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DYNAMICS OF NATURAL KILLER CELL HOMEOSTASIS – IMPLICATIONS FOR CELL-BASED CANCER IMMUNOTHERAPY

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family

ABSTRACT

Natural killer (NK) cells comprise a central role within the innate immune system, eliminating virally infected, foreign and transformed cells through their natural cytotoxic capacity. Release of their cytotoxic granules is tightly controlled through the balance of a large repertoire of inhibitory and activating receptors, and it is the unique combination of these receptors on individual cells that confers them their immense diversity both in phenotype and functionality. This thesis aimed to investigate the mechanisms sustaining NK cell homeostasis with the aim of translating these findings into more efficient NK cell-based immunotherapies against cancer.

In **paper I**, we set out to define a transcriptional timeline for NK cell differentiation through the use of single-cell RNA sequencing of unique differentiation subsets ranging from $CD56^{bright}$ to adaptive $NKG2C^{+}CD56^{dim}$ NK cells. Transcriptional differentiation was concentrated within the surprisingly diverse $CD56^{bright}$ subset which gradually transitioned into $CD56^{dim}$ NK cells before terminal differentiation into adaptive $CD56^{dim}$ NK cells.

The vastly diverse yet unique NK cell repertoire within an individual is surprisingly stable over time considering the constant renewal of these cells at steady state. In **paper II**, we performed an in-depth analysis of homeostatic proliferation in human NK cells. We identified a high degree of intra-lineage plasticity combined with transcriptional reprogramming associated with the acquired phenotype as the underlying mechanisms maintaining repertoire stability at steady state.

In **paper III**, we examined the role of NK cells in a setting of perturbed homeostasis, namely patients with high-risk myelodysplastic syndrome undergoing immunomodulatory treatment with 5-azacytidine. We identified a role for 5-azacytidine in modifying the global NK cell repertoire, as uptake of the drug by proliferating NK cells resulted in increased expression of killer cell immunoglobulin-like receptors (KIR) and improved functionality.

In **paper IV** we identified a dose-dependent cytokine addiction in IL-15 expanded NK cells, leading to the induction of apoptosis upon cytokine withdrawal. A proliferation-dependent induction of the short splice variant of BIM, combined with an altered BCL-2/BIM ratio resulted in sensitization to cell death post withdrawal.

This thesis provides new insights into the dynamic nature of NK cell homeostasis, from understanding NK cell differentiation at the transcriptional level to perturbations after cytokine stimulation and immunomodulatory therapies.

LIST OF SCIENTIFIC PAPERS

I. **Pfefferle A**[*], Netskar H[*], Ask EH, Lorenz S, Sohlberg E, Clancy T[‡], Malmberg KJ[‡]. A temporal transcriptional map of human natural killer cell differentiation. *Manuscript*.

II. **Pfefferle A**, Jacobs B, Ask EH, Lorenz S, Clancy T, Goodridge JP, Sohlberg E, Malmberg KJ. Intra-lineage plasticity and functional reprogramming maintain natural killer cell repertoire diversity. *BioRxiv*. 2019. *Manuscript*.

III. Sohlberg E, **Pfefferle A**, Andersson S, Baumann BC, Hellström-Lindberg E, Malmberg KJ. Imprint of 5-azacytidine on the natural killer cell repertoire during systemic treatment for high-risk myelodysplastic syndrome. *Oncotarget*. 2015 Oct 27;6(33):34178-34190.

IV. Jacobs B, **Pfefferle A**, Clement D, Berg-Larsen A, Sætersmoen ML, Lorenz S, Wiiger MT, Goodridge JP, Malmberg KJ. Induction of the BIM short splice variant sensitizes proliferating NK cells to IL-15 withdrawal. *Journal of Immunology*. 2019 Feb 1; 202(3):736-746.

[*‡] These authors have contributed equally.

LIST OF ADDITIONAL RELEVANT PUBLICATIONS

I. Goodridge JP, Jacobs B, Sætersmoen ML, Clement D, Hammer Q, Clancy T, Skarpen E, Brech A, Landskron J, Grimm C, **Pfefferle A**, Meza-Zepeda L, Lorenz S, Wiiger MT, Louch WE, Ask EH, Liu LL, Oie VYS, Kjällquist U, Linnarsson S, Patel S, Taskén K, Stenmark H, Malmberg KJ. Remodeling of secretory lysosomes during education tunes functional potential in NK cells. *Nature Communications*. 2019 Jan 31;10(1):514.

II. Liu LL, Béziat V, Oie VYS, **Pfefferle A**, Schaffer M, Lehmann S, Hellström-Lindberg, E, Söderhäll S, Heyman M, Grander D, Malmberg KJ. Ex vivo expanded adaptive NK cells effectively kill primary acute lymphoblastic leukemia cells. *Cancer Immunology Research*. 2017 Aug;5(8):654-665.

III. Liu LL, **Pfefferle A**, Oie VYS, Björklund AT, Béziat V, Goodridge JP, Malmberg KJ. Harnessing adaptive natural killer cells in cancer immunotherapy. *Molecular Oncology*. 2015 Dec;9(10):1904-1917.

CONTENTS

LIST OF ABBREVIATIONS

5-aza	5-azacytidine
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
ATAC	Assay for transposase-accessible chromatin
BiKE	Bi-specific killer engager
BIM S	BIM short (splice variant)
BM	Bone marrow
CAR	Chimeric antigen receptor
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CIS	Cytokine induced SH2-containing protein
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CR	Complete remission
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
DR	Death receptor
Eomes	Eomesodermin
ER	Endoplasmic reticulum
Fab	Fragment, antigen-binding
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
Fc	Fragment, crystallizable
Fv	Fragment, variable
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GMP	Good manufacturing practice
GVL	Graft-versus-leukemia
HLA	Human leukocyte antigen
HMA	Hypomethylating agents
HR-MDS	High-risk myelodysplastic syndrome
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IPSS-R	International prognostic scoring system - revised
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
KIR	Killer-cell immunoglobulin-like receptor
LFA-1	Lymphocyte function-associated antigen 1
LR-MDS	Low-risk myelodysplastic syndrome
mAb	Monoclonal antibody
MAGIC	Markov affinity-based graph imputation of cells
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MICA/B	MHC class I polypeptide-related sequence A/B
MIP-1β	Macrophage inflammatory protein-1beta
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NCR	Natural cytotoxicity receptor
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PVR	Poliovirus receptor

RNA	Ribonucleic acid
scRNA-seq	Single-cell RNA sequencing
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
T_h	T helper
TNF	Tumor-necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TriKE	Tri-specific killer engager
T_{reg}	T regulatory
t-SNE	t-distributed stochastic neighbor embedding
ULBP	UL16 binding protein

1 INTRODUCTION

Every day we are exposed to countless attacks by pathogens, but thanks to our immune system we are blissfully unaware. Well most of the time. We experience symptoms when our immune system is activated, such as fever or a runny nose, but it is only in the rare instances when our immune system fails that we have to deal with the serious consequences. The army of cells protecting us from bacteria, viruses, fungi, protozoa, prions and cells that have gone rogue are termed leukocytes or white blood cells.

Our immune system can be divided into two main arms, termed the innate and the adaptive immune system. The innate immune system is our body's first line of defence against any new pathogen and it achieves this through its arsenal of defences, ranging from physical and chemical barriers to its own army of specialized cells. Innate immune cells include mast cells, phagocytes (macrophages, dendritic cells (DC) and neutrophils), basophils, eosinophils, γδ T cells, innate lymphoid cells (ILC) and natural killer cells. Together they identify and eliminate foreign substances that have entered the body, providing the main line of defence against any pathogen our body has never encountered before. Additionally, they train the adaptive immune system to remember this newly encountered pathogen. This allows the adaptive immune cells, comprised of T and B lymphocytes, to respond faster and more efficiently after any subsequent encounter with the same pathogen[1].

1.1 BASIC CONCEPTS OF NK CELL BIOLOGY

In the early 1970s a new granular cell type capable of killing tumor cells was described and aptly named natural killer (NK) cell[2–5]. True to their name, NK cells can unleash their stored cytotoxic potential to kill foreign, transformed or infected cells. Compared to other cytotoxic cells, NK cells are not restricted by the need for prior sensitization and furthermore have the ability to orchestrate the early phase of the adaptive immune response. These characteristics result in NK cells playing a key role in the innate immune system.

The frequency of NK cells in the blood of healthy adult humans is 5-20% of all lymphocytes. Within tissues, the frequency varies depending on tissue type, with NK cells found in significant numbers in the bone marrow, liver, lymphoid organs, lung and uterus[6]. In humans, NK cells are characterized by the expression of CD56 and lack of CD3 expression. Based on the surface density of CD56, they are further divided into $CD56^{bright}$ and $CD56^{dim}$ NK cells. The ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells varies depending on their location, with $CD56^{bright}$ NK cells predominantly found in secondary lymphoid organs and tissues, while $CD56^{dim}$ NK cells account for the majority (90%) of peripheral blood NK cells[7].

1.1.1 NK cell development

Our understanding of NK cell development has increased in recent years, updating the initial four stage model to include a 5th stage of development[8–11]. NK cell progenitors (stage 1) develop into pre-NK cells (stage 2), become immature NK cells (stage 3), followed by CD56^{bright} NK cells (stage 4a) that acquire NKp80 expression (stage 4b) and eventually differentiate into CD56^{dim} NK cells (stage 5)[11].

NK cells develop from CD34^{+} hematopoietic stem cells (HSC) and the common lymphoid progenitor (CLP) in the bone marrow, which also gives rise to other ILCs, as well as T and B cells[12]. Identification of NK cell precursors outside the bone marrow, namely fetal thymocytes (CD34^{+}CD3^{-}CD4^{-}CD8^{-}) and fetal liver cells (CD34^{+}CD38^{+}) has put into question whether their development is in fact restricted to the bone marrow[13,14]. Commitment to the NK cell lineage requires the transcription factors ID2 and E4BP4 along with IL-15 signaling[15–20]. The search for an NK cell restricted precursor cell has identified CD34^{+}CD38^{+}CD45RA^{+}CD7^{+}CD10^{+}CD123^{-}CD127^{-} cells which can give rise to T-bet^{+} and Eomes^{+} NK cells, two transcription factors comprising a central checkpoint for NK cell maturation in mice[21,22]. Expression of these two transcription factors induces CD122 (encoded by *IL2RB*) expression on NK cells, a component of both the IL-2 and IL-15 receptor allowing for survival and effector function signaling to occur[22,23]. The importance of IL-15 signaling in NK cell development is best observed through mutations in the receptor components (CD122, CD132) which, together with mutations in the downstream signaling molecules JAK3, present as immunodeficiencies characterized by a lack of NK cells[24–27].

1.1.2 NK cell killing

Upon target cell recognition, NK cells can exert their cytotoxic potential by forming an immune synapse and releasing their cytotoxic granules which contain pore-forming perforin and apoptosis-inducing granzymes. Target cell recognition can occur through direct recognition of the target cell mediated by activating and inhibitory receptors or through antibody-dependent cellular cytotoxicity (ADCC) mediated via ligation of the CD16 receptor expressed on CD56^{dim} NK cells[28]. Additionally, NK cells can induce apoptosis of target cells via death receptor (DR) ligation through Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) expression[28].

NK cells largely exert their cytotoxic effect through the release of perforin and granzyme containing cytotoxic granules. Cytotoxic granules belong to the secretory lysosomes and are formed through the fusion of different vesicular structures[29]. The two main components of cytotoxic vesicles are perforin and granzyme B, although CD56^{dim} NK cells can also produce

granzyme A and M, with CD56^{bright} NK cells producing granzyme K[30]. Produced in the endoplasmic reticulum (ER), perforin is sorted into granules through the Golgi complex and then cleaved by cathepsin L to be activated[31,32]. Once released at the immune synapse, perforin attacks the target cell's membrane, a process requiring calcium, and then oligomerizes, forming a pore for granzymes to enter the cell[33,34]. Within the cytotoxic granule, perforin is kept inactive through the pH and by binding to serglycin and calreticulin[35,36]. At the immune synapse, the NK cell's plasma membrane is protected from released perforin through the protein LAMP-1 (CD107a) which coats the membrane[37]. Formation of a pore in the target cell's membrane allows for granzymes to enter and induce apoptosis, both in a caspase-dependent and independent manner, leading to production of reactive-oxygen species as well as DNA and mitochondrial damage[38]. Granzymes are sorted into the cytotoxic granules as pro-enzymes which need to undergo further cleavage by cathepsins to become fully functional[39–42].

