Impact of NK cell repertoires on immunotherapy in acute myeloid leukemia

Elin Bernson

Department of Infectious Diseases
Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2017

Cover illustration: NK cell interaction with myeloid cells by Elin Bernson

elin.bernson@gu.se

ISBN 978-91-629-0338-1 (print), 978-91-629-0339-8 (electronic)
The thesis is available at: http://hdl.handle.net/2077/52867

Printed in Gothenburg, Sweden 2017
Brandfactory AB, Kållered

ABSTRACT

Natural killer (NK) cells are lymphocytes endowed with cytotoxicity against aberrant cells, including transformed and virus-infected cells. NK cell function is dictated by a fine-tuned interplay between activating and inhibitory receptors expressed on the NK cell surface. While the different activating receptors interact with unique ligands present on healthy or transformed cells, inhibitory NKG2A and killer immunoglobulin-like receptors (KIRs) invariably recognize HLA class I molecules. The purpose of this thesis was to elucidate how interactions between inhibitory NK cell receptors and HLA class I impact on anti-leukemic functions of NK cells and on NK cell-mediated termination of inflammation. In a phase IV trial, 81 AML patients received histamine dihydrochloride and low-dose interleukin-2 (HDC/IL-2) for the prevention of recurrence of leukemia after the completion of chemotherapy. The trial comprised immunophenotyping of serial blood samples along with KIR/HLA genotyping and assessment of cytomegalovirus (CMV) serostatus. Results from papers I and II imply a beneficial role of NK cell subsets that are less inhibited by HLA while prior CMV infection, which promotes the expression of additional KIRs, impacted negatively on relapse risk and survival. Additionally, a single nucleotide polymorphism in *HLA-B* that dictates NK cell inhibition to be preferentially mediated by NKG2A impacted positively on outcome in this trial (paper III). The relevance of the interplay between activating and HLA-mediated inhibitory signaling was further illustrated in a non-malignant setting in paper IV, where modulation of NK cell receptor ligands expressed by inflammatory neutrophils was associated with enhanced susceptibility to NK cell cytotoxicity. In conclusion, these studies support i) that low-grade KIR-mediated inhibition of NK cells is relevant for the benefit of relapse-preventive immunotherapy in AML and ii) that NK cells participate in the resolution of inflammation.

Keywords: Natural killer cells, Acute myeloid leukemia, Immunotherapy, Killer-cell immunoglobulin-like receptor, human leukocyte antigen class I molecules

SAMMANFATTNING PÅ SVENSKA

Immunförsvaret är uppbyggt av celler och signalmolekyler som skyddar oss från bakterier, virus och andra smittämnen. Denna avhandling handlar om NK-celler ("natural killer cells" på engelska), som är immunförsvarsceller med förmåga att döda celler som på grund av infektion, canceromvandling eller av annan anledning är farliga eller oönskade. NK-celler tycks även vara viktiga i reglering av immunsystemet. När immunsystemet har utfört sin uppgift kan NK-celler således bidra till att involverade immunceller oskadliggörs. Förmågan att eliminera oönskade immunceller är troligtvis en bidragande orsak till att NK-celler har en viktig roll vid flera typer av blodcancer, så kallade leukemier. Akut myeloisk leukemi (AML) är den vanligaste leukemin hos vuxna och en stor andel av de patienter som diagnosticeras med AML överlever inte tre år efter diagnos. En viktig anledning till detta är att många patienter drabbas av återfall i leukemi trots att de inledningsvis svarar gynnsamt på cellgiftsbehandling. Betydande ansträngningar görs därför för att utveckla behandlingar som kan förhindra återfall i AML. En strategi är att stimulera patienternas egna immunceller med så kallad immunterapi. Ett exempel är kombinationen histamindihydroklorid och interleukin-2 (HDC/IL-2) som har utvecklats vid Göteborgs universitet. HDC/IL-2 stimulerar immunceller, däribland NK-celler, så att dessa blir bättre på att känna igen och eliminera maligna celler. I tre av de fyra delarbeten som ingår i avhandlingen undersöktes prover från en internationell klinisk studie i vilken 81 AML-patienter behandlades med HDC/IL-2 med målet att klargöra vilka faktorer hos NK-celler som påverkar behandlingens effekter vid AML.

NK-celler uttrycker flera receptorer på cellytan vilka binder till ligander på målceller. NK-cellsreceptorer receptorer är antingen aktiverande eller inhiberande, och när dessa receptorer binder till sin respektive ligand kan NK-cellen antingen aktiveras, och därmed döda målcellen, eller inhiberas. NK-cellers avdödande av en främmande cell avgörs således av dess uttryck av aktiverande eller inhiberande receptorer, och vilka av dessa receptorers ligander som uttrycks på målcellens yta. De resultat som redovisas i avhandlingens tre första delarbeten talar för att en subgrupp av NK-celler, så kallade olicensierade NK-celler som inte uttrycker de inhiberande receptorerna NKG2A och KIR, kan bidra till att förhindra återfall i AML. Resultaten ger också stöd för att patienter vars NK-celler främst kontrolleras av NKG2A-inhibition har bättre överlevnadsprognos efter behandling med HDC/IL-2. Tillsammans antyder resultaten att NKG2A bidrar till en lägre grad av NK-cellsinhibering än inhibitoriska KIR, vilket

gör att NK-celler effektivare kan aktiveras av immunterapi, och därmed döda maligna celler. I delarbete II visas därtill att patienter som tidigare har genomgått cytomegalovirusinfektion saknar NK-celler som inte uttrycker inhibitoriska KIR, vilket kan förklara varför dessa patienter uppvisade hög risk för återfall. Sammanfattningsvis talar dessa fynd för att patienter med låg-gradigt inhiberade NK-celler svarar gynnsamt på immunterapi med HDC/IL-2.

För att undvika kronisk inflammation som kan leda till vävnadsskada är det angeläget att att aktiverade immunceller elimineras när ett infektiöst agens har eliminerats. I det fjärde delarbetet belystes NK-cellers roll för avslutandet av en immunreaktion. Inflammatoriska neutrofiler befanns förändra sitt utryck av ligander så att aktiverande receptorer levererade en avdödningssignal till NK-celler. Därigenom underlättades NK-cellsförmedlad elimination av de inflammatoriska celler som har utfört sin uppgift och därmed inte längre behövs. Ökad kunskap om hur en inflammationsprocess avslutas kan leda till förbättrad behandling av kronisk inflammation, t. ex. vid autoimmuna sjukdomar.

