On the role of Natural Killer cell immunogenetics for the outcome of immunotherapy in acute myeloid leukemia

Sahlgrenska Center for Cancer Research
Department of Medical Biochemistry and Cell Biology
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2022

Cover illustration: Gene variants driving NK cell interaction with target cells by Brwa Ali Hussein

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brwa.hussein@gu.se

ISBN 978-91-8009-600-3 (PRINT)
ISBN 978-91-8009-601-3 (PDF)
http://hdl.handle.net/2077/70227

Printed in Borås, Sweden 2022
Stema Specialtryck AB

“To my family, colleagues, friends, and patients”

ABSTRACT

Numerous studies have revealed that natural killer cells have fundamental roles as effector cells in myeloid leukemias. Thus, understanding NK cell-related biomarkers that influence clinical outcome are warranted. This thesis comprises in-depth dissection of how NK cell biology impacts on survival during histamine dihydrochloride/IL-2 therapy in acute myeloid leukemia (AML), with an emphasis on role of genetics of NK cell receptors and HLA. We studied AML samples from the Re:Mission trial using flow cytometry and PCR-based techniques. In addition, various *in vitro* NK cell functional assays have been performed using healthy donors and CRISPR-edited cell lines. In ***paper I***, we sought to examine the effects of an HLA-B dimorphism, which affects HLA-E presentation, on NK cell function and on NK cell responses to leukemic cells. Results suggested that individuals with a presentable HLA-B variant harbored better-educated NKG2A^{+} cells. Furthermore, AML patients with this variant showed superior clinical outcome after HDC/IL-2 therapy in comparison to patients with non-presentable HLA-B leader peptides. In ***paper II***, we investigated the potential impact of gene variants of NKG2D, DNAM-1 and NKp30 on receptor expression and survival of AML. Findings demonstrated that an NKG2D SNP was associated with increased expression and better clinical outcome of HDC/IL-2 immunotherapy. However, this polymorphism is in linkage disequilibrium with other polymorphisms in adjacent genes. Thus, in ***paper III***, we first aimed to determine the clinical impact of NKG2A variants on outcome of AML after immunotherapy; secondly, to define whether the NKG2A gene variants affect function of NK cells. AML patients with high-expressing NKG2A alleles had a more immature NK cell repertoire, higher granzyme B content and superior clinical outcome. Taken together, this thesis provides new insights into NK cell biology, and their potential applicability to predict outcome of immunotherapy in AML.

Keywords: Natural killer cells, acute myeloid leukemia, immunotherapy, HDC/IL-2, NKG2A, NK education, SNPs, NK activating receptors, HLA class I

SAMMANFATTNING PÅ SVENSKA

Immunoterapi är en behandlingsform som syftar till att stärka kroppens egna immunförsvar mot transformerade celler som annars kan leda till tumörväxt och cancer. Sedan immunterapi infördes som behandling mot cancer har diagnosen för flera cancertyper förbättrats avsevärt, och 2018 belönades forskning kring immunterapi med Nobelpriset i medicin. I den här avhandlingen har NK-celler, en typ av immunceller, och deras inverkan på immunterapi vid akut myeloisk leukemi (AML) studerats. AML är den vanligast förekommande varianten av leukemi och har en dålig prognos, vilket gör att det finns ett stort behov av förbättrade immunoterapeutiska strategier för att behandla AML. En av anledningarna till den dåliga prognosen vid AML är att många patienter, trots att de svarar på primär cytostatika-behandling, återfaller. Detta beror på att en liten andel cancerceller finns kvar i kroppen även efter genomgången cytostatika-behandling. En strategi för att förhindra återfall är därför att genom immunterapi aktivera det egna immunförsvaret för att det i sin tur ska kunna slå ut och förgöra de kvarvarande cancercellerna. En befintlig behandling med detta syfte är en kombinationsbehandling med histamin dihydroklorid och interleukin-2 (HDC/IL-2), som har utvecklats vid Sahlgrenska akademin. Behandlingen leder till en aktivering av bland annat NK-celler, där IL-2 stimulerar NK-cellerna medan HDC förhindrar att fria syreradikaler från myeloida celler hämmar NK-cellernas funktion. Avhandlingens arbeten bygger på studier från Re-Mission-studien där 84 AML patienter som först genomgått cytostatika-behandling sedan behandlades med HDC/IL-2 under 18 månader. Patienterna följdes under minst 2 år för att utvärdera hur behandlingen påverkade deras immunförsvar, och hur detta korrelerar med återfall och överlevnad. Avhandlingen belyser vikten av inhibitorisk NK-cellssignalering, framför allt *via* den inhibitoriska NK-cellsreceptorn NKG2A, för NK-cells funktion vid AML. Resultaten visar att genvarianter som påverkar den inhibitoriska signalvägen mellan NKG2A och HLA-E påverkar risken för återfall och överlevnadstid för AML-patienter som behandlats med HDC/IL-2. Vidare indikerar resultaten att NK-celler som uttrycker NKG2A spelar en viktig roll vid eliminering av cancerceller vid AML. Sammanfattningsvis bidrar arbetena i den här avhandlingen till en djupare förståelse för NK-cells biologi, samt hur kunskapen om NK-celler kan ligga till grund för att bättre kunna avgöra både vilka patienter diagnosticerade med AML som har störst risk för återfall, och vilka patienter som har nytta av NK-cellsinriktad immunterapi.

LIST OF PAPERS

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

I. Hallner A, Bernson E, **Hussein BA**, Sander FE, Brune M, Aurelius J, Martner A, Hellstrand K, Thorén FB.

The HLA-B -21 dimorphism impacts on NK cell education and clinical outcome of immunotherapy in acute myeloid leukemia. *Blood* 2019; 133(13): 1479-1488.

II. **Hussein BA**, Hallner A, Vennström L, Brune M, Martner A, Hellstrand K, Bernson E, Thorén FB.

Impact of NK cell activatory gene variants on receptor expression and outcome of immunotherapy in acute myeloid leukemia. *Frontiers in Immunology* 2021;12:796072.

III. **Hussein BA***, Kristenson L*, Pesce S, Hallner A, Nilsson M, Nilsson S, Brune M, Hellstrand K, Elin Bernson, Tang KW, Thorén FB.

Impact of NKC locus gene polymorphisms on natural killer cell function and outcome of immunotherapy in acute myeloid leukemia. *In manuscript.*

*Authors contributed equally

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ABBREVIATION

ADCC	Antibody-dependent cellular cytotoxicity
AlloSCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
CD	Cluster designation
CMV	Cytomegalovirus
CR	Complete remission
DC	Dendritic cells
GVHD	Graft versus host disease
GvL	Graft versus leukemia
HDC	Histamine dihydrochloride
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IL	Interleukin
ILC	Innate Lymphoid Cells
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITAM	Immunoreceptor tyrosine-based activation motif
KIR	Killer immunoglobulin receptor
LFS	Leukemia-free survival
LMP	Lymphoid multipotent progenitors
LRC	Leukocyte receptor complex
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
NKC	NK complex
NOX2	NADPH oxidase type 2
OS	Overall survival
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism

PREFACE

Cancer is characterized by growth and expansion of abnormal cells that divide uncontrollably and can infiltrate and damage different parts of the human body. Cancers that begin in the blood forming tissues of the bone marrow are called leukemias. Today, cancer is considered the second cause of death in the world, however, in several cancer types there is now an improvement in survival due to developments in cancer treatment approaches.

There was a huge advancement in the treatment of cancer since nitrogen mustard was first approved by Food and Drug Administration back in 1949. Since then, many new therapies have been developed. However, it appears progressively evident that there will not be a single cure, but that patients will have to be treated individually according to their precise prerequisites. There are currently new approaches to tame the immune system in the fight against cancer, and the capability of rewiring the human immune system has surely generated enormous expectations (1), with a hope that cancer becomes a curable disease in the future.

One of the common types of leukemias is known as acute myeloid leukemia (AML) which is characterized by expansion of abnormal white blood cells that accumulate in blood and bone marrow. The significance of the immune system in AML has attained an increasing interest, leading to the development of immunotherapies. One immunotherapy in AML is a combination of interleukin-2 (IL-2), that stimulates the lymphocytes, and histamine dihydrochloride (HDC), that decreases the production of immunosuppressive reactive oxygen species (ROS). Thus, HDC/IL-2 can activate natural killer (NK) cells to employ an anti-leukemic response. With improved understanding of the genes controlling the immune system, and more precisely the NK cells, NK cell-based immunotherapy can become a more effective option for AML treatment, with the aim to improve survival. A major goal of this thesis is to understand how genes of the inhibitory NK cell receptor NKG2A, and its ligand influence the function of NK cells and outcome of immunotherapy in AML. Taken together, this thesis provides novel observations of NK cell biology that may change the view of NK cell function and their implementations in predicting outcome of AML.

1 INTRODUCTION

Immunity demands the detection and eradication or containment of infectious organisms. This is attained by two systems roughly split into innate immunity and adaptive immunity. The innate immunity is less specific and characterized by germline-encoded receptors and composed of cells and molecules that can differentiate host cells from infectious agents, and they are activated within hours of contact. On the other hand, the adaptive immune system is generally very diverse, where cell receptors are generated by somatic recombination and the cells can recognize unrestricted number of targets but become operative only 2-4 hours after first encounter with a target cell. Later, lymphocytes develop memory and can persist for a long time. Innate immunity encompasses skin, saliva, cytokines, complements, lysozymes, gut flora, mucosa, and a group of cells of the myeloid lineage, including dendritic cells (DCs), monocytes, macrophages, polymorphonuclear cells, mast cells, and innate immunity also includes innate lymphoid cells (ILCs), such as natural killer (NK) cells that can mount a rapid immune response to an external stimulus through a limited number of germline-encoded receptors. Innate immunity is usually considered as first-line of defense against pathogens and is provided essentially by phagocytic cells which release cytokines and chemokines leading to amplification of immune response, and subsequently activate adaptive immunity. The adaptive immune system includes B and T lymphocyte cells that can express a wide range of antigen-specific receptors. These antigen receptors comprise the T cell receptor (TCR) and the B cell receptor (BCR). T lymphocytes kill virus-infected cells and trigger other cells of immune system whereas B lymphocytes secrete very specific proteins called antibodies that can recognize and bind to extracellular microbes and promoting their ingestion and destruction. Normally, naïve T cand B cells meet antigens in lymphoid organs and experience a process of cell division and maturation before they produce an immune response (2).

Natural killer (NK) cells are essential effector cells of innate immunity, and they are characterized by their ability to kill virally infected and cancerous cells without prior sensitization. NK cells have been the focus of curiosity for many research groups during the last two decades. For a long time, NK cells were acknowledged merely as primitive killers, but

they are now considered major players in innate immune response, and they also influence the shape of adaptive immunity. In addition to being a potent innate source of IFN-γ, NK cells possess cytoplasmic granules containing perforin and granzymes which are involved in NK cell-mediated cytotoxicity. A group of morphological features, expression of lymphoid markers and the cell origin will all mark NK cells as lymphocytes. There is evidence that frequency of NK cells, NK cell infiltration into tumor microenvironment and NK functional capacity can impact on survival of cancer patients (3-5). A pile of facts indicates that NK cells play a major role in the control of hematological malignancies during early phases and progression.

1.1 NK CELLS: SENTINELS OF IMMUNITY

Since their discovery in the 1970s, several studies have shown that NK cells can provide first line host defense and exert antitumor cytotoxic response. NK cells represent 5-20% of human circulating lymphocytes (6). They are innate lymphoid cells (ILCs), and they are acknowledged as producers of inflammatory and immunosuppressive cytokines. IFN-γ secreted by NK cells can shape T cell immune response either by direct interaction between T cells and NK cells migrated to lymph nodes or by an indirect effect on dendritic cells (7-9). From a biological outlook, IFN-γ is a pleiotropic cytokine with multiple properties comprising antitumor, antiviral, and immunomodulatory roles and can act as a cytotoxic cytokine to induce apoptosis in tumor cells (10, 11). NK cells can also produce proinflammatory tumor necrosis factor-α (TNF-α), immunosuppressive IL-10 and growth factors such as granulocyte colony-stimulating factor (G-CSF). In addition to cytokines, NK cells secret chemokines like CCL2-5, XCL1, and CXCL8 (12).

NK cells are prototypic cells of innate immunity due to their rapid and effective immune response. NK cells lack somatically rearranged antigen receptors and can directly cause death of tumor and virus-infected cells without prior activation (13, 14). In addition, they can regulate adaptive immunity via cytokines and chemokines that they release. NK cells have also demonstrated various developmental and functional features analogous to adaptive immune cells. Studies during the last decade showed that NK cells can acquire long-lived memory like population of cells that are capable of augmented recall response (15). There is an extensive heterogeneity and plasticity of different NK cell subsets due to disparities in their phenotypes and functional characteristics and these subpopulations of NK cells can play distinct roles in cancer.

1.1.1 BIOLOGY OF NK CELLS

In human, NK cells are classically recognized by the lack of CD3 and the expression of CD56 on the cell surface. They are found both in blood (5%-15% of circulating lymphocytes) and in different lymphoid and non-lymphoid organs like spleen, liver, and lung (16, 17). Human NK cells in peripheral blood can be distinguished based on the expression of CD56 and CD16. The $CD56^{bright}$ $CD16^{dim/-}$ subset of NK cells is believed to be a precursor to the CD56 dim cells. Nevertheless, $CD56^{bright}$ cells can produce high amounts of cytokines in response to cytokines but with limited cytolytic activity, whereas $CD56^{dim}$ $CD16^{+}$ NK cells are more mature cells with high cytotoxic competence. Majority of NK cells in peripheral blood are $CD56^{dim}$ cells, whereas bright cells only make up 10%-15% of peripheral blood NK cells. The fraction of $CD56^{bright}$ and $CD56^{dim}$ NK cells in peripheral blood is different from that of tissues (16). Tissue-resident NK cells display tissue-specific markers and functionally differ from conventional peripheral blood NK cells (18). Although, bright CD56 NK cells are generally more efficient cytokine producers, $CD56^{dim}$ NK cells can also contribute to IFN-γ production. Thus, $CD56^{dim}$ NK cells can pledge a swift and complete NK cell response during early phases of the innate response (19, 20). Earlier studies revealed that human NK cells comprise of two major subsets (CD56 bright and CD56 dim)(21-23)}. Using mass cytometry, one study demonstrated high level of heterogeneity in NK cell population beyond the classical two subsets (24). Furthermore, single-cell RNA based transcriptome analysis demonstrated complex heterogeneity of NK cells with diverse functional profile (25). Hence, the paradigm of dividing NK cells into only bright and dim cells is clearly too simplistic.

However, presence of numerous NK cell subsets in the peripheral blood of human and being dissimilar both phenotypically and functionally together with their pattern of receptor expression which changes not only in cancer but also in healthy individuals by ageing and during CMV infection, demand a better understanding of the biology of these cells because this will massively help in invention of successful NK cell-based therapies.

1.1.2 DEVELOPMENT OF HUMAN BLOOD NK CELLS

In recent years, considerable familiarity has been added by studying NK cell development in parallel with the closely related ILCs. There is now amplified perception of the plasticity of hematopoietic progenitor cells and their competence for differentiation into multiple lineages. Collectively, all innate lymphoid cells (ILCs) are divided into three groups according to the expression profile of transcription factors and cytokines (26). It is important to appreciate that NK cells represent one subgroup of ILC family (27). All ILCs share mutual characters which include dependance on transcription factor like ID2 and cytokines signaling through common-gamma chain shared by IL-2 and IL-15 (28, 29). NK cells are related to group 1 of ILCs and like NK cells, ILC1 cells express T-BET and produces interferon-gamma, but lack NK cell lineage related markers, like CD16, cytolytic granules, EOMES, MHC class-I binding receptors and NKp80 (30-32). In addition, ILC1 devoid of cytolytic function that mimic those of $CD8^+$ cytotoxic T cells (33).

Development of NK cells is regulated by an array of transcription factors and extrinsic cytokine (34-36). Transcription factors like E4BP4, T-BET, EOMES, and GATA3 can regulate NK cell differentiation and maturation (37-41). The development and function of NK cells are steered by diverse cytokines, such as fms-like tyrosine kinase 3 ligand, kit ligand and IL-3 and they are critical for survival and proliferation of HSCs and normal NK cell development (42, 43). In addition, NK cells are nearly not present in IL-$15^{-/-}$ or IL-$15R\alpha^{-/-}$ mice, which indicates an imperative role for IL-15 during NK cell differentiation and precisely IL-15 is essential for transition from $CD122^+$ NK cell progenitors to mature NK cells (44, 45).

Several studies in both human and mice displayed that NK cells can develop in various tissues including bone marrow, secondary lymphoid tissues, liver, uterus, and thymus (46). In human, the primary niche of NK cell development and maturation is still a matter of controversy. Despite our better understanding of process of NK cell development, there are still mysterious questions about this process including whether human NK cells originate from a separate set of clonal precursors, or if they arise from multipotent progenitors. As illustrated in Figure 1, hematopoietic stem cells (HSC; Lin- CD34+) differentiate into lymphoid-primed multipotential progenitors (LMP) which are

characterized by expression of CD45RA. Subsequently, LMPs by expressing CD38, CD7, CD10 and CD127 can transit into common lymphoid cells (CLP) and the latter can make lineage commitment to Pro-B, Pre-T, NK cell progenitor (NKP) and ILCs. Expression of CD122 marks the transition of CLPs into NK lineage and this transition is considered an irreversible decision step. Appearance of CD56 indicated transition of immature NK cells into mature NK cells which include minor population of $CD56^{bright}$ cells that can differentiate into the major NK population of $CD56^{dim}$ cells. $CD56^{bright}$ NK cells are characterized by acquiring CD94 expression whereas $CD56^{dim}$ NK cells show downregulation of CD94 and start acquiring CD16 and KIRs. The suggested transition from $CD56^{bright}$ to $CD56^{dim}$ NK cells is based on studies where they revealed the developmental relationship between these two subsets of NK cells (22, 47, 48). For instance, in a study sorted $CD56^{bright}$ NK cells were cocultured and stimulated with synovial fibroblasts and the former cells showed downregulation of CD56 and developed a phenotype close to $CD56^{dim}$ cells (21). Moreover, there is evidence that immature NK cells can give rise to $CD56^{dim}$ cells as well (49-51). Less mature $CD56^{bright}CD16^{-}$ regulatory peripheral blood NK cells express CD94 and the NKG2A inhibitory receptor (48, 52). $CD56^{bright}$ NK cells are prominently reactive to cytokine priming, have a greater proliferative potential and superior cytokine-production capacity (53). As NK cells mature, they start downregulating expression of CD56 and up-regulating CD16, becoming $CD16^{+}CD56^{dim}$ NK cells (54).

In addition to the NK cell model of development mentioned above, there is evidence of a non-linear (branched) model of NK cell development entailing some central truths. For instance, the notion that multipotent progenitor cells become lineage limited and further mature. LMPs differentiate into either common lymphoid or myeloid progenitors and both progenitors can give rise to NK progenitors. Later, these NK progenitors differentiate into $CD56^{bright}$, $CD56^{dim}$, or adaptive NK cells (51).

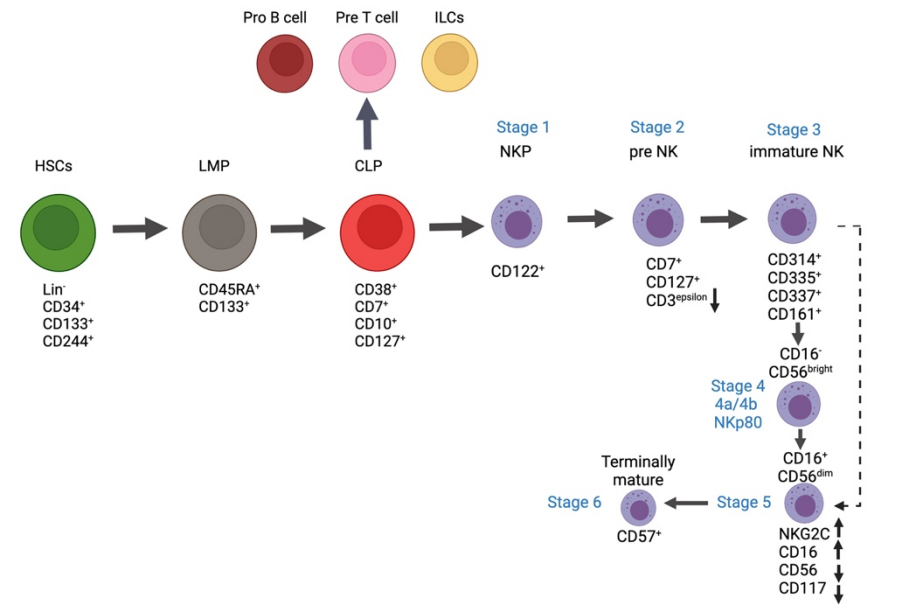

Figure 1. Developmental stages of human NK cells. In the linear model of human NK cell development, hematopoietic stem cells (HSC) differentiate into lymphoid-primed multipotential progenitors (LMP), which then give rise to common lymphoid progenitors (CLP). Lineage commitment happens at the NK cell precursor stage. The latter cells develop into $CD56^{bright}$ NK cells and then $CD56^{dim}$ NK cells. Differentiation into adaptive NK cells could consequently arise in comeback to viral infections. Dashed line indicates hypothetical paths of NK cell development. Figure is created with BioRender.com.

