DEVELOPMENT OF A NOVEL *IN VITRO* SCREENING METHOD USING GENETICALLY MODIFIED NK-92 CELLS AGAINST VARIOUS TUMOR CELLS

by

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ABSTRACT

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Keywords: NK cells, cancer immunotherapy, NK-92 cell line, NK cell surface receptors

Natural killer (NK) cells of the innate immune system are recognized for their ability to potently kill tumor cells. NK cell-mediated lysis is maintained by an intricate balance between several activating and inhibitory receptors that either trigger or dampen effector functions upon ligand engagement. In this study, we aim to dissect this complex balance by developing a cell-based screening tool to identify receptor specific anti-tumor responses. As the character of the heterogeneous tumor cell populations differs among patients, such a tool may be instrumental in developing patient-tailored cancer immunotherapies.

Genes encoding 20 NK cell surface receptors were cloned into lentiviral vectors for genetic modification of the NK-92 cell line. Genetically modified (GM) NK-92 cells were enriched and overexpression of receptors was confirmed by flow cytometry. We analyzed the effector functions of all GM NK-92 cells against human cancer cell lines as well as against primary human sarcoma explants.

Overall, genetic modifications did not hamper cytotoxic capacity of GM NK-92 cells; rather induced enhanced tumor cell targeting by receptors such as DNAM-1 and NKG2D. We further confirmed that this response was indeed DNAM-1 or NKG2D-dependent by using blocking antibodies. We also evaluated the synergistic response of prominent receptors in triggering degranulation and cytotoxicity by co-expressing DNAM-1 and NKG2D.

Our results show the feasibility of an *in vitro* genetic screening approach to identify response-triggering receptors in genetically modified NK cells expressing different activating receptors. This tool has the potential to rapidly identify patient-specific targets for adoptive immunotherapy of cancer.

ÖZET

ÇEŞİTLİ TÜMÖR HÜCRELERİNE KARŞI GENETİK OLARAK DEĞİŞTİRİLMİŞ NK-92 HÜCRELERİ KULLANILARAK YENİ BİR *IN VITRO* TARAMA YÖNTEMİNİN GELİŞTİRİLMESİ

Didem Özkazanç Ünsal

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Doğal bağışıklık sisteminin doğal öldürücü (NK) hücreleri, tümor hücrelerini güçlü bir şekilde öldürme kabiliyetleri ile tanınırlar. NK hücre aracılı öldürme, ligand bağlanması ile efektör fonksiyonları tetiklenen veya baskılanan birçok aktivasyon ve inhibisyon reseptörleri arasındaki karmaşık denge ile korunur. Bu çalışmada reseptöre özgül anti-tümör yanıtlarını tanımlamak için hücre bazlı bir tarama aracı geliştirerek bu karmaşık dengeyi incelemeyi hedefliyoruz. Heterojen tümör hücre popülasyonlarının karakteri hastalar arasında farklılık gösterdiği için böyle bir araç hastaya özel kanser immünoterapilerinin geliştirilmesinde yararlı olabilir.

20 farklı NK hücre yüzey reseptörünü kodlayan genler, NK-92 hücre hattının genetik modifikasyonu için lentiviral vektörlere klonlanmıştır. Genetik olarak modifiye edilmiş (GM) NK-92 hücreleri saflaştırılmış ve tek bir özgül reseptörü yüksek düzeyde ifade ettikleri akan hücre ölçer ile doğrulanmıştır. Tüm GM NK-92 hücrelerinin efektör fonksiyonları insan kanser hücre hatlarına ve sarkom hastalarından elde edilen tümör hücrelerine karşı analiz edilmiştir.

Sonuçta, yapılan genetik değişikliklerin GM NK-92 hücrelerinin sitotoksik kapasitelerine zarar vermediği ve hatta DNAM-1 ve NKG2D gibi reseptörler aracılığıyla arttırılmış bir tümör hedeflenmesini tetiklediği gözlemlenmiştir. Ayrıca, bloke edici antikorlar kullanılarak bu cevabın DNAM-1 ve NKG2D kaynaklı olduğu doğrulanmıştır. Ek olarak, DNAM-1 ve NKG2D'yi birlikte ifade ederek degranülasyon ve sitotoksisiteyi tetiklemede ön plana çıkan reseptörlerin sinerjik tepkisi de değerlendirilmiştir.

Sonuçlarımız, farklı aktivasyon reseptörlerini ifade eden genetik olarak modifiye edilmiş NK hücrelerinde yanıt üreten reseptörleri tanımlamak için *in vitro* genetik tarama yaklaşımının uygulanabilirliğini göstermektedir. Bu araç, adoptif kanser immünoterapisinde kullanılabilecek hastaya özgül hedeflerin hızla belirlenmesini sağlama potansiyeline sahiptir.

To my grandmother and husband…

Sevgili anneanneme ve eşime…

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α	Alpha
β	Beta
γ	Gamma
κ	Kappa
µ	Micro
µL	Microliter
µM	Micromolar
ADCC	Antibody-dependent cellular cytotoxicity
AKT	Activating Receptor tyrosine kinase
AML	Acute Myeloid Leukemia
ALL	Acute Lymphocytic Leukemia
APC	Allophycocyanin
BV	Brilliant Violet
CIAP	Calf Intestine Alkaline Phosphatase
CML	Chronic Myeloid Leukemia
CTLA-4	cytotoxic T-lymphocyte associated protein 4
CO_2	Carbon dioxide
DC	Dendritic Cell
ddH_2O	Distilled deionized water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxade
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule
DPBS	Dulbecco's phosphate-buffered saline
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EPCAM	Epithelial cell adhesion molecule
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FcεRIγ	High-affinity IgE receptor-γ
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GFP	Green Fluorescent Protein
Gly	Glycine
GvHD	Graft versus host disease
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human Leukocyte Antigen
HS	Heparan Sulfate
IFN	Interferon
IL	Interleukin
IgG	Immunoglobulin G

IRES	Internal Ribosome entry site
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITT	Immunoreceptor tyrosine tail
KIR	Killer Immunoglobulin-like Receptor
LAK	Lymphokine-activated killer cell
LB	Luria Broth
LeGO	Lentiviral Gene Ontology
LIR-1	Leukocyte immunoglobulin-like receptor subfamily B member 1
mAb	Monoclonal antibody
MCS	Multiple cloning site
MDS	Myelodysplastic Syndrome
MEM	Minimum Essential Media
MHC	Major Histocompatibility Complex
MIC	MHC class I polypeptide-related sequence
MM	Multiple Myeloma
MOI	Multiplicity of Infection
NCR	Natural cytotoxicity receptors
NEAA	Non-essential Amino Acid
NKG2	Natural Killer Group 2
OXO	(5Z)-7-Oxozeaenol
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed cell death protein 1
PI	Propidium Iodide
PI3K	Phosphoinositide-3-kinase-protein
PIPES	Piperazine-N,N′-bis (2-ethanesulfonic acid)
Puro	Puromycin
RPMI	Roswell Park Memorial Institute
Rpm	Round per minute
RT	Room Temperature
SEM	Standard Error of Mean
SFFV	Spleen Focus Forming Virus
SLAM	Signaling lymphocytic activation molecule
Syk	Spleen tyrosine kinase
TAA	Tumor associated antigen
TCR	T-cell Receptor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TME	Tumor microenvironment
TNF	Tumor Necrosis Factor
TRAIL	TNFα-Related Apoptosis-Inducing Ligand
Treg	Regulatory T cells
ULBP	UL16-binding protein
VSV-G	Vesicular stomatitis virus G
WT	Wild Type

1. INTRODUCTION

1.1. Natural Killer (NK) Cells

Experiments aimed at characterizing cell-mediated cytotoxicity against tumor cells unwittingly revealed the existence of a subset of lymphocytes with spontaneous anti-tumor response (Kiessling, Klein, and Wigzell 1975; Kiessling et al. 1975; Herberman, Nunn, and Lavrin 1975; Herberman et al. 1975). Although these original observations made in the 1970s were initially presumed to be an artefact or background cytotoxicity, scientists began to explore this previously uncharacterized distinct sub-population throughout the next decade (Oldham 1983). Owing to their intrinsic ability to kill cellular targets without any prior sensitization, they were named as natural killer (NK) cells.

NK cells are recognized as large granular lymphocytes that constitute approximately 5-20% of all circulating lymphocytes, residing in multiple lymphoid tissues such as the bone marrow (BM), lymph nodes (LN) and non-lymphoid tissues such as skin, gut, uterus, liver, and lungs, each with their unique effector functions and expression of surface markers (Carrega and Ferlazzo 2012; Jacobs et al. 2012). Even though NK cells make up a small percentage of the lymphoid compartment, their therapeutic potential have been explored in a wide variety of contexts, including but not limited to autoimmunity, pregnancy, infectious diseases and cancer.

In several mammalian species, NK cells are phenotypically characterized as $CD3^-$ $NKp46^+$ lymphocytes. Human NK cells are distinguished as $CD56^{dim}$ or $CD56^{bright}$ subsets based on their differential levels of CD56 [neural cell adhesion molecule (NCAM)] expression (Moretta et al. 2002). Approximately 90% of peripheral blood (PB) and spleen NK cells belong to the $CD56^{dim}$ $CD16^+$ subset with a great diversity of cytotoxic capacity upon interacting with target cells and diminished cytokine secretion function (Cooper, Fehniger, and Caligiuri 2001). In contrast, a minority of NK cells belong to the $CD56^{bright}$ $CD16^-$ subset and exhibit a less mature state with minimal cytotoxicity, albeit robust cytokine secretion profile (Morvan and Lanier 2016; Cooper, Fehniger, and Caligiuri 2001).

Advances in the field of immunology have demonstrated that immune cells are highly plastic and rapidly can undergo phenotypic and functional changes and that they are dynamic players in the intricate microenvironment of cellular and chemical interactions. This intertwined microenvironment is simplified and catalogued as innate and adaptive defense or immune systems; however, NK cells seem to straddle these two branches of the immune system. They are considered to be key players of the early innate immune defense in clearance of bacterial and viral infections, and tumor cells. Yet they differentiate from lymphoid progenitors that also give rise to T and B lymphocytes of the adaptive immune response. In fact, it has recently been appreciated that NK cells are prototypic innate lymphoid cells (ILCs) owing to their rapid activation, expression of key transcription factors and signature cytokine secretion profiles that direct the developing immune responses or contribute to tissue homeostasis (Ebbo et al. 2017; Artis and Spits 2015).

This rapid action stems from the expression of a multitude of pre-defined receptors that can identify self over pathogen-derived or stress-induced ligands and endows NK cells with innate abilities. Similar to other lymphoid counterparts such as other ILC subsets and T, B lymphocytes of adaptive immunity, NK cells derive from common lymphoid progenitors. NK cells are cytolytic ILCs with a basic cytotoxic machinery of lytic granules and other secretory components resembling those of cytotoxic $CD8^+$ T lymphocytes (Y. Zhang and Huang 2017; Cruz-Muñoz et al. 2019). However, unlike T cells, NK cells fail to express the CD3 surface antigen and the clonotypic receptor specific for MHC presented peptidic antigens (e.g. T

cell receptor (TCR)) and its related signal-transducing adaptor, CD3ε. Furthermore, recent experiments show that NK cells also can possess memory-like functionality contributing to the perception that NK cells are similar to adaptive immune cells with long-term memory features (T. E. O'Sullivan, Sun, and Lanier 2015). Owing to these attributes, NK cells are recognized as central and dynamic players in maintaining, providing and shaping immunity.

1.1.1. NK cell Effector Functions

Natural killer cells execute their protective functions through many effector mechanisms. Firstly, NK cells can distinguish healthy cells from nascent transformed or infected cells and directly lyse these targets. This cytolytic function can act through many processes such as degranulation and death receptor ligation, which result in target cell clearance (Smyth et al. 2005b; Moretta et al. 2002). Furthermore, NK cells can also function indirectly by producing various cytokines and chemokines, upon activation by receptor stimulation and inflammatory cytokine-induced activation signaling. Cytokine secretion by NK cells not only contributes to the clearance of target cells, but also regulates both the innate and adaptive immune responses (Fauriat et al. 2010).

The primary mechanism for NK cell-mediated cytotoxicity critically depends on the directed release of lytic substances towards the target cell, a process known as NK cell degranulation (Vivier et al. 2008). NK cells store these lytic substances in cytolytic granules that are delivered and fused to the cell membrane in the immunological synapse upon target cell recognition (Cruz-Muñoz et al. 2019). During degranulation, lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) and -2 (LAMP-2 or CD107b) which normally face the lumen of the cytoplasmic granule, are transiently translocated to the NK cell surface. This LAMP-1 expression on the NK cell surface can be used as an indirect measurement of cytolytic effector function and is routinely used in this work (Alter, Malenfant, and Altfeld 2004). The specialized proteins contained within these granules, including the pore-forming glycoprotein, perforin; and a class of serine proteases known as granzymes, Fas ligand (FasL, CD178), and TNF-related apoptosis-inducing ligand (TRAIL, CD253) are secreted to the immunological synapse

formed between the target cell and the NK cell. Granzyme B and perforin are especially critical components of the lytic granules. Perforin polymerizes and mediates pore formation on the target cell membranes, facilitating the entry of granzymes into the cytoplasm of the target cell. Once inside the target cell, Granzyme B can induce cell death by apoptosis dependent or independent of the caspases (Topham and Hewitt 2009).

Another mechanism by which NK cells mediate cytotoxicity is through the activation of death receptor-induced target cell apoptosis. The receptors present on the surface of the target cell include TRAIL-receptor (TRAIL-R) and Fas, engage with their cognate ligands, TRAIL and FasL, expressed by NK cells (Smyth et al. 2005a). This interaction induces a conformational change in the cytoplasmic tail of the death receptor and results in the recruitment of adaptor proteins that drives caspase-dependent cell death by apoptosis (Thorburn 2004). The surface expression of death receptors can be induced on target cells by IFN-γ secretion from NK or other immune cells and their activation can initiate several pro-apoptotic signaling events (Screpanti et al. 2001; Degli-Esposti 1999).

Additionally, NK cells are able to kill their targets by antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells express the activating type CD16 (or FcRγIII), a cell surface receptor for the highly conserved Fc portion of immunoglobulin G (IgG) antibodies which enables recognition of antibody-coated target cells (Vivier et al. 2008). When the Fab moiety of an IgG antibody binds to specific antigens displayed on infected or transformed cells, the Fc portion of these antibodies are recognized by CD16 and thereby trigger NK cell activation and cytolysis of antibody-coated target cells (Bryceson et al. 2005).

NK cells can also secrete pro-inflammatory and immunosuppressive cytokines as well as chemokines that shape immune responses (Abel et al. 2018). The release of inflammatory cytokines is different from the aforementioned cytotoxic granule secretion; instead, NK cells impose activation-induced signaling intermediates for the regulation of these two different functions (Reefman et al. 2010). Depending on the inflammatory environment, NK cells can produce a wide-range of cytokines and various chemokines (Vivier and Ugolini 2009; Vivier et al. 2011). They mainly

secrete Th1-type cytokines upon contact with tumor ligands or intracellular pathogens (Vivier et al. 2011). NK cells are early and potent producers of interferon gamma (IFN-γ), but they can also secrete tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) which recruit T cells and other innate immune mediators such as dendritic cells, macrophages, and neutrophils to the inflammation site and induce the activation and proliferation of these cells (Walzer et al. 2005; BOSCH et al. 2008; Abel et al. 2018). In addition, NK cells produce chemokines including chemokine (C-C motif) ligand 3 (CCL3), CCL4, CCL5, chemokine (C motif) ligand 1 (XCL1), and chemokine (C-X-C motif) ligand 8 (CXCL8) which can attract effector lymphocytes and myeloid cells to the site of inflammation (Walzer et al. 2005). Therefore, they are considered as not only effector lymphocytes but also significant regulators of immune responses.

1.1.2. NK Cell Receptors

NK cells are armed with germline-encoded receptors that can distinguish infected or transformed cells from normal. The molecular basis for target cell recognition by NK cells was postulated by the "missing-self" hypothesis several years ago (Kärre et al. 1986). Accordingly, NK cells are thought to preferentially kill targets that lack surface MHC-I (self) expression, while sparing normal cells with proper surface MHC-I expression levels. This self-tolerance was thought to be exclusively maintained by inhibitory receptor signaling upon MHC-I engagement (Figure 1.1). However, this presents a conundrum, because there are many other cells in the body with rather low levels of MHC class I expression which are yet safe from NK cell responses. Therefore, this hypothesis has evolved and acknowledged the requirement for additional activating receptors even before the discovery of receptors implicated in the recognition (Ljunggren and Kärre 1990).

Today, we know that NK cell function is controlled by an intricate balance between an array of inhibitory and activating receptors expressed at the cell surface and their corresponding ligands from a list of MHC class I and MHC class I-related molecules (Table.1.1)(Figure 1.1). The coupling of an NK cell receptor to its ligand

provides signals and a logic sum function of these signals will dictate whether an NK cell is activated or not (Figure 1.2). Indeed, it is now accepted that a lack of inhibitory signals *per se* cannot alone drive the cytotoxicity and that an activation signal is almost exclusively required, shedding light on the question of why some cells are deemed as targets despite expressing high amounts of MHC class I molecules (Leibson 1997).

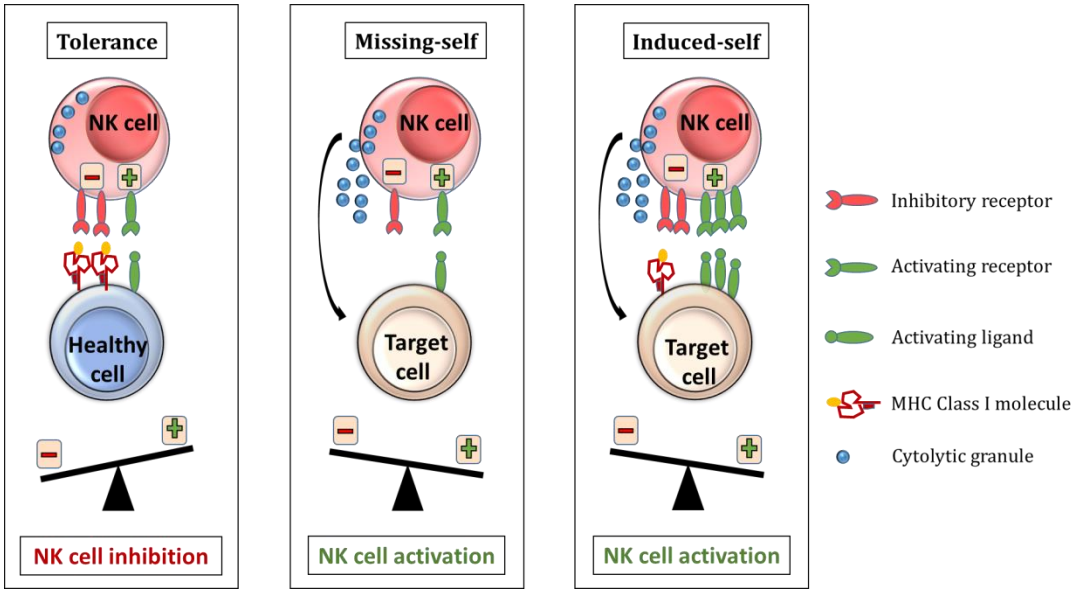


Figure 1.1. Mechanism of NK cell target recognition. MHC Class I (inhibitory ligand) is expressed by all nucleated cells to mediate tolerance to self in the presence or absence of activating ligands (left panel). When a cell is transformed or virally infected, it loses MHC Class I molecule expression (missing self in the middle panel) or increase expression of stress-induced ligands (stress-induced self in the right panel) and eventually becoming a target for NK cells. This recognition activates NK cells and induces release of cytolytic granules to kill the target cell (Adapted from (Vivier et al. 2012)).

1.1.3. NK Cell Receptor Families

Even though several receptors modulating NK cell actions have been recognized, the contribution of signals from these receptors to different aspects of NK cell biology demands further investigation (Watzl and Long 2010; Cruz-Muñoz et al. 2019). Based on current knowledge, NK cell receptors can be categorized under certain groups according to their impact on effector functions.

Receptors that favor NK cell activation by means of adhesion, degranulation or granule polarization can be categorized as activating receptors. Under normal conditions, independent from the surrounding cytokines, the balance towards the activation side can be shifted by the synergy of at least two activating receptors stimulated simultaneously (Bryceson, Ljunggren, and Long 2009). A wide selection of activation, co-activation, and adhesion receptors such as the natural cytotoxicity receptors (NCRs), DNAX accessory molecule (DNAM)-1, CD16, NKG2C and NKG2D receptors induce NK cell-mediated killing upon binding to ligands upregulated on stressed or infected cells (Vivier et al. 2008). The only exception to this rule of engaging multiple activating receptors seems to be CD16, which is able to fulfill all the requirements to promote efficient effector functions without the contribution of any synergy from other receptors (Bryceson et al. 2005). In addition, LFA-1 activating receptor engagement to its ligand (ICAM-1) is necessary in most cases to assist adhesion to target cells and provide signals to direct the cytotoxic granules towards the immunological synapse, where they release for efficient target cell cytolysis (Bryceson et al. 2005).

The varied nature of these receptors and the absence of signaling domains in their cytoplasmic tails mandates the recruitment of receptor-associated adaptor molecules for positive signal transduction (Lanier 2008). Activation signals are transduced through the adaptor transmembrane proteins such as CD3ζ, the high-affinity IgE receptor-γ (FcεRIγ), and DAP12, which signal via Immunoreceptor Tyrosine-based Activating Motifs (ITAMs) contained in their cytoplasmic tails. While CD16, NKp46 and NKG2D strictly follow these adaptors, NKG2D can also signal via the YINM motif present within the adaptor DAP10 (Lanier 2009). Interestingly, NKp80 receptor signals through an atypical tyrosine module named as the hemi-ITAM (or hemITAM), that recruits the kinase Syk, to trigger cellular cytotoxicity (Welte et al. 2006; Bauer and Steinle 2017; Steinle, Dennehy, and Klimosch 2011).

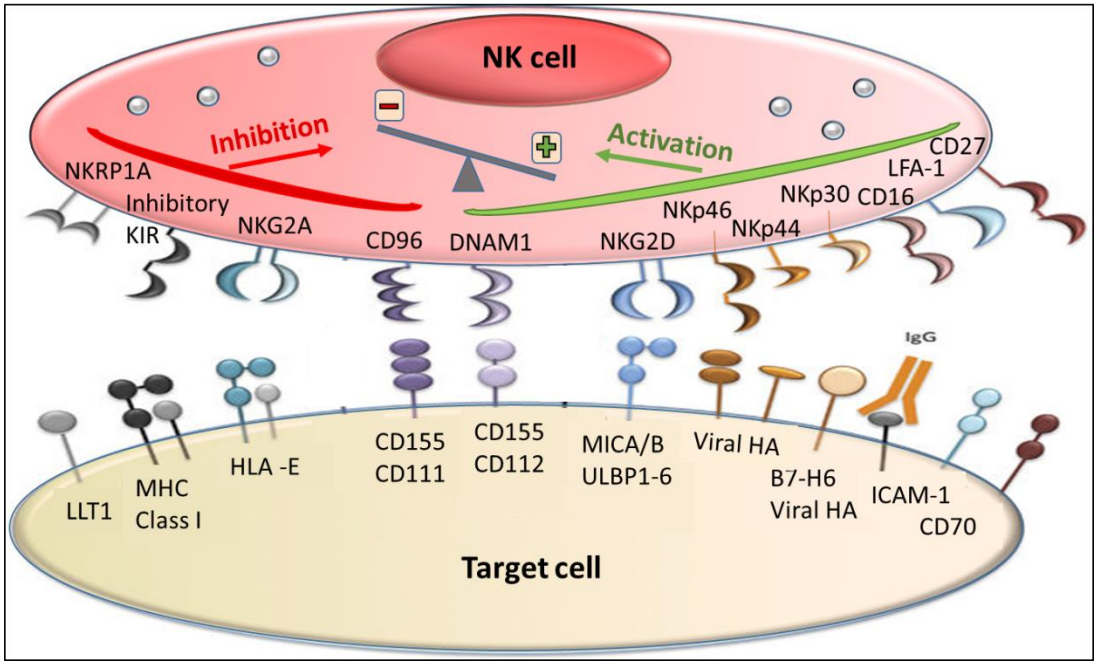


Figure 1.2. The balance between activating and inhibitory receptors determines the NK cell response. NK cell-mediated lysis relies on this intricate balance between several activating and inhibitory receptors that either triggers or dampens NK cell effector functions upon engaging their ligands. NKRP1A, inhibitory killer immunoglobulin-like receptor (KIR), NKG2A and CD96 are some of the inhibitory receptors that transduce inhibitory signals, while DNAM1, NKG2D, NKp46, NKp44, NKp30, CD16, LFA-1 and CD27 are some of the activating receptors that engage to their corresponding ligands to induce activating signals (Adapted from Martinet et al. *Cell Death and Differentiation*, 2014).

NKp30 (NCR3), NKp44 (NCR2), NKp46 (NCR1) and NKp80 (KLRF1) are part of the NCR family. NKp30, NKp46 and NKp80 are constitutively expressed on both resting and activated NK cells, while NKp44 is solely expressed upon activation (e.g. IL-2 induction)(Fuchs et al. 2005). A wide variety of NCR ligands have been reported for these receptors, starting with ligands encoded by viral genes, bacteria and parasites, but ongoing studies are still being pursued for defining new ligands (Kruse et al. 2014; Barrow, Martin, and Colonna 2019). The ligands for NKp30 and NKp44 have been identified on several types of tumors (Byrd et al. 2007). Also, together with NKp46; NKp44 has been suggested to interact with hemagglutinin of influenza virus and thus, to be able to lyse influenza-infected cells (Arnon, Markel, and Mandelboim 2006). Although all NCRs widely interact with components of viral hemagglutinins, NKp80 can only bind to activation induced C-type lectin (AICL) as a ligand (Welte et al. 2006).