LIST OF PAPERS

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

I. **Bernson E**, Hallner A, Sander F E, Wilsson O, Werlenius O, Rydström A, Kiffin R, Brune M, Foà R, Aurelius J, Martner A, Hellstrand K, Thorén F B.
Impact of killer-immunoglobulin-like receptor and human leukocyte antigen genotypes on the efficacy of immunotherapy in acute myeloid leukemia.
Leukemia, 2017, in press

II. **Bernson E**, Hallner A, Sander F E, Nicklasson M, Nilsson M, Christenson K, Aydin E, Liljeqvist J-Å, Brune M, Foà R, Aurelius J, Martner A, Hellstrand K, Thorén F B.
Cytomegalovirus regulates autoreactive NK cells and prognosticates the outcome of IL-2-based immunotherapy in acute myeloid leukemia.
Submitted

III. Hallner A, **Bernson E**, Hussein B A, Sander F E, Brune M, Foà R, Aurelius J, Martner A, Hellstrand K, Thorén F B.
Impact of HLA-B -21 dimorphism on clinical outcome of IL-2-based immunotherapy in acute myeloid leukemia.
In manuscript

IV. **Bernson E***, Christenson K*, Pasanen M, Amirbeagi F, Bylund J, Thorén F T.
Dynamic modulation of NK cell receptor ligands in inflammatory neutrophils.
In manuscript

*Authors contributed equally

Additional publications not part of the thesis:

SI. Sander F E, Rydström A, **Bernson E**, Kiffin R, Riise R, Aurelius J, Anderson H, Brune M, Foà R, Hellstrand K, Thorén F B, Martner A.
Dynamics of cytotoxic T cell subsets during immunotherapy predicts outcome in acute myeloid leukemia.
Oncotarget 2016;7(7):7586-7596.

SII. Sander F E, Nilsson M, Rydström A, Aurelius J, Riise R, Movitz C, **Bernson E**, Kiffin R, Ståhlberg A, Brune M, Foà R, Hellstrand K, Thorén F B, Martner A.
Role of regulatory T cells in acute myeloid leukemia patients undergoing relapse-preventive immunotherapy.
Cancer Immunology Immunotherapy 2017;66(11):1473-1484.

SIII. Rydström A, Hallner A, Aurelius J, Sander F E, **Bernson E**, Kiffin R, Thorén F B, Hellstrand K, Martner A.
Dynamics of myeloid cell populations during relapse-preventive immunotherapy in acute myeloid leukemia.
Journal of Leukocyte Biology 2017;102(2):467-474.

SIV. Riise R, **Bernson E**, Aurelius J, Martner A, Pesce S, Della Chiesa M, Marcenaro E, Bylund J, Hellstrand K, Moretta L, Moretta A, Thorén FB.
TLR-stimulated neutrophils instruct NK cells to trigger dendritic cell maturation and promote adaptive T cell responses.
The Journal of Immunology 2015;195(3):1121-1128.

CONTENT

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
Allo/Auto-SCT	Allogeneic/Autologous stem cell transplantation
AML	Acute myeloid leukemia
CD	Cluster of differentiation
CMV	Cytomegalovirus
CR	Complete remission
GvHD	Graft vs host disease
GvL	Graft vs leukemia
HDC	Histamine dihydrochloride
HLA	Human leukocyte antigen
IL	Interleukin
ITAM/ITIM	Immunoreceptor tyrosine-based activation/inhibition motif
KIR	Killer-cell immunoglobulin-like receptor
LFS	Leukemia-free survival
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
OS	Overall survival
ROS	Reactive oxygen species

PREFACE

Cancer is a group of diseases characterized by uncontrolled growth of cells that may spread within the body and invade essential organs. The dysregulation of cell growth that is characteristic of cancer can emerge in almost all cell types in the body. Thus, cancer that originates from cells in the blood give rise to blood cancer, or leukemia, whereas other cancer forms may arise from e.g., lung or breast cells. Cancer treatment was greatly advanced with the introduction of chemotherapy in the early 1940's. The first chemotherapeutic drug was developed based on observations made from the use of mustard gas during World War I, where the gas was found to suppress formation of new blood cells (1). More than 40 years later, a novel form of cancer treatment emerged, based of activation of immunity for improved elimination of malignant cells. Since then, cancer immunotherapy has been used successfully in treatment of several malignancies, and received the "breakthrough of the year"-designation in *Science* 2013 (2).

A common form of leukemia, acute myeloid leukemia (AML), is characterized by clonal expansion of malignant myeloid cells in bone marrow and blood. As the malignant cells themselves belong to the immune system, it is conceivable that immunotherapeutic approaches may be successfully targeting the disease. Immunotherapies that aim to activate the immunity can be designed either to stimulate the immune cells, or to remove breaks that inhibit immune cells. A form of AML immunotherapy combines these two properties by using the cytokine interleukin-2 (IL-2) to activate lymphocytes, together with histamine dihydrochloride (HDC) that reduces the formation of immunosuppressive reactive oxygen species. Thereby, the HDC/IL-2 treatment regimen may stimulate natural killer (NK) cells to exert anti-leukemic activity. With modern techniques allowing for more detailed studies of NK cell biology, it has become evident that the NK cell population is highly heterogeneous. A major aim of this thesis was to define NK cell repertoires that are of importance during immunotherapy in AML. The results presented in the thesis imply that NK cell repertoires characterized by low-grade inhibition, as determined by ligation of their surface receptors, are significant effector cells in HDC/IL-2 immunotherapy.

THE HUMAN IMMUNE SYSTEM

The human immune system is made up by an array of effector cells and molecules that protect us from infectious agents and aberrant cells. All immune cells originate from a common hematopoietic stem cell (HSC). In a process known as hematopoiesis, cells proliferate, mature and differentiate to form the cellular part of the immune system, as depicted in figure 1.

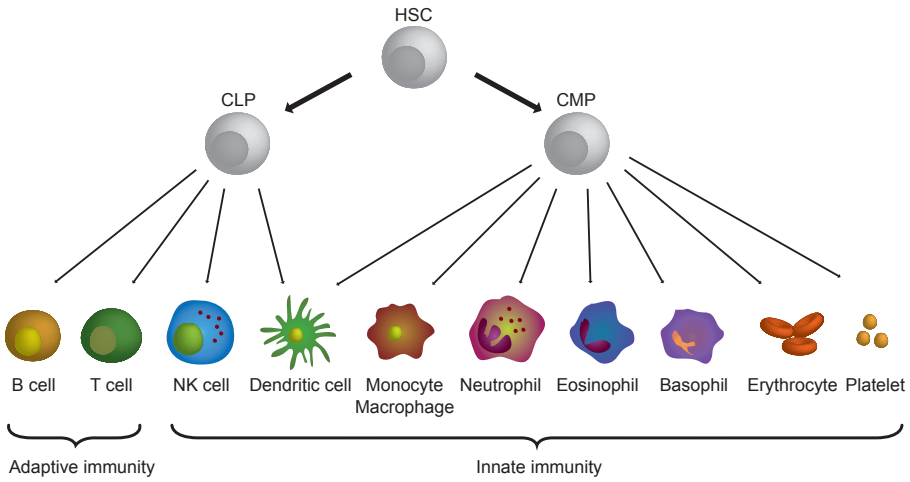

Figure 1. **Hematopoiesis**. Hematopoietic cells originate from a hematopoietic stem cell (HSC) that differentiates to either common lymphoid progenitor (CLP) cells or common myeloid progenitor (CMP) cells. Further differentiation and proliferation will generate cells that form the cellular part of the innate and adaptive immune system.