The activating receptor CD16, encoded by *FCERG3A*, allows NK cells to bind to the Fc-domain of IgG antibodies found on target cells while its intracellular tail can associate with FcRγ and the CD3ζ chain containing immunoreceptor tyrosine-based activation motifs (ITAM)[43]. This killing mechanism is termed ADCC and allows NK cells to identify and eliminate opsonized cells mediated via antibody production from B cells, an example of the innate and adaptive immune system coordinating their efforts. This mechanism of cell killing can also be utilized to regulate inflammation-associated immune responses by eliminating antigen presenting cells and T cells[44].

The final mechanism by which CD56^{dim} NK cells can induce apoptosis in target cells is via death receptor (DR) ligation. NK cells can express TRAIL and FasL on their surface, with TRAIL being the corresponding ligand for DR4 and DR5, and FasL ligating the Fas receptor (CD95). TRAIL-induced apoptosis is dependent on caspase 8 activation while FasL induces apoptosis through formation of the death-inducing signaling complex[45]. Both TRAIL and FasL can be upregulated upon type I interferon (IFN) stimulation, an example of how the cytokine environment, mediated by secretion from other immune cells (T cells, DCs, macrophages), can shape the NK cell response[46,47]. While type I IFNs increase cytotoxicity, IL-2 and IL-15 promote proliferation and survival in differentiated NK cells, with IL-12 and IL-18 enhancing IFNγ production by NK cells[44]. Tissue and tumor cells can also influence NK cells through the release of IL-10 and TGFβ, both of which suppress NK cell function[44]. Similarly, NK cells can produce cytokines, chemokines and even growth factors to influence their environment and direct the immune response. These include IFNγ, MIP1α, MIP1β, RANTES, CCL3, CCL4, CCL5 and GM-CSF[48,49].

1.1.3 NK cell receptors

An NK cell's response upon encountering another cell is based on the receptor mediated input received, or lack thereof (**Figure 1**). A combination of inhibitory and activating receptors expressed on their surface provide the necessary information to identify the encountered cell either as a healthy cell or a potential target. While the net signaling input determines the NK cell's response, in order to maintain tolerance, inhibitory signals dominate over activating signals. Major histocompatibility complex (MHC) class I molecules function as ligands for inhibitory receptors, allowing NK cells to sense 'self', whereby the loss of MHC class I on the cell surface triggers NK cell activation. This is termed the 'missing-self hypothesis' and was proposed by Kärre and Ljunggren in the late 80s[50]. In order to evade T cell-mediated killing, transformed cells downregulate MHC class I, which in turn sensitizes them to NK cell-mediated killing due to a lack of inhibitory signaling (**Figure 1**).

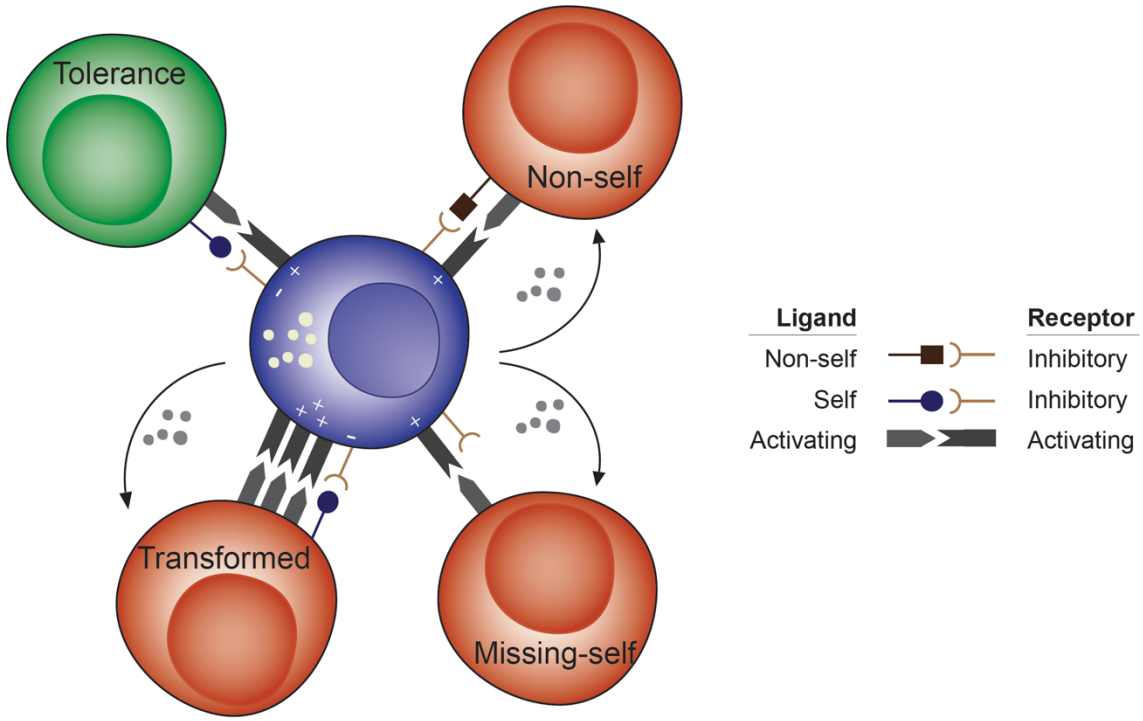

Figure 1. **Target cell interaction.** Overview of different functional outcomes of an NK cell (blue) encountering potential target cells (green, red), based on receptor input received through activating and inhibitory receptors.

1.1.3.1 Killer-cell immunoglobulin-like receptors

Killer cell immunoglobulin-like receptors (KIR) constitute the main group of inhibitory receptors expressed on human NK cells. Located on chromosome 19, stochastic expression of the KIR genes is epigenetically controlled via the KIR promoter[51]. The KIR nomenclature is based on the length of the cytoplasmic tail, short (S) or long (L), and the number of extracellular Ig-like domains (2 or 3). While the long cytoplasmic tail receptors contain immunoreceptor

tyrosine-based inhibitory motifs (ITIM), the short tails contain immunoreceptor tyrosine-based activation motifs (ITAM) that aid in binding to the adaptor molecule DAP12 (**Figure 2**). Phosphorylation of ITIMs on inhibitory receptors results in the recruitment of tyrosine-phosphatases which in turn dephosphorylate adaptor molecules associated with activating receptors[52,53]. This ensures inhibitory receptor signaling dominating over activating receptor signaling. KIR bind to specific allelic variants of human leukocyte antigen (HLA) A, B and C, the human equivalent of MHC class I proteins[54]. Non-classical HLA-F and HLA-G have also been identified as interacting with KIR receptors[55–60]. As the highly diverse KIR locus is both polygenic and polymorphic, many ligands for this large repertoire of KIR receptors still remain to be discovered[61].

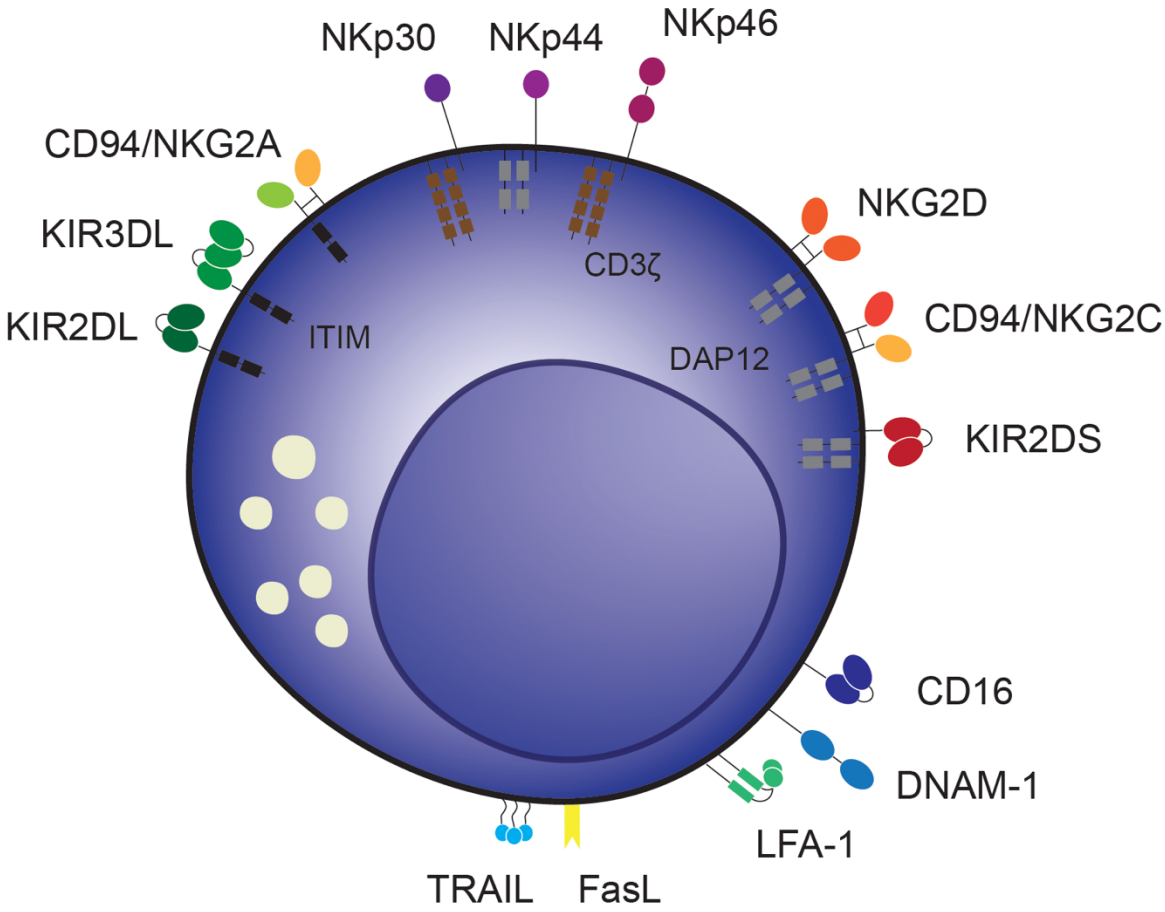

Figure 2. **NK cell receptors and ligands**. Visualization of the inhibitory/activating receptors and their intracellular signaling components, as well as ligands expressed on CD56^{dim} NK cells which are discussed in this thesis.

For simplicity, two KIR haplotypes are used to group KIR genotypes within individuals[62]. Haplotype A contains a restricted number of inhibitory receptors and one activating receptor, KIR2DS4. The less common haplotype B includes a larger repertoire of both inhibitory and activating receptors[61]. On top of the stochastic expression via epigenetic regulation of the KIR gene promoter, variation in terms of KIR gene copy number furthers adds to the diversity[63–65].

The three main inhibitory receptors commonly studied include KIR2DL1, KIR2DL3 and KIR3DL1. KIR2DL3 and KIR2DL1 bind to HLA-C allotypes with either an asparagine (C1) or lysine (C2) at position 80, respectively[66]. KIR3DL1 binds HLA-A and B with Bw4 at position 77-83[67]. Other notable KIR-ligand interactions include KIR3DL2 binding to HLA-A3/A11 and HLA-F and the activator receptors KIR2DS1 binding to HLA-C2 and KIR3DS1 binding to HLA-F[56,68,69]. Activating ligands, in particular, are still largely undiscovered.

1.1.3.2 NKG2-receptors

Within the C-type lectin NKG2-receptors, NKG2A-H exist and are located on chromosome 12[70,71]. NKG2A and NKG2C both form a heterodimer with CD94 despite NKG2A being an inhibitory receptor containing ITIMs and NKG2C being an activating receptor associating with DAP12 for signaling (**Figure 2**)[72,73]. They share a common ligand, HLA-E, with CD94/NKG2A having higher binding affinity compared to CD94/NKG2C[74]. Most likely this is to ensure tolerance. While NKG2C is mainly expressed by adaptive NK cells, NKG2A expression is associated with naïve NK cells, but can also be upregulated in activated NK cells in response to viral infection[75,76]. NKG2D is another activating receptor found on NK cells and is one of the few homodimers within the NKG2-receptor family. Ligands for NKG2D include ULBP1-4 and MICA/B which are upregulated on target cells experiencing cellular stress[77]. This makes NKG2D an important activating receptor aiding in tumor surveillance. NKG2E, like NKG2A/C, forms a heterodimer with CD94 and like NKG2F, its function is still largely unknown. NKG2B and NKG2H, meanwhile, are splice variants of NKG2A and NKG2E respectively[78].