DNAM-1 is both an activating receptor and an adhesion molecule. It is expressed not only by NK cells but also subsets of T and B lymphocytes, and myeloid cells (Martinet and Smyth 2015). It binds to poliovirus receptor CD155 (PVR) and to the nectin adhesion molecule CD112 (PVRL2), both of which are significantly upregulated in tumor cells and CMV infected cells (Castriconi et al. 2004). DNAM-1 has been shown to direct NK cell-mediated cytotoxicity against melanoma, sarcoma and also, its knockout mouse model, to be more susceptible to primary and transplantable tumors (Iguchi-Manaka et al. 2008; Gilfillan et al. 2008). Therefore, these studies elucidate its essential role in preventing spontaneous tumor formation and controlling tumor growth. Although the exact signaling pathway has not been well-characterized yet, it is known to send positive signals via an immunoreceptor tyrosine tail (ITT)-like motif that pairs DNAM-1 to Grb2 and to downstream effectors such as Vav-1 which is a central modulator of NK cell signaling pathways (Z. Zhang et al. 2015).

Another receptor family is the CD94- the Natural Killer Group 2 (NKG2) receptor system, CD94-NKG2 (Lanier 2005). In this complex of the C-type lectin family, a type II integral membrane glycoprotein (CD94) forms a disulfide-linked heterodimer with different members of the NKG2 family (CD94-NKG2A/C/E). The heterodimers such as CD94-NKG2C and CD94-NKG2E are associated with DAP12 and its cytoplasmic ITAM; consequently, they act as activating receptors. On the other hand, NKG2A contains Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) in its cytoplasmic domain by which it is classified as an inhibitory receptor (Lanier et al. 1998; Kabat et al. 2002). These receptors are expressed predominantly by NK cells and considered important for the recognition of nonpolymorphic MHC class I molecules (e.g. HLA-E in humans). However, it appears that NKG2A has a higher affinity for HLA-E; thus, when NKG2A and NKG2C are co-expressed by the same NK cell, the inhibitory signals can prevail (Béziat et al. 2011). NKG2C expression is correlated with NK cells expansion during human cytomegalovirus infection, while the exact role for NKG2E remains unclear (Gumá et al. 2006).

Besides NKG2C/E, there is another activating receptor, NKG2D, that forms a homodimer in its active form and does not heterodimerize with CD94, contrary to

its counterparts (Lanier 2015). NKG2D associates with DAP10, which recruits either phosphatidylinositol-3 kinase (PI3K) through its p85 subunit or Grb2 adaptor to induce positive signals for NK cell activation, as previously mentioned (C. Chang et al. 1999). NKG2D is constitutively expressed on NK cells and subsets of T cells and recognizes stress-inducible ligands on transformed, virus-infected or DNA damaged cells (Lanier 2015). These MHC-I related ligands can be listed as UL16-binding protein (ULBP) 1-6 and MICA/B and are not normally expressed by healthy tissues suggesting a significant role in cancer immunosurveillance (Spear et al. 2013).

The key receptors on NK cells controlling the 'self-recognition' are MHC class I-binding receptors, including the Killer Cell Immunoglobulin-like Receptor (KIR) family, the NKG2 receptor system and Leukocyte immunoglobulin-like receptor subfamily B member 1 (LIR-1) (Vivier et al. 2008). They can recognize ligands that are MHC class I molecules, as well as non-MHC class I molecules. A major group of inhibitory receptors are the KIRs which are members of the Ig superfamily (Lanier 2005). This family of receptors recognizes allelic forms of HLA-A, -B and -C (allotypes). All well-characterized inhibitory KIR receptors encompass a conserved ITIM. As soon as inhibitory receptors are cross linked with their cognate ligands, the tyrosine residues in ITIMs get phosphorylated and initiate a cascade of events that dampen NK cell activation by counterbalancing signals that are necessary for recruitment and phosphorylation of activating receptors effecting also their downstream elements (Long 2008). Because MHC class I molecules are expressed by virtually all nucleated cells, their recognition by inhibitory receptors are required for acquisition of functional competence during development, as well as for a state of tolerance. The inhibitory KIRs and NKG2A receptor are, therefore, assigned in a functional maturation process called NK cell education that allows self-inhibited NK cells to get a "license-to-kill" (Anfossi et al. 2006). Indeed, before encountering a target cell, NK cells undergo a functional calibration –or become licensed- during which engagement of these inhibitory receptors by self MHC molecules enables them to respond robustly to target cells with decreased MHC I and/or increased stress-induced ligand expression.

In addition to these inhibitory receptors specific for MHC-I molecules, a novel group of receptors is classified as inhibitors of NK cell functions based on a system of recognition independent of the missing-self hypothesis that includes receptors such as T cell immunoreceptor with Ig and ITIM domains (TIGIT), CD96, T cell immunoglobulin mucin-3 (Tim-3) and programmed cell death protein 1 (PD-1). TIGIT and CD96 contain ITIM motifs mediating inhibitory signals and compete with DNAM-1 for binding their shared ligands, hence counterbalance the activation signals generated by DNAM-1 engagement (Chan et al. 2014). In NK cells, TIGIT is expressed upon activation, while CD96 is constitutively expressed (Martinet and Smyth 2015). Tim-3 on NK cells can induce cytokine secretion and establish cytolytic responses and has at least 4 known ligands, but its immunological relevance in NK cells remains largely unknown (Ndhlovu et al. 2012). Its interaction with one of its ligands, Galectin-9, results in NK cell hyporesponsiveness, while overexpression of Tim-3 can cause dysfunction. This suggests a prominent attenuating role of this receptor over NK cell functions.

PD-1 is expressed in immune cells including T lymphocytes and NK cells. Even though its function in tumor-specific T lymphocytes has been extensively studied in the past decade, PD-1 expression on NK cells has only recently gained attention. Upon engagement with its ligands PD-L1 and/or PD-L2, PD-1 can constrain granule polarization and dampen NK cell activation (Pesce et al. 2017). PD-1 upregulation on NK cells from patients with multiple myeloma, sarcoma and lymphoma is associated with NK cell dysfunction and immune suppression (Beldi-Ferchiou et al. 2016; Pittari et al. 2017; Vari et al. 2018). PD-1 signals through one ITIM and one tyrosine-based switch motif (ITSM). When coupled to its ligand, these two tyrosine-based motifs are phosphorylated by Src family kinases and subsequently result in recruitment of SHP-1 and SHP-2 tyrosine phosphatases that turn off activation signals (Cruz-Muñoz et al. 2019).

In addition to receptors with well-defined functions, NK cells also possess receptors with dual functions that can either promote or inhibit the functions of primary activating receptors on their surfaces. These receptors belong to a group as signaling lymphocyte activation molecule (SLAM) family and their expression on NK cells are important to prevent self-killing. These receptors such as 2B4, NTBA, CRACC, CD2 harbor at least one ITSM. This motif undergoes phosphorylation to

recruit members of the SH-2 domain containing SLAM-associated protein (SAP) family.

Table 1.1. NK cell receptors and their ligands.

Receptor	CD number	Activating	Inhibitory	Ligands
LFA-1	**CD11a**	X		ICAM-1,-2,-3,-4,-5
LFA-2	**CD2**	X		CD58 (LFA-3)
FcRγIII	**CD16**	X		Fc domain of IgG
NKp30	**CD337**	X		B7-H6, HA*, HS*, HCMV pp65*
NKp44	**CD336**	X		HA*, HS*, HN*, PCNA*, unknown bacterial components
NKp46	**CD335**	X		HA*, HS*, HN*, unknown bacterial components, extracellular ligands
NKp80	**-**	X		AICL*
TNFRSF7	**CD27**	X		CD70
NKG2A	**CD159a**		X	HLA-E
NKG2C	**CD159c**	X		HLA-E
NKG2E	**CD159e**	X		HLA-E
NKG2D	**CD314**	X		MICA/B, ULBP 1-6
Tactile	**CD96**		X	CD155
TIGIT	n/a		X	CD112, CD155
DNAM-1	**CD226**	X		CD112, CD155
Tim-3	**CD366**		X	Galectin-9
PD-1	**CD279**		X	PD-L1, PD-L2
2B4	**CD244**	X	X	CD48
NTBA	**CD352**	X	X	NTBA
CRACC	**CD319**	X	X	CRACC
KIRs	**CD158**	X	X	MHC Class I molecules
BY55	**CD160**	X		HLA-C
NKR-P1A	**CD161**	X		LLT1

IgG, immunoglobulin G; HA, hemagglutinin; HS, heparan sulfate; HCMV, human cytomegalovirus; HN, hemagglutinin neuramidase; PCNA, proliferating cell nuclear antigen; AICL, activation-induced C-type lectin; HLA-E, human leukocyte antigen-E; MIC, major histocompatibility complex (MHC) class I polypeptide-related sequence; TIGIT, T cell immunoreceptor with Ig and ITIM domains; n/a, not applicable; Tim-3, T cell immunoglobulin mucin-3; PD-1, programmed cell death protein 1; PD-L, PD ligand; KIR, killer cell immunoglobulin-like receptor; LLT1, lectin like transcript 1. *These natural cytotoxicity receptor ligands have not been fully confirmed yet (Morvan and Lanier 2016; Konjević et al. 2007).

Besides the ITSM, the cytoplasmic tail of SLAM receptors contain tyrosine residues targeted for phosphorylation by Src family kinases, thus, preparing these tyrosine residues for recruitment of phosphatase containing SH2 domains. Studies on ITSM revealed that the SAP-related adaptor proteins are crucial elements that determine NK-mediated functions for SLAM family receptors (Veillette 2006). Apart from 2B4 and CD2, both of which recognize CD48 (the other member of the SLAM family) and CD58 respectively as ligands; NTBA and CRACC serve as

self-ligands in the context of homotypic cell interactions amongst NK cells. The expression of these receptors are largely restricted to the hematopoietic compartment. The recognition of 2B4, CRACC and NTBA ligands on MHC-I positive cells (e.g. neighboring NK cells) bypasses NK cell cytotoxicity but they cannot prevent NK cell targeting in the case of MHC-I loss (Veillette 2010). Here, it is important to note that NTBA and 2B4 may also function as a co-receptor amplifying NK cell responses activated by NCRs or cytokines (Marcenaro et al. 2011).

1.2. NK Cells in Cancer

1.2.1. Role in Tumor Immunosurveillance

The concept of immunosurveillance was first proposed in 1909 by Paul Ehrlich, suggesting that the immune system continuously eliminates arising tumors and thus, prevents the occurrence of cancer (Ehrlich 1909). The formal hypothesis on cancer immunosurveillance was shaped by Burnet and Thomas in 1957 (Burnet, Walter, and Hall 1957). Nevertheless, the concept failed to promote much acceptance until the observations of increased susceptibility to chemically induced tumors in immunodeficient mice were presented decades later (Kaplan et al. 1998; Shankaran et al. 2001). It was shown that these highly immunogenic tumors can be eradicated, when transplanted to an immunocompetent host. These results unmask the role of the immune system in cancer immunoediting, which comprises of three phases; elimination, equilibrium and escape (Dunn et al. 2002). As proposed earlier, stressed aberrant tumor cells are detected and eliminated by immune cells in the process of immunosurveillance. As the scavenging continues, the aberrant cells and immune cells reach to a state of truce where the tumor cells do not grow but cannot be eliminated by the immune system either. This dynamic equilibrium resembles a microevolutionary process and is maintained by the Darwinian selective pressure of the immune system exerted on the tumor cells; where tumor cells attain sufficient mutations or phenotypic changes to evade from detection. Hence, the immune edited tumor cells can now escape and outgrow the immune control.

The role of NK cells in cancer immunosurveillance is further revealed, when the anti-tumor responses of *RAG2*$^{-/-}$ mice which lacked adaptive immunity (T and B lymphocytes) are compared to *RAG2*$^{-/-}$*x [gamma]c*$^{-/-}$ mice which lacked adaptive immunity and NK cells (T. O'Sullivan et al. 2012). The latter exhibited higher incidence of sarcoma upon methylcholanthrene (MCA)-induced sarcomagenesis and thus, provides formal proof that NK cells control tumor growth in the absence of adaptive immunity. In line with this evidence, there are correlation studies in human cancers, where low NK cell counts and cytotoxic ability compromises individuals with familial incidence of cancer (Strayer, Carter, and Brodsky 1986; Bovbjerg and Valdimarsdottir 1993). Moreover, an 11-year prospective study traced the high and moderate cytotoxic activity of peripheral blood derived mononuclear cells (PBMC) and found that individuals with medium-high PBMC cytotoxic activity had a lower risk of developing cancer and vice versa (Imai et al. 2000). More evidence from animal models emphasize that knock-out of key NK cell receptors such as NKG2D and DNAM-1 results in a higher incidence of tumors compared to wild-type mice (Guerra et al. 2008; Iguchi-Manaka et al. 2008). Although the role of NK cells in the elimination of tumors has been controversial for many years, the observations in experimental models significantly correlate with clinical data for both solid and hematological malignancies suggesting that NK cells play essential roles in tumor immunovigilance and they are beneficial against cancer progression.

1.2.2. NK Cells in Cancer Patients

NK cells constitute the first line of defense against transformed cells. When hematopoietic cells undergo malignant transformation as a result of DNA damage during unregulated cell division, the DNA damage responses get activated, which induces NKG2D and DNAM-1 ligands on several tumor cells (Gorgoulis et al. 2005; Soriani et al. 2009).

NK cells are circulating lymphocytes that are attracted to the tumor milieu, where they are confronted with immunosuppression in the context of tumor evasion mechanisms. Besides poor NK cell homing and infiltration into the tumor microenvironment (TME), NK cells are also challenged with an assortment of cell

types that downmodulate the immune responses. TME harbors fibroblasts, tumor-associated macrophages, stromal cells, regulatory T cells and myeloid derived suppressor cells (MDSCs) that force specific changes in the cytotoxic phenotype, epigenetic and transcriptional profile and metabolic program of infiltrating immune cells (Whiteside 2008). Also, the TME induces several other inhibitory effects such as secretion of immunomodulatory cytokines and soluble factors such as TGF-β and IL-10, as well as upregulation of inhibitory checkpoint ligands (e.g. PD-L1 or HLA-E) or downregulation of activating receptor ligands (NKG2D) and hypoxia (Balsamo et al. 2013; Gasser et al. 2005; Li et al. 2009; Ghiringhelli et al. 2005). While these can suppress NK cell functions in the TME, being exposed to conditions prevalent in the TME or dysregulated NK cell receptor signaling may also induce a functional exhaustion of NK cells that impairs their anti-tumor activity. The NK cell exhaustion phenotype is marked by the downregulation of effector cytokines such as IFN-γ, impaired degranulation, downregulation of activating receptors such as NKG2D or upregulation of inhibitory receptors such as NKG2A, along with the downregulation of key transcription factors such as T-bet and Eomes (Bi and Tian 2017).

1.2.2.1. NK Cells in Hematological Malignancies

While the role of NK cells in tumor immunosurveillance comes from the indirect evidence of cohort studies, clinical observations from hematological malignancies indicate that a ligand repertoire expression on leukemic blasts of the myeloid lineage favoring NK cell activation is positively linked to better outcome of patients after chemotherapy (Mastaglio et al. 2018). However, NK cells in acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and myelodysplastic syndrome (MDS) are faced with several challenges in the TME (e.g. the bone marrow) and are often found to be dysfunctional especially in patients with advanced disease (Table 1.2).

Table 1.2. The role of NK cells in the pathogenesis of hematological malignancies.

Malignancy	NK cell phenotype	Clinical Relevance	References
ALL	Strong NK cell effector phenotype	Correlation with minimal residual disease	(Sullivan et al. 2014)
CLL	NK cell number	Correlation with disease stage and prognosis	(Apostolopoulos, Symeonidis, and Zoumbos 1990; Palmer et al. 2008)
CLL	NKp30 downregulation	Correlation with poor prognosis	(Hadadi et al. 2019)
AML	NKp30, NKp44, NKp46 downregulation	Correlation with poor prognosis	(Lion et al. 2012)
AML	NKG2A upregulation	Reduced effectiveness of chemotherapy	(Stringaris et al. 2014)
MDS	Reduced NK cell function and NKG2D downregulation	Association with high-risk disease	(Epling-Burnette et al. 2007)
CML	NKG2D downregulation	Imatinib restored NKG2D expression	(Maghazachi 2010)
Burkitt lymphoma	Reduced cytotoxicity and NKp46, NKp30 and CD160 expression	Correlation with poor prognosis	(Forconi et al. 2018)
T cell lymphoma	Higher NK cell numbers	Correlation with poor prognosis	(Mundy-Bosse et al. 2018)
MM	Soluble MICA production	Correlation with poor prognosis	(Rebmann et al. 2007)

ALL, Acute Lymphocytic Leukemia; CLL, Chronic Lymphocytic Leukemia; AML, Acute Myeloid Leukemia; MDS, Myelodysplastic Syndrome; CML, Chronic Myeloid Leukemia; MM, Multiple Myeloma (Adapted from (Gonzalez-Rodriguez et al. 2019)).

Several studies show a link between poor NK cell functions and altered NK cell phenotype, subset composition and competence to form an efficient immunological synapse (M. Carlsten et al. 2010; Costello et al. 2002; Epling-Burnette et al. 2007; Stringaris et al. 2014; Khaznadar et al. 2014). Most of these studies share common downregulation of key activating receptors such as the NCRs, DNAM-1 and NKG2D, independent from the subtype of MDS or AML and the loss of these receptors positively correlates with the disease burden (Costello et al. 2002; Sanchez-Correa et al. 2019). However, NK cell function in these settings can be fully or partially be restored in patients achieving complete remission after chemotherapy (Fauriat et al. 2007).

Furthermore, the presence of soluble molecules in the tumor microenvironment secreted by AML blasts and even AML stem cells, can be another factor that

triggers the loss of the activating receptor, NKG2D (Song et al. 2006; Hilpert et al. 2012; N. Boissel et al. 2006). The presence of soluble NKG2D ligands, including MICA, MICB, ULBP1, ULBP2, shed by the tumor cells, has a particularly powerful immunosuppressive effect by downregulating surface NKG2D expression on NK cells. Targeting these molecules has a great deal of therapeutic potential.

While downregulation of activating receptors is a common mechanism to escape from elimination, one other strategy is put forward by the myeloid malignancies along the same receptor-ligand axis. Data show that leukemia cells downregulate ligands of the DNAM-1 receptor to render the cells resistant to NK cell mediated killing (Kearney et al. 2016). Even though leukemic blast in AML patients express very low levels of ULBP and NCR-specific ligands, treatment with differentiation-promoting myeloid growth factors and IFN-γ can revert the resistance from NKG2D and NCRs on NK cells (Nowbakht et al. 2005). In all, intriguing strategies can be elicited by myeloid malignancies to trigger NK cell suppression, reduced NK cell numbers in the vicinity of tumor cells and evade from NK cell-mediated recognition.

1.2.2.2. NK Cells in Solid Tumors

The extent of NK cell infiltration into a solid tumor is a point of discussion, although the consensus is that it can contribute to good prognosis (Habif et al. 2019). Even though studies in head and neck, lung, breast, renal cell tumors demonstrate NK cells in tumor sites with possible clinical benefits, the majority of the tumors show almost complete dismissal of NK cell infiltration at the primary tumor sites (Halama et al. 2011; Muntasell et al. 2019; Eckl et al. 2012; André et al. 2018; Lavin et al. 2017).

A study on melanoma showed poor $CD56^{+}$ NK cell infiltrations in skin lesions, even though highly cytotoxic $CD56^{dim}KIR^{+}CD57^{+}$ NK cells were found in tumor-infiltrated lymph nodes (LN) (Erdag et al. 2012; Ali et al. 2014). NKG2D and NKp30 expression were inversely correlated with the number of tumor cells in the infiltrated LN. Also, the downregulation of activating receptors such as NKp30, NKp44 and NKG2D at the surface of PB NK cells in addition to their magnitudes

were also associated with tumor growth in cervical cancer patients (Garcia-Iglesias et al. 2009).

Furthermore, when tumor-infiltrating NK cells (NK TILs) in lung carcinoma were analyzed, it was found that they had profoundly impaired cytotoxicity, despite having expression of activating NKp44, CD69 and HLA-DR markers (Lavin et al. 2017). However, the source of failed NK TIL function might be explained by the radical downregulation of other activating receptors such as NKp30 and NKp80 (Platonova et al. 2011). In non-small cell lung carcinoma (NSCLC) samples, NK TILs were found to express higher levels of CD96 and PD-1 than those in the circulating NK cells (Carrega et al. 2008). Overall, circulating cytotoxic NK cells have the ability to penetrate into some solid tumors and this is usually associated with good prognosis and better survival. However, most of the cases demonstrate that NK cells are forced to attain an unresponsive phenotype by either downregulation of activating receptors or upregulation of inhibitory receptors at the tumor sites.

Another evasion strategy employed by solid tumors can be the modulation of ligand expression evident on a selection of sarcoma tumors. Ewing sarcoma (EW) cell lines, as well as primary explants have been shown to express the ligands for the activating NK cell receptors, DNAM-1 and NKG2D and could be conferred susceptible to lysis after an *ex vivo* activation of NK cells *in vitro* (Verhoeven et al. 2008). Consistently, osteosarcomas and rhabdosarcoma cell lines have been shown to express CD155 and/or CD112, the ligands for the activating DNAM-1 receptor (Kiany, Huang, and Kleinerman 2017; Boerman et al. 2015). However, many tumor cells express the PCNA molecule, an inhibitory ligand for NK cells, that correlates with poor prognosis, indicating that they express enough PCNA to at least partially inhibit NK cell-mediated killing at the tumor site (Wang et al. 2017).

1.3. NK Cells in Cancer Immunotherapy

Cancer immunotherapy is emerging as a beneficial tool for cancer treatment by activating the body's own immune system to produce anti-tumor effects. In recent

years, the use of targeted therapies with antibodies, small molecules and cell-based (adoptive) immunotherapy has culminated in many clinical breakthroughs in cancer treatment. The impact of such molecules in cancer therapy was appreciated by the Nobel Prize committee for Physiology or Medicine in 2018.

The importance of NK cells in tumor recognition and destruction combined with their pivotal immunoregulatory capacity confer NK cells a promising outlook for cancer immunotherapies. Because they are endowed with intrinsic abilities to preferentially target even stem-like tumor cells without priming, NK cells have been nominated as promising agents for cell-based therapies (Grossenbacher, Canter, and Murphy 2016). Therefore, small molecules and targeted antibodies are included in the development of novel protocols to effectively manipulate NK cell activity and overcome NK cell paralysis in cancers for ultimate integration in NK cell-based cancer immunotherapy.

1.3.1. Modulation of NK Cell Activity

Cytokine-activated NK cells

Cytokines such as interleukin (IL)-2, -12, -15, -18 and -21 serve as small molecule agents to promote differentiation, proliferation, survival and activation of lymphocytes (Floros and Tarhini 2015; Srivastava et al. 2013; S. A. Rosenberg et al. 1989; Phillips et al. 1987; Steven A. Rosenberg 1984). *Ex vivo* manufacturing of NK cells relies on the use of these cytokines in order to expand NK cells in sufficient clinical doses and boost anti-tumor reactivity. Among them, IL-2 is the most studied cytokine, since its first use in patients with advanced cancers (S. A. Rosenberg et al. 1989; Phillips et al. 1987; Steven A. Rosenberg 1984). The initial attempts of high-dose IL-2 therapy have comprised systemic administration of recombinant human (rh) IL-2 alone or in conjunction with adoptive transfer of PB derived autologous NK cells stimulated with IL-2 before injecting into patients. Although NK cells turn into lymphokine-activated killer (LAK) cells upon stimulation, exhibit enhanced proliferation and cytotoxicity owing to upregulation of NKp44, NKG2D, FasL, TRAIL, perforin, granzymes *in vitro*, their anti-tumor efficacy are not consistently effective in most cancer patients due to adverse

systemic complications and selective expansion of regulatory T cells (Tregs) that are activated by their IL-2 high affinity receptor (IL-2αβγ) (Rodella et al. 2001; Sim et al. 2014). These results lead to the development of alternative forms of IL-2. An updated recombinant IL-2 has been constructed with increased *in vitro* affinity for the IL-2/15Rβ subunit expressed only by NK cells, to exclude Tregs (Levin et al. 2012). Alternatively, other mutant forms of IL-2, such as F42K, show reduced affinity for IL-2Rα, but pronounced activating NK cell functions (Sim et al. 2016). A superior alternative to IL-2 is IL-15, which can preferentially induce NK cell maturation and survival (Cooper et al. 2002). Indeed, administration of exogenous IL-15 has been shown to maintain NK cell survival, *in vivo* persistence and therapeutic efficacy, without inducing Treg expansion (Waldmann 2006). However, short half-life and poorly tolerated doses in patients might jeopardize the clinical use of rhIL-15. The biological function of rhIL-15 inspires new clinical trials where it is used in combination with other cytokines and antibodies (NCT03388632:nivolumab and ipilimumab)(Waldmann et al. 2020). Moreover, a superagonist of IL-15 called ALT-803, in which an IL-15Ra sushi domain fused to IgG1Fc complexed with IL-15 mutein (N72D), was shown to potently enhance NK cell survival and cytotoxicity in many clinical settings, in comparison to its native form (Felices et al. 2017; Romee et al. 2018). This fusion protein not only stimulates typical *trans* presentation of IL-15, but also prolongs its half-life, which may lead to enhanced ADCC against human carcinoma cells (Fantini et al. 2019). Results from phase I and II clinical studies with ALT-803 proved to be safe, well-tolerated with no severe dose-limiting toxicities and GVHD in patients with hematological (relapsed/refractory MM, AML, ALL and MDS) and solid tumors. Also, all patients exhibited expansion and activation of circulating NK and $CD8^+$ T cells with minimal stimulation of Tregs. These clinical trials involving patients with both hematological malignancies and solid tumors or in combination with adoptive therapy of NK cells, or with nivolumab are currently being investigated more thoroughly (Margolin et al. 2018; Wrangle et al. 2018; Fantini et al. 2019).

Notably, the use of IL-15 in combination with IL-2 shows a synergic impact on viability and proliferation of an *ex vivo* expanded NK cell product (Siegler et al. 2010). This underlines the requirement of cytokine cocktails to achieve potency in terms of cell numbers and efficacy. Therefore, new expansion methods are being

exploited for pre-activation of PB-derived NK cells using cytokine combinations. Interestingly, one such cocktail of IL-12, -15, -18, was observed to prime NK cells with long-lasting enhanced NK cell functions enduring even after withdrawal (Romee et al. 2016). These cytokine-induced memory-like NK cells with robust anti-tumor capacity against leukemia targets and have phenotypic expression of NKG2A, NKG2C and CD94, but reduced inhibitory KIRs. The reports from clinical trials in patients with AML described the peculiar features of this artificial NK cell product as antigen unspecific with pronounced proliferative capacity, *in vivo* persistence up to 3 months, superior IFN-γ production and cytotoxicity upon *ex vivo* restimulation (Romee et al. 2016; 2012). Although cytokine regimens hold great promise for adoptive NK cell therapy, their limitations to dose-dependency, cytokine-induced NK-cell apoptosis and long-term cytokine exposure causing NK cell exhaustion should carefully be evaluated (Chambers, Lupo, and Matosevic 2018).