The immune system is commonly divided into two principal entities known as innate and adaptive immunity. Innate immunity is the evolutionarily oldest system and is considered responsible for the initial response occurring immediately after invasion of an infectious agent. Innate immunity comprises phagocytic cells including macrophages and neutrophils, antigen-presenting cells (dendritic cells and macrophages) along with innate lymphoid cells (ILCs including natural killer cells, NK cells). Upon infection, inflammatory signals alert innate immune cells to migrate to the site of inflammation

where they form a first line of defense. The innate system signals to dendritic cells (DCs) in peripheral tissues that continuously sample the extracellular environment. Activated DCs migrate to lymph nodes where they shape an adaptive immune response unique to the infectious agent. Antigen presentation by DCs thus form the basis for clonal expansion of antigen-specific T cells that in turn produce cytokines, which promote maturation of B cells that produce antigen-specific antibodies. Together, these processes lead to a highly specific immune response and commonly to the eradication of pathogens and infected cells. When pathogens are eliminated, the majority of the immune cells will undergo apoptosis to resolve inflammation at the site of infection, but a minority of the adaptive cells remain to form a pool of memory T and B cells that respond vigorously upon re-infection. Today, the strict division between innate and adaptive immunity is smudged, as certain innate cells (both macrophages and NK cells) have been proposed to develop immunological memory, as first reported in mice and later also in humans (3-5).

NATURAL KILLER CELLS

NK cells are innate lymphocytes with an inherent capacity to kill infected or transformed cells, which is achieved by the release of cytotoxic granules contained in the NK cell cytoplasm. In contrast to T cells, NK cells may target aberrant cells without prior sensitization; instead, the engagement of activating and inhibitory NK cell receptors, with cognate ligands expressed by a target cell, determines whether or not NK cells release cytotoxic granules to eliminate an encountering cell. Thus, if the target cell expresses ligands that bind to activating NK cell receptors, a lytic synapse is formed between the NK cell and the target cell followed by the release of lytic granules into the synaptic cleft (6). A schematic description of NK cell receptors, their intracellular downstream signaling pathways and the signal integration that may, or may not, lead to a cytotoxic response, is provided in chapter I.

Besides their cytotoxic capacity, NK cells produce cytokines and chemokines and thereby influence immune responses. By virtue of their capacity to destroy foreign cells and their role in immune regulation, NK cells have been ascribed a role in defense against viruses and malignant cells. Several studies have thus correlated a congenital deficiency in NK cell function or development with increased susceptibility to severe virus infections (7-10). Moreover, NK cells are implicated in surveillance of a wide range of malignant cells, including leukemic cells (11-13).

NK cells are phenotypically heterogeneous, where immature NK cells carry the $CD56^{bright}$ $CD16^{-}$ phenotype, while more mature NK cells down-modulate CD56 and upregulate CD16 ($CD56^{dim}$ $CD16^{+}$). The NK cell phenotypes further differ in their

expression of inhibitory and activating receptors as well as in their responsiveness to activation. Notably, intrinsic factors, i.e., genotype, and extrinsic factors, such as a viral infection, will shape an individual's NK cell repertoire. The shaping of the NK cell repertoire, along with attempts to define the role of NK cells and their receptors for the course of human leukemia, is a main focus of this thesis.

THE "MISSING SELF" THEORY

NK cells were first discovered as a background noise in cytotoxicity assays of lymphocyte activity against tumor cells. This "noise" was caused by NK cells that, unexpectedly and in contrast to T cells, killed tumor cells without prior sensitization. The first reports on the biology of NK cells were published in 1975 (14, 15). In the early 1980's, Klas Kärre forwarded the "missing self" hypothesis in his doctoral thesis (16). The hypothesis describes how the reduced expression or absence of "self" major histocompatibility complex (MHC) class I molecules, rather than the presence of a triggering agent, leads to NK cell recognition and elimination of aberrant cells. The proposed mechanism was further evaluated and established in subsequent work by his group at the Karolinska Institute. With the "missing self" theory, it was explained why NK cells, in stark contrast to T cells, could kill tumor cells lacking expression of MHC class I. Accordingly, mutated tumor cell lines lacking MHC class I were sensitive to NK cell mediated lysis *in vitro* and were rejected in an *in vivo* setting (17, 20).

Despite that the "missing self" hypothesis seemed to fit with former and new experimental findings, the identification of inhibitory receptors binding to MHC class I molecules in the 90's by Yokoyama and co-workers (mouse) and Moretta and co-workers (human) provided significant further support to the theory (21-23). However, the "missing self" theory did not account for the fact that a complete lack of ligands is sometimes not sufficient to promote NK cell killing of a target cell. In the last years of the 90's, activating receptors expressed on resting or stimulated NK cells were discovered (24-26). The identification of activating and inhibitory receptors and the subsequent intracellular signaling leading to NK cell activation revealed how NK cells may distinguish between healthy and diseased cells and how they, depending on what receptors are engaged, can kill infected cells or tumor cells. Until recently, the NK cell response has been thought of as a balance between activating and inhibitory signals. However, this view is changing, as it is now assumed that the NK cell response involves a complex integration of signals from several receptors at multiple stages of the signaling process (27), as described in more detail below.

INHIBITORY NK CELL RECEPTORS

In the 90's, Moretta and co-workers identified the p58 receptors, recognized by the antibody clones GL183 (28) and EB6 (29) (later called killer-cell immunoglobulin-like receptor (KIR) -2DL3 and -2DL1/KIR2DS1, respectively) to induce inhibitory signaling through binding to specific human MHC class I molecules (human leukocyte antigen; HLA class I) on target cells (30). Later KIR3DL1 was identified as a receptor recognizing certain HLA-B alleles (31). Twenty-five years and extensive research later, *KIR* genes encoding 14 NK cell surface receptors have been identified; seven of them with long intracytoplasmic tails transducing inhibitory signaling via two immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and seven with short cytoplasmic tails allowing these receptors to associate with DAP12, a key accessory protein that conveys activating signals via immunoreceptor tyrosine-based activating motifs (ITAMs) (32). One exception is KIR2DL4 that, in addition to carrying an ITIM within the long cytoplasmic tail, also associates with an ITAM-bearing protein and therefore transduces both activating and inhibitory signals (33). For a detailed list of KIRs expressed on human NK cells, their corresponding ligands and whether they convey inhibitory or activating signaling, see figure 2.