1.1.3.3 Activating receptors

Along with NKG2C and NKG2D, a number of other activating receptors exist which play important roles in regulating the cytotoxic capability of NK cells. Notably these include CD16, DNAX accessory molecule-1 (DNAM-1) and the germ-line encoded natural cytotoxicity receptor (NCR) family consisting of NKp30, NKp44 and NKp46 (**Figure 2**).

DNAM-1, also known as CD226, functions both as a coactivating receptor for NK cells and as an adhesion molecule binding to the poliovirus receptor (PVR, CD155) and Nectin-2 (CD112), a tumor ligand[79]. DNAM-1 expression correlates with education, as well as adaptive-like NK cells in mice[80–82]. In humans, DNAM-1 expression is coordinated with lymphocyte function-associated antigen 1 (LFA-1) undergoing conformational changes, as they co-localize at the immune synapse[80].

NKp30 (*NCR3*) and NKp46 (*NCR1*) are ubiquitously expressed on resting NK cells in peripheral blood, while NKp44 (*NCR2*) is upregulated on activated NK cells in response to IL-

2 stimulation[83,84]. NKp46, evolutionarily conserved in mammals, contains two extracellular Ig domains, similar to Ig-like receptors, while NKp30 and NKp44 only contain one domain each. All three receptors signal via coupling to adaptor molecules, either FcεRIγ and CD3ζ (NKp30, NKp46) or DAP12 (NKp44)[73,78,85]. B7-H6, the ligand for NKp30, is expressed on tumor cell lines as well as on neutrophils and monocytes after toll-like receptor and pro-inflammatory cytokine stimulation[86]. Similar to CD16, NKp30 also has immune-regulatory functions on top of its important role in immune surveillance[86]. The ligands for NKp44 and NKp46 have been suggested to be viral hemagglutinins[87,88].

1.1.4 NK cell differentiation

A combination of phenotypic, functional and transcriptional studies identified immature CD56^{bright} NK cells as precursors of CD56^{dim} NK cells[8,89–91]. However, despite studies in mice lacking NK specific transcription factors, as well as lineage tracing in macaques and in humans with immunodeficiencies, it is still unclear how the numerous intermediate cell stages of NK cell differentiation are transcriptionally regulated and connected[92]. Although transcriptional NK cell studies are lagging behind, intermediate NK cell subsets have been well defined functionally (**Figure 3**).

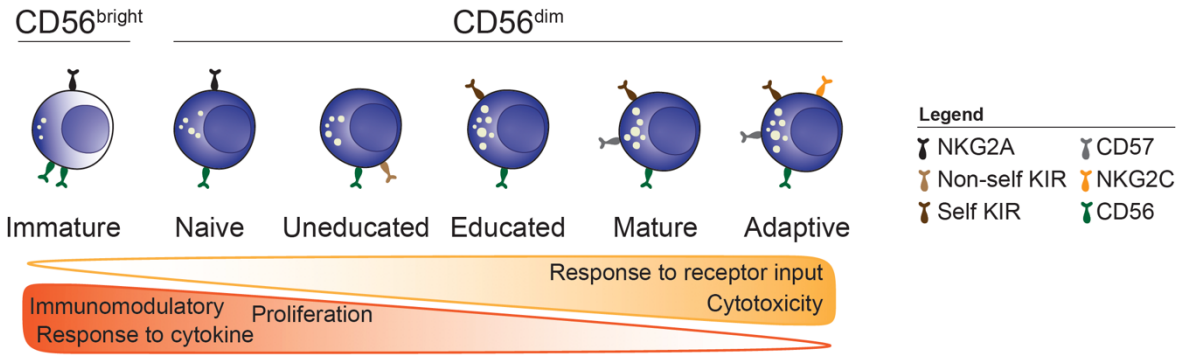

Figure 3. **NK cell subsets**. Overview of the distinct stages of NK cell differentiation based on phenotypic and functional properties.

Immature CD56^{bright} NK cells are highly responsive to cytokine priming and fulfill an immunoregulatory role. Expression of CCR7, CD62L, CXCR3, CCR5, CCR2 and CXCR4 allows CD56^{bright} NK cells to home to secondary lymphoid tissues, the liver, skin and the bone marrow, where they represent the dominant NK cell subset[6,93–96]. Conversely, cytotoxic CD56^{dim} NK cells, which prioritize activating and inhibitory receptor input over cytokine priming, mainly express CX3CR1 and CXCR1[95]. CD56^{dim} NK cells also have shorter telomers compared to CD56^{bright} NK cells, evidence for having undergone more cell divisions[97]. In line with this conclusion, CD56^{bright} NK cells have an increased proliferative capacity compared to CD56^{dim} NK cells. It has been shown that CD56^{bright} NK cells can acquire CD16 expression,

effectively transitioning into CD56^{dim} NK cells[97]. This was corroborated by the identification of an intermediate functional stage of NK cells, namely CD16^{+}CD56^{bright} NK cells which can account for up to 30% of CD56^{bright} NK cells in individual donors[98]. Furthermore, CD56^{bright} NK cells are the first lymphocyte population to reconstitute after stem cell transplantation, with CD16 acquisition, decreased surface expression of CD56 and cytotoxic effector functions following at a later time point[99–101]. However, in response to cytokine stimulation CD56^{dim} NK cells have also been observed to adopt a 'bright-like' phenotype via upregulation of CD56 expression[102].

Within the CD56^{dim} NK cell population, further distinctions of individual subsets based on phenotypic and functional characteristics can be made. Without a transcriptional basis, a defined differentiation path remains to be determined, with subsets instead being placed on a spectrum of maturation and functionality[103]. Cells expressing NKG2A are found on the immature end of the spectrum, in line with CD56^{bright} cells being NKG2A^{+}. Expression of KIR is associated with further differentiation, giving rise to educated and uneducated NK cells with varying functional potential. Generally, NKG2A and KIR are inversely expressed, but co-expression does occur. CD57, a carbohydrate epitope of unknown binding, is associated with terminal maturation, reduced proliferative capacity and increased functional potential[104]. Although the combination of NKG2A, KIR and CD57 expression is commonly used to define NK cell subsets in humans, this is a simplified model considering that up to 100,000 unique subsets exist within healthy individuals[105]. At the mature end of the spectrum is a unique group of NK cells termed adaptive or memory-like NK cells[106,107]. Adaptive NK cells can be found in approximately 40% of cytomegalovirus (CMV) seropositive individuals, whereby CMV accelerates the generation of this mature and highly functional subset[108–113]. Due to its heightened cytotoxic capacity and its longevity, this subset is of great interest for adoptive cell therapy and has therefore been the focus of recent work[114]. They are characterized by single self-KIR expression, epigenetic downregulation of intracellular signaling molecules, expression of the activating receptor NKG2C and the terminal maturation marker CD57[108].

1.1.5 NK cell homeostasis

For a long time, NK cells were assumed to be a population of cells with a short lifespan, high turnover and a stable phenotype and function. These beliefs have since been abandoned with new discoveries shedding light on their intricately regulated functionality and vast diversity. Although NK cells belong to the innate immune system, many aspects of T cell biology share a striking similarity with NK cells[115].

IL-15 is the main cytokine required for NK cell development, but also for survival, proliferation, metabolism and functionality. Immune cells, including DCs, monocytes and

other non-hematopoietic cells trans-present IL-15 on the IL-15Rα chain, which binds to the heterodimer consisting of IL2Rβ (CD122) and the common γ-chain (CD132) found on the NK cell's surface. Downstream signaling is mediated via JAK1/3, allowing for recruitment and activation of the transcription factor STAT5, a survival signal for NK cells[27]. A downstream target of STAT5 is the cytokine induced SH2-containing protein (CIS, encoded by *CISH*), which functions as a negative feedback loop by inhibiting the upstream JAK1[116]. *Cish*$^{-/-}$ knockout mice presented with increased anti-tumor activity and proliferative capacity as a result of being hyper-responsive to IL-15 signaling[116]. In an attempt to better understand the impact of IL-15 receptor signaling on proliferation, mathematical modeling was implemented. Increasing the expression of IL-15Rα on the cell surface accelerated the formation of IL-15/IL-15R complexes, particular at low IL-15 concentrations[117]. Once an IL-15 saturation level had been reached, no further augmentation of the proliferative response was achieved.

However, it was unclear how a single cytokine, such as IL-15, could have such a broad and varying effect on NK cell homeostasis as a whole. The identification of the role metabolism plays in regulating activation and functionality of immune cells shed some light on the importance of IL-15 signaling. Mouse studies identified a dose-dependent downstream signaling pathway, where high dose IL-15 activated the mammalian target of rapamycin (mTOR) as well as STAT5. mTOR, a serine/threonine kinase consisting of the two complexes mTORC1 and mTORC2, is a master regulator in cells. mTORC1 senses the microenvironment for nutrients to control metabolism while mTORC2 is involved in controlling the cytoskeletal organization of the cell[118–120].

Metabolic reprogramming due to environmental cues has been identified as a key regulator mechanism behind immune cell differentiation and function in NK cells and other immune cells[118–122]. In mice, increased cytokine priming led to metabolic reprogramming, as the cells increased their metabolic activity, thereby switching their energy source from oxidative phosphorylation to glycolysis. An increase in metabolism allowed for IFNγ and granzyme B production, conferring increased functionality which could be reversed through the use of rapamycin, an mTOR inhibitor[119]. These studies could be repeated in mice using murine CMV infection instead of IL-15 signaling, proving that viral infection could also activate mTOR leading to metabolic reprogramming[122]. In both studies, along with increased functionality, increased proliferation was also observed. In a tumor setting, a lack of available glucose due to high glycolytic activity by the tumor cells could lead to functional inhibition due to lack of mTOR activation[119,123].

1.1.6 NK cell education

NK cell education is the process whereby NK cells are functionally tuned via inhibitory interactions mediated between self-MHC and KIR or NKG2A. This is further fine-tuned by the signal strength determined by the number of inhibitory interactions[57,124]. As NK cells do not undergo positive or negative selection, it was initially assumed that they would express a minimum of one inhibitory receptor in order to maintain tolerance to self[125]. Disproven by the discovery of $NKG2A^{-}KIR^{-}$ cells in mice and humans, this population of NK cells was found to circulate in a hypo-responsive state, thereby ensuring tolerance to self[126–128]. Furthermore, NK cells have the ability to undergo re-education after transfer from one MHC class I environment to another, further validating the need for sustained inhibitory interactions in order to retain functionality[129,130].

Despite education being a dynamic process that forms an important cornerstone in NK cell functionality, the intracellular mechanism underlying education remained elusive until recently. Multiple models were proposed, including the arming, the disarming and the rheostat model without a general consensus being reached[57,131,132]. Discriminating between educated and uneducated NK cells required a functional readout or sequencing of the HLA genes, as no phenotypic readout existed. Recent work from our lab identified granzyme B retention as a sensitive and specific phenotypic readout for education, putting the core cytolytic machinery itself in the spotlight in the search for a potential underlying mechanism behind NK cell education[133]. Transcriptionally, educated NK cells are identical to uneducated NK cells, but phenotypically they accumulate granzyme B in dense-core secretory lysosomes located close to the centrosome. After target cell interaction, these large granules containing granzyme B were released, in line with increased cytotoxicity compared to uneducated cells lacking these particular granules. Pharmacological inhibition of the protein kinase PIKfyve and genetic silencing of its downstream target, the lysosome-specific calcium channel TRPML1, suggested a model where unopposed activating receptor input leads to remodeling of the lysosomal compartment and loss of dense-core secretory lysosomes in cells that lack self-specific receptors. Downstream of such morphological changes, signaling from acidic calcium stores may fine-tune the cell's functional potential through inter-organelle communication with the endoplasmic reticulum.

In addition to mediating NK cell functionality via modulation of the cellular metabolism leading to increased granzyme B expression, mTOR may serve as a functional rheostat during NK cell education[118,134]. Educated NK cells exhibited higher basal mTOR activity, which was further increased upon activating receptor ligation and also correlated with the number of inhibitory receptors expressed. Expression of SHP-1, a phosphatase required to convert

inhibitory receptor input into functional responsiveness, was required for increased mTOR activity in educated cells[135]. Conversely, continuous activating receptor input in the absence of inhibitor input dampened mTOR activity. Although education is not transcriptionally regulated in human NK cells, mTOR activity is dependent on its localization to the lysosomal compartment which in turn can be negatively regulated by TRPML1[136,137].