Tumor-Specific Antibodies to Mediate ADCC of NK cells

Tumor-specific antibodies are monoclonal antibodies (mAbs) that work partially by inducing NK cell-mediated ADCC through the binding of the IgG Fc entity and the activating CD16 receptor on NK cells. While they lead a preponderant role in cell-mediated immunity, NK cells also modulate adaptive responses and provide long-term protection through ADCC (Michaud et al. 2014). Leading evidence on the importance of ADCC comes from anti-CD20 (rituximab)-treated non-Hodgkin's lymphoma patients, and anti-HER2 (herceptin)-treated metastatic breast cancer patients (Iannello and Ahmad 2005; Sondel and Alderson 2011). Intriguing modifications are applied to monoclonal antibody designs to alter antibody structure to reduce complement activation and augment NK cell-ADCC function, while at the same time refraining from antibody-induced allodynia (Sorkin et al. 2010). Humanized anti-GD2 mAb can induce ADCC and simultaneously reduce toxicity associated with recombinant anti-GD2 therapy (Navid, M. Santana, and C. Barfield 2010).

Some studies focus on improving the binding affinity for Fc receptors through mutagenesis or glycosylation. Having the substitution S239D/I332E, Fc optimized

CD133 antibody had higher affinity to NK cells, subsequently, showing potent degranulation against human AML blasts in a xenograft model (Koerner et al. 2017). Recently, mAb 7C6 directed against the sites responsible for proteolytic shedding of important immunostimulatory ligands such as MICA/B and prevent their loss on cancer cells (De Andrade et al. 2018). These antibodies provided evidence that NK cells can be redirected against tumor targets by activation of NKG2D and CD16 and induce elevated anti-tumor immunity in a metastasis model pretreated with human NK cells.

Releasing the Breaks on NK cells: Targeting the Immune Checkpoints

Monoclonal antibodies targeting immune checkpoint receptors or ligands aim to unleash a blocked response and increase anti-tumor effects. One of the main inhibitory receptors is the inhibitory KIRs, which can be targeted by fully human anti-KIR antibodies (e.g. IPH2101 or IPH2102) (Benson et al. 2015; Vey et al. 2018). IPH2101 and IPH2102 are engineered immunoglobulin G4 (IgG4) clinical grade mAbs that have high affinity for inhibitory KIR2D moieties, blocking its interaction with HLA-C ligands. *In vitro*, they dose-dependently augment NK cell-mediated killing of autologous HLA-C expressing and antibody coated tumor cells (Vey et al. 2018). The safety and tolerability of IPH2101 were reported in phase I clinical trials in patients with AML or relapsed refractory MM (Benson et al. 2012; Vey et al. 2018). However, phase II clinical studies unexpectedly demonstrated that systemic infusion of IPH2101 significantly reduced KIR2D expression on NK cells and provoke a hyporesponsive state on NK cells, probably due to trogocytosis of NK surface expression, in multiple myeloma and smoldering multiple myeloma (Mattias Carlsten et al. 2016; Korde et al. 2014). These results suggest that IPH2101 antibody mediated KIR2D blockade *in vivo* might be restricted by loss of KIR2D on NK cells and thus, failed to show therapeutic value. Moreover, high NKG2A expression is a hallmark of tumor-infiltrating T and NK cells which leads to poor prognosis and exhaustion phenomenon in many malignancies such as liver cancer (C. Sun et al. 2017). IPH2201, is a novel anti-NKG2A mAb which demonstrated elevated *in vivo* NK cell-mediated cytotoxicity to engrafted human primary leukemia cells or Epstein-Barr virus (EBV) cell lines (Ruggeri et al. 2016). In particular, the interim results of a phase II trial combining IPH2201 with cetuximab

(epidermal growth factor receptor (EGFR) antibody) in combination with PD-1 axis blockade reported boosted tumor immunity by rescuing the effector functions of both NK and T cells against B cell lymphoma, solid tumors and T cell lymphoma)(André et al. 2018).

Other inhibitory receptors such as PD-1, TIGIT, CD96, Tim-3 are often induced or upregulated on tumor-infiltrating lymphocytes and affect NK cell functions in tumor settings, resulting in dysfunction or functional exhaustion. Therapeutic mAbs are designed to block these receptors or their ligands in order to ameliorate anti-tumor activity, which can further improve NK cell-based immunotherapy protocols. Blocking PD-1/PD-L1 signaling considerably improves cytotoxicity and cytokine production of NK cells and suppresses tumor growth *in vivo* (Liu et al. 2017). The inhibitory receptor, TIGIT, competes with DNAM-1 to bind their shared ligands (CD112 and CD155) while CD96 shares binding of CD155 with DNAM-1, as previously mentioned. The balance between these three receptors determines NK cell reactivity towards tumor targets. In cancer patients, NK cells in the tumor microenvironment upregulate TIGIT and CD96 expression and this leads to NK cell exhaustion, accompanied by poor cytolytic potential (H. Sun et al. 2019; Zhou et al. 2018). Blocking TIGIT with mAbs can abolish this functional paralysis and rescue NK cell-dependent anti-tumor responses in several tumor-bearing mouse models (Q. Zhang et al. 2018). Intervening CD96-CD155 axis can potentially reverse both NK and T cell exhaustion and restore their effector functions. At present, the safety and efficacy of anti-TIGIT mAbs, alone or in conjunction with anti-PD-1 or anti-PD-L1 Abs, are being tested in clinical trials in patients with advanced or metastatic solid tumors (NCT03119428, NCT03563716, NCT03628677)(Sanchez-Correa et al. 2019). Still, the dearth of evidence in different disease settings does not dismiss their potential value for combined therapies. Further investigation should examine the efficacy of mAb derivatives targeting the inhibitory checkpoint receptors in various disease settings.

Immunomodulatory drugs (IMiDs)

In addition to cytokines (discussed above), immunomodulatory drugs (IMiDs) are used as exogenous agents to directly or indirectly manipulate NK cells and provide

better targeting of cancer cells (Dahlberg et al. 2015). Many clinical trials with IMiDs have been reported in the context of both hematological and solid malignancies (Pan and Lentzsch 2012). In multiple myeloma, thalidomide triggers NK cell-mediated blast lysis by activating their cytotoxicity and ADCC functions (Hayashi et al. 2005). Some of these drugs can induce expression of activating ligands on tumor cells or promote the recruitment of NK cells to the tumor microenvironment (TME) by increasing secretion of selected chemokines (Cifaldi et al. 2017). Lenalidomide is proved to be the most effective to overcome suppression of NK cells by TME-associated IL-6 and TGF-β. It lowers the threshold of MICA for NKG2D-mediated NK activation and induces TRAIL upregulation on NK cells, thereby improving expansion and anti-tumor activity (Jungkunz-Stier et al. 2014; Zhu et al. 2008; Xu et al. 2013). However, in the clinical setting, lenalidomide is usually used in combination with other modalities such as monoclonal antibodies anti-inhibitory KIR antibody therapy to sustain better yield in NK cell recognition and lysis in patients with MM (Benson et al. 2015).

1.3.2. Adoptive NK Cell Immunotherapy

1.3.2.1. Autologous NK Cells

Adoptive transfer of autologous NK cells has been tested in clinical trials for malignancies ranging from lung cancer, colon cancer, breast cancer to lymphoma (Cifaldi et al. 2017; Burns et al. 2003; Krause et al. 2004; deMagalhaes-Silverman et al. 2000). In general, this strategy serves as the safe option without excessive toxicity, especially in case of *ex vivo* pre-activation by cytokines or heat-shock protein 70-peptide. Although these provided an *in vitro* boost to NK cell lytic activity and *in vivo* persistence in circulation, patients with metastatic melanoma or renal cell carcinoma could not yet exhibit tumor regression due to significant downregulation of activating NKG2D receptor expression (Parkhurst et al. 2011). Only minor anti-tumor efficacy was observed in glioma, while no improvement was observed in relapsed lymphoma or breast cancer (Ishikawa et al. 2004; deMagalhaes-Silverman et al. 2000; Burns et al. 2003). The main reason is that the

inhibitory receptors on autologous NK cells match their self MHC I ligands on tumor cells and this recognition of the self inevitably suppress autologous NK cells *in vivo*. Besides, as mentioned before, autologous NK cells obtained from cancer patients are in an immunosuppressed state with impaired functions (e.g. dysfunction due to exhaustion) explaining this dismal performance. Blocking autologous NK cell expressed inhibitory receptors specific for MHC class I by using anti-KIR mAbs can unleash this NK cell inhibition, which is currently being tested in phase I clinical trials (Vey et al. 2018).

1.3.2.2.Allogeneic NK cells

Expanded NK cell therapy gains a lot of attention with the use of allogeneic cell products in the context of leukemia, lymphoma, renal cell carcinoma and melanoma. Here, the major risk is the development of graft-versus-host (GvHD) which can be avoided to some extent using immunosuppression or by high purity CD3 depletion. However, in a study by Ruggeri et al., it has been confirmed that donor natural killer cell alloreactivity, due to KIR ligand incompatibility, could eliminate relapse and graft rejection bypassing GvHD in AML patients receiving HLA mismatch donor transplantation (Ruggeri et al. 2002). Therefore, this is later adapted to immunotherapy of *ex vivo* activated allogeneic KIR/KIR ligand mismatched PB-derived NK cells in AML patients (Miller et al. 2005). Since then, use of alloreactive PB-derived NK cells for immunotherapy has been examined in clinical trials. As such, phase I clinical trials of non-small cell lung cancer with repeated dose of allogeneic NK cells have shown no sign of toxicity and adverse reaction, but significant clinical effectiveness when coupled to chemotherapy (Iliopoulou et al. 2010). This haploidentical transplantation (mismatch) can prove effective with good prognosis, only if a strict two-step purification of leukapheresis is applied maintaining CD3 depletion and CD56 enrichment (Miller et al. 2005). Rules for an effective allogeneic NK cell infusion have been reviewed by Leung et al. (Leung 2014).

1.3.2.3. Adoptive transfer of the NK-92 cell line

NK cells are potent effectors not only for their cognate cytotoxicity but also for orchestrating subsequent immune responses. Therefore, they are regarded as strong candidates for cellular therapy of patients with malignancies or severe viral infections. However, efforts to manufacture therapeutic NK cells for adoptive immunotherapy faced certain challenges so far. Using autologous NK cells isolated from peripheral blood are not very effective, as they are functionally silenced upon encountering self-MHC antigens. On the other hand, infusing KIR-mismatched allogeneic NK cell infusions can avoid suppression by the missing-self recognition in certain cases, although it has the risk of compromising patients for GvHD reactions, surprisingly even after CD3 lymphocyte depletion (Shah et al. 2015). There is also a limit to the number of *ex vivo* expanded NK cells than can be obtained and the issue of donor-dependent variations which raises concerns for logistics and cost. For these reasons, a continuously growing NK cell line, providing predictable numbers of highly cytotoxic NK cells on expansion, was established. The NK-92 cell line (an NK cell leukemia cell line) has been investigated most thoroughly and has already been applied in clinical settings (T. Tonn et al. 2001; Klingemann, Boissel, and Toneguzzo 2016). NK-92 cells express many hallmark activating receptors such as NKp30, NKp44, NKp46, NKG2D and yet, lack most of the inhibitor receptors such as TIGIT and PD-1, except for CD94-NKG2A and low levels of KIR2DL4 (Gong, Maki, and Klingemann 1994; Maki et al. 2001). This unique phenotype confers a broad range of malignant cells susceptible to highly cytotoxic NK-92 cells. General safety and *in vivo* persistence of infused gamma-irradiated NK-92 cells have already been established in several clinical trials (Arai et al. 2008; Torsten Tonn et al. 2013). As allogeneic NK cells, NK-92 is the only cell line to be approved for clinical trials and exhibited no toxicity or tissue specific side effects in patients with advanced melanoma and renal carcinoma (T. Tonn et al. 2001). In fact, this established cell line has the potential to be a molecularly and functionally well-characterized cell product expanded under good manufacturing practice (GMP)-compliance as an off-the-shelf therapeutic product (Suck et al. 2016). Unfortunately, the NK-92 cell line lacks CD16 expression and cannot mediate ADCC. Therefore, the use of NK-92 cell line

should be avoided in case of monoclonal antibody studies, unless they are genetically modified to express CD16 receptor (Jochems et al. 2016). However, the robust *ex vivo* expansion of homogenous cell populations to large-scale production, exquisite safety, in addition to the ease of genetic manipulation renders this cell line superior to primary NK cells and an ideal platform for NK cell-based adoptive immunotherapy.

1.4. Genetic Modification (GM) of NK cells

It has been more than a decade now since the first reports have established the anti-tumor efficacy of NK cells in patients with cancer (Miller et al. 2005; Ruggeri et al. 2002; Kärre 2002). Despite this revelation, uncertainties about NK cells' *in vivo* persistence, lack of antigen specificity and relinquished ability to home to tumor niches following infusions obscure their therapeutic potential for cancer immunotherapy. Tumor cells develop escape strategies to resist attacks from endogenous NK cells which can be overcome through modulation of NK cell activity by *ex vivo* activation and expansion. However, genetic modification of NK cells to overcome the suppression in tumors, to improve cytotoxicity or to enhance tumor target recognition may hold the key to advance the efficacy of NK cell-based cancer immunotherapy.

1.4.1. GM Methods for Immunotherapy

Genetic modification strategies aim to genetically reprogram the cells in an effort to maximize their anti-tumor potential and eventually, improve the outcome of adoptive immunotherapy. Until relatively recently, studies on genetically engineering NK cells have proven to be rather challenging due to poor transgene delivery and method-related NK cell apoptosis. However, among many different modalities of gene delivery into NK cells, viral transduction seems to successfully sustain long-term desirable changes. As it can be naturally anticipated, NK cells are inherently resistant to viral transduction. NK cells possess innate immune receptors such as pattern recognition receptors that recognize foreign genomic material and

they are likely to trigger apoptosis upon viral transduction which can be modulated by small molecule inhibitors of innate immune signaling (Sutlu et al. 2012). Studies on viral transduction of NK cells shows that high transduction efficiencies can only be achieved using either NK cell lines such as NK-92 cell line or primary NK cells that have undergone *ex vivo* expansion. Although viral transduction of primary resting human NK cells typically demonstrates substantial gene delivery efficiencies, the use of retro- and lentiviral vectors have been proven to be a safe and most effective tools to genetically modify NK cells (Bari et al. 2019).

Lentiviral Gene Delivery

Most recent studies on genetic modification of NK cells have reported that lentiviral vectors ensure efficient and robust gene delivery (Waller et al. 2017; Colamartino et al. 2019; Müller et al. 2020). The most prominent power of lentiviral vectors over retroviral vectors is that they incorporate transgenes into both dividing and non-dividing cells. It has been shown that pseudotyped lentiviral vectors encoding eGFP provide a transduction rate of above 20% in in freshly isolated human NK cells with a sustained transgene expression for at least 21 days (Colamartino et al. 2019).

Additionally, lentiviral vectors induce transgene alteration without causing any distortions to phenotypic and functional characteristics of NK cells. Although most studies on lentiviral transduction of NK cell lines have reported efficiencies of 15 – 40%, it highly varies from a few percent to almost 100% in case of primary NK cells (Micucci et al. 2006; Savan, Chan, and Young 2010). In such cases of poor transduction efficiencies, multiple rounds of transduction (Micucci et al. 2006) or use of inhibitors against pattern-recognition receptors (Sutlu et al. 2012) may be used to compensate for acceptable transgene expression. Unfortunately, these studies rarely report the viability of NK cells after lentiviral gene delivery. Therefore, optimized protocols are required to achieve maximum transgene delivery without incurring any deleterious effects on NK cell viability, phenotype, or function. Nevertheless, lentiviral transduction of NK cells does deliver stable transgene expression which, depending on how they are genetically modified, might provide durable and long-term clinical response.

Table 1.3. Overview of genetic modification strategies followed for improving anti-tumor efficacy of NK cells in pre-clinical models.

Modality	Strategy	Molecule	Method	Reference
***In vivo* persistence**	Cytokine stimulation	IL-2	RV	(Nagashima et al. 1998; Konstantinidis et al. 2005)
		IL-15	RV, LV	(Imamura et al. 2014; Sahm, Schönfeld, and Wels 2012; W. Jiang, Zhang, and Tian 2008; Wen Jiang et al. 2014)
Cytotoxicity	Redirected targeting	α CD19 CAR	RV, LV	(L. Boissel et al. 2012; 2009; Shimasaki et al. 2012)
		α NKG2D-L CAR	RV	(Parihar et al. 2019)
		TCR	RV, LV	(Mensali et al. 2019; Parlar et al. 2019)
Enhanced activation	Receptor silencing	NKG2A (shRNA)	LV	(Figueiredo, Seltsam, and Blasczyk 2009)
		NKG2A (PEBLs)	RV	(Kamiya et al. 2019)
	Receptor activation	NKG2D	RV, LV	(Kamiya, Chang, and Campana 2016; Parihar et al. 2019; Y. H. Chang et al. 2013)

RV, retroviral transduction; LV, lentiviral transduction; CAR, chimeric antigen receptor.

1.4.2. Genetically Modified (GM) NK cells in Cancer Immunotherapy

Challenges associated with genetic manipulation of NK cells have delayed the debut of how NK cells can be modified and implemented in clinics for cancer immunotherapy. Unraveling the strategies to genetically manipulate NK cells has introduced countless possibilities not only to study the role of NK cells in tumor targeting but also to improve their anti-tumor cytotoxicity. Although further optimization of clinically compliant methods for genetic modification of NK cells is still required, today, it is easier to design modification strategies to generate engineered NK cells with improved *in vivo* persistence, retargeted tumor

cytotoxicity, or enhanced activation by overexpressing activating receptors or diminishing inhibitory receptors (Table 1.3).

Strategies to redirected tumor cytotoxicity

Enhanced antigen specificity is a prerequisite for immune cells to induce tumor regression without causing damage to healthy tissue. Even though NK cells possess immense anti-tumor capacity, NK cell-mediated tumor immunosurveillance was limited in most of the malignancies mostly due to their lack of target specificity. Recent studies have been exploring ways to harness the intrinsic anti-tumor capacity of other immune cells and apply those to genetically modify NK cells for the recognition of antigens specifically expressed on tumor cells (or tumor associated antigens, TAA). So far, two such strategies have been further examined to equip NK cells for treating hematologic and solid malignancies; chimeric antigen receptors (CARs) or T cell receptors (TCR)(Figure 1.3).

The majority of studies have reported that NK cells can be genetically modified to express CARs. CARs are engineered receptors that have extracellular antigen-specific variable part of a tumor antigen antibody fused with intracellular signaling adaptors (e.g. CD3ζ, CD28, 4-1BB) to enable high antigen specific recognition of tumor antigens and tumors. CAR designs are extensively studied on T cells and a significant breakthrough success was recorded for anti-CD19 CAR T cell therapy in patients with B cell malignancies. This prompted the research community to investigate a wide array of CARs against multiple different epitopes on several types of tumors (Dotti et al. 2014). Importantly, these receptors do not rely on stimulation from any activating or co-receptors to trigger robust anti-tumor cytotoxicity. Therefore, the effect of these CARs expression have been explored and characterized in several NK settings including primary cells isolated from donor blood, *ex vivo* expanded primary cells, and NK cell lines (C. Zhang et al. 2017) (Table1.3). Among these designs, the use of gene-modified natural killer cells bearing a CAR receptor with extracellular expression of the activating receptor NKG2D were redirected against ligand on leukemia and solid tumors, which transcended the efficacy of this approach (Parihar et al. 2019; Y. H. Chang et al. 2013).

An alternative method to redirect NK cells for specific tumor antigens can reside in TCR engineering strategies. TCR gene delivery can supply large populations of $CD8^+$ T lymphocytes targeted against intracellular antigens. However, the use of NK cells in TCR engineering brings certain sophistication to the technique, since NK cells avoid mispairing of endogenous TCR chains with genetically transferred TCR subunits that is presented in T cells. Studies involving TCR engineered NK cells, more specifically the NK-92 cell line that is FDA-approved for allogeneic use as mentioned before, showed that ectopic expression of αβ TCR can induce the expression of a functional antigen-specific TCR directed against an HLA-A2 restricted tyrosinase-derived melanoma epitope (Parlar et al. 2019). Similarly, another study provided evidence that these TCR-NK-92 cells, compared to unmodified NK-92 cells, have a novel gene expression pattern which resembled those of primary effector T and NK cells (Mensali et al. 2019). In fact, upon TCR engineering, the NK-92 cells acquired the phenotypic, metabolic, and functional characteristics of T cells, while maintaining the typical effector functions of NK cells.

Overall, introduction of genes that equip NK cells with CAR or TCR can redirect them against surface molecules or intracellular antigens expressed by tumor cells. Several studies have shown that CAR or TCR engineered NK cells hold great promise for clinical use in cancer immunotherapy. However, only a few of them have been translated into clinical studies. Therefore, it is crucial to further optimize the construct designs, as well as to characterize their effect in the TME, in order to pave their way for potential applications in NK cell-based cancer immunotherapy.

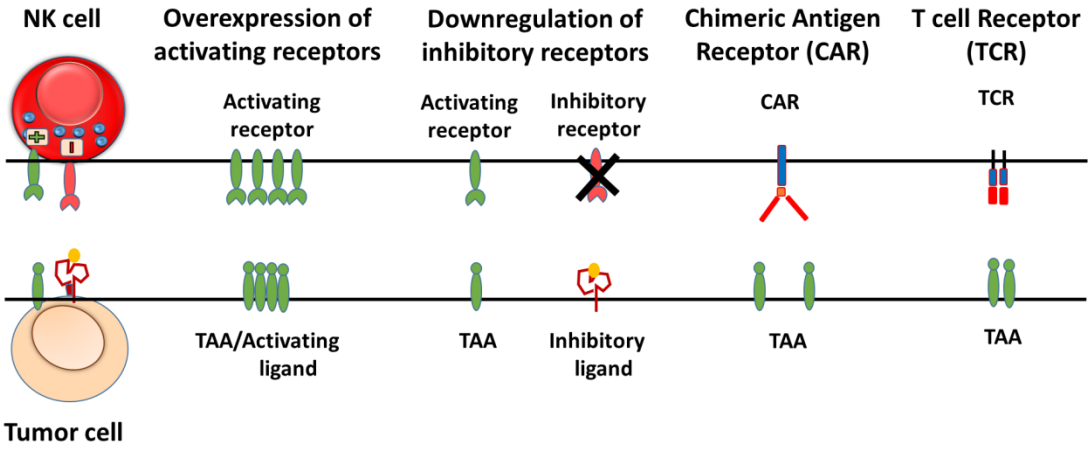


Figure 1.3. Strategies to genetically modify NK cells against tumor cells.
(Adapted from (Dahlberg et al. 2015)).

Strategies to enhance NK cell activation

As NK cell degranulation is regulated by a balance of activating and inhibitory signals from well-defined cell surface receptors, it may be possible to modulate the receptor expression through genetic modification, thereby fine tuning the NK cell responses for favorable NK-tumor cell interactions. Overexpression of activating cell surface receptors in NK cells can augment their anti-tumor responses by increasing sensitivity towards activating ligands expressed on tumor cells (Figure 1.3). As mentioned earlier (in section 1.2.2.1), tumor cells may shed activating ligands in order to escape from elimination. In such cases, higher expression of the activating receptor (e.g. NKG2D) can make saturation by shed ligands less likely, and hence, less susceptible to their potentially dampening effects. A recent report indicates that NK cells endowed with NKG2D-CD3ζ receptor expression have the abrupt capacity to target myeloid-derived suppressor cells in the tumor microenvironment, decreasing their inhibition of $CD8^+$ T cells and increasing antitumor capacity of even CAR T cells (Parihar et al. 2019). NKG2D-modified NK cells showed substantially greater cytotoxicity against a wide range of tumor cell lines *in vitro* and in xenograft models, while toxicity towards non-transformed cells such as other lymphocytes, remained low (Y. H. Chang et al. 2013; Kamiya, Chang, and Campana 2016).

Moreover, another way to tilt the balance in favor of activation is to circumvent NK cell inhibitory signals induced upon interaction with tumor cells (Figure 1.3). For this purpose, genetic reprograming of NK cells can be directed to achieve specific protein silencing. Early studies have focused shRNAs to knock-down expression of the HLA-E-binding inhibitory NK cell receptor NKG2A (Figueiredo, Seltsam, and Blasczyk 2009). Using lentiviral vectors containing shRNA sequences, the killing capacity of IL-2 activated NK cells were increased by 40% against HLA-E expressing K562 cell line. Later, this CD94-NKG2A complex was targeted by using protein expression blocker. These constructs contain a single-chain variable fragment derived from an anti-NKG2A antibody linked to endoplasmic reticulum (ER)-retention signals that hold the newly synthesized NKG2A in the ER and prevent its transport to the cell membrane (Kamiya et al. 2019). After transducing human peripheral blood NK cells, these NKG2A protein expression blockers (PEBLs) were shown to abrogate NKG2A expression, which resulted in more potent cytotoxicity than interference with an anti-NKG2A blocking antibody.

2. AIM OF THE STUDY

The overall aim of this study was to establish a novel *in vitro* cell-based genetic screening platform in genetically modified NK-92 cells expressing different activating receptors in order to identify targetable response-triggering receptors for various applications in patient-tailored cancer immunotherapy approaches.

With the following delineated sub-aims listed:

- Molecular cloning of NK cell surface receptors into lentiviral vectors.
- Generation of genetically modified (GM) NK-92 cells constituting the screening platform.
- Phenotypic and functional evaluation of the GM NK-92 cells.
- Assessment of the feasibility of the cell-based screening platform against well-established human cancer cell lines.
- Application of the cell-based screening platform against primary human sarcoma explants.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

All the chemicals used in this thesis are listed in Appendix A.

3.1.2. Equipment

All the chemicals used in this thesis are listed in Appendix B.

