NK cell recognition of malignant cells

– a CRISPR approach to define novel mediators

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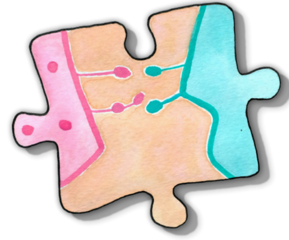

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Det är underligt med vägar och floder, funderade Sniff, man ser dem gå förbi
och får en hemsk lust att vara nån annanstans.
Att följa med och se var de slutar…

ABSTRACT

Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system, capable of eliminating malignant cells. Their activity is intricately regulated through a balanced interplay between activating and inhibitory receptors that interact with molecules on their prospective target cells. This thesis employed genome editing to delve deeper into genes and molecules that influence these dynamic interactions. A loss-of-function genome-wide CRISPR screen using the leukemic cell line K562 with NK cell cytotoxicity as the selective pressure, unveiled genes impacting target cell susceptibility. *TMEM30A* depletion (**paper I**) rendered target cells partially resistant to NK-cell-induced lysis. Subsequent investigations elucidated its role in phospholipid transport within the plasma membrane. Loss-of-function mutations in *TMEM30A*, observed in certain cancers, upregulated phosphatidylserine on the cell surface enabling interaction with inhibitory NK cell receptor TIM-3, providing protection from NK cells. *BAP1*, another gene identified in the CRISPR screen (**paper II**) was found to support MHC class I expression through involvement in interferon-γ signalling. Depletion of *BAP1* increased target cell sensitivity to NK cells by eliminating the inhibitory signal. The CRISPR/Cas9 technique was further employed to suppress the expression of crucial ligands for activating NK cell receptors. This manipulation allowed the investigation of alternative receptor-ligand interactions and provided a model to decipher the impact of a single nucleotide polymorphism (SNP) in the receptor NKG2D gene on NK cell function (**paper III**). Notably, the identified SNP in the linked gene for NKG2A emerged as the key driver of NK cell function and additionally influenced the clinical outcome of immunotherapy in acute myeloid leukemia. Leveraging this established model cell line, dominantly killed via NKp46, a subsequent genome-wide CRISPR/Cas9 screen was conducted to identify potential ligand candidates for the NKp46 receptor (**paper IV**). In conclusion, CRISPR/Cas9 technology proved to be instrumental in uncovering molecular mechanisms that regulate the interaction between NK cells and their target cells, which may pave the way for therapeutic interventions in cancer.

Keywords: Natural killer cells, CRISPR/Cas9 screen, *TMEM30A*, TIM-3, Phosphatidylserine, *BAP1*, acute myeloid leukemia, immunotherapy, HDC/IL-2, NKG2A, NKp46 ligand

SAMMANFATTNING PÅ SVENSKA

Kroppens immunförsvar består av en uppsjö av molekyler och celler vars uppgift är att skydda oss både från yttre hot så som virus och bakterier men även inre hot i form av cancer. Den här avhandlingen fokuserar på en specifik celltyp som kallas naturlig mördarcell på svenska och mer känd som NK-cell på engelska, vilken har väckt intresse för nya former av immunterapi för cancerbehandling. NK-celler övervakar och dödar kontinuerligt celler som visat sig vara virusinfekterade eller canceromvandlade. För att särskilja sjuka celler från friska har NK-celler en uppsättning aktiverande och inhiberande receptorer på sin yta, vilka interagerar med proteiner och strukturer på målcellen. Dessa så kallade ligander kan regleras upp och ned på målcellen beroende på olika omständigheter. Friska celler uttrycker proteiner på sin yta som skyddar dem mot attacker från NK-celler. Å andra sidan tenderar cancerceller att uttrycka stressrelaterade proteiner, vilka kan binda till aktiverande receptorer på NK-celler och resultera i en dödande attack. Mycket av det som påverkar interaktionen mellan NK-celler och målceller är fortfarande okänt och det finns ligander som ännu inte upptäckts.

För att identifiera nya ligander och strukturer som är involverade i interaktionen mellan cellerna så utförde vi en omfattande kartläggning med hjälp av gensaxen CRISPR. Genom undersökningen fick vi kunskap om gener som på olika sätt påverkar interaktionen, så som *TMEM30A* som är involverad i förflyttning av olika lipider i cellmembranet. Mutationer i denna gen har upptäckts i vissa cancersorter, och i delarbete I kunde vi visa att dessa mutationer kan utgöra en mekanism för cancercellerna att undkomma avdödning av NK-celler genom att inhiberande strukturer ökar på cellernas yta. *BAP1* identifierades också i undersökningen, och i delarbete II visades att mutationer i denna gen påverkar förmågan att öka uttrycket av vissa inhiberande strukturer på cellens yta, vilket kan påverka deras interaktion med både NK-celler och andra celler i

immunförsvaret. CRISPR användes även för att skapa en genmodifierad cancercell som kunde dödas med hjälp av två specifika aktiverande receptorer. Med hjälp av de cellerna kunde vi i delarbete III belysa vikten av vissa genvarianter för överlevnad efter behandling av akut myeloisk leukemi (AML) med immunterapi. Genom att manipulera interaktionerna mellan NK-cellen och denna cancercell med antikroppar kunde vi skapa en experimentell modell där avdödningen av cancercellerna nästan uteslutande sker med hjälp av den aktiverande receptorn NKp46. Genom en ny CRISPR-undersökning med denna modell kunde vi identifiera nya ligandkandidater till denna viktiga NK-cellsreceptor.

LIST OF PAPERS

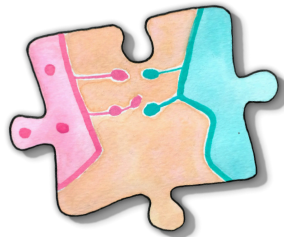
This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Kristenson L**, Badami C, Ljungberg A, Islamagic E, Tian Y, Xie G, Hussein BA, Pesce S, Tang KW, Thorén FB.
Deletion of the *TMEM30A* gene enables leukemic cell evasion of NK cell cytotoxicity.
Proc Natl Acad Sci U S A. 2024. Accepted for publication.

II. Badami C, **Kristenson L**, Svensson F, Kathirkamanathan T, Thorén FB.
BAP1 deletion disrupts IFNγ signaling and sensitizes cancer cells to NK cell cytotoxicity.
In manuscript.

III. Hussein BA*, **Kristenson L***, Pesce S, Wöhr A, Tian Y, Hallner A, Brune M, Hellstrand K, Tang KW, Bernson E, Thorén FB.
NKG2A gene variant predicts outcome of immunotherapy in AML and modulates the repertoire and function of NK cells.
J Immunother Cancer. 2023 Aug;11(8):e007202.
*Equal contribution

IV. **Kristenson L**, Islamagic E, Badami C, Rockstein L, Lind S, Hussein BA, Svensson F, Pesce S, Johansson L, Tang KW, Hellstrand K, Thorén FB.
Identification of natural cytotoxicity receptor ligands using a CRISPR-engineered target cell line.
In manuscript.



Additional publications not part of this thesis:

i. Chuang TP, Lai WY, Gabre JL, Lind DE, Umapathy G, Bokhari AA, Bergman B, **Kristenson L**, Thorén FB, Le A, Doebele RC, Van den Eynden J, Palmer RH, Hallberg B. ALK fusion NSCLC oncogenes promote survival and inhibit NK cell responses via *SERPINB4* expression. *Proc Natl Acad Sci U S A. 2023 Feb 21;120(8):e2216479120.*

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ABBREVIATIONS

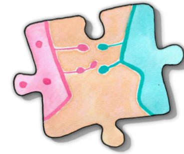
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CML	Chronic myeloid leukemia
CR	Complete remission
CRISPR	Clustered regularly interspaced short palindromic repeats
DCs	Dendritic cells
DD	Death domain
DISC	Death-inducing signalling complex
GPI	Glycolsylphosphatidylinositol
gRNA	Guide RNA
HDC	Histamine dihydrochloride
HLA	Human leukocyte antigen
IFNγ	Interferon-gamma
IL	Interleukin
IS	Immunological synapse
ITAM/ITIM	Immunoreceptor tyrosine-based activation/inhibition motif

KIR	Killer-cell immunoglobulin-like receptor
KO	Knockout
LFS	Leukemia-free survival
LRC	Leukocyte receptor complex
MDCS	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKC	Natural killer complex
OPG	Osteoprotegerin
OS	Overall survival
PtdSer	Phosphatidylserine
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
tKO	Triple-knock-out
TNFα	Tumour necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TRAIL-receptor
WT	Wild-type

PREFACE

Cancer is a group of diseases that constitutes a leading global cause of mortality. It is characterized by uncontrolled growth of the body's own cells that are capable of spreading to other parts of the body. Over the past several decades, conventional cancer treatments have involved irradiation, chemotherapy and surgical interventions. In more recent years, strategies to harness the endogenous immune system have been developed to combat cancer. This approach has shown success in treatment of several malignancies, with the groundbreaking discovery of checkpoint inhibitors being honoured with the Nobel Prize in 2018. Many of these immunotherapeutic treatments benefit from natural killer (NK) cell involvement. Much remains unknown regarding the interplay between NK cells and cancer cells. To enable development of novel therapies, more knowledge is imperative. This thesis endeavours to contribute to the ongoing quest to decipher the cancer puzzle. By the investigation of genes and molecular mechanisms that influence the dynamic interactions between cancer cells and NK cells, it can hopefully broaden our understanding and potentially pave the way for the development of future therapies.

THE IMMUNE SYSTEM



In a world full of pathogens of various kinds, a protective system is needed. The human immune system comprises a complex network of cells and molecules that have evolved to protect us from external threats like viruses and bacteria. All variants of immune cells originate from hematopoietic stem cells located in the bone marrow, and are generated in a process known as haematopoiesis, as illustrated in figure 1 (1).

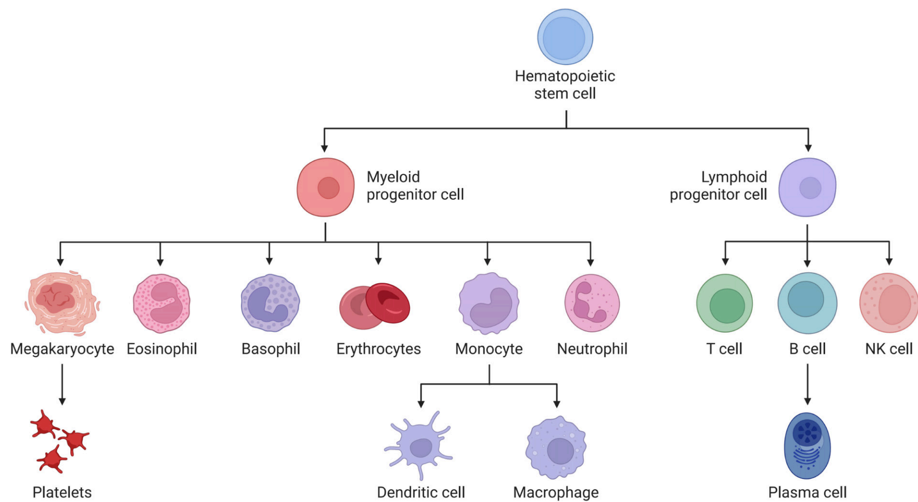


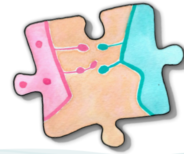
Figure 1. Haematopoiesis. Immune cells from the innate and adaptive immune system develop from myeloid or lymphoid progenitor cells, originating from a hematopoietic stem cell. Created with Biorender.com

The classification of immune cells into the innate or adaptive immune system, although traditionally distinct, has become nuanced in recent times, as it has been discovered that certain innate cells possess traits typically associated with adaptive immunity (2). The adaptive immune system is composed of two key lymphocytes: B and T cells. These cells recognize an extensive array of antigens through their highly variable antigen receptors. The generation of this diverse specificity results

from stochastic rearrangements of gene segments, allowing for virtually limitless variations to recognize a wide range of pathogens. Cells that recognize self-antigens are typically inactivated or eliminated during their maturation in the bone marrow and thymus. The T cell receptor recognize antigens bound to a major histocompatibility complex (MHC) (1). The peptides presented on the MHC molecules are derived from the continuous degradation of intracellular proteins. Subsequently, these peptides are transported into the endoplasmic reticulum lumen by transporter proteins TAP1 and TAP2, where they undergo trimming by the ERAAP protein. Proper folding of the MHC molecule is dependent on peptide binding before it is transported to the cell surface (1). The activation of adaptive T cells relies on cells from the innate immune system. Antigen-presenting cells (APCs) such as dendritic cells (DCs), internalize pathogen structures and degrade them to enable presentation of peptides on their MHC class I or II molecules. Following pathogen engulfment, dendritic cells migrate to secondary lymphoid organs where they can activate T cells (1).

In addition to dendritic cells, the innate immune system encompasses various additional myeloid cells such as macrophages, neutrophils, eosinophils and basophils. These cells recognize pathogens or pathogen-induced damage through the germline-encoded pattern recognition receptors (PRRs) of the innate immune system. The evolutionary conserved structures associated with microorganisms and recognized by these receptors are termed pathogen-associated molecular patterns (PAMPs), which can include mannose-rich oligosaccharides and peptidoglycans found in the bacterial cell wall. The PRRs include both transmembrane receptors such as Toll-like receptors recognizing extracellular structures, and cytoplasmic receptors such as NOD-like receptors recognizing intracellular structures (1). A fundamental function of the innate immune system is performed by the phagocytic cells, including macrophages, granulocytes and dendritic cells, which have the capability to engulf and degrade pathogens. Another component of the innate immune system are the innate lymphoid cells (ILC), including natural killer (NK) cells (1).

NATURAL KILLER CELLS



Section in brief:

NK cells are important immune cells, capable of eliminating cancer cells which they recognize through their repertoire of inhibitory and activating receptors. Their inhibitory receptors recognize MHC class I molecules which are expressed on all healthy nucleated cells, protecting them from harm. This constant inhibitory signalling helps the NK cells gain full functionality in a process called education. Malignant cells often downregulate MHC class I molecules and upregulate ligands for activating NK cell receptors, exposing them to potential eradication by NK cells.

NK cells were discovered in the 1970s and have since then proven to be a pivotal component of the immune system, playing a crucial role in defending the body against infections and malignancies (3-5). These cells exhibit both a unique ability to eliminate virus-infected or transformed cells without the need for prior sensitization, as well as an important role in immune regulation, positioning them as essential members of the innate immune response. In contrast to T cells, NK cells express a diverse array of inhibitory and activating receptors that enable them to distinguish between healthy and malignant cells. Stress or transformation can lead to upregulation of ligands for activating NK cell receptors. Upon engagement, these interactions prompt NK cells to release their lytic granule content, which induces apoptosis in the designated target cell. In contrast, healthy cells typically express ligands for inhibitory NK cell receptors, allowing them to evade NK cell cytotoxicity (6, 7).

NK cells are defined as $CD3^{-}CD56^{+}$ cells and can be further classified based on their expression level of CD56 and CD16. The immature $CD56^{bright}CD16^{lo/-}$ are generally considered superior cytokine producers, while the more mature

$CD56^{dim}CD16^{+}$ cells exhibit enhanced cytotoxic capacity. Interestingly, upon stimulation with target cells, $CD56^{dim}$ cells have been observed to display notable cytokine-producing capabilities, challenging the traditional perception of their functional attributes (8). The phenotypic diversity of NK cells extends to their expression of inhibitory and activating receptors, contributing to their heterogenous nature (9-11).

NK cells play a crucial role in modulating the immune system by producing cytokines and chemokines, including the chemokines CCL3, CCL4 and CCL5 and the cytokines GM-CSF, IL-5, IL-13 and IL-10 as well as the proinflammatory tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) (11, 12). Upon an activating interaction with target cells or by cytokine stimulation, NK cells start production of IFNγ via the transcription factors STAT4, T-bet, AP-1, Eomes and Jun (13). IFNγ can induce apoptosis in tumour cells and stimulate the upregulation of MHC class-I expression, thus facilitating T cell recognition (13, 14). By IFNγ secretion, NK cells promote both maturation of DCs and priming of $CD4^{+}$ T helper type 1 cells (6, 15). The functional IFNγ receptor comprises two IFNGR1 ligand-binding α-units and two signal-transducing IFNGR2 β-units. Upon binding of IFNγ to the receptor, the associated adaptor molecules JAK1 and JAK2 are activated leading to the phosphorylation of the transcription factor STAT1. Binding of STAT1 to interferon-γ activation sites facilitates transcription of genes related to antigen presentation, transcription and proliferation (13).

THE "MISSING SELF" HYPOTHESIS

Initially, it was thought that recognition by NK cells was independent on the MHC class expression of the target cell. However, Kärre *et al.* demonstrated that MHC class I molecules ("self") actively inhibit NK cells (16). The "missing self" hypothesis was thus proposed, suggesting that NK cells eliminate cells with downregulated MHC class I – a phenomenon frequently observed in virus-

infected or malignantly transformed cells (17). However, this hypothesis did not explain why certain cells lacking MHC class I, for example human erythrocytes, remained unharmed by circulating NK cells (7). The understanding of the details of NK cell recognition expanded with the identification of activating and inhibitory receptors expressed by NK cells, proving an explanation for how NK cells recognize target cells. Consequently, the engagement of these receptors with specific ligands expressed by the target cells creates a complex integration of signals, determining the cytolytic activity of NK cells (18).

