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# IMPROVING NK CELL-BASED IMMUNOTHERAPY OF CANCER BY EXPLORATION OF MIGRATION AND INHIBITORY RECEPTOR-LIGAND INTERACTIONS

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Cover illustration: Hand-drawn illustration for this thesis by Mélanie Lambert depicting how leukemic cells are being targeted by NK cells in the bone marrow.

# Improving NK cell-based immunotherapy of cancer by exploration of migration and inhibitory receptor-ligand interactions

## Thesis for Doctoral Degree (Ph.D.)

By

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The thesis will be defended in public at Erna Möllersalen, Blickagången 16, Huddinge, Friday 26th of May 2023 at 09.00.

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*To my beloved past, present and future family members*

# Popular science summary of the thesis

Over the course of a human life span, approximately one out of three people are diagnosed with cancer. It is therefore a group of diseases that is highly present in our lives and many people are either directly or indirectly affected. Acute myeloid leukemia (AML) is a particularly dangerous type of cancer. Patients that have been diagnosed with AML have a difficult journey ahead of them as the standard treatment primarily consists of several rounds with high doses of chemotherapy. As can be imagined, these treatments are extremely tough and associated with severe side effects. In addition, the patients also have to go through these treatments knowing that there is a high risk that they might not be cured, as only one out four AML patients lives five years after diagnosis.

Due to this, there is an immense need for new and more effective treatment options against AML. Natural Killer (NK) cells are a type of immune cells specialized in recognizing and killing cancer cells. Clinical trials have indicated that NK cells can be used to treat patients with AML. However, there are certain roadblocks to overcome in order for the treatment to be highly efficacious. One such roadblock is the fact that the infused NK cells are a bit lost, and have a hard time finding their way to the cancer (in this case in the bone marrow compartments) once infused into the blood stream. Some of the infused NK cells do find their way there, but too many of them unfortunately wander off in the wrong direction and end up in other organs such as the spleen, liver and lungs.

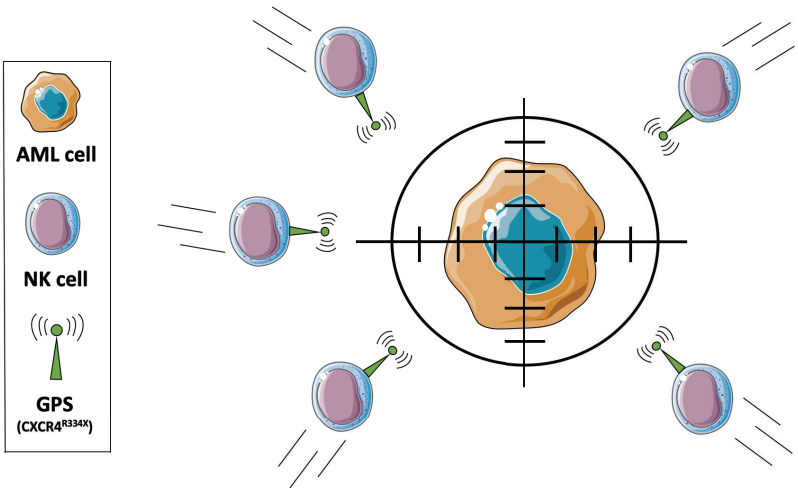


**Illustration** of how NK cells better can find and attack AML cells after being engineered to express the $CXCR4^{R334X}$ receptor, here illustrated as a GPS device guiding them to the right location. Created using images provided by Servier Medical Art. Link: smart.servier.com. Creator: Filip Segerberg.

The ability for a cell to migrate to a certain tissue is in our field referred to as homing, and the potential to enhance bone marrow homing as a means to improve NK cell-based therapy against AML has not been extensively explored. However, in **papers I and II** of this

thesis we have found that NK cells can be engineered to express a cell surface receptor, known as $CXCR4^{R334X}$, which makes them more likely to go to the bone marrow. This receptor can be thought of as a kind of GPS, that has been programmed with the coordinates of the bone marrow. We have then provided the NK cells with this GPS prior to injection. By doing this, we have found that the NK cells not only migrate better to the bone marrow compartments of mice, but also prevents leukemia growth and prolongs the life of these mice compared to control NK cells that have not been equipped with the GPS. An **illustration** of this process can be observed on the previous page.

An important feature of an NK cell is their ability to distinguish between normal cells and cancer cells. NK cells are prevented to attack normal cells as these cells express so called HLA class I molecules on their surface, which NK cells can bind to via their inhibitory receptors and interpret as self-molecules. Cancer cells often lose these molecules and can then be attacked by the NK cells as they recognize that these self-molecules are missing (referred to as missing self) on the cancer cells. How strongly NK cells will attack tumor cells that have lost these molecules will depend on how well the NK cells have been trained to recognize loss of that particular molecule. This training is actually referred to as NK cell education and is a process that likely occurs constantly when NK cells bind and unbind normal cells. In **paper III**, we have for the first time identified that the LIR-1 inhibitory receptor, which is expressed on the cell surface, can educate NK cells that have been activated with a specific protocol. We have further found that a group of activated NK cells expressing the LIR-1 receptor are particularity good at killing tumor cells that have lost this self HLA class I molecules. NK cells can also bind directly to antibodies that already have bound to the tumor cells, which usually results in that the NK cells can kill the tumor cells even more effectively, a process known as antibody-dependent cellular cytotoxicity (ADCC). In this paper, we also show that the group of activated LIR-1 expressing NK cells are particularly good at ADCC, and can when combined with antibody therapy even kill tumor cells that do not downregulate HLA class I. We speculate that this new group of activated NK cells that express LIR-1 can be used to treat various types of cancer, including AML.

Cancer usually develops from normal healthy cells as a consequence of mutations that have occurred in the DNA. This gives cancer cells a growth advantage over normal cells, which eventually leads to the development of a tumor or leukemia. In many cases, several different types of mutations can be detected in the cancer cells and the types of mutations may also differ between patients. The so-called IDH mutations are relatively common in AML as they can be detected in approximately 20% of the patients. Nowadays there are specific drugs available for patients with IDH mutations which targets and inhibits the mutated protein. However, only 30-40% of the patients responds to this treatment. In **paper IV**, we have discovered that IDH mutations causes cancer cells to lose these self HLA class I molecules mentioned in relation to the previous paper. As a

consequence of this loss of HLA class I molecules, we have then been able to show that AML cells with an IDH mutation are more easily killed by NK cells compared to AML cells that do not have the mutation. This is highly relevant as IDH mutated AML patients that do not respond to treatment with the specific IDH targeting drug potentially could be offered NK cells as an alternative treatment.

Overall, this thesis reveal that NK cell-based immunotherapy against AML can be improved by directing NK cells to the bone marrow where the leukemia resides. Furthermore it highlights that a group of activated NK cells that express the LIR-1 receptor are educated and potentially very attractive to use against various types of cancer including AML. Lastly, it describes that IDH mutated AML cells are particularly sensitive to NK cells, a novel finding that hopefully can be used to help some of these patients. In conclusion, the findings in this thesis have the potential to be used in order to advance NK cell-based immunotherapy against AML.

# Abstract

The possibility to utilize the immune system as a mean to target malignantly transformed cells has emerged as a novel treatment modality during the last decades and is a field that is under rapid development. Natural Killer (NK) cells are immune cells with an intrinsic ability to recognize and lyse tumor-transformed cells. Their role in immune surveillance of cancer is well established and clinical studies have also highlighted their treatment potential. However, the efficacy has so far been suboptimal and means to further improve NK cell-based immunotherapy against cancer is under intensive investigation. This thesis mainly focuses on how to enhance NK cell treatment efficacy against acute myeloid leukemia (AML), a disease with poor prognosis that is in urgent need for novel and more efficacious treatment options.

The potential to better home NK cells to the tumor environment is a highly appealing, but so far relatively unexplored approach to improve treatment efficacy for adoptive NK cell transfer. In **Papers I and II**, we have utilized a clinically approved mRNA transfection technique to explore how *ex vivo* expanded NK cells can be engineered to express the gain-of-function bone marrow (BM) homing receptor variant $CXCR4^{R334X}$. We have shown that $CXCR4^{R334X}$-expressing NK cells display an increased *in vitro* migration potential towards the ligand SDF-1α without compromising functionality in any other aspect. We have further highlighted the importance of the CXCR4-SDF-1α axis for *in vivo* BM homing using both $CXCR4^{R334X}$ mRNA transfected cells and CXCR*4* knocked-out cells generated using the CRISPR/Cas9 technology. Finally, we showed that $CXCR4^{R334X}$-expressing NK cells can be utilized to improve leukemia clearance *in vivo*, and furthermore have the potential to mediate a survival benefit in this context.

Education is a complicated process in which NK cells are dynamically tuned by surrounding HLA class I molecules facilitating a strong effector response against target cells lacking such molecules. Members of the killer cell immunoglobulin-like receptor (KIR) family and the natural killer group 2 member A (NKG2A) receptor have previously been shown to mediate education. In **paper III,** we have shown for the first time that the LIR-1 receptor can mediate education in *ex vivo* expanded NK cells. We have identified an educated LIR-1-expressing NK cell subset that display an elevated degranulation capacity towards HLA class I deficient or low tumor targets and further showed that this subset also has a phenotype which resembles that of educated cells. In addition, we have displayed that the subset can be detuned after blockade with specific LIR-1 monoclonal antibodies in line with the rheostat model. Finally, we revealed that this subset has a potent ADCC capacity that even can overcome inhibitory signals from cognate HLA class I ligands. We have postulated that these traits make this subset highly attractive for NK cell-based immunotherapy and could be utilized in various cancer settings, including AML.

Isocitrate dehydrogenase (IDH) 1 and 2 are frequently mutated in AML. IDH inhibitors (IDHi) have emerged as novel therapeutic agents. However, only a portion of the patients respond to treatment and the relapse rate is high. In **paper IV**, we have employed the TF-1 AML cell line overexpressing the hotspot mutation IDH2R140Q as a model system to study the epigenetical and transcriptional landscape before and after treatment with an IDHi. We have shown that TF-1 IDH2R140Q cells display a hypermethylated DNA profile leading to differential gene expression, which can be further linked to a dysregulated transcriptional network involving myeloid-related transcription factors. We further displayed that this, at least in part, can be reversed upon treatment with the IDHi. Moreover, we showed that both TF-1 IDH2R140Q cells and primary AML cells carrying IDH mutations have hypermethylated HLA class I genes leading to HLA class I downregulation. This was associated with an increased sensitivity to NK cell-mediated responses against the IDH2R140Q mutated TF-1 cells compared to wild-type (WT) TF-1 cells. Lastly, we showed that HLA class I genes in IDH mutated primary AML cells of patients that did not respond to treatment with IDHi remained hypermethylated, indicating that this patient group may benefit from treatment based on adoptive NK cell infusions.

Overall, this thesis provides evidence that NK cell migration can be modulated to redirect NK cells to bone marrow compartments for improved leukemia clearance. Furthermore, it includes a paper where the inhibitory receptor LIR-1 for the first time is shown to mediate education of *ex vivo* expanded NK cells, and identifies a unique subset of highly educated NK cells with a robust ADCC capacity that have the potential to be utilized against various cancer types including AML. Finally, the thesis includes a manuscript which shows that mutations in IDH can confer an increased NK cell sensitivity, and that IDH mutated AML patients resistant to treatment with IDHi constitute a subgroup of AML that may specifically benefit from NK cell-based immunotherapy. In conclusion, the thesis provides novel insights on how NK cells can be utilized to improve treatment for AML and potentially also other types of BM residing malignancies.

# List of scientific publications included in the thesis

I. Emily Levy, Robert Reger, **Filip Segerberg**†, Mélanie Lambert†, Caroline Leijonhufvud†, Yvonne Baumer, Mattias Carlsten‡, Richard W. Childs‡.
Enhanced Bone Marrow Homing of Natural Killer Cells Following mRNA Transfection With Gain-of-Function Variant CXCR4$^{R334X}$.
*Frontiers in Immunology*. 2019;10:1262.

II. **Filip Segerberg**†, Mélanie Lambert†, Laura Sanz-Ortega, Agneta Andersson, Richard W. Childs, Mattias Carlsten.
Improved leukemia clearance following adoptive transfer of natural killer cells engineered with the bone marrow homing receptor CXCR4-R334X.
*Manuscript*.

III. Caroline Leijonhufvud, Robert Reger†, **Filip Segerberg**†, Jakob Theorell, Heinrich Schlums, Yenan T. Bryceson, Richard W. Childs, Mattias Carlsten.
LIR-1 educates expanded human NK cells and defines a unique antitumor NK cell subset with potent antibody-dependent cellular cytotoxicity.
*Clinical & Translational Immunology*. 2021;10(10):e1346.

IV. Anna Palau, **Filip Segerberg**†, Michael Lidschreiber†, Katja Lidschreiber, Aonghus J. Naughton, Maria Needhamsen, Lisa Anna Ljung, Maja Jagodic, Patrick Cramer, Sören Lehmann‡, Mattias Carlsten‡, Andreas Lennartsson‡.
Perturbed epigenetic transcriptional regulation in AML with IDH mutations causes increased susceptibility to NK cells.
*Manuscript*.

# List of additional relevant scientific publications not included in the thesis

i. **Filip Segerberg**†, Christian Lundtoft†, Sarah Reid, Karin Hjorton, Dag Leonard, Gunnel Nordmark, Mattias Carlsten‡, Niklas Hagberg‡.
Autoantibodies to Killer Cell Immunoglobulin-Like Receptors in Patients With Systemic Lupus Erythematosus Induce Natural Killer Cell Hyporesponsiveness.
*Frontiers in Immunology*. 2019;10:2164.

ii. Mélanie Lambert, Caroline Leijonhufvud, **Filip Segerberg**, J. Joseph Melenhorst, Mattias Carlsten.
CRISPR/Cas9-Based Gene Engineering of Human Natural Killer Cells: Protocols for Knockout and Readouts to Evaluate Their Efficacy.
*Methods in Molecular Biology*. 2020;2121:213-239.

# Contents

# List of abbreviations

| | |
|---|---|
| ADCC | Antibody-dependent cellular cytotoxicity |
| AML | Acute myeloid leukemia |
| BCL-2 | B-cell lymphoma 2 |
| BLI | Bioluminescence imaging |
| BM | Bone marrow |
| BiKE | Bispecific killer engager |
| BiTE | Bispecific T-cell engager |
| B7-H6 | B7 family member H6 |
| CAR | Chimeric antigen receptor |
| Cas9 | CRISPR-associated protein 9 |
| CB | Cord blood |
| CCL | C-C chemokine ligand |
| CCR | C-C chemokine receptor |
| CD | Cluster of differentiation |
| CIML | Cytokine-induced memory-like |
| CLP | Common lymphoid progenitor cell |
| CRS | Cytokine release syndrome |
| CTLA-4 | Cytotoxic T-lymphocyte-associated antigen 4 |
| CMV | Cytomegalovirus |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CXCR | C-X-C motif chemokine receptor |
| CX3CR | CX3C motif chemokine receptor |
| DAP12 | DNAX-activating protein of 12 kD |
| DNAM-1 | DNAX accessory molecule-1 |
| EBV | Epstein-Barr virus |
| Fc | Fragment crystallizable |
| FLT-3 | FMS-like tyrosine kinase 3 |
| GM-SCF | Granulocyte-macrophage conlony-stimulating factor |
| GO | Gemtuzumab ozogamacin |

| | |
|---|---|
| GOF | Gain-of-function |
| GvHD | Graft-versus-host disease |
| GvL | Graft-versus-leukemia |
| HDC | Histamine dihydrochloride |
| HDR | Homology directed repair |
| HLA | Human leukocyte antigen |
| HSC | Hematopoetic stem cells |
| HSCT | Hematopoietic stem cell transplantation |
| IB | Intrabone |
| ICANS | Immune effector cell associated neurotoxicity syndrome |
| IDHi | IDH inhibitors |
| IDH | Isocitrate dehydrogenase |
| IFN | Interferon |
| IgG | Immunoglobulin of type G |
| IL | Interleukin |
| ILC | Innate lymphoid cell |
| IL-1R | Interleukin-1 receptor |
| IL-1RAP | interleukin-1 receptor accessory protein |
| iNK | Immature NK cell |
| IV | Intravenous |
| iPSC | Induce pluripotent stem cell |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| ITIM | Immunoreceptor tyrosine-based inhibitory motif |
| KIR | Killer cell immunoglobulin-like receptor |
| KLRC1 | Killer cell lectin-like receptor C1 |
| KLRG1 | killer cell lectin-like receptor G1 |
| KO | Knock-out |
| LCL | Lymphoblastoid cell line |
| LFA-1 | Lymphocyte function-associated antigen 1 |
| LIR-1 | Leukocyte immunoglobulin-like receptor 1 |

| | |
|---|---|
| mAb | Monoclonal antibody |
| MDS | Myelodysplastic syndrome |
| MFI | Mean fluorescence intensity |
| MHC | Major histocompatibility complex |
| MICA | Major histocompatibility complex class I-related chain A |
| MICB | Major histocompatibility complex class I-related chain B |
| NCRs | Natural cytotoxicity receptors |
| NKG2A | Natural killer group 2 member A |
| NKG2C | Natural killer group 2 member C |
| NKG2D | Natural killer group 2 member D |
| NKp30 | Natural cytotoxicity triggering receptor 3 |
| NKp44 | Natural cytotoxicity triggering receptor 2 |
| NKp46 | Natural cytotoxicity triggering receptor 1 |
| NKP | NK progenitor cell |
| NK cell | Natural killer cell |
| NSG | NOD scid gamma |
| ON | Overnight |
| PB | Perhipheral blood |
| PBMC | Peripheral blood mononuclear cells |
| PDX | Patient-derived xenograft |
| PD-1 | Programmed cell death protein 1 |
| PD-L1 | Programmed death ligand 1 |
| PIR-B | Pirb paired immunoglobulin-like receptor B |
| PSGL-1 | P selectin glycoprotein ligand 1 |
| PVR | Poliovirus receptor |
| SDF-1α | Stromal cell-derived factor-1α |
| SHP-1 | Src homology 2 domain–containing phosphatase-1 |
| SLE | Systemic lupus erythematosus |
| SP | Singe-positive |
| S1P5 | Sphingosine-1-pentophosphate 5 receptor |

| | |
|---|---|
| Syk | Spleen tyrosine kinase |
| Tactile | T cell-activated increased late expression |
| TET2 | Ten-eleven translocation methylcytosine dioxygenase 2 |
| Tigit | T cell immunoreceptor with Ig and ITIM domains |
| TCR | T cell receptor |
| TME | Tumor microenvironment |
| TNF | Tumor necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TriKE | Triscpecifik killer engager |
| ULBP | UL-16 binding protein |
| UMAP | Uniform manifold approximation and projection |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VLA-4 | Very late antigen-4 |
| WHIM | Warts, hypogammaglobulinemia, infections and myelokathexis |
| WT | Wild-type |
| ZAP70 | Zeta-chain-associated protein kinase 70 |
| 2B4 | Natural killer cell receptor 2B4 |
| 2-HG | 2-hydroxyglutarate |
| 5hmC | 5-hydroxymethylcytosine |
| 5mC | 5-methylcytosine |

# 1 Introduction

## 1.1 Immunotherapy against cancer – a new era

Human cancers are a group of diseases that are both common and often life threatening. According to recently published data, the combined global cumulative risk for a person to develop as well as to die from a cancer diagnosis before the age of 75, are 21.4% and 17.7% respectively, with slightly higher incidence and mortality rates for men compared to women. Therefore it is one of the most common causes of death worldwide (1). Treatment regimens for cancer have traditionally relied on chemotherapy, radiation, and surgery (2). Although patients can be cured from cancer by these interventions, they are associated with severe side effects and often limited in their efficacy (3). Because of these reasons, efforts have been made to improve and expand the treatment arsenal for cancer during the past decades. Today, targeted therapies as well as immunotherapy are considered as the fourth and fifth pillar of cancer therapeutics respectively (4).