3.1.3. Buffers and solutions

Calcium Chloride ($CaCl_2$) Solution: 60mM $CaCl_2$ (diluted from 1M stock), 15% Glycerol, 10mM PIPES (pH 7.00) were mixed and sterilized by autoclaving at 121℃ for 15 minutes and stored at 4 ℃.

Agarose Gel: For 100 ml 1% w/v gel, 1 g of agarose powder was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide was added to the solution.

Tris-Borate-EDTA (TBE) Buffer: For 1 L 5X stock solution, 54 g Tris-base, 27.5 g boric acid, and 20 ml 0.5M EDTA (pH 8.00) were dissolved in 1 L of ddH_2O. The solution is stored at room temperature (RT) and diluted 1 to 10 with ddH_2O for working solution of 0.5X TBE.

HBS solution (2X): 280 mM NaCl, 50mM HEPES, and 1.5 mM Na_2HPO_4 adjust pH to 7.1 with 10 M NaOH, sterilize by passing through with 0.22 µm filter. Store at -20 ℃.

3.1.4. Growth media

Luria Broth (LB): For 1 L 1X LB media, 20 g LB powder was dissolved in 1 L ddH2O and then autoclaved at 121°C for 15 minutes. For puromycin selection, at a final concentration of 100 µg/ml ampicillin was added to liquid medium just before use.

LB Agar: For 1X agar medium in 400 ml in 1 L glass bottle, add 8 g LB powder and 6 g bacterial agar powder were dissolved in 400 ml ddH2O and then autoclaved at 121°C for 15 minutes. Then, autoclaved LB agar is mixed with antibiotic of interest at a desired ratio onto sterile Petri dishes. Sterile agar plates were kept at 4°C.

Complete DMEM for sarcoma explants: Sarcoma explants were cultured in complete Dulbecco's Modified Eagle's medium (DMEM, high glucose GlutaMAX; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 1X MEM non-essential amino acids (NEAA), 1X antibiotic-antimycotic and 25 mM HEPES.

Complete DMEM for cell lines: HEK293FT, MeWo, SK-MEL-28, Capan-2 cells were maintained in DMEM (GlutaMAX; Gibco) supplemented with 10% heat-inactivated FBS, 0.1 mM NEAA, 25mM HEPES solution, 2 mM L-glutamine and 1 mM sodium pyruvate.

Complete EMEM for cell lines: SH-SY5Y cells were cultured in Eagle's Minimum Essential medium (EMEM; ATCC) supplemented with 10% heat-inactivatedFBS and Caco-2 cells were maintained in EMEM (ATCC) supplied with 20 % FBS.

Complete RPMI 1640: NK-92 cell line is maintained in culture in Roswell Park Memorial Institute (RPMI1640; Gibco) supplemented with 20% heat-inactivated FBS, 10^3 U/mlhuman recombinant Interleukin-2 (IL-2, Proleukin, Novartis) is culture every 2 days, 25mM HEPES, 2mM L-Glutamine, 1X MEM vitamins, 0.1mM NEAA, 1mM sodium pyruvate, and 0.1 mM 2-mercaptoethanol(Sigma-Aldrich, St. Louis, MO,USA).

K562, THP-1, LNCaP, PC-3, ARH-77, RPMI8226, DM6 and A375 cell lines were maintained in culture in RPMI1640 supplemented with 10% heat-inactivated FBS,

while U266 cells were cultured in RPMI1640 (GlutaMAX; Gibco) supplied with 15% FBS.

Complete McCoy's 5A medium for cell lines: Saos-2 and U-2 OS cell lines were maintained in McCoy's 5A mediumsupplied with 15 % and 10 % FBS, respectively. A549 cells were cultured in F-12K medium (ATCC) supplemented with 10% heat-inactivated FBS.

All culture media listed above were stored at +4 ℃ and brought to room temperature prior to use. Cell culture media were carefully monitored for any pH change and freshly used according to suggested expiration dates. All supplements used in culture media were purchased from Gibco, until specified otherwise (see Appendix A for detail).

Freezing medium: All the cell lines were frozen in heat-inactivated FBS containing 6% DMSO (v/v).

3.1.5. Commercial kits used in this study

Table 2.1. List of commercial kits used in this study.

Commercial Kit	**Vendor**
Nucleo Spin® Gel and PCR Clean-up Kit	Macherey-Nagel, Germany
Nucleo Spin® Plasmid Midiprep Kit	Macherey-Nagel, Germany
Nucleo Spin® Plasmid Miniprep Kit	Macherey-Nagel, Germany
Calcium Phosphate Transfection Kit	Sigma-Aldrich, St. Louis, MO,USA

3.1.6. Enzymes

Table 3.2. List of enzymes used in this study.

Enzyme Name	**Vendor**
BamHI - HF	New England Biolabs, MA, USA
EcoRI - HF	New England Biolabs, MA, USA

3.1.7. Antibodies

Table 3.3. List of antibodies used in this study.

Antibody Identity	Clone	Isotype Control Antibody	Vendor
APC Anti-human CD337 (NKp30)	P30-15	APC Conjugated Mouse IgG1 κ	Biolegend
PE-Cy7Anti-human CD336 (NKp44)	p44-8	PE-Cy7 Conjugated Mouse IgG1 κ	Biolegend
BV510Anti-human CD335 (NKp46)	9E.2	BV510 Conjugated Mouse IgG1 κ	Biolegend
PEAnti-human NKp80	5D12	PE Conjugated MouseIgG1 κ	Biolegend
APC Anti-human CD160	BY55	APC Conjugated Mouse IgM κ	Biolegend
PE-Cy7 Anti-human CD161	BY55	PE-Cy7 Conjugated Mouse IgG1 κ	Biolegend
BV510 Anti-human CD27	T271	BV510 Conjugated Mouse IgG1 κ	Biolegend
PE Anti-human CD244 (2B4)	C1.7	PE Conjugated Mouse IgG1 κ	Biolegend
APC-Cy7Anti-human CD2	RPA-2.10	APC-Cy7 Conjugated Mouse IgG1 κ	Biolegend
APC Anti-human CD226 (DNAM-1)	11A8	APC Conjugated Mouse IgG1 κ	Biolegend
PE-Cy7 Anti-human CRACC	162.1	PE-Cy7 Conjugated Mouse IgG2b κ	Biolegend
AF647 Anti-human NKG2A/CD159a	131411	AF647 Conjugated Mouse IgG2a	R&D Systems
APC Anti-human NKG2C/CD159c	134591	APC Conjugated Mouse IgG1	R&D Systems
BV510 Anti-human NKG2D	1D11	BV510 Conjugated Mouse IgG1 κ	Biolegend
PEAnti-human CD352 (NTB-A)	NT-7	PE Conjugated Mouse IgG1 κ	Biolegend
BV421Anti-human	NCAM16.2	BV421 Conjugated Mouse	Biolegend

CD56		IgG1 κ	
PE Anti-human CD56	5.1H11	PE Conjugated Mouse IgG1 κ	Biolegend
BV421Anti-human CD56	NCAM16.2	BV421 Conjugated Mouse IgG2b κ	BD Pharmingen
PE Anti-human CD107a	H4A3	PE Conjugated Mouse IgG1 κ	BD Pharmingen
PE Anti-human CD107a	H4A3	PE Conjugated Mouse IgG1 κ	Biolegend
APC Anti-human TNF-α	MAb11	APC Conjugated Mouse IgG1 κ	Biolegend
APC-Cy7Anti-human IFN-γ	B27	APC-Cy7 Conjugated Mouse IgG1 κ	Biolegend
Purified anti-human CD155 (PVR)	SKII.4	Mouse IgG1 κ	Biolegend
Purified anti-human CD226 (DNAM-1)	DX11	Mouse IgG1 κ	BD Pharmingen
Purified anti-human CD314 (NKG2D)	1D11	Mouse IgG1 κ	Biolegend

3.1.8. Bacterial strains

Escherichia coli (*E.coli*) Top10 strain is used for plasmid amplifications and lentiviral construct amplifications.

3.1.9. Mammalian cell lines

HEK293FT: Human embryonic kidney 293 (HEK293) cell line derivative that stably expresses the large T antigen of SV40 virus and has fast-growing specificity (Invitrogen R70007).

NK-92: Human natural killer cell line was derived from a non-Hodgkin's lymphoma patient isolated in the year 1992(ATCC® CRL 2407™).

K562: K562 is an established human immortalized myelogenous leukemia line is a model cell line derived from a 53 years old female chronic myelogenous leukemia patient in blast crisis (ATCC® CCL- 243™).

THP-1: Human acute monocytic leukemia cell line provided leukemic blasts isolated from peripheral blood of 1-year old infant (ATCC® TIB-202™).

U266: Human B cell lymphoma cell line was derived from peripheral blood of 53 years old male plasmacytoma patient (ATCC® TIB-196™).

ARH-77: Human B lymphoblast cell line was isolated from plasma cell leukemia patient and immortalized by Epstein-Barr virus (EBV) transformation (ATCC® CRL-1621™).

RPMI8226: Human B lymphocyte cell was derived from peripheral blood of a plasmacytoma patient (ATCC® CRM-CCL-155™).

A549: Human primary lung adenocarcinoma cell line was derived from epithelial lung tissue of a 58 years old carcinoma patient (ATCC® CCL-185™).

Caco-2: Human primary epithelial cell line that was originally derived from a colon carcinoma (ATCC® HTB-37™).

Capan-2: Human pancreatic ductal adenocarcinoma cell line was established in 1975 from primary tumor of a 56 years old patient with pancreatic ductal adenocarcinoma (ATCC® HTB-80™).

PC-3: Human prostate cancer cell line that was isolated from metastatic epithelial tissue from 62-year-old adenocarcinoma patient (ATCC® CRL-1435™).

LNCaP: Human metastatic prostate carcinoma cell line that was originally isolated in 1977 from the lymph node of a patient with prostate cancer (ATCC® CRL-1740™).

A375: Human malignant melanoma cell line was isolated from 54-year-old patient. It was a kind gift from Prof. Michael Nishimura (Loyola University Chicago, IL, USA).

DM6: Human melanoma cell line was initiated through explant culture of a solid tumor in lymph node tissue in Duke University Medical Center, Durham, USA. It was kindly provided as a gift from Dr. Hillard F. Seigler (Department of Immunology, Duke University Medical Center).

MeWo: Human melanoma cell line was initiated in 1974 from 78 years old malignant melanoma patient (ATCC® HTB-65™).

SK-MEL-28:Human patient-derived melanoma cell line was isolated from 51 years old patient (ATCC® HTB-72™).

SH-SY5Y: Human metastatic neuroblastoma cell line was a subline of the SK-N-SH cell line that was established from in 1970 from a bone marrow biopsy of a metastatic neuroblastoma patient (ATCC® CRL-2266™).

U-2 OS: Human osteosarcoma cell line was cultivated in 1964 from the bone marrow tissue (tibia) of a patient suffering from osteosarcoma (ATCC® HTB-96™).

Saos-2: Human osteosarcoma cell line was isolated from the primary osteosarcoma patient in 1973 (ATCC® HTB-85™).

Human primary sarcoma explants: Freshly excised primary sarcoma tumor samples from patients were dissociated immediately after surgical excision with tumor dissociation kit using the gentleMACS Octo Dissociator with heaters (Miltenyi Biotec, Germany) (Translational Research and Economic Development Unit, Nova Southeastern University, FL, USA). These samples were processed and cultured at least for 12 weeks by serial passaging inorder to obtain a sarcoma explant from various tissues.

3.1.10. Plasmids and oligonucleotides

Table 3.4. List of plasmids used in this study (see Appendix C for plasmid maps).

Plasmids	**Purpose**	**Source**
LeGO-iG2 Puro	Lentiviral construct for eGFP expression with IRES	A kind gift from Prof. Boris Fehse (University Medical CenterHamburg-Eppendorf, Hamburg, Germany)
pMDLg/pRRE	Virus production/packaging plasmid (Gag/Pol)	Addgene (#12251)
pRSV-REV	Virus production/packaging plasmid (Rev)	Addgene (#12253)
phCMV-VSV-G	Virus production/packaging plasmid (Env)	Addgene (#8454)

Table 3.5. List of oligonucleotides used in this study.

Oligo Name	Sequence (5'to 3')	Purpose of Use
SFFV_Forward	TGCTTCTCGCTTCTGTTC	Sequencing
IRES_Reverse	GCCCTCACATTGCCAAAA	Sequencing

3.1.11. DNA ladder

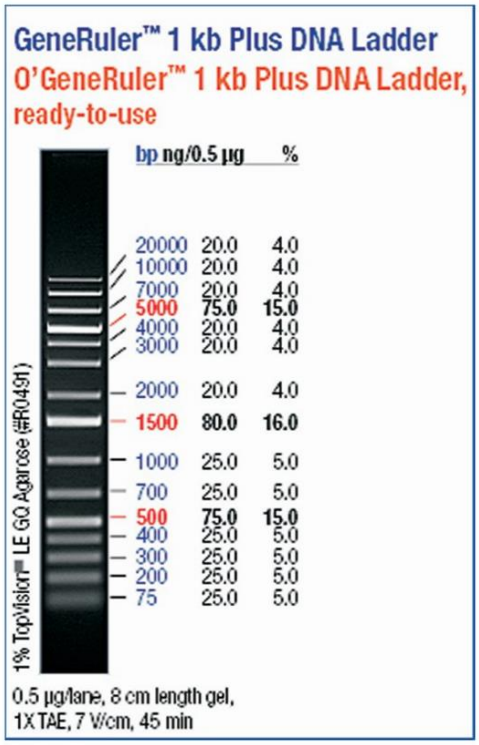


Figure 3.1. 1 kb GeneRuler DNA ladder mix used in this study (Thermo Fisher Scientific).

3.1.12. DNA sequencing

Sequencing service was commercially provided by McLab, CA, USA.

3.1.13. Software

Table 3.6. List of software used in this study.

Software	Acknowledged Corporation	Purpose of Use
CLC Main Workbench	CLC Bio, Denmark	Vector map construction, control digest planning, DNA sequencing analysis
FlowJo_v10.6.1	Tree Star Inc., BD Biosciences, USA	Displaying and analyzing flow cytometry data
GraphPad Prism 8	GraphPad Software Inc., CA, USA	Data analysis, graphing, biostatistics

3.2. Methods

3.2.1. Bacterial cell culture

Bacterial culture growth: Top10 *E.coli*bacteria were cultured in LB media with 1:1000 dilution of Ampicillin and cultured at 37 ℃, for 16 hours and at 220 rpm shaking. Cells were spread on Ampicillin Petri dishes by the use of glass beads and incubated at 37 ℃, for 16 hours. At the end of the incubation, at least five single colonies were picked from the plates. For glycerol stocks of bacteria, a single colony was grown at 3 ml LB media at 37 ℃, for 16 hours and at 220 rpm shaking overnight, culture was diluted 1:3 at the end of incubation and then, culture for an additional 3 hours at 37 ℃, 220 rpm shaking for glycerol stock preparation. Bacteria culture were halted at the log phase of growth and mixed with glycerol in a total volume of 1ml at final concentration of 10% (w/v) and stored in cryotubes at -80 ℃ deep freezer.

Preparation of competent bacteria:An aliquot glycerol stock of competent Top10 *E.coli* cells were grown in 3 ml LB without any antibiotics at 37 ℃, for 4 hours, at

220 rpm shaking. Later, they were transferred into a 250ml culture flask with 50 ml LB and grown overnight at 37 ℃, 220 rpm shaking. Next day, 4 ml of the culture was added into 400 ml of LB without any addition of antibiotics in a 2L culture flask and incubated at 37 ℃, 220 rpm shaking until optical density 590 (OD590) measurement at spectrophotometerreached around 0.375. The culture was, then, aliquoted into eight 50ml falcon tubes and incubated on ice for 10 minutes. Cells were kept on ice and centrifuged at 1600xg for 10 minutes at 4 ℃. The supernatant was discarded, and each pellet was resuspended in 10 ml of ice-cold $CaCl_2$ solution and centrifuged at 1100xg for 5 minutes again at 4 ℃. The supernatant was discarded, and each pellet was resuspended in 2 ml of ice-cold $CaCl_2$ solution. Cells were kept on ice for 30 minutes and combined in one tube and distributed into 200 µl aliquots that were snap-frozen in liquid nitrogen and stored at -80 ℃.

Transformation of competent bacteria: Competent Top10 *E.coli*bacteria were kept in 200 µl aliquots at -80 ℃. For each transformation, plasmid DNA and competent *E.coli* cells were thaw on ice. Plasmid DNA was added to competent *E.coli* cells, cells were incubated on ice for 30 minutes. The cells were taken to heat block at 42℃ and immediately heat shocked for 90 seconds on ice. 800 µl of LB was added to each tube and competent cells were incubated at 37 ℃ water bath for 45 minutes. The cells were centrifuged at 13,000xrpm for 1 minute and the pellet was resuspended in 100 µl LB to be spread on Petri dishes. Glass beads were placed on ampicillin-LB agar Petri dishes. Plates were incubated at 37 ℃ without shaking overnight.

Plasmid DNA isolation:Commercial Macherey-Nagel Mini or Midiprep Kits were used according to manufacturer's manual. The final DNA concentrations and purities were measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

3.2.2. Mammalian cell culture

Maintenance of cell lines:HEK293FT cells were split in 2-day periods before reaching 90% confluency. Cells grown in sterile tissue culture flasks were first washed with 1xDPBS and trypsin-EDTA was added to the flask to be incubated for

5 minutes at 37 ℃, 5% CO_2. Next, they were resuspended in complete DMEM and split at 1:3 to 1:8 ratio.

NK-92 cells were cultured in complete RPMI 1640 as previously described. Cells were maintained in sterile tissue culture flasks at a density between $0.3x10^6$ to $1x10^6$ viable cells/ml. They were split every 2 to 3 days and supplied with 10^3 U/ml IL-2 every 48 hours.

K562 cells were maintained in complete RPMI 1640. Cells were maintained in sterile tissue culture flasks at a density between $0.3x10^6$ to $1x10^6$ viable cells/ml. They were split every 2 to 3 days.

Other target human cancer cell lines were cultured in their appropriate complete media (as described in 3.1.9) and maintained as suggested by manufacturer's culture protocol in sterile tissue culture flasks with filtered caps at an incubator set to 37 ℃ with 5% CO_2.

Cell freezing: Cells were split 1:2 or 1:3 for suspension cells and to a confluence of 50-70% for adherent cells prior to freezing depending on the cell growth curve of each cell line. The next day, cells were counted and at least1-$5x10^6$ cells/vial were frozen in a cryovial with a final volume of 1 ml. After counting cells were centrifuged at 300xg for 5 minutes after which supernatant was discarded, and the pellet was resuspended in 0.5ml FBS and incubated on ice for 20 minutes. When incubation is finished, cells were quickly restored in FBS with a final concentration of 6% DMSO(v/v) in 1 ml. Cryovials were stored in freezing container first in -80 ℃ for 24 hours, and then, were transferred to liquid nitrogen for long term storage.

Cell thawing: Cells were preserved in cryovial at the liquid nitrogen. A cryovial was restored from liquid nitrogen and quickly thaw. 1 ml of thaw cell suspension in 6% DMSO(v/v)in FBS was diluted in 5 ml FBS at room temperature in 15 ml falcon tube and centrifuged immediately at 300xg for 5 minutes. Resulting supernatant was discarded and cell pellet was resuspended with an appropriate complete media.

3.2.3. Production of lentiviral vectors

For production of VSV-G pseudotyped lentiviral vectors, $5x10^6$ 293FT cells were seeded into a poly-D-lysine coated 100 mm culture dish (Corning, NY, USA). Next

day cells were co-transfected with 7.5 µg of gene-of-interest plasmids along with 3.75 µg of pMDLg/pRRE, 2.5 µg of pRSV-REV and 1.25 µg of phCMV-VSV-G using calcium phosphate transfection kit (Sigma-Aldrich) in the presence of 25 µM Chloroquine (Sigma-Aldrich). 10 hours later medium was changed and thereafter virus supernatant was collected at 24-hour and 36-hour time points and stored in -80 ℃ until further use. Aliquots from each production were used to determine viral titers by transduction of 293FT cells with a serial dilution of viral supernatants. Multiplicity of infection (MOI) was calculated as the number of infectious units divided by the number of 293FT cells exposed to the vector.

3.2.4. Lentiviral transduction of NK-92 cells

$1x10^6$ cells were cultured in a T25 culture flask (Sarstedt, Germany) and lentiviral vector supernatant at MOI=5 was added to the culture in the presence of a final concentration of 8 ***µ***g/ml of protamine sulfate (Sigma-Aldrich), 10^3 U/ml IL-2 and 1.5 mM (5Z)-7-Oxozeaenol (Sigma-Aldrich) in a final volume of 6 ml. The flasks were incubated at 37 ℃, 5% CO_2 overnight. At the end of incubation, cells were transferred to a 15 ml falcon and spun down at 300xg for 5 minutes at room temperature. Then, virus supernatant was replaced with fresh complete growth medium. Cells were maintained for at least 3 days before eGFP expression was evaluated by flow cytometry. Genetically modified cells were enriched by puromycin (1µg/ml) selection 10 days post-transduction. If eGFP expression detected by flow cytometry is above 95%, the new cell line is frozen for later use or otherwise, is subjected to phenotyping or functional assessments. 17 cell lines of iG2 Puro backbone were produced and frozen for further use.

3.2.5. Flow cytometry

Surface staining: Cells were first counted and then, washed once with 1xDPBS. For each staining, $0.25x10^6$ cells per tube was centrifuged and pellets were resuspended with 100 µl 1xDPBS by a gentle vortex. Appropriate amount of monoclonal antibody was added by pipetting and incubated at +4 ℃ for 30 minutes in dark.

Appropriate isotype-matched antibodies were used as a control for each molecule of interest. List of antibodies used in this study is given in Table 3.1. Cells were, then, washed with 1.5 ml 1xDPBS and centrifuged (300xg, 5 minutes). Supernatant was discarded and cells were resuspended in 100 µl 1xDPBS by a vortex. Cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences, USA). All GM NK-92 cells were analyzed by flow cytometry by gating on; FSC-A vs SSC-A for P1 population, FSC-A vs FSC-H for single cells, SSC-A vs $CD56^+$to mark live $CD56^+$ NK-92 cells and lastly, eGFP vs dTomato for $eGFP^+$ NK-92 cells before and after puromycin selection (See appendix 1). Analysis was carried out by FlowJo software (Tree Star Inc., BD Biosciences, USA).

Fluorescence-activated cell sorting (FACS): NK-92 cells were counted and $5x10^6$ cells were collected in a sterile tube. Cells were washed once with 5 ml 1xDPBS and supernatant was discarded. Then, they were resuspended in appropriate volume of 1xDPBS supplemented with 0.1% FBS and isotype control or monoclonal antibody was added. After incubating at room temperature for 30 minutes in dark, they were washed once more with 5 ml 1xDPBS. Cells were resuspended as $10x10^6$ cells/ml and filtered using 70 µm sterile strainer to remove aggregates. $NKG2D^+$ and/or $DNAM\text{-}1^+$ cell populations were sorted on a BD FACS Aria II flow cytometer by gating on FSC-A vs SSC-A, followed by single cells via FSC-A vs FSC-H, then eGFP vs SSC-A and lastly, on NKG2D vs DNAM-1 (BD Biosciences, USA). The resulting purified $NKG2D^+$, $DNAM\text{-}1^+$ and $NKG2D^+/DNAM\text{-}1^+$ GM NK-92 cells were resuspended in appropriate volumes of complete media supplemented with 10^6 U/ml IL-2 and 1% Penicillin-streptomycin for at least 10 days. GM NK-92 cells were maintained in the cell culture as described before, until they reached adequate numbers where they were frozen for future use.

3.2.6. Analysis of NK cell degranulation on genetically modified NK-92 cell

In order to investigate effector functions of genetically modified (GM) NK-92 cells, their degranulation capacities were evaluated against target cells. 0.2×10^6 effector NK-92 cells were co-cultured with target cells on a 1:1 ratio in a final volume of 200 µl in a V-bottom 96-well plate. As soon as the cells were set-up, fluorochrome-

conjugated anti-CD107a monoclonal antibody was added in 1 µl. All well contents were resuspended, and plate was centrifuged at 50xg for 2 minutes. After incubation at 37 ℃, 5% CO_2 for 1 hour, 2mM Monensin (Biolegend) was added into all wells and resuspension of all well contents was repeated followed by a plate centrifuge. Plates were incubated at humidified incubator for 3 more hours. Later, centrifuge was set to +4 ℃ and plate was centrifuged at 400xg for 5 minutes. All well content was transferred to a flow cytometry tube for surface staining. Fluorochrome-conjugated anti-CD56 antibody was added in 1 µl and incubated on ice for 30 minutes at dark. When incubation is finished, cells were washed with 500 µl cold 1xDPBS and centrifuged (300xg, 5 minutes). Finally, cells were resuspended in 300 µl ice-cold 1xDPBS to proceed with acquisition at flow cytometry.

Blocking experiments were set-up similar to degranulation assay. Target cells were counted and pre-incubated with the corresponding 25 µg/ml blocking antibodies (e.g. anti-CD155) for 15 minutes on ice prior to co-culture. Fluorochrome-conjugated anti-CD107a monoclonal antibody was added at the initiation of the assay. After 1 hour of co-incubation, GolgiStop (BD Biosciences) was pipetted at a 1:300 dilution to all wells and incubated for 3 more hours until proceeding to surface staining before acquisition on flow cytometer.