KIR – HLA recognition

Among the major inhibitory KIRs (iKIRs), KIR2DL1 recognizes the C2 group of HLA-C whereas KIR2DL2/L3 recognizes HLA-C1, and to some extent certain HLA-C2 alleles although with lower affinity (34). C1 and C2 of HLA-C are distinguished either by a lysine (C2) or arginine (C1) at position 80 in the HLA-C molecule (34). Experiments designed to clarify the degree of NK cell activation in individuals homozygous for either C1 or C2 indicate that the HLA-C2 – KIR2DL1 interaction delivers a stronger inhibitory signal than HLA-C1 – KIR2DL2/L3 ligation (35), resulting from KIR2DL2/L3 – C1 interactions being more peptide-selective than KIR2DL1 – C2 interactions (36). HLA-C2 also serves as a ligand to the activating KIR2SD1, although with a weaker binding affinity than KIR2DL1 (37). KIR3DL1 recognizes the Bw4 epitope present in certain alleles of HLA-A and HLA-B with an arginine at position 83 (34). Moreover, due to a dimorphism at position 80, HLA-Bw4 binds to KIR3DL1 with different affinity; presence of an isoleucine (80Ile) at position 80 allows for a stronger interaction than threonine (80Thr) (38). Interestingly, a correlation between the stronger HLA-C2 and HLA-Bw4-80Ile has been identified, and correspondingly HLA-C1 associates with the weaker HLA-Bw4-80Thr (39). In addition to receptor-ligand recognition, the KIR/HLA binding affinity and subsequent inhibition is influenced by the peptide presented by the HLA class I molecule (40).

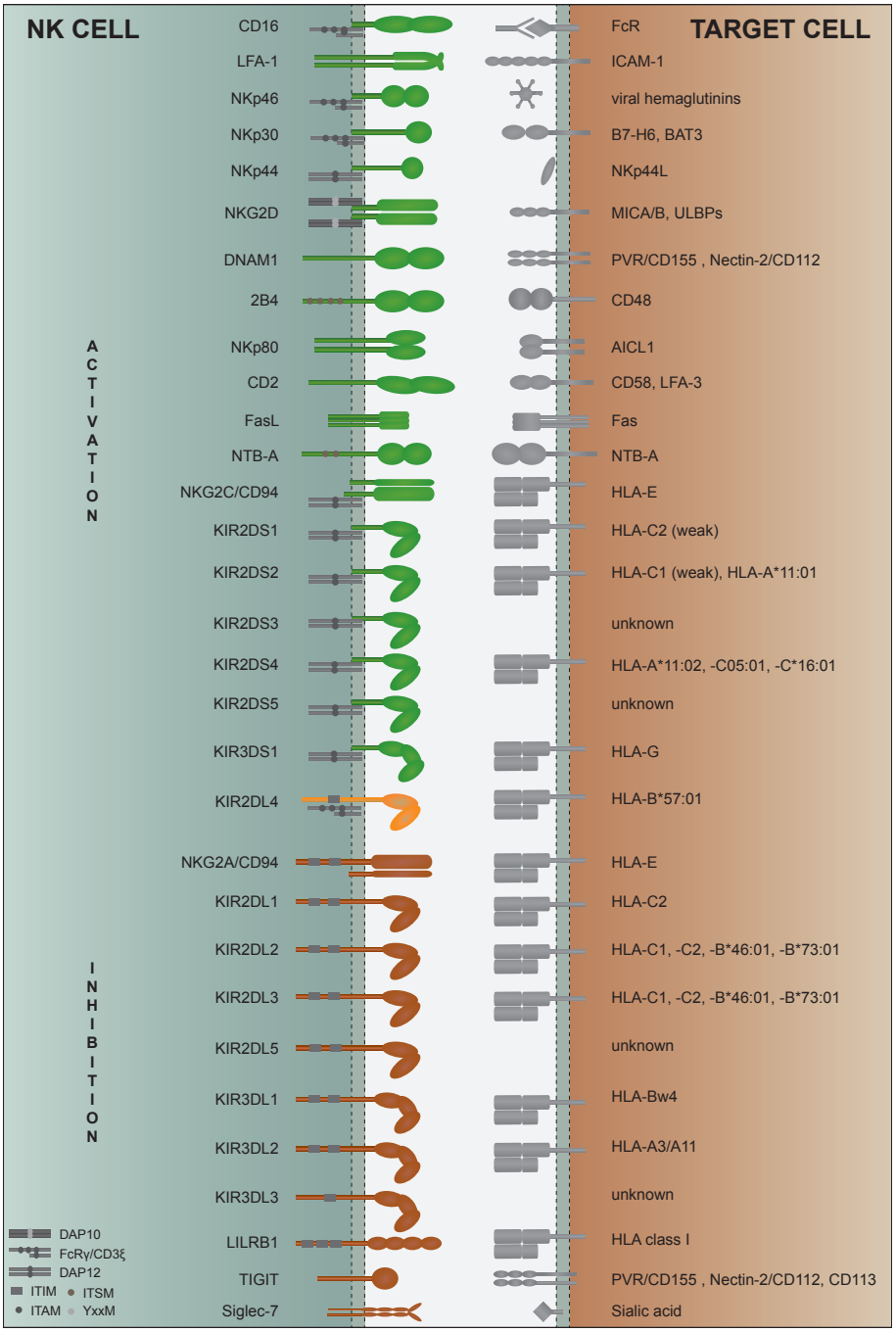

Figure 2. NK cell receptors and their ligands. NK cells express activating (green) and inhibitory (red) receptors that bind to ligands on target cells.

KIR haplotypes

The *KIR* locus encodes 15 genes (of which two are pseudogenes) and is highly polymorphic, polygenic and complex, generating a substantial number of haplotypes. Broadly, *KIR* gene combinations comprise two haplotypes; groups A and B. The group A haplotype is non-variable and encodes mainly inhibitory KIRs in addition to the framework *KIR* genes. Group B haplotypes are more variable in terms of number and combinations of *KIR* genes, and encode at least one activating KIR. The group A and B haplotypes also segregate at the allele level (41). There are between 16-158 alleles identified for each *KIR* gene, and while most alleles encode KIR proteins expressed on the NK cell surface, some intact alleles are either non-transcribed or not functionally transported to the cell surface (42). Moreover, allele variations in *KIR* genes may affect their ligand affinity (43).