NK CELL GENES

The inhibitory and activating receptors expressed by NK cell belong to two major receptor families: the immunoglobulin superfamily and the C-type lectin superfamily. These receptor genes are typically clustered together with the other family members on two main genomic locations. The genes of the immunoglobulin superfamily are situated in the leukocyte receptor complex (LRC) on chromosome 19 and encompass genes for NKp46 and Killer cell immunoglobulin-like (KIR) receptors. Conversely, a ~2Mb region on chromosome 12, known as the natural killer complex (NKC), harbours the c-type lectin genes such as *CD94* and the NKG2 family (19). Numerous allelic variations, marked by single base substitutions, have been identified in genes within the NKC region, with a frequency exceeding 10%. Certain of these single nucleotide polymorphisms (SNPs) in the NKG2D and NKG2A genes have been associated with natural cytotoxicity activity and with cancer incidence. These SNPs are in linkage disequilibrium, suggesting a high likelihood of being inherited together (20).

INHIBITORY RECEPTORS

To ensure tolerance to autologous healthy cells, NK cells are equipped with inhibitory receptors (depicted in figure 2). Two major families recognize classical and non-classical MHC class I molecules. KIRs recognize HLA-A, HLA-B HLA-C, while the heterodimer NKG2A/CD94 (referred to as NKG2A) engage with non-classical HLA-E (7, 21).

Peripheral blood NK cells constitute a heterogenous population with varying expression of several markers, including NKG2A and KIRs. The receptor expression varies over the course of NK cell development. NKG2A defines more immature cells and as $CD56^{dim}$ NK cells mature, NKG2A is downregulated. However, cytokine stimulation can still induce NKG2A expression. Conversely, KIRs and CD57 are lowly expressed by immature NK cells and are acquired during maturation of $CD56^{dim}$ cells (22, 23).

Killer-cell immunoglobulin-like receptors

The human KIR gene family comprises 15 genes, yet the number of KIR genes present in any individual varies on group level, with only a limited set commonly shared. These highly polymorphic genes encode type I transmembrane glycoproteins characterized by two or three extracellular Ig-like domains, denoted as KIR2D or KIR3D respectively. The cytoplasmic tails of these receptors occur in varying lengths, designated long (L) or short (S). Long tails harbour one or two immunoreceptor tyrosine-based inhibitory motifs (ITIMs), facilitating inhibitory signalling. Conversely, receptors with short tails associate with the adaptor protein DAP12 that enables activating signalling (7).

These receptors engage with both the α_1 and α_2-helices of the HLA molecule as well as the bound peptide, providing a possibility of peptide-dependent modulations of the interaction (24). The KIRs recognize three main epitopes, distinguished by polymorphisms in the α_1 helix. Epitopes C1 and C2 of HLA-C are defined by two different amino acids at position 80, while residues 77-83

define the Bw4 motif in HLA-A and -B molecules. Each person can possess one, two or three of these epitopes and variations in the KIR genes and the associated peptide enables further diversifications of the interactions (25).

NKG2A/CD94

The type II transmembrane heterodimeric NKG2A engages the non-classical HLA-E molecule (26, 27). It is encoded by the *KLRC1* gene on the NK gene complex (NKC) region of chromosome 12 (21). The cytoplasmic tail of NKG2A harbours two ITIMs, facilitating inhibitory signalling (7). HLA-E binds peptides derived from the leader sequences of HLA-ABC, establishing an additional layer of protection of normal cells as it monitors the levels of other HLA molecules (26, 27). A dimorphism in the HLA-B leader peptide has been identified which impacts the HLA-E expression. This is caused by the presence of either a methionine (M) or a threonine (T) at residue -21 where methionine alone provides a strong anchorage to the HLA-E molecule that enables proper folding and subsequent surface expression (25, 28). Despite all HLA-ABC leader peptides being presented on HLA-E, individuals carrying at least one -21M allele exhibit increased surface expression. In contrast, T/T individuals demonstrate stronger KIR-dependent inhibitory signalling (25).

Non-HLA-specific inhibitory receptors

There are additional receptors expressed by NK cells that limit their function. These are not restricted to binding HLA molecules.

A family of nectin and nectin-like-binding receptors has emerged as novel NK cell immune checkpoints. This group includes the inhibitory receptors **TIGIT**, **CD112R**/PVRIG, **CD96**/TACTILE and the activating receptor DNAM-1 (described under the *Activating receptors* section). These receptors interact with PVR/CD155 and Nectin-2/CD112 with varying affinity. The ligands are typically found in adhesion junctions, particularly among epithelial and neuronal cells and have been observed to be upregulated on cancer cells. TIGIT is expressed on 40-60% of human peripheral blood NK cells, with higher levels observed on $CD56^{dim}$

cells (29). The level of expression is suggested to correlate with maturation and serves as a marker for activation but has not been correlated with exhaustion (29-31). TIGIT exhibits the strongest affinity for PVR among the NK cell inhibitory receptors with a weaker affinity for nectin-2 (32). It initiates inhibitory signalling through its ITIM-containing cytoplasmic domain, like the other inhibitory receptors of this family. In contrast, CD112R demonstrates the strongest affinity Nectin-2, while CD96 interacts with PVR more strongly than DNAM-1 but less so than TIGIT (32). Ongoing research aims to elucidate their function and intricate relationship.

The T cell immunoglobulin and mucin domain 3 (**TIM-3**) is an additional co-inhibitory receptor. TIM-3 is encoded by the gene *HAVCR2* and is a type I transmembrane protein, belonging to the TIM family of which TIM-1 and TIM-4 also are expressed in humans (33, 34). TIM-3 was first described to be expressed by IFNγ-secreting T helper 1 cells and has later also been identified to be present on $CD8^{+}$ T cells, dendritic cells, monocytes as well as NK cells, with the latter demonstrating the highest transcription levels in PBMC (35-41). It is constitutively expressed by virtually all $CD56^{dim}$ NK cells and heterogeneously by $CD56^{bright}$ NK cells. Cytokine stimulation can upregulate the expression on both subsets (42). Several ligands are reported for TIM-3, including galactin-9, phosphatidylserine, high motility group protein B1 (HMGB1) and carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1) (43-47). TIM-3 is often considered a marker for T cell exhaustion (39, 48). For NK cells however, the role of TIM-3 is less defined and needs further exploration. Ndhlovu *et al.* suggested that TIM-3 is an activation and/or maturation marker for NK cells as its expression was induced on $CD56^{bright}$ cells upon cytokine stimulation (42). However, Da Silva *et al.* studied TIM-3 in metastatic melanoma where TIM-3 defined a population of NK cells with a dysfunctional phenotype (49). Additionally, the functional consequence of TIM-3 stimulation also remains incompletely understood as TIM-3 has not only demonstrated induction of inhibitory signalling but also enhancement of IFNγ production (50). This

ambiguity is further augmented by its lack of canonical inhibitory signalling motifs in the cytoplasmic domain (51). Thus, the function of TIM-3 appears context-dependent, both regarding the disease setting and the nature of the presented ligand.

IRp60/CD300A is an inhibitory receptor that, like TIM-3, recognizes phosphatidylserine as well as phosphatidylethanolamine and has been demonstrated to strongly inhibit NK cell cytotoxicity (52-54). Its expression extends across various cell types, including NK cells. Though higher level of expression can be seen in $CD56^{bright}$ NK cells, it is prevalent in most blood NK cells (53). In humans, it belongs to an eight-member family with both activating and inhibitory characteristics (55).

Inhibitory signalling

Inhibitory signalling in NK cells rapidly occurs upon engagement of the inhibitory receptors and is necessary to block potential activating signals at an early stage. As reviewed in detail later, the receptors aggregate at an inhibitory synapse after which the tyrosine residue within their intracellular ITIM motif undergoes phosphorylation by Src family kinases. The tyrosine phosphatases SHP-1 and SHP-2 are subsequently recruited to the ITIM through their SH2 domains, resulting in the blockade of activating pathways by dephosphorylation of critical proteins such as Vav1 (18, 56, 57).

Education

Results chieved in β_2-microglobulin-deficient mice initially challenged the "missing self" theory as the autologous MHC class I-deficient cells remained unharmed by circulating NK cells (58, 59). Since also healthy nucleated cells may express ligands for activating receptors, a secondary control system is required to prevent autoreactivity. In the normal setting, all healthy cells express MHC class I, preventing elimination through interaction with its cognitive inhibitory receptor on the NK cell. However, not all NK cells express inhibitory receptors that recognize self-MHC class I. Since KIR genes and MHC class I genes are located

on different chromosomes and thus inherited independently, there is no safeguarding mechanism to ensure that all NK cells express a KIR that engages with a self-MHC class I. Indeed, approximately one tenth of all NK cells do not express inhibitory receptors for self-MHC class I (60, 61). To protect the host from NK cell-mediated harm, NK cells continuously undergo a process known as education or licensing. It is a process that fine-tunes NK cell activity with regards to its current environmental inhibitory input. Throughout their developmental journey, inhibitory receptors play a pivotal role in educating NK cells and those NK cells that do not express at least one receptor that recognize self-MHC class I molecules become hyporesponsive (62). NK cells adapt to their current MHC environments, as illustrated by the restoration of functionality in hyporesponsive NK cells transferred from MHC class I-deficient mice to a wild-type (WT) setting (63). The education process could be visualized as a bow that needs to be tightened with negative strain to generate full force upon release. The interaction between KIRs and NKG2A and their respective ligands complements each other, as individuals with a weak HLA-allele for KIR often harbour an allele supporting strong NKG2A-HLA-E interactions and vice versa (25). Moreover, $TIGIT^{+}$ mouse NK cells exhibit heightened responsiveness towards PVR^{neg} target cells compared with $TIGIT^{-}$ cells, suggesting the potential involvement of TIGIT in the education of mouse NK cell (30). Inhibitory receptors serve as indispensable components for maintaining the functionality of NK cells, enabling them to respond effectively to target cells with reduced MHC class I levels.

Several models have been proposed to describe NK cell education. According to the *arming model*, functional maturation of NK cells requires inhibitory input obtained from the interaction with self-MHC class I. Cells that lack this signal hence remain immature and hyporesponsive (60, 64). Conversely, the *disarming model* portrays NK cells in a constantly reactive state, leading to loss of responsiveness due to continuous stimulation in the absence of inhibitory signals from self-MHC class I (60, 65). The *rheostat model* provides an analogous perspective on the education process, contrasting with the more binary "on-off"

views. It suggests that all the inhibitory signals of varying strength are summed up and collectively modulate the responsiveness of the cell (66). These models do not represent completely separated views and could, when combined, provide a comprehensive understanding of NK cell education.

The education process results in highly functional NK cells, but there are a few phenotypic characteristics that discriminate educated and uneducated NK cells. One study has demonstrated that self-KIR-expressing educated NK cells hold larger Granzyme B granule levels. This phenomenon may be attributed to the constant inhibitory input that allows granule stores to accumulate, while uneducated cells constantly loose granules due to the continuous stimulation (67). It has also been reported that unlicensed cells, despite forming fewer conjugates with target cells, exhibit normal lytic granule polarization upon conjugation. The hyporesponsiveness was instead partially attributed to diminished signalling from activating receptors to adhesion receptor LFA-1 (68, 69), which aligns with the study that demonstrated that educated cells exhibited more active LFA-1 after target cell stimulation. A stronger control of LFA-1 conformation was also demonstrated in educated cells upon cytokine stimulation. Additionally, the expression of the coactivating receptor DNAM-1 has been associated with NK cell education, believed to confer heightened effector functions to educated NK cells. This illustrates that the activity of NK cells is regulated at multiple levels to ensure minimal harm to self, given that the potentially reactive, educated cells, possess a stronger control system (70-72).

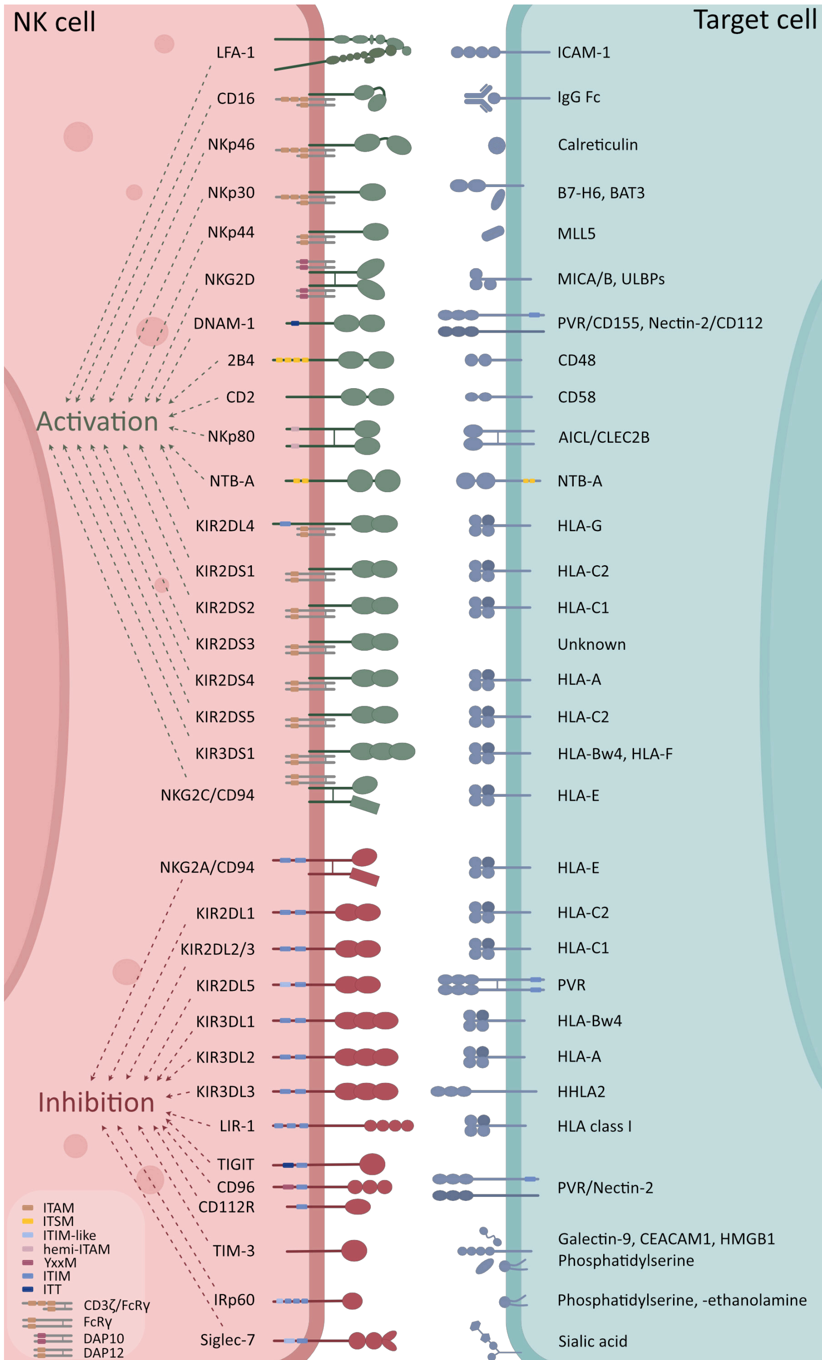


Figure 2. NK cell receptors and their corresponding ligands. Activating and inhibitory receptors expressed by NK cells and the ligands they interact with on a target cell.

ACTIVATING RECEPTORS

In addition to inhibitory receptors, NK cells also express a large repertoire of activating receptors that initiate activating signals upon engagement, leading to degranulation of cytotoxic granules, culminating in NK cell-mediated target cell killing. These receptors engage with ligands expressed by potential target cells, which are typically upregulated on virus-infected and transformed cells but may also be expressed in healthy tissues. Receptors and corresponding ligands are illustrated in figure 2.

The natural cytotoxicity receptors (NCRs) are regarded as pivotal activating receptors on NK cells and include **NKp46** (NCR1/CD335), **NKp44** (NCR2/CD336) and **NKp30** (NCR3/CD337) (73-75). Using blocking antibodies, the NCRs have been demonstrated to be important mediators of NK cell cytotoxicity against many cancer cell lines. Their surface density has been correlated to the NK cell ability to kill target cells and the critical function of NCRs in cancer immunosurveillance has been demonstrated in several studies (**paper II**; (73-83)). The NCRs have also been implicated in defence against microbial pathogens including viruses (84-86).

NKp46 is highly conserved across species and is the only known NCR expressed in mice. NKp46 and NKp30 are found on both resting and activated NK cells, are upregulated on stimulated cells but downregulated on adaptive NKG2C^{+} NK cells. NKp44 expression is absent on resting NK cells but become expressed in response to cytokine stimulation (87, 88). NKp46 is expressed on all mature NK cells, ILC1, a subset of ILC3 and γ/δ T cells (76, 89).