Intracellular cytokine staining (ICS) for IFN-γ and TNF-α in genetically modified cells

ICS assay was a similar set-up with degranulation procedure. GM NK-92 cells, as well as wild-type NK-92 and backbone control (iG2 Puro without any gene-of-interest) cell lines, were co-cultured with K562 cells on a 1:1 ratio in a V-bottom 96-well plate. All well contents were re-suspended, and plate was centrifuged at 50xg for 2 minutes. After incubation at 37℃, 5% CO_2 for 1 hour, 2mM Monensin was added into all wells and resuspension of all well contents was repeated together with plate centrifuge. Plates were incubated at humidified incubator for 3 more hours. Later, centrifuge was set to +4 ℃ and plate was centrifuged at 400xg for 5 minutes. All well content was transferred to a flow cytometry tube for monoclonal

antibody staining. Fluorochrome-conjugated anti-CD56 antibody was added in 1 µl and incubated on ice for 30 minutes at dark. When incubation is finished, cells were washed with 500 µl cold PBS and centrifuged (300xg, 5 minutes). For cell fixation and permeabilization, 1% paraformaldehyde (PFA), 1x perm buffer (0.1% saponin) in 1x DPBS was used to treat the cells in 250 µl. After incubation on ice for 15 minutes, cells were washed with 1x perm buffer and centrifuged (300xg, 5 minutes). This maintains permeabilized cells ready for IFN-γ or TNF-α monoclonal antibody staining. 1.25 µl of fluorochrome-conjugated anti-IFN-γ or anti-TNF-α antibody was added to samples and incubated on ice for 30 minutes at dark. Cells were, then, given a final wash with 1x perm buffer and centrifuged (300xg, 5 minutes). Before acquisition on flow cytometry, 300 µl ice-cold 1x DPBS was used to resuspend the cells.

3.2.7. Analysis of NK cell cytotoxicity by xCELLigence real-time cell analysis (RTCA)

Real-time cell viability experiments were performed using the xCELLigence RTCA DP device (ACEA Biosciences, CA, USA) placed in a humidified incubator at 37°C and 5% CO_2. The E-16 plates were incubated with 100 µL of cell-free growth medium (10% and 15% FBS containing McCoy's 5A medium (GE)) at room temperature for 15 minutes. After incubation, background impedance signal was measured to control all the connections. The target cells were seeded into plates as $5x10^3$cells in 100 µL forU-2OS and Saos-2 cell lines. The plates were mounted to the device after incubation at room temperature for 30 minutes before starting the experiment. The target cells were allowed to settle for 15-17 hours before adding effector cells. The following day, the effector cells were added onto the target cells at an E:T ratio of 1:1. Real-time measurements were performed by recording the Cell index (CI) every 15 minutes for a period of 40 hours. Data analysis was carried out with the RTCA software (version 1.2, Roche Diagnostics, Basel, Switzerland).

3.2.8. Statistical analysis

Graphs and statistical data analysis were prepared by using GraphPad Prism (GraphPad Software Inc. La Jolla, CA, USA). Values were expressed by arithmetic mean plus or minus standard deviation (SD) or standard error of the mean (SEM), where find appropriate. Statistical differences were determined by a two-way ANOVA with Dunnett's multiple comparison test or Tukey's post-hoc analysis, where ns, *, **, ***, **** indicates not significant, $p < 0.05$, $p < 0.01$, $p < 0.005$, $p < 0.0001$.

4. RESULTS

4.1. Generation of Genetically Modified (GM) NK-92 Cell Lines Overexpressing Different NK Receptors

4.1.1. Design and Production of Lentiviral Vectors

Dissection of NK and tumor cell interactions may reveal novel targets for NK cell-based cancer immunotherapy. In order to evaluate the capacity of different NK cell activating receptors, a cell-based screening platform can be set up to efficiently trigger target cell recognition, exempting the need to identify heterogeneous ligand repertoires on tumor cells. For this purpose, we aimed to overexpress one NK cell receptor at a time on a model NK cell line, NK-92. In order to ensure the efficiency of gene expression, codon-optimized sequences encoding 20 different NK cell surface receptors were used in this study. These sequences were cloned into Lentiviral Gene Ontology (LeGO) vector backbone, LeGO.iG2 Puro or LeGO.iT2 Puro, which is a 3rd generation self-inactivating lentiviral vector utilizing the spleen focus-forming virus (SFFV) promoter to express a bicistronic transcript coding for the gene-of-interest (GOI) and a fluorescence and/or selection marker (Figure 4.1A).

Each gene-of-interest (DNAM-1, NTBA, 2B4, CD2, NKG2A, NKG2C, NKG2D, NKG2E, NKp30, NKp44, NKp46, NKp80, CRACC, CD160, CD161, CD27,

CD16a, CD94, Siglec7 or Siglec9) was cloned into the multiple cloning site (MCS) of these lentiviral vectors and the resulting plasmids, including the empty vector controls, were used to produce lentiviral particles.

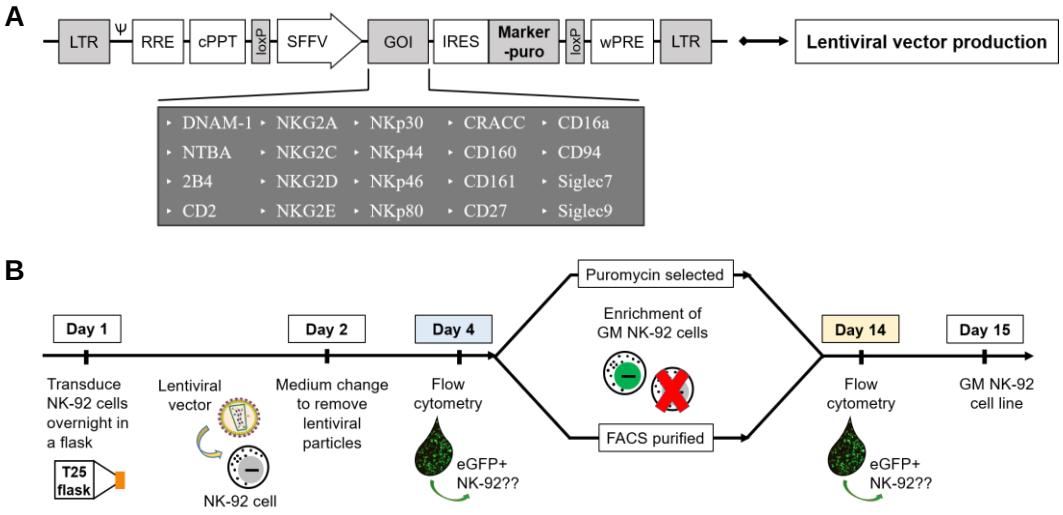
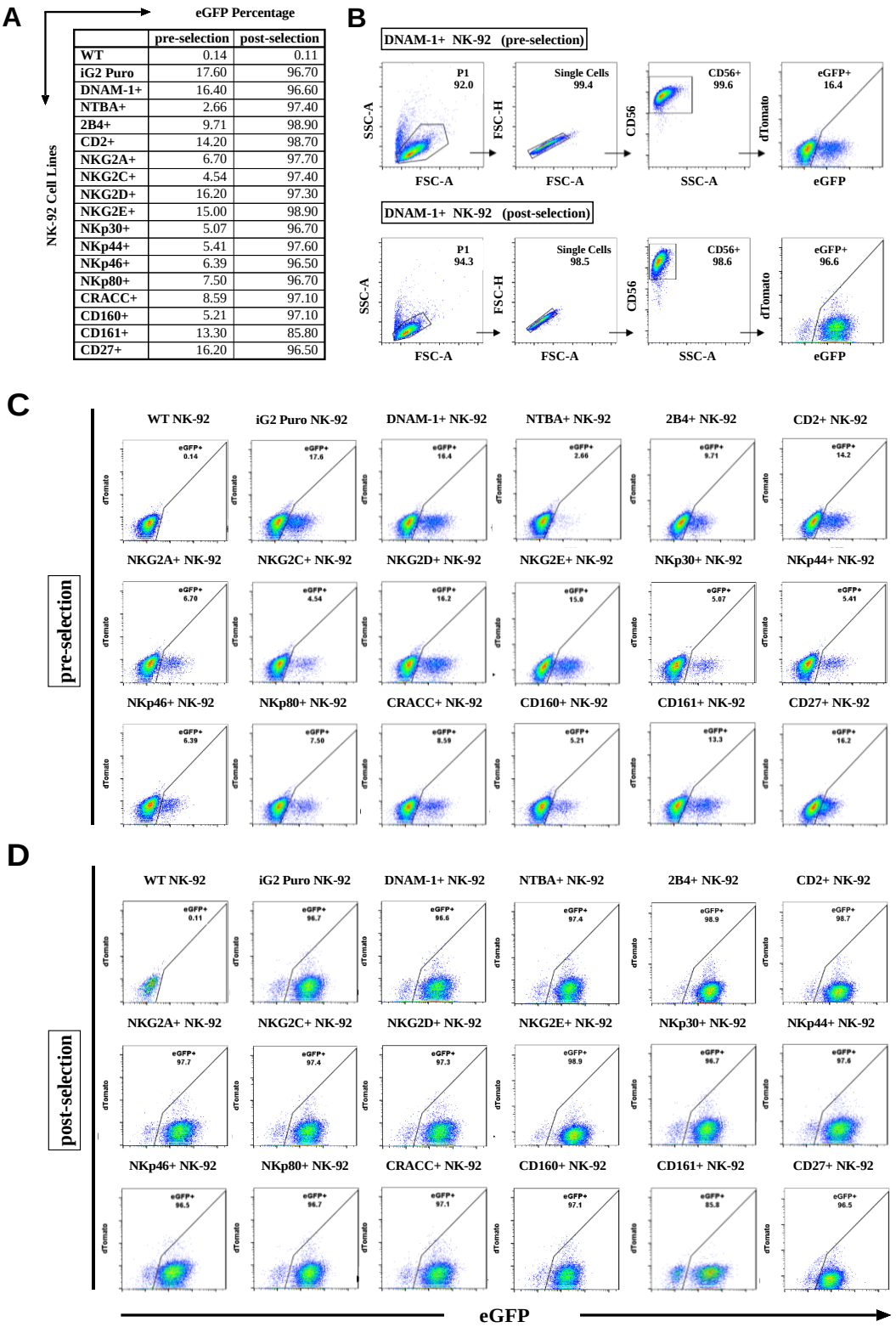


Figure 4.1. Generation of GM NK-92 cell lines. **(A)** Genes encoding 20 different NK cell surface receptors were cloned into lentiviral vector backbones to produce lentiviral particles expressing each NK receptor. **(B)** 15-day timeline to obtain GM NK-92 cells each overexpressing a single NK cell receptor. NK-92 cells were transduced with one vector at a time (MOI=5) and enriched either using FACS purification or puromycin selection. Transduction efficiencies were confirmed by detection of $eGFP^+$ cells by flow cytometry before (at day 4) and after (at day 14) enrichment.

LeGO.iG2 Puro or LeGO.iT2 Puro vector backbones express either of the two fluorescent markers; eGFP or dTomato, respectively, that enable the tracing of transduction efficiency. Additionally, in order to be able to select positively transduced cells, vectors were carefully chosen to have an antibiotic resistance gene (puromycin resistance gene or "puro") fused to the fluorescent marker gene (indicated as Marker-puro) (Figure 4.1A). This design allowed us to enrich genetically modified (GM) NK-92 cells either using fluorescence-activated cell sorting (FACS) or simply by puromycin selection when a FACS machine was not available (Figure 4.1B). For the sake of simplicity, only the enrichment process of NK-92 cells engineered with LeGO.iG2 Puro-based vectors obtained through puromycin selection is provided here.

In order to generate a GM NK-92 cell line which stably overexpresses a gene-of-interest, NK-92 cells were processed in a 15-day timeline (Figure.4.1B). Firstly, NK-92 cells were transduced with one lentiviral vector at a time at a multiplicity of infection of 5 (MOI=5) and lentivirus containing medium was washed away after an overnight incubation in the presence of protamine sulfate and (5Z)-7-Oxozeaenol (OXO) for increased transduction efficiency. Detection of eGFP expression percentages by flow cytometry at day 4 (pre-selection) and at day 14 (post-selection) revealed the efficiency of transduction (Figure 4.2A). All GM NK-92 cells were analyzed by flow cytometry and the representative gating strategy is shown (Figure 4.2B) (See appendix 1). Following puromycin enrichment for 10 days, GM NK-92 cells were confirmed to be positively transduced, because all cell lines showed at least 96.50% eGFP expression except for the CD161 transduced NK-92 cell line (85.80%) and untransduced WT NK-92 cells (0.11%) (Figure 4.2A). Representative plots of all newly generated NK-92 cell lines for only eGFP expression pre-selection and post-selection are presented in Figure 4.2C and Figure 4.2D, respectively. Hence, each GM NK-92 cell line, overexpressing a specific gene, was confirmed to be positively transduced and ready for prospective use in functional assays against target cells.

It is important to note here that this study was originally designed to test 20 different receptor genes. Although the planned cloning was achieved for all genes in both vector backbones (LeGO.iG2 Puro and LeGO.iT2 Puro); CD94, CD16a, Siglec7 and Siglec9 expressing lentiviral vectors were omitted from the analysis because the large gene insert did not allow efficient lentiviral production. Therefore, DNAM-1^{+}, NTBA^{+}, 2B4^{+}, CD2^{+}, NKG2A^{+}, NKG2C^{+}, NKG2D^{+}, NKG2E^{+}, NKp30^{+}, NKp44^{+}, NKp46^{+}, NKp80^{+}, CRACC^{+}, CD160^{+}, CD161^{+}, CD27^{+} and only iG2 Puro expressing NK-92 cell lines were generated and their results are presented in this thesis.



	pre-selection	post-selection
WT	0.14	0.11
iG2 Puro	17.60	96.70
DNAM-1+	16.40	96.60
NTBA+	2.66	97.40
2B4+	9.71	98.90
CD2+	14.20	98.70
NKG2A+	6.70	97.70
NKG2C+	4.54	97.40
NKG2D+	16.20	97.30
NKG2E+	15.00	98.90
NKp30+	5.07	96.70
NKp44+	5.41	97.60
NKp46+	6.39	96.50
NKp80+	7.50	96.70
CRACC+	8.59	97.10
CD160+	5.21	97.10
CD161+	13.30	85.80
CD27+	16.20	96.50

Figure 4.2. Evaluation of the selection process on successfully transduced NK-92 cells. **(A)** eGFP percentages of NK-92 cell lines were obtained by flow cytometry pre-selection and post-selection. **(B)** Representative gating strategy. $eGFP^+$ percentages on successfully transduced cells were evaluated by flow cytometry **(C)** pre-selection and **(D)** post-selection for each GM NK-92 cell line.

4.1.2. Phenotypic Confirmation of Overexpression

While analysis of eGFP expression enabled the tracking of GM cells, additional confirmation of full vector functionality was carried out for each sample by analyzing the expression level of the NK receptor encoded by the lentiviral vector. Therefore, GM NK-92 cells were stained with antibodies specific for the corresponding receptor or isotype control antibodies and surface expressions of the proteins were measured by flow cytometry (Figure 4.3A). Fold mean fluorescence intensity (MFI) values of a given receptor was calculated by normalization (Figure 4.3B). WT NK-92 cells had baseline expression of certain markers such as NTBA, CRACC and CD160, but were negative for markers such as DNAM-1, NKp80 and NKG2D. Lentiviral gene delivery was successful in inducing a dramatic overexpression of DNAM-1 (1000-fold), NTBA (10-fold), NKG2D (10-fold), NKp80 (100-fold) and CD161 (10-fold) molecules on NK-92 cells, while the induction of CD2, 2B4, NKp30, NKp44, NKp46 and CRACC were at more moderate levels (Figure 4.3C).

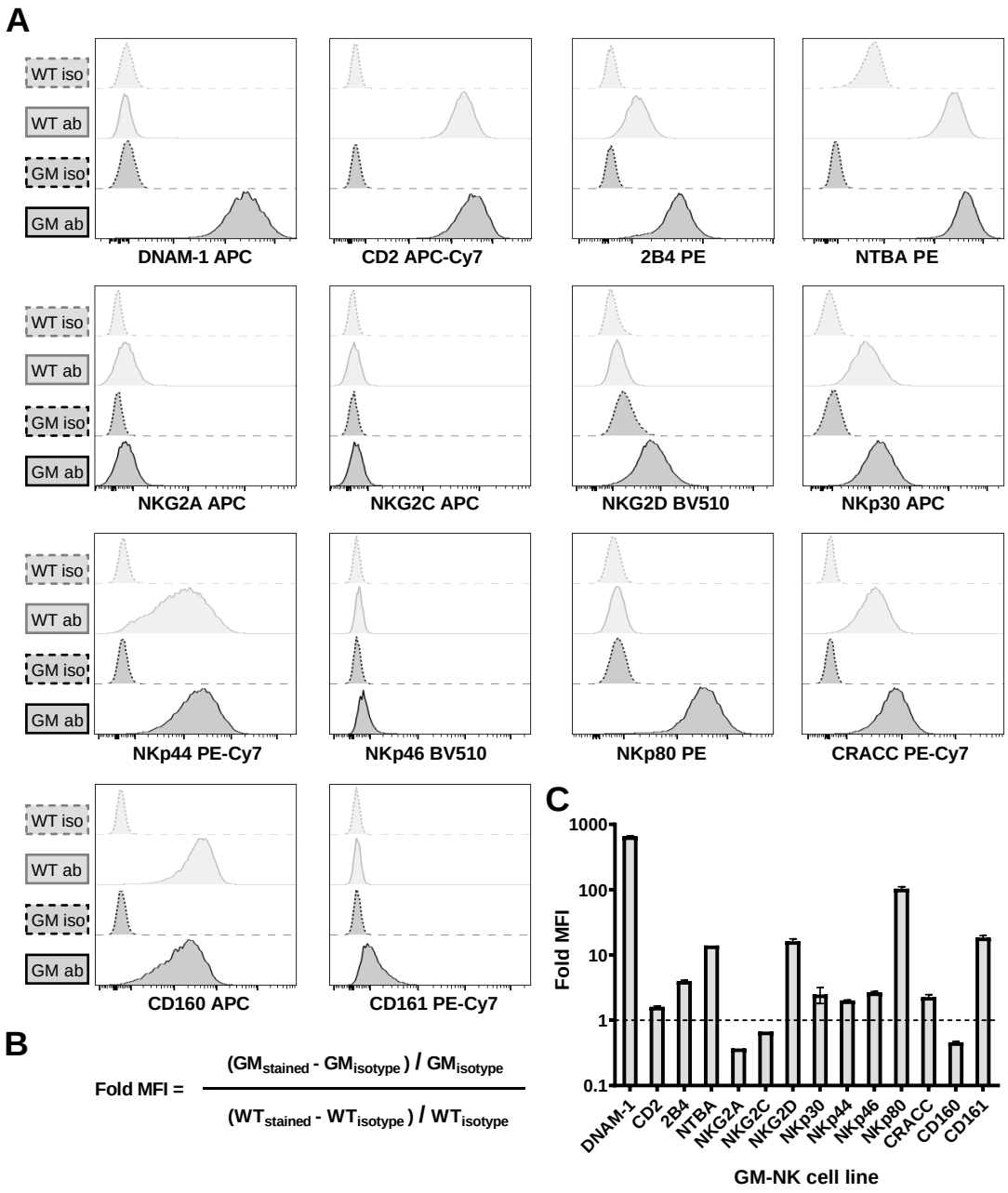


Figure 4.3. Surface receptor expression profiles of GM NK-92 cells were verified by flow cytometry. **(A)** Representative figures showed expression of cell surface receptors in GM NK-92 cells compared to WT (dashed line: isotype staining (iso), filled histogram: antibody staining (ab)). Fold MFI value of a given receptor expression on a GM NK cell line was **(B)** calculated by normalization to WT and **(C)** graphed accordingly. Error bars indicate SD of two independent staining experiments.

4.1.3. Functional Confirmation of GM NK-92 Cell Lines

In order to evaluate the functional responses of the GM NK-92 cell lines in a screening platform, they were co-cultured at a 1:1 effector to target (E:T) ratio with K562 target cells together with wildtype (WT) NK-92 and empty vector (iG2 Puro) as controls. Degranulation capacity was measured by flow cytometry based on surface CD107a expression and was evaluated in comparison to no target as negative control and unspecific activators PMA and ionomycin (PMA/IO) as a positive control (Figure 4.4A). Lentiviral genetic modifications did not disrupt or hamper the cytotoxicity of NK-92 cells but rather induced enhanced degranulation (Figure 4.4B). Degranulation against the K562 cell line showed marked increase especially in DNAM-1^+ and NKG2D$^+$ GM NK-92 cells compared to WT cells. Interestingly, CD2^+ NK-92 cells degranulated significantly the lowest among all GM NK-92 cells.

While no specific correlation between responses from PMA/IO induction and K562 co-cultures was observed between NK-92 cells modified with different receptors, technical variations in both the negative and positive controls prompted the need for a normalization methodology that will empower the integral comparison between GM NK-92 cells in the screening platform without underestimating the degranulation response against different target cells. Therefore, degranulation was normalized by setting the PMA/IO responses for each GM NK-92 cell line to 100% and degranulation against different target cells were normalized to this response for each GM cell line (Figure 4.4C). Normalization revealed a parallel trend with previous analyses where degranulation levels between DNAM-1^+, NKG2D$^+$ and CD2^+ GM NK-92 cells were all similar. Thus, this normalization methodology was affirmed and applied to all flow cytometric analysis where the screening platform is used from this point forward.

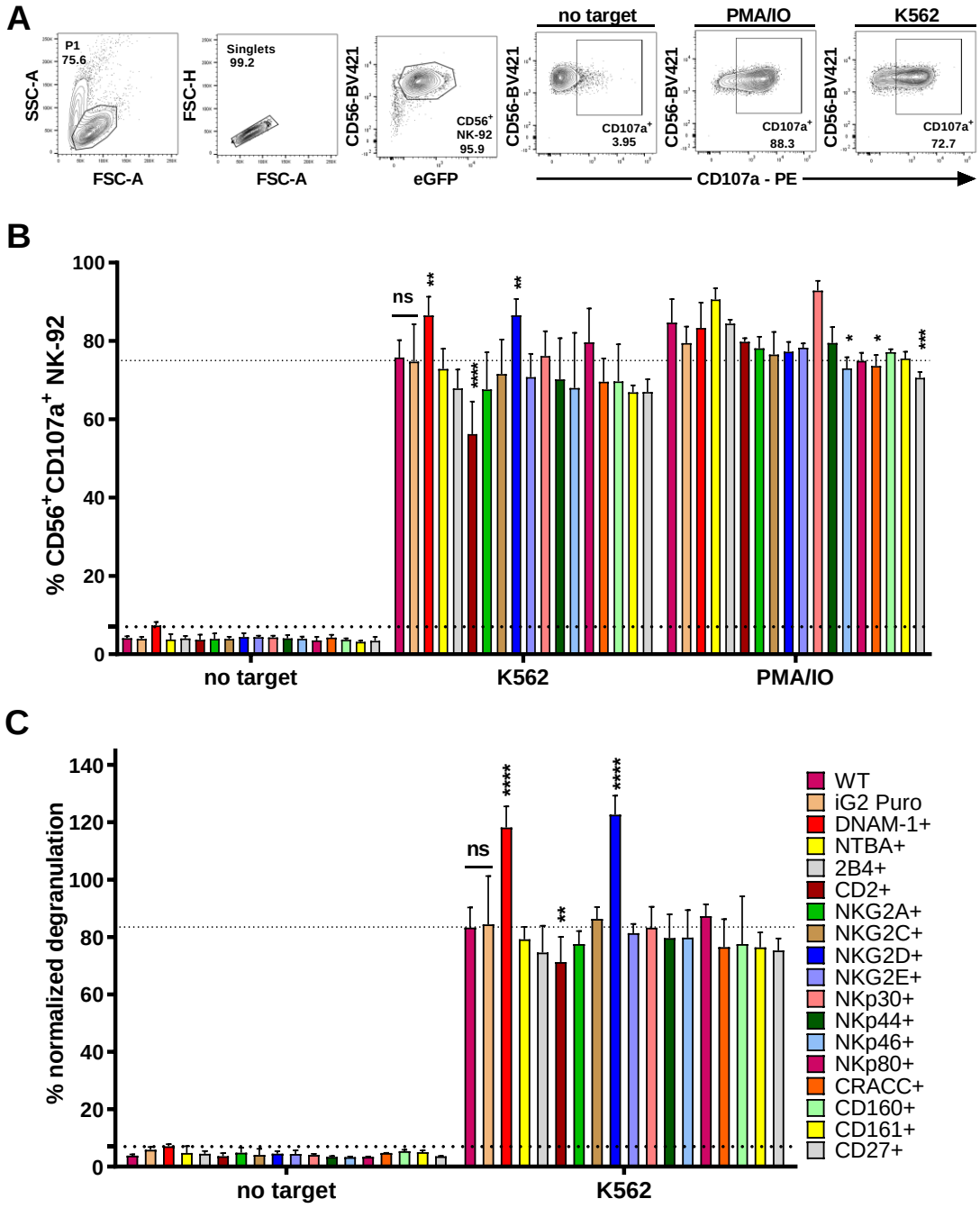


Figure 4.4. Degranulation of WT and GM NK-92 effector cells against K562 target cells. NK-92 cells were co-cultured with K562 target cells at 1:1 (E:T) ratio for 4 hours. All degranulation analysis by flow cytometry followed **(A)** the represented gating strategy: FSC-A vs SSC-A, FSC-A vs FSC-H, GFP vs CD56 and lastly, CD107a vs CD56 cells. Degranulation assays run with negative control (no target), positive control (PMA/IO) and against NK cell's natural target, K562 cell lines (K562). Percentage of **(B)** $CD56^{+}CD107a^{+}$ NK-92 cells or **(C)** normalized degranulation were plotted (normalization was done by taking each cell line's PMA/IO response as 100%). Mean of three independent experiments, run in duplicates, were plotted with SD. Dashed line indicates WT degranulation against K562.