1.2 NK CELLS IN THE DISEASE SETTING

1.2.1 Myelodysplastic syndrome

Myelodysplastic syndrome (MDS) is a group of clonal stem cell disorders characterized by aberrant HSC differentiation within the bone marrow (BM) (**Figure 4**). As a result, MDS patients develop various cytopenias depending on the exact differentiation block, leading to an increased risk of disease progression to acute myeloid leukemia (AML). MDS progresses to AML in approximately one third of patients. Although cigarette smoke, benzene exposure and previous chemo- or radiotherapy treatment can cause MDS, it generally occurs as an age-related disease, with the average age of onset being 76 years[138–140].

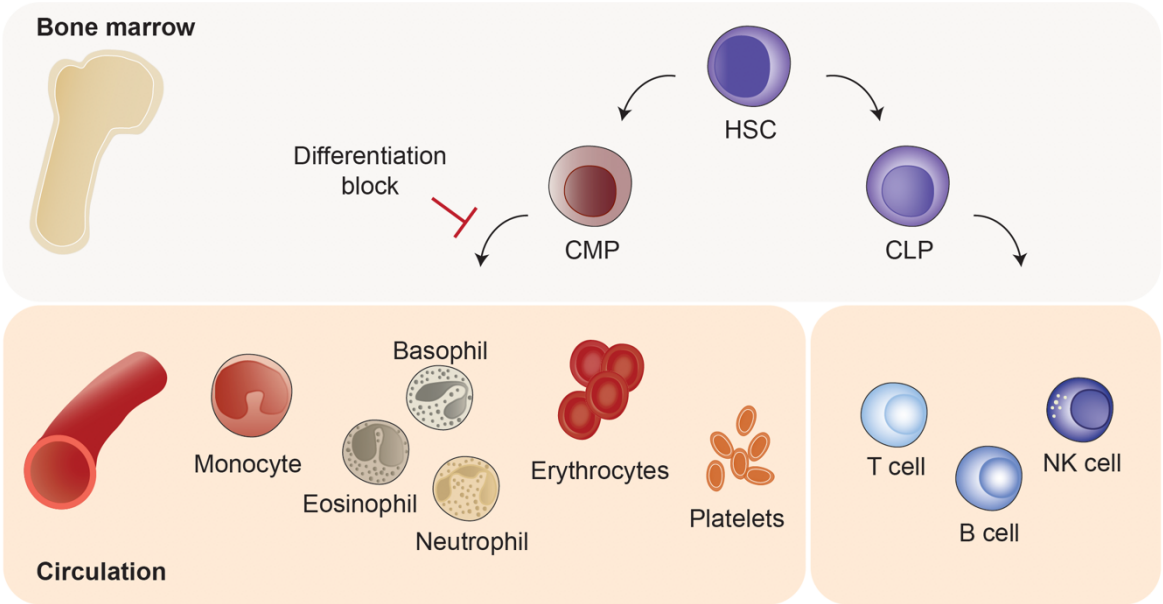

Figure 4. **Hematopoietic stem cell differentiation**. Simplified overview of the main differentiation steps from a hematopoietic stem cell (HSC) to the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) as well as the immune subsets they give rise to within the periphery. MDS arises due to a differentiation block downstream of the CMP whereby patients develop various cytopenias depending on the exact location of the block.

1.2.1.1 Prognosis, risk groups and treatment

MDS is a very heterogenous disease as its manifestation is influenced by a variety of mutations leading to varying differentiation blocks, hence resulting in very variable outcomes for

patients[141]. The revised-international prognostic scoring system (IPSS-R) groups patients into risk groups based on their predicted outcome[142,143]. This is based on five parameters, namely hemoglobin, platelet count, neutrophil count, BM blast percentage and cytogenetics, creating four risk groups[142]. Treatment options are based on these risk groups, whereby the two lower risk groups, referred to as low-risk MDS (LR-MDS), are grouped together and the two higher risk groups, high-risk MDS (HR-MDS), are grouped together. LR-MDS patients usually present with various cytopenias, which are treated with either growth factors or lenalidomide, depending on the genetic mutations[144,145]. HR-MDS patients have a poor prognosis as the median survival is less than one year if the disease goes untreated[142,146]. Treatment consists of hypomethylating agents (HMA), with the aim of delaying the onset of AML and thereby prolonging survival time[147]. The only available cure for HR-MDS patients is an allogeneic hematopoietic stem cell transplant (HSCT), but due to the late age of onset, other comorbidities and the risks associated with a HSCT, many patients do not qualify[148–150]. For many HR-MDS patients, HMAs are therefore the standard treatment, which consist of either 5-azacytidine (5-aza) or decitabine[151]. 5-aza has been shown to increase survival by 9.5 months on average, but only 50% of patients are responding to this treatment[151,152]. Furthermore, failure after HMA treatment is common, and although switching to a different HMA or to lenalidomide is being investigated, no good standard treatment options remain for these patients[153–157].

1.2.1.2 5-azacytidine

Although 5-aza is commonly used to treat HR-MDS patients, its exact mechanism of action remains elusive[158]. 5-aza is a cytidine analogue lacking a methylation site that can incorporate during replication in RNA and DNA. Additionally, it has cytotoxic properties and can therefore directly affect malignant cells. MDS patients often have silenced tumor suppressor gene promotors through hypermethylation of important CpG sites of these genes. Hence, 5-aza's hypomethylating properties are assumed to be the main mechanism of action in the MDS setting[159,160].

It has been proposed that 5-aza may also directly affect the immune system, allowing for better immunological control of the malignant clones[161]. Here NK cells are of particular interest, considering their cytotoxic capabilities, high turnover and methylation-sensitive regulation of their effector function via inhibitory and activating receptor input. Methylation has been shown to inhibit ligand expression for activation receptors on NK cells, such as NKG2D[162]. Furthermore, KIR genes are expressed in their de-methylated state, as the KIR promoter is epigenetically regulated[163]. Hence NK cell mediated control of the malignant clone may be a contributing mechanism of action of 5-aza considering their high turnover *in vivo*, allowing for uptake of the drug, resulting in a potentially modified NK cell repertoire.

1.2.2 Adoptive NK cell therapy

In 1909 Paul Ehrlich first proposed his hypothesis of cancer immunosurveillance, a hypothesis that would not be proven until many years later[164,165]. Mouse studies cast light on the power the immune system possesses in eliminating cancerous cells, whereby mice lacking immune cells showed increased susceptibility to chemically induced tumors[165]. From these initial ground-breaking studies, the concept of immunotherapy was developed, which has exploded in recent years, from being recognized as the 'Breakthrough of the Year' in 2013 by *Science* magazine, to James P. Allison and Tasuku Honjo being awarded the Nobel Prize in Medicine in 2018 for their work on immune checkpoint inhibitors.

1.2.2.1 The concept of immunoediting

Constant interactions between immune cells and potential malignant cells allow these threats to be eliminated before they can overpower the body in the form of cancer. This process is termed immunoediting and consists of three phases, immunosurveillance, equilibrium and escape[166]. During immunosurveillance, immunogenetic tumors, tumors that are sensitive to immune cell killing, are eliminated. In the equilibrium phase, non-immunogenic tumors co-exist with immune cells which are constantly exerting a selective pressure on the tumor cells. This allows the survival of immune escape variants, which during the escape phase can further develop to form a cancerous tumor by evading detection[166]. Mouse studies have identified a central role for NK cells in immunosurveillance. Compared to $RAG2^{-/-}$ mice lacking adaptive immune cells, $RAG2^{-/-}$ x $\gamma c^{-/-}$ mice which also lacked NK cells developed chemically induced sarcomas more rapidly[167].

1.2.2.2 NK cells and the tumor microenvironment

The tumor microenvironment is a hostile place. Cells within this environment, including fibroblasts and infiltrating immune cells, are remodeled to aid in tumor development and reduce immune cell function[168]. Myeloid-derived suppressor cells and T_{regs}, as well as the release of TGFβ, adenosine, prostaglandin E2 and IDO are all able to dampen NK cell cytotoxicity at the tumor site[168–175]. Furthermore, NK cells first need to home to the tumor microenvironment, a challenge in itself. Together, this results in poor NK cell infiltration in many solid tumors[176]. Hematological malignancies and settings of metastasis provide a more favorable environment for NK cells to exert their cytotoxic potential[177]. In particular, NK cells have great potential in eliminating minimal residual disease, characterized by quiescent cancer-stem cells which are resistant to standard treatments[178].

1.2.2.3 NK cells in HSCT and adoptive cell therapy

In the setting of hematological malignancies where patients are treated with HSCT, NK cells are the first lymphocyte population that can be detected following engraftment[179]. Their ability to mediate graft-versus-leukemia (GVL) effects is vital for elimination of residual disease, as increased number of NK cells after transplantation result in better treatment outcome[180,181]. Insights into the specificity of NK cell alloreactivity, determined by specific combinations of KIR and HLA, paved the way for the ground-breaking discovery of a potential role of NK cells in mediating GVL in haploidentical HSCT against AML[61,177]. Studies aiming at harnessing NK cell alloreactivity in the context of HSCT have recently been reviewed[182,183]. The indication that NK cells may deliver a potent GVL effect in the setting of HSCT inspired the whole NK cell community to develop adoptive NK cell therapy based on transfer of 'KIR ligand mismatched' NK cells across HLA barriers to promote missing self-recognition. Whereas many studies did not find a beneficial effect of genetic KIR ligand mismatch, calculation of the functional dose of KIR ligand mismatched NK cells was associated with less relapse after NK cell therapy against AML[184–186]. Currently there are 397 open clinical trials exploring different types of NK cell products for a variety of diseases (clinicaltrials.gov).

NK cells utilized for adoptive cell-based therapies are usually cytokine-primed and often expanded to ensure activation of effector functions and to obtain the required cell numbers. A variety of activation and expansion protocols have been proposed and tried in clinical settings, usually relying on supra-physiological levels of cytokines, include any combination of IL-2, IL-15, IL-12 and IL-18[187,188]. One negative effect of stimulation with high levels of cytokines is the reduction the cells experience in cytokine concentration upon infusion, as severe side-effects prevent patients from being treated with the same cytokines[189–192]. Studies in non-human primates given daily doses of *in vivo* human IL-15 treatment resulted in an initial expansion of NK cells starting on day 8 and peaking at day 13-15. However, after IL-15 treatment was stopped on day 12, NK cell numbers quickly diminished back to baseline by day 22[193]. In line with these findings, one major bottleneck with adoptive NK cell therapy has been ensuring persistence after infusion to create a time-window long enough for the activated NK cells to eliminate their targets[194]. Another downside of using cytokines to drastically induce NK cell proliferation is the naïve phenotype achieved by these expansion protocols[114]. Proliferation capacity decreases with NK cell maturation and correlates inversely with functionality[103]. Furthermore, highly expanded NK cells have reduced metabolic activity, further affecting their functionality[118]. Focus has now shifted towards guided-expansion protocols and genetically modified NK cells, not only resulting in large cell numbers but also in specific phenotypes and functional properties[114].

1.2.2.4 Modulating NK cells to enhance anti-tumor functionality

A number of different methods, other than cytokine priming, are currently being investigated to increase NK cell anti-tumor functionality in adoptive cell therapy. These range from the use of monoclonal antibodies (mAb) to chimeric antigen receptors (CAR) to bi- and tri-specific killer engagers (BiKE, TriKE)[195,196]. mAbs, such as trastuzumab, cetuximab and rituximab, are already used in the clinic to successfully treat a variety of tumors[197]. Treatment effect is mediated by the Fab fragment inhibiting surface receptors on the tumor itself, which are important for survival, while the Fc portion is able to bind to CD16 on NK cells, resulting in ADCC[198]. In lymphoma patients treated with rituximab, a mAb against CD20 expressed on B cells, treatment outcome correlated with increased NK cell numbers in the blood[199]. Another use of mAbs has been to block the inhibitory NKG2A receptor on NK cells. Many tumor types upregulate HLA-E expression, the ligand for NKG2A on NK cells[200]. This can result in NK cell inhibition whereby blocking would help unleash their cytotoxic potential, which has been demonstrated in clinical trials using monalizumab[201].