Several ligands have been identified for the NCRs. Viral molecules, such as influenza-derived haemagglutinin, have been shown to interact with NKp46 and NKp44 (90, 91). All three NCRs bind different epitopes of the glycosaminoglycan heparan sulphate and it has been speculated that, although found on normal cells surfaces, these interactions function as a sensor for environmental changes due

to malignant transformation (92, 93). Intracellular proteins have also been found to interact with NCRs through surface expression caused by stress or tumour transformation. Nuclear BAT3/BAG6 has been reported to stimulate NK cell cytokine secretion through NKp30 by translocating to the membrane upon heat shock (94, 95). Similarly, both MLL5 and PCNA are nuclear proteins that were shown to translocate to the cell surface to function as a ligand for NKp44 (96, 97). The type I transmembrane protein B7-H6 is another ligand for NKp30 (98). It is not expressed on healthy cells but has been found upregulated on a large variety of cancer cells, a process aided by the proto-oncogene Myc (**paper I;** (98, 99)). Soluble forms of the ligand have been found in several cancer types and has been shown to force downregulation of NKp30 (100-103). Nidogen-1 is another soluble ligand reported to bind NKp44 (104). The identity of cellular ligands for NKp46 have remained largely elusive, despite extensive efforts. The participation of the intracellular cytoskeletal protein vimentin in NKp46-dependent killing of *Mycobacterium tuberculosis*-infected monocytes has been proposed, although it could not be established if it acted as a primary ligand to NKp46 (105). Additionally, the complement factor P/properdin has been shown to bind NKp46 although it was shown to induce a different NK cell response than the canonical pathway of activation (106). Likewise, CD4 and Siglec-6-8 were proposed to be binding partners to NKp46 in a large screening effort but their capacity to induce NK cell activation remains uncertain (107). Recently, ER-stress-induced externalized calreticulin was suggested as an NKp46 ligand (108). It remains to be determined whether these identified ligands account for the numerous unidentified NKp46Ls (109-114).

The structure of the NCRs comprises an extracellular portion of two C2-type Ig-like extracellular domains (NKp46) or a single IgV-like domain (NKp44 and NKp30), linked with a stalk region, followed by a transmembrane region and a short intracellular domain (75, 76, 87, 115, 116).

NKp46 has been shown to bind ligands through its membrane proximal domain (D2), which features several glycosylation sites of which one sugar-carrying residue, Thr225, plays a crucial role in the interaction of NKp46 with both viral and tumour ligands (86, 117, 118). Like NKp46, attachment of sialic acids to glycosylation residues on NKp44 has been shown to be important for recognition of viral ligands (91). For NKp30, the stalk region has been identified as crucial for ligand binding (119). Both NKp30 and NKp46 were shown to form homo-dimers or -oligomers which can provide a stronger ligand-binding affinity and contribute to target cell lysis (120, 121).

NKG2D is a major activating NK cell receptor of relevance for elimination of malignant cells (122). It is a type II transmembrane protein encoded by the *KLRK1* gene within the NKC on chromosome 6 and can be found on all human circulating NK cells as well on $CD8^+$ and γ/δ T cells. NKG2D differs from other NKG2 family members in that it is expressed as a disulfide-linked homodimer and does not associate with CD94 molecules (123, 124).

The ligands for NKG2D are stress-induced molecules of cellular origin that are upregulated on infected, stressed or transformed cells. These ligands are structurally related to MHC class I-molecules although they do not associate with β_2-microglobulin or bind peptides. MHC class-related sequence (MIC) A/B and UL16-binding protein (ULBP) 4/5 are type I transmembrane proteins, while ULBP1-3/6 are membrane-bound through a glycolsylphosphatidylinositol (GPI)-anchor, though ULBP2 and ULBP5 exist in both transmembrane and GPI-anchored versions (123, 124). Their genes are highly polymorphic which expands the variety of ligands even further (125). There is likely functional redundancy among the NKG2D ligands as there are individuals harbouring a null allele for MICA and/or MICB without any apparent phenotypic consequences (126). Yet, presence of diverse NKG2D ligands and the development of multiple evasion mechanisms by viruses and cancers, aimed at circumventing NKG2D-dependent recognition, underscores the importance of the NKG2D axis. Virtually all cells

can express one or more ligands and solid tumours commonly express two or more NKG2D ligands, which bind NKG2D with varying affinities (124, 127-129). However, it remains incompletely understood which NKG2D ligands are upregulated on specific cancers or by certain pathogens.

Additional receptors contributing to NK cell activation include **2B4**/CD244, **DNAM-1**/CD226 and **CD2**. Although regarded as co-receptors, incapable of independently inducing cytolytic functions in NK cells, they have demonstrated roles in NK cell cytotoxicity (53, 130-132). Findings from both our genome-wide CRISPR screen and functional assays involving blocking antibodies, as outlined in **papers I** and **IV**, underscore the involvement of CD2 for recognition of leukemic cells (**papers I**, **IV**). These receptors are believed to play a crucial role in target cell adhesion (130, 133, 134). 2B4, DNAM-1 and CD2 are transmembrane proteins with two extracellular domains, expressed on various immune cells, including NK cells. 2B4 binds the GPI-anchored CD48, which also serves as a ligand for the adhesion molecule CD2, albeit with lower affinity than its primary ligand CD58/LFA-3 (135, 136). The ligands to DNAM-1 include Nectin-2/CD112 and PVR/CD155, expressed by cancer cells but also to some extent by healthy epithelial and endothelial cells (137). As mentioned, these ligands interact with stronger affinities with other NK cell receptors, including the inhibitory receptors TIGIT and CD112R, creating a dynamic relationship between activating and inhibitory signalling with varying affinities (138).

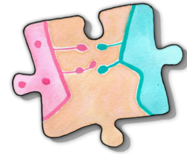
Redirected killing assays suggest a hierarchy among these receptors in co-activating NK cells, with 2B4 inducing the strongest activation, followed by DNAM-1 and CD2 as the weakest inducer (132). This hierarchy could be linked to the expression patterns of their corresponding ligands, given that LFA-3/CD58 exhibit broad expression, while CD48 expression is limited to hematopoietic cells and a specific subset of endothelial cells (132). Co-activation may thus be essential to limit NK cell alloreactivity.

CD16 is a low-affinity Fc receptor that enables NK cells to recognize IgG1 and IgG3 molecules for antibody-dependent cellular cytotoxicity (ADCC). This transmembrane protein comprises two Ig-like C2-type extracellular domains, a transmembrane domain and a short cytoplasmic tail. The density of CD16 is correlated with ADCC activity of NK cells (139). The engagement of CD16 contribute to NK cell cytotoxicity, cytokine and chemokine production as well as to IL-2-stimulated NK cell's function and proliferation (140-142).

Activating signalling

Activating receptors transmit signals intracellularly through tyrosine-based phosphorylation. The activating NK cell receptors with a short cytoplasmic tail, thus lacking signalling capacity, associate with adapter proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs). There are three ITAM-containing adapter proteins expressed in NK cells: FcεRIγ, CD3ζ and DAP12 (143-145). FcRγ and CD3ζ forms homodimers and heterodimers to associate with CD16 and NKp46 and NKp30, where a charged arginine residue in the transmembrane domain of the latter two enables the interaction (144, 146, 147). Instead, a lysine residue in the transmembrane domain of NKp44 allows association with the homodimeric DAP12 (18, 148). Upon signalling, the two tyrosines within the ITAM motif undergo phosphorylation by members of the Src-kinase family, facilitating binding of the SH2 domain of the tyrosine kinases Syk and ZAP70. This results in activation of PI3K, PLC-γ and Vav 2/3. This process leads to Ca^{2+} influx, degranulation and transcription of chemokine and cytokine genes. NKG2D associates with the adapter molecule DAP10, which contains a tyrosine-based-motif that differs from the ITAM. Upon phosphorylation, it binds either to the p85 subunit of PI3K or the adaptor Grb2 and Vav1, both resulting in activation of Vav1 (7, 18, 149).

SYNAPSE FORMATION



Section in brief:

One of the main functions of NK cells includes elimination of malignant cells. As NK cells encounter potential target cells, the integration of signals received from inhibitory as well as activating determines whether to proceed with lysis of the encountered cell (18). Upon the interaction, the NK cell initiates the formation of an immunological synapse (IS), which is an intricately coordinated process that involves distinct steps, described in detail below.

Following the encounter of a potential target cell by the NK cell, the formation of the immune synapse (IS) unfolds in a highly organized, sequential process with specific stages and checkpoints, depicted in figure 3 (131, 150, 151). The rigidity of the target cell membrane has been shown to influence the ability of IS formation and the lipid composition impacts the cell interactions (**paper I;** (152)). The major purpose of the IS is to establish close proximity to the target cell to facilitate efficient cytotoxicity for the NK cell. Additionally, the formation of a tight synaptic cleft, typically 10-30 nm deep, serves to protect neighbouring cells from potential damage (153).

As lytic granules are preformed in resting NK cells, unlike resting cytotoxic T cells (CTLs), the events leading up to degranulation needs precise regulation. The IS formation, culminating in target cell killing, is conceptually divided into three stages: recognition, effector and termination stage (69). The first step of the recognition stage involves the first contact between the cells by "tethering" receptors (154). Firmer adhesion is enabled through interactions between integrins, such as MAC-1 and LFA-1 and its ligand ICAM-1, providing an initial activation signal via phosphorylation of Vav1, which is essential for downstream

cytoskeletal reorganization and lipid raft clustering (150, 155-158). As opposed to CTLs, resting NK cells have been found to express LFA-1 in its active conformation which could bring light to the more dynamic nature of NK cells' interaction with target cells (130, 151, 159). The activation of LFA-1 is further increased by a second activation signal through a receptor such as NKG2D, 2B4, DNAM-1 or CD2 (69, 130).

Inhibitory immunological synapse

The inhibitory IS is distinct from the lytic IS and represents a contact point between the two cell types where signalling functions to terminate the cytotoxic commitment (155, 160). Its formation occurs upon interaction between inhibitory NK cell receptors and their cognitive ligands on the target cell (130, 161, 162). The inhibitory receptors have been demonstrated to cluster at the inhibitory IS, without the need for actin reorganization (163-165). Vav1, being the substrate of SHP-1, undergoes dephosphorylation, thereby blocking further activating signalling due to the critical role of Vav1 in actin reorganization (155, 166). The recruitment of phosphatases rapidly leads to inhibition of multiple additional points of activation, including conjugate formation, LFA-activation, IS clustering of activating receptors and lipid rafts and Ca^{2+} mobilization (130, 155, 157, 163, 165-169).

Actin reorganization

Without overruling inhibitory signals, the effector stage is initially characterized by actin reorganization, followed by clustering of activation receptors, lipid raft aggregation and lytic granule polarization (169, 170). Filamentous actin (F-actin) is formed from cellular globular actin, dependent on Wiskott-Aldrich syndrome protein (WASp), thus creating a radially symmetric stable cell-cell contact site with its typical cleft (151, 154, 161, 171).

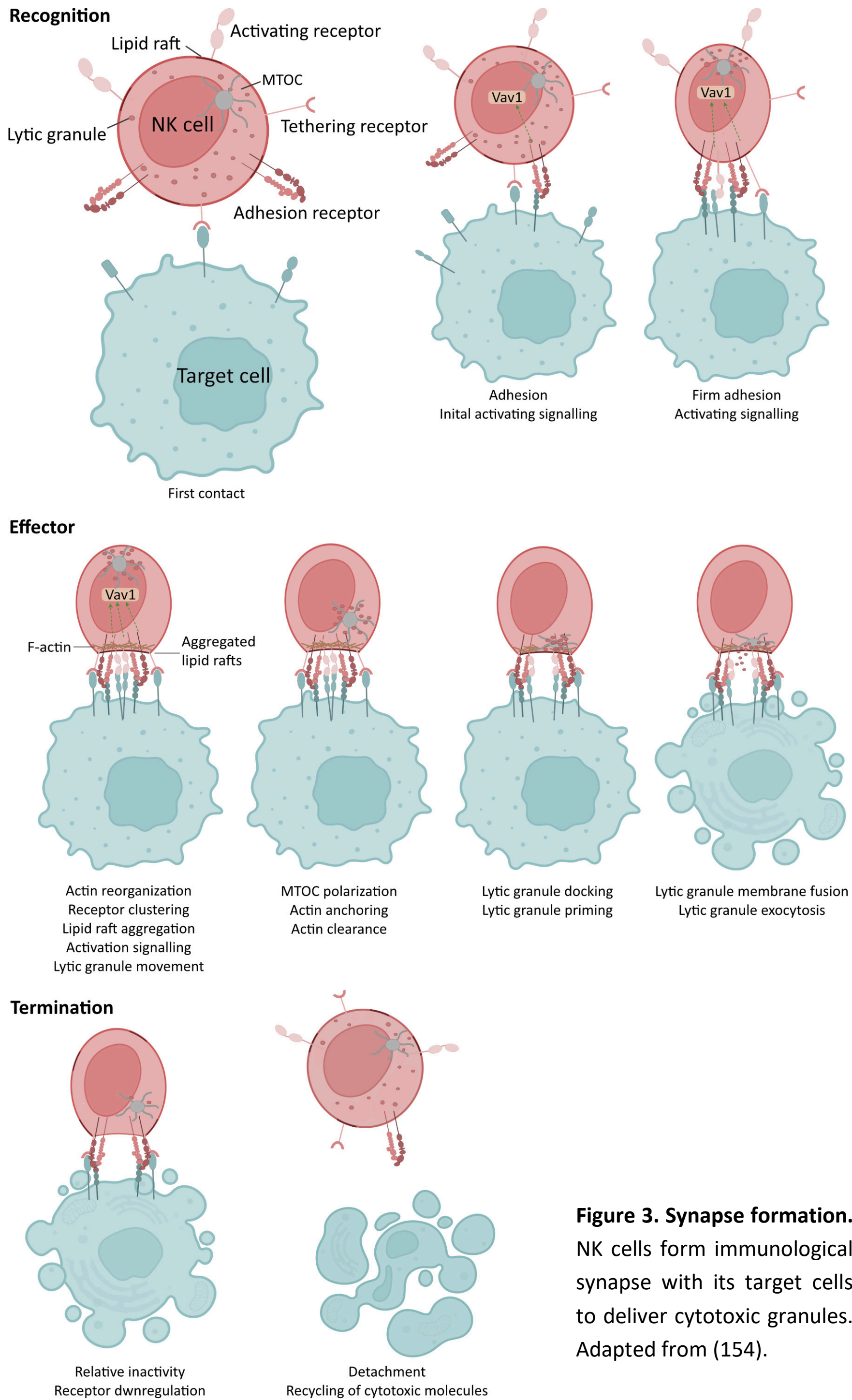


Figure 3. Synapse formation. NK cells form immunological synapse with its target cells to deliver cytotoxic granules. Adapted from (154).

Actin rearrangement has been shown to be crucial for both lipid raft polarization, cell-cell adhesion as well as activating signalling (155, 165). Indeed, polarization and clustering of CD2, LFA-1 and MAC-1 at the IS was shown to be restrained by inhibition of actin polymerization (150). Instead, NKp46 knockdown experiments revealed the importance of the receptor in actin rearrangement (172).

Receptor clustering

Receptor aggregation in the IS is important for generation of robust signalling in NK cells (173). Adhesion receptors such as LFA-1, MAC-1, 2B4 and CD2 have been found to cluster in the peripheral supramolecular activation cluster (SMAC), contributing to the ring shape of the IS together with F-actin (150, 174). Also the major activating receptors NKG2D, NKp46 and NKp30 have been found to cluster in the IS (163, 172, 174, 175). The nature of ligands that are presented on the target cell also influence aggregation as ULBP1 was shown to induce larger NKG2D receptor nanoclusters compared to MICA (176). The spatial arrangement of receptors may also be influenced by the lipid microenvironment. Lipid rafts have been observed in the periphery of the T cell synapse, potentially forming signalling platforms for activating receptors (169, 177). Following activating receptor signalling, PI3K-ERK2 and PLCγ-JNK pathways facilitate mobilization of ions, in particular Ca^{2+}, which is needed for granule exocytosis (178-180). PLCγ-generated IP3 is involved in release of Ca^{2+} from intracellular stores in the endoplasmic reticulum and from extracellular environment by activating calcium channels (181, 182).

Granule polarization and exocytosis

Cytotoxic granules appear highly heterogenous and can contain granzymes, perforin, granulysin, death ligands and small anti-microbial peptides (182-184). Granzymes are serine proteases and the five variants expressed in humans (Granzyme A, B, H, K and M) exhibit different cleaving affinities (182, 184). Granzyme A and Granzyme K are thus defined as trypsins; Granzyme B aspase; Granzyme H chymase and Granzyme M metase. They are all synthesized as pro-

enzymes and are cleaved to their active form by cathepsin C or H in the granules (182). Perforin is a pore-forming protein, essential for the lytic function of cytotoxic lymphocytes, to deliver granzymes to the target cell (185). It is expressed at lower levels in CD56^{bright} NK cells compared to CD56^{dim}. An acidic environment in combination with the proteoglycan serglycin that form complexes with granzymes keep the lytic molecules inactive and prevent damage to the NK cell (186-189).

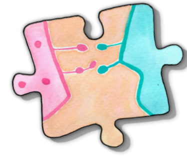
The preformed lytic granules move along microtubules to the microtubule organizing centre (MTOC) (190, 191). The MTOC is simultaneously polarized towards the synapse. Actin rearrangement as well as ERK phosphorylation, Vav1 activation and Pyk2 activity are required for MTOC polarization (192-194). Following vesicle priming, the granules fuse with the plasma membrane to be released into the cleft between the two interacting cells. Two modes of fusion are known to occur: complete and incomplete fusion. The complete fusion involves complete granule content discharge into the cleft whereas during incomplete fusion, only a portion of the content is released through a transient membrane pore (195). This practice, allowing for rapid recycling of granule content is suggested to be implicated in serial-killing, which is further explained in *Killing dynamics* (174, 196).