NK cells go through several maturation steps as mentioned above, gradually losing NKG2A but gaining expression of killer-cell immunoglobulin-like receptors (KIRs) and CD57 until attainment of terminal maturation stage. Terminally differentiated NK cells characterized by $CD56^{dim}NKG2A^{-}KIR^{+}CD57^{+}$ demonstrated lower proliferative capacity but high functional potential as they preserve high degree of cytotoxic potential (52, 55). We currently have minute information regarding the development of adaptive NK cells and from where they originate. It has been shown that liver is the primary site where these cells originate (56). Adaptive NK cells might originate from either NK cell precursors or from a unique precursor cell (51, 57). Human NK cells can develop into memory-like effector cells with

clinical applications in leukemia. These cells revealed enhanced IFN-γ production upon-re-exposure to stimuli (58). Additionally, memory-like NK cells can upregulate CD25 (59) and revealed demethylation of IFN-γ promoter regions (60). Human CMV is one of the main viruses that can cause adaptive NK cells to undergo contraction but persist for long-term and expand and causes numerous epigenetic reprogramming in these cells (60, 61). Furthermore, CMV-driven adaptive like features of NK cells can lead to high production of IFN-γ after re-exposure to the virus (62, 63). It has been shown that the CMV-induced adaptive like NK cell subset may prevent disease relapse after transplantation and provide protection against infection diseases (64). Nevertheless, our studies included in this thesis uncovered that less mature NK cells responding to cytokine stimulation can be very functional and these cells noticeably exhibited an impact on the clinical outcome of immunotherapy in acute myeloid leukemia. Moreover, a study by Wagner *et al*. showed that after stimulation of peripheral blood $CD56^{bright}$ NK cells with IL-15, these cells not only had superior cytokine production compared to $CD56^{dim}$ peripheral blood NK cells, but also displayed greater degranulation and killing in response to tumor cell targets (65).

All in all, there are many unresolved questions that scientists are still curious about, like whether phenotype or metabolic fingerprints decide on the functional fate of NK cells. Further dynamic studies and development of new techniques should shed light on unresolved questions related to NK cell development. Recently it has been realized that in terms of development, phenotype and functional profile, NK cells are more diverse than previously thought. Thus, the conventional perception of the NK cell lineage as composed of a comparatively homogenous group of cells with analogous functions and durability is not precise anymore. Rather, the NK cell lineage is remarkably diverse. The functional profile of NK cells from different tissues is dissimilar and this can lead to the question of what the characteristics of NK cell lineage are.

1.1.3 NK CELL RECEPTORS

NK cells are equipped with a diverse group of activating, co-stimulatory and inhibitory receptors on the cell surface and they play a substantial role in NK cell response towards virally infected and transformed cells (Figure 2). Here, briefly different types of receptors are described.

HLA-specific inhibitory receptors. Imperative regulation of NK cell function is controlled by a sophisticated set of inhibitory receptors. Consequently, binding of HLA class I molecules on self, healthy cells to inhibitory receptors expressed on NK cells prevents self-cell cytolysis. Human NK cells express two major different sets of inhibitory receptors: killer immunoglobulin-like receptors (KIRs) and the CD94/NKG2A heterodimer (66, 67). KIRs are type I transmembrane receptors that can bind to specific HLA-A, B and C molecules (68-72). The genes encoding the KIRs is located on chromosome 19 within the leukocyte receptor complex. There are domains present in cytoplasmic tails in both types of inhibitory receptors allowing transduction of inhibitory signals and called immunoreceptor tyrosine-based inhibitory motif (ITIM). Inhibitory KIRs are recognized by long cytoplasmic tails ("L" as in "long", as shown in different inhibitory KIR's nomenclature). Each KIR contain two or three extracellular Ig domains designated KIR2D and KIR3D, respectively. Same as HLA, KIRs are described by high level of polymorphism, which vastly affects the interaction between the KIRs and their respective HLA class I ligands.

On the other hand, NKG2A is a type II transmembrane heterodimeric inhibitory receptor expressed by some $CD8^+$ T-cells and a subset of NK cells, and it binds to HLA-E, a non-classical MHC class I recognized by limited polymorphism (73). NKG2A belongs to the lectin-like receptor family and is encoded by a gene, *KLRC1,* located in the NK gene complex (NKC) region on chromosome 12 (73). The cytoplasmic tail of NKG2A receptor contains two ITIMs that transmit an inhibitory signal. In addition to inhibitory KIRs, NKG2A is considered another key inhibitory receptor in the education process of NK cells (74). Another MHC-specific inhibitory receptor but with broader specificity is the type 1 transmembrane leukocyte Ig-like receptor (LIR-1). Although it binds to most HLA class molecules, both classical and non-classical, its affinity to the ligands is largely considered weaker in comparison to KIRs and NKG2A (75, 76). Additionally, LIR-1 can also bind the

human CMV MHC class I homolog called UL-18 peptide (77-79). In common with KIRs, LIR-1 contains inhibitory motifs in the cytoplasmic tails and has association with SHP-1 during transduction of inhibitory signaling (80).

Recent studies revealed that signaling through inhibitory receptors can occur through two independent pathways. First pathway is the dephosphorylation of Vav-1 by SHP-1 (81). After engagement of inhibitory receptors with the respective ligand, inhibitory signal through the ITIM cause phosphorylation of latter and recruit phosphatases like SHP1, SHP2, and SHIP molecules which then can neutralize the activation signal by dephosphorylating the Vav-1 molecule attached to the immunoreceptor tyrosine-based activating motif (ITAM)(82-84). The second pathway involves c-Abl-kinase mediated phosphorylation of CrK and latter leads to severance of CrK from the signaling complex generated during activation of NK cells (85). Both pathways of inhibitory receptors can prevent actin-dependent signals of the activation signaling pathway. The importance of SHP1 molecule in NK cell responses towards target cells was investigated in a recent paper and findings displayed that reduction in SHP1 was linked to a greater NK cell response (86).

KLRG1 is an additional C-type lectin-like inhibitory receptor with a single ITIM. The expression of KLRG1 is attained during the development of NK cells and is linked with a drop in proliferative capacity (87). Engagement of KLRG1 with its ligand, E-cadherin, has been revealed to dampen human ILC2 function ex vivo; still the genuine function *in vivo* is not well described (88-90). In addition to KLRG1, NK cells also express LAG-3 which is a negative costimulatory receptor alike CD4 that can ligate HLA class I on antigen presenting cells with high affinity. While function of LAG-3 is evidently documented in activation, proliferation and homeostasis of T cells, its impact on NK cells remains ambiguous (91). IL-1 receptor can act as a regulator of downstream signaling of IL-1 receptor family and Toll-like receptor (92). Blocking IL1R8 showed to promote antitumor competence of NK cells (93, 94).

In addition to HLA class I specific inhibitory receptors that govern function of NK cells and prevent damage of healthy cells, there are other NK cell inhibitory checkpoints which are accountable for maintenance of homeostasis including PD-1, CD96, TIM-3 and TIGIT. These receptors are upregulated in pathological conditions and subsequently, affects the response of NK cells towards target cells (95, 96).

Activating receptors and coreceptors. Human NK cells express diverse activating and co-stimulatory receptors which can stimulate cytotoxic activity upon exposure to target cells. Natural cytotoxicity receptors (NCRs) are among the major activating receptors of NK cells. NCRs are type I transmembrane receptors that belong to immunoglobulin superfamily including NKp30, NKp46, and NKp44. NCRs are considered key receptors for initiation of NK cell cytotoxic activity. The NCRs were primarily discovered by the group of Moretta almost 20 years ago in a series of redirected lysis experiments using NK cells from human blood (97-99). NKp46 and NKp30 are expressed on almost all resting human NK cells, and they are upregulated after stimulation of NK cells, but downregulated in adaptive like NK cells in seropositive CMV individuals, as expression goes down with differentiation (100). Although NKp30 and NKp46 are constitutively expressed by peripheral blood NK cells in resting state, NKp44 is upregulated on NK cells after cytokine stimulation, in particular on $CD56^{bright}$ NK cells (99-101). NCRs can interact with both soluble and membrane bound ligands. Recent studies regarding identification of different ligands for NCRs enlightened the biological functions that this key group of receptors play in NK cell immune surveillance of cancer. It was previously reported that low expression of NKp46 on NK cells was remarkably linked with reduced overall survival in AML (102). Furthermore, high expression of NKp46 on $CD16^{+}$ NK cells was shown to independently predict both overall survival and leukemia-free survival in AML patients (103). The findings from the aforementioned studies offer opportunities to design NK cell-based therapy against tumors. Nevertheless, cellular pathways accountable for placing the NCR ligands on the cell surface remain to be well described molecularly, as knowing these might help to understand how different NCR ligands regulate the function of NK cells.

Another essential activating receptor is NKG2D, a type II transmembrane and C-type lectin-like receptor. NKG2D can ligate its ligands which encompass ULBPs 1-6, and MICA/B. These ligands are structurally related to HLA class I; upregulation of these ligands is typically seen on tumor cells, infected and/or stressed cells, promoting antitumor responses of NK cells (104).

NKG2C is also an activating NK cell receptor, which like its inhibitory counterpart NKG2A, can bind to HLA-E but with lower binding affinity (105, 106). Individuals with CMV seropositive displayed skewed repertoire of NK cells, and NKG2C was found to be highly expressed among NK cell subsets adapted to CMV (61, 107). Moreover, Hammer *et al.* showed adaptive NKG2C+ NK cells can recognize HCMV strains encoding variable UL40 peptide and this in turn could control expansion and differentiation of these cells (108). The term memory-like NK cells is currently implemented to NK cells possessing a $CD56^{dim}CD57^{+}KIR^{+}NKG2A^{-}NKG2C^{+}$ phenotype, and this memory-like subset of NK cells is commonly expanded in individuals previously exposed to CMV and can express certain trademarks of adaptive immune system including longevity, clonal expansion, effector antitumor and antiviral activity (109, 110).

Other receptors like 2B4, NTB-A, DNAM-1, CD2, and NKp80 function principally as co-receptors for NK cell function. These costimulatory receptors can contribute to amplification of function of NK cells induced by major activating receptors (111-116). Finally, CD16 expression on $CD56^{dim}$ NK cells can induce antibody-dependent cellular cytotoxicity after engagement with the Fc portion of IgG opsonized on target cells (117).

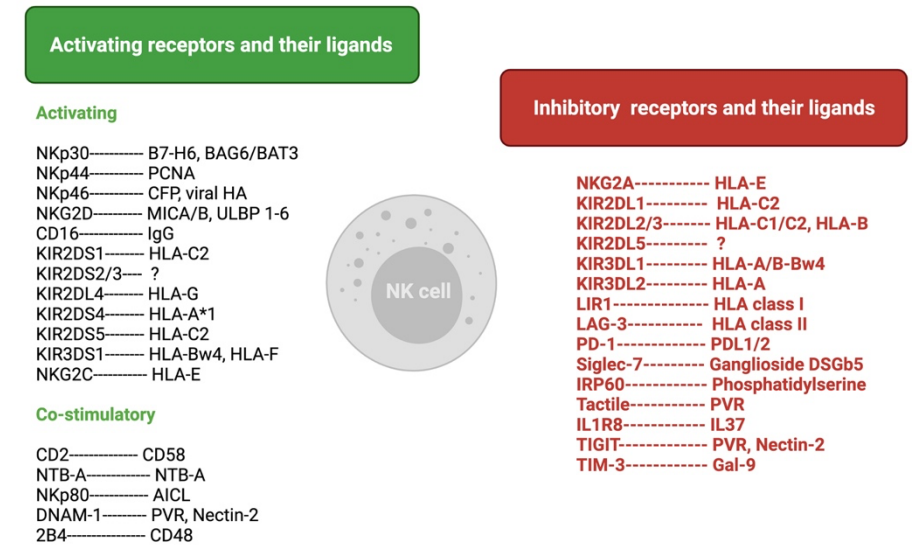

Figure 2. Outline of receptors and ligands of human NK cells. Inhibitory receptors are marked with red and activating/co-stimulatory receptors in green.

1.1.4 THE "MISSING SELF" THEORY

NK cells were termed for their ability to elicit spontaneous natural cytotoxicity toward virally infected cells and transformed cells when there is reduced or no expression of self-major histocompatibility (MHC) class I. The aim behind downregulation of MHC by target cells is to evade recognition by T cells, but NK cells can thereby overcome this immunological Achille's heel (118-120). Moreover, Klas Kärre and colleagues introduced the concept of missing self which is involved in process of NK cell recognition (118, 121, 122).

Whilst determining inhibitory receptors as molecular means used by NK cells to distinguish healthy cells from tumor cells lacking MHC class I expression, subsequent studies exhibited that lack of self-MHC class I molecules is not sufficient to cause lysis of target cells. NK cells also require stimulation by ligands expressed by target cells to activate NK cells via specific receptors and promote antitumor capacity of NK cells (104, 123). We now understand that the process of NK cell recognition and killing of target cells embroil incorporation of signals transduced by activating and inhibitory receptors of NK cells (124, 125).

The natural function of the inhibitory-ligand interaction is to preserve tolerance to self by limiting autoreactivity against tissues expressing MHC class I. The expression of self-inhibitory receptors is firmly connected to the capacity of NK cells to maintain functional competence in a process known as education or licensing (126). Subsequently, around 10% of NK cells in mice and human lack known self-MHC specific inhibitory receptors are considered hyporesponsive as they are poorly responding to MHC class I deficient cells or cross-linking of stimulatory receptors (127, 128).

1.1.5 SELF-TOLERANCE AND EDUCATION

Expression of inhibitory receptors specific for MHC class I prevents many NK cells from attacking self-cells. However, other studies strongly indicate that not all NK cells express inhibitory receptors for self MHC class I (129). On the other hand, inhibitory receptors can enhance basal responsiveness of NK cells in a process called education. It was believed that the "at least one" hypothesis entails that an education route acts during development of NK cells to safeguard that only NK cells with self-MHC class I-specific inhibitory receptors are indorsed to mature and prime NK cells for enhanced effector function (130). Nevertheless, NK cells lacking inhibitory receptors for self MHC class I can have a mature phenotype, but they are hypofunctional when encountering target cells lacking MHC class I expression (126, 131). It is well identified that recognition of self-MHC class I molecules via inhibitory receptors is critical for the attainment of functional competence by NK cells (132-136). However, the underlying mechanisms governing self-tolerance and education are not clearly understood yet. Contrasting T and B cells, NK cells use germline-encoded receptors to create variety and attain self-tolerance by offsetting reactive potential with sensitivity to inhibition by self-MHC. Killing capacity of NK cells was initially thought to be inherent, and switched off by inhibitory receptors, thus killing target cells lacking self MHC class I expression (118). Nowadays the understanding is progressed, and we know that the environmental MHC is crucial in tuning of NK cells prior to encounter the target cells. Furthermore, education process was shown to necessitate co-expression of ligands to activating receptors of NK cells to ensure NK cell reactivity (129). Education is not exclusively restricted to bone marrow where NK cells stem from but can also occur throughout their journey and it is influenced by numerous environmental factors (137).

Several models have been developed to describe the regulation of human NK cell function. In the arming or licensing model, engagement of inhibitory killer immunoglobulin-like (KIRs) receptors of NK cells with their respective MHC class I ligands can provide functional maturation and superior effector capacity to NK cells (Figure 3). In support of importance of inhibitory signals for education, multiple studies revealed that deletion of key mediators of inhibitory signaling pathway can restrict the education of NK cells and can cause buildup of immature

NK cells (138-141). It has been uncovered that increased inhibitory signals, when compared to activating signals, can lead to superior responsiveness of NK cells towards target cells and vice versa (142). In other words, stronger inhibitory signal input proved greater efficacy in education process of NK cells. The strength of interaction between inhibitory KIRs and the cognate ligands can affect education and accordingly it has been speculated that copy number of KIR genes can have an impact on NK cell education and overall functional responsiveness at the level of NK cell repertoire (143).

According to the disarming model, NK cells are equipped to kill target cells and secrete cytokines by default, and in absence of expression of self-specific inhibitory receptors NK cells become hyporesponsive due to unrestrained positive signal via their activating receptors which can induce a state of anergy. In the disarming model, NK cells may firstly respond but later can become hyporesponsive if they do not receive inhibitory signal as the latter can preserve the potential reactivity of NK cells (Figure 3) (129). One study showed that continuous stimulation of activating NKG2D receptor can lead to development of hyporesponsive NK cells (144). Another study revealed that NK cells expressing inhibitory KIRs that can bind self-MHC class I ligands are loaded with more granzyme B granules when compared to uneducated NK cells and this indicates the significant role of inhibitory signals during the process of NK cell education which fits with the aforementioned education models (145).

Intriguingly, researchers proposed another model which combines the concepts of arming and disarming in a single model, and it has been argued that human NK cell education is not an ‘on and off’ procedure but rather is a tunable process along the continuum determined by the strength of inhibitory input that each NK cell receives (Figure 3). Thus, each NK cell unveils graded intensities of responsiveness that parallels with their quantitative sensitivity for inhibition by self-MHC class I molecules. Depending on the MHC expression in the environment, the tuning upward or downward can govern the functional set-point of NK cells offering the drive behind education, with the aim to educate these cells in how to distinguish self from non-self and at the same time granting each NK cell with the utmost responsiveness. In fact, the latter model termed the rheostat model considers variation in responsiveness

of NK cells quantitatively depending on the amplitude of inhibition they receive, rather than being only a two-state mode (responsive vs. hyporesponsive) (146-148).

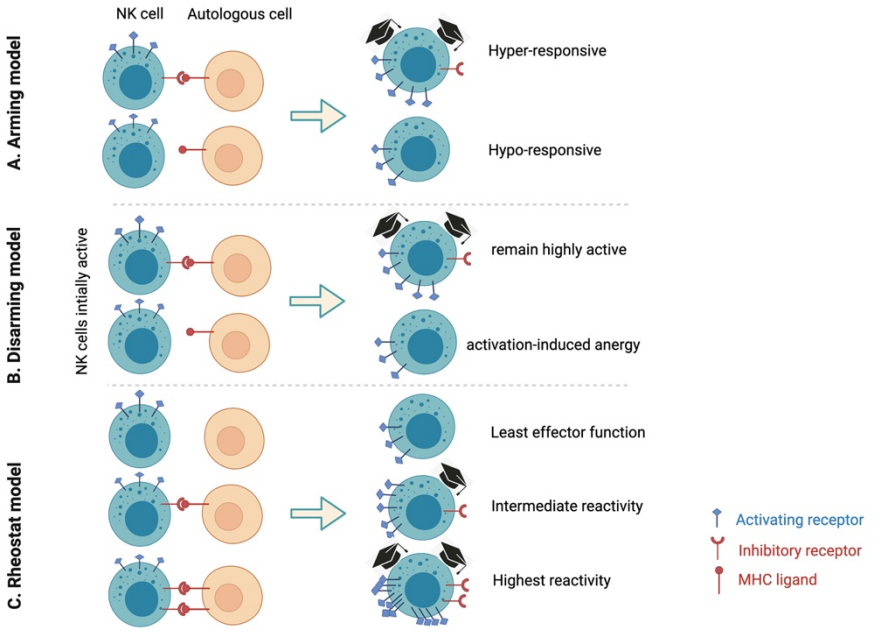

Figure 3. Models of NK cell education. (A) The arming model hypothesizes that only NK cells with capacity to bind self-MHC class I ligands will be educated and can become cytotoxic. (B) In disarming model, all developing NK cells are responsive in first place, but only those meeting inhibitory receptor ligands can be rescued from a state of anergy caused by persistent stimulation and stay responsive. (C) Rheostat model is related to the quantity of inhibitory signals each NK cell receives. Those cells with less interactions with self MHC class I display the least effector function. The change in local environment of MHC class I can fine-tune NK cell response in a tuning model which is fitting with A, B, and C. Figure is created with BioRender.com

It is vital to appreciate what factors determine the response potential of educated NK cells because despite the proposed models of education, there are still existing gaps regarding the molecular mechanisms controlling this process. One of these factors can be that clustering of activating receptor like NKG2D and NKp46 towards the immunological synapse, created during cell-cell contact, can lower the threshold of NK cell activation and regulate the polarization of lytic granules of NK cells to the immune synapse (149, 150). Moreover, adhesion molecules like LFA-1 are also critical for education of NK cells as they play a role in the compartmentalization of inhibitory and activating receptors and offer stable conjugate formation, thus ensure potential NK cell reactivity. Both the distribution of NK cell receptors and cell adhesion are essential for optimal regulation of NK cells and their responsiveness (151-153).