It is well established that the immune system plays a pivotal role when it comes to cancer immunosurveillance (5, 6). In the field of immunotherapy, the aim is to boost natural defense mechanisms already existing within a host, or by other means utilizing cells or components originating from the immune system, to target and treat cancer. Sometimes it may even be necessary to replace an impaired immune system with a new one that originates from an allogeneic donor. Main immunotherapeutic approaches include cancer vaccines, oncolytic virus therapies, cytokine therapies, anti-tumor antibodies, immune checkpoint inhibitors as well as adoptive cell transfer (7). Immune checkpoint inhibitors have gained significant attention over the last couple of years due to their potential to block inhibitory signaling pathways, often utilized by malignantly transformed cells to escape immune cell recognition, which then revoke dampened immune responses (8, 9). Two of the most recognized examples are blockade of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and interruption of the programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) interaction (10, 11).

Although adoptive cell transfer was first described in the mid 1980s (12), major breakthroughs within the field has only been made in more recent years, much due to the reason that we now have the possibility to genetically modify immune cells for improved functionality (13). The most renowned and frequently-used approach is perhaps the construction and introduction of chimeric antigen receptors (CARs) into T cells. A CAR construct combines the antigen binding domain from an antibody with the signalling domain of a T cell receptor (TCR), which enables T cells to circumvent their normal HLA-restricted antigen recognition (14). Although early clinical trials with the first generation of CAR T cells were promising (15), the big breakthrough came years later when costimulatory molecules were implemented into the CAR constructs and they were

directed towards the B cell antigen CD19 (16-19). Apart from CD19, there are now also anti-BCMA CARs approved for clinical use against multiple myeloma (20).

Although T cells are the most explored and utilized immune cells within the field of immunotherapy against cancer, other immune cells may also prove to be beneficial for these purposes. One such cell, that possess potent anti-tumor properties and have gained an increased amount of attention over the past couple of years, are Natural Killer (NK) cells (21).

## 1.2 Basic NK cell biology

### 1.2.1 Introduction to NK cells

NK cells were initially discovered in the mid 1970s by two independent research groups and identified as cells that could lyse malignantly transformed cells without the need for prior antigen recognition (22-25). Phenotypically, human NK cells are identified as CD56+CD3- and constitute approximately 5-15% of the total number of peripheral blood mononuclear cells (PBMCs) (26). NK cells have primarily been considered to be part of the innate immune system, although evidence suggest that they can exhibit memory-like/adaptive features (27). From a functional perspective, NK cells can release cytokines such as tumor necrosis factor (TNF)-$\alpha$, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-$\gamma$, which enables them to communicate with other cells of both the innate and adaptive immune system (28). However, as NK cells are highly cytotoxic cells they are perhaps mostly recognized for their intrinsic ability to eradicate virally infected cells, and even more importantly from the perspective of this thesis, their potential to lyse malignantly transformed cells and their role in cancer immunosurveillance (29). The most studied mechanism is the degranulation pathway, in which NK cells can release vesicles containing highly cytotoxic molecules such as perforin and granzymes. However, NK cells can also induce target cell death by directly binding to a group of receptors that may be expressed on certain tumor cells referred to as death receptors, which include the TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 and the CD95 (APO-1/Fas) receptor (30).

### 1.2.2 NK cell development, migration and tissue homing

NK cells are known to develop from CD34+ hematopoietic stem cells within the bone marrow (BM) compartment, and to go through several maturation stages before becoming fully competent cells (31). In more recent years, it has been discovered that NK cells belong to a larger family of cells known as innate lymphoid cells (ILCs), all lacking diversified antigen recognition receptors (32, 33). According to the prevalent linear model, human hematopoietic stem cells (HSCs) first develop into common lymphoid progenitor cells (CLPs) which have the potential to differentiate into precursor populations of T cells, B cells and ILCs including NK progenitors (NKPs). Expression of the interleukin-1 receptor

(IL-1R) is an early marker of commitment towards the NK linage whereas CD122 is considered to be expressed by fully committed cells. NKPs then develop into immature NK cells (iNK). These cells usually express high levels of IL-1R in combination with other common NK cell activation markers such as the natural killer group 2 member D (NKG2D) receptor and the natural cytotoxicity receptors (NCRs) including the natural cytotoxicity triggering receptor 3 (NKp30), 2 (NKp44) and 1 (NKp46) which all will be covered in more detail later on in the introduction of this thesis. iNK cells will eventually differentiate into mature NK cells expressing the NK cell marker CD56 (34-36). Mature NK cells obtained from peripheral blood can be grouped into $CD56^{bright}CD16^{dim}$ or $CD56^{dim}CD16^{bright}$ NK cells. The former is considered to be more prone towards producing cytokines upon stimulation and constitute roughly 5% of the NK cells found in peripheral blood whereas the latter is more cytotoxic, expressing the killer cell immunoglobulin-like receptors (KIRs) which also will be covered in much more detail later on in this thesis, and make up the remaining 95% of the NK cells in the peripheral blood (34).

The leukocyte adhesion cascade is a well-studied process which consist of a series of events including initial tethering and rolling, cell activation, arrest and transendothelial migration. Selectins, such as P-selectin and E-selectin expressed by endothelial cells, are highly involved in mediating the initial capture and rolling of cells by binding to glycosylated ligands, such as P selectin glycoprotein ligand 1 (PSGL-1). Chemokines can then activate the bound cells further by binding to their specific chemokine receptors as exemplified below. Integrins, such as very late antigen-4 (VLA-4) also participate in the initial rolling but go through conformational changes upon chemokine mediated activation. This causes them to increase both in numbers and in binding strength leading to firm adhesion and cell arrest on the endothelial wall. The integrins are then also involved in transendothelial migration where the leukocyte finally pass through the endothelial barrier and into the targeted tissue (37).

NK cell migration is governed by a complex machinery of events and molecules including the above mentioned selectins, chemokines and integrins (34). The exact molecules involved will depend on the context and which tissue that the NK cell might be migrating towards or are currently retained within. The C-X-C motif chemokine receptor (CXCR) 4 receptor is a seven transmembrane G protein coupled receptor that recognizes and binds to a chemokine known as stromal cell-derived factor-1α (SDF-1α) (38, 39). It is since long known to be expressed on HSCs and leukemic cells within the BM and to be important for effective BM homing of infused HSCs in the setting of hematopoietic stem cell transplantation (HSCT) (38, 40-42). The important role in relation to this is further emphasized by the knowledge that plerixafor, a known CXCR4 antagonist, causes CD34+ hematopoietic stem cells to egress from the BM compartment and enter the blood stream which enables harvest from donors prior to allogeneic HSCT (43, 44). Interestingly, both NKPs and iNKs mentioned above express high levels of the CXCR4 receptor which

likely helps to retain them in the BM compartment during development (45, 46). VLA-4 is another molecule that through binding to vascular cell adhesion molecule 1 (VCAM-1) helps to retain NK cells within the BM compartment (34). During maturation, CXCR4 becomes downregulated which likely contributes to the extravasation of NK cells form BM to the blood. The egress of NK cells from the BM to the circulation is likely also regulated by other chemokine receptors and molecules and has been linked to the upregulation of CXCR3, CXCR6, CX3C motif chemokine receptor (CX3CR) 1 and sphingosine-1-pentophosphate 5 receptor (S1P5) (45, 47). **Figure 1** below illustrates how NK cells can egress from the BM during development and migrate to various tissues in the body, and furthermore depicts which molecules that so far have been reported to govern these processes.

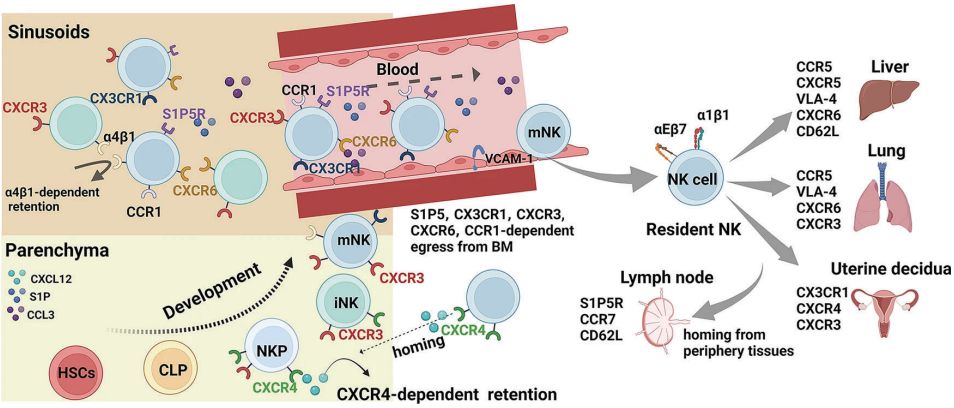


**Figure 1.** "NK cell trafficking and homing at steady state". From: Ran GH, Lin YG, Tian L et al. Natural killer cell homing and trafficking in tissues and tumors: from biology to application. *Signal Transduct Target Ther*. 2022;7(1):205. This figure was reprinted under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes has been made. Link: https://creativecommons.org/licenses/by/4.0/.

Apart from the BM and blood, NK cells can also be found in several other tissues and organs such as the lymph nodes, spleen, liver, lungs and in the uterus (47, 48). Migration to these tissues will be governed by other sets of molecules. L-selectin (CD62L) expressed on NK cells can bind to CD62L ligands and initiate migration to lymph nodes (34). Lymph node homing is also known to be regulated by the expression of the C-C chemokine receptor (CCR) type 7, which binds to the C-C chemokine ligand (CCL) 19 and 21 (49, 50). Similar to what has been shown in BM, upregulation of S1P5 stimulates egression (45). Trafficking to the liver and to the lung has linked to the expression of CXCR6, CCR5 as well as VLA-4 (34). NK cells can also permanently reside in tissues and are then referred to as tissue-resident NK cells. These type of cells are usually defined by the expression of CD69, CD103 and CD49a as well as various chemokines receptors and integrins (51, 52). These type of NK cells will however not be discussed further in this thesis.

### 1.2.3 NK cell activation and the 'missing self' hypothesis

The discovery of NK cells and their natural cytotoxic potential provided an explanation to the previously incomprehensible background killing of tumor cells that many research groups at the time had observed within their experimental setups (53). However, it remained unclear how this cytotoxic activity was regulated. In 1986, Kärre et al. postulated the 'missing self' hypothesis which was the first ground-breaking attempt to understand the underlying principles of NK cell-mediated cytotoxicity (54). According to the hypothesis, NK cells have the ability to identify and eradicate cells that are unable to present self-major histocompatibility complex (MHC) class I molecules, referred to as human leukocyte antigen (HLA) class I in humans, on their surface (55, 56). As most healthy human cells express HLA class I molecules, whereas virus-infected and tumor-transformed cells often downregulate these molecules, it is a system that allows NK cells to discriminate between self and non-self (55, 57). Although we still do not understand the exact underlying mechanism for NK cell activation and target recognition, studies over the past years have gradually expanded our knowledge. Today, it is known that NK cell activation and target recognition are regulated through a dynamic balance from both activating and inhibitory signals (57-59). **Figure 2** below illustrates how NK cells can discriminate between self and non-self, including the missing self-concept.

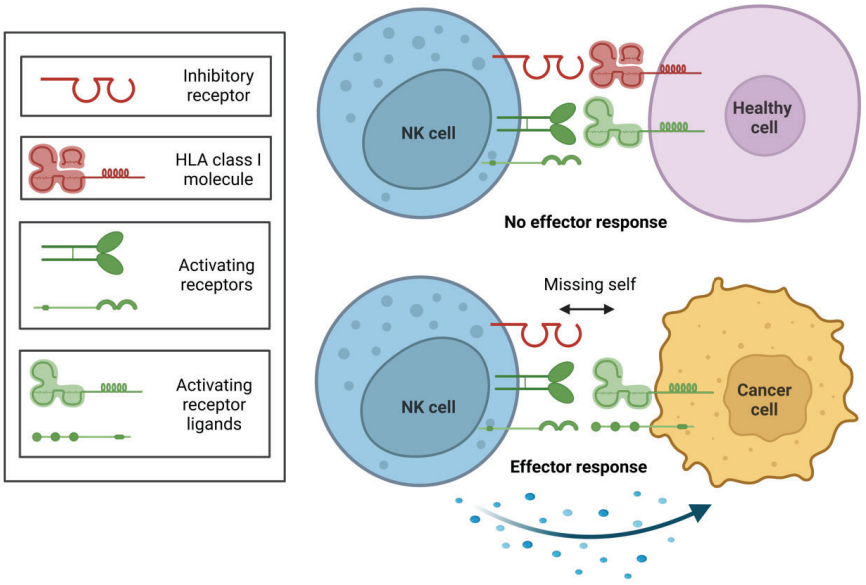


**Figure 2.** Schematic illustration of how NK cells recognize targets cells through activating and inhibitory receptors. The NK cell in the top is inhibited by the healthy cells through HLA class I. The NK cell in the bottom can mount a strong effector response due to missing self-recognition in combination with stimulatory input from activating receptors. Created with BioRender.com. Creator: Filip Segerberg.

Most of the well-studied inhibitory receptors expressed on NK cells bind to classical and non-classical HLA class I molecules (59, 60). Ligation results in phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM), known to be present in the intracellular domain of inhibitory NK cell receptors. This leads to the recruitment of tyrosine-specific phosphatases such as Src homology 2 domain–containing phosphatase-1 (SHP-1) which can inhibit NK cell effector responses through dephosphorylation of key molecules within certain activating signaling pathways (57, 61). In contrast, many of the activating NK cell receptors instead use an immunoreceptor tyrosine-based activation motif (ITAM) associated with adapter proteins such as FcεRIγ, CD3ζ and DNAX-activating protein of 12 kD (DAP12) (62-64). Phosphorylation of ITAM further results in the recruitment of the tyrosine kinases spleen tyrosine kinase (Syk) and Zeta-chain-associated protein kinase 70 (ZAP70) which initiates downstream events (57).

### 1.2.4 NK cell-mediated target recognition

NK cells possess an array of activating receptors on their surface. NKG2D is a transmembrane glycoprotein expressed on all human NK cells. It binds to certain stress-induced glycoproteins on target cells, such as the major histocompatibility complex class I-related chain A (MICA) and B (MICB) as well as to members of the UL-16 binding protein (ULBP) family (57). Ligands for NKG2D are known to be important for NK cell-mediated recognition of both tumors and viruses (65, 66). Another activating receptor known to be involved in cancer immunosurveillance and the recognition of several different tumor types is the DNAX accessory molecule-1 (DNAM-1), also known as CD226 (67-71). DNAM-1 belongs to the immunoglobulin-superfamily and is known to be dependent on complex formation with lymphocyte function-associated antigen 1 (LFA-1) in order to function properly (72). The ligands are the poliovirus receptor (PVR) and nectin-2, also often referred to as CD155 and CD112, respectively (73). Other important activating receptors are the natural killer cell receptor 2B4 and the before mentioned NCRs including NKp30, NKp44 and NKp46. The 2B4 receptor, also known as CD244, binds to CD48 (57, 74). Although the ligands for the NCRs to a large extent are unknown, some ligands related to both infections and cancer have been identified, such as B7 family member H6 (B7-H6) binding to NKp30 (75). Lastly, it is important to mention that a majority of all NK cells express the low-affinity receptor for IgG, CD16 (FcγRIII), which can bind to the fragment crystallizable (Fc) part of immunoglobulins of type G (IgG) that has bound to target cells (76). Ligation of IgG bound to a target cell induces a strong activation signal that can trigger both degranulation, through a mechanism known as antibody-dependent cellular cytotoxicity (ADCC), and cytokine secretion. Interestingly, activation of NK cells via this mechanism can trigger these responses independent of simultaneous co-activating signals from other activating receptors which normally is required in NK cells that have not been pre-activated with cytokines (76, 77).