CARs, originally developed for T cells, have been applied to NK cells for redirecting their cytotoxic capacity towards specific tumor targets. Compared to T cells, NK cells have the advantage that they are short-lived, avoiding the need for a suicide gene, and that they can recognize targets having downregulated MHC class I[202,203]. On the other hand, NK cells have proven to be difficult to transfect and the half-life of the CAR has also been a limiting factor in utilizing this treatment effectively in the clinic[204,205].

Another recent development is the design of BiKEs, and more recently TriKEs[206]. BiKEs consist of fusing the Fv portions of mAbs recognizing a tumor specific antigen, such as CD133, to CD16[207]. TriKEs, which have shown increased ADCC and cytokine release compared to BiKEs, utilize IL-15 to link the two Fv domains[208]. These small molecules allow for redirected lysis of tumor cells by directly cross-linking CD16 on NK cells and have shown promising results in *in vitro* models and *in vivo*[196].

Although NK cell immunotherapy has made a huge leap forward in the past decade, better understanding NK cell biology in a homeostatic setting will provide knowledge that can be implemented to improve current therapies and develop future treatment strategies.

2 AIMS

This thesis aimed to gain insights into the fundamental mechanisms that shape human NK cell homeostasis and to understand how NK cell repertoire diversity influences outcomes of immunomodulatory therapies.

Paper I. NK cell diversity stems from a combination of differentiation, homeostatic interactions and adaptive responses to the environment. In **paper I** we aimed to identify the regulatory gene-circuits driving functional diversification and specialization during NK cell differentiation.

Paper II. An individual's NK cell repertoire is made up of a unique combination of subsets and is stable over time. In **paper II** we set out to identify how NK cell repertoire diversity is maintained during homeostatic proliferation by delineating cellular and molecular programs involved.

Paper III. A standard treatment for high-risk myelodysplastic patients is 5-azacytidine, a hypomethylating agent with an unknown mechanism of action. NK cells have a high-turnover *in vivo* and KIR expression on NK cells is epigenetically regulated via methylation of the promoter regions. This paper aimed to investigate if *in vivo* cellular uptake of 5-azacytidine could be monitored in NK cells through repertoire changes and determine the functional consequences of *in vitro* uptake in proliferating NK cells.

Paper IV. Protocols used for adoptive NK cell therapy often involve supra-physiological levels of IL-15 to induce large-scale expansion. Upon transfer into patients, the cytokine-dependent cells undergo sudden cytokine withdrawal resulting in the induction of apoptosis. In this paper we set out to study the molecular mechanisms of IL-15 withdrawal in NK cells.

3 RESULTS AND DISCUSSION

3.1 NK CELL DIFFERENTIATION

Classification of individual NK cell subsets is based on phenotypic and functional characteristics with the exact differentiation pathway still under debate. Clear functional and phenotypic differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells identified these as the two main NK cell subsets[10,97,209]. Further characterization of $CD56^{bright}$ NK cells identified them as the probable immature precursor to $CD56^{dim}$ NK cells[8,89–91]. Despite being commonly accepted, this has not been proven to date. A study in macaques using NK cell lineage tracing attempted to challenge this assumption, stating that $CD56^{bright}$ and $CD56^{dim}$ NK cells represent two distinctly separate lineages[92]. Due to rather large differences in the NK cell biology between macaques and humans, including receptor repertoires and definition of $CD56^{bright}$ and $CD56^{dim}$ subsets, these results need to be interpreted with caution.

3.1.1 The regulome of human NK cell differentiation as we knew it

Transcriptionally, NK cell differentiation has not been as well described. Although mouse studies have identified the importance of T-bet and Eomes in the differentiation step from immature $CD27^{+}CD11b^{-}$ to mature $CD27^{-}CD11b^{+}$ NK cells, the downstream signaling pathway remains to be characterized[22]. Other transcription factors involved in NK cell differentiation include ZBTB32, IRF2 and IKZF3 which were identified through mouse models[210–212]. Bulk sequencing, combined with ChIP sequencing, of human $CD56^{bright}$ and $CD56^{dim}$ NK cells identified the TCF1-LEF-MYC axis within the $CD56^{bright}$ population and the PRDM1-MAF-ZEB2 axis within $CD56^{dim}$ NK cells[213]. The recent rise in single-cell technologies also saw the commercialization of single-cell RNA sequencing (scRNA-seq). The first scRNA-seq study in human NK cells was focused on characterizing the heterogeneity within peripheral blood and organs in both mice and humans, without going in detail into NK cell differentiation[214]. In **paper I** we generated a unique scRNA-seq dataset to delineate the temporal transcriptional regulation of human NK cell differentiation.

3.1.2 A temporal transcriptional map of NK cell differentiation

Healthy donor buffy coats were screened for education status and the presence of adaptive NK cells. From each donor we FACS sorted six populations from freshly isolated NK cells, namely $CD56^{+}$ (bulk), $CD56^{bright}$, $NKG2A^{+}CD56^{dim}$, self $KIR^{+}CD56^{dim}$ (educated), non-self $KIR^{+}CD56^{dim}$ (uneducated) and either adaptive NK cells or self $KIR^{+}CD57^{+}CD56^{dim}$ NK cells depending on the donor. Transcriptionally, the five sorted NK cell subsets covered the entire transcriptional landscape of bulk $CD56^{+}$ NK cells. We therefore focused our analysis on the

individual subset samples which provided equal cell numbers for analysis, which was vital for the CD56^{bright} NK cells as they are found only in low frequencies within the blood. Confirming phenotypic and functional studies, we identified two main transcriptional islands which corresponded to the CD56^{bright} and CD56^{dim} NK cell populations. Intriguingly, they were connected by a narrow bridge which, based on RNA velocity analysis (**BOX 1**), identified a transition from the CD56^{bright} to CD56^{dim} island[215]. This was further corroborated by pseudotime analysis (**BOX 1**) which provided a time component to the expression patterns of individual genes[216].

BOX 1. Single-cell RNA sequencing analysis

RNA velocity

Single-cell RNA sequencing data only provides a snapshot in time, but the amount of spliced and unspliced mRNA of individual genes within cells is indicative of the rate at which gene splicing and degradation is occurring. The ratio between spliced and unspliced mRNA can therefore be used to calculate a high-dimensional vector termed RNA velocity, which provides the time derivative of expression states of individual genes. RNA velocity can therefore be implemented to predict the future state of each cell in terms of time, adding directionality to a traditional t-SNE plot to help identify cell lineages.

Pseudotime

Since differentiation is asynchronous, single-cell RNA sequencing provides a snapshot of cells at different differentiation stages. These cells can then be ordered along differentiation trajectories based on their gene expression, which is termed pseudotime. The Palantir algorithm orders cells in pseudotime based on possible identified differentiation trajectories, whereby the probability of each cell to differentiate into each terminal state is identified. This provides the relative distance of each cell from the initially identified starting cell.

Surprisingly, CD56^{bright} NK cells dominated the transcriptional timeline, whereby two out of three transcriptional checkpoints occurred within this small population. These transcriptional checkpoints represent a stage in differentiation where gene expression is tightly controlled, potentially mediated by important transcription factors to progress to the next stage of differentiation (**Figure 5**). Global gene trends identified increased variation in the late stage of pseudotime, corresponding with CD56^{dim} differentiation, as CD56^{dim} specific gene trends were to a certain degree uncoupled from CD56^{bright} dominating global trends. Furthermore, despite having only sorted NK cells with very high CD56 expression for the CD56^{bright} subset, we could identify two unique transcriptional clusters within this population while CD56^{dim} NK cells distributed over only three clusters despite the larger phenotypic and functional diversity within this second population. Transitioning from cluster 1 (early CD56^{bright}) to 2 (late CD56^{bright}) was associated with a decrease in gene expression, while cluster 3 and 4 within the conventional CD56^{dim} population were similar in transcription, with one cluster representing an activated version of the other. Adaptive cells formed a third CD56^{dim} cluster which also contained the terminal cell identified by pseudotime analysis.

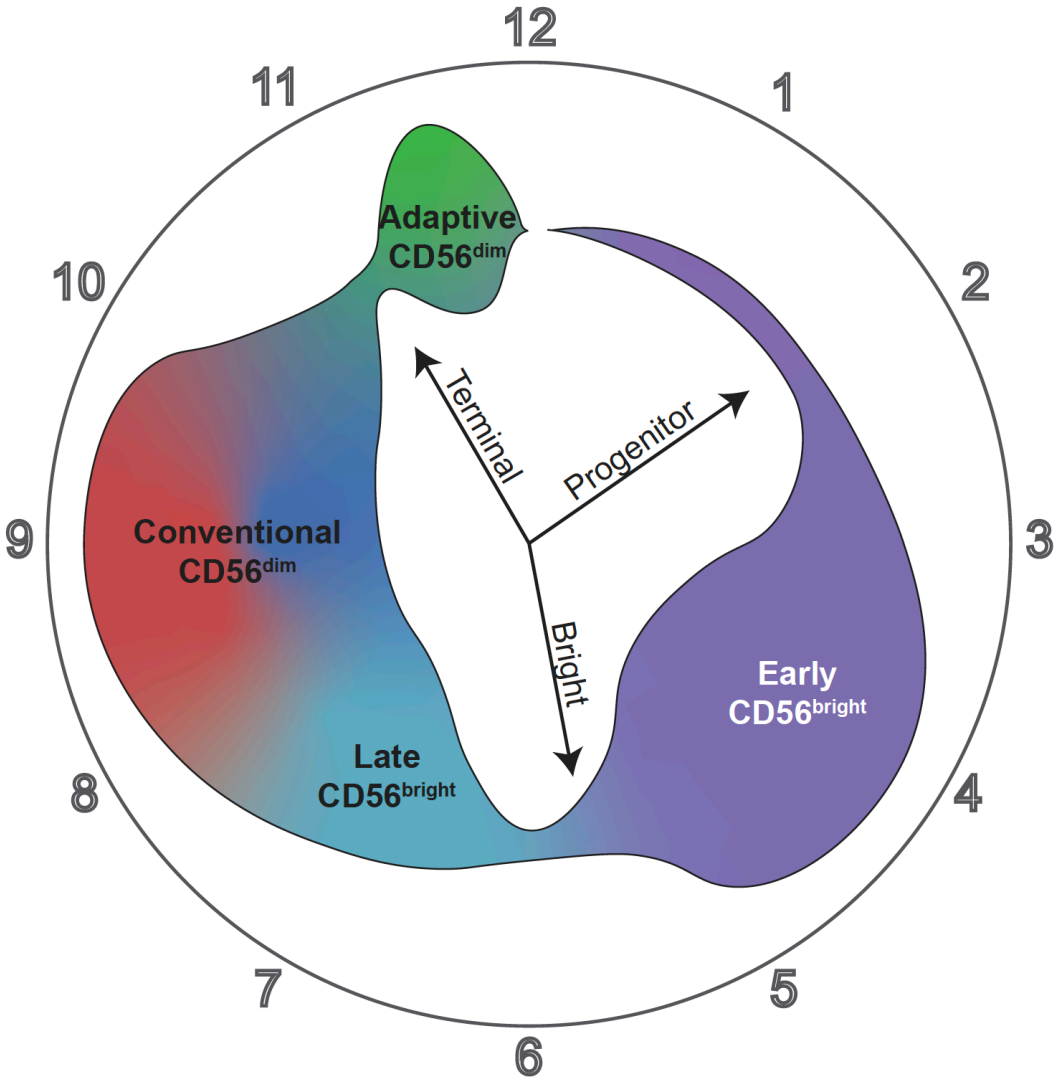

Figure 5. **Summary of paper I**. A clock model of NK cell differentiation, denoting the transcriptional clusters in time along with the differentiation checkpoints. The arms of the clock indicate the three transcriptional checkpoints, the color coding refers to the transcriptional clusters and the 'time' is indicative of pseudotime.

3.1.3 The bridge connecting CD56^{bright} to CD56^{dim} NK cells

We identified a substantial proportion of NKG2A^{+}CD56^{dim} NK cells exhibiting a CD56^{bright} transcriptional profile. These unique cells were concentrated near the bridge but could also be identified within the early CD56^{bright} cluster in pseudotime. Although we cannot exclude that a small fraction of NKG2A^{+}CD56^{bright} NK cells contaminated this sample based on the sorting gate, the low frequency of CD56^{bright} NK cells within the total NK cell population prior to sorting cannot account for this observation. Examination of the most proximal cells on each side of the bridge region identified a significant proportion of sorted NKG2A^{+}CD56^{dim} NK cells prior to the transition. The bridge transition itself was therefore transcriptionally 'non-dramatic' with major transcriptional changes occurring just prior to this region as identified by RNA velocity.