Lytic granule delivery and detachment

The termination stage describes the period when the NK cell remains relatively inactive and the IS is preserved in order to protect surrounding cells while maintaining a high concentration of cytotoxic effector molecules to ensure target cell death (154). NK cells have been reported to safeguard themselves from lytic molecules through the externalization of LAMP-1/CD107a and the cytosolic expression of serine protease inhibitor B9 (197-199). Studies also suggest that the tightly packed lipids, found in the membrane of the granules itself and exposed on the plasma membrane after exocytosis, reduce the perforin-binding capacity (200, 201). After interaction, activating receptors are internalized or shredded to

enable detachment from the target cell (202, 203). The interaction of inhibitory receptors with apoptosis-induced PtdSer on the target cell may enable termination signalling (**paper I**). Establishment, maintenance as well as dissociation of the IS are highly regulated processes (156). Detachment of the NK cell from the target cell is regulated by several factors and is a determining factor of the serial-killing capacity of NK cells (202, 204). Resistant target cells or incomplete killing prolonged the detachment process that was induced by target cell loss of surface proteins and reduction in activating signalling in NK cells (69, 204-206).

TARGET CELL DEATH



Section in brief:

NK cells employ two primary pathways to induce apoptosis in target cells. The first involves the use of granzymes that are stored in lytic granules, while the second relies on ligation of death receptors. These pathways exhibit distinct dynamics, determining the circumstances under which NK cells favour one method over the other (206).

PERFORIN/GRANZYME PATHWAY

After lytic granule exocytosis from the NK cell into the synaptic cleft, perforin has the ability to insert itself into the target cell membrane. Pores in the membrane can be generated by perforin oligomerization, in a calcium- and pH-dependent process. Depending on local concentration of perforin and lipid composition, both smaller, transient pores and larger, stable, ring-shaped pores form (182, 187). Two mechanisms for the entry of granzymes into the target cell cytoplasm have been proposed. The first suggests that granzymes enter through plasma membrane pores formed by perforin. Alternatively, a perforin-serglycin-granzyme complex is endocytosed by the target cell, potentially using the mannose 6-phosphate receptor, and perforin instead disrupts the endosomal membrane, allowing granzyme to diffuse into the cytoplasm, where it induces apoptosis via caspase-dependent or independent mechanisms. Several studies favour of both mechanisms (185, 207-209).

Granzymes initiates several pathways (depicted in figure 4), all ultimately leading to cellular apoptosis, which can be described as ATP-dependent programmed caspase-mediated cell death. It occurs normally during development, cell

population maintenance and in response to cell damage, induced by cytotoxic immune cells. It induces minimal inflammation and damage to surrounding tissue (210). The caspase-dependent initiation of apoptosis involves direct granzyme cleavage and activation of caspases, such as granzyme B activation of caspase-3 and -8 (182, 211). Granzymes can further induce apoptosis through other mechanisms such as granzyme B- or granzyme K-mediated cleavage of the pro-apoptotic protein Bid, a member of the Bcl-2 family that control and regulate the apoptotic mitochondrial processes (182, 212). Cleavage of Bid lead to membrane permeabilization, causing release of mitochondrial proteins, like cytochrome c and smac into the cytosol (213, 214). Upon binding to cytochrome c, the procaspase-activating adaptor protein Apaf1 polymerizes, forming a wheel-shaped heptamer called apoptosome together with the initiator procaspase-9 (215, 216). This clustering activates caspase-9, which, in turn activates downstream executioner procaspases, including caspases-3/6 and -7 (217). Additionally, granzymes exert other functions through which they enhance apoptosis. Cleavage of DNA-associated proteins by granzyme A/B/K/M causes DNA damage and reduces DNA repair capacity, further amplifying the pro-apoptotic impact (218-220). Activated execution caspases degrade nuclear and cytoskeletal proteins and material by activating cytoplasmic endonucleases and proteases (221). Cytoskeletal reorganisation, dependent on caspase-3, leads to membrane blebbing, eventually resulting in formation of apoptotic bodies. The membrane also undergoes additional transformations during apoptosis (215). High Ca^{2+} concentration inhibits the activity of the P4-ATPase flippase and caspase-3 cleavage of the α-unit further inhibits its function (222, 223). Along with irreversible activation of the Xkr8 scramblase, this leads to exposure of PtdSer on the outside of the apoptotic cells (224). Phagocytes recognize PtdSer as an "eat-me" signal to ensure clearing of dead cells before their membranes are compromised (225). This process is termed efferocytosis and although non-professional phagocytes, like epithelial cells, can clear dead cells, most apoptotic cells are cleared by macrophages (226-228).

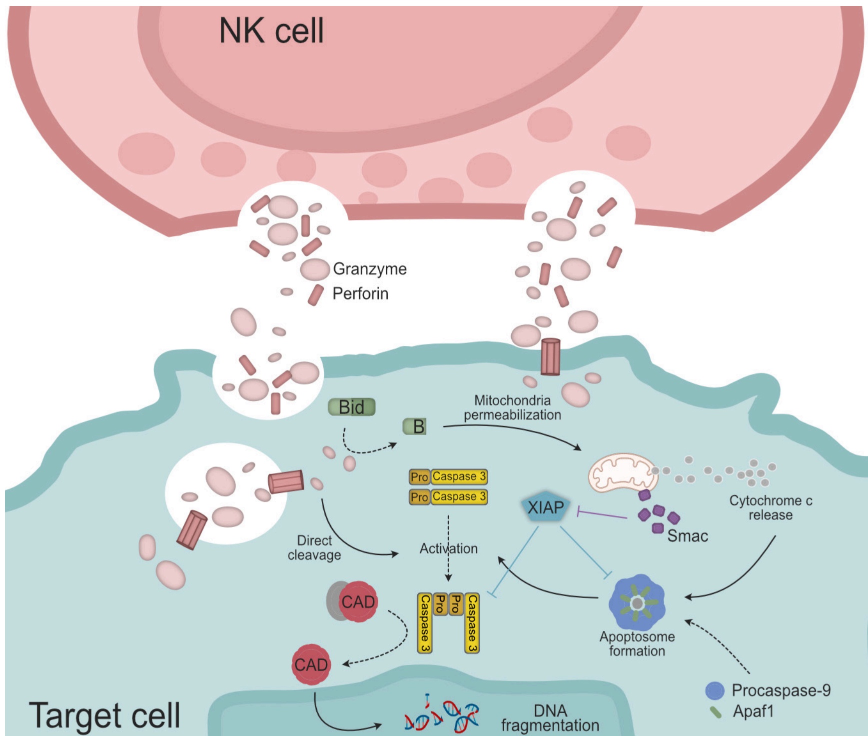


Figure 4. Perforin/granzyme killing pathway. NK cells can kill their target cells by secretion of their cytolytic granule content containing perforin and granzymes.

DEATH-RECEPTOR MEDIATED PATHWAY

In addition to utilizing their activating receptors to trigger degranulation, NK cells possess an alternative mechanism for targeting and eliminating cells – employing their repertoire of death ligands. These ligands engage death receptors expressed on the surface of target cells. This arsenal includes the TNF family of ligands Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). The binding of these ligands to their respective receptor on a target cell initiates the extrinsic apoptotic pathway, culminating in cellular apoptosis (184, 206). Expressed as homotrimeric type II transmembrane proteins, FasL and TRAIL contain a conserved C-terminal domain known as TNF homology domain that facilitates

self-association and interaction with their cognative receptors (229). FasL has been shown to associate with lipid rafts, which is reportedly crucial for its activity (230, 231). While one study suggested TRAIL association with lipid rafts in K562 cells, additional confirmation is required (232). FasL and TRAIL can also be cleaved from the cell surface by endopeptidases to form soluble versions (206, 233-235). Unlike FasL, soluble TRAIL has been shown to retain its activity albeit less potent than its membrane-bound form (206, 233, 236).

Both FasL and TRAIL are expressed on NK cells, with very low levels of TRAIL on resting NK cells, and both are induced upon cytokine stimulation (**paper IV**; (237, 238)). Additionally, TRAIL and FasL are present in intracellular lytic granules that are secreted upon activation with distinct kinetics (182, 183, 239, 240). These ligands are proposed to play a crucial role in NK cell-mediated control of cancer and virus infections as well as immunological self-tolerance (236-238, 241-243).

The Fas/CD95 receptor binds FasL while five receptors capable of binding TRAIL have been identified in humans: TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/Decoy receptor 1, TRAIL-R4/Decoy receptor 2 and the soluble receptor osteoprotegerin (OPG) (184). TRAIL-R2 and two decoy receptors have been identified in mice (184). The human membrane-bound receptors are expressed by the genes *TNFRSF10A-D* (238). The structural details of the TRAIL-receptors are depicted in figure 5. TRAIL-R1, R2 and R4 are all transmembrane proteins whereas TRAIL-R3 is anchored to the membrane by glycosylphosphatidylinositol. Notably, TRAIL-R1 and TRAIL-R2 are the only receptors equipped with intracellular signalling moieties, known as death domains (DD), which are capable of inducing apoptosis. Conversely, the lack of signalling capabilities in TRAIL-R3, TRAIL-R4 classifies them as anti-apoptotic decoy receptors. The trimeric TRAIL and FasL binds three corresponding receptors. TRAIL-R1 and -R2 form homotrimeric or heterotrimeric clusters, creating a receptor-ligand complex with the Zn^{2+} ion-stabilized TRAIL trimer at the centre

(240, 244-246). Inclusion of TRAIL-R4 in the heterotrimer complex disrupts signalling due to its intracellular truncated death domain (247, 248). Studies suggest further clustering into larger organized networks through dimerization of receptor trimers, facilitating full activation of intracellular signalling (246, 249). Supramolecular cluster formation is proposed to rely on the stability provided by the stalk domain of membrane-bound TRAIL, potentially explaining the lower potency of soluble TRAIL (250). Despite TRAIL-R2 having a stronger affinity for TRAIL at physiological temperatures compared with the other membrane-bound receptors, TRAIL-R1 is preferentially used for apoptosis-induction (245, 251-253). The distinct requirements for membrane-bound TRAIL between TRAIL-R1 and -R2, where the former does not seem to require it, may be attributed to the need for TRAIL-R1 localization to lipid rafts for functionality, unlike TRAIL-R2 (240). In addition to inducing apoptosis by binding Fas-associated death domain protein (FADD), TRAIL-R1, -R2 and -R4 has been demonstrated to induce gene transcription by activating NF-kB signalling through binding to TRADD (244, 254, 255).

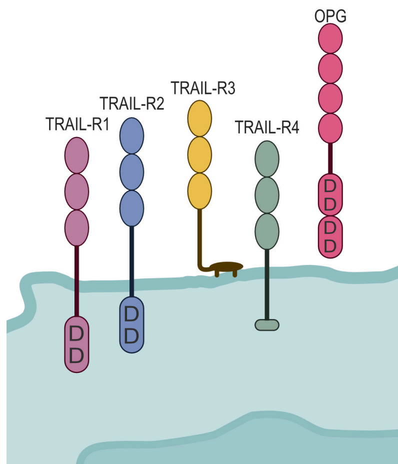


Figure 5. Receptor structure. The TRAIL-receptors TRAIL-R1-4 and Osteoprotegerin (OPG) differ in their structure. TRAIL-R1 and TRAIL-R2 both have a functional intracellular death domain, while TRAIL-R4 has a truncated version. TRAIL-R3 in associated to the membrane via a GPI-anchorage and OPG is a soluble receptor.

Once the active TRAIL or Fas receptor trimer complex is assembled, the death-inducing signalling complex (DISC) is recruited, as illustrated in figure 6. The DISC comprises Fas-associated death domain (FADD), which binds to the receptors through interactions between their DDs, and procaspases-8 binding to FADD through death effector domains (DEDs). The role of caspase-10 is not fully established but only caspase-8 appears to be essential for apoptosis (184, 206, 211, 247, 256-258). The stoichiometric ratio of the DISC components remains incompletely understood but a study suggests multifold more caspase-8, compared to FADD, which allows DED-chain formation, a crucial step for caspase-8 activation through autoproteolytic cleavage (259). Once activated, caspase-8 can in turn cleave and activate the executioner procaspases, culminating in target cell apoptosis (260).

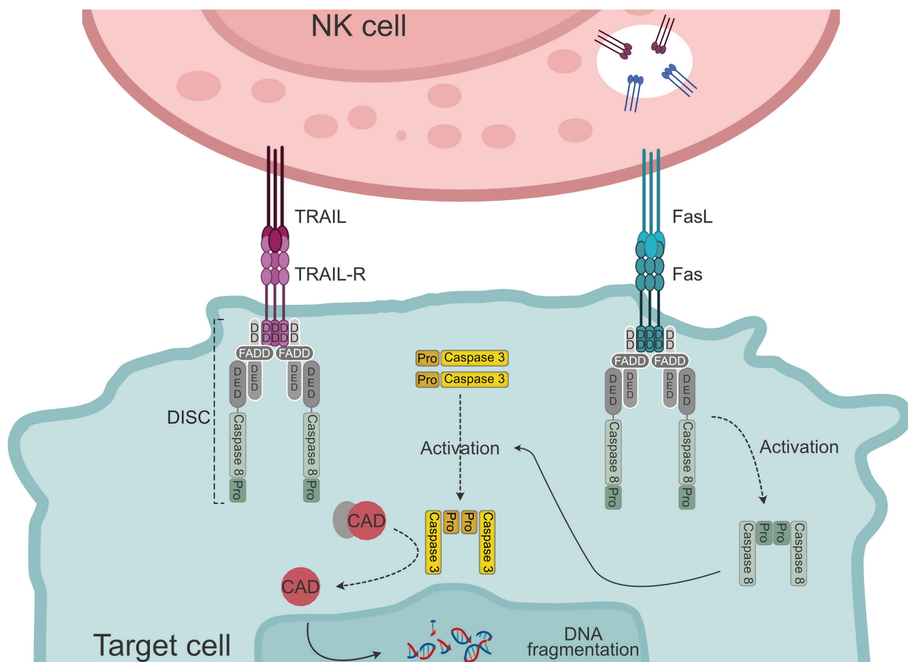


Figure 6. Death-receptor induced killing. NK cells can kill their target cells by engaging death receptors using their death ligands TRAIL and FasL.

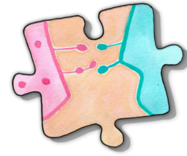
c-FLIP, encoded by the gene *CFLAR,* serves as a negative regulator of death receptor-induced apoptosis. Three isoforms are predominately expressed, among which the two short versions exhibit anti-apoptotic function and the long version act both pro- and anti-apoptotic, depending on expression levels. All isoforms contain DEDs, enabling them to bind to the DISC and outcompete procaspase-8, thereby inhibiting apoptosis (261). NK cells express two isoforms of c-FLIP to protect themselves from death receptor-induced apoptosis. Additionally, NK cells express TRAIL-R4 and upregulate TRAIL-R3 upon activation as a protective measure (262).

KILLING DYNAMICS

The two killing pathways, death receptor-induced killing and granzyme/perforin-mediated killing, exhibit substantial differences in kinetics. Death receptor-induced killing is demonstrated to occur after approximately 90 minutes, while cytotoxic granule-mediated killing takes place within a few minutes (263). Potential explanations for this disparity include differences in granule localization and secretion dynamics. Moreover, variation in threshold levels required for the cytotoxic inducers adds another layer of complexity. The granzyme/perforin pathway necessitates only two to four granules to be released for target cell elimination, contrasting with the death ligand pathway which may require a higher number of death ligands for effective apoptosis induction (206, 264). Differences in kinetics of intracellular signalling pathways are also noteworthy. Granzyme B, upon cytoplasmic entry, efficiently cleaves caspase-3, triggering the apoptosis cascade. Conversely, death ligands form larger clusters upon receptor engagement before the DISC assembly, leading to apoptosis. This potentially slower process has been demonstrated to be even less efficient in specific cells, resulting in delayed induction of apoptosis (206).

It has been demonstrated that NK cells use the two different methods with distinct dynamic timing. This is evident in studies of so-called serial-killing NK cells, which constitutes a small subset of NK cells that appears to account for the majority of target cell killing events. These cells exhibit the capability to eliminate as many as seven target cells within a 12-hour period (204, 265). For this phenomenon to occur, specific dynamics and regulated processes come into play. The detachment from one target cell has been identified as crucial to enable the formation of a new synapse (202, 204). During this process, activating receptors are downregulated or shed which can pose a limiting factor for further serial-killing (202, 203). Another limiting factor is granule depletion, as it has been demonstrated that NK cells use 10% of their total lytic granule content in a single killing event, despite that only two to four degranulation events are needed (264). Stimulation with IL-2 or IL-15 has been shown to assist in restoring granule content (265). A study by Prager *et al.* revealed that serial-killing NK cells initially employ the faster and more efficient granzyme/perforin-mediated killing, which is gradually replaced by the slower death ligand-mediated killing as the granule content is depleted (266).

NK CELLS IN CANCER



Section in brief:

NK cells are important players in the battle against cancer, with the most established role in acute myeloid leukemia. Thus, cancer cells develop various mechanisms to evade NK cell recognition. To combat the evasion strategies, immunotherapeutic interventions are under development, with the aim of boosting the immune system. These include stimulation by cytokines, the transfusion of immune cells, potentially enhanced by genetic engineering and antibodies for various purposes.