KIRs are a family of transmembrane glycoproteins expressed on NK cells (60). Structurally, they can have either two or three extracellular Ig-like domains (referred to as KIR2D or KIR3D) followed by a transmembrane fragment and a cytoplasmic tail that can be either long (L) or short (S). Although KIRs are perhaps mostly known to transmit inhibitory signals, there are in fact both activating and inhibitory receptors within the KIR family. KIRs with a long cytoplasmic domain (KIR2DL and KIR3DL) are inhibitory and have either one or two ITIM motifs associated to their intracellular domain, whereas KIRs with a short cytoplasmic tail (KIR2DS and KIR3DS) are activating and instead seem to associate with DAP12 (57, 78). All KIRs are encoded for by the KIR genes located on chromosome 19 (79). In total there are 15 genes plus 2 pseudogenes and high polymorphism has been reported in several of the genes. However, all genes are not present within every individual and the number of genes that an individual possess can vary (80, 81). The set of KIR genes present within a given individual can be classified into two main haplotypes, referred to as A and B, depending on if the genes mainly code for activating or inhibitory KIRs (57, 78). The KIRs are also known to be stochastically expressed in cells, with the consequence that an individual will have NK cells with a varying KIR repertoire (82, 83). This seems to be a result of epigenetic regulation through DNA methylation patterns as well as probabilistic bidirectional promotor switches (84-86).

Inhibitory KIRs are known to bind to classical HLA class I molecules of the A, B and C subtype (61). As with the KIRs, there is substantial polymorphism within the HLA genes resulting in differential HLA class I recognition among the KIRs (87, 88). HLA-C molecules can be grouped into HLA-C1 or HLA-C2 depending on whether they possess an asparagine or lysine residue at position 80 in the $\alpha$-domain of the HLA molecule. KIR2DL1 recognizes HLA-C2 molecules, whereas KIR2DL2 and KIR2DL3 recognizes HLA-C1 molecules (89, 90). KIR3DL1 is instead known to bind to the Bw4 serological epitopes, defined by amino acids in position 77-83 within the $\alpha$-domain of the HLA molecule, that is present in certain HLA-A and HLA-B molecules. On the other hand, HLA molecules of the Bw6 serotype are not recognized by KIR3DL1 (91). KIR3DL2 have been reported to bind to HLA-A3 and HLA-A11 (92). Although binding specificity of the inhibitory KIRs is well defined, less is known about the ligands for activating KIRs. However, some ligands have been identified but studies have shown that they either bind to HLA class I molecules with lower affinity than inhibitory KIRs, or not at all (57).

In contrast to the KIR family, the natural killer group 2 member A (NKG2A) is a receptor that is strictly inhibitory and signals through an ITIM (57). It recognizes non-classical HLA class I molecules of the E subtype (93). It is a type-II transmembrane protein of the C-type lectin-family and forms a disulfide-linked heterodimer with CD94 (CD94/NKG2A) (94). The killer cell lectin-like receptor C1 (KLRC1) gene, encoding for NKG2A, is present on chromosome 12 and unlike the KIR genes, there is little polymorphism within the gene (95, 96). Another common inhibitory receptor is the leukocyte immunoglobulin-like receptor

subfamily B member 1 (LILRB1/LIR-1) (97). The LIR-1 gene is located on chromosome 19 and the receptor is known to be expressed on 5-80% of all NK cells in peripheral blood of healthy donors (98, 99). It is a surface glycoprotein that possesses 4 extracellular Ig-like domains and 4 ITIM molecules in the cytoplasmic tail (57). LIR-1 also recognizes HLA class I molecules, but has a broader binding specificity compared to the KIR and the NKG2A receptors (100). It is known to be able to recognize HLA class I molecules of various subtypes, however with lower affinity compared to KIR and NKG2A which seem to result in weaker inhibition (101). This is due to its capacity to bind conserved regions shared between several HLA class I molecules (57, 101). The strongest affinity has been reported for HLA class I molecules of the G subtype (102).

Apart from the above-mentioned receptors, several additional receptors of both activating and inhibitory nature have been described. Such receptors include for example natural killer group 2 member C (NKG2C), CD2, T cell-activated increased late expression (Tactile/CD96) and T cell immunoreceptor with Ig and ITIM domains (Tigit/CD113), killer cell lectin-like receptor G1 (KLRG1) as well as the more recently described family of Siglec receptors shown to recognize hypersialylated structures on target cells (57, 103-105). **Figure 3** below displays inhibitory and activating NK cell receptors, and their respective cognate ligands, which are discussed throughout this thesis.

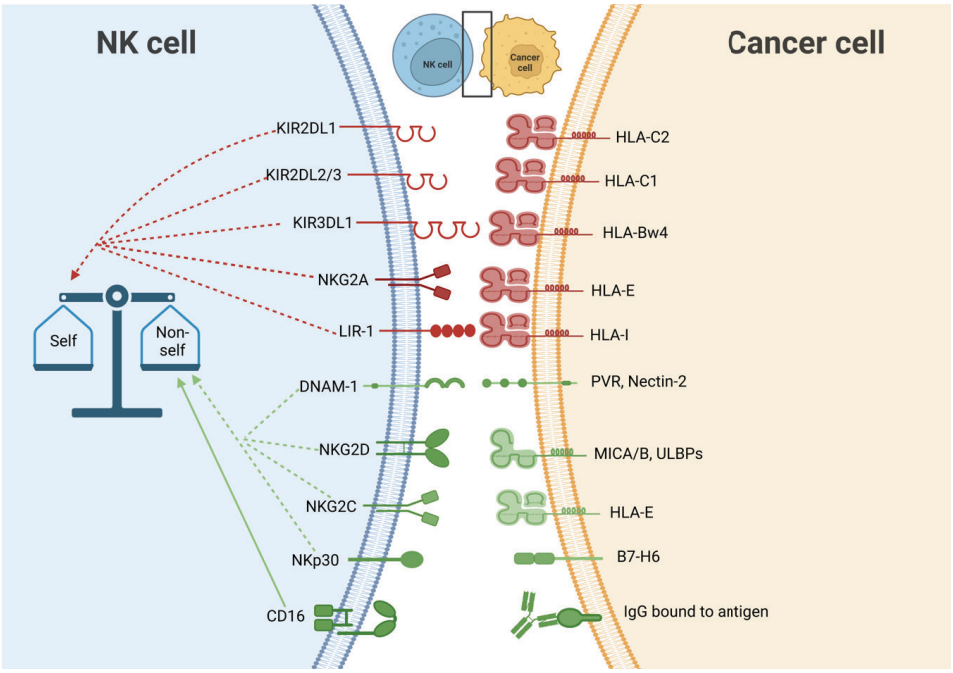


**Figure 3.** Illustration of NK cell inhibitory and activating receptors as well as their cognate ligands important for this thesis. The illustration also show how these interactions are involved in governing the overall NK cell response towards a cancer cell. Created with BioRender.com. Creator: Filip Segerberg.

### 1.2.5 NK cell education

Although NK cells are acknowledged for being able to eradicate target cells without the need for prior sensitization of a specific antigen, it has become evident that they need to go through a process, referred to as "licensing" or "education", in order to acquire full functional maturation (106). NK cell education (the term used hereafter) refers to the process by which certain inhibitory receptors present on the cell surface of NK cells engage with corresponding HLA class I molecules in the environment, endowing NK cells with improved effector functions. Evidence has revealed that it is absolutely pivotal for this process to occur prior to target cell encounter, if an NK cell is to mount an effective immune response following recognition of missing self in such situation (107-109). **Figure 4** below illustrates the education process and how it endows NK cells with the ability to mount a potent effector response due to missing self.

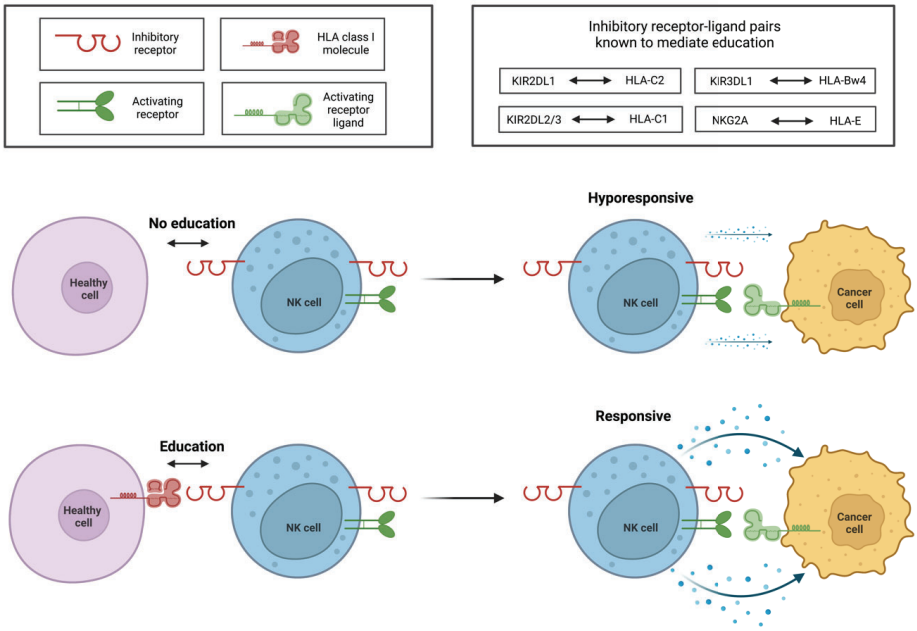


**Figure 4.** Schematic illustration of education and the subsequent effect of the process upon target recognition. The NK cell in the top has not been educated through cognate HLA class I binding prior to encountering a cancer cell, and therefore remains hyporesponsive. The NK cell in the bottom on the other hand has been educated and is therefore able to mount an effective response. Created with BioRender.com. Creator: Filip Segerberg.

Although researchers within the field have been in agreement regarding the fact that education is dependent on the interaction of inhibitory receptors and their cognate HLA class I ligands, there has been a lack of consensus regarding how these interactions actually results in education (110) and for this reason, two major models were initially postulated. The arming model suggests that all NK cells are naive to begin with, and

thereby incapable of mounting strong effector responses. Binding to cognate HLA class I molecules initiates intracellular programs endowing NK cells with an increased effector potential. The disarming model on the other hand postulates that all NK cells are functionally competent, but that lack of inhibitory signals will exhaust the NK cells and put them in a state of anergy. Evidence has been provided for both models, but a concord has not been reached (106, 107, 110).

Although researchers have been in disagreement regarding the fundamental principles of how education is achieved, more recent evidence suggests that there is a higher degree of plasticity in the system compared to what was previously thought, which has unified researchers over what is known as the rheostat model (107). According to this model, education is a dynamic process that can be tuned up or down depending on the environment (111). It has been shown that educated NK cells can lose education when put in an HLA class I deficient milieu (112), whereas uneducated NK cells likewise can become educated when put in an HLA class I rich environment (113). The rheostat model also emphasizes that NK cells can be co-educated from several inhibitory receptors simultaneously, and that the total number of interactions calibrates the overall education potential in a quantitative manner (106, 114). Further, more recent evidence suggests that the high polymorphism seen within both the KIR and the HLA genes has consequences on education as certain allele combinations bind better or worse to one another (114, 115). Receptors considered to mediate education in human NK cells are certain members of the inhibitory KIR family as well as the NKG2A receptor, whereas the LIR-1 receptor has not been evidenced to mediate education. The role and importance for activating receptors in NK cell education is less studied. However, there is evidence suggesting that the activating KIRs, and in particular KIR2DS1, can detune NK cells in certain settings (116). Although not a requirement for NK cell education *per se*, DNAM-1 has also been linked to NK cell education as expression of the molecule seems to correlate with the educational state of NK cells in mice (117).

Even if NK cell education was first mentioned almost 20 years ago, and substantial research has been devoted to understanding the basic principles of how and why NK cell education arises, very little is known about the molecular mechanisms governing this process. The importance of the tyrosine phosphatase SHP-1 was highlighted a couple of years ago as a mediator for inhibitory signals, and both the abundance and localization of SHP-1 has recently been shown to differ in educated versus non-educated NK cells (118-120). Another study has indicated differences in basic metabolism due to education, where educated NK cells rely more on glycolysis compared to uneducated NK cells, which instead use mitochondrial respiration to a higher degree (121). Recent evidence also suggests that educated NK cells go through structural changes of the lysosomal compartment and accumulate the serine protease Granzyme B (122). Interestingly, this remodeling was not found to be related to changes in transcriptional

programs, supporting previous knowledge that NK cell education cannot be linked to a specific transcriptional signature (122, 123). As it is important to better understand the molecular basics for education, it is also important to deepen our knowledge regarding the kinetics of the system, both from a basic scientific view and if the system is to be further explored and utilized in the settings of immunotherapy against cancer. Currently available evidence indicates that detuning likely occurs within hours, whereas retuning of previously educated NK cells seems to take a couple of days (124, 125). However, the experiments in these studies were conducted *in vitro* under specific conditions, and should perhaps therefore not be considered as universal evidence.

To conclude this section, there is undoubtedly great need for more detailed evidence regarding both the molecular mechanisms, as well as the kinetics that governs education. An *ex vivo* model where NK cell education easily could be induced would greatly help to achieve this. Although it was recently shown that KIR3DL1+ human NK cells from HLA-Bw4- individuals could be *in vitro* educated through co-culture with HLA-Bw4+ target cells, the effects were quite small and the methodology cumbersome (126). A cleaner, more efficient and preferably also transient system is warranted in order to properly dissect the underlying mechanism and kinetics of education. In relation to this, I have as a side project during my PhD studies explored whether non-educated NK cell subsets, lacking their cognate HLA class I ligands, could be educated *ex vivo* through electroporation of mRNA encoding their specific, but non-self HLA class I molecules. Preliminary data from this project have indicated that these non-self HLA class I molecules can be expressed on the cell surface after electroporation, but has so far not resulted in any education. Future efforts will have to delineate the true potential of this approach.

## 1.3 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a severe and heterogenous disease characterized by the presence of highly proliferative and abnormally differentiated hematopoietic cells primarily located within the BM compartment and in the blood (127). A series of complex genetical events is responsible the pathogenesis of the disease (128). The definitive diagnosis and risk classification is based on the genetic landscape including potential structural abnormalities and a set of various types of mutations (129). Examples of common mutations in AML include NPM1, FLT3, DNMT3A, NRAS or KRAS, RUNX1 and TP53 (128). Mutations in isocitrate dehydrogenase (IDH) 1 or 2 are also frequent as they occur in approximately 20% of all patients (130). Under normal circumstances, the IDH enzymes mediates the conversion of isocitrate to α-ketoglutarate (αKG) (131). The epigenetical regulator ten-eleven translocation methylcytosine dioxygenase 2 (TET2) is dependent on αKG to mediate demethylation of DNA by the hydroxylation of 5-methycythosine (5mC) into 5-hydroxymethylcytosine (5hmC) (132). Mutated IDH further converts αKG into the oncometabolite 2-hydroxyglutarate (2-HG) which leads to the depletion of αKG and the

competitive inhibition of αKG dependent enzymes including TET2 (131, 133, 134) Indeed, IDH mutated patients display a hypermethylated DNA profile which has been linked to the afore-mentioned pathways (135) **Figure 5** below illustrates the function of normal and mutated IDH and the subsequent effects of IDH mutations.

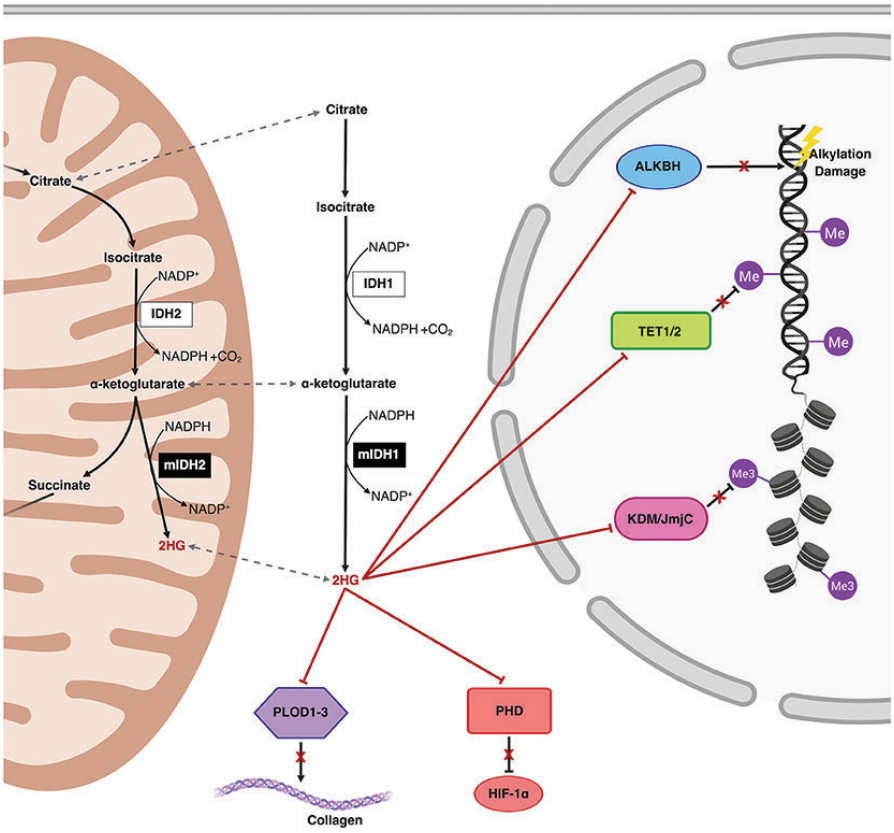


**Figure 5.** "Schematic representation of the mutIDH1 and mutIDH2 pathways and molecular mechanisms related to oncogenesis". From: Golub D, Iyengar N, Dogra S et al. Mutant Isocitrate Dehydrogenase Inhibitors as Targeted Cancer Therapeutics. *Front Oncol*. 2019;7:417. This figure was reprinted under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes has been made. Link: https://creativecommons.org/licenses/by/4.0/.