Around 20% of healthy human peripheral blood $CD56^{dim}$ NK cells lack expression of CD94/NKG2A and KIRs (154, 155) and this is considered theoretically a large proportion of uneducated NK cells. However, recent studies discovered that education of NK cells can also happen even through non-MHC molecules in addition to classical and non-classical MHC mediated education (Figure 4) (151, 156). It was recently shown that LIR-1 as another NK cell inhibitory receptor can educate expanded LIR-1^+ NK cells *ex-vivo*, but not freshy purified or short-term preactivated NK cells. LIR-1 mediated education of NK cells was found to be related to higher expression of DNAM-1 and granzyme B (157).

The capability of NK cells to mount an immune response while keeping tolerance to self is considered vital for the role of NK cells in cancer immunosurveillance, eradication of pathogens and pregnancy. NK cell education was originally thought of as a single developmental incident, but there is now a pile of data supporting the notion that education is an ongoing calibration activity to maintain no reactivity towards self-cells. Despite noteworthy observations in finding distinctive markers of NK cell education, the underlying molecular mechanisms of this process are not well understood. Studies in the field of immunometabolism have displayed a role of cellular metabolism in NK cell education and modulating effector function (158). Low levels of glycolysis and oxidative phosphorylation are preserved when NK cells are not stimulated. Yet, upon cytokine stimulation both metabolic pathways

become upregulated and indicates important role of cellular metabolic pathways in regulating NK cell functional capacity. It has been shown that educated and uneducated NK cells are dissimilar in terms of amplified uptake of nutrients and there was an increase in metabolic activity in the former cells. Moreover, NK cell educated through NKG2A revealed higher metabolic activities and these cells were more resistant to drop in oxidative phosphorylation when compared to KIR-educated cells (159).

Recent evidence show that educated and uneducated NK cells play roles in human immune system, with educated NK cells providing protection against MHC-deficient cancer cells and uneducated NK cells providing protection against viral infections and tumours that keep MHC expression (160-162) Of clinical significance, uneducated human conventional NK cells can also be pre-activated with IL-12, IL-15, and IL-18 to revert baseline hypo-responsiveness and develop enhanced functionality in response to CD16 ligation and AML blast cells (163). Taken together, understanding immunogenetic diversity of NK cell inhibitory receptors and their interactions with the respective ligands and further dissecting role of metabolic programming during education process may unveil novel targets to enhance functional competence of NK cells to be used in treatment of cancer patients.

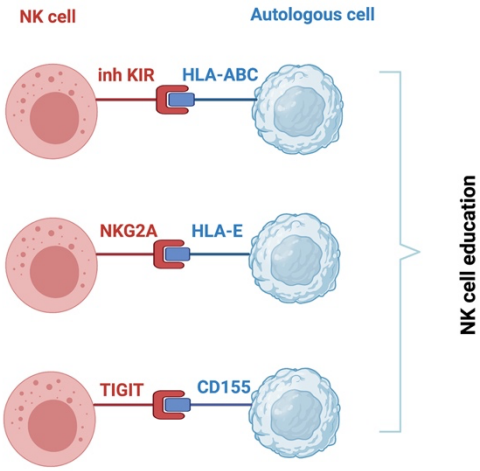

Figure 4. Signaling pathways of NK cell education. Classical NK cell education is mediated through the interaction of inhibitory receptors with self HLA-ABC molecules (classical MHC-I dependent) or HLA-E (non-classical MHC-1 dependent). In addition, interaction of TIGIT receptor on NK cells with non-MHC-I ligand called (CD155) can modulate NK cell education (non-MHC dependent). Figure is created with BioRender.com.

1.1.6 NK CELLS AND TARGET CELL LYSIS

In contrast to T cells, NK cells identify and eradicate target cells lacking HLA class I in a process called missing-self recognition as mentioned above (118). Thus, in the absence of inhibition, signaling from activating receptors such as natural cytotoxicity receptors, NKG2D, 2B4 and DNAM-1 is vital to fuel NK cell cytotoxicity (124). To attain degranulation and consequently elimination of target cells, NK cells necessitate synergistic activation through co-engagement of receptors. The regulation of effector function of NK cells is warranted by a vibrant equilibrium between inhibitory and activating receptors. Here, response of NK cells toward target cells and how NK cells recognize target cells are described (see summary in Figure 5).

Lytic granule-mediated killing. The major mechanism by which NK cells kill virally infected or transformed cells is through the release of lytic granules in a process called degranulation. These lytic granules are lysosomal-related organelles (164-168). The formation of these lytic granules commences during the development and maturation of NK cells (169). The content of these granules is separated from the cytoplasm by a double layer membrane. Lytic granules are transported along the microtubules using dynein motor proteins and are then polarized toward the immune synapse created with the target cell. Later, at the formed synapse the granules fuse with the plasma membrane of the target cell, enter the target cells and induce apoptosis (170-172). The process of exocytosis of granules is a multi-step process that is induced by the contact between the NK cell and a target cell leading to the formation of the immune synapse (173, 174). Once the immune synapse is formed, NK cell activating receptors engage their respective ligands on target cells. LFA-1 mediates polarization of cytotoxic granules of NK cells toward the target cells and permits an efficient NK cell cytotoxicity (175, 176). Th Granzymes are serine proteases that are expressed in T and NK cells (177). Granzymes are produced into the endoplasmic reticulum (ER) as a pre-pro-protein containing a signal peptide that helps guiding into ER (178). In the ER, the pre-pro-protein will be changed to an inactive proenzyme. Afterwards, further modifications leads conversion to an active granzyme which will be stored in complex with serglycin (179-182). Inside the granules, the activity of granzymes is dampened due to the low pH (183). On the other hand, perforin is important for the activity of lytic granules of NK cells as it helps

delivery of granzymes and later their release into the cytoplasm of the target cell (184, 185). It has been demonstrated that reduction in the expression of perforin is linked with diminished cytotoxic activity of NK cells (186).

Lytic granule mediated killing of target cells occurs through two distinct pathways, which include osmotic lysis and necrosis mediated by perforin and granzyme-induced apoptosis (187). There are two paradigms regarding how exactly granzymes delivered into the target cells. The classical model is that perforin can bind to cell membrane of target cell and with the aid of Ca^{2+} they make transmembrane channels which facilitate entrance of granzyme molecules (188, 189). An alternative pathway is through a slower process termed endocytosis by the target cell (190) where both perforin and granzymes are taken up into endosomes. Subsequently, granzyme escapes the endocytic vesicles and enter the cytosol with assistance of perforin-mediated endosomal membrane damage (185).

How NK cells protect themselves from perforin and granzymes during the process of synthesis, storage and release has been investigated and various mechanisms of resistance have been proposed regarding self-protection once there is a release of the granule content. First, NK cells were described to possess an inhibitor in their cytosol called SB9 which protects NK cells from misallocated granzyme B that getaways the exocytotic pathway (191-193). Another mechanism is related to the presence of lysosomal-associated membrane protein-1 (LAMP-1) in the granules. During NK cell degranulation, LAMP-1 is externalized and lines the plasma membrane of NK cells at the immune synapse created with target cell, thus preventing binding and damage mediated by perforin (194). Reduction in LAMP-1 expression was shown to be associated with disturbance in the movement process of lytic granules, decreased level of perforin and inhibition of granule-mediated killing of target cells (195). Moreover, tight packaging of lipid layers is another factor that has been proposed to prevent intercalation of perforin to the lipid layer (196). Another proposed mechanism that can prevent NK cells from degradation by cytolytic granules is through cathepsin B which can reach the membrane of NK cells, cleave, and inactivate the perforin (197).

Death receptor-mediated killing of target cells. In addition to lytic granule release, NK cells can mediate killing of target cells through surface expression of ligands for engagement of death receptors including FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (198-200). Engagement of death receptors leads to induction and recruitment of caspases which trigger cell death of the stressed cell through apoptosis (201). Activation of caspase 8 and 10 at the death-inducing signaling complex formation after ligation of death receptors can eventually lead to apoptosis (202). After apoptosis of target cells via release of granule or death receptor engagement, NK cells detach from the target cell and consequently may mediate killing of other adjacent cells in a process called serial killing (203). It is still debatable how many target cells will be killed by each individual NK cell; it is becoming obvious now that a small responsive subset of NK cells is accountable for the majority of killing events (204). Characterizing and isolating the NK cell subset with a serial killing property may be advantageous for NK cell-based therapy. However, there is a limit to how many target cells an individual NK cell can kill. Gwalani and Orange answered a fundamental question in their study where they showed that an NK cell requires only 2-4 degranulation events to cause cell death of a target cell. Furthermore, NK cells discharge nearly one tenth of their total lytic granule pool upon encounter with a single target cell, but they need just over one-hundredth of the total lytic granule to kill target cell (167). It was recently proposed NK cells use granules in process of primary serial killing, whilst they shift to death receptor-mediated killing when the pool of granules declines (205).

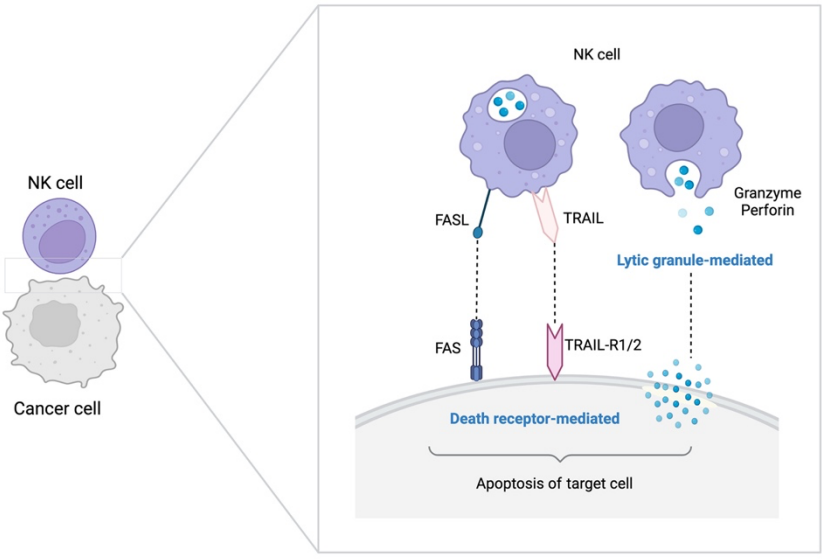

Figure 5. NK cell-mediated lysis of tumor cells. NK cells can induce cell death in target cells using two different mechanisms: lytic granule-mediated or death receptor-mediated. Figure is created with BioRender.com.

1.1.7 GENES DRIVING NK CELL RESPONSE

NK cells are characterized by a diverse expression of inhibitory and activating receptors, hitherto the functional effects and the developmental origin of this diversity is not well known. Hence, a better understanding of this diversity can provide us with an improved competence to harness them therapeutically. There are multiple factors influencing the NK cell repertoire, such as variation in MHC class I, KIR genes, NK complex genes and a prior CMV infection. Thus, one aspect to better understand the NK cell response is to dissect immunogenetics of various NK cell receptors and their respective ligands.

The major histocompatibility complex and genes encoding different NK receptors, the NK complex of lectin-related genes on chromosome 12 (NKC) and the leukocyte receptor complex of immunoglobulin-related genes on chromosome 19 (LRC), are large, dense clusters of immune loci. These genetic complexes share some features, like excessive levels of polymorphism. The interactions between these genetic complexes have inferences for improved understanding of predisposition to infection, autoimmune diseases, and cancer. Figure 6 illustrates major genes of the three genetic complexes mentioned above.

Phenotypic diversity of NK cells is influenced by the difference in genetics among individuals and environmental factors. Genetic factors are strongly affecting the expression pattern of inhibitory receptors involved in self-tolerance and education of NK cells, whereas environmental cues like pathogens and cancers can affect the expression profile of activating receptors. Understanding how these factors govern and modulate diversity of NK cell populations could be of much help in harnessing these different NK subsets for NK cell-based therapy in different settings like infection, cancer, reproduction, and transplantation (24).

Interaction of NK cell receptors and HLA class I is regulated by three genomic complexes located on different chromosomes (206). In addition to polymorphic MHC gene locus on chromosome 6 (207), which encodes class I ligands, the natural complex (NKC) region on chromosome 12 encodes lectin-like NK cell receptors (208, 209), and the leukocyte receptor complex (LRC) on chromosome 19 encodes KIR

receptors (210, 211). Here, we briefly describe these genomic complexes.

MHC genes. The human MHC genes spans approximately 4 Mbp on chromosome 6p21.3, involves 200 identical loci and comprise replicated chunks of ~100 kb, each including an HLA class I gene (212). There are six existing functional genes of HLA class I encompassing classical A, B, C, and non-classical E, F, and G (213). Each one of these HLA genes is present on every HLA haplotype. The human *HLA* locus strikes an equilibrium between highly polymorphic genes encoding HLA-A, B, and C and less polymorphic HLA-E, F, and G. All these 6 HLA class I molecules are ligands for one or more of NK cell receptors (67). High polymorphic feature of HLA class I genes provides numerous advantages to the human immune system. For instance, possession of a couple of functionally different allotypes of HLA-A, B, and C can provide wide immune response to any stressed or transformed cells. The ligands for KIRs include four epitopes of HLA-A, B, and C which are A3/A11, Bw4, C1, and C2. The way the HLA epitopes are distributed among the three principal HLA allotypes, every individual can express 1, 2, 3, or all 4 epitopes (214). KIRs bind to HLA class molecules and create interaction with the amino-terminal part and the carboxyl-terminal part of α1 helix and the bound peptide (215). Major polymorphisms in α1 helix can define the three major epitopes of HLA that are recognized by KIRs. The C1 epitope of HLA class I is characterized by an asparagine residue at position 80, whereas HLA-C2 possesses lysine at this position instead. In this way, every allotype of HLA-C either has a C1 or a C2 epitope and is a KIR ligand. In contrast, only a fraction of HLA-A and B allotypes are ligands for KIRs. This function is bestowed by a sequence at residues 77-83, which establishes the Bw4 epitope by selected HLA-A and B allotypes (216). HLA-C1 is a ligand for KIR2DL2 and KIR2DL3, while HLA-C2 can bind KIR2DL1. Nevertheless, studies presented that KIR2DL2 and to less extent KIR2DL3, can also bind HLA-C2 (217). KIR3DL1 can bind to HLA-Bw4 epitope of some of HLA-A and many HLA-B molecules. Both HLA-A3 and A11 are considered ligands for KIR3DL2.

The most significant function of HLA molecules is the initiation and regulation of host immune response. HLA molecules present peptides to T cell receptors for commencing tolerance and activation of T cell

response (218). At the same time these molecules have substantial role in NK cell development, equipping them with the functional competence. There is an immense variation between individuals due to the polymorphism in MHC gene. The end product of this variation is a raise in number of antigen peptides that can be presented at the level of population and consequently making species survive pathogens. Whilst it is now clear that KIRs have specificity for HLA class I allotypes, the expression of HLA on cells is quite heterogenous due to the diverse repertoire of their peptides. Although HLA peptides are considered essential for detection by T cells (219), the exact mechanism of how HLA peptides manipulate the process of NK cell recognizing HLA is not well known (220).

On the other hand, HLA-E is a non-classical MHC molecule with a limited polymorphism with two alleles varying at position 107 (221). HLA-E is considered the oldest and most conserved of HLA class I isotype ligands, engages the NKG2A receptor on NK cells and can educate a huge population of NK cells (73, 222). HLA-E requires loading of peptides from signal sequences of classical MHC class I for proper folding, transportation, and expression on cell surface (73, 223, 224). Consequently, the interaction of HLA-E bound to leader sequence peptide derived from HLA class I with NKG2A receptor on NK cells denote a way of sensing the global expression of classical MHC class I molecules. There is a dimorphism at the -21 position of HLA-B, which translates into a leader peptide with either a threonine (T) or a methionine (M) in position 2 (105, 225). In humans, leader peptides from HLA- A and C always carry a methionine, whereas most HLA-B allotypes have a T and only a minority an M. Only leader peptides with an M at position 2 allows for HLA-E surface expression.

Due to the HLA-B -21 dimorphism (rs1050458), education of NK cells was found to be regulated by two complementary systems. In one study published by Horowitz *et al.*, -21M HLA-B alleles were hardly found in haplotypes where genes encode ligands for KIR3DL1 (HLA-Bw4) or KIR2DL1 (HLA-C2) are present but was rather present together with genes encoding for the weaker KIR2DL2/3 ligand (HLA-C1). HLA-B M/x individuals showed to have better educated $NKG2A^{+}$ whereas individuals who cannot provide peptide (HLA-B TT) for HLA-E presentation, normally have education of their NK cells through the KIR

pathway. NK cell education determined by NKG2A resulted in phenotypically more varied subsets of NK cells with greater functional potential (216). The single nucleotide polymorphism rs1050458 (A or G) which encodes the two isoforms of the leader peptide of HLA-B -21 M and T, respectively, has been examined in the context of HIV control and risk of developing graft versus host disease (226, 227). Additionally, HLA-B -21 dimorphism revealed a striking influence on the outcome of immunotherapy in AML (paper I).

Leukocyte receptor cluster genes. Fifteen KIR genes have been recognized in humans and they are encoded in one of the most variable regions on chromosome 19q13.4 called leukocyte receptor cluster (206, 228, 229). Differences in number, content and polymorphisms in KIR related alleles can create huge diversification of the KIR phenotype (230). Both functional and clinical consequences of such diversity on NK cell response demand more explorations. A characteristic feature of KIR genes is the attainment of a remarkable number of polymorphisms (231). For instance, KIR3DL1 showed to have 110 alleles and this generated 66 allotypes (232). Studies revealed that such allelic variations can regulate NK cell activation and influence the frequency of NK cells expressing KIR3DL1 (230, 233). KIRs have two or three extracellular Ig domains, named 2D or 3D, that can bind HLA surface after forming a V-shaped interface (234). Inhibitory and activating KIRs are designated L and S, respectively, based on the length of their cytoplasmic tails that affect signaling potential of these receptors. In addition to haplotypic variations, there is a significant genetic diversity in the *KIR* loci, and such variation can lead to remarkable dissimilarity in the repertoire of KIR receptors between individuals (229). There are two haplotypes of KIRs; the haplotype A that includes inhibitory KIRs and KIR2DS4 genes and the haplotype B which includes 6 activating KIR genes in addition to inhibitory KIR genes (229, 231, 235). The complex interaction of inhibitory KIRs with the respective HLA class I ligands could define the capability of NK cells to respond to an activating signal regardless if the target cell expressed MHC class ligand or not (236).

Natural killer gene complex. There are 28 genes within the 2 Mb of chromosome 12p13 demarcated as NK complex (NKC) region, where 18 of these genes encode lectin-like receptors, and of which 13 are

transcribed by NK cells (MAFA-L, NKR-P1A, LLTI, CD69, AICL, KLRF1, CLEC-2, CD94, NKG2A/B, NKG2C, NKG2E/H, NKG2D and NKG2F). These lectin-like receptors are important in NK cell biology and known to be involved in recognition of target cells (237-239). As anticipated from duplicated genes, the structures of C-type lectin receptors encoded by NKC loci are greatly related.

Most members of C-type lectin family form heterodimers with CD94, except NKG2D (240, 241). Genes traversing the NKC locus are categorized into clusters encoding very allied molecules and many of them are vastly related to NK cell function and involved in the process of recognition of target cells. NKC demonstrated marked polymorphism and has been linked with resistance to viral infections and target specificity (208). Genes of lectin-like receptors can be grouped into families, where each family encompasses molecules that are vastly related i.e., more than 80% amino-acid identity (208). NK cell receptors encoded by NKC that have been studied so far are either activating or inhibitory. Both DNA and protein expression homology study comparisons revealed that genes of NKG2C, -F, and -E are highly similar whereas NKG2D possesses low resemblance to other NKG2 family member genes (242).

NKC locus genes showed a close association with NK cytotoxicity and incidence of cancer (243). There is a locus of 270 kb within the 2 Mb of NK complex region that encompasses key NK cell receptors, such as the CD94 gene and lectin-like receptor genes as shown in Figure 6. Several studies suggested an association between single nucleotide polymorphisms in the components of NKC locus NK cell receptor genes and outcome of cancer (243-245). Despite all the present knowledge we have about the NKC locus, further dissection is going on regarding this locus and its genes as this offer deeper insights into the biology of NK cells. Impact of different NK cell receptors gene variants will be reviewed further in next section.