3.1.4 Formation of the functional template for education

In line with previous reports in mice and human, stratification of NK cells based on education, e.g. the expression of self or non-self KIRs, did not reveal any transcriptional differences between the two subsets[133]. Our lab recently described that inhibitory interactions during education are associated with non-transcriptional remodeling of the lysosomal compartment, which accounted for the increased functionality in educated NK cells through the accumulation of dense-core secretory granules. These findings led us to perform a global analysis of genes associated with lysosomal biogenesis, expression of which was increased within the $CD56^{dim}$ transcriptional island, with a gradual increase from early to late $CD56^{bright}$ NK cells. Furthermore, genes important for vesicle formation and trafficking, such as *RAB27A*, were higher expressed within the $CD56^{dim}$ population, with highest expression identified in the activated $CD56^{dim}$ cluster. Mutations in *RAB27A* cause Griscelli syndrome type 2, resulting in a degranulation effect[217], as Rab27a is recruited to the lytic granules by LFA-1 stimulation, aiding the granule in docking to the plasma membrane[218,219]. Hence, $CD56^{dim}$ NK cells are poised for modulation of the lysosomal compartment mediated via inhibitory and activating receptor input received at the cell surface, resulting in fine tuning of their functionality.

3.1.5 Methodological considerations for scRNA-seq analysis

Our scRNA-seq dataset allowed us to identify a transcriptional timeline for NK cell differentiation which only partially overlapped with the phenotypic model. Most importantly, the data highlighted the heterogeneity and the important contribution of $CD56^{bright}$ NK cells to the differentiation process. Sorting of individual subsets prior to sequencing combined with the single-cell resolution was essential in making these observations, but also provided some challenges. Compared to other immune cells, resting NK cells are transcriptionally inactive. Furthermore, the 10X Genomics single-cell sequencing platform we used in this study is less sensitive in terms of gene transcripts detected per cell when compared to other platforms such as Smart-seq2 which generates full-length cDNA libraries[220]. The combination of these two results in many zero values in the obtained data, which are difficult to deal with, as it is not obvious whether these represent missing values or actual zero expression of the genes. With the recent rise in scRNA-seq datasets being generated, the bioinformatic pipelines dealing with the downstream analysis of these immense datasets are rapidly developing and improving. In particular, algorithms aimed at inferring missing values within scRNA-seq datasets due to technical limitations of the sequencing have being developed[221].

We implemented the Markov affinity-based graph imputation of cells (MAGIC) algorithm (**BOX 2**) in order to be able to visualize gene expression across the t-distributed stochastic neighbor embedding (t-SNE) map generated[222]. While MAGIC and other similar algorithms are immensely valuable by reducing the number of gene dropouts due to missing values within individual cells, data generated by them needs to be interpreted with caution. We did observe differences in expression of NK cell associated genes between our donors, which could be due to false imputations by MAGIC. It is important to point out that this only concerned a small subset of genes investigated, with the majority showing identical expression patterns. Furthermore, t-SNE analysis, PhenoGraph-based clustering (**BOX 2**), differential gene expression analysis by SCDE (**BOX 2**), RNA velocity and calculation of pseudotime by Palantir downstream of choosing the starting cell was performed without MAGIC imputation[215,216,222–224]. Lastly, we are validating the MAGIC imputed gene expression through bulk RNA sequencing results, allowing us to discriminate between true zero expression genes and falsely imputed values.

BOX 2. Analysis algorithms

MAGIC

A computational method for identifying gene expression within individual cells in scRNA-seq that was lost in the sequencing process due to drop-out. MAGIC utilizes information gained from neighboring cells to restore gene expression successfully through imputation, effectively maintaining original cluster structures while restoring two- and three-dimensional gene interactions.

PhenoGraph

PhenoGraph identifies cellular populations within single cell data, taking the high-dimensionality of the dataset into account. Compared to dimensionality reduction algorithms reducing single-cell data to two-dimensions, PhenoGraph instead implements a graph-based method to identify densely-connected nodes representing individual populations.

SCDE

A statistical method, based on the Bayesian interpretation of probability, to identify differential gene expression within single-cell RNA sequencing data. SCDE effectively reduces the background noise typical of scRNA-seq data by fitting error models to individual data points, thereby improving identification of differential gene expression between cell groups.

3.2 NK CELL HOMEOSTASIS

At the donor level, the NK cell repertoire is vastly diverse and unique[105]. However, once the NK cell repertoire has fully formed, it is well-maintained over time considering the rather rapid turnover of the cells[108,225]. Proliferation therefore plays an important role in replenishing the NK cell pool at steady state and in maintaining a stable repertoire. How homeostatic NK cell proliferation leads to subset repertoire stability was investigated in **paper II**. We examined the perturbed NK cell homeostasis in a cohort of MDS patients undergoing immunomodulatory treatment in **paper III**, characterizing how proliferation in combination with 5-azacytidine

modulated the NK cell repertoire. In **paper IV** we examined the implications of cytokine stimulation for immunotherapeutic purposes.

3.2.1 Subset repertoire stability

Proliferation has mainly been examined in the viral or disease setting, despite being one of the essential processes NK cells undergo regularly[225]. Recent interest in NK cell expansion protocols for adoptive cell therapy have shone a spotlight on the importance of understanding this fundamental process. In **paper II** we asked the question of how proliferation is able to maintain stable NK cell repertoires at steady state. We hypothesized that the observed stability was either the result of self-renewal from an immature pool of progenitor cells followed by differentiation or the result of intra-lineage plasticity. Intra-lineage plasticity (**BOX 3**), defined as phenotypic and functional changes occurring within a given cell lineage, has been observed in other immune cells[226,227]. Although environmental influences on NK cell functionality, such as cytokines, chemokines, growth factors and immunosuppressive molecules, are well known, NK cell plasticity has largely remained unexplored[228].

BOX 3. Cellular plasticity
Plasticity refers to phenotypic and functional changes occurring within populations of cells. Intra-lineage plasticity, also known as functional plasticity, refers to cells of a given lineage adapting to their surroundings in response to cytokine or receptor input which is translated into transcriptional changes resulting in an altered phenotype and modified functionality. An example of this is macrophages transition between an M1 and M2 phenotype, T cells transitioning from T_h to T_{reg} phenotype or ILC subsets transitioning between ILC1-3 phenotypes.

In **paper II** we combined high-resolution flow cytometry and fluorescence-activated cell sorting (FACS) with scRNA-seq to delineate the cellular and molecular changes occurring at the single cell level during homeostatic proliferation. 16-color flow cytometry provided a population-based readout with single-cell resolution allowing for high-dimensional assessment of phenotype, functionality, division state and intra-cellular signaling. Furthermore, we utilized scRNA-seq (10X Genomics) to identify transcriptional changes associated with varying proliferation kinetics in a defined subset of NK cells and compared these to baseline transcriptional signatures of individual NK cell subsets.

3.2.1.1 An in vitro model for homeostatic NK cell proliferation

We developed a simple model with minimal external influences in order to generate robust data allowing us to interpret the role IL-15 plays in inducing NK cell proliferation. Purified NK cells obtained from isolated peripheral blood mononuclear cells (PBMC) from healthy blood donors were cultured in the presence of low-dose IL-15. IL-15 was chosen due to its central role in regulating NK cell homeostasis. All components of the cell culture protocol were optimized,

including dose and dosing schedule of IL-15, starting cell concentration, medium source and replenishment schedule, as well as the presence or absence of feeder cells to induce a linear onset of proliferation with maximal subset retention to mimic homeostatic proliferation. This was as opposed to inflammation-induced or lymphopenia-induced proliferation, which is associated with rapid cell turnover resulting in subset skewing towards naïve NK cells with higher proliferative potential[114]. We implemented a cell tracking dye to monitor the onset of proliferation and subsequent cell divisions, allowing us to stratify our readouts by the number of divisions a cell had undergone.

3.2.1.2 Subset retention through intra-lineage plasticity

In order to induce proliferation in both naïve and terminally mature NK cell subsets, daily addition of 5ng/mL IL-15 was required. 5ng/mL was low enough to prevent excessive proliferation of naïve NKG2A$^+$ NK cells, but high enough to induce mTOR activation as well as linear cell proliferation at the rate of one division per 24 hours, after the initial onset on day 3. Interestingly, 10ng/mL every two days did not yield the same results, evidence of IL-15 being tightly regulated *in vivo*. While subset distribution at the bulk population level only minimally changed over the course of 7 days, we did observe subset-specific proliferation kinetics which correlated with mTOR activation. IL-15-induced mTORC1 upregulation prior to proliferation onset could predict downstream proliferation three days later at both the donor and subset level. Repeated sampling of the same blood donors over time confirmed stable NK cell repertoires, but also an intrinsic metabolic set point determining the level of mTOR activation in response to IL-15 stimulation, accounting for the donor-specific proliferation kinetics observed.

Despite subset-specific proliferation kinetics, the actual subset frequencies at the population level remained largely stable. This suggested that the repertoires were maintained through intra-lineage plasticity during homeostatic proliferation *in vitro* (**BOX 3**)[226,227]. Indeed, adding an additional step of sorting individual NK cell subsets prior to our proliferation protocol revealed a surprising degree of cellular plasticity in both naïve and mature subsets. Further analysis identified functional changes associated with the acquisition of NKG2A and CD57, whereby NKG2A acquisition was associated with increased proliferative potential and decreased functionality, while the reverse was true for CD57 acquisition. Surprisingly, even previously assumed terminally differentiated CD57$^+$ NK cells could acquire a naïve phenotype (NKG2A$^+$) and start proliferating, provided that CD57 expression was lost. This functional dichotomy between NKG2A$^+$ and CD57$^+$ cells skewed the subset distribution within individual generations. The functional CD57$^+$ cells predominantly identified as slowly cycling cells (0-1 cell divisions after 5 days) while NKG2A$^+$ cells identified as rapidly cycling cells (≥2 cell

divisions after 5 days). Rapidly cycling cells therefore exhibited lower cytotoxic potential compared to slowly cycling cells. It is important to remember that this is a pure cytokine stimulatory environment, effectively priming the cells but lacking all receptor-based input from other cells. CD56^{bright} NK cells are very cytokine receptive but poorly cytotoxic, while cytotoxic CD56^{dim} NK cells require activating and inhibitory receptor input. Hence, rapidly cycling cells may acquire functional potential through further receptor input. In our setting, rapidly cycling educated NK cells underwent transcriptional reprogramming, resulting in a more immature signature, while slowly cycling educated NK cells acquired a more mature signature when compared with baseline subsets.