Standard treatment for AML relies on high-dose chemotherapy that for a subgroup of eligible patients also can be followed by hematopoietic stem cell transplantation (HSCT) (136). Although the overall survival has improved over the years, much due to improvements in supportive care and lower treatment related mortality (127, 137), the prognosis is still dismal. Approximately 35-40% of adult patients under the age of 60, and 5-15% above the age of 60, are cured of the disease (127, 138). Further, chemotherapy can be very burdensome for the patients due to side effects, many of which are often a consequence of therapy-induced neutropenia (139). Due to the severe side effects associated with standard treatment, and the poor overall survival rates, new treatment

modalities for AML are highly warranted. Although the treatment arsenal for AML has not changed substantially over the past 40 years (140), new and more specific drugs are currently being investigated for approval and broader use (127). This is much due to the advancements in genetic profiling of the disease which has not only improved prognosis prediction, but also identified several new targets for treatment development (127, 141). Examples of new and more targeted drugs are different types of FMS-like tyrosine kinase 3 (FLT-3) inhibitors such as midostaurin, the B-cell lymphoma 2 (BCL-2) inhibitor venetoclax, the IDH1 and IDH2 inhibitors ivosidenib and enasidenib, as well as the hedgehog signaling pathway inhibitor glasdegib (142-146).

Additionally, immunotherapy-based approaches to improve treatment of AML are being explored. Administration of exogenous cytokines, such as interleukin (IL)-2 and interferon-α is one approach that has been used to restore immune cell function, as it is known to be hampered by AML (147). Although meta-analysis has indicated that the use of these interventions as mono-therapy in AML fail to improve outcome (148, 149), combining cytokine stimulation with other therapies looks promising (147). It could also be so that certain subgroups of AML patients may respond better to exogenous cytokine-based treatment than others. A study has revealed that a dimorphism at position 21 in the HLA-B gene can predict response to combined treatment with IL-2 and histamine dihydrochloride (HDC) in AML patients (150). This is especially interesting from an NK cell perspective as the dimorphism is known to impact NK cell education (151). Another approach being explored is the use of monoclonal antibodies (mAbs). Despite the fact that naked mAbs such as Lintuzumab (anti-CD33) have shown disappointing results in clinical trials (152), the use of conjugated mAbs to either radioactive compounds or cytotoxic agents holds promise (147). Gemtuzumab ozogamacin (GO) is an anti-CD33 mAb conjugated to the cytotoxic agent calicheamicin that has proven to be a beneficial add on to standard induction treatment for AML patients with a favorable risk profile (153). Further, the use of bispecific T-cell engagers (BiTEs) co-targeting CD3 with known AML antigens such as CD33 in order to recruit T cells is another interesting approach (154). The use of AML vaccines, either utilizing peptides, GM-CSF or dendritic cells, has also been explored in AML with various results (147).

However, the most successful example of immunotherapy against AML is perhaps HSCT, where immune cells are known to mediate the graft-versus-leukemia (GvL) effect, primarily through a mismatch in HLA molecules between recipient and donor (147). Based on this knowledge, the use of adoptive cell transfer in AML is attractive. As CAR T cell therapy has been successful in B cell malignancies, efforts have been made to develop CAR T cells also for AML. Examples of antigens that have been explored are CD123, the Lewis antigen, CD33 and interleukin-1 receptor accessory protein (IL-1RAP) (155-158). However, heterogeneity of the disease and the targeting of normal hematopoietic cells due to difficulties in identifying tumor specific antigens are issues that so far are limiting

the efficacy of this approach (147). Nevertheless, the impressive efficacy that CAR T cell-based therapy has displayed against B cell malignancies has highlighted the role that immune cells may play in future cancer therapy, and indeed also resulted in intensified research to utilize NK cells for this purpose (21).

## 1.4 NK cell-based immunotherapy

### 1.4.1 Role in immunosurveillance of cancer

NK cells are well known to be involved in the surveillance of cancer. Apart from early studies revealing potent responses against several tumor cell lines *in vitro*, a link between tumor targeting and NK cell functionality has been observed in several preclinical mouse models (159). Similarly, cancer development has been associated with certain primary NK cell immunodeficiencies in humans (21, 160, 161). Further, a prospective study including 3500 individuals that were followed for 11 years has highlighted the importance of NK cell-mediated immunosurveillance as it was shown that poor cytotoxicity towards the erythroleukemic cell line K562, known as the golden standard NK cell target for cytotoxicity, at the time of inclusion correlated with higher risk of developing cancer (162). Another layer of evidence stems from the observations that the infiltration of CD57+ immune cells, likely to be NK cells, in certain tumors seems to be of prognostic value (163-165) Other indirect evidence of NK cell-mediated immunosurveillance comes from findings related to immune escape mechanisms. For example, it has been shown that tumor cells can induce downregulation of DNAM-1 expression on tumor-associated NK cells (166). New evidence further highlights the importance of NKG2D in immunosurveillance as it was recently shown that leukemic stem cells can be defined by their absence of NKG2D ligands (167). Together with previous knowledge regarding downregulation of NKG2D ligands in cytomegalovirus (CMV) (168), this suggests that evasion of NKG2D-mediated NK cell recognition is an important immune escape mechanism for both tumors and viruses.

### 1.4.2 Therapeutic relevance and current limiting factors

The revelation that NK cells, apart from being implicated in immunosurveillance, should also be intensively explored for more direct immunotherapeutic purposes stems from a groundbreaking study published by Ruggeri et al. in 2002. The authors displayed that a mismatch in KIR and KIR ligands between donors and recipients significantly reduced the risk of relapse in patients undergoing allogeneic HSCT because of an AML diagnosis (169). Indeed, a few years later it was shown that adoptive transfer of haploidentical NK cells was safe and could induce remission in poor prognosis AML (170). Since then, the efficacy of adoptively transferred NK cells has been explored in several clinical studies and in various settings (21, 159, 171). Although adoptive transfer of NK cells holds great promise, with studies that successfully have shown a clear benefit from the treatment (172, 173),

the efficacy has so far been limited due to poor *in vivo* persistence and proliferation, insufficient homing and tumor localization as well as impaired tumor targeting (171).

### 1.4.3 Means to further bolster NK cell-based immunotherapy

Intensive research is being conducted in order to overcome the before-mentioned limitations and to improve NK cell-based immunotherapy against cancer (171). This includes pre-activation with various different cytokine and expansion protocols, the exploration of alternative cell sources, the potential to combine NK cell infusions with mAbs, bi- or tri-specific killer engagers (BiKEs or TriKEs), blockade of inhibitory signals, as well as genetical engineering (21, 171).

Many of the clinical studies until today have used peripheral blood-derived NK cells that prior to infusion have been short-term (12-16 hours) activated with IL-2 (21, 171). In more recent years, various protocols are now allowing us to expand NK cells *ex vivo* to enhance the cell numbers and increase the cytotoxicity of the cells (174-177). Adoptive transfer of NK cells in the afore-mentioned clinical studies has often also been followed by additional IL-2 administration to support temporary engraftment (21). Although it has been shown that IL-2 stimulation leads to both proliferation and improved cytotoxicity in NK cells (171) it is also known that IL-2 can stimulate regulatory T cells which in turn can suppress and potentially abrogate the NK cell-mediated effect (178). Furthermore, studies have shown that the proliferative effect that IL-2 mediates is rather short-lived (179, 180). Therefore, the potential of other cytokines such as IL-12 and IL-15, as well as combinations of different cytokines, is currently being explored (171, 181). For example, a combination of IL-12, IL-15 and IL-18 has been used which not only shows promising results, but also seems to generate cytokine induced memory-like (CIML) NK cells with enhanced anti-leukemic potential (182, 183). In addition to the various protocols using peripheral blood-derived NK cells, alternative cell sources are also being explored. This includes cord blood (CB)-derived NK cells and induced pluripotent stem cell (iPSC)-derived NK cells, both with various advantages and disadvantages compared to peripheral blood-derived NK cells (184-187). Apart from cytokines, several drugs could also be used to further bolster the functionality of infused NK cells. This includes checkpoint inhibitors that for example block the PD-1/PDL-1 interaction as PD-1 is known to also be expressed by NK cells, the use of classical mAbs or the more recently developed engagers (BiKEs or TriKEs) that for example can link CD16 to a tumor antigen, as well as drugs that can make malignant cells more sensitive to NK cells such as bortezomib (188-192)

Genetic manipulation is another highly interesting method that could be used to improve the functionality of NK cells before injection (21, 171, 193), but has traditionally been complicated and associated with low efficacy and poor viability. However, recent improvements with regards to both transduction and transfection have facilitated a higher success rate. It is now possible to study the effect of transiently expressed

proteins in cells through the introduction of chemically modified RNA, or to generate more permanent genetical modifications such as gene knock-out (KO) through the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system. Permanent introduction of new genes into the DNA can also be achieved using for example retro- or lentiviral transduction, or through the more recently developed transposon-based techniques such as the sleeping beauty and the piggybac systems (193-196). A disadvantage with these techniques is that genes are inserted randomly into the DNA with increased risk of insertional mutagenesis and, although unlikely, potentially cancer transformation compared to directed insertion (194, 197). Interestingly, it was recently shown that by utilizing homology directed repair (HDR) in combination with the CRISPR/Cas9 system, site-specific gene insertion can be achieved in primary human NK cells, which holds great promise for future trials (198).

The afore-mentioned methods have already started to be utilized in several ways to boost NK cell-based immunotherapy. For example, it has been shown that *ex vivo* expanded human NK cells can be genetically engineered to express a high-affinity variant of the CD16 receptor through mRNA electroporation, leading to improved ADCC against lymphoma cell lines *in vitro* (199). Apart from naturally being able to recognize and lyse tumor cells, the potential of also equipping NK cells with different types of CARs has brought a lot of excitement into the field of NK cell-based cancer immunotherapy. Indeed, NK cells have certain advantages over T cells as they can recognize tumor cells by several different ways as detailed further up in this introduction and furthermore because they do not induce graft-versus-host disease (GvHD) and give rise to significantly less cytokine release syndrome (CRS) as well as immune effector cell associated neurotoxicity syndrome (ICANS) (21, 169, 170, 184, 200). In one of the initial CAR NK cell trials, Imai et al. demonstrated that a second generation anti-CD19 CAR can be expressed on *ex vivo* expanded NK cells and significantly improve NK cell-mediated lysis of leukemic B cells *in vitro* (201). Several other CAR constructs directed towards different antigens have since then also been tested in NK cells (21). One of these recently published articles is interesting as it combines the introduction of a CAR targeting the CD38 antigen, known to be expressed in multiple myeloma, with simultaneous CRISPR/Cas9 mediated knock-out of CD38 in expanded NK cells as a mean to avoid fratricide since CD38 is also expressed on NK cells (202). Another recent article has instead of just knocking out CD38 utilized the HDR technology mentioned above and replaced it with a high-affinity CD16 receptor for improved targeting when combined with the CD38 targeting mAb daratumumab (203). NK cells expressing CARs are also starting to be explored in the context of AML. For example, Du et al. recently showed that the piggyBac system could be utilized to co-express an NKG2D CAR together with ectopic IL-15 resulting in an improved anti-leukemic effect together with enhanced persistence (196). Dong et al. instead took advantage of the before-mentioned CIML NK cells which they through

lentiviral transduction equipped with a CAR directed towards mutated NPM1 and also observed promising results (204).

The ability of infused NK cells to adequately home to the tumor environment is another very important, and so far relatively unexplored, subject that needs attention if NK cell-mediated therapy is to be successful (171). The above-described improvements regarding genetic manipulation can however also be utilized for these purposes. For example, it has been shown that *ex vivo* expanded NK cells can be electroporated with mRNA to overexpress the lymph node homing receptor CCR7, which significantly improved the homing capacity of these cells towards the known ligands CCL 19 and 21 *in vitro* (199). Effective BM homing is likely to be very important for efficient NK cell-mediated targeting of AML and other BM residing malignancies, but will be dependent on other receptors and chemokines as mentioned earlier in the introduction. One such receptor that likely can impact on adoptively infused NK cells ability to home to the BM compartments is CXCR4. In a recent study performed by our collaborators at the NIH, it was however shown that *ex vivo* expanded NK cells home poorly to BM compartments when infused into rhesus macaques (205). These NK cells were, as in many protocols, pre-activated with IL-2 and previous studies have shown that both cell isolation and IL-2 stimulation can lead to a decrease in CXCR4 expression, which may explain these results (206, 207). Interestingly, in a recently published clinical trial performed at the Karolinska University hospital where short-term IL-2 activated haploidentical NK cells were evaluated for safety and treatment efficacy in patients suffering from refractory or relapsed AML or high-risk myelodysplastic syndrome (MDS), it was shown that patients receiving donor NK cells with higher levels of CXCR4 compared to those receiving NK cells with lower CXCR4 levels had a better chance of responding, highlighting the potential role of this receptor in immunotherapy (173). **Figure 6** on the next page provides an overview of the above-discussed methodologies that are currently being explored to improve NK cell-based immunotherapy against cancer.

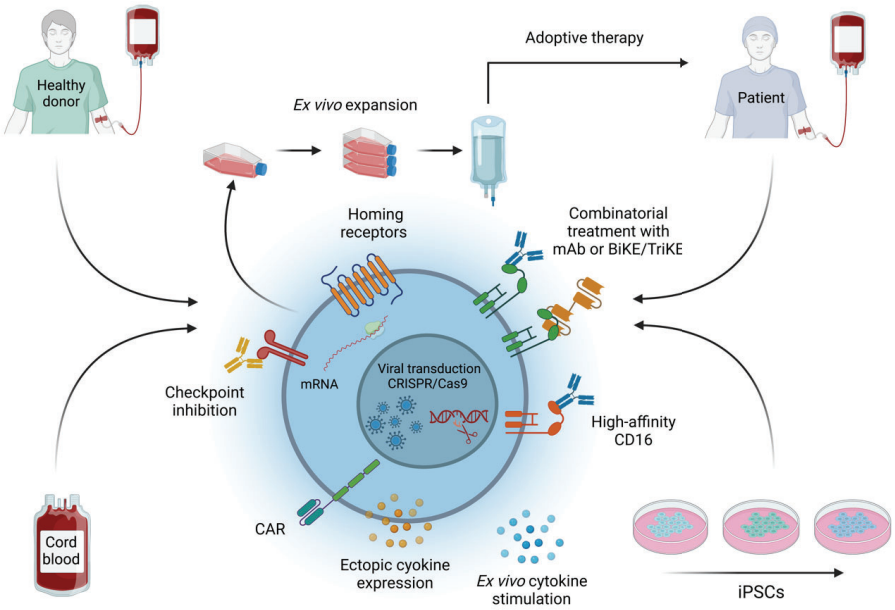


**Figure 6**. Illustration over various means to enhance NK cell based immunotherapy against cancer. Created with BioRender.com. Creator: Filip Segerberg.

To summarize, it is evident that NK cells are immune cells with a potent intrinsic capacity to recognize and kill malignantly transformed cells. This is governed by a complex network of activating and inhibitory receptors as well as other molecules which tunes NK cell responsiveness. Their role in immunosurveillance is well established and ongoing research is currently also assessing their therapeutic potential. So far, it looks very promising but means to further enhance the efficacy is still required in order for these cells to reach their full potential. This thesis explores how NK cell migration as well as inhibitory receptor-ligand interactions can be utilized as a mean to further improve NK cell-based immunotherapy against cancer, with a particular focus on AML.

# 2 Research aims

The overall aim of this thesis has been to investigate whether modulation of homing and exploration of inhibitory receptor-ligand interactions can lead to insights that can be utilized to improve NK cell-based immunotherapy against AML.

**Paper I:** To elucidate how means to prepare NK cells for adoptive transfer impact on basic expression of the bone marrow homing receptor CXCR4, and to determine if the gain-of-function variant $CXCR4^{R334}$ can be expressed on *ex vivo* expanded NK cells to improve their bone marrow homing capacity.

**Paper II:** To investigate whether *ex vivo* expanded human NK cells, engineered to overexpress the gain-of-function bone marrow homing receptor $CXCR4^{R334X}$ can be utilized for improved leukemia clearance *in vivo*.

**Paper III:** To study education and cytotoxic potential within *ex vivo* expanded NK cells and to identify a subset particularly suited for NK cell-based immunotherapy of cancer.

**Paper IV:** To explore if IDH mutations in AML alters the HLA class I expression and if this impact on NK cell cytotoxicity in order to potentially identify a subgroup of AML patients that could particularly benefit from adoptive NK cell infusions.

# 3 Ethical considerations

Although I work with NK cells derived from healthy deidentified blood donors, there are still ethical considerations associated to my research projects. For example, in both **paper I** and **paper II**, we have performed experiments on mice in order to provide answers to our research questions. This research has of course been conducted after ethical approval and in accordance with the three-R-rule to replace, reduce and refine. Nevertheless, the use of animals in medical research is a highly sensitive and frequently debated subject. To constantly have discussions on this topic is both natural and important to ensure use of animals only when absolutely necessary. For this reason, I will elaborate in the upcoming paragraph on my thoughts on the matter and how I motivate the use of animals to answer our research questions.

In the end of 2019, I took a doctoral course in medical research ethics at KI. In the course, we were taught about the 4 fundamental principles of modern medical research ethics, first communicated in the Belmont report, which can be used for guidance in making ethical decisions. These 4 principles are the principle to do good, the principle to avoid doing harm, the principle of autonomy and the principle of justice. As I see it, we have actually transgressed several of these fundamental ethical principles by performing our mice experiments. First, we have violated the principle of not doing harm by inoculating a portion of these mice with leukemia. This principle was further violated by the fact that all of the mice were subjected to injections which likely gave rise to pain. Secondly, we have violated the principle of autonomy since the mice had no possibility to say no to participation. Lastly, we have also violated the principle of justice seeing that these experiments will not benefit the mouse species at all. Instead, we performed the experiments in order to benefit the health of humans. Regarding the principle to do good, I would of course like to make the argument that this principle has been fulfilled, as these studies have highlighted the importance of CXCR4 for human NK cell BM homing and leukemia clearance. Nevertheless, it is clear that 3 out of these 4 principles have been violated and the obvious follow up question is of course whether these violations make these experiments unethical? My answer to this is yes, but only if we are to consider the life of an animal and the life of a human as equally important. However, to me, a human life will always have a much bigger moral weight compared to an animal's life. This type of statement is however not based on logical reasoning, but rather on deep-rooted emotional feelings. From a logical perspective, this type of statement makes no sense at all because there is no evidence that can support it. Who am I to say that a human has a greater right to live compared to an animal? Still, I stand by my emotional viewpoint and argue that a human life will always be worth more than the life of an animal. I think this is really how I can motivate research on animals and also why I believe that even though 3 out 4 Belmont principles are violated, the research is still morally justified. With this said, I would like to make it clear that I do care deeply about all kinds of animals and I am greatly

looking forward to the time when we have identified other means to answer our research hypotheses. Unfortunately we are not there yet and animals are still our best model system to study certain scientific mechanisms.