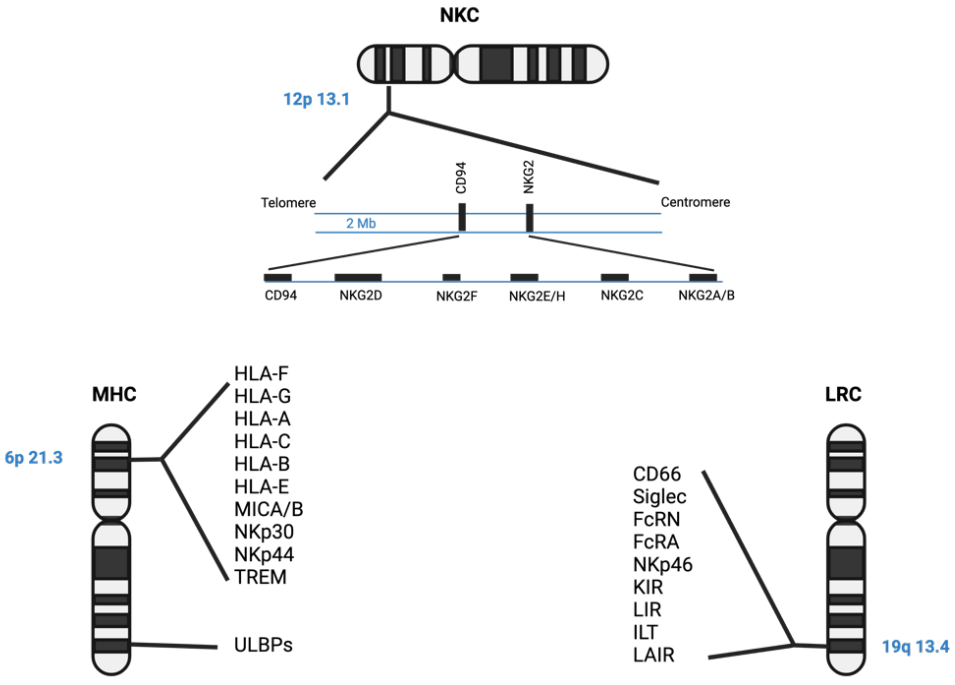

Figure 6. Human MHC, NKC and LRC gene complexes. Figure is adapted from (246) and modified with BioRender.com.

1.1.8 NK CELL RECEPTOR GENE VARIANTS

Gene variants or single nucleotide polymorphisms (SNPs) are considered one of the most common types of genetic differences in the human genome. SNPs were found to be important in cancer development as variants controlling mismatch repair of DNA, regulation of cell cycle, immunity and cell metabolism were shown to be linked with susceptibility to cancer development (247-252). From a clinical point of view, gene variants can be used as a diagnostic and response biomarker in the setting of cancer.

The location where SNPs occur in the gene, could be in promoters, intron, exon, and 3′ or 5′ untranslated regions (UTR) of the genes. Consequently, the impact on susceptibility to cancer varies according to the location of the SNPs. SNPs located in promoter regions can affect expression of the gene by changing the activity of the promoter. Gene variants in the core promoter i.e., TATA box, was shown to inhibit promoter activity (253). Moreover, changes in epigenetic mechanisms such as DNA methylation, chromatin alteration, histone alteration and silencing of genes can result from gene variants occurring in the promoter region (254). Methylation of DNA occurs mainly in the island of CpG of the promoter region. SNPs related to DNA-methyltransferase-3A of the promoter region were associated with risk of developing esophageal cancer (255).

Gene variants in exonal regions of chromosomes can be categorized into non-synonymous or synonymous based on their capacity of replacing the encoded amino acids. By changes in hydrogen bonding and phosphorylation, non-synonymous SNPs can substitute amino acid which in turn affects the function and structure of the protein. As a result of these alterations, there will be modifications in signaling pathways and proteins related to oncogenes and tumor suppressor genes (254). On the other hand, synonymous SNPs were previously known to not be important as they do not affect the amino acid sequence. Though, some studies indicated that synonymous SNPs can affect both expression and function of genes by alterations in neighborhood genes. Synonymous gene variants affect structure mRNA and its stability and folding of proteins. Consequently, synonymous SNPs results in response to different target therapies (256).

Introns encompass enhancers that can prompt transcription initiation or elongation and they can affect the expression of gene and splicing of mRNA. SNPs located in the intron regions can influence risk of cancer by both genetic and epigenetic mechanisms (254).

SNPs related to UTR regions of mRNA including 3′ and 5′ are crucial as they can govern translation process (257). The 3′-UTR set the expression level of the gene through mRNA degradation and translation. Consequently, variants occurring in the 3′-UTR region are involved in many diseases. Gene variants in 3′-UTR can modify process of translation suppression mediated by microRNA. Gene polymorphism within the 5′-UTR has been linked with multiple diseases as they control mRNA processing, stability, transportation, and translation initiation (254).

In this thesis gene variants of four NK cell receptors will be described including NKG2D, DNAM-1, NKp30, and NKG2A. The focus will be on influence of these SNPs on NK cell function and receptor expression and their impact on clinical outcome of AML. NKG2D is a C-type lectin transmembrane type II receptor and one of the major NK cell activating receptors. The gene encoding NKG2D, *KLRK1*, is located within a cluster of genes called NK complex (NKC) that encompasses several genes expressed by NK cells (CD94, NKG2F, NKG2C, NKG2E, and NKG2A) (258). In addition to NK cells, NKG2D is expressed by other immune cells like $CD8^+$ T-cells, a subpopulation of $CD4^+$ T-cells, iNKT and γδ T cells (104, 259). NKG2D is linked with DNAX-activatory protein-10 (DAP-10). Crosslinking of NKG2D ligands causes dimerization of two monomers of the receptor which in turn phosphorylates DAP-10 and induces activation of NK cells (260). Cytokines like IL-2 and IL-15 can cause upregulation of the NKG2D receptor on NK cells, whereas TGF-β reduces the expression of NKG2D (258). Several studies demonstrated impact of NK cell gene variants of NKC region on incidence of cancer. The initiating work by Imai and colleagues displayed a key role of NKG2D gene polymorphism in cancer immunosurveillance and preventing development of cancer (243, 261). In an 11-year follow up study including more 3,500 healthy Japanese donors, low cytotoxicity of peripheral blood lymphocytes was associated with higher incidence of cancer and vice versa (261). Afterwards, the variation in natural cytotoxic activity of peripheral

blood lymphocytes was attributed to the NKC region on chromosome 12. Furthermore, they showed that there are eight gene variants in the NKC associated with function of NK cells. All these 8 SNPs are linked with NKG2D except one for NKG2A. These 8 SNPs were divided into two haplotypes, NKG2D hb1 and NKG2D hb2. Each block consisted of two main alleles, a low cytotoxicity allele (LNK) and a high cytotoxicity allele (HNK). Individuals with HNK1/HNK1 from NKG2D hb2 displayed lower incidence of cancer (243). Furthermore, HNK1/HNK1 showed lower incidence of colorectal cancer (262) and aerodigestive tract cancer (263). Patients with chronic myeloid leukemia harboring HNK1/HNK1 attained deeper molecular remission after dasatinib more rapidly when compared to LNK1/LNK1 patients and this allows this subset of patients to discontinue TKI safely (264). Impact of NKG2D rs1049174 is in 3′-UTR and studies showed the LNK allele of NKG2D was more frequent among HPV-related cancer cases when compared to a healthy cohort. Furthermore, NKG2D LNK had higher affinity to microRNA1245 which in turn negatively regulates NKG2D expression (265). There is only a limited number of studies which addressed functional influence of NKC locus SNPs and basically all these studies attributed the impact on cancer to effects of NKG2D. One of the main aims of this thesis was to find out if the effect of NKC locus on cancer outcome is purely driven by NKG2D or driven by NKG2A gene variants located in the promoter region of the gene.

The gene encoding NKp30 is in the extremely polymorphic end of MHC class III region of chromosome 6 and can be transcribed into three mRNA splice variants referred to as (A, B, and C) by alternative splicing, with different biological impact (97, 266). Both NKp30 A and B are immunostimulatory isoforms and they can enhance NK cell cytotoxicity and IFN-γ release, whilst the NKp30C isoform is considered immunosuppressive due to induction of IL-10 secretion. Abundance of the NKp30C isoform was found to be associated with unfavorable outcome of some malignancies (267-269). A combination of three NKp30 SNPs (rs986475, rs1052248 and rs11575836) was reportedly shown to affect the expression of NKp30 splice variants which in turn affects NK cells function and outcome of gastrointestinal stromal tumor (269). Both rs986475 and rs1052248 are in 3`-UTR whereas rs11575836 is in the promoter region (269). We studied the

impact of NKp30 gene variants on outcome of immunotherapy in AML in **paper II**.

DNAM-1 (CD226) is a transmembrane glycoprotein and belong to family of Ig superfamily. The gene encoding CD226 is mapped to human chromosome 18 (113, 270). DNAM-1 is expressed on T-cells, NK cells, monocytes, and a subgroup of B-cells (113). Recently a non-synonymous coding variant, rs763361 (C/T) (Gly307Ser), related to DNAM-1 has been shown to be involved in vulnerability to diabetes and numerous autoimmune diseases (271, 272). The precise consequence of this gene variant is not well understood. Several hypotheses claimed that DNAM-1 SNP can alter an exon-splicing enhancer or RNA splicing, resulting in a non-functioning protein (273). There are studies where they connected the DNAM-1 gene variant to increased susceptibility to gastric and cervical cancers (274, 275). Also, T allele of rs763361 was found to increase risk of non-small lung cancer (276). Nevertheless, there are reports showing no impact of this SNP on disease susceptibility (277). How this DNAM-1 polymorphism affects outcome of IL-2 based therapy is investigated in **paper II**.

1.2 NK CELLS AND CANCER

NK cells are crucial cells of the innate immunity which play a key role in cancer immunosurveillance. Major phenotypic changes of NK cells in patients with cancer can have an impact on functional capability of NK cells by reducing the cytolytic activity of these cells (278). Two decades ago, an 11-year follow up study in over 3,500 healthy Japanese using biochemical and immunological parameters, indicated that the incidence of cancer was lower among individuals with higher natural cytotoxic activity (261). Due to heterogeneity of NK cells, their roles in eradication of cancer becomes debatable and is largely dependent on the cancer types (279, 280).

The role of NK cells in controlling growth and spread of tumors has been investigated and validated in animal models (279, 281), but their contribution to cancer immunosurveillance in human is still not fully established. Though, observations from various experimental models have clearly indicated a role of NK cell response in different hematological malignancies (281, 282). The major cause of death in patients with acute leukemia, a type of leukemia that will be discussed more in detail below, is disease relapse which is due to resistance to chemotherapies and/or escape of leukemic cells from immune surveillance (283). On the other hand, reduced expression of MHC molecules or upregulation of ligands of NK cell activating receptors on leukemic cells showed to induce increased killing capacity of NK cells and the significant role of these cells in eradication of leukemic cells (284).

Due to their less complicated cues of activation, NK cells have gained a lot of attention in the field of cancer immunotherapy. Nevertheless, the anti-tumor response of NK cells faces multiple boundaries in the context of cancer immunotherapy, and strategies to restore NK cell effector function which will be further discussed in coming sections.

1.2.1 ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is an aggressive malignancy of the bone marrow characterized by unrestrained proliferation of undifferentiated myeloid cells (AML blasts) with high degree of heterogeneity. Hematopoietic stem cells are the origin of the AML blasts, where the stem cells have undergone genetic and/or epigenetic modifications to keep a self-renewal feature to sustain the disease activity (285, 286). A study involving more than 1500 AML cases revealed that the driver of AML usually is due to mutations in genes involved in epigenetic regulations (287). The founder clones responsible for initiation of establishment of leukemic cells can additionally attain further mutations generating subclones, each with distinctive cellular and functional morphology (288).

According to the cell type that proliferates and its maturation status, AML has historically been classified by French-American-British group (FAB) into different morphological subtypes (M0-M7). Nevertheless, this type of classification does not have a prognostic value. Hence, the World Health Organization (WHO) founded a new classification for tumors of hematopoietic and lymphoid tissues in 2008, which is based on the cytogenetics of AML. It was further updated in 2016 to include distinctive biomarkers related to some myeloid neoplasms and acute leukemias. The new additions in the WHO 2016 revised version are mainly derived from the genomic landscape and next-generation sequencing data (289).

AML, which was first described as being one entity of acute leukemia, has now deviated to be considered a more complex and heterogenous disease described by dissimilar pathophysiological mechanisms, clinical patterns and molecular phenotypes that can benefit from individualized therapies. Fit AML patients usually receive intensive induction chemotherapy which includes 7 days of cytarabine and 3 days of anthracycline, and around 60-80% of patients younger than 60 years attain complete remission (CR) after induction therapy (290). CR after induction is less frequent among patients older than 60 years. Once CR attained, consolidation chemotherapy (2-3 rounds) is given to eliminate residual leukemic cells and prevent disease relapse. Young AML patients with adverse cytogenetics are recommended allogeneic stem cell transplantation to avoid risk of relapse. On the other hand, elderly

patients are usually ineligible for intensive chemotherapies due to low performance, comorbidities, or high risk of infection after chemotherapy, and studies have demonstrated a 5-year survival of around 35% for these patients (291). There are a wide range of approved and ongoing novel therapies implemented in the treatment of AML. Examples are the use of hypomethylating agent and venetoclax for unfit patients, addition of FLT-3 inhibitor to induction therapy or distribution of hypomethylating agents for patients harboring FLT-3 mutations (291). After receiving induction and consolidation therapies, the natural course of AML is associated with high relapse. Thus, with the aim to reduce the relapse risk, a combination treatment including histamine dihydrochloride and IL-2 has shown promising results as a relapse preventative measure and is currently the only approved treatment beyond chemotherapy that aims to maintain remission in AML (292). Chemotherapeutic agents can employ pro-oxidant effects to induce apoptosis but toxicity, high risk of relapse and treatment refractoriness linked with these kinds of treatments can lead to increased death rates (293). Furthermore, despite potential clinical efficacy of HDC/IL-2 in prevention of relapse in AML, the benefit of this combination of treatment in various genetic subgroups of AML needs further exploration and discovery of novel immunotherapeutic strategies can further enhance clinical outcome and quality of life in AML.

Despite huge developments seen in the field of AML treatment, the outcome is still relatively dismal. A better understanding of the pathophysiology of AML and development of multiple new therapies is now happening at a swift pace. During the last decade, the great accomplishments in AML treatments have been those approaches aimed at restoring the immune response to enhance the eradication of leukemic cells and reduce the risk of relapse. Furthermore, NK cells have become of specific interest in immunotherapies targeting AML, as these cells are key cytotoxic and cytokine producing cells of the innate immunity, making them crucial for an immunotherapy aiming at eradicating cancer cells (294, 295).

1.2.2 NK CELLS IN THE EYES OF AML

In patients with myeloid leukemias, the functional capacity of NK cells is frequently dampened at baseline and when the disease progresses or relapses, but NK cells can reinstate their effector functions once these patients reach a state of complete remission (296, 297). There is a clear association between frequency of NK cells and possession of highly activated NK cells at diagnosis and during remission, and the clinical outcome of leukemia (298). Studies performed *in vitro* showed that NK cells can eradicate myeloid leukemic cells (299). According to a pile of studies, NK cells possess a key role for the outcome of different hematological malignancies. One of the earliest pieces of evidence for the substantial role of NK cells in extermination of AML blasts was noticed when AML patients showed lower relapse rate after hematopoietic stem cell transplantation encompassing alloreactive NK cells (300). Here, I cover some findings from different studies indicating the significant roles of NK cells in controlling leukemic cells and the underlying mechanisms leading to impaired recognition of leukemic cells by NK cells (Figure 7).

Patients with AML commonly display an altered phenotype of NK cells. For instance, reduced expression of NKp46 was found to be linked with poor outcome in AML (102). Additionally, Stringaris *et al.* showed that NK cells from AML patients have reduced expression of NKp46, upregulated expression of NKG2A, and reduced cytotoxic effector function (301). These findings were mainly seen in NK cells recovered at diagnosis but the expression of NK cell activating receptors and degranulation ability was found to be restored 6 weeks after receiving chemotherapy (297). Furthermore, in the same study they suggested reduced antitumor activity of NK cells is driven by AML blasts. (297). In addition to above mentioned studies in relation to NK cells in AML, our group previously demonstrated that in elderly patients with AML the high expression of NKp30 and NKp46 on $CD16^{+}$ NK can predict survival (302). Also, presence of unlicensed NK cells among AML patients harboring high expression of NKp46 was shown to be linked with favorable outcome after receiving histamine dihydrochloride and IL-2 (162). On the other hand, the expression pattern of NK cell activating receptor ligands showed an association with the outcome of AML. For instance, the low expression of NKG2D ligands on the surface of AML cells due to aberrant epigenetic mutations reduced

recognition of AML blasts and consequently lysis of these leukemic cells by NK cells (303).

Another example of NK cell dysfunction in AML patients is the elevated frequency of immature NK cells ($CD56^{bright/dim}$ KIR^{-} $CD57^{-}$), which is associated with reduced relapse-free and overall survival (304). Defective maturation of NK cells in AML was proposed to be related to high levels of microRNA-29b, which can prevent expression of transcription factors required for NK cell development such as T-bet and EOMES (305).

Immune checkpoint receptors can maintain adequate NK cell response and they are considered crucial in self-tolerance. One of the factors that can contribute to failure of NK cell recognition of AML cells is enhanced expression of immune checkpoint inhibitors and their ligands. It has been shown that increased DNA methylation of the *PD-L1* gene, which is linked with reduced expression of PD-L1 in AML, was found to be associated with improved survival (306).

Another aspect playing an important role in modulating both the functional and differentiation capacity of NK cells is the immunosuppressive environment in the bone marrow of AML patients. The ability of tumors to control the immune-mediated response has been lately included in the list of cancer pledges, and such immunosuppressive mechanisms represent key targets for designing novel therapies. In addition to soluble immunosuppressive factors like IL-10 and TGF-β, there are numerous cells causing hypofunctional NK cells such as regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) (307, 308). Furthermore, reactive oxygen species (ROS) can cause reduced NK cell function and even apoptosis in patients with AML (309-311).

Further understanding of the mechanisms underlying the immune dysfunction seen in AML is warranted to develop new immunotherapies. Firstly, how the tumor microenvironment suppresses NK cell function. Secondly, how treatments affect the maturation and function of NK cells. Thirdly, understanding mechanisms used by leukemic cells to evade NK cell cytotoxicity.

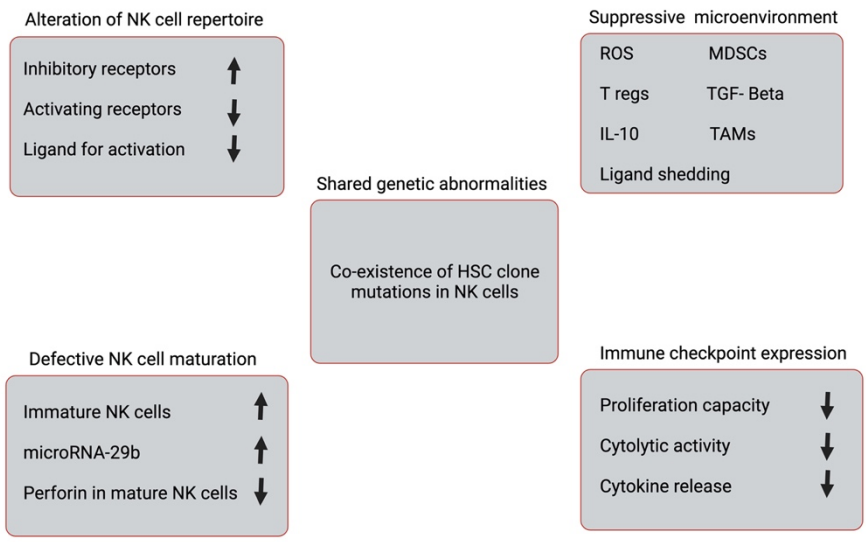

Figure 7. Mechanisms causing impaired recognition and eradication of leukemic blasts by NK cells. Adapted from (285, 298) and created with BioRender.com.