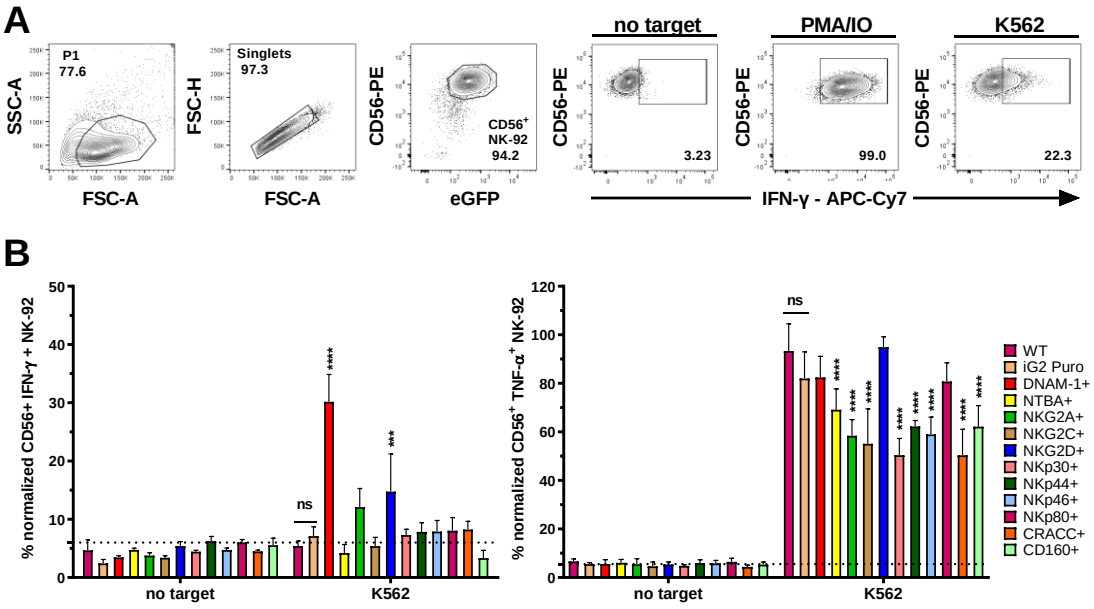


Figure 4.5. Pro-inflammatory cytokine profiles of WT and GM NK-92 cells against K562 target cells. WT or GM NK-92 cells were co-cultured with K562 target cells at 1:1 (E:T) ratio for 4 hours and analyzed by flow cytometry **(A)** by gating on: FSC-A vs SSC-A, FSC-A vs FSC-H, GFP vs CD56 and lastly, IFN-γ or TNF-α vs CD56 cells. Percentage of normalized **(B)** $CD56^{+}IFN\text{-}\gamma^{+}$ or **(C)** $CD56^{+}TNF\text{-}\alpha^{+}$ NK-92 cells were shown. Normalization was calculated by taking each cell line's PMA/IO response as 100%. Means of two independent experiments, run in duplicates, were plotted with SD.

In addition to the degranulation assay, NK cell-mediated cytotoxicity is associated with pro-inflammatory cytokine release upon contact with a target cell. Therefore, measurement of IFN-γ and TNF-α is a proven signature of NK cell activation. We used this expression as means to evaluate the effect of genetic modifications on NK cell functionality. Flow cytometric analysis of IFN-γ expression is represented in Figure 4.5A. Accordingly, a selection of GM NK-92 cell lines produced for this study were shown to function as much as WT and control cells and genetic modification did not mitigate these functions (Figure 4.5B). While DNAM-1^{+} and $NKG2D^{+}$ GM NK-92 cells demonstrated a remarked increase only in IFN-γ expression against K562 cells, they failed to induce TNF-α expression. Surprisingly, only iG2 Puro, DNAM-1^{+}, $NKG2D^{+}$ and $NKp80^{+}$ GM NK-92 cells showed similar expression of TNF-α compared to WT NK-92 cells (Figure 4.5B). These degranulation and cytokine profile analyses provided evidence that GM NK-

92 cell lines in the screening platform were functionally responsive and that they have the capacity to trigger the full range of effector functions.

4.2. Developing a Novel GM NK-92 Cell-based Screening Platform

GM NK-92 cells were shown to be powerful in triggering elevated degranulation responses compared to WT NK-92 cells. While K562 cells conventionally serve as a natural target of NK cells, the efficacy and feasibility of the screening method required to be tested on a panel of other well-established human cancer cell lines, as well as human primary sarcoma explants. This work was conducted in collaboration with Dr. Adil Doğanay Duru's lab at Nova Southeastern University in Florida/USA (as described in section 3.1.9 of the Materials and Methods).

The screening platform of GM NK-92 cells was subjected to testing for their degranulation responses against a panel of well-characterized human cancer cell lines (Figure 4.6). This target cell line panel included a selection of hematological malignancies such as leukemia (K562 and THP-1), B cell lymphoma (U266), myeloma (ARH-77 and RPMI8226) and various solid tumors such as primary lung adenocarcinoma (A549), primary colorectal adenocarcinoma (Caco-2), primary pancreatic ductal adenocarcinoma (Capan-2), metastatic prostate carcinoma (LNCaP and PC-3), melanoma (A375, SK-MEL-28, MeWo and DM6), metastatic neuroblastoma (SH-SY5Y) and osteosarcoma (Saos-2 and U-2 OS) (Figure 4.7, right panel). The 16 different GM NK-92 cell lines constituting the cell-based screening platform were responsive against 16 different target cell lines, although the responses varied greatly depending on the target (Figure 4.6). All NK-92 cells (with or without genetic modification) degranulated against hematological malignancies (THP-1, U266 (in particular), ARH-77 and RPMI8226) at higher levels when compare to their responses against solid tumor cell lines (except for A375 and DM6 melanoma cells).

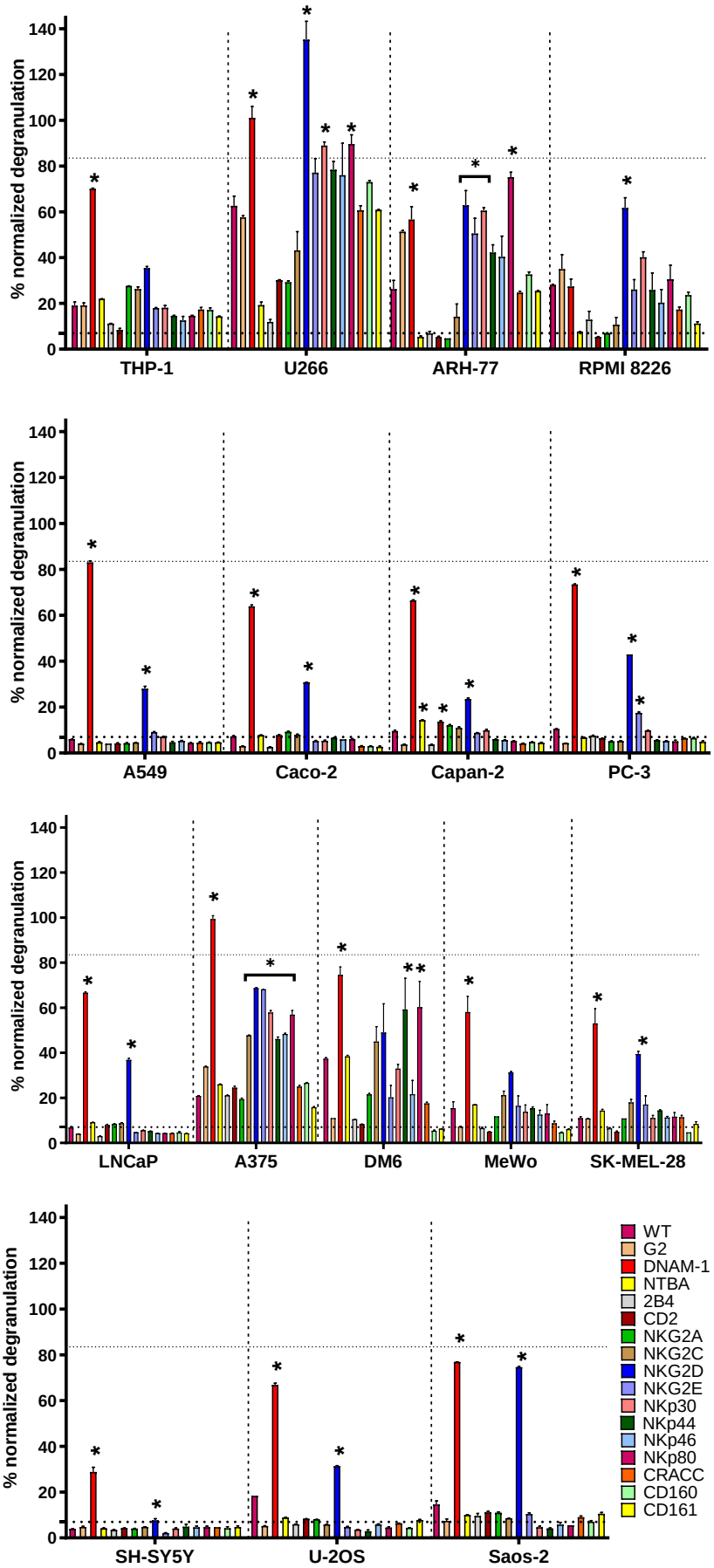
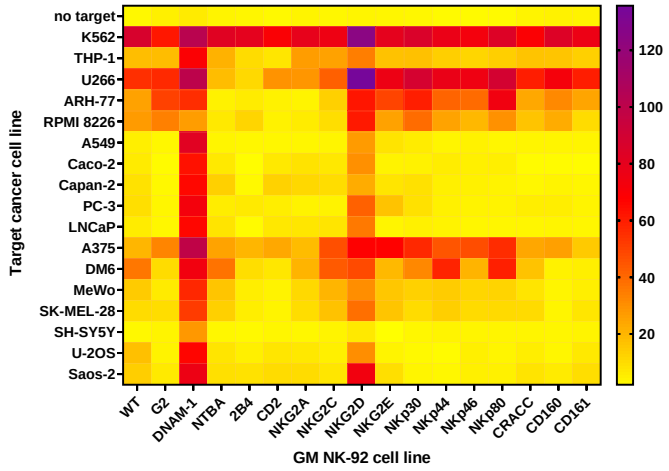

% normalized degranulation
0
20
40
60
80
100
120
140
THP-1
U266
ARH-77
RPMI 8226
A549
Caco-2
Capan-2
PC-3
LNCaP
A375
DM6
MeWo
SK-MEL-28
SH-SY5Y
U-2OS
Saos-2
WT
G2
DNAM-1
NTBA
2B4
CD2
NKG2A
NKG2C
NKG2D
NKG2E
NKp30
NKp44
NKp46
NKp80
CRACC
CD160
CD161

Figure 4.6. GM NK-92 cell-based screening platform tested against well-established human cancer cell lines. WT and GM NK-92 cells were co-cultured with target cell lines at 1:1 (E:T) ratio for 4 hours in degranulation assays. Percent normalization was calculated by taking each cell line's PMA/IO response as 100%, results from one representative experiment, plotted as means of technical replicates with SD.

The degranulation capacity of the GM NK-92 panel showed that compared to WT NK-92, NKG2D^{+} GM NK-92 cells had enhanced degranulation against the majority of target cell lines except for THP-1, DM6 and MeWo. Additionally, DNAM1^{+} GM NK-92 cells showed promiscuously enhanced response against almost all cancer cell lines except for RPMI8226.



Cell line	Origin
K562	Chronic myelogenious leukemia
THP-1	Acute monocytic leukemia
U266	B cell lymphoma
ARH-77	Myeloma
RPMI8226	Myeloma
A549	Primary lung adenocarcinoma
Caco-2	Primary colorectal adenocarcinoma
Capan-2	Primary pancreatic ductal adenocarcinoma
PC-3	Metastatic prostate adenocarcinoma
LNCaP	Metastatic prostate carcinoma
A375	Malignant melanoma
DM6	Malignant melanoma
MeWo	Malignant melanoma
SK-MEL-28	Melanoma
SH-SY5Y	Metastatic neuroblastoma
U-2 OS	Osteosarcoma
Saos-2	Osteosarcoma

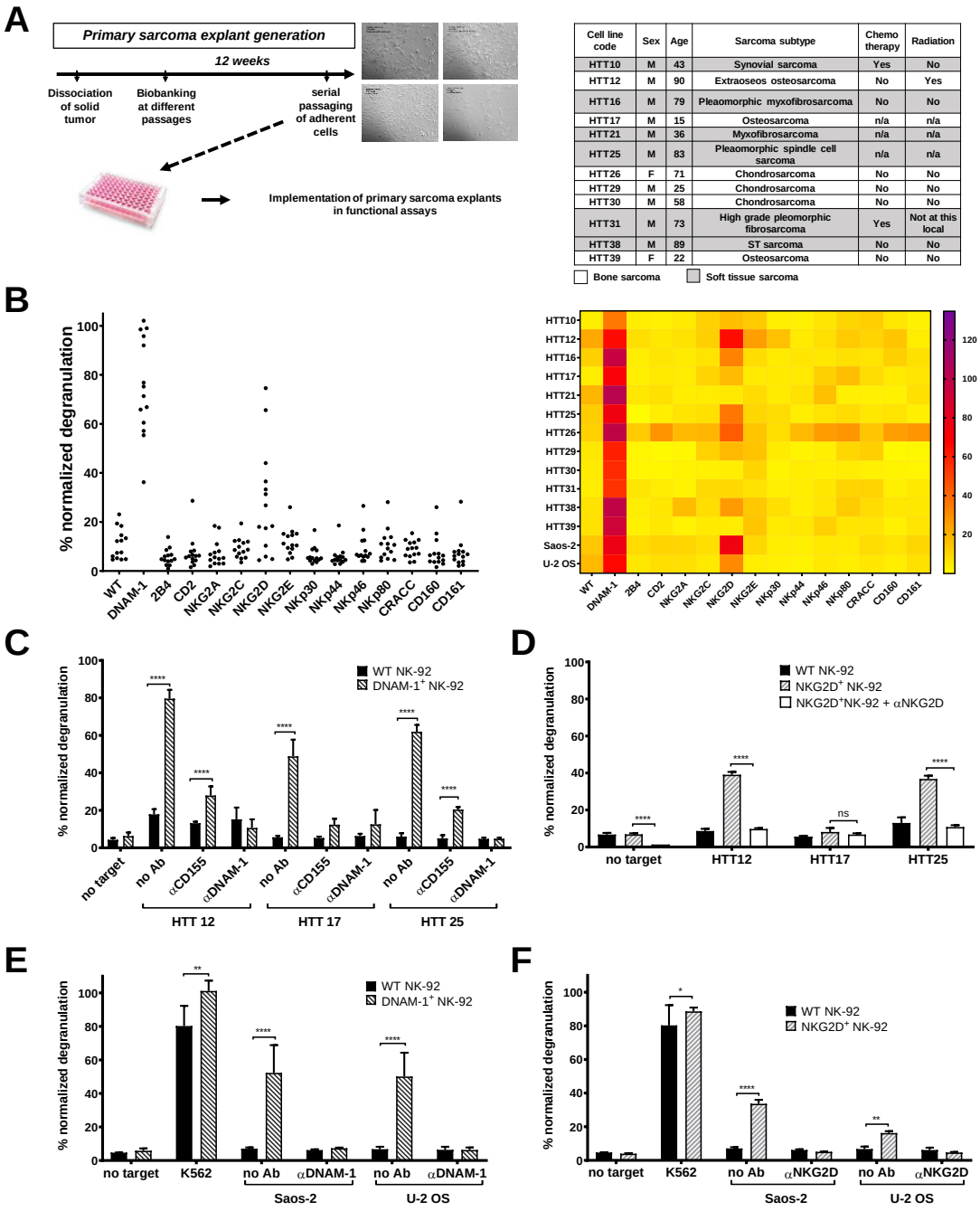
Figure 4.7. Heat map of the GM NK-92 cell-based screening platform against well-established human cancer cell lines. WT or GM NK-92 cells were co-cultured with target cells at 1:1 (E:T) ratio for 4 hours in a degranulation set up. Heat map (left panel) showing normalized %CD56^{+}CD107a^{+} WT and GM NK-92 cells against human cancer cell lines (normalization was done by taking each cell line's PMA/IO response as 100%, results from one representative experiment, plotted as means of technical replicates). The screening platform included a list of well-established human cancer cell lines with given origins (right panel).

Additionally, all the normalized degranulation data from the screening platform across various target cell lines were plotted as a heat map which allowed compact and comparative analysis (Figure 4.7, left panel). The heat map representation enhanced the identification of differential expression receptor-mediated responses against tested cell lines such as ARH-77, RPMI8226, A375 and DM6, and validated the applicability of the NK cell-based screening platform to identify the targetable NK cell receptor-ligand interactions.

Furthermore, we evaluated the functional response of the GM NK-92 cells in triggering degranulation against human primary sarcoma explants to validate the NK-92 cell-based screening system. In order to exploit novel sarcoma patient samples, freshly excised primary sarcoma tumor samples from patients undergoing surgery were isolated and processed to generate a panel of primary sarcoma explants (as described in materials and methods 3.1.9) (Figure 4.8A). Sarcoma explants stem from various tissue types and hence, possibly represent heterogeneity of phenotypic and genetic characteristics of tumor cells (Figure 4.8A).

In order to functionally test the cell-based screening approach, the GM NK-92 panel was co-cultured at a 1:1 (E:T) ratio with 12 selected sarcoma explants and two well-established sarcoma cell lines (U-2 OS and Saos-2) with control NK-92 cells in order to provide a comparative analysis of degranulation (Figure 4.8B). DNAM-1^+ GM NK-92 cells degranulated significantly against all 12 primary sarcoma explants and cell lines, while NKG2D^+ GM NK-92 cells were responsive (more than 20%) when exposed to 10 out of the 12 explants. Thus, the degranulation responses from these co-cultures demonstrated that DNAM-1 and NKG2D induced enhanced anti-tumor responses when compared to WT or controls.

Next, we wanted to further confirm that this augmented NK cell response was indeed DNAM-1 or NKG2D-dependent by interfering with either the receptor on NK-92 cells or the corresponding ligand interaction using blocking antibodies (against DNAM-1 or CD155, a ligand of DNAM-1). Normalized degranulation responses against sarcoma explants (HTT12, HTT17, HTT25) were completely abrogated upon blocking DNAM-1 on DNAM-1^+ NK-92 cells (Figure 4.8C). While in the case of CD155 ligand blocking on target sarcoma explants, partial response by DNAM-1^+ NK-92 cells was still observed, which is probably due to the presence of other DNAM-1 ligands, (such as CD112) on the tumor cells. Thus, GM NK-92 degranulation response was confined with DNAM-1 receptor mediated engagement against primary sarcoma explants.



Cell line code	Sex	Age	Sarcoma subtype	Chemo therapy	Radiation
HTT10	M	43	Synovial sarcoma	Yes	No
HTT12	M	90	Extraoseos osteosarcoma	No	Yes
HTT16	M	79	Pleaomorphic myxofibrosarcoma	No	No
HTT17	M	15	Osteosarcoma	n/a	n/a
HTT21	M	36	Myxofibrosarcoma	n/a	n/a
HTT25	M	83	Pleaomorphic spindle cell sarcoma	n/a	n/a
HTT26	F	71	Chondrosarcoma	No	No
HTT29	M	25	Chondrosarcoma	No	No
HTT30	M	58	Chondrosarcoma	No	No
HTT31	M	73	High grade pleomorphic fibrosarcoma	Yes	Not at this local
HTT38	M	89	ST sarcoma	No	No
HTT39	F	22	Osteosarcoma	No	No

Figure 4.8. GM NK-92 cell-based screening platform validated by degranulation assay using human sarcoma explants. **(A)** A panel of primary sarcoma explants (at right) was generated by serial passaging (on the left). **(B)** Degranulation analysis of GM NK-92 cells against 12 sarcoma explants were evaluated by flow cytometer by co-culturing at 1:1 (E:T ratio) for 4 hours. Normalized % CD56+CD107a+ NK-92 cells were shown (normalization was done as described earlier). WT, DNAM-1+ or NKG2D+ NK-92 cells were treated with **(C)** anti-CD155 (αCD155), anti-DNAM-1 (αDNAM-1) or **(D)** anti-NKG2D (αNKG2D) blocking antibodies prior to degranulation against HTT12, HTT17 and HTT25. NK-92 effectors were co-cultured with U-2 OS and Saos-2 sarcoma cell lines at 1:1 (E:T) ratio for 4 hours after treatment with **(E)** anti-DNAM-1 or **(F)** anti-NKG2D blocking antibodies.

Also, NKG2D receptor on NKG2D$^+$ NK-92 cells was blocked by antibodies prior to co-culture with sarcoma explants (HTT12, HTT17, HTT25) to verify NKG2D involvement in the observed degranulation responses (Figure 4.8D). When treated with NKG2D blocking antibody, NKG2D$^+$ NK-92 cells demonstrated mitigated degranulation, comparable to the responses of WT NK-92 cells against HTT12 and HTT25. NKG2D blocking did not trigger degranulation of NKG2D$^+$ NK-92 cells against HTT17, as NKG2D mediated response was already nominal compared to HTT12 and HTT25 and was selected as a negative control.

In parallel, blocking of DNAM-1 or NKG2D receptors on GM NK-92 cells was also assessed against human sarcoma cell lines (U-2 OS and Saos-2) (Figure 4.8E, F). Both DNAM-1 blocking on DNAM-1$^+$ NK-92 cells and NKG2D blocking on NKG2D$^+$ NK-92 cells decreased degranulation to the background levels against sarcoma cell lines. Therefore, we show that the degranulation response of GM NK-92 cells overexpressing DNAM-1 and NKG2D are solely due to the abundant and functional interaction between the respective receptors and their corresponding ligand pairs. Hence, the degranulation responses of GM NK-92 cells in the screening platform provided evidence for functional interactions between receptors on effector cells and ligands on target tumor cells. In summary, this work demonstrates the feasibility of an *in vitro* genetic screening approach to identify response-triggering receptors in GM NK-92 cells.

4.3. Generation of Dual Vectors: NKG2D$^+$/DNAM-1$^+$ NK-92 Cell Line

Because the results from our screening platform consistently underline the importance of the DNAM-1 and NKG2D receptors against a variety of target cells, we wanted to exploit potential synergies between the two receptors by co-expressing DNAM-1 and NKG2D on GM NK-92 cells.

4.3.1. Sorting Strategy and Phenotyping

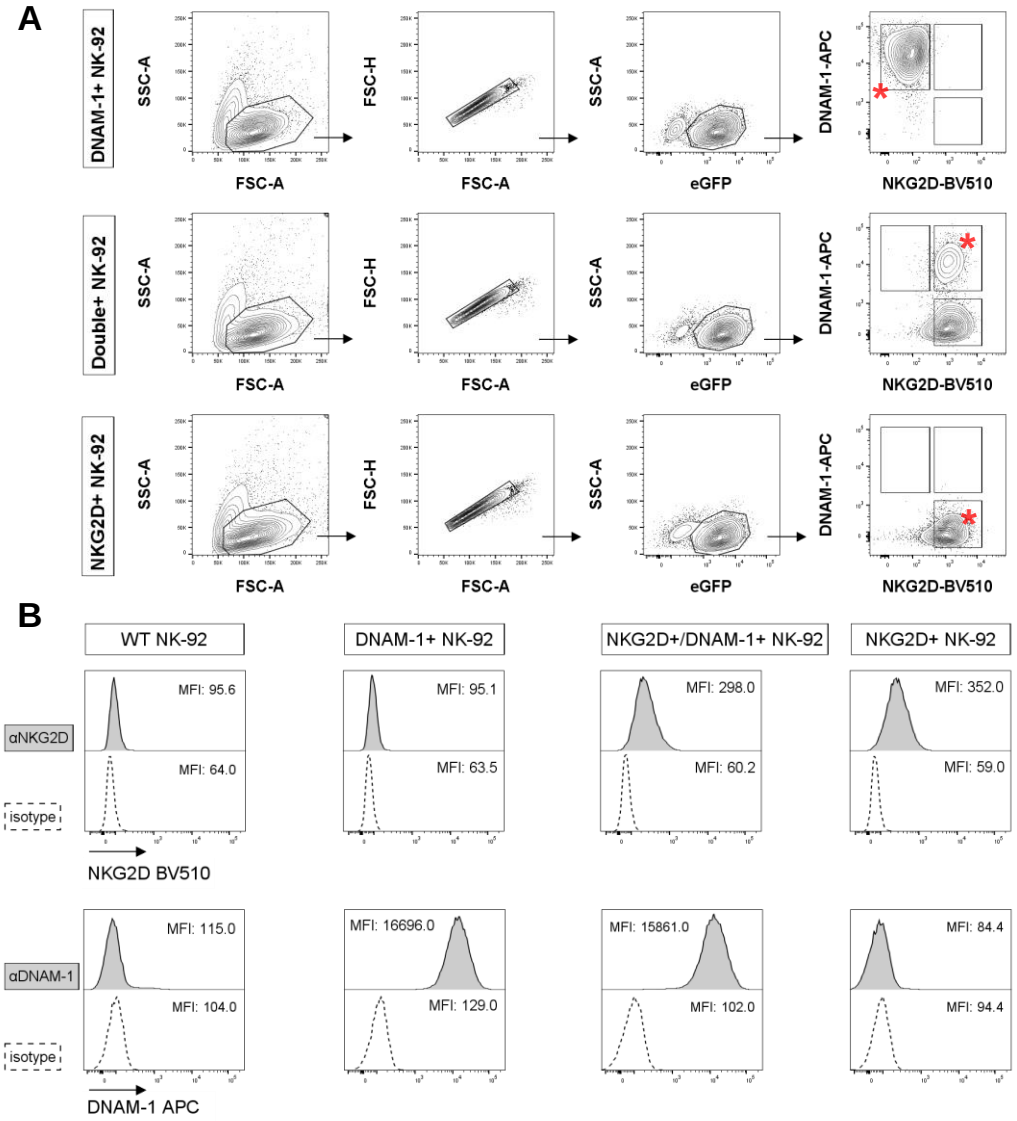


Figure 4.9. Generation of NKG2D and DNAM-1 co-expressing GM NK-92 cells. NKG2D^{+} and/or DNAM-1^{+} cell populations (shown by red asterisk) were sorted with fluorescence-activated cell sorting (FACS) **(A)** by gating on FSC-A vs SSC-A, followed by FSC-A vs FSC-H for singlets, then eGFP vs SSC-A and lastly, on NKG2D vs DNAM-1. **(B)** WT and GM NK-92 cells were stained with αCD56 and, αNKG2D or αDNAM-1 antibodies and MFI of each staining was measured by flow cytometer (dashed line: isotype control, filled histogram: stained).