Apart from the ethical consideration given to mouse work above, the CRISPR/Cas9 system is another method present in my research which I believe is highly important to discuss from an ethical point of view. The gene editing tool has inarguably provided researchers with the ability to do good. For example, the technique has been explored in the context of agriculture, as it potentially can be used to genetically modify crops and thereby also solve starvation issues around the world. Another obvious approach is to use it to knock-out certain disease-causing genes and thereby prevent onset of disease. In **paper II**, we have used the CRISPR/Cas9 system to knock-out the CXCR4 receptor. In our study, this was primarily done as a proof-of-concept to highlight the role for the CXCR4/SDF-1α axis in NK cell BM homing. However, our group may be interested in using the system to boost the functions of NK cells in many different ways in future studies. For example, we could use the technique to knock out certain checkpoint inhibitory receptors for improved cytotoxic capacity, or potentially even knock-in genes that could improve both *in vivo* persistence, homing and tumor targeting.

It is clear that the CRISPR/Cas9 technique has the potential to help the research community to achieve incredible things. However, the development of the technique has also raised ethical concerns. One such concern, which hopefully can be abrogated as the technique continues to develop, is the risk for off-target effects. In worst case scenario, off-target gene-editing could potentially induce mutations favorable for cancer development. Even if we are not injecting any gene-edited cells into humans within the scope of my projects, we have injected mice with CRISPR/Cas9-edited cells and the overall future aim is of course to be able to inject humans. Although the risk for CRISPR/Cas9-mediated off-target mutations is relatively small if guide selection is done properly, we always check for intact basic functionality post gene-editing. For knocking-out CXCR4, which was done in **paper II**, this included making sure that the edited NK cells had an intact cytotoxic capacity against an array of AML cell lines. Another ethical aspect to take into consideration regarding CRISPR/Cas9 is the potential risk that the technique could be used for the wrong purposes. For example, if the tool is continued to be developed, it could be used to predefine what type of traits that we would like newborns to have. Indeed we have already heard of examples that have crossed the line of what is ethically defensible. Based on this, one could question whether we should contribute to develop a technique that could be used for such purposes. On the other hand, there is a risk that almost all new technical inventions can be used for evil purposes if falling into the wrong hands. Therefore, I do not think that this is an argument that should prevent us from using the system. Instead I believe that all projects where CRISPR/Cas9, or any other gene-editing technique, is used should be thoroughly examined before they are initiated.

Further, I believe that all serious researchers must take their individual responsibility to prevent misapplication.

Although there may be other ethical concerns related to my projects, I believe that the two above-discussed methodologies are the most important ones from an ethical perspective.

# 4 Results and discussion

## 4.1 Paper I-II

### 4.1.1 Study rationale

As described in the introduction of this thesis, there is a plethora of evidence highlighting the role for NK cells in immune surveillance of cancer and furthermore the potential to utilize these cells in a therapeutic setting. Although clinical studies have revealed that NK cells can successfully be used as a treatment option, the efficacy has so far been suboptimal (171-173). However, technological advancements are now enabling researchers to genetically engineer NK cells for enhanced functionality and means to further improve NK cell-based immunotherapy are being investigated intensively (193). As it stands, most of these efforts have so far been focused on improving the direct cytotoxic ability of NK cells. Although relevant, other aspects may be equally important to consider in order to obtain durable clinical responses. Insufficient homing to the tumor environment has previously been suggested to limit the efficacy (171), but little research has so far been devoted to overcome this problem.

In the context of leukemia and other BM residing malignancies, the potential of newly infused immune cells to home to the BM compartments is likely to be of importance for clinical efficacy. However, a study from our collaborators at the NIH have revealed that NK cells that have been *ex vivo* expanded in high doses of IL-2 for 12-14 days in fact display poor BM homing when adoptively infused into Rhesus Macaques (205). CXCR4 is a BM homing receptor previously described to have importance for proper homing and engraftment during HSCT (38-42). Apart from being expressed on hematopoietic stem cells, CXCR4 is also expressed on lymphocytes (206). A recent clinical trial performed at the Karolinska University Hospital highlighted the potential therapeutic relevance of the CXCR4-SDF-1α axis as it was shown that high-risk AML and MDS patients displayed improved responses to adoptive NK cell therapy if the donor NK cells expressed high levels of the CXCR4 receptor on their surface (173). However, previous studies have also indicated that IL-2 stimulation, commonly used before and during adoptive NK infusion in order to activate the cells, can downregulate CXCR4 surface expression and thereby potentially decrease their migration potential towards BM compartments (206, 207).

Warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome is a rare autosomal dominant disease. Patients with WHIM syndrome can have various mutations in the C-terminal portion of the CXCR4 receptor, resulting in a truncated receptor variant with gain-of-function (GOF) properties (208). Thus, newly produced immune cells in WHIM patients have been reported to be retained within the BM compartments, and thereby giving rise to the various symptoms indicated by the acronym of the disease (209). **Figure 7** on the next page depicts both the WT and the mutated receptor.

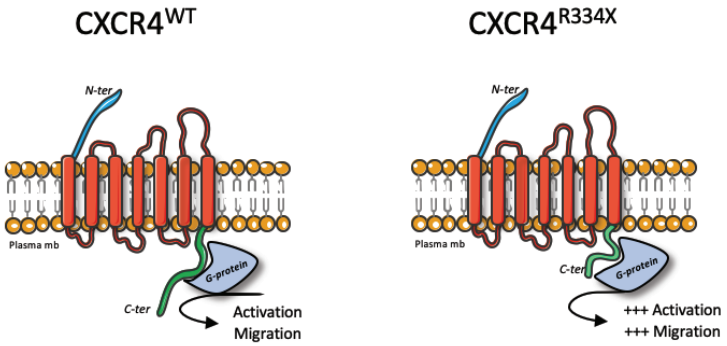


**Figure 7.** Schematic Illustration of both the CXCR4$^{WT}$ and the CXCR4$^{R334X}$ receptor. Created using images provided by Servier Medical Art. Link: smart.servier.com. Creator: Mélanie Lambert.

Although the research that I have been conducting during my doctoral studies does not focus on WHIM syndrome *per se*, we hypothesized that the GOF receptor variant could be utilized to increase BM homing of adoptively infused NK cells and ultimately also lead to improved leukemia clearance. For this purpose, we used *ex vivo* expanded NK cells and transfected them with mRNA encoding for the most common GOF receptor variant in WHIM, known as CXCR4$^{R334X}$ (210). In **paper I,** we have carefully investigated the potential to express the receptor in healthy donor-derived NK cells, and evaluated the migration potential of these cells both *in vitro* and *in vivo* using healthy mice. In **paper II,** we further dissected the CXCR4-SDF-1α axis *in vitro*, but more importantly also evaluated the potential of these cells to improve leukemia clearance in tumor bearing animals.

### 4.1.2 Results

In **paper I**, we first investigated how common means to prepare NK cells for adoptive therapy affected CXCR4 cell surface expression levels and observed a significant decrease in CXCR4 mean fluorescence intensity (MFI) following both NK cell isolation and cryopreservation. Additionally, we also observed significantly decreased expression levels in cells that had been overnight (ON) IL-2 activated as well as in cells that had been *ex vivo* expanded for 14 days compared to non-stimulated cells. Furthermore, in subsequent *in vitro* transwell migration assays, we could show that these cytokine-activated NK cells also displayed a reduced potential to migrate towards SDF-1α. We next performed a head-to-head comparison of *ex vivo* expanded NK cells derived from healthy donors as well as from WHIM patients harboring the R334X mutation, which revealed that the WHIM patient-derived NK cells migrated significantly more to BM compartments of healthy mice while only showing a slight, yet comparable, increase of CXCR4 surface expression.

Based on this, we explored the possibility to overexpress either the CXCR4$^{WT}$ or CXCR4$^{R334X}$ GOF variant in healthy donor derived NK cells. We could then show that

electroporation with mRNA encoding both these receptor variants resulted in a dose dependent increase in CXCR4 cell surface expression, which lasted for 36-48 hours, with a peak expression after 2-8 hours for the WT variant and after 4-16 hours for the R334X variant. We further validated that neither of these modifications altered cell viability, expression of some of the more common activating and inhibitory receptors as well as the ability of the cells to degranulate and produce cytokines. As we had observed an increased receptor expression, we subsequently explored whether this would also result in an increased migration potential and could show that $CXCR4^{R334X}$–electroporated, but not $CXCR4^{WT}$–electroporated, cells migrated significantly better towards SDF-1α *in vitro* compared to control NK cells. We also confirmed that this was due to $CXCR4^{R334X}$ expression as migration was completely abolished after exposure to the CXCR4 antagonist Plerixafor.

Lastly in this paper, we investigated whether the enhanced migration capacity observed *in vitro* also would translate into an improved *in vivo* BM homing potential upon intravenous (IV) injection into healthy immunodeficient NOD scid gamma (NSG) mice. By harvesting organs from mice injected with either control or $CXCR4^{R334X}$–expressing NK cells 24 hours after injection, we could observe a significantly increased BM infiltration of NK cells in the mice that had received the $CXCR4^{R334X}$ NK cells compared to the mice that had received the control NK cells. The opposite pattern was observed when looking at cells in the blood and in the lung, whereas no differences between the two conditions were observed in the liver. These results were then further validated in a separate experiment using bioluminescence imaging (BLI) of live mice, where we again could show that $CXCR4^{R334X}$ electroporated NK cells primary localized in BM compartments such as the femur and skull 24 hours after injection, whereas control NK cells were confined to the lung and the spleen.

In **paper II**, we further dissected the CXCR4-SDF-1α axis by CRISPR/Cas9-mediated KO of the CXCR4 gene. Although the KO led to a small but non-significant reduction in CXCR4 cell surface expression, despite adequate KO efficacy, this resulted in a significantly decreased *in vitro* migration capacity compared to both control and $CXCR4^{R334X}$ mRNA electroporated cells. Furthermore, we were able to observe a similar pattern when evaluating the BM homing potential of control CXCR4-KO and $CXCR4^{R334X}$–expressing NK cells 24 hours after injection in healthy NSG-SGM3 mice. Notably, we were also able to show that neither of these modifications had an impact on the ability of NK cells to target several different AML cell lines *in vitro*.

Although further validation of the importance of the CXCR4-SDF-1α axis was important, the main objective of **paper II** was to investigate whether the enhanced BM homing capacity observed for $CXCR4^{R334X}$–expressing NK cells also could be utilized for improved leukemia clearance *in vivo*. We first wanted to investigate whether the $CXCR4^{R334X}$–expressing NK cells could mediate a reduced leukemic burden compared control NK cells.

To this end, an AML xenograft mouse model was developed in which a high dose of MOLM-14 cells were intrabone (IB) inoculated into the right hind leg of NSG-SGM3 mice. The mice were then treated with repeated NK cell injections over a period of two weeks before being sacrificed for organ harvest. As MOLM-14 cells were exclusively inoculated into a single leg, the model enabled us to determine the anti-leukemic effect in this particular leg, as well as the spread to the contralateral leg. This analysis revealed that $CXCR4^{R334X}$ electroporated NK cells was the only condition that not only could reduce leukemic burden in the inoculated leg, but also could hamper spread to the non-inoculated leg. Based on these results, we used the same model to explore whether the observed reduction in tumor burden also would translate into a survival benefit. Although a clear benefit was observed compared to untreated mice, there was no survival advantage compared to control NK cells. We speculated that the discrepancy between the two endpoints were due to model related factors and therefore developed a revised model based on the IV inoculation of a significantly reduced number of MOLM-14 cells. We anticipated that such a model would be less aggressive and simultaneously also mimic AML development in humans more accurately. After we had characterized the model and validated that the initial *in vivo* growth of MOLM-14 primarily occurred in the BM compartments, and showed that untreated mice displayed a prolonged survival compared to mice within the IB model, we again assessed how treatment with our different conditions would affect survival. After excluding events of early deaths observed in both treatment groups, we could in a subsequent analysis show that $CXCR4^{R334X}$-expressing NK cells could facilitate a significantly prolonged survival of mice compared to control NK cells.

### 4.1.3 Discussion

In **paper I**, we, revealed that common means to prepare NK cells for adoptive transfer resulted in a decreased CXCR4 cell surface expression. In **paper I** and **paper II**, we showed that transfection with $CXCR4^{R334X}$ encoding mRNA could increase the surface expression of the receptor which promoted an enhanced migration capacity towards SDF-1α *in vitro* and to BM compartments *in vivo*. Finally, and most importantly, we also showed that $CXCR4^{R334X}$-expressing NK cells were able to improve *in vivo* leukemia clearance compared to control NK cells in leukemia bearing mice.

The fact that the CXCR4 expression was downregulated after procedures which are commonly applied when preparing NK cells for therapeutic use, including stimulation with IL-2, is highly interesting as it confirms previously reported results and also identifies a potential need for improvement (206, 207, 211). This is especially so in relation to leukemia and other BM residing malignancies, as NK cells have been reported to have a poor *in vivo* BM homing potential (205), and CXCR4 expression levels have been correlated with response to NK cell treatments in a recent clinical trial (173). It was therefore highly appealing to try to improve CXCR4 expression levels, and at the same time also test the

hypothesis of taking advantage of a previously reported WHIM syndrome GOF mutation (208). Although we were able to show that electroporation with both $CXCR4^{WT}$ and $CXCR4^{R334X}$ encoding mRNA resulted in a significant increase in expression compared to control NK cells, it was surprising to see that only $CXCR4^{R334X}$ resulted in an improved *in vitro* migration capacity. It should be noted that a direct comparison between $CXCR4^{WT}$ and $CXCR4^{R334X}$ electroporated cells would be slightly unfair, as the migration experiments were carried out 8 hours after electroporation, for which the CXCR4 expression levels were higher in $CXCR4^{R334X}$-electroporated cells compared to the $CXCR4^{WT}$-electroporated cells. For this type of analysis, it would perhaps have been more appropriate to compare $CXCR4^{WT}$ cells 8 hours after electroporation to $CXCR4^{R334X}$ cells 16 hours after electroporation. Nevertheless, the $CXCR4^{WT}$ expression was a this timepoint several folds higher compared to control NK cells without increasing the migration potential. This data perhaps suggest that the $CXCR4^{WT}$ genotype does not really have the intended effect when overexpressed in NK cells. Indeed, our supplementary data in **paper I** could only show a positive correlation between CXCR4 expression levels and migration potential in $CXCR4^{R334X}$ electroporated cells, but not in $CXCR4^{WT}$ electroporated cells. It should however be pointed out that this observation was made in this particular study, and that others have reported a migration benefit when overexpressing the WT variant in NK cells (212). In retrospect, we should perhaps also have investigated the $CXCR4^{WT}$ variant alongside the $CXCR4^{R334X}$ variant in our *in vivo* based experiments. Nevertheless, although we could not observe a benefit after electroporation with $CXCR4^{WT}$, our data clearly indicates that utilizing cells electroporated with $CXCR4^{R334X}$ have the intended effect.

In relation to our *in vivo* data, where we have showed that $CXCR4^{R334X}$ electroporated cells have a superior BM homing potential as assessed by BM harvests 24 hours after infusion, one can also in retrospect argue that we perhaps should have conducted more in-depth studies to facilitate better mechanistic insights. Although we have carefully explored the kinetics of the receptor expression *in vitro*, it could have been interesting to also assess this *in vivo*. CXCR4 is known to become internalized upon ligand interaction, but alongside enhanced signaling through increased calcium-flux, less internalization has been reported as another mechanism by which the GOF variants retain cells in BM compartments in WHIM patients which potentially could have enabled such staining (209, 210). A perhaps more important set of experiments to conduct in order to have a better understanding of the *in vivo* migration pattern over time would have been to harvest mice at several more time points in addition to our 24-hour time point, or to explore this through *in vivo* tracking using BLI or $^{89}Zr$ (205). However, as our primary aim was to show proof-of-concept for that the CXCR4-SDF-1α axis can be used to improve BM homing and thereby promote an enhanced anti-leukemic effect, these types of question remain but should be more thoroughly investigated in future studies. Nevertheless, our lab has, in relation to a different project, evaluated NK cell homing 48 hours after electroporation with the

CXCR4$^{R334X}$ encoding mRNA and at that time point could not observe a difference compared to control NK cells. It should be pointed out that this experiment, as our 24-hour homing experiment, was performed in healthy mice and it can be speculated that it would look different in tumor bearing mice as engagement with tumor cells *per se* may promote BM retainment, something that potentially also could have been investigated. It would then have been interesting to assess the functionality of those migrated cells, as NK cells at least in the tumor microenvironment (TME) of solid tumors have displayed an impaired functionality (213). To assess the *in situ* function within the BM compartment is also relevant in relation to the CXCR4$^{R334X}$ expression, as BM retained cells in WHIM patients have been reported to undergo apoptosis (209, 214-216). However, one can argue that the reduction in tumor burden, along with the survival benefit observed in mice treated with CXCR4$^{R334X}$ compared to control NK cells, at least indicate that the cells are not severely hypofunctional *in vivo*.

Despite a relatively clear reduction in tumor burden observed with the IB model, we could not detect any differences in survival using that model, which we speculated was a result of the high number of MOLM-14 cells that were inoculated in combination with the previously reported aggressive growth pattern of this cell line and others in NSG mice (217). As pointed out in **paper II**, survival is an endpoint which is quite seldom reported on when evaluating NK cell treatment efficacy in AML xenografted mice, likely because this type of data is difficult to generate and due to the lack of well-established and optimized models for this purpose. However, to base conclusions solemnly on tumor burden is potentially problematic since tumor burden and survival does not always correlate perfectly, which we have learnt from our data using the MOLM-14 IB model. As our aim has always been to identify a product which would have the potential to be translated into a clinical setting, we were determined to evaluate survival using an additional model before making any conclusions in relation to the true therapeutic potential of this approach.