1.2.3 APPROACHES TO RESTORE NK CELL EFFECTOR FUNCTION

During past several years, there has been several breakthroughs in the process of engineering effective immune cells as therapy for many cancer types. T cell chimeric antigen receptor (CAR) therapy has been extensively examined and used in patients with lymphoid malignancies with noticeable clinical results (312). Yet, there are some factors limiting the use of such a promising treatment approach like cost, risk of GVHD, and suppressive tumor microenvironment (313). In parallel to the development of different T cell-based treatment modalities, a lot of research is oriented towards the development of adoptive NK cell therapies. In general, NK cells have some advantages over T cells due to their ability to promptly recognize and kill target cells but at the same time with a limited damage of healthy cells. In addition, unlike T cells, they do not cause GVHD (300) (314-316).Therefore, NK cells hold promise as an off-the-shelf product for cancer immunotherapy.

NK cells play an important role in the defense against cancer and viruses. The cytotoxic granules including granzyme B and perforin present within NK cells, as well as the swift production of IFN-γ and TNF-α upon cell stimulation, allow their prompt response towards aberrant cells (13, 317-320). Within the last few years, NK-based immunotherapy has emerged as a credible approach for treating various cancer types in clinic (313, 321).

There are multiple factors limiting the implementation of NK cells in human cancer trials. In addition to factors mentioned in previous section that potentially led to the escape of cancer cells from NK cell response, there are other factors hindering the application of NK cell therapy. For example, the presence of dysfunctional NK cells in the context of myeloid malignances (301), limited persistence *in vivo* (322), and migration and homing of NK cells to tumor tissues (323) are considered significant hurdles limiting the efficacy of NK cell-based therapy in cancer. Diverse methods have been implemented to enhancee and restore NK cell response toward tumour cells. Hence, better understanding of the cellular and molecular interactions between the immune system and different types of tumour cells allows the rising number of approaches that are being actively exploited to manipulate

immune cells to develop immunotherapy. There are several approaches to boost and restore functional capacity of NK cells towards AML and here briefly some approaches will be explained separately (Figure 8).

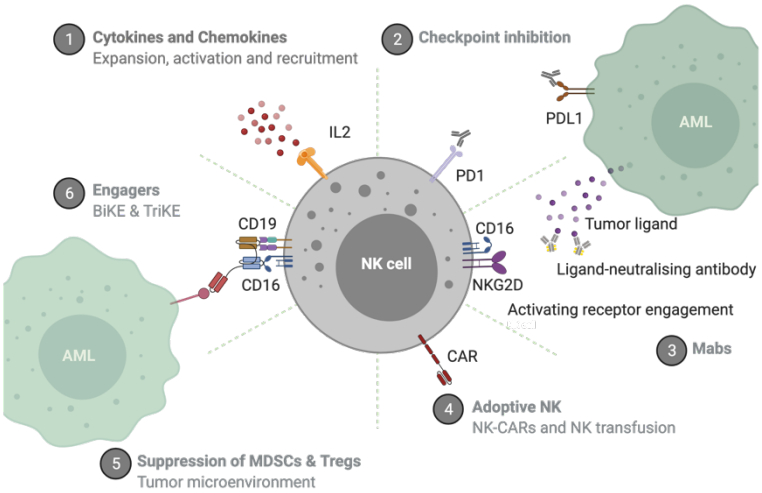

Figure 8. Strategies for restoring NK cell effector function. Figure is created with BioRender.com.

Activating NK cells with cytokines. Cytokines like interleukin 2, 12, 15, 18, and 21 showed important roles in proliferation, stimulation and enhancing effector function of NK cells (324). *In vitro* stimulation of NK cells using IL-2 or IL-15 were demonstrated to induce expansion of NK cells and enable recovery of dysfunctional NK cells in AML patients because these cytokines can induce upregulation of NKp30, NKp46, NKG2D and NKG2C receptors (325-327). Stimulation of anergic NK cells with IL-21 has been exhibited to effectively revert NK cell cytotoxic capacity and induce production of IFN-γ, TNF-α, and chemokines that can recruit T cells (328).

High-dose of IL-2 was first approved in 1998 for patients with metastatic melanoma and showed response only in a subgroup of patients (329). IL-2 as a monotherapy did not display striking clinical impact in patients with AML and MDS (330-334). In contrast, when IL-2 was combined with HDC in a phase III trial including 320 AML

patients in remission significant improvement in leukemia-free survival comparing to standard of care was observed for the treatment arm (292, 335). HDC can reduce the generation of reactive oxygen species (ROS), and consequently, enhance the stimulatory effects of IL-2 (302, 336). Later, 84 patients with AML were enrolled in a phase IV trial, called the Re:Mission trial, where patients received multiple cycles of HDC/IL-2 as a maintenance therapy after induction and consolidation chemo-therapy cycles. Peripheral blood mononuclear cells were collected from each patient at start and end of first and third treatment cycle, as shown in Figure 9. Findings from the Re:Mission trial showed a treatment-induced expansion of NK cells. Also, HDC/IL-2 induced high expression of NKp30 and NKp46 on $CD16^{+}$ NK cells which associated with improved clinical outcome (302). Furthermore, expansion of $CD56^{bright}$ NK cells at the beginning of treatment envisaged reduction in relapse of AML (103, 302). Treatment with IL-2 has been demonstrated increment in number of regulatory T cells (Tregs) and reduction in GVHD in patients receiving allogeneic stem cell transplantation (337, 338). Surprisingly, Treg expansion was not associated with inferior LFS in the Re:Mission (339). Nevertheless, it cannot be excluded that targeting CD25 on Tregs or using IL-15 instead of IL-2 might enhance the anti-leukemic property of HDC/IL-2 even further.

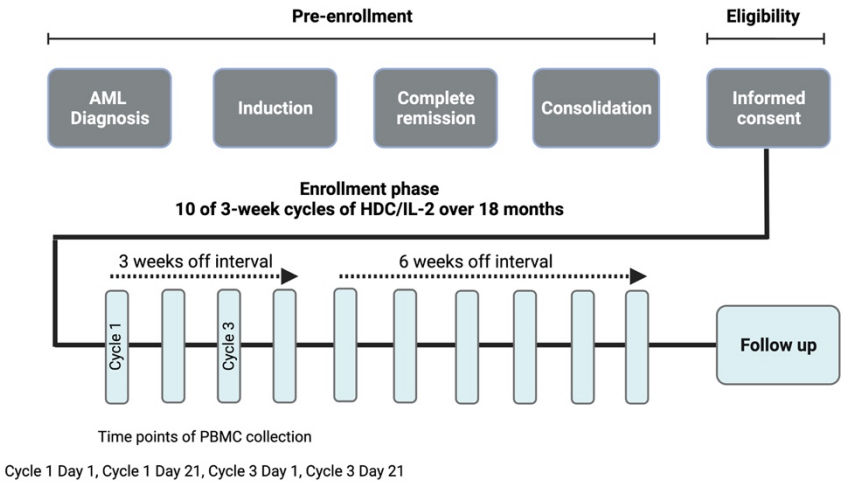

Figure 9. Scheme of phase IV Re:Mission trial. PBMCs from enrolled patients were collected at beginning and end of cycles 1 and 3. Figure is created with BioRender.com.

Since novel therapies are warranted for patients with hematological malignancies who relapse after receiving hematopoietic stem transplantation. IL-15 can stimulate both $CD8^+$ T-cells and NK cells and consequently augment antitumor response. A super agonist version of IL-15 was tested in elderly patients who had relapsed AML after transplantation in a phase I clinical trial and results showed that this treatment was well-tolerated and safe (340). Moreover, engineered autologous AML cells have various leukemia or patient-related antigens that embrace a potential target for vaccine treatment for AML. Nevertheless, prior usage of vaccines in context of AML has not been fruitful and partially explained by presence of inhibitory influence generated by AML blasts. Hence, alternative way is genetically encoded AML cells expressing IL-12 or IL-15 and this approach was shown to be less toxic compared to systemic administration of these cytokines (341, 342).

Cytokines are also implemented in priming of NK cells with the aim to increase proliferation and expansion. Yet, the short-lasting effect of the cytokine stimulation and short-term tenacity of NK cells might limit their clinical effects. It has been shown that a combination of triple cytokine including IL-12, 15, and 18 generated a subset of NK cells with enhanced and long-lasting anti-leukemic activity. These so-called cytokine-induced memory-like cells (CIML) are characterized by being antigen non-specific, have enhanced proliferation, elevated cytotoxic activity and higher IFN-γ production after *in vitro* re-stimulation (343). Moreover, these cells can be implemented as an adoptive transfusion in relapsed/refractory elderly AML, and they exhibited efficacious induction of remission without development of cytokine release syndrome and graft-versus host disease (58, 344-347).

Immunomodulatory agents. AML cells can change the expression profile of their ligands to evade NK cell-mediated killing, which may unfavorably affect the clinical outcome. There are agents that can restore expression of ligands on AML blasts to NK cell receptors rendering them more vulnerable to NK cell cytotoxicity (348). Hypomethylating agents like azacytidine and decitabine can induce upregulation of NKG2D ligands on AML cells and enhance eradication by NK cells (349). Furthermore, hypomethylating agents enhanced cytolytic activity of NK cells towards AML cells by reducing shedding of NKG2D ligands on AML cells (350). Other immunomodulatory drugs with potential clinical impact in AML include lenalidomide and pomalidomide. Leukemic cells treated with these drugs showed downregulation of HLA class I on AML cells, consequently boosting the NK cell anti-leukemic response by decreasing the inhibitory signaling (351). Since the glycogen synthetase kinase beta (GSK3β) expression is high in NK cells from AML patients, inhibition of GSK3β displayed restoration of the NK cell activity mediated by upregulation of LFA expression on NK cells (352). The immunomodulatory drugs might be used as novel therapies to potentiate the cytotoxicity of NK cells.

Monoclonal antibodies. CD16, or Fc γ receptor IIIa, is considered one of the most potent activating receptors expressed by human NK cells (353, 354). CD16 can bind the Fc domain of IgG and transduce activation signals, release of lytic granules, and induce killing of target

cells opsonized by antibodies (355, 356). NK cell antibody-dependent cell-mediated cytotoxicity (ADCC) is involved in killing of target cells. Taking benefit of this property, monoclonal antibodies become another approach to enhance NK cell antitumor response. The advantage of therapeutic antibodies is not only boosting the functionality of NK cells but also redirecting NK cells to cancer cells. Rituximab is nowadays used in the frontline treatment of different B cell malignancies (357) and can mediate NK cell ADCC response. The only approved drug conjugate for treatment of $CD33^+$ AML is now Gemtuzumab ozogamicin which is composed of anti-CD33 antibody and calicheamicin (358-360). The presence of leukemic stem cells in AML can sustain disease activity and lead to refractoriness and relapse, and antibodies targeting stem cells markers like IL3Rα expressed on AML cells revealed antileukemic responses (361).

Engagers. Bi-specific (BiKE) and tri-specific (TriKE) killer cell engagers are recombinant antibodies that can link NK cells and tumor cells. A full humanized BiKE binding to CD16 on NK cells and CD33 on AML cells mediated NK cell cytotoxicity and triggered cytokine release. Furthermore, the NK cell response with this BiKE was further enhanced by adding the ADAM17 inhibitor which can prevent shedding of CD16 (362). Afterwards, the same researchers who developed this BiKE integrated human IL-15 cross-linker into the BiKE, and thus generated a TriKE. Supplementing BiKE with IL-15 can promote proliferation, persistence, and activation of NK cells, and the 161533TriKE showed superior NK cell function and reverted defective NK cells in AML patients post allogeneic stem cell transplantation (363).

Immune checkpoint inhibitors. Ligation of inhibitory receptors expressed on NK cells is one mechanism by which target cells evade immune response. In addition to KIRs, NKG2A and LIRs, NK cells also express other immune checkpoint inhibitors that have been linked with NK cell dysfunction. These checkpoints include PD-1, TIM-3, TIGIT, Siglec7/9, CD96 and CD200 (364). T-cell related immune checkpoint inhibitors are less effective in myeloid malignancies due to relatively low frequency of mutations and consequently neoantigens. Yet, blockade of interaction between PD-1 and PD-L1 showed activation of NK cells, indicating a crucial role for NK cells in cancers with low MHC

expression (365). The potential therapeutic role of PD-1 blockade is still in the exploratory phase in treatment of AML. The distribution of Nivolumab, an anti-PD-1 antibody, in combination with induction therapy of idarubicin and cytarabine displayed promising results in AML patients (366). Furthermore, nivolumab was examined in a phase II trial as a maintenance therapy in high-risk AML in complete remission and it was found to be safe (367).

Recent approaches aimed at optimizing NK cell-based therapy against hematological malignancies included therapeutic use of anti-KIR antibodies. Lirilumab, an antibody targeting KIR2D, was used in a phase I trial in patients with multiple myeloma in combination with Lenalidomide and showed to be safe, tolerable and with signs indicating being efficacious (368).

One major biological concern reported while using anti-KIR antibody is the prompt drop in NK cell function (369), that could limit their potential role in NK cell-based therapy. To overcome this issue, a combination of anti-KIR antibody and other therapeutics might be able to overcome this hurdle and boost NK cell response. There are many ongoing trials evaluating this drug as a monotherapy or in combination with other drugs. According to findings presented in paper III in this thesis, using anti-KIR antibodies in patients harboring NKG2A GG or CC genotype would be interesting to see if this will have any impact on clinical outcome.

Blocking LIR-1 or NKG2A led to enhanced cytotoxicity of KIR^- NK cells towards AML cells(370). It has been suggested that approaches combining blockade of LIR-1, NKG2A and KIR alone or in combination maybe a viable option for NK cell therapy in acute leukemias (370). Nevertheless, detuning of NK cells might become a hurdle for such kind of therapy. On the other hand, in another study they targeted NKG2A and demonstrated strong NK cell response towards leukemic cells in mice (371).

Overcoming tumor environment induced-NK cell suppression. Tregs and MDSCs can limit NK cell antitumor immune responses. Hence, a promising strategy to restore NK cell function is to target MDSCs using a TriKE agent as the latter can target CD33 expressed on

both leukemic cells and MDSCs at same time (372). Activated Tregs accumulate in bone marrow of leukemia patients. Strategies to interrupt gathering of Tregs in bone marrow hold promise as a therapeutic potential for leukemia immunotherapy to enhance NK cell function since Tregs can suppress NK cell cytotoxicity (373).

Adoptive NK cell therapy. The idea of adoptive NK cell transfer stemmed from favorable effects of alloreactivity of NK cells after allogeneic stem cell transplantation. Mismatch between donor NK cell KIRs and the recipient's HLA class I expression can trigger alloreactivity of NK cells (300, 374). Alloreactive NK cells can target leukemic cells through graft-versus-leukemia, thus supporting engraftment and preventing graft-versus-host disease through donor T-cell ablation (375).

Adoptive transfer of NK cells has been extensively investigated and explored in context of AML. Moreover, adoptive NK therapy might be used as a bridging step when AML patients are not eligible for transplantation or in cases where the risk of disease relapse post-transplant is high. There are different ways that adoptive NK cells can be applied. In a phase I clinical trial, patients with acute leukemia received infusion of donor CD3-depleted NK cells pre-activated with both IL-15 and IL-21 after HLA-haploidentical transplantation. The study showed that donor NK cells are well tolerated and there was reduced disease progression when compared to the group who did not have NK cell infusion (376). Infusion of donor NK cells can also be administered prior to HSCT. In a phase I study, donor NK cells as part of preparatory regimen for allogenic HSCT displayed strong association between relapse-free survival and number of infused NK cells (377). Donor-derived NK cells can also be implemented as a bridge therapy prior to HSCT in relapsed/refractory AML as it exhibited reduction in disease burden (378).

Adoptive transfer of NK cells has been tested in AML patients outside HSCT setting as well. Haploidentical related-donor NK cells infused to patients with relapsed/refractory AML in conjunction with high dose of cyclophosphamide, fludarabine and subcutaneous IL-2 resulted in complete remission in 5 out of 19 patients with an unfavorable risk group (314). Transfusion of haploidentical KIR-HLA mismatched

donor NK cells is not only used to induce complete remission but also to maintain remission (315, 379-382). Studies showed an association between number of donor alloreactive NK cells and the clinical outcome. Hence, expansion of NK cells *ex vivo* and *in vivo* to attain adequate number of donor NK cells is highly demanded (381). Amplifying functional capacity and number of NK cells is achievable through cytokine stimulation or co-culturing with feeder cells expressing membrane bound cytokines (383-386). The first clinical trial using *in vivo* recombinant human IL-15 to enhance haploidentical donor NK cell transfusion in patients with relapsed/refractory AML revealed better NK cell expansion and higher rate of remission when compared to clinical trials where IL-2 was used (316). Due to easy cultivation, expansion, cost effectiveness, high therapeutic doses, possible genetic modifications and feasibility of repeated infusions, transfusion of activated human NK92 cell line in relapsed/refractory AML patients was found to be safe and feasible and it is an alternative option to primary human NK cells transfusion (387).

CAR-modified-NK cells. The capacity of NK cells to kill target cells during adoptive transfusion is mainly dependent on the engagement of NK cell receptors with their respective ligands expressed on target cells. Hence, using genetically modified NK cells harboring a construct encoding a chimeric antigen receptor (CAR) to boost specificity and cytotoxicity of NK cells night be a valuable option. In CAR-NK, NK cells are genetically modified to express CARs that can identify and recognize specific antigen expression on tumor cells.

The approval of anti-CD19 CAR- T-cell, tisagenlecleucel, for treatment of relapsed pediatric B-ALL was groundbreaking in the field of leukemia therapy (388). Yet, the promising effect of CAR-T-cell therapy has not been translated for treatment of AML (389). Short-living NK cells are considered an alternative to T-cells to generate a novel CAR approach with lower cost and a more favorable side effect profile (390). Identification of markers related to AML for CAR-NK therapy is a major barrier as AML and normal hematopoietic stem cells share some phenotypic markers. CD33 is a popular target for employing novel therapies in treatment of AML as it is expressed on majority of AML cells and on leukemic stem cells (391). First-in-human trial of CD33-CAR-NK cell demonstrated that this treatment strategy is safe in

patients with relapsed/refractory AML (392). There are other aspects of CAR-NK cell to be exploited including target optimization, structure design and identifying ideal patients who get benefit from such therapies.

All in all, NK cells represent effective immune cells which are fortified with fast-acting and prompt anti-tumor response. Adoptive NK cells transfusion was proven promising in hematological malignancies. Though, there are a list of limiting factors that might hinder the progression of such kind therapies including attaining of large amount of NK cells, *in vitro* expansion and maintaining *in vivo* survival. Despite these challenges, currently with new tools of genetic engineering approaches there are many pre-clinical and clinical trials ongoing in relation to NK cell therapy.

2 AIMS

This thesis was designed to provide new insights about how genes of NK cell receptors and HLA shape and control human NK cell immunity and influence the outcome of immunotherapy in acute myeloid leukemia.

Paper I aimed at defining the role of the dimorphism at position -21 of the HLA-B on the function of NK cells and outcome of immunotherapy in acute myeloid leukemia.

Paper II aimed at defining the impact of gene variants of NK cell activating receptors on the expression level and clinical outcome of immunotherapy in acute myeloid leukemia.

Paper III aimed at dissecting how gene variants within the NK complex region affect NK cell responses and their influence on outcome of IL-2 based therapy in acute myeloid leukemia.

3 MATERIALS AND METHODS

Samples

Paper I-III present data from a phase IV open-label single-arm Re:Mission trial that included eighty-four patients with AML (18-79 years) in first complete remission. After receiving induction and consolidation chemotherapies, each study participant received 10 consecutive cycles of histamine dihydrochloride (HDC; 0.5 mg, subcutaneously twice daily) and low-dose IL-2 (16 400 IU/kg, subcutaneously twice daily) in a three-week cycle for 18 months or until disease relapse or death. Enrolled cases were followed for up to 2 years after enrollment. Primary study objectives included assessment of minimal residual disease (MRD) and measuring the dynamics of immune cells including NK cells, myeloid cells, and T cells before and after treatment cycles. The trial was approved by ethics committees from each participating institution. Study participants received written informed consent before enrollment in the study. In addition, samples from healthy donors were used for purpose of *in vitro* experiments and functional assays in papers I and III. Healthy individual samples were received from the blood bank of Sahlgrenska hospital under ethical approvement and informed consent provided. Moreover, paper III demanded us to generate polyclonally active NK cells. For that purpose, NK cells were purified from a healthy individual and co-cultured with irradiated 221G cell line and allogeneic PBMC feeder cells in a cell culture medium containing IL-2 and phytohemagglutinin (PHA-M). After 5 days, the cells were kept in the same medium but without PHA-M.