NK cells are considered to play a crucial role in surveillance and defence against cancer (4). While their pivotal role in haematological malignancies is established, their presence in solid tumours also associates with improved survival in various cancer types including colorectal, lung and gastrointestinal stromal cancer (78, 79, 81, 267-269). However, the prevalence of NK cells within tumours is notably lower than that of T cells and their functionality can be dampened by the immunosuppressive tumour microenvironment (TME) (270, 271). This often results in NK cells adopting an exhausted phenotype, characterized by reduced levels of perforin and granzyme B. NK cells have been suggested to be more efficient in preventing metastasis than controlling the solid tumour growth (270, 272). Consequently, interventions aiming to modulate the immune response by alleviating suppression and enhancing NK cell infiltration, persistence and cancer cell recognition hold promise in improving cancer outcomes. NK cells exhibit diverse function across cancer types, emphasizing the need for studies that explore the impact of different NK cell phenotypes and genotypes in specific settings (273, 274). Such investigations are necessary for a comprehensive understanding of NK cell dynamics in cancer and may pave the way for more targeted and effective therapeutic approaches tailored to specific cancers.

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is the most prevalent form of acute leukemia, affecting three to eight people per 100 000 with a median age of around 70 years (275). AML is characterized by uncontrolled proliferation of immature myeloid cells which have lost the ability of normal differentiation and controlled growth. Consequently, AML entails an accumulation of undifferentiated myeloid cells in bone marrow. These cells are incapable of generating mature blood cells, which additionally prevents the proper differentiation of normal cells (275, 276). The five-year overall survival exhibits large age-dependent variations, exceeding 50% for individuals below 50 years but dropping to around 10% for those above 70 years of age (277). AML is a heterogenous disease, marked by diverse karyotypic and mutational aberrations that impact prognosis (275). Leukemic cells devoid of chromosomal alterations, exhibiting a so-called normal karyotype, display recurrent mutations in *NPM1*, *FLT3*, *DNMT3A*, *IDH1/2* and *CEBPA* (278).

Standard treatment involves immediate induction therapy post-diagnosis with the aim to eliminate leukemic cells from the bone marrow and to restore normal haematopoiesis, through high doses of chemotherapeutic drugs. The specific regimen varies globally but typically include induction cycles with three days of anthracycline and seven days of cytarabine, whereas in Sweden cytarabine is administered for only five days (275, 276, 279). Older patients may instead receive venetoclax and azacytidine in the initial phase of therapy. The primary goal of this first phase is to induce complete remission (CR), defined as less than 5% blasts among nucleated bone marrow cells along with the return of normal haematopoiesis (280). Patients in CR subsequently undergo consolidation therapy, typically involving additional cycles of chemotherapy but can also include allogeneic stem cell transplantation for selected patients (276, 277). Targeted therapies for certain subtypes of AML have recently been incorporated into treatment recommendations (281). Despite achieving CR and undergoing consolidation therapy, many patients relapse with poor prospects of long-term survival. Maintenance therapy, administrated after the completion of

conventional induction and consolidation therapy, serves as a promising strategy to sustain prolonged remission, in particular for patients ineligible for allogeneic transplantation (276). Approved therapies include 5-azacitidine for older patients and histamine dihydrochloride/IL-2 for younger patients (282, 283). The rationale behind the latter is discussed in detail later.

NK CELL INTERACTION WITH CANCER CELLS AND TUMOUR ESCAPE

NK cells play a complementary role to T cells, both possessing the ability to recognize MHC class I but in diverging manners. Cancer cells expressing MHC class I-bound peptides face the risk of detection and elimination by T cells. To evade this recognition, cancer cells often downregulate the expression of the MHC complex at various levels involving transcriptional, epigenetic and posttranscriptional mechanisms (284). Given that MHC class I is recognized by NK cell inhibitory receptors, its absence instead triggers NK cell cytotoxicity, resulting in elimination of the cancer cell. During their transformation, cancer cells tend to upregulate activating ligands for NK cell receptors. This interaction has been proven critical as the expression of activating NCRs associates with improved outcome in several diseases (81, 285, 286).

Due to the higher expression of TRAIL-Rs on malignant cells, TRAIL has been proposed as a promising therapeutic target due to its potential selectivity (287). Evidence of the protective role for TRAIL in cancer development has been generated using TRAIL/TRAIL-R-deficient mice or by antibody blockade of the interaction. Depletion of the ligand leads to higher susceptibility to tumour formation as well as metastasis formation (238, 288). The role for TRAIL-R is less straightforward however, as studies have reported diverging results from TRAIL-R-deficient models (238). The anti-tumour effect seen by TRAIL could also be attributed to targeting of immunosuppressive cells within the tumour

microenvironment (238, 289). The role for NK cells in TRAIL-mediated cancer control has been demonstrated in several studies and gained further support from studies where the effect of the neutralizing TRAIL antibody was abolished by NK cell depletion (238, 243, 288).

Escape mechanisms

Cancer cells employ various strategies to avoid recognition and elimination by NK cells. Dysregulated expression of activating and inhibitory NK cell receptors is often demonstrated in cancer patients, leading to impaired NK cell function (78, 290, 291). Inhibitory receptors like PD-1 and TIGIT are frequently upregulated in cancer settings, potentially contributing to dysfunctional NK cells (292, 293). Conversely, lower levels of activating receptor are often observed in various cancer types, as a result of different manipulation mechanisms. The release of soluble forms of ligands such as B7-H6, galactin-3, BAT3 and NKG2DLs has been associated with the downregulation of NKp30 and NKG2D (100-103, 285, 294-296). Cancer cells employ various mechanisms to achieve this, including ligand secretion, matrix metalloprotease-assisted shedding or by ligand-containing exosome release (100, 297-300).

Tumour-infiltrating NK cells are commonly scarce and the NK cells found within the tumour are highly influenced by the immunosuppressive TME (271, 301). Factors produced within the tumour promote the accumulation of myeloid-derived suppressor cells (MDSCs), which exhibit an immunosuppressive phenotype, impairing T and NK cell function through mechanisms such as consumption of essential metabolites, expression of inhibitory surface molecules and the production of radical oxygen species (ROS) and nitrogen species, inducing NK cell dysfunction and apoptosis (302, 303). Cancer cells and MDSCs secrete TGF-β to promote tumour growth while inhibiting immunosurveillance by downregulating activating NK cell receptors and promoting immunosuppressive regulatory T cell differentiation (303-305). Additionally, the production of lactic acid creates an acidic TME which inhibits NK cytotoxicity

(306, 307). PtdSer is also commonly dysregulated in the TME, contributing to immunosuppressive environment (308, 309).

Cancer cells have developed various mechanisms to evade direct cytotoxicity from NK cells, including the secretion of cathepsin B to cleave and inactivate perforin, the production of SerpinB9 to inhibit the function of granzyme B and activation of autophagy to degrade granzyme B (206, 310-314). Additionally, to avoid death ligand-induced elimination, cancer cells have developed various resistance mechanisms. Mutated forms of TRAIL receptors and altered levels of decoy receptors have been identified (315-317). Furthermore, the expression of the apoptosis inhibitory protein c-FLIP is also a common mechanism for evasion of death ligand-mediated apoptosis (318).

NK CELL-BASED IMMUNOTHERAPY

Given their importance in cancer immunosurveillance, numerous NK cell-based immunotherapies are under development in order treat these diseases and to combat the different mechanisms cancer cells can employ to evade the immune system.

Blockade of inhibition

As KIRs and NKG2A have been described as "NK cell immune checkpoints", antibodies developed to block these receptors were anticipated to exert anti-tumour efficacy, similar to the PD-1 and CTLA-4 blockade for T cells. However, the results for KIR-targeting antibodies have been somewhat disappointing (319). Nevertheless, the anti-NKG2A antibody monalizumab has shown potential benefits in combination with other treatment options and is currently undergoing evaluation in clinical trials for a spectrum of haematological and solid malignancies (29, 320). Given that NKG2A can be expressed not only by NK cells but also by $CD8^+$ T cells, the latter represents a potential contributor of the

treatment efficacy (320). The divergence in efficacy between immune checkpoints associated with T cells and KIR/NKG2A on NK cells could be attributed to distinct dynamics. Contrary to immune checkpoints associated with T cells, such as PD-1 and CTLA-4, which are upregulated on exhausted T cells to limit the immune response, KIRs and NKG2A are expressed on resting NK cells. They play a crucial role in NK cell education, contributing to the mediation of self-tolerance. Disruption of these interactions may have more complex implications, as it involves interfering with the finely tuned mechanisms that govern the discrimination between self and missing-self by NK cells (29).

The role of PD-1 and CTLA-4 in NK cells remains largely unexplored but NK cells are suggested to contribute to the therapeutic success of anti-PD-1 and anti-CTLA-4. Although, PD-1 does not appear to be a marker for exhausted NK cells, evidence indicates that it inhibits NK cell function and in vivo studies have highlighted the role of NK cells for the therapeutic efficacy of PD-1/PD-L1 blockade (321).

In PVR-expressing tumours blockade of TIGIT has shown promising results by improving NK cell cytotoxicity and cytokine secretion, where the expression of DNAM-1 appears vital (29, 322). The combined blockade against TIGIT and CD112R further enhanced NK cell responses against breast cancer cells, supporting the suggested complementary inhibitory pathways mediated by these two receptors (29, 323).

Despite contradictory results regarding TIM-3's role as co-inhibitory or activating receptor, anti-TIM-3 blockade improved NK cell effector function, as demonstrated by studies by da Silva *et al.* and *Xu et al.*, in experiments performed with cells from melanoma and lung cancer patients (49, 324). Studies utilizing TIM-3 blockade, alone or in combination with PD-1 have shown promising effects (48). Clinical studies are currently conducted but some have shown disappointing results (325).

Dysregulated PtdSer creates an immunosuppressive environment, presenting an opportunity for therapeutic interventions through targeted approaches. The use of both PtdSer-binding ligands and antibodies has exhibited anti-tumour potential by enhancing the immune response. The PtdSer-binding protein AnnexinV has been shown to augment the immunogenicity, both by pre-incubation of mice-injected apoptotic cells and by *in vivo* administration (326, 327). PtdSer-specific antibodies have demonstrated the ability to generate anti-tumour responses and to promote the polarization of immune-suppressive cells towards a more immunostimulatory phenotype (328, 329). Clinical trials investigating the antibody bavituximab has proven the drug to be safe but a phase III-trial with non-small cell lung cancer patients did not verify an effect (308, 330, 331). Recently however, a phase II-trial on glioblastoma patients, could demonstrate that bavituximab reduced the number of MDSCs, indicating potential benefits of the drug (332).

Cytokine stimulation

NK cells express receptors for various cytokines, thereby enabling responses to signals from the surroundings, such as induction of proliferation induced by IL-2, IL-12, IL-15, IL-18 and IL-21 (333). These receptors consist of subunits, with the common γ-chain shared among several receptors. The α- chain (CD25) binds IL-2 with low affinity, while the combined β (CD122)- and γ (CD132)- chains forms an intermediate-affinity complex. The heterotrimeric complex, composed by all three chains, binds IL-2 with high affinity (334). The IL-15 receptor shares the β-subunit with IL-2, and in combination with the common γ-chain and the IL-15 α-unit, forms the high affinity IL-15 receptor (333).

IL-2 has been shown to support survival and enhance NK cell functions by upregulation of perforin, granzyme B and activating receptors (333). Regulatory T (T_{reg}) cells express higher levels of the trimeric high-affinity IL-2 receptor, allowing them to be responsive to low concentrations of IL-2. In therapeutic settings, this enhanced response by T_{regs} to IL-2 risk reducing the stimulating

effect on other lymphocytes due to its immunosuppressive functions (335, 336). To counteract this, a modified IL-2 version displaying higher affinity for the β- and γ-chains is being evaluated (337).

IL-2 has been approved for use in metastatic renal cell carcinoma and metastatic melanoma (333). It has also been evaluated as monotherapy in AML, but it has not shown any benefit for treatment of AML, despite the importance of lymphocytes in combatting the disease (338-341)). The inability of IL-2 to induce an effective anti-leukemic response has been hypothesised to be caused by the immunosuppressive mechanisms found in the disease. ROS produced by monocytes can induce NK cell apoptosis. Combining low-dose IL-2 with histamine dihydrochloride (HDC) was demonstrated by Brune *et al.* to not only promote NK cell killing of leukemic cells but also protect the NK cells from monocyte-derived ROS (342). By binding histamine type 2 receptors on myeloid cells, histamine reduces generation of ROS by the myeloid NADPH oxidase NOX2 (343). Following phase I/II and III clinical trials, demonstrating safety and improved leukemia-free survival, HDC/IL-2 was approved in the EU for relapse prevention in AML (344, 345).

A phase IV trial (the Re:Mission trial; overview presented in figure 7) was conducted to evaluate the immunomodulatory effects of the HDC/IL-2 treatment regimen as a maintenance therapy in AML, administered post-induction and consolidation chemotherapy cycles. The treatment induced expansion of NK cells and enhanced expression of NKp30 and NKp46 on $CD16^{+}$ NK cells, which was associated with improved survival. It was also concluded that the number of $CD56^{bright}$ NK cells at treatment onset was associated with improved outcome (80). This treatment uses low-doses of IL-2, avoiding the severe toxicity seen in high-dose regimens, and although T_{reg} cell expansion was observed during treatment cycles, it was not associated with impaired clinical outcome (346).

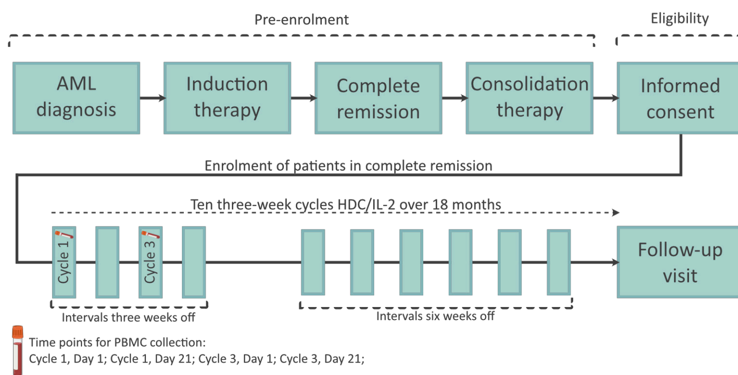


Figure 7. Overview of the Re:Mission trial. Following complete remission and consolidation therapy, AML patients were enrolled in the Re:Mission trial where they received ten three-week cycles of HDC/IL-2 over 18 months. Samples were collected before and after cycles one and three.

IL-15 has demonstrated activating effects on NK cells similar to those of IL-2 but does not expand T_{reg} cells, making it a suitable alternative to IL-2 treatment. (333, 336). Recombinant IL-15 has demonstrated enhanced expansion of NK cells and generally well tolerability in a phase I clinical trial with advanced solid tumours (347). Moreover, a modified variant of IL-15 has been engineered to extend its half-life, demonstrating promising results in a phase I clinical trial (348, 349).

NK cell engagers

Engagers are synthetic molecules engineered to facilitate binding between immune cells and their target cells. These include bi- and tri-specific antibodies and they typically target a tumour antigen on one end and one or two activating receptors on the other. Despite still being in the early stages of development for NK cells, several NK cell engagers (NKCEs) have been developed with promising results for various malignancies. Unlike T cell engagers, adverse events associated with NKCE are not commonly observed, potentially offering a safer therapeutic option. Bispecific NKCEs which target CD16 on NK cells and

various antigens, like CD30, EGFR, CD123, CD19, CD20, CD33 and CD133 on tumours have demonstrated efficacy in pre-clinical studies. Some of these are currently under investigation in clinical trials (350). The addition of an IL-15 component to create a tri-specific NKCE has shown potential for further improvement by enhancing NK cell survival (351). Both bi- and tri-specific NKCEs targeting NKG2D to various tumour-antigens are investigated in phase I/II clinical trials. As NKG2D and CD16 expression may be downregulated in cancer settings, making them potentially unsuitable as targets, NKCEs developed to target NKp46 could offer a more stable alternative. NKp46 expression has been shown to remain stable and is predominantly expressed by NK cells, providing specificity. Numerous versions of bi-, tri- and even tetra-specific NKCEs have been engineered to target NKp46 and a range of antigens (350, 352). The bi-specific CYT-338, targeting NKp46 and CD38 has proven efficacious in multiple myeloma mice models (353). Furthermore, a tri-specific NKp46-ANKET NKCE, targeting NKp46, CD16 and a tumour antigen, demonstrated activation of NK cell and improved recruitment and tumour control in haematological and solid cancer models. This approach could potentially be enhanced by the addition of a variant of an IL-2 element to create a tetra-specific NKCE (350, 354).

Death receptor engagers

TRAIL has been regarded as a promising therapeutic alternative as it can selectively trigger apoptosis in cancer cells (235, 355). Two main strategies are under clinical investigation. These include TRAIL-R agonistic antibodies that bind TRAIL-R1 and TRAIL-R2 with high affinity as well as recombinant TRAIL. Several TRAIL-R1 antibodies have been developed but only HGS-ETR1/mapatumumab has entered clinical trials where phase I and II trails have shown good safety. The efficacy is dubious despite one promising trial with follicular non-Hodgkin's lymphoma (355, 356). Several trials have been conducted with TRAIL-R2 agonists but also these studies remain inconclusive due to lack of efficacy or liver toxicity. However, a multivalent antibody that in

theory would have superior capacity to generate trimerization of the TRAIL-receptor has entered clinical phase I trials (355, 357). Despite the limitation of rapid clearance from the blood, recombinant TRAIL is still a promising therapeutic. Several versions are under investigation, of which dulanermin is the most studied. Despite several disappointing results, it has shown promise in a phase III trial for non-small-cell lung cancer (235, 358). Its questionable efficacy may be appointed failure to induce TRAIL-R trimerization and interaction with decoy receptors.