As mentioned in the manuscript, we debated whether a patient-derived xenografted (PDX) mouse model could be a potential option as such a model would more closely resemble human leukemia and have a slower growth pattern compared to a xenograft model derived from a cell line (218). However, PDX models are known to be more difficult to establish and recent evidence has highlighted that more aggressive AML subtypes are overrepresented in these models as they are more likely to engraft (218, 219). Instead, we developed an optimized variant of our MOLM-14 xenograft model based on the IV inoculation of a significantly lower number of MOLM-14 cells. This model proved to be less aggressive as untreated mice had prolonged survival. It should also be mentioned that an optimized MOLM-14 IB model was initially explored in parallel, which was also based on a reduced number of MOLM-14 cells for inoculation. Although this model, in a preliminary experiment, also proved to be less aggressive compared to our original IB model, we decided to go forward with the IV model as it was easier to handle and because we also

speculated that it resembled AML development in humans more accurately. In addition to this, we also assessed survival effects in two other models based on the IV inoculation of the HL-60 and OCI-AML3 cell lines. However, we were then unable to observe a survival benefit after adoptive NK cells transfer in any of these models compared to untreated cells, making them less relevant to explore in relation to CXCR4$^{R334X}$. The lack of efficacy in these models could perhaps have been expected based on the relatively low cytotoxicity which we have observed towards these cell lines at lower effector-to-target ratios, but it cannot be assumed that cells behave in the exact same way *in vitro* and *in vivo*, since MOLM-14 certainly can be targeted relatively easy *in vitro* as shown in **paper II**, but then also appeared to be much more difficult to control *in vivo*. As no effect of NK cell treatment was observed in either the HL-60 model or the OCI-AML3 model, one could perhaps question the *in vivo* anti-leukemic efficacy of NK cells overall. However, as outlined in the introduction of this thesis, there are substantial evidence from both mouse experiments as well as human clinical trials which contradicts this, indicating that the lack of efficacy is more related to these models being poor surrogates for primary AML.

Although we had developed an additional MOLM-14 IV-based model more suited for survival analysis, it was not a perfect model as events of early deaths were observed in mice treated with both control and CXCR4$^{R334X}$-expressing NK cells. As can be read in the manuscript, we speculated that this was an effect of reducing the initial tumor load, which resulted in larger technical variances between samples as illustrated by the relatively large spread in survival among untreated mice. To compensate for this, we performed an analysis where mice that did not survive longer than the median survival of untreated mice were excluded, and were then able to observe a significant difference in survival between mice treated with control NK cells and mice treated with CXCR4$^{R334X}$-electroporated NK cells. It should however be noted that these results were obtained using NK cells from a single donor, where control NK cells had been identified to mediate a prolonged survival compared to untreated mice, and that future studies will have to explore donor-dependent effects in more detail as well as the potential mechanisms governing this. In relation to these data, we also acknowledge that the survival benefit of mice treated with CXCR4$^{R334X}$-expressing compared to control NK cells appears to be rather small. However, this has to be put into the context of the model. Although not included in the thesis version of the manuscript, we have observed that equipping NK cells with the CXCR4$^{R334X}$ receptor resulted in a similar increase in survival length as a 4-fold higher dose of control NK cells did, highlighting that the observed effect might not be so small after all.

Nevertheless, the survival benefit can probably still be improved. It can be hypothesized that a more stable or permanent expression may increase survival further. Indeed mRNA electroporation only resulted in a transient expression of CXCR4$^{R334X}$, and it is possible that cells could migrate out of the BM once the transient expression decreases. However, this would have to be thoroughly investigated in future studies with and without tumor

burden and preferably also head-to-head with our current mRNA based approach. To continue to improve the BM homing capacity *per se* by utilizing other molecules than CXCR4 is another potential approach to further increase the anti-leukemic effect. Undoubtedly, the migration cascade is a complex process where many different molecules are involved, and it may be possible to utilize additional pathways involved (34). Unpublished but promising data from a separate project ongoing in our lab indicates a potential role for fucosylated, and therefore functional, E-selectin ligands on NK cells as a means to improve BM homing. Another appealing approach for achieving better homing to the BM compartments is to block or KO chemokine receptors involved in homing NK cells to other organs (34).

Although it is highly important to explore the above-mentioned approaches to further improve the BM homing potential of NK cells, other aspects must also be considered if this approach is to be of clinical relevance. Apart from careful investigations relating to dosing, the timing of these NK cells infusions and their relation to other therapies is likely to be important. Interestingly, within the fucosylation project mentioned above, our lab is also investigating how the TME in the BM compartments during leukemia may alter factors important for cell migration. These analyses have revealed that the SDF-1α levels appear to be negatively correlated with an increased tumor burden in an HL-60-based mouse model. In a context where SDF-1α levels are low, it may be even more important to utilize the CXCR4$^{R334X}$-variant as it has been shown to mediate increased signaling (208-210). If low SDF-1α levels is a general phenomenon in AML, and if the levels can also be restored after for example pre-treatment with chemotherapeutic agents, and what the kinetics of such potential restoration would look like, is a highly interesting question that remains to be investigated. Previous studies in multiple myeloma have showed that SDF-1α levels are decreased for that disease (220), so it is not unreasonable to think that this may also be the case in AML. Although further validations and additional experiments are required to delineate this, these observations at least indicate that the exact time point of NK cell injections could be of relevance.

Another aspect that is important to consider is the fact that the redirection of NK cells to the BM compartments, as a stand-alone approach, may not be enough for obtaining durable clinical responses. Although **paper II** indicate that the CXCR4-SDF-1α axis, as an independent modality, can be utilized for improved treatment efficacy against AML, it may be different in a clinical setting. It may then be necessary to combine this treatment approach with other interventions, such as a BiKE/TriKE, or to co-express CXCR4$^{R334X}$ with an anti-AML targeting CAR. The latter has already begun to be explored in the context of other diseases. For example, Arezoo Jamali et al. previously showed that the WT variant of CXCR4 can be co-expressed with an anti-CD19 CAR after viral transduction, leading to an enhanced migration capacity and potent killing of CD19 positive targets *in vitro* (212). Building on our data using the GOF variant, Yu Yang Ng et al. recently displayed that NK

cells co-expressing CXCR4$^{R334X}$ with an anti-BCMA targeting CAR could decrease tumor burden and prolong survival of multiple myeloma engrafted mice compared to treatment with NK cells only expressing the CAR (221). Unfortunately, they did not include a condition where CXCR4$^{R334X}$, but not the CAR, was expressed, which would have been highly interesting and potentially indicative of the potential of CXCR4$^{R334X}$ modified cells as a stand-alone approach.

While the efficacy likely can be improved, either through further enhancement of the BM homing potential itself or by combining the approach with methods to increase the direct cytotoxic potential of the cells, these two papers provide proof-of-principle for that the intrinsic migration capacity of NK cells can be altered and used as a mean to increase NK cell-mediated rejection of AML. The papers lay the foundation for future studies that can continue to build upon these data and eventually also evaluate the true therapeutic potential of the approach in a clinical trial.

## 4.2 Study III

### 4.2.1 Study rationale

As stated in the introduction, education is a dynamic process which occurs in NK cells upon signaling from specific inhibitory receptors that have bound to their cognate HLA class I ligands expressed by most healthy tissues in the body. This endows NK cells with the ability to be responsive to cells that lack these ligands, e.g. malignantly transformed cells, while remaining tolerant to self. Education has previously been reported to be mediated through specific inhibitory KIRs, which can bind to classical HLA molecules of the B and C subtypes. as well as the NKG2A receptor which binds to non-classical HLA-E molecules (106, 107). However, the LIR-1 receptor which is known to be able to bind to several different types of both classical and non-classical HLA class I molecules and similarly to KIRs and the NKG2A receptor receive inhibitory input via phosphorylation of ITIM motifs, has not been reported to mediate education (100, 101).

Most studies on NK cell education have been performed on non-stimulated NK cells (hereafter referred to as resting NK cells) in a controlled *in vitro* environment as well as in mouse studies (106-109). However, for therapeutic use, NK cells are often pre-activated with cytokines to enhance their cytotoxic capacities and to promote proliferation (171, 176, 177). The impact that these procedures have on NK cell education has not been extensively studied, but may be of importance as it potentially can help to refine these products further by for example allowing pre-selection of specific NK cell subsets for infusions. In this study, we therefore investigated how overnight (ON) IL-2 stimulation and *ex vivo* expansion for 12-14 days, two commonly used methods to prepare NK cells for adoptive transfer (170, 174), impact on education. The paper provides a detailed characterization of how different NK cell subsets from pre-selected healthy donors functionally respond to target cells after exposure and describes for the first time how

the LIR-1 receptor promotes enhanced responses, similarly to what can be observed in other educated NK cell subsets. As can be read in the result section below, we were able to further link this finding to NK cell education through detailed phenotypical characterization as well as by performing additional functional experiments. Lastly, we showed that LIR-1-expressing NK cells also mediated a potent ADCC response when combined with a mAb, and that this response even could overcome LIR-1-mediated inhibition through cognate HLA class I binding which indicates that this subset has therapeutic potential.

### 4.2.2 Results

In this study, we have utilized NK cells from KIR and KIR-ligand genotyped healthy donors to identify donors that only expressed inhibitory KIRs (haplotype A/A) or inhibitory and activating KIRs (haplotype B/x). We then stained the NK cells with fluorescently conjugated antibodies targeting common inhibitory NK cell receptors and after flow cytometry utilized a Boolean gating strategy that enabled us to identify cells expressing only one type of inhibitory receptor, but lacking all of the other inhibitory receptors that we stained for (hereafter referred to as singe-positive (SP) subsets). In this study, we have primarily compared LIR-1$^{SP}$, KIR2DL1$^{SP}$, KIR2DL2/3$^{SP}$, KIR3DL1$^{SP}$ as well as NKG2A$^{SP}$ cells within the different conditions (resting, ON IL-2 activated and expanded NK cells) to each other and to the NK cell inhibitory receptor negative subset (iNKR$^{-}$) which lacked all of the afore-mentioned inhibitory receptors.

In the study it was first shown that education, measured through the capacity to degranulate against the gold standard HLA class I deficient K562 cell line, could be observed within resting NK cells as expected. However, a similar educational pattern could also be observed in *ex vivo* expanded NK cells, but was less pronounced in ON IL-2 activated NK cells. More importantly, this initial analysis revealed that LIR-1$^{SP}$ *ex vivo* expanded NK cells also displayed a significantly enhanced response towards K562 cells compared to the iNKR$^{-}$ subset, indicating that this subset potentially had been educated. To evaluate the impact of feeder cells within the expansion protocol and to control for CMV infection as a potential bias, we also performed the experiments using NK cells from feeder free expansions as well as NK cells originating from CMV seronegative donors and could still observe enhanced responses within the LIR-1$^{SP}$ subset. Next, in line with the rheostat model (111, 114), we assessed whether co-expression with other inhibitory receptors potentially would lead to an increased responsiveness and were then able to detect that the addition of LIR-1 to either KIR2DL1$^{SP}$, KIR2DL2/3$^{SP}$ or KIR3DL1$^{SP}$ *ex vivo* expanded NK cells boosted the response towards K562 cells further. This was in stark contrast to resting NK cells, where no signs of co-education could be detected.

We then performed a uniform manifold approximation and projection (UMAP) analysis, using 8 common activation markers, of both resting and expanded NK cells to more

closely characterize LIR-1$^{SP}$ *ex vivo* expanded NK cells from a phenotypic perspective. This analysis revealed that resting LIR-1$^{SP}$ NK cells clustered similarly to uneducated KIR-expressing NK cells, which was different from that of educated KIR-expressing NK cells and NKG2A-postive NK cells. In the *ex vivo* expanded NK cells on the other hand, the educated and non-educated NK cells did not group separately based on the expression of these 8 markers. When assessing these markers individually, it also became clear that LIR-1$^{SP}$ *ex vivo* expanded NK cells displayed an increased expression of most of these markers compared to its resting counterpart. More importantly, we could also observe that LIR-1$^{SP}$ *ex vivo* expanded NK cells displayed a similar phenotype as *ex vivo* expanded NK cells educated trough KIR or NKG2A, for example with increased expression of DNAM-1 and Granzyme B. This pattern was not observed for resting LIR-1$^{SP}$ NK cells.

In an attempt to more closely link the enhanced responses and phenotypical changes observed in the LIR-1$^{SP}$ NK cells to the LIR-1 receptor specifically, we performed additional co-culture experiments against K562 cells, but this time we also included NK cells that had been pre-treated for 24 hours with a LIR-1 blocking antibody. This analysis revealed that *ex vivo* expanded LIR-1$^{SP}$ NK cells degranulated less towards K562 cells compared to the unblocked controls, indicative of potential detuning. Furthermore, this observation could not be observed in any of the other SP-subsets, except for when being co-educated by LIR-1. We also confirmed the blocking specificity in short-term cultures using ON IL-2 activated NK cells.

We next took advantage of the HLA class I$^{low}$ Epstein-Barr virus (EBV)-transformed LCL 721.221 cell line, for which we (in addition to the WT cell line) also had variants specifically transfected with either of the following HLA alleles: HLA-B58, HLA-Cw7, HLA-Cw15, HLA-E and HLA-G. We could then observe that LIR-1$^{SP}$ NK cells were inhibited by most of these variants, but to a less extent compared to KIR$^{sp}$ or NKG2A$^{sp}$ cells receiving inhibitory input form the variants that expressed their cognate HLA class I molecule. In relation to this, we then explored if expression of the LIR-1 receptor could aid KIR or NKG2A positive cells to overcome inhibition from their specific LCL 721.221 variants, which was not the case. Using the same system, we continued and investigated the potential of the LIR-1$^{SP}$ *ex vivo* expanded NK cells to perform ADCC and were able to show that the subset indeed degranulated potently towards 721.221 WT cells in the presence of the anti-CD20 mAb rituximab. To our surprise, we were also able to show that LIR-1$^{SP}$ *ex vivo* expanded NK cells could overcome inhibition mediated by all of the LCL 721.221 transfectants in the presence of rituximab, which was not the case for any of the other inhibitory NK cell subset investigated. Lastly in this study we confirmed the above mentioned ADCC capacity for the *ex vivo* expanded LIR-1$^{SP}$ NK cell subset also by using killing assays.

### 4.2.3 Discussion

In this paper, we have discovered that the inhibitory receptor LIR-1 can mediate education in *ex vivo* expanded NK cells. To the best of our knowledge, this is a unique observation that has not previously been described. In summary, we have shown that *ex vivo* expanded LIR-1$^{SP}$ NK cells have superior responses towards the gold standard HLA class I deficient NK target K562, as well as towards the WT variant of the HLA class I$^{low}$ EBV transformed LCL 721.221 cell line, altogether indicative of potential education. We have then phenotypically characterized the subset and shown that it has increased expression of markers associated with education. In specific LIR-1 blocking experiments, we further linked the observation to the LIR-1 receptor *per se*. Lastly in the paper, it was shown that the LIR-1$^{SP}$ subset also displayed a potent ADCC capacity, which ultimately was able to overcome inhibitory signals from cognate HLA class I molecules that had bound to LIR-1.

The first figure of the paper confirms what has previously been described with regards to enhanced responses in educated subsets among resting NK cells (106). However, it also reveals that a similar educational pattern can be observed after 12-14 days of expansions for the subsets that has been educated through KIR or NKG2A, which has not been described before. More importantly, the figure reveals that LIR-1$^{SP}$ NK cells, similarly to the KIR and NKG2A educated subsets, also displayed enhanced responses compared to iNKR$^{-}$ NK cells. Although cytokine stimulation protocols utilizing IL-2 and IL-15 previously has been shown to educate iNKR$^{-}$ cells by upregulating KIR expression (222), LIR-1 has not been linked to education in either resting or cytokine activated cells. According to the rheostat model (111, 114), NK cells can be co-educated by several inhibitory receptors simultaneously and it was therefore interesting to see that this pattern also held true for LIR-1$^{SP}$ cells co-expressed with different KIRs, as shown by the second figure in in the paper. By looking at the first figure in the paper, one can of course question the relevance of this subset as well as the relevance of these *ex vivo* expanded NK cells in general, seeing that the degranulation levels only seem to be marginally increased compared to resting NK cells while also being significantly lower compared to those observed for IL-2 activated NK cells. However, degranulation levels do not always reflect the killing capacity and it is already well established that NK cells expanded with this protocol display a significantly enhanced killing capacity compared to resting NK cells, potentially mediated through the upregulation of death receptor ligands (174). NK cells expanded with this protocol are also known to have an increased production and release of cytokines (174). However, the ability to produce cytokines such as IFN-ɣ was not explored in this study which is a potential limitation. It would certainly have been interesting to assess this given that the release of IFN-ɣ also is increased upon education (108, 110).

In the fourth figure of the paper, we wanted to investigate whether the enhanced responses that we had observed within the LIR-1$^{SP}$ NK cells could be specifically linked to the LIR-1 receptor, and therefore performed the LIR-1 blocking experiments mentioned in

the results section. These experiments revealed a significant reduction in degranulation towards K562 cells following a 24 hour blockade, which we interpreted as potential detuning. The kinetics of the potential detuning is corroborated by a previous study which have revealed that NK cells can detune *in vitro* within 24 hours if deprived from cognate HLA class I binding (124). In Segerberg et al. (125), one of my papers not included in the thesis to adhere to a strict focus on cancer but of relevance within the context of education, we investigated a potential detuning mechanism of NK cells in systemic lupus erythematosus (SLE). In the mentioned paper, we were able to show that a relatively large proportion of patients with SLE harbored autoantibodies to KIRs, and that autoantibodies derived from these patients, after overnight exposure, could decrease NK cell responses in healthy donor derived NK cells when co-cultured with HLA class I deficient K562 cells. We further linked this finding to a potential detuning mechanism, as we in the paper could show that the autoantibodies seemed to bind and shed KIRs from being detected by the flow cytometry antibodies that were used, and that this could be linked to a concomitant reduction in degranulation. As can be appreciated in **Figure 8** on the next page, a decreased KIR expression due to shedding after overnight incubation with autoantibodies from one patient correlated with a decreased degranulation potential, interpreted as detuning. Similarly to the other report mentioned above, a retuning phenomenon occurred over the upcoming 48-96 hours after wash-out (124, 125).