Located closely to the *KIR* genes in the leukocyte receptor complex (LRC) on chromosome 19 is the gene encoding the leukocyte immunoglobulin-like receptor B1 (LILRB1; also known as ILT2, CD85J, LIR1 or MIR7), which is expressed by a subset of NK cells and more broadly recognizes several HLA class I molecules (44, 45). Another receptor conveying inhibitory signaling is TIGIT (T-cell Ig and ITIM domain), which recognizes PVR (CD155) on target cells (46). Human inhibitory NK cell receptors additionally include the NKG2A/CD94 heterodimer (henceforth referred to as NKG2A) that ligates the non-classical HLA-E molecule (47-49). The signaling via NKG2A and HLA-E is partly determined by a genetic dimorphism, as described in next section.

-21 HLA-B dimorphism

HLA-E requires a peptide derived from the leader sequence of classical HLA class I molecules in order to properly fold and present at the cell surface. HLA-A and -C molecules constantly express such a peptide. By contrast, due to a dimorphism at position -21, only a fraction of the HLA-B alleles present a peptide allowing for HLA-E expression. The disparity is caused by the presence of either a methionine or a threonine at position -21 (-21M or -21T, respectively), where only peptides from classical HLA molecules with a -21M allow for proper folding of HLA-E (48). Individuals with a T/T genotype will hence not have any HLA-E expressed on their surface that has been mediated by their HLA-B allele (see fig. 3).

Despite that HLA-E expression is dependent on peptides from all three alleles of the classical HLA molecules (-A, -B and –C), individuals with a -21M HLA-B display higher surface staining of HLA-E (50). Thus, the presence of at least one -21M, i.e., an M/x (either M/M or M/T) genotype, is sufficient to promote HLA-E expression (50).

Interestingly, there is a striking correlation between -21T at HLA-B and the stronger HLA-C2 haplotypes. Moreover, the majority of $Bw4^+$ HLA-B alleles have -21T in HLA-B (50, 51). Together, this implies that individuals carrying the T/T genotype encoding for lower HLA-E expression have genes that encode for HLA molecules that bind iKIRs with higher affinity. Thereby, depending of the presence of either T/T or M/x, inhibitory signaling in is shifted to be either KIR-dependent in T/T carriers, or NKG2A-dependent in M/x carriers (50).

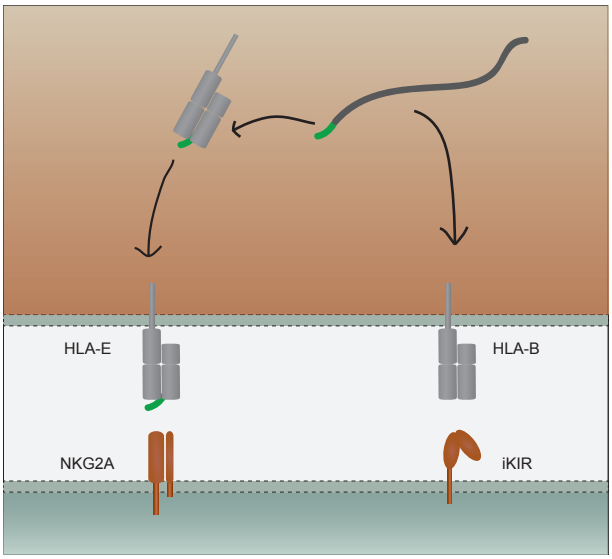

Figure 3. HLA-E expression. Surface expression of HLA-E requires a peptide derived from classical HLA molecules. A methionine at position -21 allows classical HLA to present such a peptide (green).

Inhibitory signaling

Similar to iKIRs the intracytoplasmic tail of NKG2A contains two immunoreceptor tyrosine-based inhibition motifs (ITIMs) (52). Upon ligation of inhibitory receptors, ITIMs are phosphorylated by Src family tyrosine kinases. The phosphorylated sites of the ITIMs then bind to Src homology 2 (SH2) domains of the protein tyrosine phosphatases SHP-1 and SHP-2 (53). This rapidly occurring process is independent of events required for activation, including adhesion through integrins and actin polymerization, and leads to the accumulation of iKIRs and NKG2A and microcluster formation within the synapse (52, 54). In fact, NK cell inhibitory synapses may apparently be formed independently of activating synapse formation (6). As reviewed further below, the signal transduced by iKIRs and NKG2A ligation acts by blocking activating signaling at multiple stages of the signaling cascade, from actin-dependent recruitment of activating receptors and Ca^{2+} flux to release of cytokines and cytotoxicity (6, 27). Inhibitory signaling, occurring when NK cells meet bystander cells with intact HLA class I expression, allows for recognition and avoidance of "normal" healthy cells.

NK cell licensing

Inhibitory signaling has two principal properties; it inhibits activating signals, but it also renders NK cells to be more responsive to activating signals (52), which is a central paradox addressed in this thesis. Ligation of iKIRs and NKG2A sets the functionality of NK cells in a process termed licensing, or education, which will be described in more detail in below (55, 56). More specifically, in order for an NK cell to be licensed and functional, it is generally believed that it must express at least one inhibitory receptor (iKIR or NKG2A) that recognizes self-HLA class I molecules. NK cells that lack expression of inhibitory receptors that bind to self-HLA molecules will be hyporesponsive, or unlicensed. In addition, the strength of the inhibitory signaling sets the functional responsiveness of an NK cell; the stronger inhibitory signaling an NK cell is exposed to at steady state, the more responsive will this NK cell be to activation stimuli (57).

HLA class I and KIRs segregate genetically – KIRs are encoded on chromosome 19 and HLA class I on chromosome 6 (58, 59) – and they are hence inherited independently. Consequently, a large fraction of individuals will have discordance between their set of KIR and HLA genes leading to a KIR lacking its corresponding HLA ligand (referred to as a "missing ligand" genotype). The maturation status of an NK cell determines what inhibitory receptors are expressed. Immature $CD56^{bright}$ NK cells mainly express NKG2A, while more mature $CD56^{dim}$ NK cells gradually lose NKG2A and acquire KIRs in a, at least partly, stochastic fashion (60). This means that in individuals with a missing ligand genotype, there will be a portion of NK cells expressing only KIRs that lack their cognate HLA (hereafter termed a non-self KIRs, or NS-KIRs). According to the NK cell dogma, an $NKG2A^-$ NK cell that only expresses NS-iKIR(s) will not receive inhibitory input, and hence is hyporesponsive. These cells, termed unlicensed or uneducated, remain non-responsive in terms of cytotoxicity until they upregulate expression of a self-KIR (sKIR) or NKG2A to their surface (fig. 4).