Flow cytometry analysis

One of the powerful tools in field of immunology research is flow cytometry which offers a wide range of measurement and analysis of both intracellular and extracellular components of each individual cell starting from morphology, complexity, and phenotypic profile. Flow cytometry has been used majorly in this thesis to assess phenotype of NK cells, measuring degranulation capacity of NK cells. Furthermore, cytokines like IFN-γ and TNF-α and lytic granules such granzyme B were measured using intracellular staining approach.

NK cell functional assays

The approaches to assess functions NK cells and their subpopulations that have been used in this thesis include assays evaluating degranulation, cytotoxicity, cytokine production and granzyme B level. In the degranulation assay, NK cells were stimulated overnight with IL-2 or IL-15. Next day, NK cells were coincubated with leukemic blasts from AML patients or leukemic cell lines for 3 hours in the presence of anti-CD107a antibody. In the cytokine assay, after overnight stimulation of NK cells with IL-2, protein transport inhibitor containing Brefeldin A (GolgiPlug) was added after an hour of adding the target cells to NK cells and coculture was kept for additional 4 hours. In the cytotoxicity assay, pre-activated NK cells were cocultured with target cells stained with a cell trace marker for 3 hours and afterwards viability of target cell was measured by flow cytometry. In experiments where determination of the role of NK cell activating receptors was required, effector cells were incubated with a blocking monoclonal antibody at room temperature before co-culturing with target cells.

After completion of coincubation with target cells, NK cells were stained with a master mix of antibodies to identity NK cell subsets and maturation stages. In intracellular staining experiments, NK cells were fixed and permeabilized before staining for intracellular cytokines. Stained samples were acquired and analyzed with flow cytometry using a 5-laser BD LSRFortessa.

TaqMan genotyping assay

Genomic DNA was extracted from PBMCs of AML patients and healthy individuals using the Qiagen® DNeasy Blood & Tissue kit. Gene variants included in this thesis, NKG2D rs1049174, DNAM-1 rs763361, NKp30 rs986475, NKp30 rs1052248 and NKG2A rs1983526, were genotyped using the TaqMan-Allelic discrimination technique with Applied Biosystem 7500 Fast Real-Time PCR System.

TaqMan technology uses 5′nuclease activity of *Taq* polymerase to detect fluorogenic signal generated during PCR cycles. Each TaqMan assay involves two primers for amplifying the sequence of interest and two allele-specific and differently labeled TaqMan minor groove binder (MGB) probes for allele detection. Each MGB probe has a fluorescent reporter dye (FAM or VIC) in the 5′end and a fluorescence quencher at

the 3′ end of the probe. The two MGB probes only differ in sequence at the SNP site, one probe matches the wild-type allele and the other probe to the variant allele. TaqMan genotyping is based on FRET technology where the 5′reporter dye is covalently linked to the 3′quencher dye in both probes. When the probes are intact, there will be very low or no fluorescence. The probes hybridize to the target site during annealing step and during extension the reported and quencher dyes are freed due the nuclease activity of *Taq* polymerase, and this leads to enhanced fluorescence of the reported dye. The Taq polymerase only recognizes the probe that completely hybridized to the target sequence. At the end of PCR reaction, the fluorescence signals are collected for the two reported dyes, and it will be normalized using signals collected from a third dye (e.g., ROX).

Gel electrophoresis and SSO-based typing

AML patients from the Re:Mission trial and healthy donors included in the papers of this thesis were genotyped for HLA-B -21 dimorphism (rs1050458) using two approaches which encompass an agarose gel electrophoresis-based method and a sequence-specific oligonucleotide method (SSO Labtype HD class IB locus typing kit). In brief, in the gel-based approach a 200 bp fragment containing the SNP was produced by PCR. The amplified material was then treated with NIaIII restriction enzyme. The product made from the HLA-B -21T allele is resistant to cleavage by NIaIII and only showing a single band of around 200 base pairs whereas the product generated from the HLA-B -21M-encoding allele is NIaIII digestion-sensitive and can generate two bands of approximately 100 and 50 base pairs, respectively.

Luminex-based technique has been implemented to differentiate various HLA alleles. The typing method in a reverse-SSO assay consists of multiple steps. First, PCR-amplification of target DNA is performed using a group specific primer. The PCR product is biotinylated, which permits it to be spotted using a fluorescence called R-Phycoerythren-conjugated Streptavidin (SAPE). Basically, there will be denaturation of the PCR product and then rehybridization to complementary DNA probes that are immobilized on and conjugated to fluorescently coded microspheres with the aim to identify HLA alleles. Each microsphere mixture includes positive and negative control to remove a non-specific background signal. Afterwards, the data is acquired using a Luminex

analyzer which can detect intensity of fluorescence of PE on each microsphere. Later, the HLA alleles of each sample can be determined by matching the pattern of positive and negative bead IDs using the HLA Fusion Software.

Generating cell line knockout clones using CRISPR/Cas9

The CRISPR/Cas9 is considered a potent method for quickly creating knockout cell lines. With CRISPR/Cas9 a generation of a complete loss-of-function mutations and precise alteration in the targeted gene of the cells is feasible. Additionally, CRISPR has the prospective to produce permanent and stable loss-of-function changes, letting long-term analysis of a customized cell line. A cell harboring those mutations can serve as a novel cell line and can be implemented for instance in studying essential questions in cell biology, cancer biology and genetics and consequences of loss of genes. With expression of Cas9 nuclease and a guide RNA (gRNA) in a cell, a double-strand break can be accomplished in the target gene. In brief, the process of generation of a knockout cell using CRISPR includes five steps. First, selection of knockout strategy either by using one-plasmid system (encoding both Cas9 and gRNA on the same vector) or two-plasmid system (encoding Cas9 and gRNA on various vectors). Second, selection of gRNA target sites and cloning of gRNAs targeting the gene of interest into the vector. Third, delivering of CRISPR into the cells of interest either by transfecting or transducing gRNA plasmids into a Cas9-expressing cell line. Fourth, isolation and expansion of single-cell knockout clones to generate clones that can be confirmed as pure knockouts using FACS sorting approach. Lastly, authentication of knockout genes using sequencing techniques.

K562 cells – a cell line from a patient with chronic myeloid leukemia in blast crisis – was obtained from American Type Culture Collection (ATCC). To test the NK cell functional capacity based on NKG2D gene variants and to be able to attribute the difference in NK cell cytotoxicity to gene variants related to NKG2D gene with a high resolution, a generation of an NKG2D-dependent cell line model was exceedingly demanded. For this purpose, the CRISPR/Cas9 technique was used to knockout three NK cell activating receptor ligands expressed by K562 (B7-H6, ligand for receptor NKp30 and PVR and NECTIN-2, ligands for receptor DNAM-1), that are mainly involved in NK killing of these

target cells. The details of CRISPR/Cas9 mediated gene edition of K562 can be found in paper III.

Statistics

GraphPad prism version 7.0 was used to analyze the data in all three papers. Unpaired two-sided Student's t-test was performed to compare means of two groups. Paired and unpaired t-tests were applied when required accordingly. The one-way analysis of variance (ANOVA) test followed by Bonferroni´s multiple-comparison test was performed for multiple group comparisons. Linear regression analysis was performed to determine the relationship between the impact of one allele of a gene on expression of a receptor. Statistical analysis of overall survival (OS) and leukemia free survival (LFS) was executed using the log rank test. More precisely, log rank Mantel-Cox test to compare the survival between two groups and log rank test for trends when there were more than two groups. Variables that significantly predicted LFS and/or OS were further examined by univariable and multivariable cox regression analyses.

4 RESULTS AND DISCUSSION

Paper I. The HLA-B -21 dimorphism impacts on NK cell education and clinical outcome of immunotherapy in acute myeloid leukemia

Functional competence of NK cells is regulated by inhibitory KIRs and NKG2A that can bind to HLA class I. Furthermore, it has been shown that individuals either have robust KIR-dependent regulation of NK cells or faint KIR inhibition with more inhibition via the NKG2A receptor. This dichotomy of NK cell regulation is based on a dimorphism in position -21 of the gene encoding HLA-B, which relates to amino acid number 2 in the peptide presented by HLA-E (216). Little is known regarding the consequences of this dichotomy in leukemia. We demonstrated that AML patients carrying HLA-B -21 M/x had a lower risk of relapse and showed prolonged survival after receiving IL-2 based immunotherapy, when compared to patients carrying HLA-B -21 TT (Figure 10A-B).

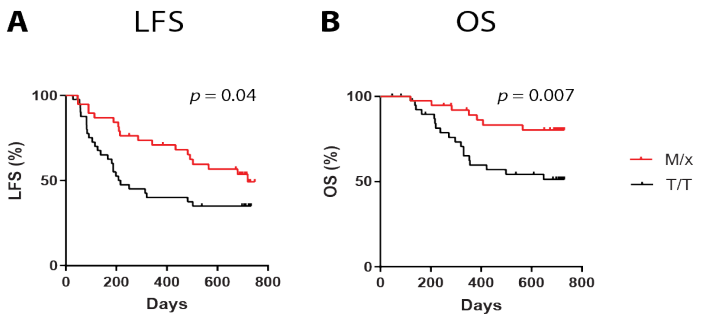

Figure 10. Impact of HLA-B -21 dimorphism on the clinical outcome of immunotherapy in AML. (A) Leukemia-free survival and (B) overall survival of AML patients with HLA-B -21 M/x (n=38) and HLA-B -21 TT (n=42) after receiving immunotherapy.

The clinical consequences of HLA-B -21 dimorphism is not well understood. Patients with M/M genotype demonstrated accelerated contraction of HIV infection, and this was associated with a compromised NK cell function mediated by the augmented NKG2A-mediated inhibition that can reduce killing of HIV-infected target cells (393, 394). Moreover, Ramsuran *et al.* demonstrated that HLA-B

haplotypes favoring NKG2A-mediated education of NK cells aggravate the disadvantageous effect of HLA-A on the control of HIV, because excessive levels of HLA-A expression leads to amplified levels of the signal peptide derived from HLA-A that can bind HLA-E and cause NKG2A-mediated impairing clearance of target cells by NK cells (226). At first sight, these findings do not go in line with what we have described in paper I, but at the same time the background of the disease states is very different. There are multiple factors behind HLA-B -21 M/M being unfavourable in HIV infection (explained in detail in paper I). One of these factors is related to the gene encoding HLA-C, as high expressing HLA-C alleles are vital for increased cytotoxic T cell response towards HIV infected cells (395, 396) and there is a strong linkage disequilibrium between HLA-C low-expressing alleles and HLA-B -21M (216); consequently, the poor outcome of HIV in patients harboring HLA-B M/M genotype may be in part related to a low expression of HLA-C.

It has been reported that AML patients in all FAB subtypes have high levels of $NKG2A^+$ NK cells (397). In addition, we observed induction of NKG2A after IL-2-based therapy in our AML cohort (398). Hence, we sought to dissect the link between superior clinical outcome associated with HLA-B -21M/x in the Re:Mission study. We identified a significant upsurge in frequency of $NKG2A^+$ NK cells in these patients before treatment start when paralleled to healthy donors (Figure 11A). The high frequency of $NKG2A^+$ cells was not only limited to KIR^- or single-KIR^+ NK cells, as NK cells of AML patients with multiple KIRs also exhibited high frequencies of $NKG2A^+$ cells (Figure 11B). Despite elevated levels of NKG2A expression in the Re:Mission trial, NK cells regulated through NKG2A revealed an association with a superior clinical outcome. In addition to induction of NKG2A, IL-2 was found to cause induction of granzyme B mainly in $NKG2A^+$ cells.

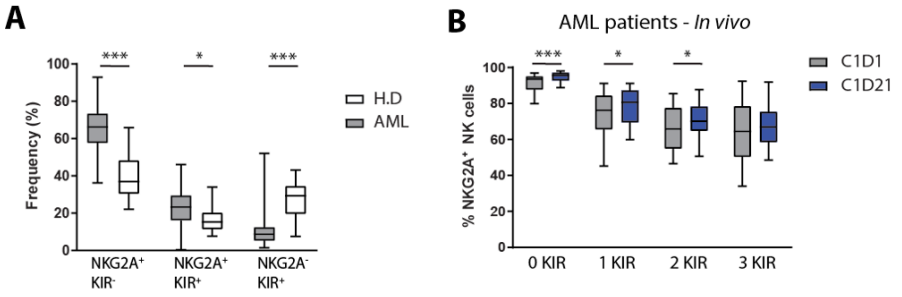

Figure 11. Expression pattern of NKG2A and granzyme B in NK cells after exposure to IL-2. (A) Frequency of NK cell subsets in AML patients before start of HDC/IL-2 therapy (n=64) and in healthy donors (n=24). (B) Frequency of NKG2A$^+$ NK cells in NK cell subsets expressing 0, 1, 2 or 3 KIRs in AML patients before and after immunotherapy (n=54).

The NKG2A-HLA-E pathway has regularly been studied using the transfected 721.221 cell line with an HLA-E construct with a linked peptide from HLA-A (referred to as .221 AEH cells) which expresses extreme levels of HLA-E that can easily inhibit degranulation response of NK cells (Figure 12A). The hindrance associated with .221AEH cells led us to take advantage of the T2E cell line, where expression of HLA-E is amendable through providing external peptides. After using various concentrations of the peptide, we observed an inverse correlation between the degranulation response of NKG2A$^+$ NK cells and the intensity of HLA-E expression by T2E cells. After stimulating NK cells with IL-2, we noted no striking reduction in degranulation of NKG2A$^+$ cells using T2E cells when compared to T2E cells without peptide (control) (Figure 12A-B). This finding indicates that IL-2 stimulation can make NK cells withstand the inhibitory effect of NKG2A-HLA-E interaction when HLA-E is expressed at levels comparable to the *in vivo* setting. As HLA-E expression is even lower on AML blasts compared to T2E cells and in line with these findings, we showed that NKG2A$^+$KIR$^-$ NK cells have significantly higher degranulation capacity toward KIR-matched AML blasts after IL-2 stimulation when compared to NKG2A$^-$KIR$^+$ NK cells (Figure 12C).

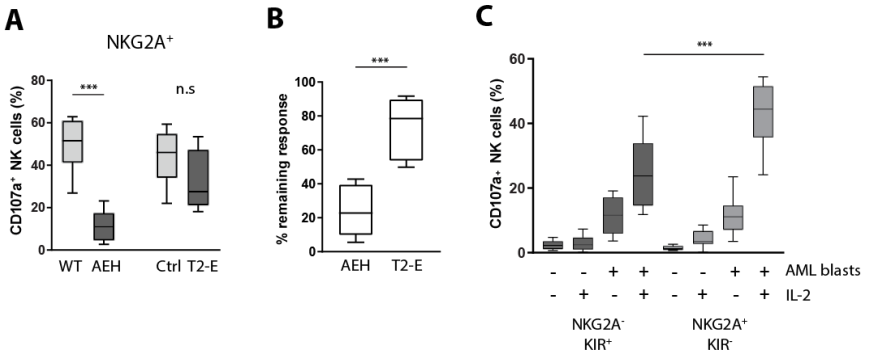

Figure 12. Response of NKG2A$^+$ cells towards cell lines expressing HLA-E and AML blast cells. (A) Degranulation response of NKG2A$^+$ NK cells in healthy donors (n=8) after pre-activation with IL-2 and coculture with .221-WT, .221-AEH, T2 cells without HLA peptide (control), and T2 cells provided with HLA peptide (T2-E). (B) Evaluation of remaining response of NKG2A$^+$ NK cells with the presence of HLA-E compared with the response observed in the HLA-E control cell line. (C) Frequency of degranulation in subsets of NK cells of healthy donors (resting, n=10; IL-2 stimulation, n=12) towards KIR-matched CD34$^+$ AML blast cells from two patients showed sizeable degranulation capability.

There are multiple factors that may contribute to NKG2A-HLA-E being an overridable pathway in the context of HDC/IL-2 therapy in AML. First, IL-2 can upregulate NK cell activating receptors, and this can cause overruling of NKG2A inhibition but not the stronger KIR-mediated inhibition in HLA-B -21 TT patients. Second, the low expression of HLA-ABC in the myeloid compartment (399) was largely found in M/x patients; this reduction of NK cell ligands may be sufficient to overcome the inhibitory signal input. Third, the organization of ITIM molecules of NKG2A and inhibitory KIR receptors in their intracellular portions might play a role as the N-terminal of ITIM is located proximally in case of inhibitory KIRs whereas distally in case of NKG2A in relation to the membrane (400). This can affect the binding of SHP1 and 2 molecules which in turn affect the strength of inhibitory signals transduced through these receptors (400-402). One hypothesis is thus that the strength of inhibitory signals through NK cells expressing high levels of NKG2A may be lower compared to NK cells expressing inhibitory KIRs. Finally, HLA-E expression is relatively lower when compared to HLA-ABC (403),

which may contribute to HLA-E-mediated inhibition being easier overridable than HLA-ABC-mediated inhibition.

Finally, individuals carrying HLA-B -21 M/x showed slightly higher HLA-E expression; accordingly, NKG2A$^+$ cell of M/x donors were better educated via their NKG2A receptor, as they displayed stronger degranulation response against KIR-ligand matched HLA class I intact AML blasts after IL-2 stimulation, and intriguingly NKG2A$^+$ cells from these patients were accountable for the superior degranulation (Figure 13A-B).

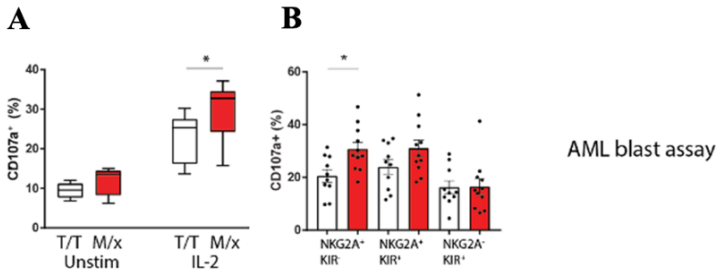

Figure 13. Dimorphism in HLA-B -21 drives education response of NK cells. (A) Degranulating capacity of NK cells and (B) their subsets towards HLA-matched CD34$^+$ AML blasts in resting and IL-2 stimulated NK cells from M/x (red, n=11) or T/T (white, n=10) donors.

In summary, the findings presented in paper I indicate how different NK cell repertoires can impact the outcome of immunotherapy in AML. Nevertheless, the high expression and larger population of NKG2A$^+$ NK cells did not reveal any unfavourable clinical outcome in our studies. Thus, NK cells educated and controlled mainly through NKG2A in M/x patients are better responders toward leukemic cells, and at the same time the cytokine stimulation may empower NK cells to override NKG2A-mediated inhibition relatively easier when compared to stronger inhibitory signal associated with KIR. It should be emphasized that the superior outcome of patients with NKG2A-regulated NK cells does not rule out the possibility that NKG2A blockade could further enhance the efficacy. However, there is a risk that NKG2A blockade

may lead to reduced activity of NK cells as described for KIR blockade. Interestingly, paper I underscores the importance of NKG2A-HLA-E interaction for NK cell functionality with emphasis on the ligand expression. In paper III, we follow up this finding to investigate whether gene variants on the receptor side influence NK cell function and clinical outcome of IL-2-based immunotherapy in AML.

Paper II. Impact of NK cell activating gene variants on receptor expression and outcome of immunotherapy in acute myeloid leukemia

There is an association between multiple single nucleotide polymorphisms of genes encoding NK cell receptors with the expression of the receptors and risk of developing cancer and autoimmune diseases (269, 272). These findings incited us to investigate whether gene variants of NK cell activating receptors, including NKG2D, DNAM-1 and NKp30, had an impact on the receptor expression and the outcome of patients with AML after receiving IL-2-based immunotherapy. It has been shown that a variation in natural cytotoxicity is driven by haplotypes of NKG2D, where several SNPs with an impact on NK cell function have been identified in the NKC locus (243). Additionally, one study stated that there is an association between the G allele of NKG2D rs1049174 gene variant and the outcome of hematopoietic stem cell transplantation in patients with hematological malignancies (265). Furthermore, NKG2D haplotypes have been shown to affect the NKG2D expression and consequently, are responsible for the variation in natural cytotoxic activity (404). In line with these studies, after genotyping our AML cohort for NKG2D rs1049174 SNP we observed that patients carrying at least one G allele were found to have significantly better overall survival, with a similar trend for leukemia-free survival (Figure 14A-B). Moreover, the G allele revealed an augmented effect on the expression of NKG2D, and this was more prominent in $CD16^{-}CD56^{bright}$ NK cells at start and after receiving immunotherapy (Figure 15A-B). Higher expression of NKG2D among $CD16^{-}$ $CD56^{bright}$ NK cells after immunotherapy was linked with a trend towards better survival. These data show that high NKG2D expression might provide a survival benefit for AML patients receiving IL-2-based immunotherapy. However, there is a linkage disequilibrium among other SNPs located in the NKC locus, and these SNPs are in turn related to NK cells and can modulate functional competence of these cells. Thus, although our findings regarding gene variant of NKG2D go in parallel with previous studies, we believe that this effect may not merely be driven by the NKG2D SNP.