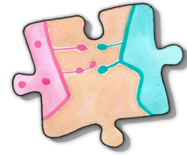
Cell therapies

The use of allogeneic and autologous adoptive NK cell transfer is an advancing field of immunotherapy which offers a high efficacy against various cancers. *Ex vivo* stimulation with various cytokines and feeder cells is a normal procedure to enhance the activity of the NK cells. The combination of IL-12, IL-15 and IL-18 generates cytokine-induced memory-like NK cells, which display heightened responses to restimulation. Cytokine-stimulated NK cells have been demonstrated to be safe and efficacious in clinical trials (350, 359).

Genetic engineering holds the potential to augment the tumour-targeting capabilities of NK cells. Chimeric antigen receptor (CAR) T cells, derived from autologous peripheral blood, have demonstrated remarkable success in haematological malignancies, with six therapies now FDA-approved for treatment of various blood cancers (360-362). As CAR T cell therapies face several challenges, such as severe toxicities and potential risk of lymphoma development, NK cells have emerged as a promising alternative (363). An anti-CD19 CAR NK product, generated from cord blood and engineered to express both IL-15 and an inducible suicide-switch, have shown promising results in phase I/II clinical trial in $CD19^{+}$ B cell maligancies (364, 365). Multiple additional CAR NK cell products are currently under development or evaluation in clinical trials. NK cells can be sourced from various origins, including cord blood, peripheral blood, NK cell lines and induced pluripotent stem cells, each having

distinct advantages and disadvantages. CRISPR/Cas9-mediated gene disruption is becoming increasingly used to deplete the CAR NK cells of immune checkpoints, further fine-tuning their efficacy (366).

GENETIC ENGINEERING



Section in brief:

Modification of DNA through genetic engineering is useful in both research and clinical applications. Clustered regularly interspaced short palindromic repeats (CRISPR) is an innovative tool that utilizes a combination of an endonuclease and a guide RNA. This guide RNA provides instructions to the Cas9 enzyme, directing it to specific locations for DNA cleavage. The continuous evolution of this technique has enabled a spectrum of procedures, including gene knockout, knock-in, silencing and base-editing.

The ability to modify the genome has long been considered a valuable tool for both research and therapy. Historically, this process posed challenges, relying on spontaneous mutations or imprecise editing induced by mutagenic substances like irradiation or chemicals. The era of targeted editing began with the development of zink-finger nucleases in the 1990s and later with transcription activator-like effector nucleases (TALEN) in the 2010s. These technologies involve DNA-binding proteins linked to endonuclease catalytic domains, inducing double-stranded breaks (DSBs) at a precise DNA target site (367, 368). The discovery of clustered regularly interspaced short palindromic repeats (CRISPR) by Emmanuelle Charpentier and Jennifer Doudna in 2012 marked a paradigm shift and was honoured with the Nobel prize in 2020 (369). CRISPR offers numerous advantages, including ease of use, cost efficiency, high specificity and efficiency, as well as being useful for high-throughput and multiplexed gene editing and screening purposes (370).

CRISPR

Originally developed as a sophisticated bacterial defence mechanism against viruses, CRISPR has been harnessed for use in research and therapeutic applications (369, 371). Bacteria utilize sequence-specific RNA molecules, along with CRISPR-associated endonuclease proteins (Cas) to destroy invading foreign nucleic acids. Through engineering, the two-molecule system of tracrRNA:crRNA in bacteria can be combined into a single guide-RNA (gRNA) molecule, facilitating straightforward genetic editing of almost any genome of interest (369, 372). The gRNA consists of a 20 bases long spacer sequence specific to the target genomic sequence, and a scaffold sequence, allowing binding to the Cas protein. The simplicity of gRNA design is a compelling feature of the CRISPR/Cas system, making it widely applicable. Considerations of on- and off-target activity are essential during gRNA design (373, 374). Crucial is also proximity of a protospacer adjacent motif (PAM) sequence, which is essential for the Cas enzyme to bind to the target DNA. For the commonly used Cas9 from *Staphylococcus pyogenes* (SpCas9), the PAM sequence is 5'-NGG-3', and although common in the human genome, Cas9 proteins from other species recognize alternative PAM sequences, allowing flexibility in target selection (369, 372, 375). New Cas proteins with novel features, such as Cas12, are continuously being discovered and hold promise for research and clinical applications (376). In **papers I**, **II**, **III** and **IV**, Cas9 endonuclease was used for knockout purposes.

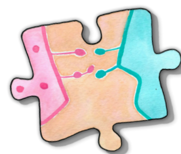
Upon combined expression, Cas9 enzyme makes several interactions with the scaffold sequence of the gRNA, forming a ribonucleoprotein complex (RNP). This binding inflicts conformation changes to the Cas9 protein, activating its DNA-binding capabilities. The seed sequence, comprising the first 10-12 bases at the PAM proximal end of the spacer sequence, initiates binding to the DNA. If the sequence matches, the spacer continues annealing (372). Mismatches hinders binding, with the tolerance decreasing closer to the PAM sequence (373). The nuclease domains of the Cas9 endonuclease, RuvC and HNH, are exposed to the DNA upon the conformational change caused by PAM and DNA binding. The

HNH domain cleaves the complementary DNA strand, and the RuvC domain cleaves the non-complementary DNA strand, resulting in a double-stranded break (DSB) 3-4 bp upstream of the PAM sequence (369, 372). The DSB can be repaired by the cell through two main repair pathways: homolog-directed repair (HDR) or non-homologous end-joining (NHEJ). Depending on the desired outcome, both pathways can be useful. HDR is a high-fidelity repair mechanism but has low efficiency and is only active during S and G2 phases. Knock-in experiments use HDR by supplying a repair template, although additional modifications may be needed to increase efficiency. On the other hand, NHEJ is much more active but error-prone often resulting in insertions and deletions (indels) at the repair site. For knockout experiments, this tendency is utilized to induce a frame-shift mutation leading to a premature stop-codon, resulting in a non-functional protein (372, 377).

Beyond the creation of gene knockouts, the exceptional versatility of CRISPR/Cas systems has facilitated various additional applications, including CRISPR-activation of gene expression, base-editing and epigenetic regulation (378, 379).

CRISPR functions as a powerful gene-editing tool in the exploration and identification of diverse carcinogenic pathways and genetic mechanisms, spanning from the initiation of cancer to metastasis. The papers within this thesis are examples of how the CRISPR technology can be utilized to expand the knowledge in various aspects of cancer escape mechanisms, sensitivities and prognostic factors.

METHODS



This chapter provides a general overview of the major methods used. More detailed descriptions can be found in each paper.

CRISPR/Cas9 KO

To generate knockout cell lines with depleted expression of the proteins of interest to study, we employed the CRISPR/Cas9 technology. In the original K562 cell line, constitutive Cas9 expression was ensured by lentiviral transduction and clones were generated via single-cell sorting. For each gene knockout, plasmid-based gRNAs were delivered to the K562 cells using electroporation and single-cell-sorting was performed for clone generation. In other cell lines, Cas9 and gRNA were delivered by electroporation or lipofection in the form of ribonucleoprotein, comprising a Cas9 protein, complexed with crRNA/tracrRNA mRNA. Protein staining was initially used for knockout verification, followed by sanger sequencing for clone validation.

Genome-wide CRISPR/Cas9 screen

The experimental design for the genome-wide CRISPR/Cas9 screen is depicted in figure 8. K562 cells underwent double lentiviral transduction to ensure adequate Cas9 expression. For the screen using our generated triple-KO (tKO) cell line, the three ligands B7-H6, PVR and Nectin2 were first depleted using CRISPR/Cas9 and a verified negative clone was generated. We used the pooled genome-wide human Brunello CRISPR knockout gRNA library. This library contains an average of four gRNAs per approximately 19 000 genes as well as 1 000 non-targeting control gRNAs (380). Both WT-K562 cells and tKO-K562 cells were subsequently transduced with lentivirus containing the Brunello gRNA library at multiplicity of infection of 0.4, to increase the chance of maximum of one gRNA per cell. Antibiotic selection eliminated untransduced cells and the

culture was split into two replicates. To prevent potential growth advantages or disadvantages associated with specific knockouts, cells were cultured for a maximum of ten days before selection initiation. Prior to NK cell selection, titration experiments were conducted to define optimal conditions. To initiate the selection procedure, the culture was mixed and split in two batches per replicate. The cells used for selection was co-cultured together with polyclonally IL-2-activated NK cell. One condition tKO-screen condition included a blocking antibody against NKG2D. The control batch was cultured in medium alone under the same conditions.

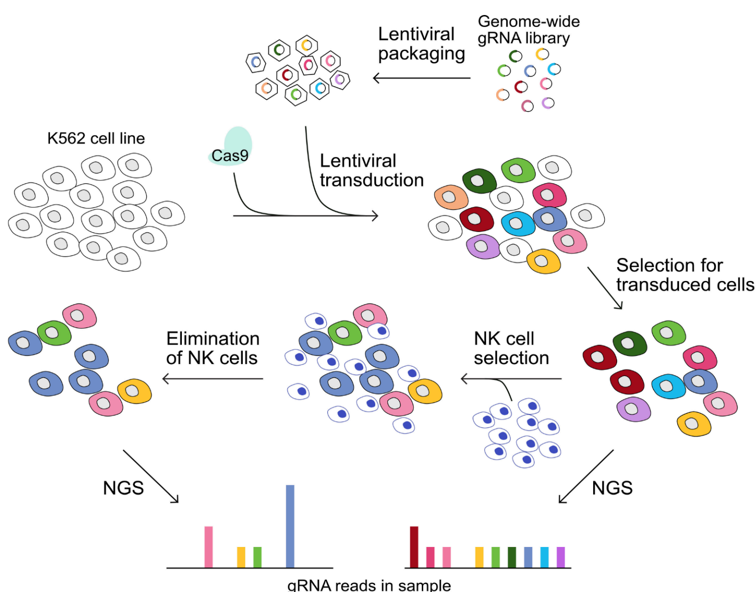


Figure 8. Overview of the genome-wide CRISPR screen. K562 cells were transduced with Cas9 and a gRNA library for stochastic KO of genes. Cells were later exposed to NK cells to select based on enhanced or reduced susceptibility to NK cell cytotoxicity. Comparison with unexposed control cells using NGS revealed genes important for the interaction.

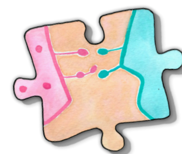
Cultures were maintained until approximately 60% of the target cells were killed. Each condition and replicate was pooled separately and cultured for an additional two days with the addition of antibiotics to remove NK cells. Subsequently, cells were pelleted and frozen. The accumulation or depletion of specific guides within

the NK cell-selected population, compared to the unexposed cells in the control arm, was analysed using next generation sequencing and the MAGeCK software.

Functional assays

The studies presented in this thesis predominantly focus on NK cell function and activity in response to specific stimuli. Flow cytometry-based assays were primarily used for these investigations, where the endpoint parameter could include the quantification of dead target cells or assessment of degranulating or cytokine-producing NK cells. The basic procedure involved co-culture of NK cells and target cells, with one cell type, usually the target cells, pre-labelled before exposure to facilitate the separation of the two cell types during analysis. In cytotoxicity assays, different cell ratios were used to ensure an optimal level of killing. Finding a balance is crucial, as excessive killing may mask true differences between samples, while insufficient killing may make differences difficult to discern. A live/dead marker, staining DNA or intracellular amines was employed to study killing, enabling the identification of cells with compromised membranes. For degranulation, an antibody binding to the granule marker CD107a/LAMP-1 was used to stain all NK cells that exposed the intragranular protein on their surface due to granule release. For cytokine production, cells were fixed and permeabilized after co-culture to allow for intracellular staining, with a blocking agent used to trap cytokines in the Golgi complex.

RESULTS AND DISCUSSION



The overall objective of this thesis work was to enhance our understanding of the factors influencing the interaction between NK cells and their target cells, with a focus on exploring novel proteins and structures involved in this process.

To identify genes associated with the NK cell and target cell interaction, we used CRISPR screens, allowing a larger-scale study of phenotypic alterations in cells as a result of genetic modulations. By using NK cell cytotoxicity as the selection method after the genetic modifications, we could gain insight into what genes that influence the interplay by providing resistance or susceptibility to NK cells upon depletion. Conducting a *genome-wide* screen broadened our investigations beyond membrane-bound proteins to include intracellular proteins with indirect effect on cell interactions. To validate and delve deeper into the mechanisms underlying the impact of specific genes on the interaction, we conducted extensive follow-up studies on selected hits from the screens, culminating in the studies included in this thesis (**papers I**, **II**, **IV**). In order to study the interactions further, we also employed the CRISPR technology to disrupt genes of interest. This enabled us to create useful models with skewed interactions that proved valuable in studies of how polymorphisms in certain genes impacted the interactions in AML (**paper III**).

In our initial CRISPR screen, we used WT-K562 cells and co-cultured them until approximately 60% of the cells had succumbed to NK cell cytotoxicity. Following the MAGeCK analysis, genes implicated in either providing protection or rendering susceptibility to NK cell cytotoxicity were identified as depleted or enriched respectively (**paper I**; figure 9).

Among the enriched gRNAs, targeting genes typically involved in NK cell elimination, the most crucial gene identified was *NCR3LG1*, encoding B7-H6,

the ligand for the activating NK receptor NKp30. Notably, previous screens utilizing K562 cells with NK cell selection consistently identified B7-H6 as the dominantly enriched gRNA target (381-383). Considering the high dependence of K562 cell killing on NKp30 (**paper I**; (384)), we deliberately applied lower selection pressure compared with previous studies. This approach enabled us to uncover additional hits such as *CD58*, encoding the ligand for activating NK cell receptor CD2 (previously identified also in (382, 383) and *TMEM30A*. CRISPR/Cas9 knockout of the genes for B7-H6 and CD58 served to validate the significance of the B7-H6-NKp30 interaction in K562 cell elimination, while also revealing a minor role for CD58.

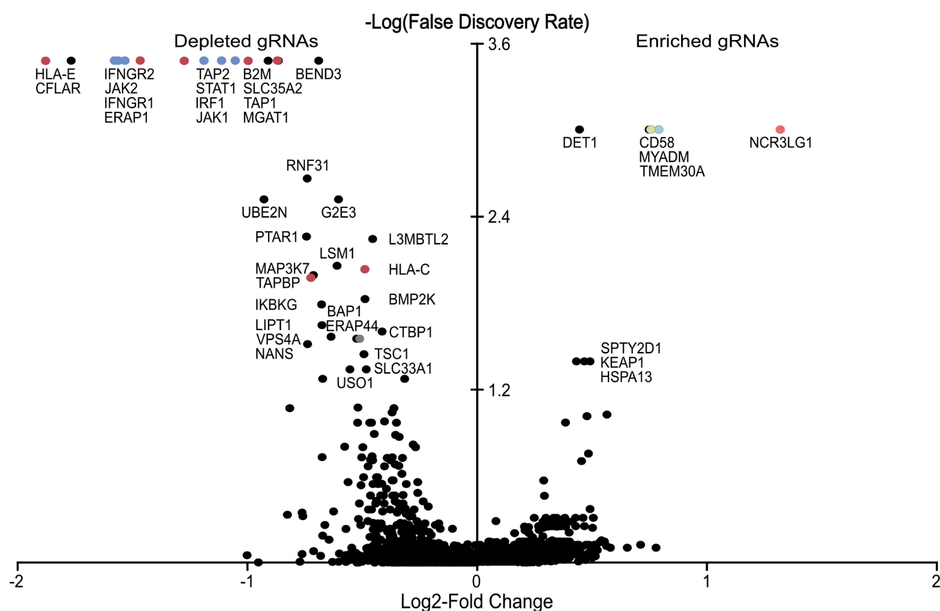


Figure 9. Genome-wide CRISPR screen results. Depleted and enriched gRNAs from one replicate obtained from analysis by MAGeCK software. Genes related for IFNγ signalling are indicated in blue and those involved in the antigen presentation pathway in red.

TMEM30A emerged as one of the highly enriched gRNA-targeted genes, indicating a protective role in cells lacking a functional gene. This gene encodes the CDC50A β-subunit, part of an P4-ATPase flippase complex, responsible for transporting phospholipids such as PtdSer and PtdEtn from the outer to the inner leaflet of the plasma membrane. Consequently, establishment of *TMEM30A* KO K562 cells allowed us to confirm that cells exhibit elevated levels of PtdSer on their surface in the absence of a functional *TMEM30A* gene (figure 10A; (385)). Although, CDC50A is one of three β-subunits, studies show that CDC50A associate with ten different α-units, whereas CDC50B and CDC50C only interact with a smaller number (386-388). Based on the screen results, the function of CDC50A appears critical, while there seems to be a redundancy among the numerous α-units as none of them emerged as top hits. In agreement with the screen findings, these *TMEM30A* KO cells demonstrated reduced sensitivity to NK cell cytotoxicity as well as lower induction of NK cell degranulation and cytokine production (figure 10B).