Despite being hyporesponsive against potential target cells, unlicensed NK cells respond to cytokine stimuli, differentiate and proliferate to a similar extent as licensed NK cells (60). Licensing is believed to protect from NK cell autoreactivity, as NK cells not sensing "self" display limited capacity to respond to stimuli and thereby do not attack self-cells. The mechanism by which inhibitory signaling "licenses" or "educates" NK cells remains to be elucidated, but recent results suggest that licensed NK cells harbor higher granular load, i.e., have both larger and more granzyme B-dense granules, compared with unlicensed NK cells (61). The difference is reportedly independent of transcription; instead, a model was proposed comprising the engagement of inhibitory receptors on licensed NK cells that hinders NK cells from constant leakage of granules

upon binding to activating receptors. Licensed NK cells may thereby accumulate granules and have a stored "charge" of granular load, while unlicensed NK cells constantly leak and thus do not build up any granular load, thus remaining hyporesponsive. According to the proposed model, the granules do not only function as a repository of cytotoxic mediators, but also make up a signaling hub due to storage of Ca^{2+}. Thus unlicensed NK cells, with limited granular load, will not only be poorly cytotoxic, but also less responsive to effector signaling. The model proposes that stimulation of unlicensed NK cells resulting in increased granular load may render these cells responsive and able to kill target cells. Accordingly, *in vitro* cytokine stimulation of unlicensed NK cells can result in enhanced responsiveness to activating stimuli (55, 56, 62).

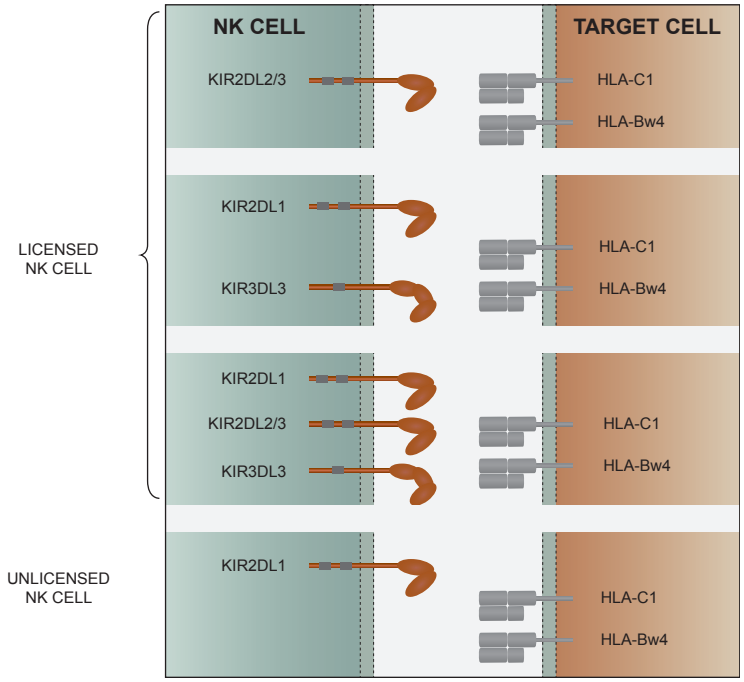

Figure 4. NK cell licensing. Expression of inhibitory KIRs that recognize cognate HLA class I molecules allows NK cell responsiveness, or licensing. Thereby, NK cells that express only inhibitory KIRs that lack cognate HLA molecules will be hyporesponsive, or unlicensed.

ACTIVATING NK CELL RECEPTORS

NK cells express a battery of activating receptors, which upon ligation transmit activating intracellular signals that may entail NK cell-mediated killing of target cells. The major activating receptors include the natural cytotoxicity receptors (NCRs) NKp30 and NKp46, expressed on resting and activated NK cells (24, 63), and NKp44, which is induced upon NK cell stimulation (25). B7-H6 (64) and BAT-3 (65) have been identified as ligands to NKp30, and NKp44L (an isoform of the mixed-lineage leukemia-5, MLL5, protein) is a cellular ligand to NKp44 (66). Viral ligands to the NCRs have been identified (67-70), but no endogenous cellular ligand to NKp46 has been discovered to date. Another major activating receptor is NKG2D (71), which is expressed as a homodimer and recognizes the stress-induced ligands MHC class I chain-related (MIC) A and MICB (26, 72), and UL16-binding proteins (ULBPs) (73). The activating Fc receptor Fcγ RIIIa (CD16) is mainly expressed on $CD56^{dim}$ NK cells and binds to the Fcγ part of IgG to mediate antibody-dependent cellular cytotoxicity (ADCC) towards cells coated with antibodies (e.g., opsonized cells) (74). Similar to CD16, NKG2C is also expressed on more mature NK cells, and generate activating signaling upon binding to HLA-E (74). In addition, several co-receptors act in concert with the major activating receptors for NK cell activation including NKp80, DNAM-1, CD2 and the SLAM family members 2B4 and NTB-A (27, 74, 75). The activating KIRs (aKIRs) include KIR2DS1, KIR2DS2, KIR2DS4, KIR2DS5 and KIR3DS1 (76, 77). aKIRs are not as well characterized as their inhibitory counterparts, but it has been shown that both classical and non-classical HLA molecules function as ligands to aKIRs; details of specific ligands for each activating receptor are depicted in figure 2.

Synapse formation

The procedure of NK cell killing can be divided into three stages: the initiation, effector and termination stage (6). The initiation stage involves cell-cell adhesion and formation of the initial synapse, in which the major receptor signaling events take place (78). Immune synapse formation involves LFA-1 (that binds to ICAM-1), and MAC1, both of which accumulate in the synapse upon initiation (79, 80). This initial adhesion results in a first outside-in activating signal, essential for tight conjugation to target cells (81) and lytic granule polarization (82). LFA-1/ICAM-1 ligation also leads to recruitment of co-activating receptors. This promotes an inside-out signaling that promotes an open conformation of LFA-1 to further enhance signaling within the synapse (82, 83).

However, LFA-1 engagement alone is insufficient for a cytotoxic response, and requires additional recruitment of activating receptors to the synapse. These are recruited to, and clustered in, the synapse in an actin-dependent process (6). When the recruited activating receptors bind to a cognate ligand on the target cell, intracellular signaling

may be mediated through several pathways, and engagement of one activating receptor can result in multiple intracellular signals, as reviewed below.