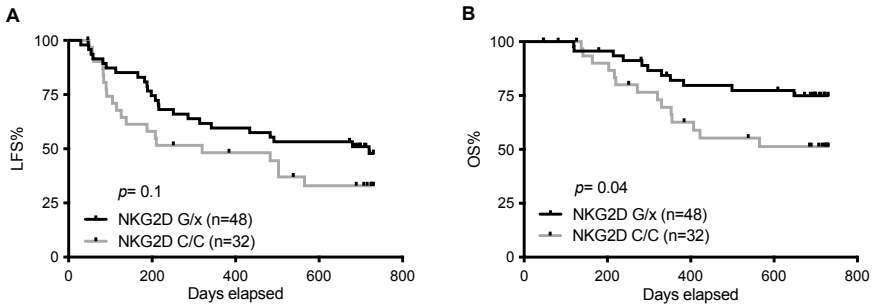

Figure 14. Impact of NKG2D rs1049174 on the clinical outcome of immunotherapy in AML. (A) Leukemia-free survival and (B) overall survival of AML patients with G/x (n=48) and CC (n=32) after receiving immunotherapy.

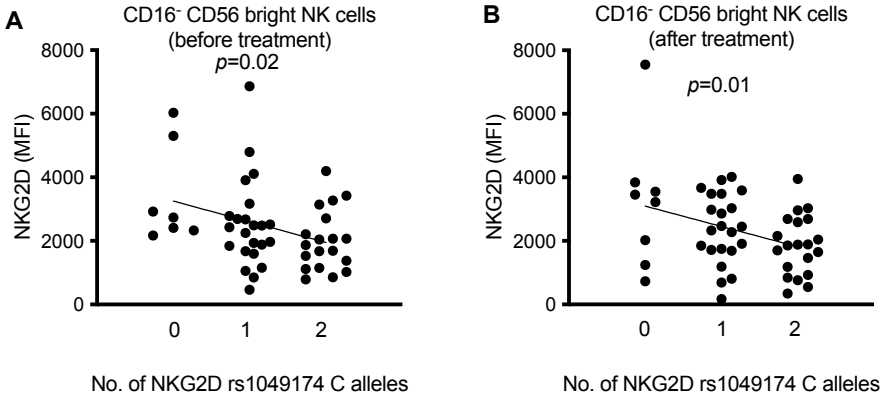

Figure 15. Impact of NKG2D rs1049174 on NKG2D expression. (A) Expression of NKG2D according to NKG2D rs1049174 in CD16-CD56bright NK cells at start (A) and after one cycle of HDC/IL-2 therapy (B). Patients in figures A and B are categorised according to number of C alleles. Numbers are equal to 7, 23 and 19 at start and 8, 23 and 20 after immunotherapy.

DNAM-1 has been reported to have principal roles in myeloid malignancies (405, 406). Hence, in paper II we looked at the correlation between DNAM-1 expression, the DNAM-1 rs763361 gene variant and clinical outcome of IL-2-based therapy in AML. Unpredictably, below median expression of DNAM-1 before treatment start was associated

with a striking improvement in leukemia-free survival but no impact on overall survival (Figure 16A-B). This finding was not related to differential expression of DNAM-1 among young and elderly patients, as low DNAM-1 expression continued to be a significant predictor of LFS in a cox-regression analysis considering for age. There are various reports showing an association between expression of DNAM-1 and education of NK cells. The coordinated regulation and colocalization of DNAM-1 and LFA-1 at the immune synapse can contribute to enhanced functional state of educated NK cells (407). DNAM-1 expression may be a factor that is required for education to happen, or DNAM-1 may be induced by education and necessary to maintain the education state of NK cells (407, 408). Furthermore, DNAM-1 is expressed during very early stages of NK cell development, and this might indicate its importance in cellular development and setting the functional state of NK cells (408). We previously reported that patients missing one of the HLA molecules that act as a ligand for inhibitory KIRs showed better survival, and this was due to the presence of an uneducated subset of NK cells in these patients (162). Hence, it is possible that our finding that low DNAM-1 expression is beneficial prior to HDC/IL-2 is related to the presence of an uneducated and autoreactive NK cell population with low DNAM-1 expression. For a better understanding of mechanisms underlying the beneficial outcome linked with low DNAM-1 in the Re:Mission trial, information about expression of TIGIT and CD96 receptors may be important, considering that DNAM-1, TIGIT and CD96 comprise an axis of paired receptors with opposite functions on both NK and T cells. However, we are lacking this information.

We observed a strong induction of DNAM-1 expression in both $CD16^{-}$ $CD56^{bright}$ and $CD16^{+}CD56^{+}$ NK cell subsets, and patients with above-median expression (MFI) between treatment start and three weeks of treatment displayed superior leukemia-free survival, with a similar trend for overall survival (Figure 16C-E). Despite the impact of gene variants of DNAM-1 on the expression level, we did not see any impact of the DNAM-1 SNP on treatment outcome in our cohort.

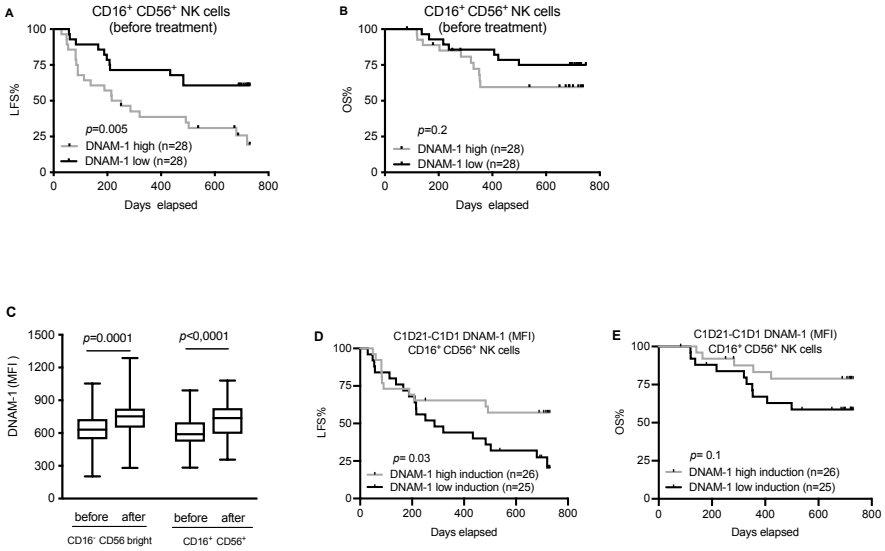

Figure 16. Correlation between DNAM-1 expression and outcome immunotherapy in AML. (A-B) Leukemia-free survival (LFS) and overall survival (OS) of AML patients before initiation of HDC/IL-2 immunotherapy categorized according to DNAM-1 median fluorescence intensity (MFI). I Influence of of HDC/IL-2 therapy on (MFI) of DNAM-1 expression in $CD16^{-}$ $CD56^{bright}$ and $CD16^{+}$ $CD56^{+}$ NK cells of AML patients at start (n=51) and after (n=59) receiving IL-2 based therapy. (D-E) Impact of induction of the $CD16^{+}CD56^{+}$ NK DNAM-1 expression on LFS and OS during first three weeks of immunotherapy in AML patients.

It has been reported that there are different splice variants of NKp30, and that these variants have differential effector functions. Interestingly, the NKp30 splicing profile was described to affect the clinical outcome of gastrointestinal stromal tumor. The NKp30 A and B splice variants are considered immunostimulatory as they can prime cytotoxicity and Th1 cytokine secretion, respectively, while on the other hand the NKp30C isoform is immunosuppressive as it induces IL-10 secretion. Furthermore, NKp30 SNPs like rs986475 and rs1052428 showed a correlation with the expression of NKp30 splice variants. (269). Half of the NKp30C isoform patients carried rs986475 AA or AG genotype whereas none of the AB patients had these genotypes (267, 269). In addition to aforementioned findings, we previously demonstrated a protective role of high expression of NKp30 in elderly patients with

AML after immunotherapy (302). Thus, to see if NKp30 gene variants are of importance in the context of immunotherapy in AML, we genotyped our patients for two NKp30 SNPs, rs986475 (A/G) and rs1052248 (T/A). We observed only a trend toward a higher expression of NKp30 among patients lacking G allele of rs986375 in different subsets of NK cells at start and after one cycle of immunotherapy, but no influence of NKp30 rs986475 on LFS and OS was identified (Figure 17A-F). No impact of NKp30 rs1052428 could be identified neither on expression, nor on the clinical outcome. In addition, when we combined both NKp30 SNPs in one model we still could not observe any clinical impact. We did not have the chance to profile our cohort of AML patients for different NKp30 isoforms to see if profile C patients do differently based on the SNPs. Of note, another possible explanation of why we don't see the impact of different gene variants of NKp30 is probably due to significant difference in the status of the diseases and treatments background comparing our studies to studies where they reported impact of NKp30 SNPs.

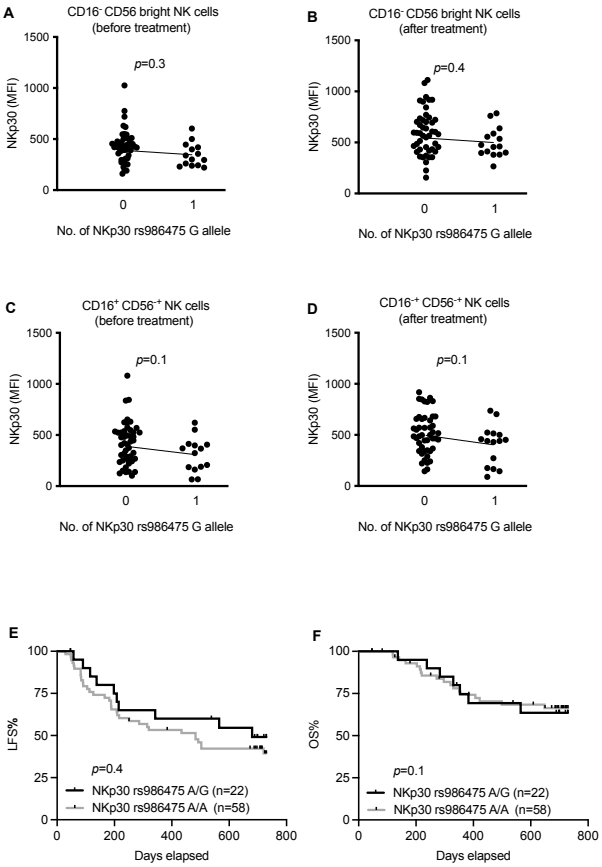

Figure 17. Impact of gene variants of NKp30 rs986475 on expression of NKp30 and clinical outcome of immunotherapy in AML. (A-D) Median fluorescence intensity of NKp30 based on genotypes of NKp30 rs986475 in AML patients in NK cell subsets including CD16$^-$ CD56^{bright} and CD16$^+$ CD56$^+$ before and after HDC/IL-2 therapy. Patients are dichotomized based on presence (14 out of 61 before therapy, and 15 out of 62 after therapy) or absence of G allele in panels A-D. (E-F) Survival curves showing impact of NKp30 rs986475 on leukemia-free survival (LFS) and overall survival (OS) of AML patients after receiving HDC/IL-2 therapy.

Paper III. Impact of NKC locus gene polymorphism on natural killer cell function and outcome of immunotherapy in acute myeloid leukemia

There are several genes located in the NKC locus, including the genes encoding NKG2D and NKG2A. As mentioned above, gene variants in the NKC locus have been demonstrated to correlate with natural cytotoxic activity and outcome of cancer, and this effect has been ascribed to NKG2D gene variants (243, 265). There are only limited number of studies focused on functional consequences of these SNPs on NK cells. As mentioned previously, we reported in paper I those patients with NK cells that are mainly educated and regulated by NKG2A have favorable clinical outcome in AML (409). Thus, in paper III our goal was firstly, to develop methods to study the impact of NKC locus gene variants on NK cell function; secondly, to determine impact of NKG2A gene variants on NK cell function; and thirdly, to assess the clinical significance of NKG2A gene variants in AML immunotherapy.

For these studies we chose to start with K562 cells, the same cell line used in the highly cited study describing the link between low NK cell activity and increased risk of cancer (261) but we could not observe the impact of high natural cytotoxicity allele of NKG2D rs1049174 (Figure 18A). NK cell cytotoxicity assays disclosed a major role of NKp30 but only a minor role for NKG2D after performing blockade of these receptors (Figure 18B). Furthermore, combination blockade of NKp30 and DNAM-1 receptors provided further protection against killing. To augment the impact of NKG2D on the NK-mediated killing of the K562 cells, and to be able to generate a model that can offer a better resolution in the difference between genotypes of NKG2D rs1049174, we used the CRISPR technique to delete the major ligands involved in killing of K562. These were B7-H6, a ligand for NKp30; and PVR and Nectin-2, ligands for DNAM-1. The resulting triple knockout *B7H6*$^{-/-}$*PVR*$^{-/-}$ *NECTIN2*$^{-/-}$ cell line generated is hereafter referred to as tKO K562 (Figure 18C). As anticipated, tKO K562 cells showed diminished vulnerability to killing by NK cells. Yet, at higher E:T ratios, tKO K562 cells were readily killed by NK cells and killing was largely NKG2D-dependent as blockade of NKG2D resulted in marked reduction of NK cell cytotoxicity (Figure 18D).

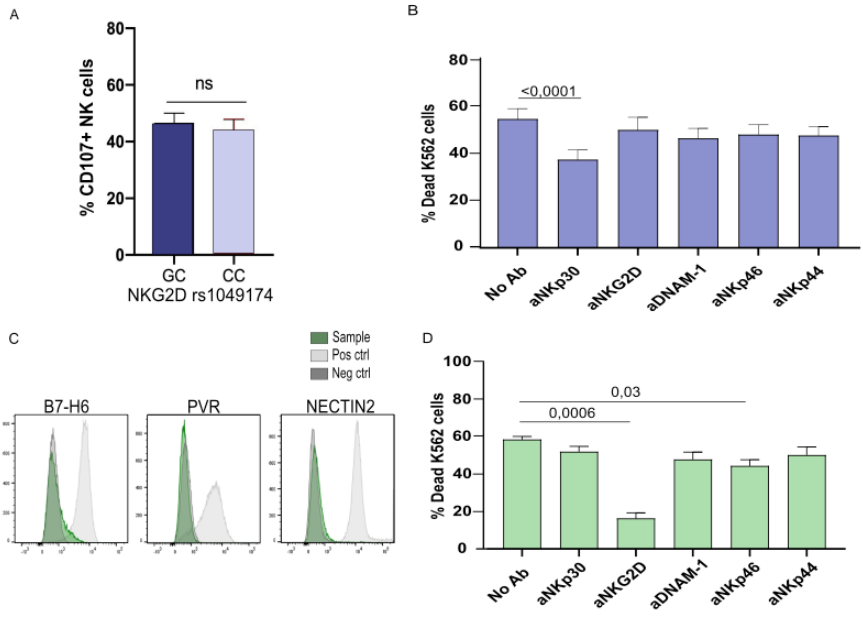

Figure 18. Generation of a NKG2D-dependent cell line model. (A) Degranulation of healthy donor NK cells according to NKG2D GC (n=11) or CC (n=12) after treatment with IL-2 and co-incubation with wild type K562. (B) Cytotoxicity of polyclonally activated NK cells from healthy individual against wild type K562 in presence and absence of various activating NK cell receptor blockade (n=9). (C) Expression pattern of triple knock-out K562. (D) Cytotoxicity assay of polyclonally activated NK cells from healthy donors against triple knock-out K562 at 8:1 effector:target ratio in presence of blockade of NK cell receptors (n=5).

In line with previous studies mentioned above, where they demonstrated an association between NKG2D haplotypes and NK cell function and consequently cancer incidence, we demonstrated the impact of NKG2D rs1049174 on the outcome of immunotherapy in AML (paper II). Nevertheless, the difference in degranulation response of healthy NK cells of individuals carrying either NKG2D Gx or CC was minor despite using the highly NKG2D-dependent model (Figure 19A). Additionally, no difference could be observed in a cytotoxicity assay (Figure 19B). Interestingly, the higher degranulation in NKG2D G/x donors was due to a relatively bigger population of the more responsive NKG2A^{+} NK cells (Figure 19C-D).

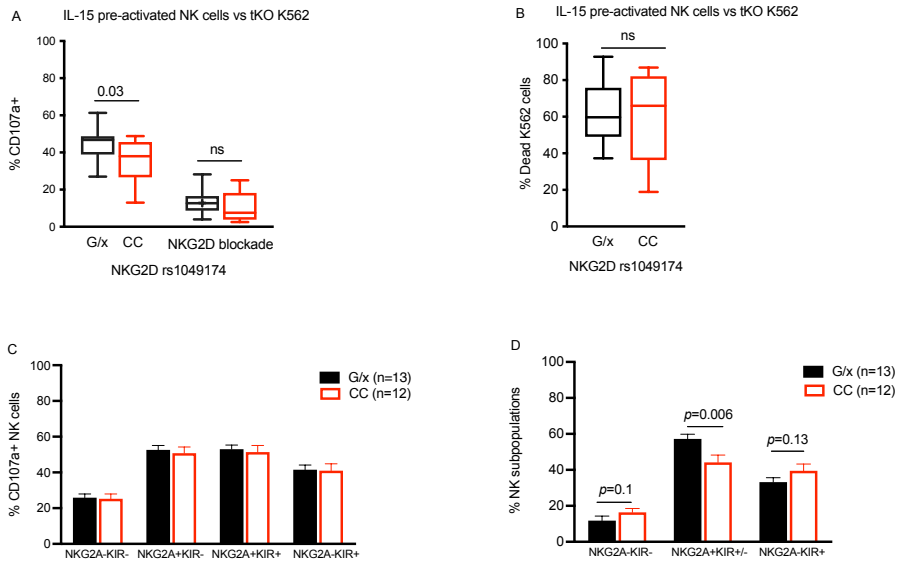

Figure 19. Measuring NK cell function according to the status of NKG2D genotypes. (A-B) Degranulation and cytotoxicity of NK cells isolated from healthy donors harboring either NKG2D G/x or CC and stimulated with IL-15 and cocultured with tKO K562. NKG2D receptor block was included in the experiment as indicate. (C) Degranulation of healthy donor NK cell subsets based on expression of NKG2A and KIR in donors carrying G/x (n=13) or CC (n=12). (D) Percentage of NK cell subsets in healthy donors based on NKG2D SNP G/x (n=13) and CC (n=12).

The finding that the enhanced degranulation response based on NKG2D rs1049174 was driven by the size of the NKG2A$^+$ subset points towards a biological and clinical role of the NKG2A SNP. Hence, to study the role of NKG2A gene variants, we genotyped healthy donors and the Re:Mission cohort of AML patients receiving HDC/IL-2 therapy for NKG2A rs1983526, located in the promoter region of *KLRC1*, the gene encoding NKG2A. We showed that healthy donors carrying NKG2A GG have a considerably larger population of NKG2A$^+$/KIR$^{-/+}$ NK cells as compared to C/x donors (Figure 20A). Moreover, the frequency of NKG2A$^+$/KIR$^{-/+}$ cells was significantly higher among AML patients with NKG2A GG after receiving HDC/IL-2 therapy when compared to C/x patients (Figure 20B). Expression of NKG2A after stimulation with IL-2 was significantly higher among healthy donors with NKG2A GG. Moreover, the same pattern was also observed in AML patients after

three weeks of IL-2-based therapy in both bright and dim CD56 NK cells.

A higher expression of NKG2A should be linked with greater inhibitory input in donors with NKG2A GG, and this increased inhibitory input may in turn lead to more responsive NKG2A$^+$ cells. However, when we performed degranulation assays towards wild type K562 cells after stimulating healthy donor NK cells with IL-2, we could not see any difference among NKG2A$^+$ cells after comparing NKG2A GG against CC. The reason for seeing no difference between the two genotypes could be due to the very high degranulation response we achieved using wild type K562. It might be worth trying out a less stimulating cell line for that purpose. On the other hand, it has been shown that educated NK cells retain higher amounts of granzyme B (145). Interestingly, after three weeks of immunotherapy, the C allele of NKG2A SNP clearly caused reduction in the load of granzyme B in CD16$^+$ CD56 dim NK cells (Figure 20C). Thus, the elevated intracellular granzyme B content in NKG2A GG patients may reflect higher educational status of NK cells among carriers of this genotype. However, when we looked at the granzyme B content in various subsets of NK cells in AML patients after immunotherapy. More interestingly, NKG2A rs1983526 (GG) donors mounted a stronger IFN-γ production when challenged with target cells. The difference between NKG2A GG and CC in terms of both granzyme B and IFN-γ was not only restricted to NKG2A$^+$ cells but was rather present in all NK cell subsets. The explanation for the latter finding could be due to that NKG2A GG leaves an epigenetic imprint that remains even after NK cells lose the expression of their NKG2A. Furthermore, a recent paper showed that the level of inhibitory SHP-1 is lower in responsive NK cells (86). Hence, another possibility is that the enhanced education of NKG2A$^+$ cells in GG patients affects the long-term expression levels of inhibitory mediators like SHP-1 in the NK cell that remains beyond the point of KIR acquisition and loss of NKG2A.

