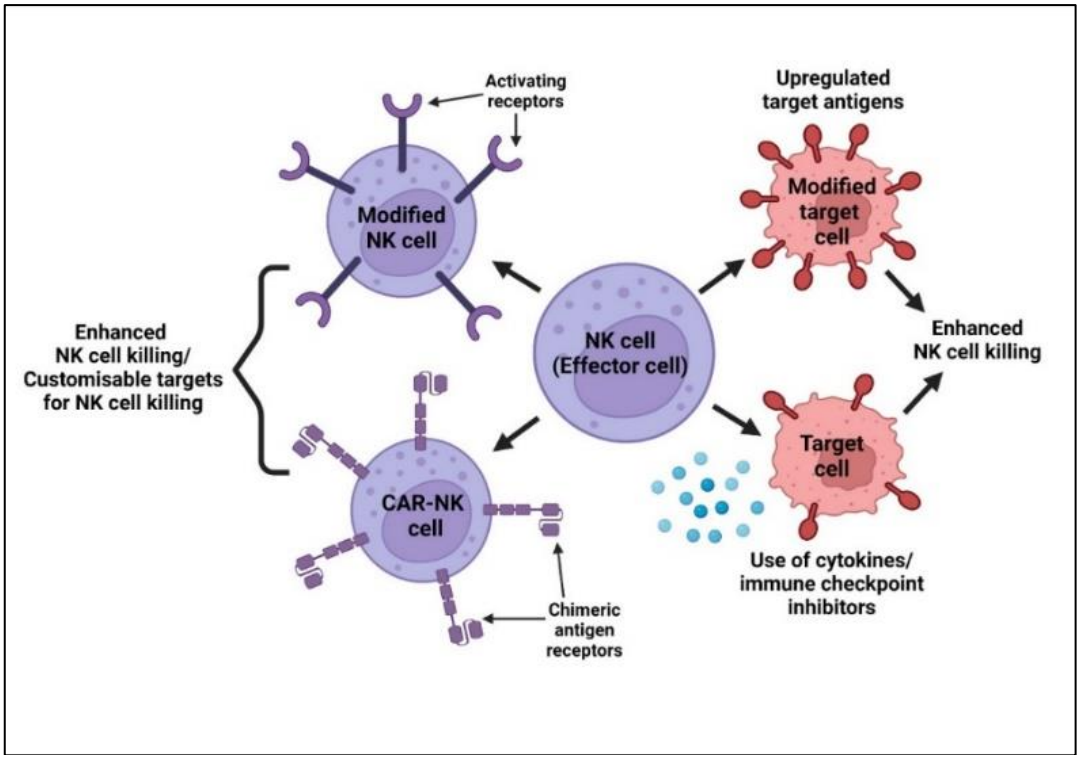

Figure 2.2 Methods for enhancing NK cell therapeutic potential.

2.6 Transgene delivery techniques

Modifications to effector NK cells may benefit cell biology studies and expand cellular functions such as cytokine secretion and ADCC. To that end, efficient gene delivery techniques are vital prerequisites for NK cell genetic modification. Transfection with either naked plasmid DNA, transposase DNA-mediated integration or mRNA by electroporation method is commonly used for cell modification. In addition to these conventional methods, viral transduction was also possible through retroviral or lentiviral vectors (Hu et al., 2018).

Being innate immune cells, NK cells are first responders towards viral infections, this role could have led them to develop mechanisms for resistance against viral infections (Lanier, 2008). Foreign viral RNA present during transduction can activate pathogen-associated molecular patterns (PAMP) in NK