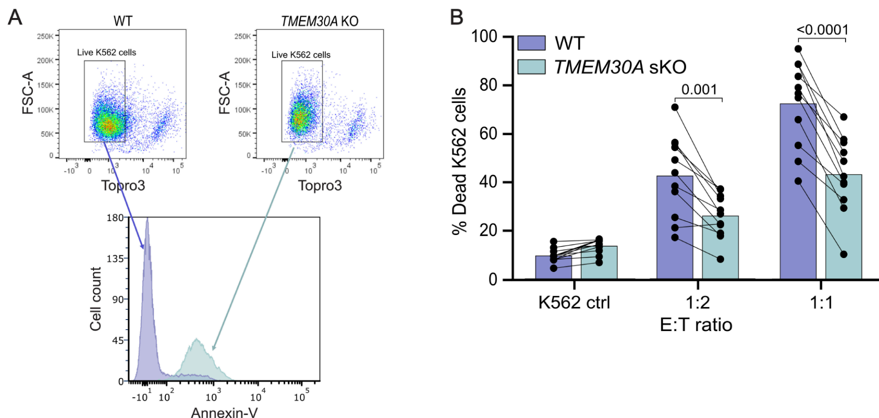


Figure 10. CDC50A depletion result in enhanced PtdSer exposure and lower sensitivity to NK cell cytotoxicity. (A) Staining for PtdSer of WT (blue) and *TMEM30A* KO (turquoise) K562 cells. (B) Cytotoxicity assay with WT and *TMEM30A* KO cells (n=11). One-way ANOVA analysis with Šidák's multiple comparisons test was used as statistical analysis. Error bars represent SEM.

Interestingly, an examination of the expression of the crucial ligands for NK cell receptors revealed that *TMEM30A* KO had no impact, except for an increase in HLA-E expression in the KO cells. Given that HLA-E engages the inhibitory NK cell receptor NKG2A, this could potentially explain the reduced sensitivity, especially considering the striking importance of HLA-E in the WT CRISPR-screen. However, an exclusive analysis of NKG2A$^-$ NK cells in the degranulation assay revealed that even in the absence of this inhibitory interaction, the *TMEM30A* KO cells induced lower degranulation, extending beyond the increased expression of HLA-E.

Given that these experiments were done in the same cell line used for the CRISPR screen, it was crucial to validate whether this finding could be extended to other cancer types. Consequently, we generated *TMEM30A* KO cells of four additional cell lines representing various haematological cancers: acute T cell leukemia (jurkat), acute promyelocytic leukemia (HL-60), diffuse large B cell lymphoma (SU-DHL-5) and Burkitt's lymphoma (Raji). We could confirm that *TMEM30A* KO versions of these cell lines were indeed less sensitive to NK cell cytotoxicity and induced lower NK cell degranulation. Examining the additional *TMEM30A* KO cell lines, we observed an increased expression of HLA-E in the myeloid cell line, HL-60, but this did again not alone account for the overall reduced susceptibility.

This observed increased expression is intriguing. Membrane lipids are known to play a crucial role in membrane protein localization and activity, either through association with proteins in lipid rafts or through interaction with transmembrane proteins (389-392). Indeed, by supplying a docking site with its negative charge, PtdSer plays a crucial role in the correct localization and activation of several intracellular proteins (308, 393-395). A study by Enneshi *et al.* indicated increased BCR motility in cells with *TMEM30A* mutations, suggesting that altered membrane composition could be a contributing factor (396). Further investigation is warranted to confirm whether changes in membrane composition

explains the altered levels of HLA-E and why this phenomenon extends to the myeloid cells.

Notably, these *TMEM30A* KO cells across various cancer types consistently displayed higher levels of exposed PtdSer compared to WT cells. To verify that the exposed PtdSer was the cause of the reduced susceptibility, we used PtdSer-binding AnnexinV to block the interaction. This intervention resulted in an increased sensitivity to NK cell cytotoxicity for the *TMEM30A* KO cells but not for WT cells (figure 11A). This confirmation highlighted the role for PtdSer, yet the underlying mechanism remained unclear. By employing blocking antibodies towards the two inhibitory NK cell receptors TIM-3 and IRp60/CD300a, known to bind PtdSer, we aimed to shed light on their involvement.

Blocking TIM-3 revealed that *TMEM30A* KO target cells became more sensitive to NK cell cytotoxicity (figure 11B). However, for IRp60, the role was less evident as the antibody exhibited an agonistic effect on the WT cells, reducing their sensitivity. In contrast, *TMEM30A* KO cells were unaffected, suggesting a complementary role for the IRp60 antibody and the overexpressed PtdSer on the KO cells. To further validate the role of the receptors, we knocked them out in primary NK cells and used them in degranulation assays with *TMEM30A* KO och WT cells. Comparing the *TMEM30A* KO-induced reduction in degranulation between the negative control NK cells and *HAVCR2* or *CD300A* KO NK cells illustrated the importance of TIM-3. The lower reduction observed with NK cells lacking TIM-3 emphasized the receptor's role, indicating that without the receptor, *TMEM30A* mutation was less critical. Regarding IRp60, a non-significant trend in the same direction was observed.

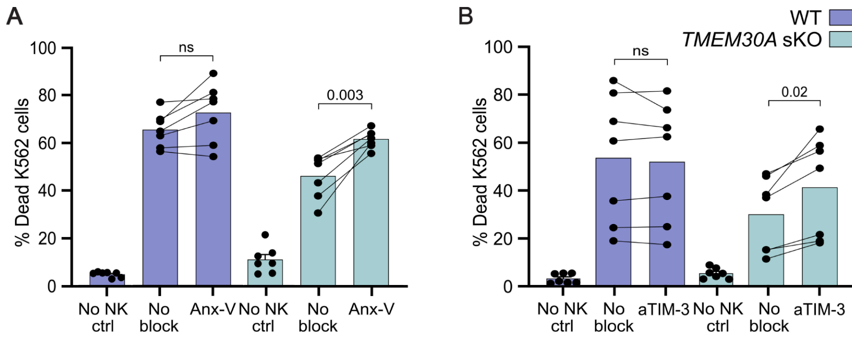


Figure 11. Enhanced exposure of phosphatidylserine protects *TMEM30A* KO cells from NK cells through interactions with inhibitory receptors. (A) and (B) Cytotoxicity assay with WT and *TMEM30A* KO K562 cells, supplemented with Annexin-V (Anx-V) for blockade of PtdSer (n=7) (A) or combined with TIM-3 for blockade (n=7). One-way ANOVA analysis with Šidák's multiple comparisons test was used as statistical analyses. ns not significant, error bars represent SEM.

Considering that PtdSer is a known "eat-me" signal to phagocytes to engulf apoptotic cells, it can seem counterintuitive that *TMEM30A* mutations could be beneficial for cancer cells in an *in vivo* setting (37, 397-401). However, studies have reported recurrent loss-of-function *TMEM30A* mutations in diffuse-large-B-cell lymphoma and follicular large B cell lymphoma (396, 402, 403). In light of our results, it is tempting to speculate that these mutations reflect an NK cell evasion strategy used by lymphoma cells. A simplified illustration depicting the potential mechanism can be found in figure 12. There are conflicting findings on whether increased exposure of PtdSer by living cells alone is sufficient for phagocytosis. Segawa *et al.* demonstrated that overexpression of the scramblase TMEM16F, leading to increased extracellular PtdSer did not result in enhanced phagocytosis, suggesting that exposed PtdSer alone might be insufficient for engulfment (404). However, subsequent research by the same group later indicated that PtdSer-expressing CDC50A-null cells were engulfed by macrophages while cells expressing a caspase-resistant flippase complex, showing no exposure of PtdSer during apoptosis, were not engulfed (223). There are indications suggesting that alterations in membrane fluidity, and consequently the mobility of PtdSer and the

corresponding potential for receptor clustering, may serve as a mechanism for phagocytes to differentiate between live and dead PtdSer-exposing cells (308, 405). Specific PtdSer receptors such as TIM-1, involved in efferocytosis, exhibit enhanced binding affinity for oxidized PtdSer. One proposed mechanism for this oxidization involves cytochrome C. Given that cytochrome C is released upon mitochondrial membrane permeabilization during apoptosis, it is conceivable that the apoptosis-induced oxidized PtdSer constitutes a more potent "eat-me" signal (308, 406). Further investigations are warranted to determine whether PtdSer exposure alone is enough to function as an "eat-me" signal.

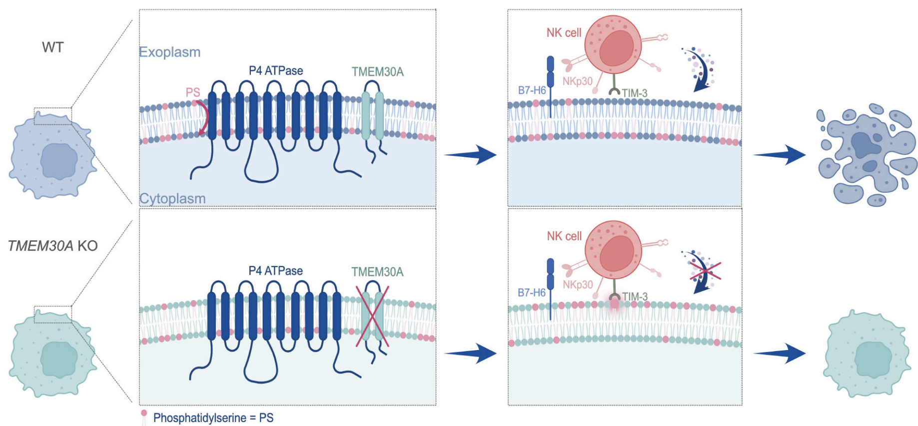


Figure 12. Potential mechanism for reduced NK cell sensitivity of *TMEM30A* KO cells. The exposed PtdSer of *TMEM30A* KO cells can interact with inhibitory NK cell receptor TIM-3, allowing the cell to escape NK cell cytotoxicity.

The specific characteristics of membrane lipids and the modulation of their asymmetry can have additional implications on cell-cell interactions that would be interesting to study further. With an increased accumulation of PtdSer in the outer leaflet of the membrane, the physical properties of the normally tightly packed layer may change, potentially influencing the interactions with NK cells (407). A study by Friedman *et al.* highlighted that changes in the stiffness of target cells impact the synapse-forming ability of the NK cells (152). Although not studied

specifically in this project, it is conservable that also phosphatidylethanolamine (PtdEtn) would be exposed to a higher degree on the outside of cells as it is also transported by the P4-ATPase complex (408). With an accumulation in the outer leaflet, the phospholipids, especially the smaller PtdEtn, could impact the curvature of the membrane, affecting processes like exocytosis and endocytosis. This, in turn, might influence synapse formation and perforin/granzyme uptake (390, 409, 410). The disruption of the tightly packed, highly ordered and rigid outer leaflet may, on the other hand, make the cells more sensitive to NK cell-generated lytic molecules.

The β-subunit is critical for the proper membrane translocation of the P4-ATPase complex (408). In the absence of the CDC50 protein, the α-unit accumulates in the cytoplasm, leading to ER-stress (411). This phenomenon could potentially impact the interaction between cells and NK cells. Interestingly, the ER-stress marker ecto-calreticulin was recently proposed to be a ligand for activating receptor NKp46 (108). While this suggests that *TMEM30A* KO cells could be more susceptible to NK cells, our observations did not support this hypothesis. Nevertheless, it would have been interesting to investigate whether our KO cells displayed increased ER-stress and if this made them more vulnerable to NKp46-dependent killing.

The evolutionary rationale behind the development of this inhibitory mechanism is intriguing to consider. It may be reasonable for NK cells to possess a sensory mechanism that enables them to discern when their target cell is undergoing apoptosis, followed by an inhibitory mechanism that facilitates detachment from the cell. A study by Anft *et al.* have indicated that detachment is swifter when the NK cell has executed a lytic hit potentially because inhibitory receptors can then recognize upregulated ligands on the target cell in response to apoptosis (205). Another possible aspect is that NK cells receive these inhibitory signals to regulate their cytotoxicity, thereby mitigating the risk of overwhelming the clearance capacity of the immune system and minimizing damage to surrounding tissue.

There are broader implications of PtdSer exposure on tumour immunity beyond NK cell function. Dysregulation of extracellular PtdSer is commonly observed in the cancer microenvironment and its blockade has shown the ability to shift tumour-associated macrophage phenotypes from myeloid-derived suppressor cells to pro-inflammatory M1 macrophages (308, 309, 385). Both PtdSer and TIM-3 antibodies have demonstrated promising potential in pre-clinical studies and are currently under evaluation in clinical trials (49, 324, 332).

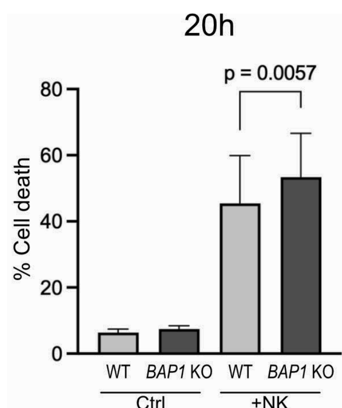


Figure 13. BAP1 depletion sensitizes cells to NK cells upon IFNγ exposure. Cytotoxicity assay with WT and *BAP1* KO K562 cells 20h with polyclonally-activated NK cells Statistical analysis using paired t-tests was performed. Error bars represent SEM.

In our analysis of the genome-wide CRISPR screen with WT-K562, we identified depleted gRNAs targeting specific genes that typically confer protection against NK cell cytotoxicity (Figure 9). One of these genes was *BAP1* which encodes a deubiquitinating enzyme, regarded as a tumour suppressor (**paper II**, (412)). Surprisingly, our generated *BAP1* KO cells did not exhibit altered sensitivity in standard cytotoxicity assays with NK cells. However, when the 20-hour long screen assay was replicated, an enhanced susceptibility of the KO cells became evident (figure 13). Discrepancies between the two assays were identified in relation to IFNγ, with ELISA experiments revealing IFNγ release only after the 20-hour long assay, not the shorter one. Considering the context of the other protective genes identified in the screen, we speculated that BAP1 might be linked to IFNγ signalling. Accordingly, the KO phenotype would only manifest in the

presence of IFNγ, affecting both sensitivity and degranulation-stimulating capacity.

To further elucidate the role of *BAP1* and its impact on the overall cellular proteome upon deletion, we conducted tandem mass tag-mass spectrometry on both WT and *BAP1* KO K562 cells, with and without exposure to IFNγ. Even without IFNγ exposure, the protein content exhibited variation between the samples, which underscores the crucial role of BAP1 in numerous cellular processes. ULBP1 and ULBP2 were found upregulated in *BAP1* KO cells. While a cellular stress-response to transfection and KO could explain this, staining could not confirm this observation, which is in line with other KO cell lines we have created.

Only upon IFNγ treatment, did it become apparent that numerous proteins induced by IFNγ signalling were depleted in the *BAP1* KO cells (figure 14). These include guanylate-binding proteins and HLA class I-related proteins such as TAPBP/1/2, β_2-microglobulin and HLA-E. Consistent with this, lower levels of extracellular HLA class I was exhibited by the *BAP1* KO cells, when exposed to IFNγ.

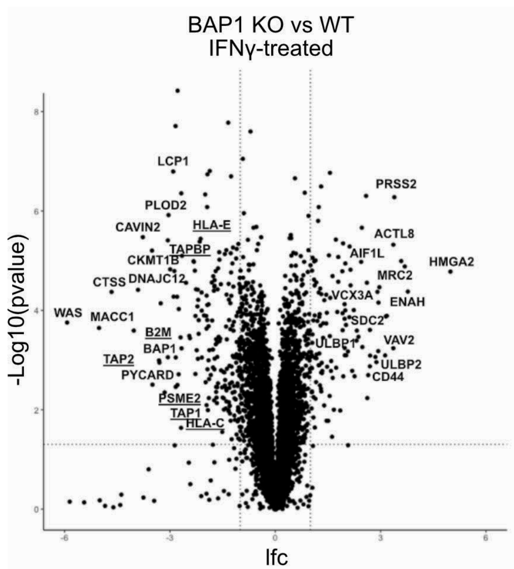


Figure 14. IFNγ and antigen presentation pathways are disrupted by BAP1 depletion. Proteome analysis displaying differentially regulated proteins of *BAP1* KO vs WT-K562 cells in IFNγ-treated condition.

This is in line with the results from the WT-K562 screen where genes associated with HLA class I expression were highly depleted (figure 9). Given that HLA class I molecules engage inhibitory NK cell receptors (7, 21), the depletion of these genes logically renders the cells more susceptible to NK cell-mediated cytotoxicity. These findings align with previous screens (381, 383, 413-415), providing additional validation for the results of our study. Moreover, in our results showed that gRNAs targeting genes involved in the IFNγ signalling pathway were also depleted. This includes genes encoding components such as *IFNGR*, *JAK* and *STAT*. The IFNγ pathway is known to regulate HLA class I expression (13, 14), providing a rationale for the observed depletion, a phenomenon previously described (381, 383, 414, 415). Interestingly, a screen conducted by Pech *et al.* presented alternative perspectives on these genes (382). Their use of the NK cell line NK92, which have a low expression of the main MHC class I-binding inhibitory receptors, resulted in distinct outcomes compared to our study using primary peripheral blood NK (416). In their screen, genes related to HLA class I and IFNγ signalling did not confer resistance. Conversely, IFNγ signalling genes were found to provide sensitivity. This discrepancy may be attributed to their identification of ICAM-1 as a top sensitivity gene, as it is regulated by IFNγ signalling (131, 417). These varying results underscore the impact of the NK cell receptor repertoire on the interactions with target cells, influencing how different genes modulate the interplay between NK cells and their targets. Consistent with this, the expression of the adhesion molecules ICAM-1 and NCAM-1/CD56 further supported the reduction in IFNγ signalling response observed in the *BAP1* KO cells.

To explore what specific stage of the IFNγ signalling pathway that is influenced by BAP1, we examined the transcription factor STAT1. During resting condition, staining revealed no impact of *BAP1* KO, neither on total STAT1 or phosphorylated STAT1.