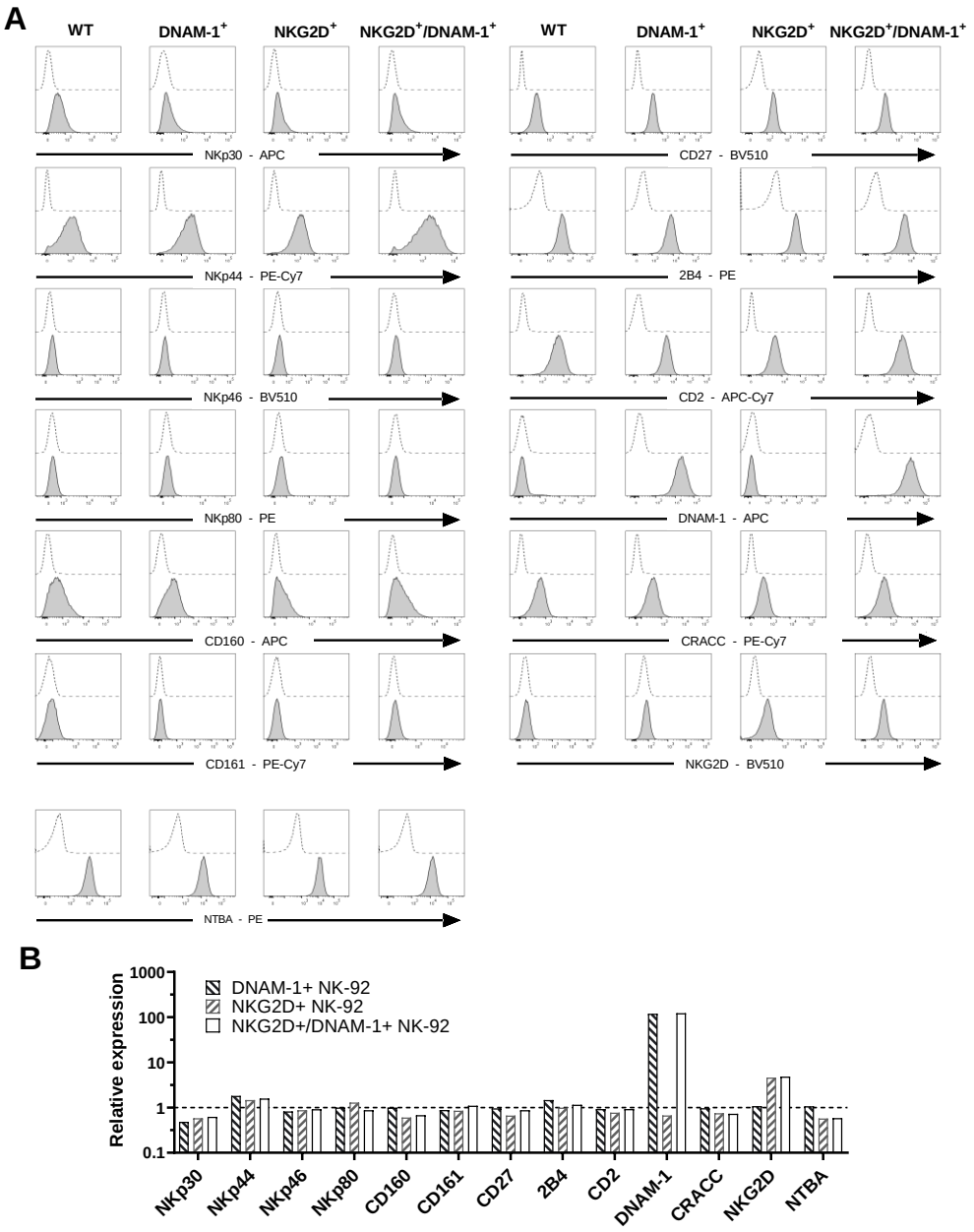


Figure 4.10. Surface expression of NK cell receptors on GM NK-92 cells. WT and GM NK-92 cells were stained with antibodies for surface expression of NKp30, NKp44, NKp46, NKp80, CD160, CD161, CD27, 2B4, CD2, DNAM-1, CRACC, NKG2D and NTBA, and analyzed by flow cytometry. (A) A single staining for each marker expression was shown for WT, DNAM-1+, NKG2D+ and NKG2D+/DNAM-1+ NK-92 cell lines (dashed line: isotype control, filled grey: antibody stained). (B) Relative expression of each single staining was calculated by the mean fluorescence intensity ratio of GM cells relative to unmodified WT NK-92 cells after normalization with isotype controls.

For this purpose, we genetically modified NKG2D$^+$ NK-92 cells with a second transduction to overexpress DNAM-1 receptor and enriched these double-transduced cells by FACS sorting to generate NKG2D$^+$/DNAM-1$^+$ NK-92 cells (or double+ NK-92) (Figure 4.9A). Analysis of single or double transduced NK-92 cells confirmed similar levels of receptor expression and assured that any observed functional differences would result from altered expression (Figure 4.9B).

A more detailed phenotyping analysis on WT, NKG2D$^+$, DNAM-1$^+$ and NKG2D$^+$/DNAM-1$^+$ NK-92 cells revealed that genetic modifications did not alter the relative expression level of any other surface receptor, but only the respective target molecules (Figure 4.10).

4.3.2. Evaluation of Functional Response against Sarcoma Cell Lines

After confirming the overexpression of both receptors on GM NK-92 cells, functional response mediated by these receptors was analyzed by their degranulation capacities and pro-inflammatory cytokine expression levels (Figure 4.11). Evaluation of degranulation response of NKG2D$^+$/DNAM-1$^+$ NK-92 cells against U-2 OS and Saos-2 sarcoma cell lines marked a moderate increase compared to NKG2D$^+$ and DNAM-1$^+$ alone transduced cells (Figure 4.11A). We further confirmed the triggering of cytokine production by NKG2D$^+$ and/or DNAM-1$^+$ expressing GM NK-92 cells upon co-culturing with sarcoma cell lines. Compared to WT NK-92 cells, NKG2D$^+$ and DNAM-1$^+$ NK-92 cells displayed increased IFN-γ and TNF-α expression in response to sarcoma cell lines which was not consistent with the degranulation responses (Figure 4.11B).

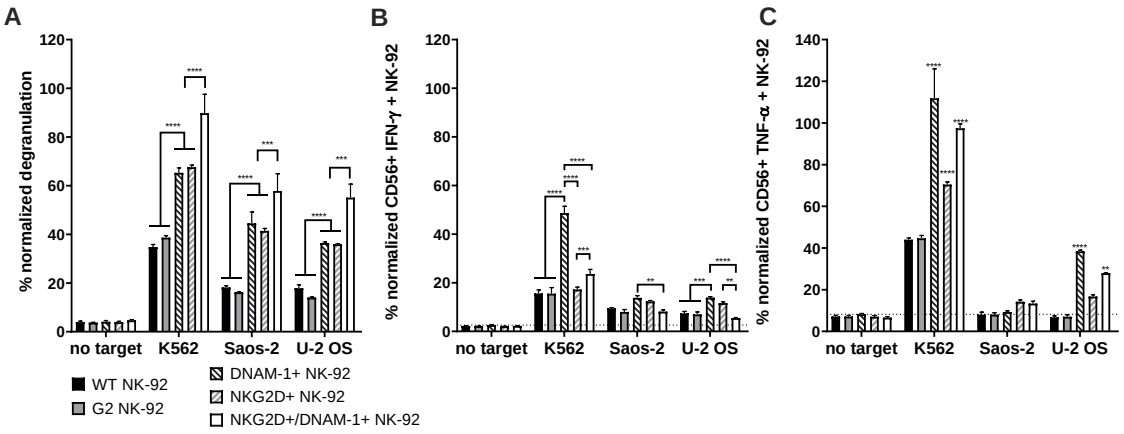


Figure 4.11. Degranulation of NKG2D and/or DNAM-1 receptor expressing NK-92 cells against K562 and sarcoma cell lines. Percent normalized **(A)** degranulation **(B)** IFN-γ and **(C)** TNF-α response of WT, G2, NKG2D and/or DNAM-1expressing NK-92 cells against K562, Saos-2 and U-2 OS cells. Mean of two independent experiments run in duplicates were plotted with SEM.

4.3.3. Cytotoxicity of NKG2D$^+$/DNAM-1$^+$ Expressing NK-92 Cells

While degranulation is a measurement for NK cell-mediated targeting, assessment of degranulation does not necessarily correlate with target cell lysis. Target cell lysis depends on not only the extent of effector cell degranulation, but also the content of secretory lysosomes. Therefore, in order to understand the functional consequences of NKG2D and/or DNAM-1 receptors in NK-92 cells; we performed electrical impedance-based cytotoxicity assays against U-2 OS and Saos-2 cells (Figure 4.12). Quantification of cytotoxic activity at 1:1 (E:T) ratio followed for 12 hours shows that DNAM-1$^+$ NK-92 cells are the most efficient in killing U-2 OS and Saos-2 cells (Figure 4.12A).

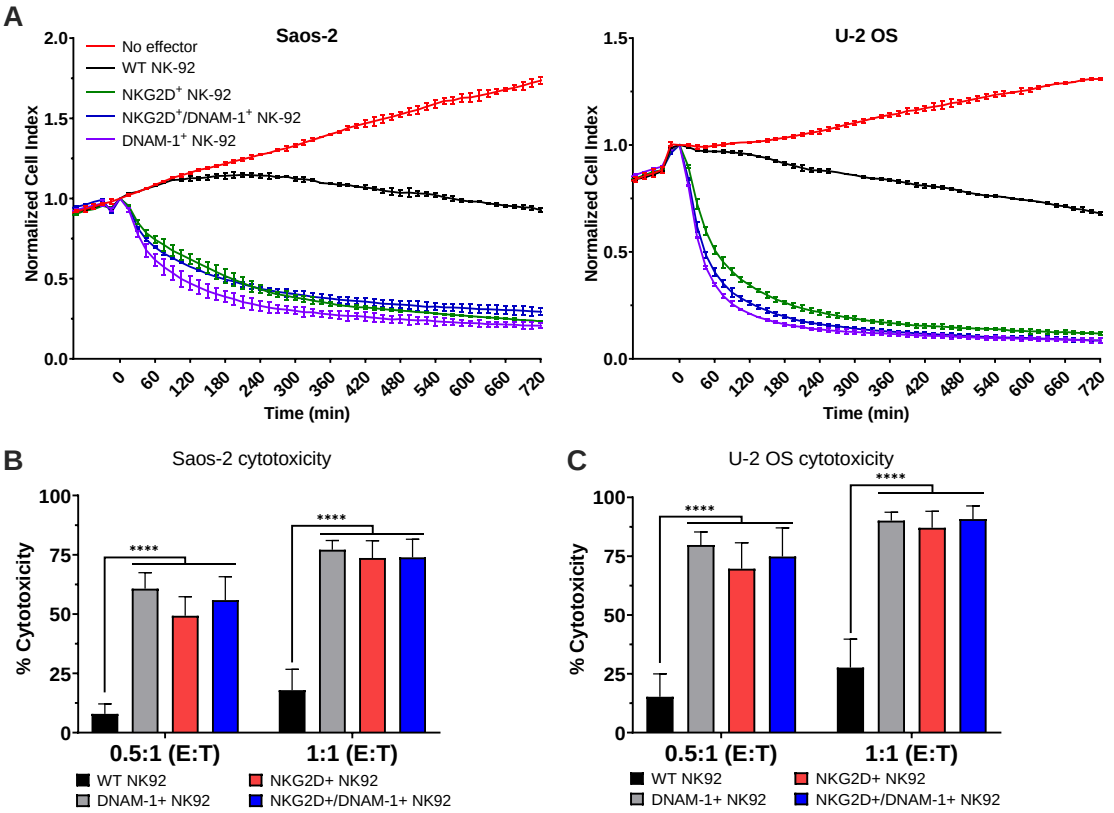


Figure 4.12. NKG2D and DNAM-1 receptor mediated cytotoxicity against sarcoma cell lines. **(A)** Representative results at a 1:1 (E:T) ratio from Saos-2 (left panel) and U-2 OS (right panel) sarcoma cell lines for real-time measurement of cell index recorded every 15 minutes using *xCELLigence RTCA platform.* Effector cells were added to cultures only after target cells reached to desired confluencies (normalized cell index) at 0 hour time point. Calculation of cytotoxicity against **(B)** Saos-2 and **(C)** U-2 OS was done after 4 hours of co-culture at a (E:T) ratio of 0.5:1 and 1:1. Results from two independent experiments, run in triplicates, were plotted with SD.

Although both NKG2D$^+$ and NKG2D$^+$/DNAM-1$^+$ NK-92 cells rapidly killed target cells at a much higher rate compared to WT NK-92 cells, co-expression of the two receptors failed to amplify the killing activity. Also, measurements with changing (E:T) ratios at 4-hour time point only slightly affected the trend in cytotoxicity levels, yet, co-expression still did not surpass the cytotoxicity of corresponding single-positive GM NK-92 cells (Figure 4.12B, C). Therefore, we conclude that the co-expression of DNAM-1 and NKG2D does not seem to be a feasible approach in further enhancing the NK-92 activity against sarcoma.

4.4. Future Directions

Developing a Cell-based Screening Tool for Patient-tailored Cancer Immunotherapy

In this study, we evaluated the feasibility of an NK-92 cell-based *in vitro* screening platform to identify targetable response-triggering receptors and assess their response to validate their applicability. Our results indicate that identification of the unique NK cell receptor response profile of various tumor cells highlights the potential importance of a cell-based screening in order to characterize targetable patient-specific NK cell/tumor interactions. This is especially valid for heterogeneous tumor cell populations where there is no available antibody to characterize ligands on patient-derived tumor cells. In such a case, this cell-based screening platform can be optimized into a tool that can rapidly and accurately identify patient-tailored candidates, focusing on the functional outcomes of genetically modified NK cells for adoptive immunotherapy of cancer (Figure 4.13).

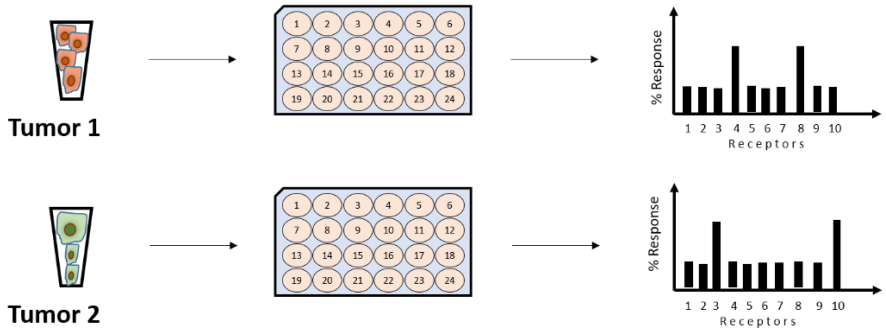


Figure 4.13. Development of a screening tool that can rapidly identify targets for patient-tailored immunotherapy of cancer.

5. DISCUSSION & CONCLUSION

Natural killer (NK) cells of innate immune system serve as a first line of defense in immunosurveillance by potently recognizing tumorigenic and virally infected cells (Alici and Sutlu 2009). They are able to directly lyse these targets cells, while sparing healthy cells with proper surface MHC-I expression levels. This cytolytic function can act through many processes such as degranulation and death receptor ligation, which results in target cell clearance (Smyth et al. 2005b; Moretta et al. 2002). Additionally, NK cells can function indirectly by producing various cytokines and chemokines, upon activation by receptor stimulation and inflammatory cytokine-induced activation signaling. Hence, NK cells not only contribute to the clearance of target cells, but also regulate both the innate and adaptive immune responses (Fauriat et al. 2010).

NK cells express several activating and inhibitory receptors at the cell surface that either promote or dampen the killing of target cells upon coupling to their corresponding ligands (Lanier 2008). NK cell-mediated lysis is determined by a complex balance between these germline-encoded receptors which dictate whether an NK cell is activated or not. In fact, it has been demonstrated that the activating receptors display a collaborative synergy during target cell engagement which amplifies the extent of response dramatically (Bryceson et al. 2006). However, these processes are tightly controlled by a group of inhibitory receptors. These receptors act as negative regulators of NK cytotoxicity and inhibit the action of NK cells against self-targets. If the target cell expressing self MHC Class-I molecules is recognized by inhibitory receptors, inhibitory signals can block the activation of cytotoxic pathways triggered by activating receptors (Long 2008).

This understanding of how NK cells recognize and profoundly eradicate target cells emphasizes their importance in cancer immunosurveillance. Therefore, dissection and fine-tuning of the complex interaction between NK cell and target cell can potentially be a valuable approach for both prognostic and therapeutic purposes in NK cell-based immunotherapy of cancer.

Gene transfer to NK cells is a promising tool in cancer immunotherapy since it is applicable to both autologous and allogenic settings (Bari et al. 2019; Dahlberg et al. 2015). The genetic modifications involving a gene that sustain and boost NK cell activity were shown to improve efficacy of tumor cell lysis (Kamiya, Chang, and Campana 2016; Nayyar, Chu, and Cairo 2019b). As described earlier, the balance between activating and inhibitory receptors determines NK cell activity on target cell. Therefore, it is a critical approach to modulate cell surface receptor expression to improve anti-tumor efficacy of NK cells.

In a setting where the NK cell remains unresponsive to presented targets such as autologous tumor cells, a balance of activating and inhibitory signals prevails. It is possible to overcome this balance via genetic modification by either upregulating activating receptors on the NK cell surface or downregulating inhibitory receptors in order to abolish the inhibitory signaling. Such an approach can be used to gain a basic understanding of the receptors involved in target cell killing or tolerance while presenting functional data regarding possible therapeutic effect of such modified cells. Therefore, we proposed to develop a screening method based on the genetic modification of NK cells by upregulating a single receptor at a time and evaluate functional readouts such as NK cell degranulation and cytotoxicity against target cancer cells. As both the character of the tumor cell population and phenotypic status of NK cells differ among patients, such a tool will be instrumental in developing patient-tailored NK cell-based cancer immunotherapy. Also, this study can shed light on NK/tumor interactions by screening of contributing receptors via genetic modification and help to understand basics of this dual interaction.

In order to establish a novel in vitro screening tool on genetically modified NK cell line (NK-92) expressing different activating receptors, we generated GM NK-92

cells, each overexpressing one NK receptor at a time and used this platform for the first time to characterize tumor-specific functional NK cell receptor signatures of well-characterized cancer cell lines as well as primary human sarcomas. We evaluated the functionality of GM NK-92 cell lines in the screening platform and showed that they were functionally responsive with the capacity to trigger the full range of effector functions. Additionally, it was observed that GM NK-92 cells that overexpress DNAM-1 and NKG2D efficiently triggered a response to various established tumor cell lines, while WT NK-92 cell responses remained comparably low. The response of GM NK-92 cells was almost strictly dependent on the corresponding receptor and ligands and cytotoxicity response of these potent receptors was further confirmed with an electrical impedance-based cytotoxicity assay.

Our results demonstrate, for the first time, the feasibility of a GM NK-92 cell-based functional screening approach in establishing and predicting personalized immunotherapies for cancer patients, while providing essential insight into how to enhance NK cell mediated anti-tumor responses. Given that NK-92 is the only NK cell line that is FDA-approved for allogeneic use, it is also possible to generate an off-the shelf GM therapeutic NK-92 libraries and predict the most efficient treatment option for each patient using such an in vitro screening tool on patient-derived tumor cells. This can rapidly and efficiently characterize a personalized adoptive immunotherapy regimen which can then be used either as a monotherapy or in combination with immune checkpoint inhibitors.

The immune system plays a significant role in disease progression and anti-tumor responses against several hematological and solid tumor malignancies. This also supports the idea that immunotherapy approaches could be a valuable treatment option, but unfortunately they have been poorly explored. While NK cells are one of the promising candidates in the development of advanced cancer immunotherapies, very few clinical trials are currently using NK cells as a therapeutic option (Barkholt et al. 2009; Locatelli et al. 2018; Mavers and Bertaina 2018). Clinical trials using chimeric antigen receptors (CAR) and T cell receptors (TCR) focus on engineering T cells for immunotherapy modalities (Parlar et al. 2019; Mensali et al. 2019). As the obstacles associated with the genetic

manipulation of NK cells are being overcome, strategies exploring the use of genetically modified NK cells are recently gaining more attention (Nayyar, Chu, and Cairo 2019a).

When NK cells are attracted to the tumor milieu, they are confronted with several challenges in the context of tumor evasion mechanisms resulting in diminished NK cell cytotoxicity. Studies on both hematological and solid tumor malignancies demonstrated that almost all tumor cells upregulate inhibitory checkpoint ligands (e.g. HLA-E, the ligand of inhibitory NKG2A receptor) or downregulate activating receptor ligands (e.g. NKG2D ligands) in the tumor microenvironment (Balsamo et al. 2013; Gasser et al. 2005; Li et al. 2009; Ghiringhelli et al. 2005). Nevertheless, there are very few studies which showed the effect of using receptor modulation to enhance NK cell anti-tumor cytotoxicity (Kamiya, Chang, and Campana 2016; Parihar et al. 2019; Y. H. Chang et al. 2013; Figueiredo, Seltsam, and Blasczyk 2009; Kamiya et al. 2019). This study provides a novel platform to predict and customize efficient immunotherapies and suggests a perspective for the development of such strategies as potential therapeutic targets for various types of tumors.

DNAM-1 and NKG2D are two activating NK cell receptors which recognize stress-induced ligands that are commonly induced by tumors. Thus, they have been implicated as key players in immunity against human tumor cells and have been extensively characterized in several settings of cancer immunotherapy (Morisaki, Onishi, and Katano 2012). As mentioned earlier, many studies have shown reduced activating receptor expression such as DNAM-1 and NKG2D on tumor infiltrating lymphocytes from cancer patients or shedding of their respective ligands from tumor cells (Nieto-Velázquez et al. 2016; Guillamon et al. 2018; Salih, Rammensee, and Steinle 2002; Iguchi-Manaka et al. 2016). This downregulation of NK cell activating receptor in the periphery may result in abrogated anti-tumor responses and can be reversed by ex vivo activation of NK cells expressing DNAM-1 and NKG2D, that have been shown to efficiently target not only primary cells, but also cancer cell lines in vitro (Cho et al. 2010; Nayyar, Chu, and Cairo 2019b; Verhoeven et al. 2008; Boerman et al. 2015). Here, we demonstrate that

primary sarcomas and other tumor cells can be efficiently targeted by genetically modified NK-92 cells overexpressing NKG2D or DNAM-1 receptors.

We also addressed whether the synergy from co-expression of DNAM-1 and NKG2D would further enhance the anti-sarcoma activity of NK-92 cells. The signaling pathways downstream DNAM-1 and NKG2D may overlap through shared activation motifs, eventually using mutual essential molecules to exert effector functions (Z. Zhang et al. 2015; Molfetta et al. 2017). Thus, it is possible to argue that simultaneous triggering of DNAM-1 and NKG2D may result in a limited enhancement of these signals, while it may also present a risk of two signals encountering a rate limiting step of either ligand engagement or signaling intermediates that restricts the amount of action possible. Our results demonstrate that while the co-expression of the two receptors affects the degranulation response, it fails to provide a significant cytotoxicity in killing sarcoma cell lines. An alternative means to assess the effect of co-expression can be the involvement of different signaling pathways such as TRAIL or FasL, but such an approach remains to be analyzed in further studies.

Paving their way for therapeutic strategies, the characterization of novel biomarkers to identify the most efficient course of treatment is becoming increasingly necessary. Therefore, efforts to develop new strategies that enable rapid and efficient generation of information on patient-specific tumors have recently intensified. In this study, we established a functional screening platform that enables the identification of NK cell receptor responses against a variety of tumor cells including both cell lines and primary human explants. This study was the first to simultaneously intercalate several receptors and assess their responses to various target cells. Therefore, valuable information emancipated from the NK-92 cell-based screening platform could be implemented as a tool to perform parallel assessment of several activating NK cell receptors and pinpoint the ones with the ability to equip NK-92 cells for cytotoxicity against the individual patients' tumor cells. This approach would contribute immensely to making efficient and patient-tailored immunotherapy treatments.

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7. APPENDIX A: Chemicals

Table 7.1. List of chemicals used in this study.

Chemicals and Media Components	**Company**
Agar	Sigma-Aldrich, St. Louis, MO, USA
Agarose	Sigma-Aldrich, St. Louis, MO, USA
Ampicillin	Corning, GellGro, USA
Boric Acid	Sigma-Aldrich, St. Louis, MO, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, St. Louis, MO, USA
Chloroquine	Sigma-Aldrich, St. Louis, MO, USA
Distilled Water	Merck Milipore, USA
DMEM	Gibco, Life Technologies, Grand Island, NY, USA
DMSO	Sigma-Aldrich, St. Louis, MO, USA
DNA Gel Loading Dye, 6X	NEB, USA
DPBS, 1X	Sigma-Aldrich, St. Louis, MO, USA
EDTA	Applichem, Germany
Ethanol	Sigma-Aldrich, St. Louis, MO, USA
Ethidium Bromide	Sigma-Aldrich, St. Louis, MO, USA
Fetal Bovine Serum	Gibco, Life Technologies, Grand Island, NY, USA
HEPES Solution, 1 M	Gibco, Life Technologies, Grand Island, NY, USA
Interleukin-2 (Proleukin)	Novartis Pharmaceuticals, Switzerland

Ionomycin	Sigma-Aldrich, St. Louis, MO, USA
Isopropanol	Sigma-Aldrich, St. Louis, MO, USA
LB Broth	Sigma-Aldrich, St. Louis, MO, USA
L-glutamine	Gibco, Life Technologies, Grand Island, NY, USA
Sodium Pyruvate	Gibco, Life Technologies, Grand Island, NY, USA
MEM Vitamin Solution, 100X	Gibco, Life Technologies, Grand Island, NY, USA
MEM Non-essential Amino Acid Solution	Gibco, Life Technologies, Grand Island, NY, USA
2-Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA
Methanol	Sigma-Aldrich, St. Louis, MO, USA
Monensin	Biolegend, CA, USA
GolgiStop	BD Biosciences, USA
NaCl	Sigma-Aldrich, St. Louis, MO, USA
(5Z)-7-Oxozeaenol	Sigma-Aldrich, St. Louis, MO, USA
Penicillin-Streptomycin	Sigma-Aldrich, St. Louis, MO, USA
PFA	Biolegend, CA, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, MO, USA
PIPES	Sigma-Aldrich, St. Louis, MO, USA
Poly-L-Lysine	Sigma-Aldrich, St. Louis, MO, USA
Protamine Sulfate	Gibco, Life Technologies, Grand Island, NY, USA
RPMI-1640	Gibco, Life Technologies, Grand

	Island, NY, USA
Saponin	Sigma-Aldrich, St. Louis, MO, USA
Trizma	Sigma-Aldrich, St. Louis, MO, USA
Trypsin-EDTA	Gibco, Life Technologies, Grand Island, NY, USA

8. APPENDIX B: Equipments

Table 8.1. List of equipments used in this study.