Signaling downstream of activating receptors

Activating receptors transduce signals intracellularly via tyrosine-based phosphorylation. Some activating NK cell receptors have only short cytoplasmic tails and are therefore dependent on associated molecules for signal transduction. CD16, NKp46 and NKp30 associate with FcRγ and/or CD3ζ, whereas NKp44, NKG2C/CD94, KIR2DS1-5 and KIR3DS1 associate with DAP12, and NKG2D with DAP10. FcRγ, CD3ζ and DAP12 all comprise intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) that harbor two tyrosine residues that, when phosphorylated by Src kinase family members, bind to SH2 domains of Syk or ZAP70 (27, 71), which activate multiple downstream signaling mediators (including the proteins LAT, PLCγ1 and PLCγ2, Vav2 and Vav3, to mention some; see below). The DAP10 molecule carries a tyrosine-based motif different from ITAM that either signals via PI3K – CrkL (a member of the Crk family proteins) via Grb2/Vav1/PLCγ2, or Vav1 activation via SLP-76 phosphorylation (27). Finally, the SLAM family members hold immunoreceptor tyrosine-based switch motifs (ITSMs) in their cytoplasmic tails that signal via SAP and EAT-2 adaptors. In summary, downstream activating signals involve a large number of signaling molecules, where Vav, PLCγ, Crk, Syk and ZAP70 are some of the proteins involved.

The signaling pathways result in varying responses dependent on the downstream signaling endpoints. ERK activation is a downstream signal shared by several signaling pathways. Activation of ERK regulates the transcription factors NF-κB that controls continuous NK cell cytotoxicity as well as cytokine production, and GSK-3β, a signaling molecule negatively regulating NK cell effector functions (84). Pathways leading to ERK activation, involving the signaling molecule Vav1, are therefore critical in regulating actin skeletal rearrangements and clustering of activating receptors in lipid rafts (85). PLCγ, another protein critical for NK cell activation, is involved in the generation of IP_3 that induces Ca^{2+} release from intracellular stores in the endoplasmatic reticulum, and also regultes Ca^{2+} influx from the extracellular environment (86). Ca^{2+} flux is essential for mediating exocytosis of cytotoxic granules (87). Together, the activating pathways translate into NK cell cytotoxicity and cytokine release (88), taking place in the effector stage.

Delivery of lytic granules in the effector stage

In the effector stage of NK cell cytotoxicity, F-actin reorganizes and preformed lytic granules polarize to and converge within the immunological synapse, which is carried out through the movement of the microtubule organizing center (MTOC) and dynein-dependent movement of granules along the microtubules (27, 89). F-actin reorganization is dependent on the adaptor protein Crk, which downstream contributes to actin reorganization and regulation of inside-out signaling through LFA-1 (90). Granule polarization is followed by lytic granule fusion with the plasma membrane and release of granule content into the synaptic cleft between the NK cell and the target cell (6, 91). The convergence and release of granules within the synapse specifically directs the cytotoxicity to a target cell and hinders damage to bystander cells (89).

The lytic granules are small vesicles containing proteins and proteoglycans, amongst them the cytotoxic protein perforin that perforates the cell membrane of the target cell (92). This allows passage of the serine protease granzyme B into the target cell, which induces apoptosis through processing of a wide range of key substrates (91, 93, 94). In addition to granzyme B and perforin, cytotoxic granules contain membrane-bound FasL- or TRAIL that are expressed on the NK cell surface upon degranulation (86). Ligation of FasL to the Fas receptor induces target cell-apoptosis in a caspase-dependent pathway. Similarly, TRAIL binding to receptors containing a "death domain" mediates target cell apoptosis in a pathway resembling that induced by FasL engagement (95). Transmembrane LAMP1 (also known as CD107a and commonly used as a marker for NK cell degranulation) expressed on the granules is essential for their transportation and release (96).

Activation signaling also induces secretion of cytokines and chemokines, the most prominent being interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Chemokine and cytokine secretion are differently controlled; chemokines are secreted promptly after activation and can be induced by single engagement of an activating receptor, whereas IFN-γ secretion requires simultaneous activation of different receptors and occurs at a later stage (97).

The termination stage is characterized by relative inactivity, detachment of the NK cell from the target cell and downregulation of some activating receptors, including NKG2D and CD16 (6). Downregulation of CD16 after target cell interaction is a cause of shedding by metalloproteinases (98); for other receptors it is unclear whether NK cells downregulate their receptors in a similar manner as T cells, i.e., by internalization, or if they utilize other mechanisms (6).

Co-engagement of activating receptors

In order for an NK cell to exert cytotoxicity towards a target cell, polarization and degranulation of lytic granules must take place. There is no single NK cell receptor that upon ligation induces both of these events; instead these processes are controlled by separate signals (99). On a similar note, even CD16 requires LFA-1 as co-factor for successful target cell killing (27). Also other activating receptors, including NKp46 and NKG2D that are considered as major activating receptors, need to be activated simultaneously with another activating or co-activating receptor in order to induce degranulation and killing of a target cell (27, 83, 100). This need of synergy between several activating receptor signals is believed to have developed as a control element, where several receptors with different signaling properties synergize in NK cell activation (27).

INTEGRATION OF INHIBITORY AND ACTIVATING SIGNALING

The activation status of an NK cell has long been thought of as a balance between activating and inhibitory signaling, with the strongest signal "winning" the competition. It is however becoming evident that this model does not correctly describe the integration of inhibitory and activating signaling. Instead, interactions between NKG2A or KIR ligation and their respective ligands likely inhibit activating signals already at an early stage of the synapse formation, by blocking both outside-in signaling mediated by LFA-1 and inside-out signaling occurring when co-activating receptors act in concert with LFA-1 (82, 83). Blocking of actin-dependent processes, including recruitment of activating receptors, receptor tyrosine phosphorylation and myosin II recruitment, the later required for degranulation (52), can be the result of inhibitory signals that inactivate the adaptor protein Crk (90). Moreover, engagement of inhibitory receptors to MHC class I ligands with the subsequent recruitment and phosphorylation of SHP-1 results in dephosphorylation of Vav1 (101), which is essential in downstream signaling of activating receptors. Inhibitory signaling also prevents NK cell activation by inactivating PLCγ and LAT, where the latter is critical for PLCγ recruitment to the immunological synapse (102). Mechanisms by which inhibitory signaling might disrupt activating signals are summarized in figure 5.