However, upon IFNγ exposure, it became evident that *BAP1* KO cells generated lower levels of phosphorylated STAT1, crucial for subsequent signalling (figure 15), which may be due to the lower expression of the receptor IFNGR1 in *BAP1* KO cells.

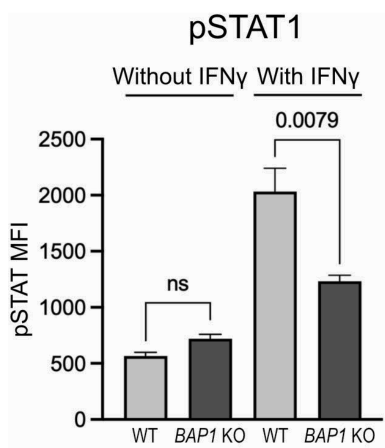


Figure 15. BAP1 depletion impairs the expression of components of the IFNγ signalling pathway. Staining of pSTAT1 without and with IFNγ treatment. Statistical analysis included Mann-Whitney test. ns not significant, error bars represent SEM.

The role of BAP1 is intricate and this study contributes a piece to the larger understanding by revealing its involvement in IFNγ signalling. Within the IFNγ signalling pathway, BAP1 emerges as a regulator across multiple steps, as evidenced by alterations in both the STAT1 and IFNGR1 levels in the BAP1-deficient model. Consistent with this, BAP1 depletion has been shown to diminish the expression of STAT2 and IRF9 in clear cell renal cell carcinoma (418). Another facet of HLA regulation by BAP1 has been identified in recent research, demonstrating its role in the epigenetic promotion of HLA expression (419).

Beyond its involvement in IFNγ signalling, our proteomic analysis unveils additional functions of BAP1 with diverse implications in various contexts. The role of BAP1 in malignancies appears complex and highly dependent on the specific disease context. Traditionally considered a tumour suppressor gene due to its role in BRACA1-dependent inhibition of breast cancer, BAP1 has also been

implicated in cancer progression (412). For instance, a study has shown its involvement in HOXA gene upregulation and myeloid leukemogenesis (420). In uveal melanoma, *BAP1* mutations correlate with a higher risk of metastatic disease and lower survival rates (421-425). Similarly, *BAP1* mutations are associated with poorer outcome and predisposition to other cancer types (426, 427). However, in a cutaneous melanoma mouse model, BAP1 depletion significantly reduced tumour growth (428).

This apparent discrepancy underscores the importance of considering the disease setting to elucidate the mechanism behind the observed phenotypic outcomes. Our study, demonstrating the inhibition of HLA class I upregulation following BAP1 depletion, enabling evasion from T cell recognition, provides a potential explanation for the unfavourable outcomes observed in certain cancer types. The reduced HLA expression renders the cells vulnerable to NK cell cytotoxicity, giving a rationale for NK cell-based immunotherapy in malignancies with *BAP1* mutations.

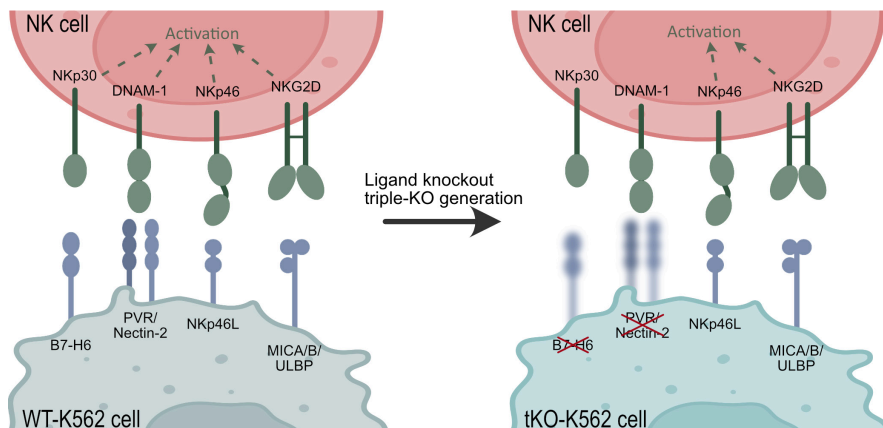


Figure 16. tKO-K562 cell generation. WT-K562 cells are killed by NK cells mainly through interactions between NKp30 and B7-H6. By depletion of the ligands for NKp30 and DNAM-1, the tKO-K562 cells could be killed via the interactions between NKG2D, NKp46 and their ligands.

Not only genes expressed by the potential target cells impact disease outcome but the effector cells' genes also play a crucial role (20, 429). A specific SNP,

rs1049174, in the gene encoding NKG2D, *KLRK1,* has been reported to impact on natural cytotoxicity and cancer development risk (20). This study used the K562 cell line to study the effect of the NKG2D alleles. However, as we saw in **paper I**, NK cell killing of WT-K562 is not dependent on NKG2D, making it challenging to discern allele-related differences. We observed that elimination of specific ligands allowed us to manipulate the interactions between NK and target cells and our B7-H6^{neg}CD58^{neg} dKO was instead killed by NKG2D and DNAM-1 (**paper I**). Similarly, albeit fairly resistant, a B7-H6^{neg}PVR^{neg}Nectin2^{neg} tKO cell line was shown to be killed via NKG2D as illustrated in figure 16. Even with this highly NKG2D-dependent target cell model, there was a surprisingly small difference in degranulation between NK cells from donors with one high cytotoxicity NKG2D allele, rs1049174 G/x, and NK cells with two low cytotoxicity alleles, C/C. Further investigation revealed that the G/x individuals harboured more NKG2A^{+} NK cells which were responsible for a higher degranulation, regardless of NKG2D allele (figure 17).

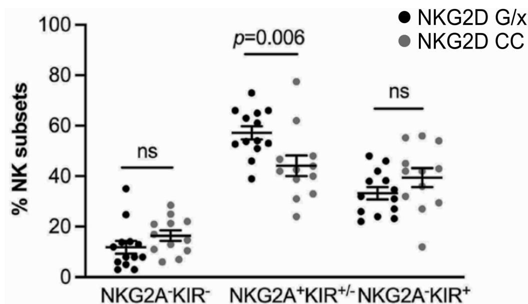


Figure 17. A NKG2D-dependent model demonstrate effect of NKG2D genotype on NK cell functionality. Frequency of NK cell subsets in healthy donors, divided on NKG2D SNP G/x (n=13) and CC (n=12). Ns non significant. Error bars represent SEM.

This observation led to analysis of the NKG2A gene, *KLRC1*, located near the *KLRK1* gene in the NKC locus, with a known polymorphism, rs1983526, in linkage disequilibrium with the NKG2D polymorphism. Healthy donors with the favourable NKG2A G/G alleles had a higher fraction of NKG2A^{+} CD56^{dim} NK cells than did C/x individuals. Further investigation on the functional implications

of the NKG2A genotype, revealed a correlation with a stepwise increase in IFNγ production and higher numbers of the G allele, which was not only restricted to $NKG2A^+$ cells but also KIR^+NKG2A^- cells.

Similar results were observed in a clinical cohort of AML patients receiving maintenance therapy with HDC/IL-2. Thus, G/G patients expressed higher frequency of $NKG2A^+$ NK cells, and the G allele was associated with high levels granzyme B, suggesting that the NKG2A G variant is associated with a higher cytolytic capacity. Surprisingly, as in healthy donors, the granzyme B expression appeared not to be confined to a specific NKG2A/KIR subgroup. This finding that not only $NKG2A^+$ NK cells from NKG2A G/G individuals displayed higher functionality, but also other subsets was intriguing. This contrasts with the rheostat educational model, which dictates that NK cell functionality relies on the inhibitory input it receives during steady state. It would be expected that $NKG2A^+$ cells in G/G donors had enhanced effector function as the increased NKG2A expression provides more inhibitory signal. However, it fails to explain the elevated IFNγ response and granzyme B expression in $NKG2A^-$ subsets. Notably, granzyme B expression levels have been a proposed marker of NK cell educational status. Thus, additional mechanisms beyond the rheostat model of education are necessary to account for the increased IFNγ response and granzyme B expression in G/G donors (430).

Leukemic blasts exhibit lower HLA-E expression (**paper III**, (431)) potentially leading to a more robust response against these cells by the larger fraction of $NKG2A^+$ cells observed in the individuals with the NKG2A G/G genotype. In contrast, HLA-ABC expression remains normal in leukemic blasts, providing consistent inhibitory input for KIR^+ cells.

When comparing the significance of NKG2D and NKG2A gene polymorphisms on the outcome of HDC/IL-2 immunotherapy, it was observed that patients harbouring a NKG2A rs1983526 G allele exhibited both better leukemia-free

survival (LFS) and overall survival (OS) (figure 18). Patients carrying the NKG2D rs1049174 G allele showed trends for improved LFS and OS.

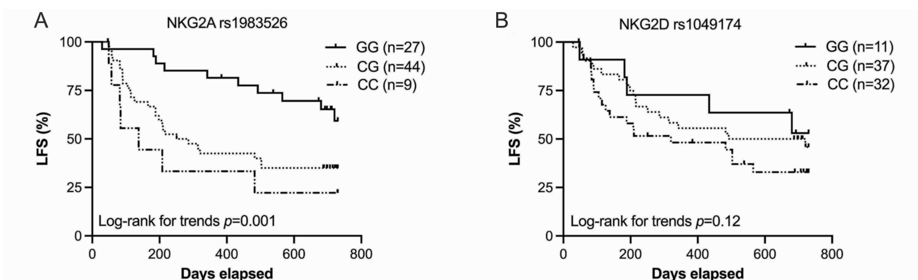


Figure 18. Impact of NKG2A and NKG2D genotypes on outcome of HDC/IL-2 immunotherapy in AML. (A) Leukemia-free survival (LFS) of AML patients divided by NKG2A rs1983526 genotypes (GG n=27, CG n=44 or CC n=9), following HDC/IL-2 treatment. (B) Leukemia-free survival of AML patients divided by NKG2D rs1049174 genotype (GG n=11, CG n=37 or CC n=32), following HDC/IL-2 treatment.

These results imply that the observed beneficial effects are likely attributed to the NKG2A genotype rather thanNKG2D. While this does not entirely invalidate the potential impact of the NKG2D genotype, the linkage disequilibrium between the SNPs raises the possibility of misdirected focus towards the wrong SNP. Numerous studies have highlighted associations between the NKG2D dimorphism and disease outcome (432-435). However, our findings suggest that these associations may be driven by the NKG2A SNP. Nevertheless, it is worth noting that the higher expression of NKG2D, as reported for the favourable NKG2D allele, could certainly influence NK cell function (432, 436). Additionally, it is essential to consider that both NKG2A and NKG2D expression is not exclusive to NK cells alone and other cell types may contribute to the observed effects of the different genotypes.

A dimorphism in the HLA-B leader peptide is another genetic factor reported to impact the function of NKG2A^{+} cells. The HLA-B-21 M allele produces a high

affinity peptide for HLA-E binding while the HLA-B-21 T allele reduces HLA-E expression by producing a peptide unable to bind HLA-E (25, 431). To investigate whether this dimorphism was somehow impacted by the NKG2A genotype, the patients were stratified based on the HLA-B-21 genotypes. The levels of granzyme B were markedly reduced for each NKG2A C allele in HLA-B-21 TT individuals but remained unaffected in the M/x patients. Similarly, studies of the outcome revealed that the M/x patients remained unaffected by the NKG2A genotype, while HLA-B-21 T/T patients exhibited significantly improved LFS if they also carried the NKG2A G/G genotype.

One hypothesis to explain these findings is that HLA-B-21 M/x individuals receive sufficient inhibitory stimulation due to the enhanced interaction between NKG2A and HLA-E thanks to its elevated levels. Hence, the NKG2A G/G genotype is redundant. For the HLA-B-21 T/T patients however, lower HLA-E levels leaves room for higher inhibitory signalling obtained from the increased NKG2A expression of the NKG2A G/G genotype. A recent study proposed that the HLA-B-21 M peptide induces high HLA-E expression but provides low receptor recognition (437). This suggests that the M/x patients may have a larger fraction of antagonistic HLA-E peptides, removing the benefit of the higher NKG2A expression of the NKG2A G/G genotype, providing a potential alternative explanation. It was intriguing to observe that patients with the NKG2A^{high} NKG2A G/G and the HLA-E^{low} HLA-B-21 T/T exhibited the most favourable LFS among all groups. The collective impact of the HLA-B-21 and NKG2A rs1983526 genotypes appear to endow these patients with well-educated KIR^{+} cells and more functional, potentially cytolytic NKG2A^{+} cells, making this an advantageous genetic combination.

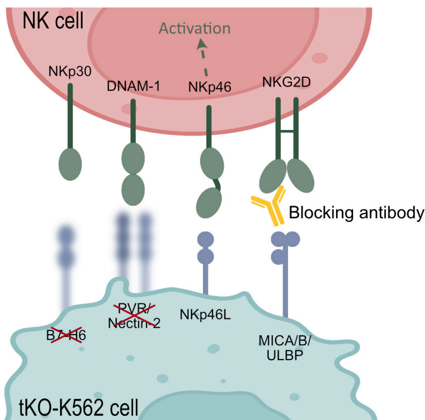
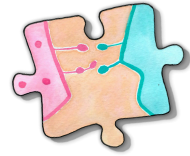


Figure 19. Illustration of a tKO-K562 cell screen setting. Given that NK cell primarily eliminate tKO-K562 cells through interactions involving NKp46 and NKG2D, the addition of a blocking antibody targeting NKG2D would shift the killing to nearly exclusively depend on NKp46.

The ability to manipulate the receptor-ligand interaction facilitated a detailed study of the NKG2D alleles. During the validation of the model, it became evident that the newly established cell line was susceptible not only to NKG2D- but also NKp46-mediated killing. Given the existing gaps in understanding the ligands recognized by NKp46, we opted to conduct another genome-wide CRISPR screen using the tKO cell line. To enhance the screen selection's dependency on the NKp46 interaction with its unknown ligand, we included a condition where tKO-K562 cells were exposed to NK cells in the presence of a blocking antibody against NKG2D (illustrated in figure 19). As described above, the most important genes for NK cell killing of WT-K562 cells were *NCR3LG1*, *CD58* and *TMEM30A*. Naturally, in the screens with tKO K562 cells, where B7-H6 was depleted, *NCR3LG1* was not identified as a top hit, while *CD58* and *TMEM30A* remained important. Instead, genes that were redundant in the WT screen suddenly appeared to be of key importance in the tKO screen. Among these genes there may be genes that encode and/or regulate the expression of NKp46 ligands. In **paper IV**, we present studies evaluating the role of such genes for NKp46 recognition of malignant cells.

CONCLUDING REMARKS



This thesis represents a piece of the intricate puzzle illustrating the larger aspects of NK cell interactions with its target cells.

CRISPR screens have proven invaluable tools for uncovering the intricacies of gene involvement in cell interactions and the continuous development of the technique introduces endless research possibilities. In this thesis, the technology has played a central role. Comparisons between screens have revealed how minor adjustments can influence cellular interplay, particularly emphasizing the critical role of killing levels. The choice of cells in a screening setting is equally vital with variations in ligand and receptor expression of both target and NK cell influencing the output. Interactions between ligand and receptor that do not occur due to lack of expression or low dependence will for obvious reasons not appear in the screen results. Similarly, since multiple ligands and receptors often have more than one interaction partner, the absence of expression of one partner may amplify the significance of another interaction The use of WT-K562 cells in a screen, unveiled indirect regulatory mechanisms influencing interactions. By mutating *TMEM30A*, cancer cells can evade immunity by upregulating surface PtdSer. This has been reported to generate an immunosuppressive environment and **paper I** illustrates how this upregulated PtdSer on *TMEM30A* KO cells interacts with inhibitory NK cell receptor TIM-3, hindering NK cell cytotoxicity. The potential vulnerability of these cells to phagocytic uptake in a cancer setting needs further exploration, as studies suggest phagocytes possess distinct mechanisms for distinguishing between live and dead PtdSer^{+} cells. **Paper II** demonstrates the impact of intracellular regulation on NK cell susceptibility. The deubiquitin protein BAP1 emerges as a positive regulator of MHC class I expression through modulation of the IFNγ pathway. Depletion of the protein,

resulted in lower expression of phosphorylated STAT1 after IFNγ stimulation, as a result of reduced levels of IFNGR.

The CRISPR technology proved useful in revealing the impact of allelic variants of the NKG2D gene in **paper III**. An engineered NKG2D-dependent model demonstrated modest differences in NK cell function between the two allelic versions. In-depth analyses instead uncovered that an associated SNP in the NKG2A gene was the actual driver of the observed effects. Interestingly, when considering the NKG2A genotype in conjunction with previously identified HLA-B genotypes, distinct variations emerged in the outcomes of immunotherapeutic interventions in AML. Notably, the impact of the NKG2A genotype was evident only in cases with the less favourable HLA-B-21 dimorphism, resulting in the most favourable patient outcome. Beyond advancing our understanding of NK cell genotypes and phenotypes, the findings from this study can serve to guide in treatment decisions by providing insights into weather specific patients are likely to benefit from interventions.

Through the targeted KO of ligands for NK cell receptors, we successfully manipulated interactions with NK cells. The depletion of three specific ligands, enabled the generation of a highly NKp46-dependent model. By addition of an antibody against NKG2D, we achieved almost exclusive killing via NKp46. Leveraging this model in an additional CRISPR screen in **paper IV**, we could identify genes for potential NKp46 ligands.

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