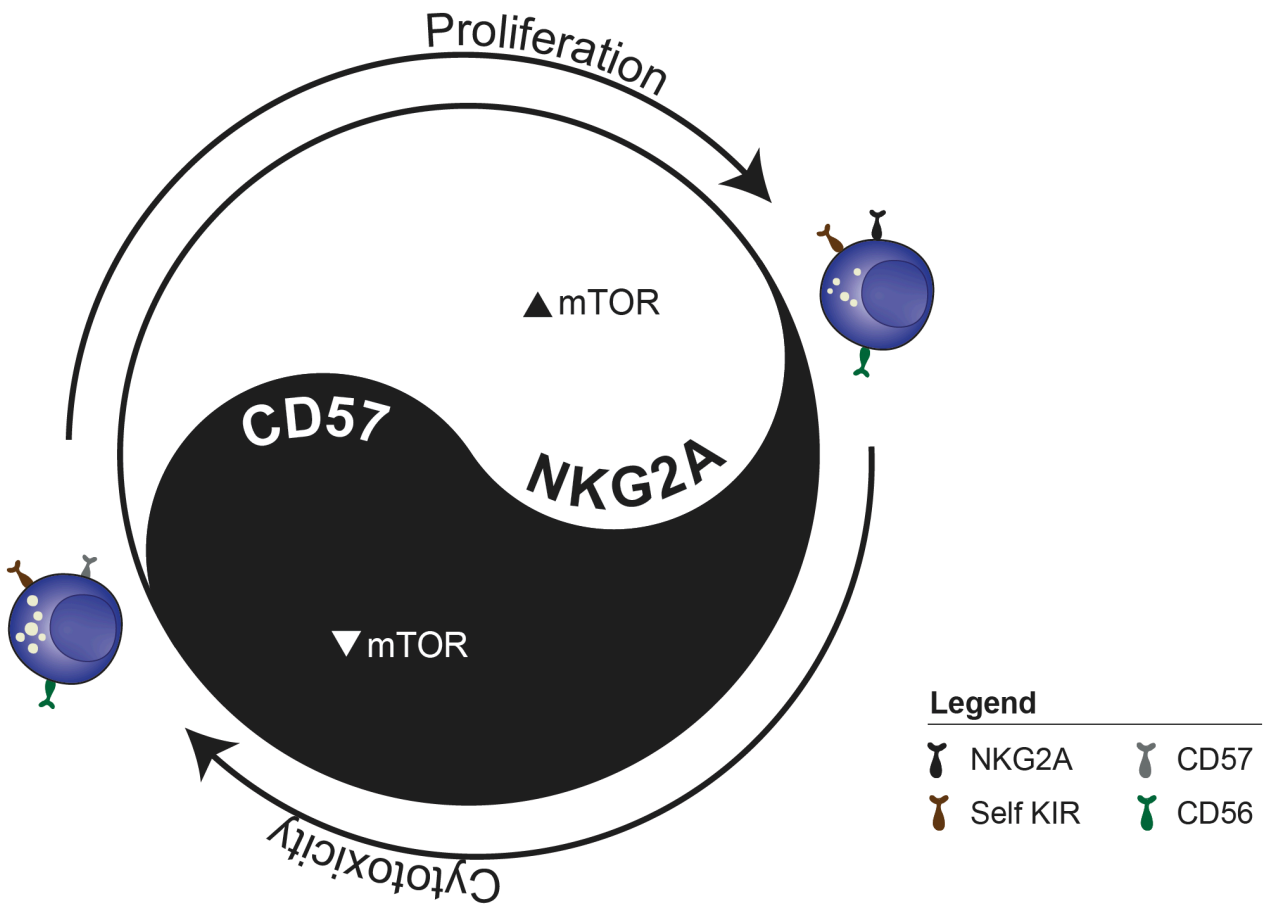

Figure 6. **Summary of paper II**. The functional dichotomy between proliferation and cytotoxicity observed during IL-15-induced homeostatic proliferation.

Our simplified *in vitro* homeostatic NK cell proliferation model allowed us to examine the central role IL-15 plays in maintaining NK cell homeostasis (**Figure 6**). The balance between strength and timing of the IL-15 signal determined the responsive subsets, whereby the degree of downstream mTOR activation dictated the proliferative response. CD57 expression negatively influenced mTOR activation and proliferation. Although it is used as a main marker for subset discrimination in NK cells, the function of CD57 remains unknown[104,229]. It is not a

receptor or protein in itself, but rather a carbohydrate epitope created by an enzyme called B3GAT1 on other cell surface proteins. In neural cells, CD57 has mainly been associated with adhesion proteins, while binding to the IL-6 receptor has also been proposed[230]. It would be interesting to further delineate how CD57 is associated with these cellular changes, whether it plays a functional role by inhibiting or activating another surface protein, or if it is simply a surrogate marker for other ongoing cellular modifications.

In addition to the differential mTOR activation in distinct NK cell subsets, we also identified a donor intrinsic component which was stable over time and thus contributed to the stability in terms of subset repertoires observed within individuals. Identifying the mechanism behind this intrinsic component would be of great value for understanding and modulating the proliferative capacity of NK cells. This is particularly true in the setting of adoptive cell therapy, where the choice of suitable HLA-matched donors is almost always limited and cellular expansion to obtain sufficient cell numbers is a necessity.

Lastly, although our readouts in this study allowed us to examine NK cell proliferation at the single-cell level, we were not able to visually observe cellular division or functional interactions with target cells. This would be of particular interest considering the asymmetric PI3K and mTOR activity post-cell division observed in T cells and its role in controlling their differentiation fate[231–236]. Based on the induced transcriptional signature in rapidly cycling cells, which included both RNA-modifying metabolic genes and actin filament organization genes, the loss of functionality in rapidly cycling cells may be due to underlying deficits at the immune synapse. Conjugate formation experiments combined with F-actin staining at the site of the immune synapse would further shed light on the loss of functionality observed.

3.2.2 Perturbations in the disease setting

Differences in terms of proliferation speed, phenotype and functionality between homeostatic and spontaneous proliferation have been investigated in murine T cells[237–239]. Spontaneous proliferation, occurring in severely lymphopenic mice, was characterized by a rapid onset of cell division which was cytokine-independent. Homeostatic proliferation, on the other hand, occurred in mildly lymphopenic mice at a slower division rate and required both cytokine and T cell receptor (TCR) stimulation. The proliferation-induced phenotype was reverted after removal of the proliferation cues and cytotoxic capacity of $CD8^+$ T cells was lost during the initial phase of intense proliferation (12 days)[237–239].

In humans, one disease associated with various cytopenias is MDS, which has the potential to progress to AML. 5-aza is able to induce a clinical response in 50% of the patients, which has mainly been attributed to its demethylating effects of previously methylation-silenced

genes[159,160]. However, 5-aza can also be considered an immunomodulatory drug leading to immunological control of the malignant clone and thereby delaying disease progression[240]. 5-aza requires uptake by the cells in order to exert its hypomethylating effects which is achieved via incorporation into DNA and RNA during cell division[241,242]. NK cells can kill stressed and malignant cells, have a relatively high turnover rate *in vivo* and possess epigenetically regulated inhibitory receptors, namely KIR, which are important in fine-tuning their function. This makes them an interesting immune cell to further investigate to decipher the mechanism by which 5-aza can mediate immunological control of malignant clones in MDS.

3.2.2.1 KIR induction partially restores altered NK cell repertoires in MDS patients

In **paper III** we monitored the KIR repertoire pre- and post-5-aza treatment in a cohort of high-risk MDS patients. We hypothesized that NK cells could be used to determine *in vivo* uptake of the drug through modulation of their KIR repertoire mediated by 5-aza. This was complemented by *in vitro* functional studies, replicating the 5-aza treatment regimen and using IL-2 stimulation to mimic the cytopenic environment *in vivo* and induce proliferation.

High-risk MDS patients presented with perturbed NK cell repertoires characterized by higher frequencies of naïve NK cells within the $CD56^{dim}$ compartment, in line with lower KIR expression and increased proliferation. Notably, after five days of 5-aza treatment, the frequency of Ki-67^{+} NK cells returned to baseline levels as observed in healthy controls. 5-aza treatment induced KIR expression, particularly KIR2DL3 and KIR3DL1, as well as co-expression of multiple KIRs which was most evident in proliferating cells, many of which were $NKG2A^{+}CD57^{-}$. 5-aza therefore could partly restore the mature NK cell repertoire in MDS patients. Our *in vitro* studies confirmed KIR upregulation, particularly in $NKG2A^{+}CD57^{-}$ proliferating cells. This is in line with 5-aza needing to be incorporated into DNA through cell division for it to mediate its hypomethylating effects[243].

3.2.2.2 Increased NK cell functionality post-5-aza treatment

In addition to demethylating effects on the malignant clone, 5-aza also modulates the NK cell repertoire which is perturbed in MDS patients, characterized by higher proliferation compared to healthy controls. In agreement with our functional results in **paper II**, cells having undergone multiple rounds of cell division after six days of IL-2 stimulation exhibited lower degranulation (CD107a) and IFNγ production. Intriguingly, this loss of functionality was reversed with the addition of 5-aza. This can be partially attributed to the reduced proliferation observed in 5-aza treated cells and the phenotypic maturation of the cells through acquisition of KIR (**Figure 7**). However, the increase in functionality is most likely a direct result of demethylation of genes mediating effector function. Identification of the NK cell specific

targets of 5-aza through assay for transposase-accessible chromatin (ATAC) sequencing would provide important information on how to boost NK cell functionality in proliferating cells. This would have important functional implications for expansion protocols prior to adoptive cell therapy, which has been identified as a potential treatment option for HR-MDS patients having failed HMA treatment. An adoptive NK cell therapy trial in HR-MDS patients showed the potential of NK cells to control the malignant clones after lymphodepletion treatment[185]. Importantly, patients entering remission, despite multiple rounds of failed standard therapy, could be bridged to a HSCT, the only cure currently available for MDS. This highlights the potential NK cells have at controlling MDS given the correct microenvironment, as well as cellular activation level and state.

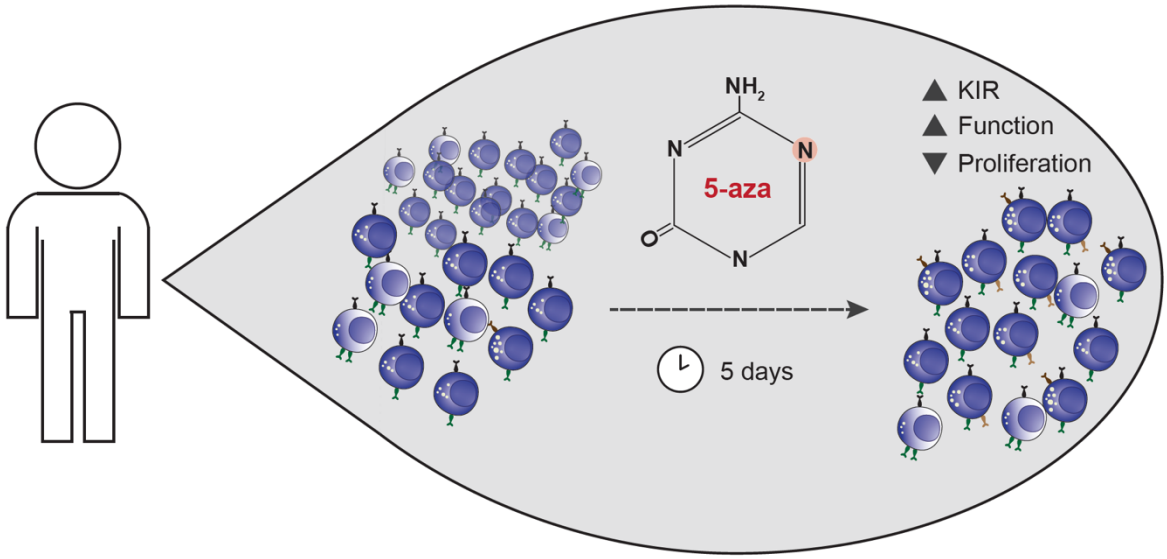

Figure 7. **Summary of paper III**. The effect of 5-aza treatment on the NK cell repertoire, resulting in decreased proliferation and increased KIR expression and functionality.

3.2.3 Implications for immunotherapy

In both **paper II** and **paper III**, cytokine-driven proliferation is associated with dramatic phenotypic and functional changes to the NK cell repertoire. In the setting of immunotherapy, this is of particular interest considering the current expansion protocols utilized to generate adoptive NK cell products for therapy. Expansion protocols for therapy often include supra-physiological levels of cytokines, including IL-15, to achieve the required target cell numbers[188]. However, this results in severe and acute cytokine deprivation post-infusion as continued stimulation through IL-15 injections are not feasible due to severe side-effects. Considering how tightly IL-15 is controlled, made evident in **paper II**, this is not surprising. It does however lead to poor cell persistence in the treated patient, often resulting in a time window that is too short to mediate the maximum or even desired effect. In **paper IV**, we set

out to characterize the mechanism behind IL-15 addiction and withdrawal in expanded NK cells.

3.2.3.1 An in vitro model to study cytokine-dependence

The backbone of this study was to implement the proliferation model developed in **paper II** and adapt it to study effects of cytokine withdrawal post IL-15 induced proliferation/activation. We first wanted to see if NK cells could become addicted to cytokine stimulation (**BOX 4**) and if this was dependent on the dose of IL-15 used for priming. 1ng/mL of IL-15 was sufficient to provide a survival signal and even induced very low levels of proliferation and was therefore chosen as the low-dose. For the high-dose, 10ng/mL of IL-15 was chosen, which induced rapid proliferation resulting in subset skewing towards a naïve phenotype as predicted. Compared to **paper II**, IL-15 was administered only every 48 hours together with complete renewal of the medium. These rather minor modifications induced large phenotypic changes at the subset level, again highlighting how minor changes in IL-15 alone can have profound effects on NK cells in culture. In line with the increase in KIR expression observed in cytokine-induced proliferation, combined with the acquisition of NKG2A in proliferating cells, a dramatic increase in the $NKG2A^{+}KIR^{+}CD57^{-}$ subset was observed.