Equipment	**Company**
Autoclave	Hirayama, HiClave HV-110, Japan
Balance	ISOLAB, 302.31.002, Germany
Centrifuge	Eppendorf, 5415D, Germany Eppendorf, 5702, Germany VWR, MegaStar 3.0R, USA Beckman Coulter, Allegra X-15R, USA
CO_2 Incubator	Thermo Fisher, Heracell Vios 160i, USA Binder GmbH, Germany
Automated Cell Counter	Thermo Fisher, Countess II FL, USA
Deep freezer	-80 °C, Forma, Thermo Electron Corp., USA -20 °C, Bosch, Turkey
Electrophoresis Apparatus	Biorad Inc., USA
gentleMACS Octo Dissociator with	Miltenyi Biotec, Germany

heaters	
Filters (0.22 μm and 0.45μm)	Merck Millipore, USA
Fluorescence-activated cell sorting	BD Aria II, USA
Flow cytometer	BD LSR Fortessa, USA
Gel Documentation	Biorad, UV-Transilluminator 2000, USA
Heater Thermomixer Comfort	Eppendorf, Germany
Hemocytometer	ISOLAB, Neubauer, 075.03.001, Germany
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert Modell 300, Germany
Laminar Flow	Heraeus, HeraSafe HS 12, Germany Heraeus, HeraSafe KS, Germany
Liquid Nitrogen Tank	Taylor-Wharton, 300RS, USA
Magnetic Stirrer	VELP Scientifica, Italy
Microliter Pipettes	Gilson, Pipetman, France Thermo Fisher Scientific, USA
Microscope	Zeiss, Primo Vert, Germany Zeiss Observer Z1, Germany
Microwave Oven	Bosch, Turkey
pH Meter	Mettler Toledo, USA
Refrigerator	Bosch, Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	New Brunswick Sci., USA NanoDrop 2000, Thermo Fischer

	Scientific, USA
Vortex	VELP Scientifica, Italy

9. APPENDIX C: Plasmid Maps

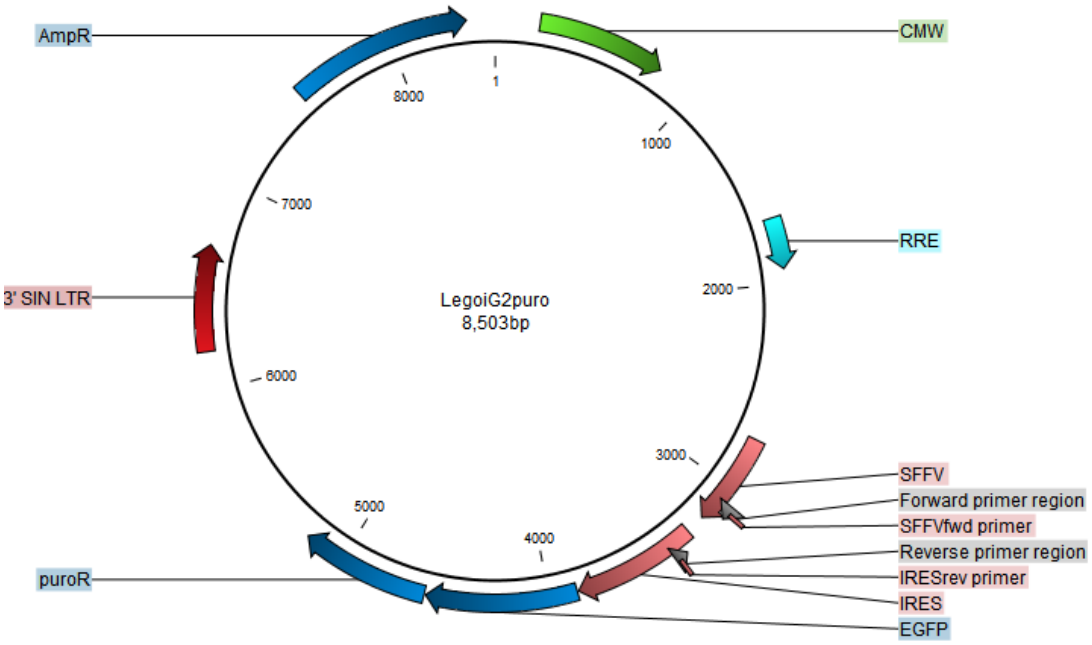


Figure C.1. The vector map of LeGO.iG2 Puro.

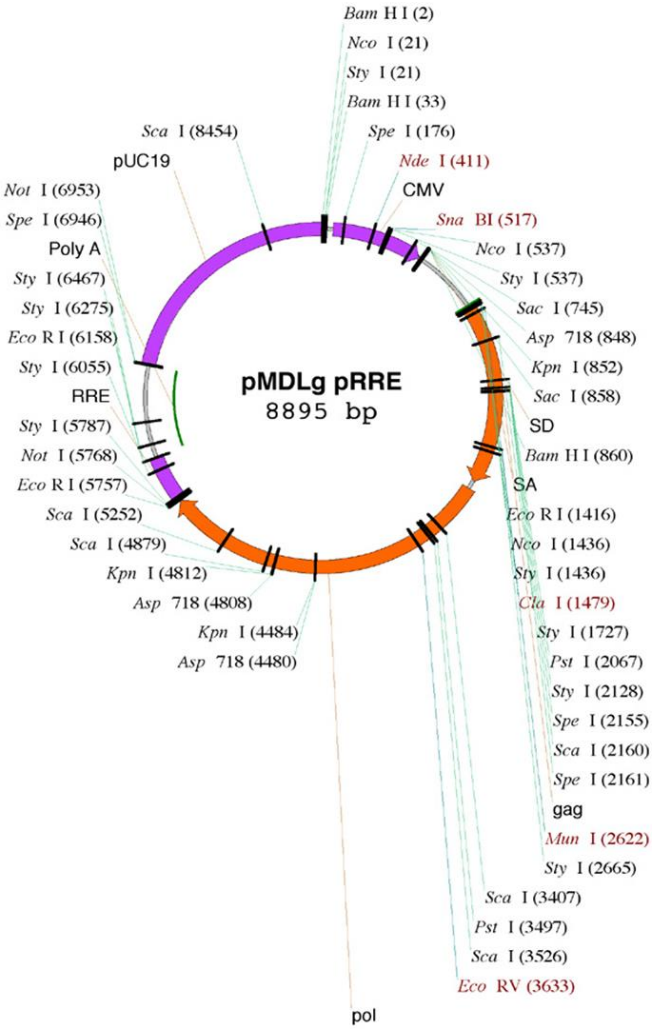


Figure 4. The vector map of pMDLg/pRRE.

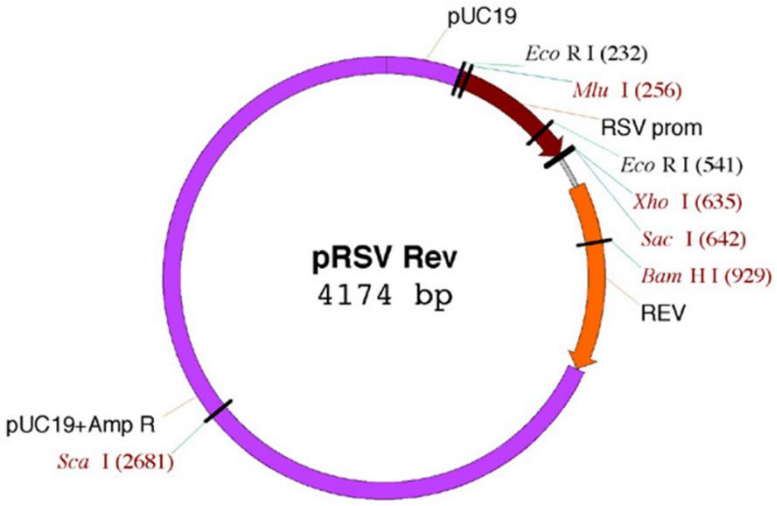


Figure C.3. The vector map of pRSV-REV.

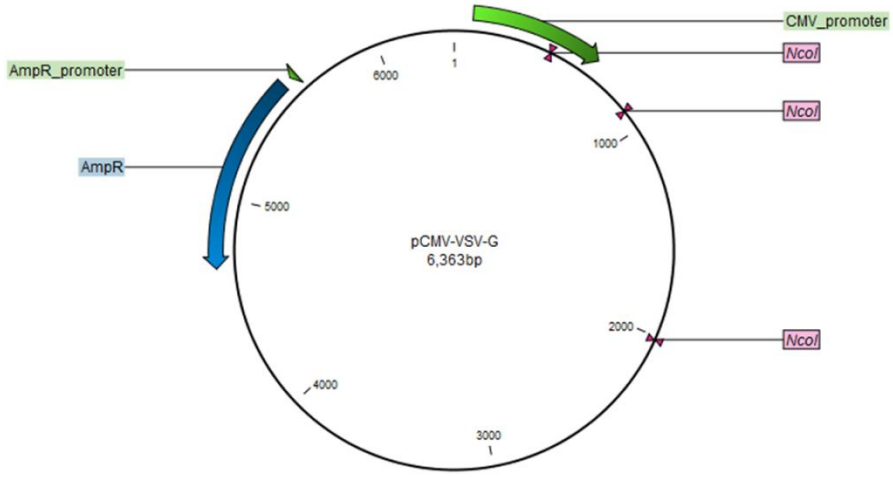
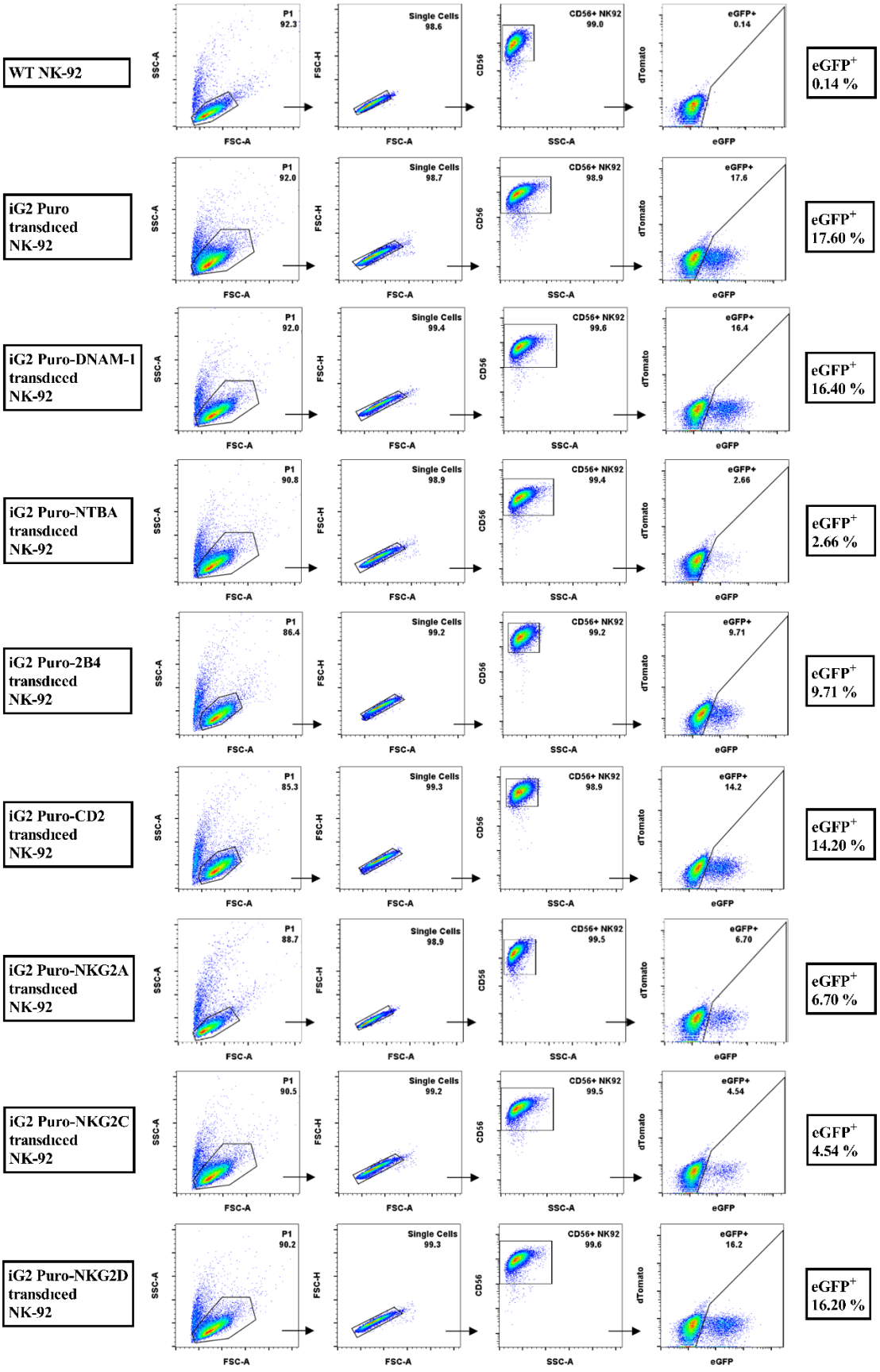


Figure C.4. The vector map of pCMV-VSV-G.

10. APPENDIX D: Generation of GM NK-92 cell lines



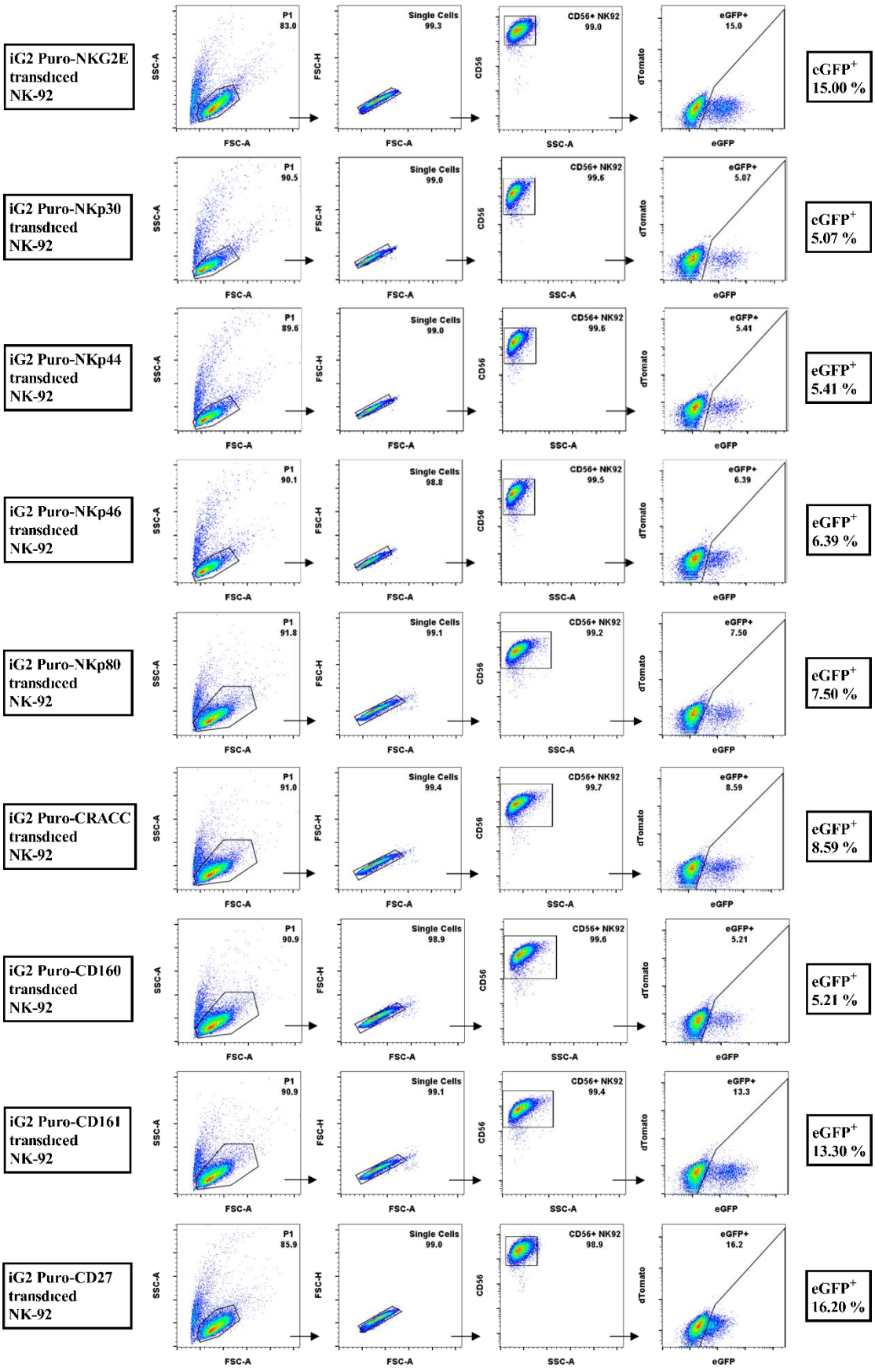
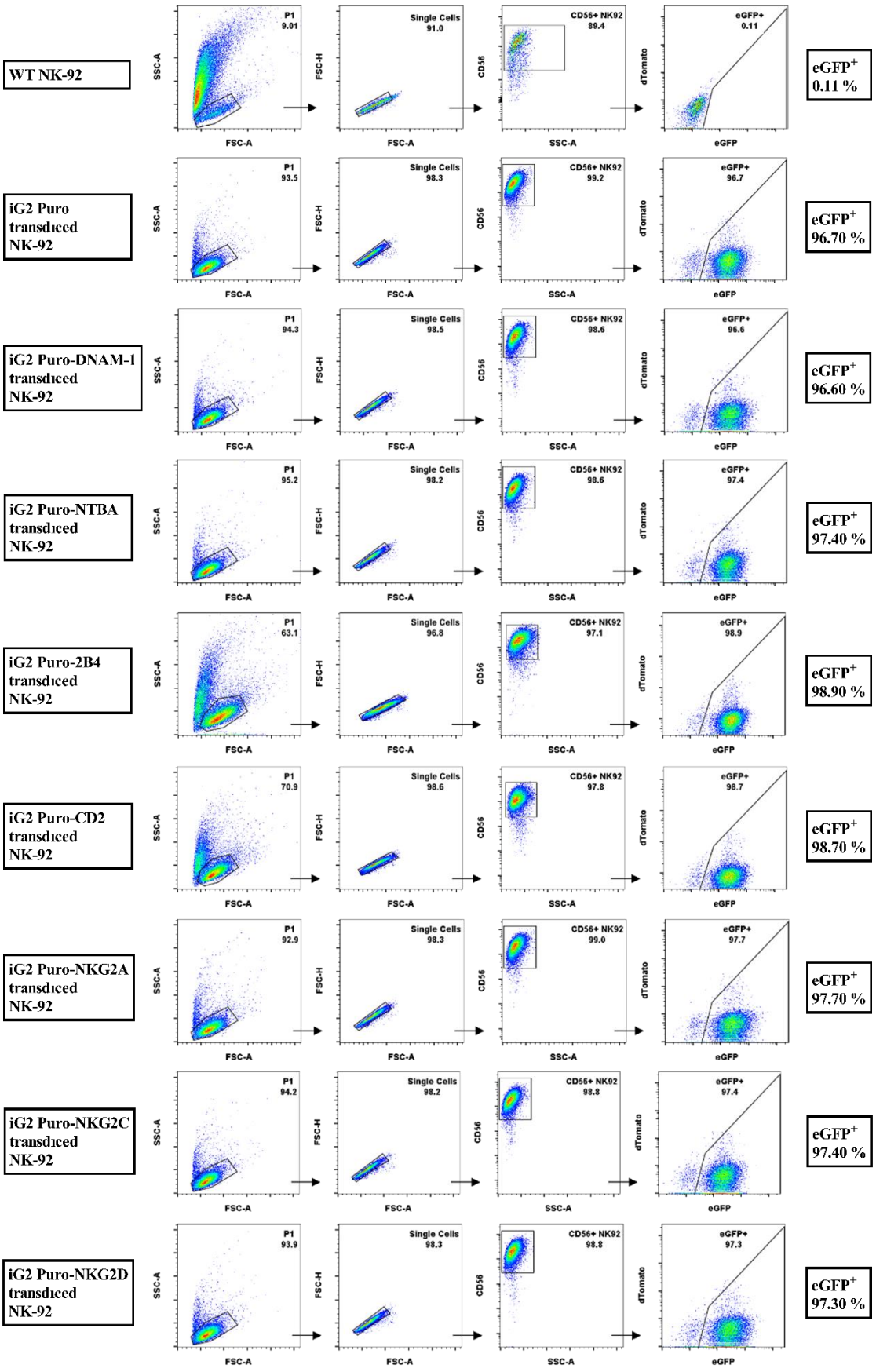


Figure D.1. Flow cytometer analysis of GM NK-92 cell lines at day 4 (pre-selection).


WT NK-92
P1
9.01
Single Cells
91.0
CD56+ NK92
89.4
eGFP+
0.11
eGFP+
0.11 %
iG2 Puro transduced NK-92
P1
93.5
Single Cells
98.3
CD56+ NK92
99.2
eGFP+
96.7
eGFP+
96.70 %
iG2 Puro-DNAM-1 transduced NK-92
P1
94.3
Single Cells
98.5
CD56+ NK92
98.6
eGFP+
96.6
eGFP+
96.60 %
iG2 Puro-NTBA transduced NK-92
P1
95.2
Single Cells
98.2
CD56+ NK92
98.6
eGFP+
97.4
eGFP+
97.40 %
iG2 Puro-2B4 transduced NK-92
P1
63.1
Single Cells
96.8
CD56+ NK92
97.1
eGFP+
98.9
eGFP+
98.90 %
iG2 Puro-CD2 transduced NK-92
P1
70.9
Single Cells
98.6
CD56+ NK92
97.8
eGFP+
98.7
eGFP+
98.70 %
iG2 Puro-NKG2A transduced NK-92
P1
92.9
Single Cells
98.3
CD56+ NK92
99.0
eGFP+
97.7
eGFP+
97.70 %
iG2 Puro-NKG2C transduced NK-92
P1
94.2
Single Cells
98.2
CD56+ NK92
98.8
eGFP+
97.4
eGFP+
97.40 %
iG2 Puro-NKG2D transduced NK-92
P1
93.9
Single Cells
98.3
CD56+ NK92
98.8
eGFP+
97.3
eGFP+
97.30 %
SSC-A
FSC-A
FSC-H
CD56
dTomato
eGFP

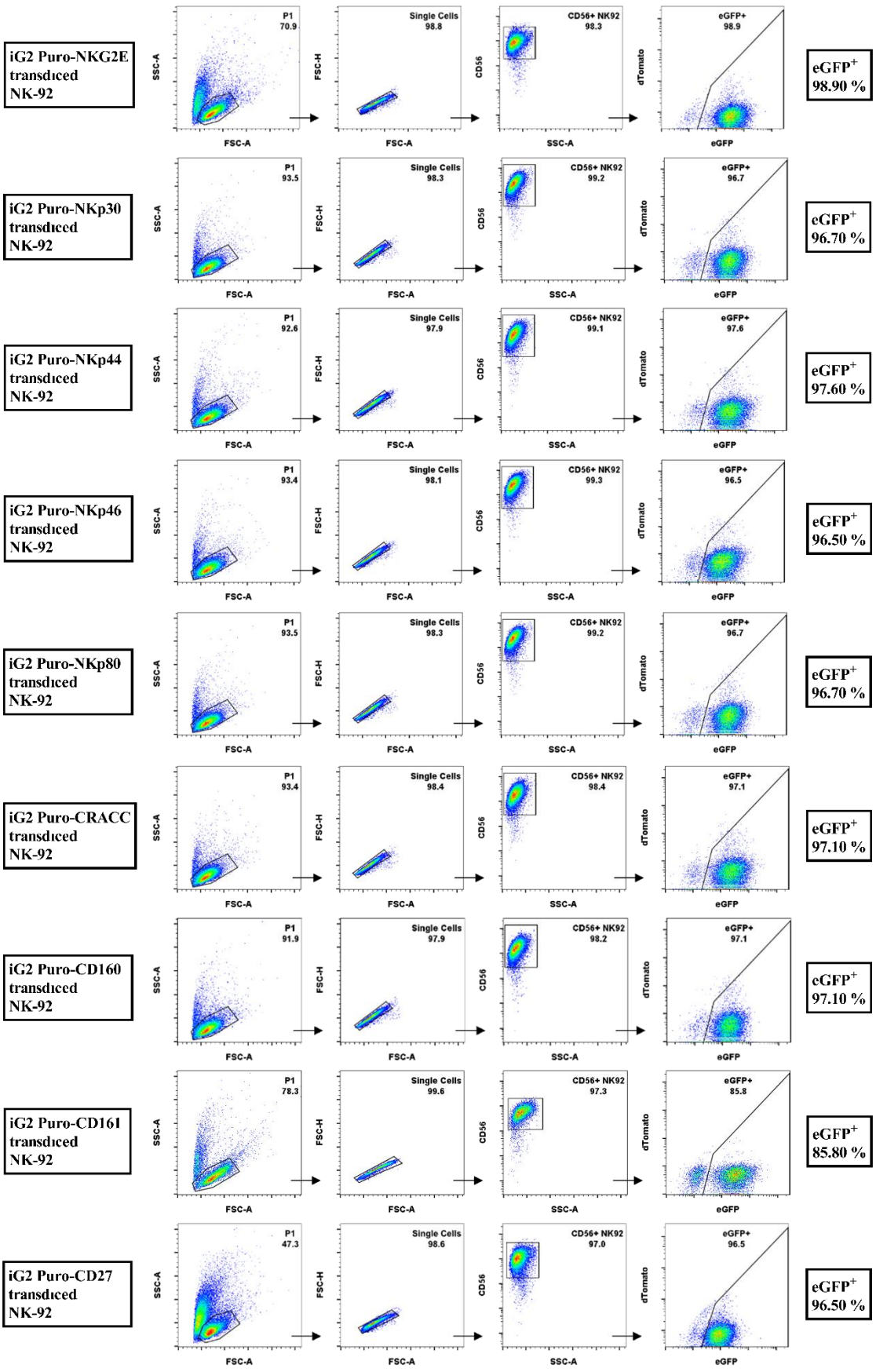


Figure D.2. Flow cytometer analysis of GM NK-92 cell lines at day 14 (post-selection).