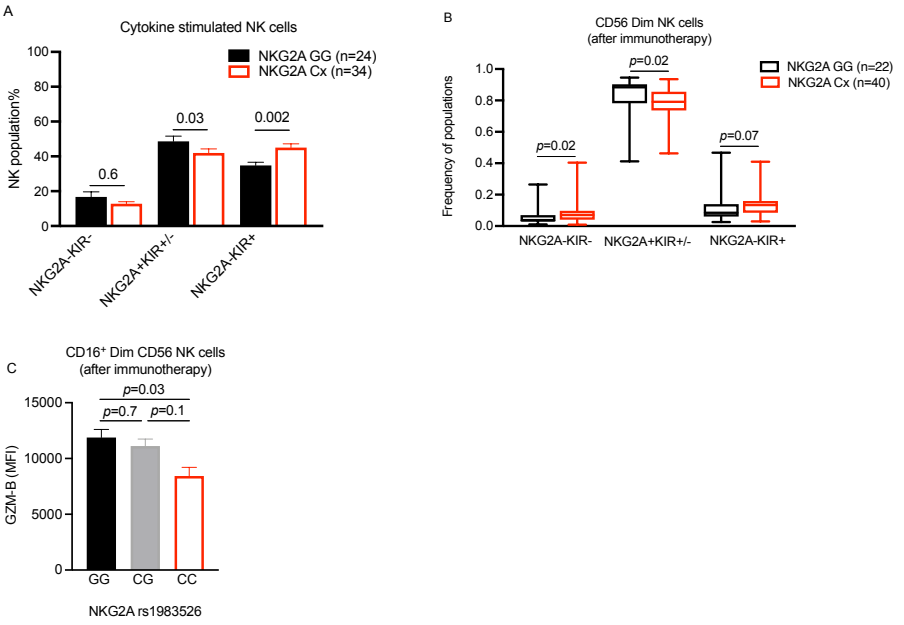

Figure 20. NKG2A and granzyme-B in NK cells based on NKG2A genotypes. (A) Distribution of various NK cell subpopulations in NKG2A GG (n=24) or C/x (n=34) donors. (B) Frequency of diverse NK cell subsets in AML patients categorized according to NKG2A GG (n=22) and C/x (n=40) after immunotherapy. (C) Influence of NKG2A rs1983526 on granzyme-B content after three weeks of immunotherapy in AML patients (GG n=21, CG n=29 and CC=8).

Subsequently, we studied to what magnitude NKC locus gene polymorphisms influence the clinical outcome of immunotherapy in AML. Patients harboring GG genotype of NKG2A rs1983526 showed to have superior leukemia-free survival, whereas a less clear association was seen in case of NKG2D rs1049174 (Figure 21A-B). Moreover, we also performed a Cox regression analysis to pinpoint the contribution of each SNP using a combinatory model involving both SNPs, where each of the risk alleles (C for both SNPs) was treated as a numerical predictor. The results sturdily implied that the impact on leukemia-free survival is associated with the NKG2A rs1983526 (p=0.004) and not the NKG2D rs1049174 (p=0.91). Furthermore, in a multi-variable analysis, which encompassed age, number of induction cycles, CMV serostatus and HLA-B rs1050458 genotype as co-variates, NKG2A rs1983526 was found to be an independent predictor of leukemia-free survival (Table 1).

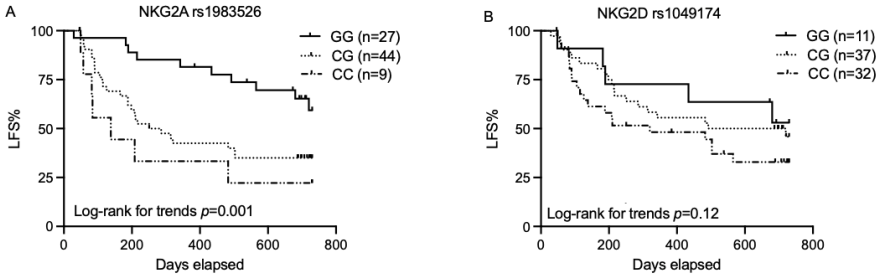

Figure 21. Correlation of NKG2A and NKG2D gene variants with clinical outcome of HDC/IL-2 in AML. (A) Leukemia-free survival of AML patients carrying NKG2A GG (n = 27), CG (n = 44) or CC (n=9) after receiving immunotherapy. (B) Leukemia-free survival of AML patients based on NKG2D GG (n = 11), CG (n = 37) or CC (n=32) after treatment with HDC/IL-2.

Table 1. Univariable and Multivariable analysis of LFS

Gene variants	*Univariate analysis*			*Multivariate analysis*		
	HR	**95%CI**	***p* value**	**HR**	**95%CI**	***p* value**
NKG2D SNP LFS	1.58	0.87–2.87	0.12	1.39	0.76-2.56	0.28
NKG2A SNP LFS	2.70	1.32-5.49	0.001	2.19	1.05-4.59	0.01

Interestingly, we also found that the NKG2A gene variants had a differential impact on clinical outcome in patients depending on the status of the dimorphism in the HLA-B -21 leader peptide. NKG2A GG rescued the outcome of AML patients carrying the unfavorable HLA-B -21 TT genotype (Figure 22A-B), while no further improvement of outcome was observed in individuals who had the favorable HLA-B -21 Mx genotype. A possible explanation to this finding is that Mx individuals "already" have well-functioning NKG2A-educated NK cells that can strike against HLA-E-low AML blasts regardless of NKG2A genotype. On the other hand, TT patients, who mainly have KIR-educated NK cells that are readily inhibited by the intact HLA class I expression on blasts, will benefit more from the NKG2A GG genotype as this allows them to also obtain highly functional NKG2A$^+$ NK cells.

In support of this hypothesis, we noted that each additional NKG2A C allele reduced the intracellular content of granzyme B in HLA-B -21 TT individuals, while the granzyme B levels were unaffected by the NKG2A genotype in HLA-B -21 M/x individuals (Figure 22C-D).

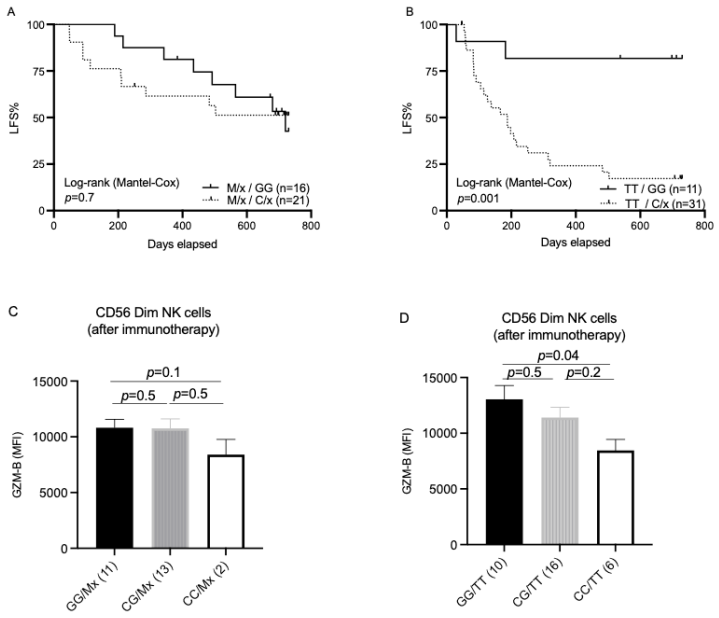

Figure 22. Effect of HLA-B -21 dimorphism and NKG2A gene variants on outcome of HDC/IL-2 in AML patients. (A) Leukemia-free survival for HLA-B -21 M/x patients divided into NKG2A GG (n=16) and C/x (n=21). Leukemia-free survival for HLA-B -21 M/x patients divided into NKG2A GG (n=11) and C/x (n=31). Influence of NKG2A SNP on Granzyme-B load in AML patients at after one cycle of IL-2 based therapy in HLA-B -21 Mx carriers (C) and HLA-B -21 TT carriers (D).

5 CONCLUDING REMARKS

The findings presented in this thesis provide new insights into the biology of NK cells, underscoring the fundamental role of the receptor ligand pair, NKG2A/CD94–HLA-E, in NK cell regulation. These studies complement the previously conducted biomarker studies of the Re:Mission trial in AML. The deep dissection of immune-related markers within this trial has demonstrated various factors impacting the clinical outcome. Although these findings need to be confirmed in future studies, the immune biomarkers may be used to prognosticate and predict the clinical outcome of patients in AML and may be used to guide therapy. The findings in this thesis may be highly relevant in a clinical setting as the NKG2A-HLA-E interaction clearly affects the clinical outcome of AML. Moreover, these findings open new avenues for adoptive NK cell transfusion.

Paper I is clearly pointing towards a role of NK cell repertoire during AML immunotherapy. NK cells that are mainly regulated by NKG2A due to a dimorphism in HLA-B -21 showed superior clinical outcome in AML after immunotherapy. This finding was mainly due to the NKG2A-HLA-E being overridable after IL-2-based therapy due to low expression of HLA-E on AML cells. All in all, findings in this paper may not be restricted to AML only but may be of significance in malignancies where the expression of HLA-E is low. Nevertheless, we genotyped a cohort of patients with ovarian cancer for the dimorphism of HLA-B -21, but we could not observe any clinical impact. Thus, it could be that this effect is driven by IL-2 based therapy or that the impact of HLA-E expression as the effect of the HLA-B -21 dimorphism might not be same in cancer settings where the expression of HLA-E is high. It would be interesting to investigate the potential impact of the HLA-B -21 dimorphism in different hematological malignancies, in the setting of immunotherapy vs no immunotherapy, transplant vs no transplant and in different oncological tumors. Furthermore, NKG2A was recently found to be another brick in the wall of the pathophysiology of COVID-19 due to its important role in implementation of adequate immune response, and it was suggested that blocking this receptor may rescue patients who have severe SARS-CoV-2 (410). The potential impact of the HLA-B -21 dimorphism in this regard would be interesting to study. Another important angle to think of NKG2A-HLA-E is from the corner

of T cells. In a recent paper by Cazzetta *et al*., there was a subset of NKG2A$^+$ Vδ2 T cells that exerted the utmost antitumor response among the entire population of Vδ2 T cells and this was found to be due to education effect of NKG2A, similar to NK cells (411). A limitation of our studies within the Re:Mission trial, is that we only had access to peripheral blood samples but not to bone marrow samples. Since the ultimate battle between NK cells and the last remaining blasts will take place in the bone marrow, samples taken from this tissue may have provided more information regarding some of the unresolved questions, especially when it comes to immune regulation and the impact of tumor microenvironment of leukemic cells.

Paper II demonstrated the clinical impact of gene variants of NKG2D rs1049174 on NKG2D expression and the outcome of immunotherapy in AML, which go in line with previous studies. Though, there is a linkage disequilibrium between NKG2D gene variants and the adjacent genes. It was difficult to purely attribute the impact on NK cell function to NKG2D. Therefore, this paper paved the road for us to dissect role of different NKC locus SNPs in detail in paper III.

Paper III Despite using a highly NKG2D-dependent model we found that NK cells from donors with a high cytotoxicity allele of NKG2D (G/x) degranulated only slightly more as compared to (CC) individuals. Furthermore, the enhanced degranulation response was due to possession of a larger population of more responsive NKG2A$^+$ NK cells. The linked NKG2A gene variant clearly skewed the NK cell repertoire towards more NKG2A-expressing NK cells. More interestingly, the GG genotype of NKG2A rescued HLA-B -21 TT patients, who have a very dismal clinical outcome. Although the granzyme B content and IFN-γ were clearly higher in NKG2A GG individuals, it was not only confined to NKG2A$^+$ cells. Therefore, I believe that the possibility of education impact is weak, but rather our data points towards imprinting effect. Nevertheless, for better understanding to see if there is a link between NKG2A gene variants and education, prospectively we are looking forward to test different cell lines with the aim to be able to find the window where we can observe if there is any impact of NKG2A genotypes on education. Another interesting question is whether there is a difference at transcription level among the genotypes of NKG2A and

transcriptional profiling at single cell level might be able to shed some light on this issue.

Indeed, nowadays a lot of interest goes into direction of transfusing adaptive like NK cells as these cells are highly differentiated and expressing self-KIRs without NKG2A. Furthermore, evidence has demonstrated the clinical benefit of using adaptive-like NK cells as a novel antitumor therapy approach. Despite notable evolvement in this field, there are many unresolved questions about adaptive NK cell therapy requiring further dissection. Though, the results in this thesis point to a direction denoting a substantial role of educated immature NKG2A$^+$ NK cells. It will be interesting to see how the field advances. It will be noteworthy to see impact of transfusing different NK cell subpopulations in different settings in future studies. For instance, in HLA-E high tumors, it may be better with adaptive NK cells expressing NKG2C. In other settings, immature cells may be better.

I believe it is important to consider the expression level of HLA-E in different tumor types to be able to tailor immunotherapies. For cancers where the HLA-E expression is too high, a combination of cell transfusion of *ex vivo* expanded hyper-responsive NKG2A$^+$ cells isolated from NKG2A GG donors and anti-NKG2A monoclonal antibody might enhance the antitumor response of NK cells by disrupting the NKG2A-HLA-E interaction. However, for tumors with low/normal levels of HLA-E expression, the transfusion of NKG2A$^+$ cells of NKG2A GG carriers without NKG2A blockade might provide an alternative approach to eradicate malignant cells rather than transfusing all NK cells.

All in all, one of the feasible employments of findings in this thesis might be in context of NK cell adoptive therapy where pools of educated and hyper-responsive NKG2A$^+$ NK cells endowed with high antitumor potential from donors harboring super functional NKG2A GG genotype might be possible for harnessing for purpose of cellular therapy in cancer.

ACKNOWLEDGEMENT

This thesis represents a milestone of almost a 5-year of work, experience, and opportunities. It has been a fantastic journey! Truth be told, there is always a finish line! Looking back to the past five years, I am filled with all kinds of feelings and memories. It would not be feasible to complete this thesis without the support and pray of many kind people surrounding me. So, it is time to gratefully acknowledge and appreciate those who have contributed to this.

First and foremost, I am extremely grateful to my supervisor, **Fredrik Bergh Thorén** for giving me the opportunity to join ThorenLab and being exposed to NK cell research. Also, thankful for your invaluable advice, continuous support, and patience during my PhD study. Your immense knowledge and plentiful experience have encouraged me a lot. I should say it is not easy to place a clinician in a research bubble at least from where I come. I still have those papers where you explained me how to prepare different dilutions of IL-2 during the very first days of joining the lab! I admit I struggled in the beginning but now coming out with flying colors. Thank you! I owe you a big hug if SARS-CoV-2 allows it!

I also would like to thank my co-supervisor, **Elin Bernson**, for your constant support, being available whenever needed despite being in Cambridge. In addition, your constructive suggestions for the papers and thesis were determinantal. You left me your thesis years back with a note saying looking forward to reading yours in some years! Here we are. Time is flying! Remember me as your first PhD student to supervise and I hope you many more.

Lovisa Vennström, as a co-supervisor someone expect to have a lot of meetings but unfortunately, we did not have that chance due to your busy clinic schedules. Nevertheless, I would like to thank you for helping with clinical studies, inputs on papers and arranging clinical samples. I am looking forward to work with you in some years when I am back to clinic. Thank you!

Enormous thanks must go to **Kristoffer Hellstrand**, for your feedback and input and your fresh eyes on different projects. By the way, playing the guitar in the Friday afternoons is very soothing and is my favorite. **Anna Martner**, thank you for all the inputs during lab meetings. Your ability to pinpoint important questions in relation to projects is fascinating.

Alexander, thank you for introducing me to FACS and sharing your experimental thoughts and ideas. I enjoyed collaborating with you. Interesting

fact, both of our theses point towards the significance of NKG2A but from different directions. **Ali,** thank you for teaching me the cell isolation techniques in the very beginning of this journey. **Linnea**, my old neighbor in the writing room, we shared some early mornings with CRISPR screen set ups. Hope these will turn out fruitful very soon. I have enjoyed discussions and exchanging ideas with you. **Hana**, thank you for spending a lot of your time trying to fix or helping with fixing the LSR. You are so kind and consistently go the extra mile for not just work but your colleagues. Grateful for taking me through Rhapsody. Looking forward to results of our latest collaborative single cell project! **Hanna**, thank you for always being available. You are a fantastic asset for TIMM lab. **Malin Nilsson**, your peaceful mind is admiring. I thank you for introducing me to TaqMan genotyping. **Ebru**, I enjoyed those little chats during incubation times about the migration regulations. **Junko,** thank you for chatting about cats. I guess Milo and Piccadilly well occupied parts of our brains. **Silvia**, one of the most wonderful human beings I have ever met. I learnt so much from you. Thank you for gathering us at yours for a dinner in Mölndal. I guess you miss Bus 25 a lot. The jam jar from your mom's farm was delicious! **Roberta**, thank you for providing the loveliest atmosphere at work and for organizing all TIMM lab orders. **Sanchari**, thank you for extending pair of hands whenever demanded. I hope our permit discussions soon will be over. **Mike**, the same birthday guy! Enjoyed all the nice discussions we had over coffee breaks. **Andreas**, thank you for being always helpful and for creating a nice environment in the lab. **Belson,** your beautiful smile and your kind heart is something will stay in the back of my mind for good. You are one of the kindest persons I have ever seen. **Mohammad Arabpour**, thank you for sharing the office and for off the topic little chats. **Anne and Chiara**, great that you both joined Thorén lab with such an enthusiasm. You are both hard-working and I am sure you both will come out with flying colors. Chats about personal number and BankID something I will not forget. **Veronika**, thank you for your positive energy. I still do not think I can pronounce the polish word Szczęsny correctly!

I would like to thank all other past and present lab members (Johan Aurelius, Anna Rydström, Beatrice Hallgren, Malin Pasanen, Britta Claesson, Annica Andersson, Anna Nilton, Meriem Echbarthi,, Theebiga Kathirkamanathan, Isabel Runnberger, Kennedy Ross, Anella Naluai, Andreas Erlandsson, Anthon Ivarsson, Hamodi, Yassir Abdulrahim, Harsha Meghadri, Jonathan Bjorkquist, Frida Svensson, Emma Stoopendahl, Nuttida Issdisai, Elin Blick, Zahra Sheybani, Mustafa Kaya and Angelica Ljunberg) of **TIMM lab.** It is their kind help and support that have made my PhD period a wonderful time. Each one of you contributed to success of this story differently. Even an early morning smile is counted! Such an incredible work environment made leaving bed in early mornings and biking to work easy!

Ka-Wei Tang and **the crew (Alan, Isak, Guojiang, Diana, Yarong, and Mariana**) thank you for making work environment nice and always saying yes for any kind of request. Looking forward to joining you in few months and exploring new adventures.

I will forever be thankful and grateful for all the teachers from primary school to medical school who have made me the person who I am today.

To my wonderful friends, I express my gratitude for your unconditional friendship, love, encouragement, support, and patience throughout these years. On top of that, the long phone chats we had between now and then were quite relieving and joyful as well.

Dr Rezan Kadir, huge thanks to you for paving the road to get me the clinical research position at Royal Free in London back in 2010 because this provided me the first taste of research which I cherish it for the rest of my life.

To my Nanakali node (**Dr Aria**, **Dr Araz** and **Dr Narin**), thank you for all the years of friendship, love and support. You are amazing human beings, and remember you are blessings for cancer patients.

Last, but not least, my family (**Daya, Baba**, **Lale**, **Srwa** and **Bryar**) had to grudgingly learn to accept my separation from them and still gave me nothing but support, day after day, both emotionally and spiritually: my love and gratitude for you can hardly be expressed in words. Daya and Baba, what you have done for both of us is beyond imagination. I am forever indebted to you for giving us countless opportunities and experiences. I love you with all my heart!

Lale, you are the blossom and the blessing of our family. You were sent to us as a gift during early years of this thesis and you brought us a lot of joy. FaceTime calls provided me energy every day. Love you, Jargoka!

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