In the light of these data, it seems reasonable to think that LIR-1$^{SP}$ NK cells could also detune within 24 hours. Furthermore, it would of course have been interesting to investigate a potential retuning mechanism also in the LIR-1$^{SP}$ subset within this paper, but this was unfortunately beyond the scope of this study. Additional evidence for the observed reduction in degranulation potential after 24 hours of LIR-1 blockade being a result of detuning can perhaps instead be drawn from one of the supplementary figures in the paper, where short-term blockade of ON IL-2 activated NK cells did not lead to decreased degranulation against K562 cells. Instead, an enhanced response was observed against HLA class I-expressing targets, highlighting the specificity of the receptor as it specifically resulted in missing self among the LIR-1$^{SP}$ subset. In retrospect, and from a scientific standpoint, it would perhaps also have been valuable to assess the effect of a 24 hour LIR-1 blockade in resting and ON IL-2 activated NK cells. It would also have been interesting to phenotype the cells with regards to the activation markers used to characterize non-blocked cells in order to assess whether education markers such as DNAM-1 and granzyme B also would be altered.

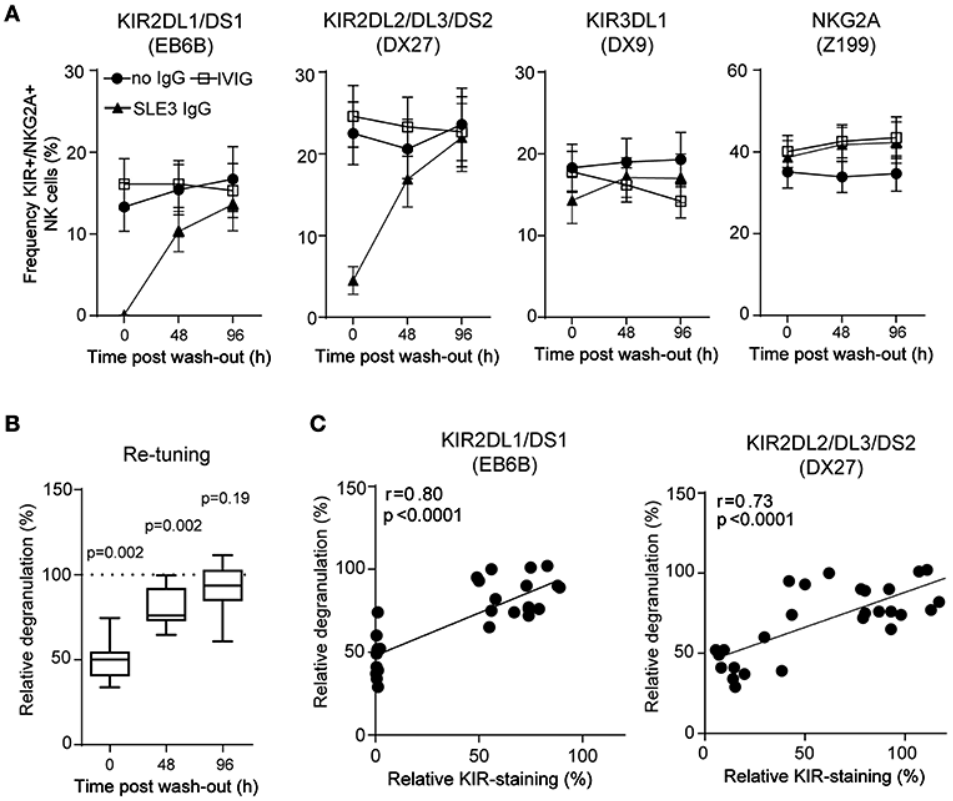


**Figure 8. "**NK cells retune following wash-out of IgG". From: Segerberg F, Lundtoft C, Reid S et al. Autoantibodies to Killer Cell Immunoglobulin-Like Receptors in Patients With Systemic Lupus Erythematosus Induce Natural Killer Cell Hyporesponsiveness. *Front Immunol.* 2019;10:2164. This figure was reprinted under the Creative Commons Attribution 4.0 International (CC BY 4.0) license. No changes has been made. Link: https://creativecommons.org/licenses/by/4.0/. A) Flow cytometry-based cell surface stainings of the denoted KIR receptors in healthy donor derived NK cells after overnight exposure to autoantibodies from one SLE patient as well as after 48 respective 96 hours post wash-out. B) Degranulation potential against K562 cells in healthy donors derived NK at the above mentioned timepoints. C) Correlation between A and B.

In the following figures of the paper, we took advantage of the EBV transformed LCL 721.221 cell line and utilized both the WT form as well as transfected variants expressing single HLA class I alleles, to further investigate the potential of the LIR-1$^{SP}$ expanded NK cell subset. First, we were able to confirm that the observations we made using the K562 cell line also could be detected against 721.221 WT cells, as the LIR-1$^{SP}$ expanded NK cell subset again displayed an enhanced degranulation capacity compared to the iNKR$^{-}$ subset. We then investigated whether LIR-1-expression could support NK cells that expressed KIRs or NKG2A to overcome inhibition mediated by these transfectants, which was not the case. However, this is perhaps not so surprising given how strongly the KIR$^{SP}$ subsets and the NKG2A$^{SP}$ subsets are inhibited by these transfectants. More surprising was the finding illustrated in the following figure, were LIR-1$^{SP}$ NK cells not only mediated potent ADCC against the HLA class I$^{low}$ 721.221 WT variant in the presence of rituximab, but also were

able to overcome inhibitory signals from all of the investigated transfectants. However, this finding is perhaps logical given that the data in this paper show that LIR-1-expressing NK cells are less inhibited by their cognate HLA class I ligands compared to other subsets, and the fact that the LIR-1 receptor previously has been shown to mediate weaker inhibition as compared to other receptors (101). Whether this observation simply is a result of weaker inhibition, or if the ability to overcome inhibition is mediated through some other mechanism, remains to be determined. In addition, it would also be highly valuable to explore whether the same observations can be made using other mAbs or even BiKEs or TriKEs. Testing this capacity using a BiKE or TriKE directed towards CD33 would for example be attractive in relation to AML and this thesis (223, 224).

Although the LIR-1$^{SP}$ subset was the only subset that could overcome inhibition by the transfectant cells expressing the cognate HLA class I ligands in the presence of rituximab, it should be noted that the HLA expression levels observed in these transfectants likely is significantly higher compared to what can be expected in an *in vivo* human setting. It is therefore not unlikely to think that also the other subsets potentially could overcome inhibition by for example autologous tumor cells in an *in vivo* scenario. In light of this, it would not only have been valuable to test several different mAbs, but also against target cells expressing more physiological HLA class I levels or perhaps even patient-derived material. In retrospect, it would also have been interesting to explore the effect of some additional HLA class I allele variants for the different HLA molecules that were explored in this context. Indeed, a large polymorphism exists within both HLA class I molecules, the different KIRs and also for the LIR-1 receptor (80, 81, 87, 88, 225, 226), and different variants of KIRs expressed on NK cells has for example been shown to bind different HLA class I allele variants with different affinity (115).

The reason why LIR-1 would mediate education in *ex vivo* expanded NK cells, but not resting NK cells, as well as how this mechanistically is regulated, is undoubtedly intriguing but also difficult to answer. Weaker inhibitory signals compared to KIRs and NKG2A could potentially be the reason for that LIR-1 have not been reported to mediate education in resting NK cells (101). However, seeing that a similar, or potentially even weaker inhibition was observed in LIR-1-expressing NK cells in our study, this cannot be the reason why *ex vivo* expanded LIR-1$^{SP}$ NK cells appear to be educated. As pointed out in the manuscript, another possible explanation may be that the intracellular signaling is different in *ex vivo* expanded compared to resting NK cells. As the abundance and localization of SHP-1 has been recently shown to be important for education, it would certainly have been interesting to look at SHP-1 in the context of both resting and *ex vivo* expanded LIR-1$^{SP}$ cells to determined potential differences (118-120).

In the manuscript, we also explored whether LIR-1-mediated education was influenced by the feeder cells used in the expansion protocol by expanding NK cells without feeder cells. Upon co-culturing these cells with K562 cells, we were still able to detect an enhanced

response in the LIR-1$^{SP}$ cells, indicating that the feeder cells at least cannot be the whole explanation. Nevertheless, the response within LIR-1$^{SP}$ cells was still much higher in feeder containing medium, potentially indicating that cells have a role, at least with regards to the magnitude of the response. Although LIR-1 has not been reported to mediate education in resting NK cells, we were still able to detect a small yet significant increase in degranulation against K562 cells compared to iNKR$^{-}$ cells. Hence, a potential hypothesis is of course that LIR-1$^{SP}$ resting NK cells in fact are educated, but that proper stimulation through the LIR-1 receptor or some other unknown mechanism potentiates the education. Although we have not HLA-phenotyped the lymphoblastoid cell line (LCL) SMI-LCL which were used as feeder cells, and therefore do not know exactly which HLA molecules it expresses, it may be that a chronic LIR-1 stimulation for a period of 12-14 days either potentiates an already established but discrete education, or initiates de novo education. In relation to this and to the finding in general, it would be valuable to explore if a completely different expansion protocol, for example utilizing K562 cells expressing membrane bound IL-21, also would result in education within the LIR-1$^{SP}$ cells (177).

How much of the observed enhanced responsiveness of the expanded LIR-1$^{SP}$ NK cell subset that can be attributed to education is another interesting question worth contemplating. Although our phenotypic characterization reveals that expanded LIR-1$^{SP}$ NK cells possess a phenotype resembling that of educated NK cells, with increased expression of DNAM-1 and Granzyme B (117, 122), most activation markers included in the analysis were upregulated within the subset during expansion. It may of course be that LIR-1 expression is just a surrogate marker, and that the enhanced response primarily is due to the accumulation of these activation markers. However, an NK cell that has not been educated through any inhibitory receptor should in theory be hyporesponsive, contradicting this argumentation (106). Furthermore, antibody mediated blockade of the LIR-1 receptor resulted in a decreased degranulation potential, indicating that LIR-1 likely is not just a surrogate marker but involved in dictating the response. However, it should be noted that blocking LIR-1 did not detune the LIR-1$^{SP}$ NK cells to the same level as the iNKR$^{-}$ subset, which could just be the result of lack of antibody specificity, but could also imply that other mechanisms potentially are involved. The accumulation of activating receptors could be an explanation to this, but also altered signaling from other inhibitory receptors. In our study, we have only stained for KIRs that previously have been shown to mediate education, but as *ex vivo* expansion evidently can lead to education through LIR-1, it may also have a similar effect on other KIRs which we have not stained for. If LIR-1 co-express these KIRs to a higher degree than the iNKR$^{-}$ subset, this could also be a contributing factor. To summarize this section, and to reiterate what already was pointed out in the introduction of this thesis, the mechanisms that governs NK cell education are poorly understood and until we have a better understanding of this, I believe it will be utterly difficult to fully comprehend the phenomena that we have observed in this study.

The possibility to utilize the expanded LIR-1$^{SP}$ NK cells for immunotherapeutic purposes is an interesting avenue to explore. The subset certainly has the potential to mediate robust responses against cancers with downregulated HLA class I, as illustrated by our data against HLA class I deficient or low cell lines, but also against tumors with cognate HLA class I expression if simultaneously combined with a mAb, and possibly also a BiKE or TriKE that can engage CD16. As some tumors are known to upregulate HLA-G, blocking antibodies have already been explored in an attempt to induce missing self within LIR-1-expressing NK cells (227, 228). A potential limitation of this is that the blocked cells may eventually detune. Instead, it should be possible to take advantage of the fact that LIR-1 binds most HLA class I molecules, which may prevent potential detuning of the LIR-1$^{SP}$ subset *in vivo*. A disadvantage with binding to several HLA molecules is of course that a mismatched situation, where the donor expresses an inhibitory receptor for which the cognate HLA class I ligand is missing within the recipient and which previously has proven beneficial with regards to NK cell therapy (169, 170), can likely not be achieved. The possibility to utilize the cells in an allogeneic mismatched scenario would therefore have to be investigated. As discussed in the paper, an ideal next step would be to further explore the LIR-1 education as well as the anti-tumor potential using mouse models. However, this was not done in the current paper as it is potentially challenging seeing that mouse NK cells do not express the human LIR-1 ortholog Pirb paired immunoglobulin-like receptor B (PIR-B) (229). Xenograft models, like the ones utilized in **paper II** of this thesis, could be an option but it is not certain that LIR-1$^{SP}$ cells would be able to stay educated in such a model, thereby making them less relevant for this particular purpose. Another option is to utilize fully humanized mice (218), but this comes with the problem of establishing cancers in an immunocompetent host.

Other potential aspects that would need to be addressed before this subset could be used in a clinical setting is to better understand the donor variability that we have observed when looking at LIR-1$^{SP}$ NK cells after expansion. We have noticed that the responsiveness among LIR-1$^{SP}$ NK cells can vary, and that not all donors have LIR-1$^{SP}$ NK cells that appears to be educated. Polymorphism in the LIR-1 gene may impact on this (225, 226), but would require investigation. Furthermore, it would also be important to address how to generate a large quantity of LIR-1$^{SP}$ cells. As shown in one of the supplementary figures within the paper, the absolute number of LIR-1$^{SP}$ cells is low as NKG2A is expressed by most of the expanded NK cells. Sorting LIR-1$^{SP}$ cells prior to the start of expansions has been investigated in relation to this study and could enrich for LIR-1 positive cells, but the majority of these cells also expressed NKG2A. However, it may be that LIR-1 can be co-expressed with other inhibitory receptors in expanded NK cells and that these subsets still would be highly attractive. In fact, we have shown a co-educational pattern in this study. Exactly which receptors that should be co-expressed with LIR-1 would have to be carefully investigated and may depend on which disease that is to be targeted. However, LIR-1 expression on NK cells that expressed other inhibitory

receptors was also decreased during the expansion, and LIR-1 could only be detected in approximately 15-20% of the expanded NK cells. If this number of LIR-1 positive NK cells would be enough would also have to be investigated. Although not included in the manuscript, preliminary data has been generated which indicate that LIR-1 expression may be increased in *ex vivo* expanded NK cells upon electroporation with LIR-1 encoding mRNA. However, these results need to be verified with additional future experiments, including detailed kinetics. From a therapeutic perspective, it might be more beneficial to generate more stable LIR-1 expression through for example viral transduction, in which LIR-1 could be placed under a more active promotor.

In summary, this paper provides evidence that the LIR-1 receptor can mediate education in *ex vivo* expanded NK cells, and furthermore indicates that the subset has attributes which are attractive for immunotherapeutic purposes. In the light of this thesis, it would be interesting to further explore the ability of this subset to be utilized in an AML context. Future studies will have to address the subsets potential to target specific cancer forms, including AML, as well as to provide more mechanistic insights.

## 4.3 Study IV

### 4.3.1 Study rationale

IDH mutations are detected in approximately 20% of all AML patients (130). IDH inhibitors (IDHi) have emerged as a new treatment option for this patient group (230). However, the initial response rates are approximately 30-40% with the vast majority of these patients eventually becoming resistant to therapy, resulting in an increased survival length of less than a year (145, 230-232). Thus, the efficacy can be improved and the need for new and more effective treatment options for this patient group remain. The objective for this study was to better characterize the epigenetic and transcriptional landscape of IDH mutated AML in the absence and presence of a novel IDHi. Findings would have the potential to improve our understanding of the molecular network involved in IDH mutated AML, and lead to insights that can be utilized for therapeutic purposes. For this reason, we took advantage of the TF-1 AML cell line, modified to overexpress either the WT or a mutant variant of IDH2. In addition, we also utilized pre-existing patients co-cohorts. As detailed in the introduction of this thesis, mutations in IDH ultimately leads to inhibition of the epigenetic regulator TET2, which mediates DNA methylation and has been reported to cause a hypermethylated DNA profile in IDH mutated patients (133, 135). In summary, the first part of this paper verifies this finding using the described model system, and further reports on a dysregulated transcriptional network involving myeloid-related transcription factors, which in part seemed to be reversed upon treatment with IDHi. In the second part of the paper, we show specifically how methylation of the HLA gene cluster within our TF-1 model leads to the downregulation of HLA class I molecules on the surface of the cells, and describe the resultant sensitizing to NK cell-mediated killing.

Lastly, we show that HLA class I seems to remain downregulated in IDHi treated non-responding patients, and postulate that this patient group may particularly benefit from adoptive NK cell therapy. As I specifically have been involved in the second part of the paper, focusing on the HLA downregulation and subsequent enhancement in NK cell sensitivity, the result and discussion section below will be more focused on these parts, which also is in line with the scope of the thesis.

### 4.3.2 Results

For this study, we have utilized the TF-1 cell line overexpressing either the IDH2WT or the well characterized IDH2R140Q mutant variant as a model system. We initially showed that the TF-1 IDH2R140Q variant produced high levels of 2-HG, and displayed a hypermethylated DNA profile with a subsequent loss of 5hmC. We then identified that this affected transcription and characterized differential gene expression of both up and downregulated genes, but with a higher proportion of downregulated genes, in TF-1 IDH2R140Q compared to TF-1 IDH2WT cells. We were then able to link these epigenetic and transcriptional changes to an IDH-AML subtype specific transcription factor network. Treatment with the IDH-2 inhibitor enasidenib (AG-221) partially reversed the above-mentioned pattern, with a decrease in 2-HG levels and a regain of 5hmC levels when we measured four respectively seven days after drug exposure. We could also observe a treatment effect with regards to gene expression, with both up and downregulated genes, and link this to a myeloid transcription factor driven response.