BOX 4. Cellular addiction
Cytokine priming results in intracellular signaling changes occurring within cells. Continuous stimulation with non-physiological cytokine levels can result in an altered cellular state, which requires further cytokine stimulation to support survival. This can be referred to as cytokine-dependence or addiction, whereby cytokine withdrawal can lead to detrimental consequences to the cell.

To identify if six days of IL-15 stimulation was sufficient to induce cytokine dependence, we cultured the cells for an additional 48 hours after complete cytokine removal and compared this to a control arm receiving continued cytokine stimulation. Addiction, translating to a decrease in cell number due to the induction of apoptosis (as measured by the induction of caspase-3 expression), was observed in the cells having undergone withdrawal and this was dose-dependent. Cells addicted to high-dose IL-15 exhibited the biggest drop in cell number, which correlated with proliferation, whereby subsets expressing NKG2A were most affected.

3.2.3.2 The balance between pro- and anti-apoptotic molecules

Numerous pro- and anti-apoptotic genes make up the apoptosis network and it is the fine balance between these two opposing forces that dictates the outcome of the cell during various types of stimulations[244]. Within resting NK cells, BCL-2 has been identified as an important anti-apoptotic protein which can be further upregulated through IL-15 stimulation, leading to downstream STAT5, but not mTOR activation[118,194]. In actively proliferating NK cells,

MCL-1 expression is vital in maintaining viability[245]. BIM is a pro-apoptotic molecule and its downstream target BAX is directly inhibited by BCL-2[246]. In murine effector $CD8^+$ T cells, increased BIM levels are balanced by increased BCL-2 levels, expression of which dictates the amount of BIM that can be tolerated[247]. Similarly, in murine NK cells, the BCL-2/BIM ratio was influenced by IL-15 stimulation and withdrawal, whereby changes in the ratio could render the cells sensitive to cell death[247–249]. In line with these mouse studies, we observed an IL-15 dose-dependent increase in BCL-2, MCL-1 and also BIM expression. BCL-2 and MCL-1 were both crucial for survival in NK cells stimulated with high-dose IL-15 as shown through blocking experiments.

After cytokine withdrawal, the expression of anti-apoptotic proteins decreased over 48 hours, leading to an altered BCL-2/BIM ratio due to a less substantial decrease in BIM expression. Further investigation into the splice variants of BIM revealed preferential upregulation of the BIM short (BIM S) splice variant with IL-15 stimulation, one of the potent apoptosis-inducing splice variants[250,251]. BIM S was preferentially upregulated in proliferating cells stimulated with high-dose IL-15 and remained highly expressed until 24 hours after cytokine withdrawal. When compared to BCL-2 levels, which halved 24 hours after cytokine withdrawal, this severely altered the pro/anti-apoptotic ratio, exposing rapidly cycling cells to high levels of toxic BIM S within 24 hours after cytokine withdrawal (**Figure 8**).

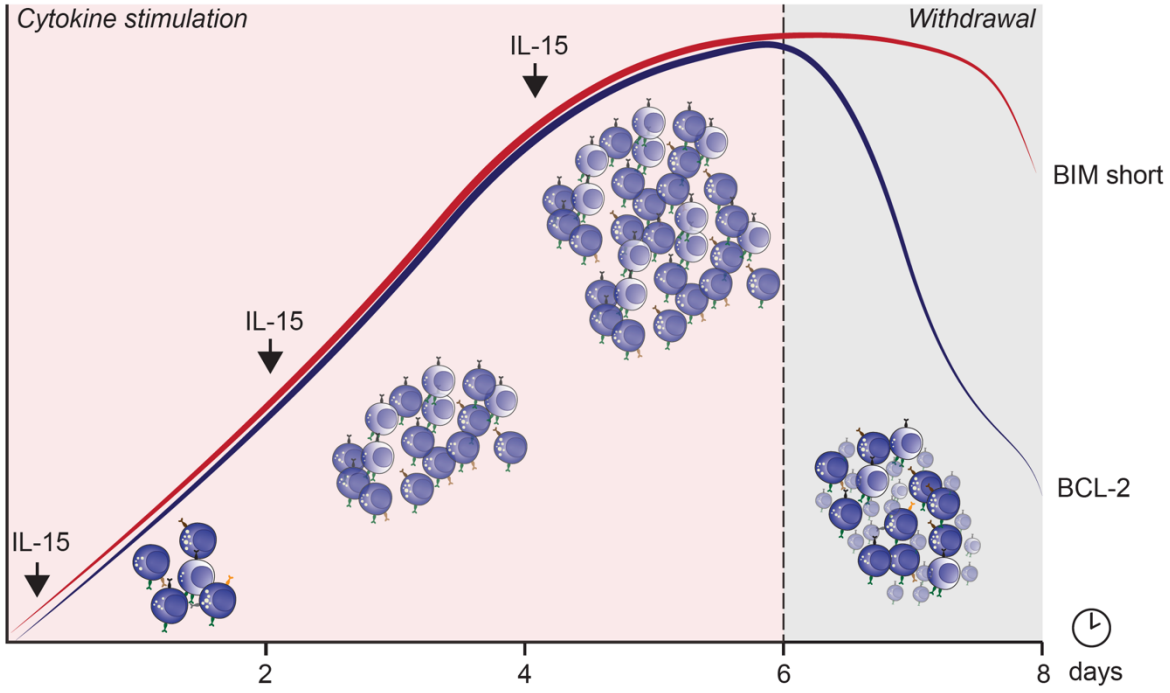

Figure 8. **Summary of paper IV**. The mechanism by which apoptosis is induced in cycling NK cells after IL-15 induced cytokine dependence and subsequent withdrawal. The curves represent expression of BIM short (red) and BCL-2 (blue) over culture time.

In **paper IV** we identified a mechanism by which apoptosis is induced within 48 hours in IL-15 stimulated rapidly cycling NK cells undergoing cytokine withdrawal (**Figure 8**). This has potentially important implications for current cell therapy protocols in which NK cells are expanded with high levels of IL-15 prior to infusion into the patient. As in **paper II**, this study used purified NK cell cultures with only cytokine stimulation. While we did not evaluate the inclusion of feeder cells on the BCL-2 and BIM expression levels, studies based in mice observed similar upregulation of these apoptotic proteins in response to cytokine simulation *in vivo*[248]. Irrespective of the culturing conditions, it may be helpful to monitor the levels of BCL-2 and BIM in expansion protocols to avoid the induction of apoptosis upon transfer into the patient.

3.2.3.3 A metabolically optimized NK cell activation protocol for adoptive cell therapy

Our group has previously completed a Phase I/II clinical trial where patients with primary chemotherapy-refractory MDS, secondary AML (MDS/AML) and *de novo* AML were infused with short-term IL-2 activated haploidentical NK cells[185]. Of the 16 patients infused, a complete remission (CR), marrow CR, or partial remission was observed in six patients. Infusion of the NK cell product allowed for five patients to be bridged to an allogeneic HSCT, with three patients still remaining disease free more than five years after treatment. This study identified MDS as a promising target for adoptive NK cell therapy, as five of the six patients responding to treatment had refractory or secondary MDS. Unfortunately, we could not achieve an expansion of infused NK cells which has been shown to correlate with disease clearance in AML[184,252]. Limited NK-cell chimerism could be observed in all evaluable responders but was only evident in 50% of non-responders. Hence, improving NK cell persistence post-infusion could potentially lead to improved clinical outcome.

Based on the findings in **paper II** and **paper IV**, this led us to develop and validate an IL-15 based clinical activation protocol designed to induce proliferation without leading to cytokine dependence. We hypothesize that by infusing NK cells undergoing homeostatic proliferation, they would be able to continue proliferating within the patient, leading to better persistence and functional outcome.

By stimulating NK cells daily for two days with 4 ng/mL IL-15, we were able to induce homeostatic proliferation. In order to avoid IL-15 dependence and decreased functional potential through transcriptional reprogramming in rapidly cycling cells, the amount of IL-15 was reduced to 2 ng/mL on day 3, followed by two more days of culture without any additional cytokine addition. The cells would then be harvested on day 6 and infused into the patient. This protocol induced steady proliferation which was maintained during the final two days without extra cytokine addition. We also did not observe a decrease in functionality without additional

cytokine stimulation in the final two days. It is important to point out that this was not a setting of cytokine withdrawal, as the medium was not refreshed. Residual amounts of IL-15 still present in the media most likely provided the necessary survival cues to the proliferating cells. This is a more physiologically relevant environment for the product prior to infusion and yielded sufficient NK cells numbers to be used for treatment. We have completed the GMP validation runs and are hoping to treat the first high-risk MDS patient in the near future.

4 CONCLUDING REMARKS

This thesis provides new insights into the dynamic nature of NK cell homeostasis, from understanding NK cell differentiation at the transcriptional level to perturbations after cytokine stimulation and immunomodulatory therapies. Listed below is a summary of the key findings from each of the four papers.

- $CD56^{bright}$ NK cells consist of two distinct transcriptional populations dominating the transcriptional timeline of NK cell differentiation, including two out of three transcriptional checkpoints identified (**paper I**).
- Transcriptionally $CD56^{bright}$ NK cells gradually transition into $CD56^{dim}$ NK cell which undergo a further transcriptional checkpoint prior to terminal maturation into adaptive NK cells (**paper I**).
- Global repertoire diversity is maintained through a high degree of intra-lineage subset plasticity during IL-15-driven homeostatic proliferation *in vitro*, whereby subset-specific proliferation kinetics correlate with mTOR activation (**paper II**).
- Subset plasticity at the phenotypic level is tightly linked to the functional fate of the cell and associated with transcriptional reprogramming defining the acquired phenotype (**paper II**).
- *In vitro* 5-aza treatment has profound and replication-dependent effects on KIR expression and NK cell functionality towards tumor target cells (**paper III**).
- Increased frequencies of KIR^{+} NK cells in MDS patients undergoing 5-aza treatment indicates drug uptake during *in vivo* cell division (**paper III**).
- *In vitro* expansion of human NK cells with IL-15 leads to a dose-dependent addiction, resulting in caspase-3 induced apoptosis due to a dysregulated BCL-2/BIM ratio following IL-15 withdrawal (**paper IV**).
- Withdrawal-induced apoptosis in IL-15 activated NK cells was linked to a proliferation-dependent induction of BIM short, a pro-apoptotic splice variant of BIM (**paper IV**).

5 FUTURE OUTLOOK

NK cells circulate in a pre-primed state full of effector molecules, such as granzyme B and perforin, and have a natural ability to kill cancer cells. Based on their cytotoxic capacity they hold great potential in the clinic as a cancer treatment, made evident by the number of ongoing clinical trials. However, to date most completed and ongoing clinical trials are based on the transfer of cytokine-activated polyclonal NK cell populations from donors with very variable NK cell repertoires. To fully harness the clinical potential of NK cells, future trials need to be founded on recent breakthroughs in our understanding of the vast repertoire diversity and the fundamental mechanisms that govern the intrinsic functional potential of distinct NK cell subsets at steady state and following cytokine stimulation.

The vast heterogeneity of NK cells at steady state within individuals has become evident through the use of single-cell technologies, such as multi-parameter flow cytometry, mass cytometry and scRNA-seq. Understanding how NK cells repertoires are formed, maintained over time and what functional roles individual cell subsets perform at steady state are important for generating the ideal NK cell product. This could either involve modifying existing cells to improve functionality, expanding highly cytotoxic subsets while ensuring retention of functionality or designing a 'synthetic' genetically engineered killer cell from induced pluripotent stem cells.

Furthermore, we need to understand how NK cells are functionally shaped by their surroundings. The soluble factors, metabolic cues, fluctuations in oxygen levels and pH encountered by an NK cell in the tumor microenvironment are very different from steady state and their impact on NK cell function and persistence cannot be underestimated. This is particularly difficult to study in the human setting, with mouse models only providing an approximation.

By understanding the basic biology, from development to differentiation to receptor and cytokine input, we will build up our tool kit which can then be applied to design and develop effective treatment strategies. After all, the 'natural' killing capacity is there, we just need to understand how to harness it.

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