One specific set of genes that were downregulated upon hypermethylation in the TF-1 IDH2R140Q cells were genes within the HLA cluster and our RNA sequencing data revealed that several HLA class I and class II genes were affected. This finding was further validated in a large cohort of IDH mutated AML patients, where genes encoding both HLA class I and HLA class II showed a high degree of hypermethylation leading to transcriptional downregulation. We next validated that the reduction in transcripts observed in the TF-1 IDH2R140Q cells also would translate to a reduced amount of HLA class I surface molecules, and observed a significant decrease in pan-HLA class I. When staining for individual HLA class I molecules, we could further detect a large and significant decrease in HLA-C as well as HLA-Bw6, and a smaller but still significant decrease in HLA-Bw4, which together with HLA-E was expressed at very low baseline levels in the TF-1 WT cells. As already explained in detail throughout this thesis, HLA class I is highly involved in regulating NK cell functionality. Naturally, after confirming that TF-1 IDH2R140Q cells also displayed a reduced amount of HLA class I molecules on their surface, we investigated how NK cells would functionally respond to these cells if put together in a co-culture assay. These results revealed that NK cells both degranulated to a higher degree, and displayed an increased production of IFN-$\gamma$ and TNF-$\alpha$ upon being co-cultured with TF-1 IDH2R140Q cells compared to TF-1 IDH2WT cells. Furthermore, we were able to confirm an enhanced responsiveness with specific killing assays. Similar to **paper III**, we next

performed an analysis using a Boolean gating strategy which enabled us to determine the contribution of specific NK cell subsets. The grouping was once again based on the expression of inhibitory receptors and included the following subsets: iNKR$^{-}$, LIR-1$^{SP}$, KIR2DL1$^{SP}$, KIR2DL2/3$^{SP}$, KIR3DL1$^{SP}$ and NKG2A$^{SP}$. This analysis revealed an enhanced responsiveness among all of the investigated subsets. KIR-ligand genotyping of TF-1 cells prior to the start of these experiments had showed that the TF-1 cells expressed HLA-C molecules bellowing to the C1 group, but not the C2 group, and we therefore performed a sub analysis were we specifically compared KIR2DL2/3$^{SP}$ cells to KIR2DL1$^{SP}$ and iNKR$^{-}$ cells which again showed an increase response in all subsets when co-cultured with TF-1 IDH2R140Q compared to WT, but with the largest response from the KIR2DL2/3$^{SP}$ subset.

We next sought to validate the HLA class I downregulation and subsequent increase in sensitivity to NK cell-mediated killing in another system. For this purpose, the HL-60 AML cell line was pre-treated with a membrane-permeant 2-HG precursor known as octyl-D-2-HG for 14 days. As with the TF-1 model, this resulted in a decrease in HLA class I molecules and an increased sensitivity to NK cells, which was not observed in the HLA class I deficient K562 cell line used as a control. We then went back to the TF-1 model system and showed that pre-treatment with IFN-ɣ for 48 hours resulted in a significant increase of HLA class I molecules on the cell surface of the TF-1 IDH2R140Q cells, which completely abrogated NK cell degranulation as well as cytokine production, and prevented NK cell-mediated killing. Lastly in this paper, we re-analyzed data from a recent study by Wang et al. (233) to determine methylation levels in IDH mutated AML patients after treatment with IDHi and were able to show that non-responding patients remained hypermethylated in HLA class I genes, indicating that they may be sensitive to NK cells and therefore benefit from NK cell-based immunotherapy.

### 4.3.3 Discussion

In this paper, we have used a model system based on the overexpression of IDH2WT or IDH2R140Q in TF-1 cells to mimic IDH2 mutated AML. We have shown that TF-1 IDH2R140Q display a hypermethylated DNA profile resulting in differentially expressed genes linked to a dysregulated transcriptional network involving myeloid-related transcription factors, which in part can be restored after treatment with IDHi. The main novel finding of the paper is that the HLA class I cluster are among the hypermethylated genes, and that this results in the downregulation of HLA class I molecules which sensitizes IDH mutated TF-1 cells to NK cell-mediated killing. Furthermore, it was finally shown that IDHi resistant patients remain hypermethylated in HLA class I, suggesting that this patient group could benefit from NK cell-based immunotherapy.

Initially in the paper, we showed that the TF-1 IDH2R140Q mutated cells produced high levels of 2-HG, which also is known to occur in IDH mutated patients (234). Furthermore, a hypermethylated DNA profile with gain of 5mC and subsequent loss of 5hmC was

observed in an inverse correlative pattern which, as mentioned in the results section, resulted in a differential gene expression. Upon treatment with AG-221, a reversed pattern could be observed with loss of 5mc and subsequent gain of 5hmC, in line with what has previously been reported (235). However, treatment with AG-221 only partially restored gene expression, indicating that 7 days of treatment may be insufficient.

The fact that the hypermethylation also affected several of the HLA genes and that we could detect a consequent HLA class I downregulation followed by an increased sensitivity of the TF-1 IDH2R140Q mutated cells to NK cell-mediated killing is highly interesting and relevant. Although NK cell-based immunotherapy against AML holds promise, and means to further increase the efficacy are currently being investigated as exemplified by **paper I** and **paper II** in this thesis as well as by others (184, 236, 237), the efficacy has so far been suboptimal. However, it may be that certain subgroups of patients are more sensitive to NK cells than other, and therefore would benefit from such treatment to a higher degree. The data presented in this study is indicative of this, and has taken the first step in delineating a subgroup of AML patients that may benefit in particular from NK cell-based immunotherapy, all in line with a more personalized treatment approach which may be necessary for diseases such as cancer.

In this study, we have provided evidence that IDH mutations sensitize AML cells to NK cell-mediated killing. However, the mechanisms governing this will need to be further delineated in future studies. Although we have linked the increased NK cell sensitivity to HLA class I downregulation, which is a reasonable conclusion to make based on the data presented and since HLA class I is well established to be involved in dictating the NK cell response to target cells (54, 55, 57), it cannot be ruled out that other factors may have contributed to our observation. Indeed, we did observe increased responses in all explored subsets against TF-1 cells harboring IDH2R140Q compared to WT cells within the Boolean gating analysis that we performed. This could, at least in part, be explained by potential lack of specificity among the HLA class I antibodies that were used, which may not cover all HLA class I molecules making the subset data more difficult to interpret. It should also be noted that in this paper, in contrast to **paper III**, we did not use KIR haplotype A/A donors thereby potentially introducing more confounding factors within our system. Furthermore, although our panel used to detect inhibitory receptors should cover the most common and important inhibitory receptors, it does not include all receptors that potentially could mediate inhibition. For example, it has recently been shown that HLA-G can regulate NK cells in breast cancer via KIR2DL4 (238, 239), and neither of these two molecules were included in our panel. Nevertheless, other potential mechanisms cannot be excluded. One such mechanism could be signals mediated through various activating receptors, as its not unreasonable that ligands for common activating receptors also could be differentially expressed in the TF-1 IDH2R140Q variant. For example, our RNA-sequencing data indicated that B7-H6, the ligand for NKp30, was

slightly upregulated in the TF-1 IDH2R140Q cells compared to the TF-1 IDH2WT cells. It would have been interesting to also stain the TF-1 cells for this ligand as well as other ligands binding to activating receptors. In a preliminary experiment (data not shown in the paper), we did stain TF-1 IDH2WT and TF-1 IDH2R140Q cells for the DNAM-1 ligands PVR and nectin-2, as well as the NKG2D ligands ULBP1, ULBP2, ULBP3 and MICA/B which did not reveal any dramatic changes. This preliminary experiment was however performed with an unoptimized flow panel and within an experiment where the TF-1 cells displayed an altered sensitivity to NK cells compared to how they normally would respond, thereby making it difficult to draw reliable conclusions. Furthermore, neither of these molecules were increased in the RNA sequencing data either, suggesting that these particular molecules may not be involved. Even so, future studies should dissect this further in repeated experiments using an optimized panel covering the above mentioned ligand as well as others.

Although our data indicate that other mechanisms potentially are involved, the link between HLA class I downregulation and subsequent enhancement of NK cell-mediated responses remain. The subanalysis of the KIR2DL2/3$^{sp}$ subset mediating the strongest response after HLA class I downregulation in TF-1 IDH2R140Q cells, together with the reduced response for NK cells against this cell line upon HLA class I upregulation after IFN-ɣ treatment, indicate that HLA class I is at least partially involved in mediating this effect. Furthermore, the fact that HL-60 cells also downregulated HLA class I after Octyl-D-2-HG treatment resulting in a similarly enhanced sensitivity to NK cells as was observed in the TF-1 system, which was not observed against K562 cells used as a control, also implies that HLA class I downregulation is important in mediating the effect. Nevertheless, future studies will have to dissect the exact mechanism in more detail.

In retrospect, there are a few other limitations in our study which are important to be aware about, and potentially need to be addressed in future studies. For example, the link between the hypermethylation profile and the differentially expressed genes could have been further investigated, as well as the correlation between hypermethylation and gene expression before and after treatment. Additionally, as already pointed out, the treatment duration of 7 days is rather short, and it would have been interesting to evaluate responses to prolonged treatment. It would also have been highly interesting to explore the effects on HLA after treatment with AG-221, both in terms of methylation and expression, and to evaluate how that would have affected the NK cell sensitivity. In relation to NK cell-mediated responses, additional killing experiments could also have been performed to increase power, but then the main objective with those experiments were to confirm that the enhanced degranulation capacity and cytokine production also resulted in an improved killing. An additional avenue that potentially could have been explored in this study, and may yet be explored in future studies, is the potential effect on T cells. Our

data also showed that HLA class II genes were downregulated and how this would influence T cell-mediated immunity was not explored in our current study.

Although the TF-1 model used in this study seemed to resemble many features of how IDH mutated AML behaves in a human setting, it was limited in certain ways and therefore not completely optimal. For example, as the IDH2R140Q mutation were not stably expressed by the cell line, it would disappear over time which made it difficult to assess effects of for example long term AG-221 treatment. Such a system may potentially also have introduced some degree of variability within the data, especially as the IDH2R140Q expression was evaluated by measuring 2-HG levels in the supernatant prior to the experiments. In our system, we specifically mimicked AML carrying the IDH2R140Q mutation. Although a model cannot cover all genetic variants associated with a disease, it would have been interesting to also evaluate mutations in R172, described as the other hotspot mutation in IDH2. Indeed studies have shown that R172 mutated IDH2 may result in even higher 2-HG levels (240). In relation to 2-HG, it would furthermore have been interesting to more closely investigate the association between 2-HG levels and TET2 inhibition, as well as how this correlates to HLA class I downregulation and increased NK cell sensitivity. In addition, it would have been highly valuable to also look at the effects of IDH1 mutations, as we only evaluated effects caused by an IDH2 mutation in our model system. Overall, our study could have benefitted from assessing the findings with another model, either carrying a different variant of the IDH2 mutation or an IDH1 mutation. Although we evaluated the HLA class I downregulation with a different system by looking at HL-60 AML cells after octyl-D-2-HG treatment, these analyses did not cover additional mutations.

As a next step, it would be interesting to validate the findings in patient material carrying either IDH1 or IDH2 mutations. However, such experiments would have to be carefully designed as AML is a heterogenous disease where several types of mutations are present simultaneously (129). Properly matched controls in terms of mutational burden would be an absolute requirement. Furthermore, although our RNA sequencing data indicates that patients with AML mutations have downregulated HLA class I, the level of downregulation on an individual basis would be rather difficult to assess without also having matched material from before the disease onset, as both the type of HLA molecules as well as the level of baseline expression may differ between individuals. It may be possible to simply compare HLA levels in IDH mutated and non-mutated patients if properly matched in terms of other mutations, but it would then require a rather large cohort to enable adequate conclusions. In addition to patient material, it would also be important to validate our findings and provide proof-of-concept for that IDH mutated AML would be more sensitive to NK cells compared to non-IDH mutated AML in an *in vivo* model. For this purpose, a xenograft model utilizing for example a cell line with and without a stable IDH mutation could be considered.

Although certain limitations have been identified, where future studies will have to dissect the underlying mechanisms in more detail and confirm our findings using patient-derived material and an *in vivo* model, the data presented in this study have revealed that mutated IDH2 results in HLA class downregulation which in turn seem to confer an increased sensitivity to NK cell-mediated killing. An interesting finding of potential clinical relevance was the fact that IDH mutated AML patients that had not responded to treatment with IDHi remained hypermethylated in HLA class I genes, possibly identifying a subgroup of patients which may particularly benefit from treatment with NK cells. Interestingly, in a recent clinical trial where high-risk MDS and AML patients were treated with adoptive NK cell infusions, one out of nine non-responding patients harbored an IDH mutation whereas this number was 50% (three out of six) of the responding patients (173). Although the study size was small and the finding could be confounded by other factors or simply be a coincidence, it supports the data in this study and the hypothesis that IDH mutated patients may be more sensitive to NK cells. If IDH mutations confer an increased sensitivity to NK cells, it is possible that other types of cancers also could be particularly sensitive to NK cell-mediated lysis. IDH mutations are frequent in glioma patients and it would therefore be interesting to explore the sensitivity to NK cells for that malignancy as well (241). However, although IDH mutations are frequent in gliomas, one study has shown that they may mediate escape from NK cell recognition through downregulation of the NKG2D ligands ULBP1 and ULBP3 (242), and the sensitivity would therefore have to be explored in detail for that disease. Another interesting research question to investigate as a follow up to this study is if TET2 mutations, common in both AML and MDS (141, 243), lead to a similar downregulation in HLA class I and potentially also increased sensitivity to NK cells. Interestingly, a recent study showed that autologous NK cells from MDS patients harboring TET2 mutations exhibited a reduced cytotoxic potential (244). The authors did not investigate HLA class I levels nor education in relation to this, but a putative detuning mechanism as a result of HLA class I downregulation cannot be excluded as a contributing mechanism.

In summary, this paper describes a novel finding in which mutations in IDH seem to result in HLA class I downregulation and increased sensitivity to NK cell-mediated killing. Based on the data presented in this paper, we hypothesize that IDH mutated patients that do not respond to IDHi therapy represent an AML subgroup that may benefit particularly from NK cell-based treatments. Future studies will have to dissect the mechanisms more in detail and to further explore this concept using patient materials and *in vivo* models.

# 5 Conclusions

This thesis provides evidence for that migration, as an independent factor, can be modulated to improve BM homing and leukemia targeting of adoptively infused *ex vivo* expanded NK cells. Furthermore, it details how a previously reported hyporesponsive subset of NK cells appears to be educated after *ex vivo* expansion, and that this subset has the potential to be utilized in a therapeutic setting due to its strong baseline killing and ADCC capacity. Lastly, the thesis describes how a specific mutation in AML entails an enhanced sensitivity to NK cell-mediated responses, and that AML patients carrying this mutation may particularly benefit from NK cell treatment. Overall, this thesis presents original and unique findings which may be utilized to improve NK cell-based immunotherapy against AML and potentially also other diseases. Specific conclusions from the constituting articles are found below.

**Paper I-II**

- Common means to prepare NK cells for adoptive infusions, such as cell isolation and cryopreservation, short-term IL-2 activation and *ex vivo* expansion results in a low CXCR4 cell surface expression and a reduced migration capacity.
- The gain-of-function $CXCR4^{R334X}$ BM homing receptor variant can be expressed in *ex vivo* expanded NK cells, resulting in an increased BM homing potential without negatively impacting on NK cell viability, phenotype or cytotoxic potential.
- Expression of $CXCR4^{R334X}$ on adoptively infused *ex vivo* expanded NK cells can improve *in vivo* leukemia clearance and prolong survival of AML-xenografted mice.

**Paper III**

- The LIR-1 receptor seems to be able to mediate both education and co-education in *ex vivo* expanded NK cells resulting in a robust cytotoxic baseline potential and other features indicative of education.
- $LIR\text{-}1^{SP}$ NK cells has a potent ADCC capacity that even can overcome inhibitory signals from cognate HLA class I ligands and consitute a subset particularly suited for NK cell-based immunotherapy of cancer.

**Paper IV**

- Mutated IDH leads to a hypermethylated HLA gene cluster and subsequent downregulation of HLA class I molecules resulting in an increased sensitivity to NK cell-mediated responses.
- IDH mutated AML patients not responding to IDHi appears to remain hypermethyalated in HLA class I genes, suggesting that these patient group could benefit from NK cell-based immunotherapy.

# 6 Future perspectives

Although this thesis describes several new insights that may be employed to enhance NK cell-based immunotherapy against AML and potentially also other malignancies, it further highlights that additional studies are required. These studies should focus on delineating some of the underlying mechanisms more carefully as well as validating the therapeutic potential of the findings and whether there are ways to further improve the efficacy. With regards to **papers I-II,** we have now provided proof-of-concept for the approach. However, there is still room for improvement and it would therefore be highly interesting to evaluate if permanent expression of $CXCR4^{R334X}$ can increase the efficacy further. In relation to that, more in-depth homing studies over time with both transient and permanent expression should be performed. To explore the potential to combine $CXCR4^{R334X}$ expression with means to enhance persistence as well as the direct targeting capacity, for example through a BiKE/TriKE or an AML targeting CAR is also highly appealing. Follow up studies to **paper III** should perhaps investigate the true therapeutic potential of the $LIR\text{-}1^{SP}$ subset in relation to other subsets, and if any particular malignancies are more suitable to target. From the perspective of this thesis, it would of course be of interest to evaluate efficacy against AML more specifically. Such studies should include *in vivo* models, and would then also have to address ways to enrich for the subset as the number of cells likely would have to be increased to enable for adoptive infusions. Additional mechanistic insights would also be of interest, but such studies may yet be difficult to conduct due to our limited knowledge of how education in fact is regulated. In relation to **paper IV,** it would be valuable to confirm the results with additional *in vitro* and *in vivo* models, where the NK cell sensitivity conferred by both IDH1 and IDH2 mutations could be evaluated in detail. Such studies should also try to better define the impact of HLA class I downregulation in relation to potential other mechanisms contributing to the enhanced sensitivity. Additionally, it would be highly valuable to confirm the findings using patient-derived material.

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# 8 References


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