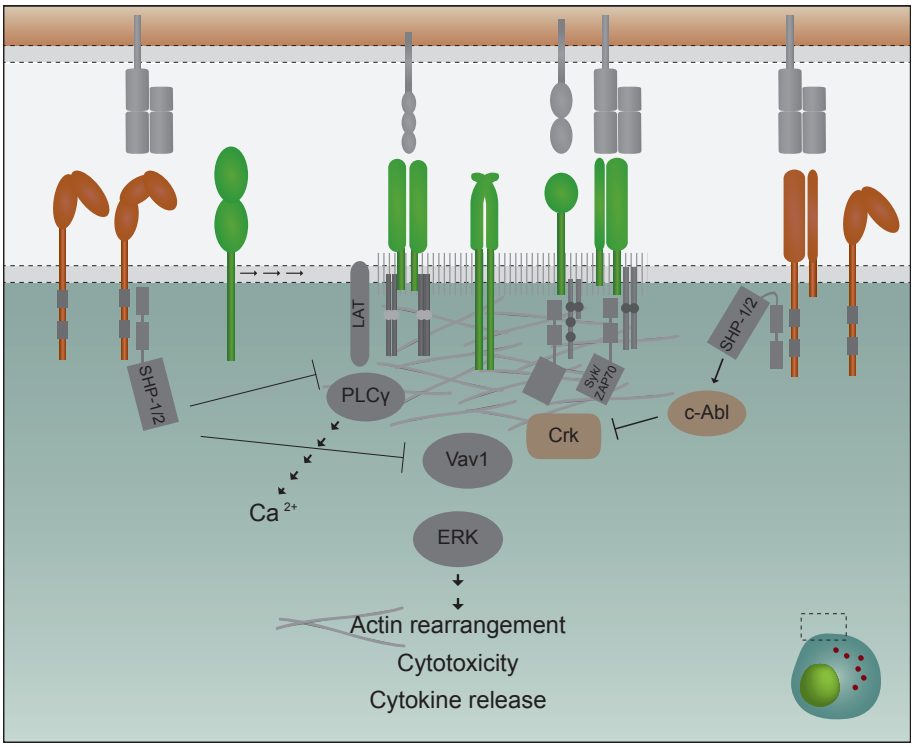

Figure 5. NK cell integration of receptor signals. Ligation of activating (green) and inhibitory (red) receptors present on the surface of NK cells results in intracellular signaling cascades. A fine-tuned interplay between these signals determines the activation status of the NK cell.

Formation of inhibitory microclusters occurs within seconds upon ligation of KIR to a cognate HLA, which induces rearrangement of the actin cytoskeleton, suppresses formation of new activating receptor microclusters and blocks initiation of Ca^{2+} flux upon simultaneous ligation of NKG2D (103). Moreover, ITIM-mediated signaling is required for retraction of an NK cell from a target cell. Collectively, these data imply that inhibition occurs upstream of actin-dependent signals, and prevents activating signaling at an early stage before they can take action (27). With these seemingly robust inhibitory mechanisms, one can ask how NK cells are able to mediate cytotoxicity *in vivo*. However, NK cells are implicated significant effector cells in several malignancies, including acute myeloid leukemia where the leukemic blasts mostly express HLA class I (**Paper I**, 12, 13, 104), suggesting that NK cells can kill also in the presence of inhibitory receptor ligands and that the inhibitory signaling may be overridden by activating signals in certain settings.

Although NK cell activation is controlled through the inability of a single activating receptor to induce NK cell-mediated killing without co-activating receptors, the abundance of activating receptors potentially involved in NK cell activation provides multiple opportunities for an NK cell to be activated. NK cells utilize combinations of several signaling pathways to induce cytotoxic responses (105), and might therefore be capable to evade inhibition by taking alternative "routes" for delivering the activation signal downstream. Similarly, the presence of multiple regulatory pathways coupled to inhibitory receptors imply that the ability of inhibitory signaling to interrupt NK cell activation depends on cooperation between different inhibitory pathways (102). For example, NKG2A is only able to inhibit inside-out signaling when co-engaged with single activating receptors, but not when simultaneously co-engaged with NKG2D and 2B4 (83). This suggests that the ability of NKG2A to inhibit an activating signal might depend on the strength of the activating signal. Indeed, there are examples where the presence of inhibitory ligands is not sufficient to inhibit NK cell cytotoxicity (106). In another report, Abeyweera *et al.* observed that KIR2DL2 ligation was insufficient to impair an already ongoing Ca^{2+} response (103). Even though co-clustered inhibitory receptors may block the activation locally by interrupting an activating signal upstream the initiation of Ca^{2+} flux (27), inhibitory signaling may not be sufficient to target the Ca^{2+} response.

NK CELL-MEDIATED IMMUNE REGULATION

NK cells respond to a wide range of stimuli and also produce cytokines, chemokines and growth factors that regulate other aspects of immunity (107). The interplay between NK cells and immune cells takes place within several organs; in addition to peripheral blood and bone marrow, NK cells also reside in secondary lymphoid tissues. Immature $CD56^{bright}$ NK cells are mainly found in lymph nodes and tonsils, whereas $CD56^{dim}$ cells represent the dominant pool of NK cells in the spleen (108, 109). Moreover, tissue-resident NK cells are found in the liver, lung, uterus, intestine and skin. Despite the intriguing biology of tissue-resident NK cells along with the purported role of these cells in health and disease (109), including the importance of uterus NK cells during pregnancy (110), an in-depth discussion of tissue-resident NK cells falls beyond the scope of this thesis.

Approximately 90% of NK cells in blood display low expression of CD56 ($CD56^{dim}$ phenotype), while a smaller fraction present a $CD56^{bright}$ phenotype (108). Traditionally, $CD56^{bright}$ NK cells have been ascribed immunomodulatory properties by virtue of cytokine production whereas cytotoxicity is purportedly restricted to NK cells of the $CD56^{dim}$ phenotype. Lately, several reports have challenged this view, suggesting that $CD56^{dim}$ and $CD56^{bright}$ NK cells, depending on the extracellular environment, might be equally potent cytokine producers and that both of these NK cell phenotypes are endowed with cytotoxicity (97, 111, 112).

NK cells respond to stimuli delivered by innate and adaptive immune cells. Thus, cytokines such as IL-1, IL-2, IL-12 and IL-15 along with IL-18 produced by innate antigen-presenting cells (APCs) may prime NK cell cytokine production and/or differentiation. Moreover, IL-2 released by activated T cells in secondary lymphoid tissues contributes to NK cell activation (108). The supply of cytokines from other immune cells is essential for NK cell function and NK cells rely on cytokines produced by other cells for their endurance, as exemplified by IL-15 that is fundamental for NK cell survival and differentiation (107, 108).

NK CELL RESPONSE TO INFLAMMATORY SIGNALS

In response to stimulation, NK cells rapidly secrete immunomodulatory cytokines and chemokines (108). In particular, NK cells constitute a significant source of interferon-γ (IFN-γ) that promotes immune responses through several mechanisms; it activates APCs that upregulate MHC class I expression and thereby triggers the formation of cytokines of relevance to the killing of intracellular pathogens. Via dendritic cell (DC)-activation, IFN-γ subsequently shapes T cell responses (107, 108, 113). Thus, IFN-γ secreted by NK cells is important for functional immune responses, as has been shown in the response against cytomegalovirus infection in mouse (MCMV) (114) and in NK cell-mediated control of murine melanoma (115). In addition to IFN-γ, NK cells produce proinflammatory cytokines including TNF-α, the growth factors GM-CSF, G-CSF and IL-3, along with chemokines such as MCP-1, MIP-1α, RANTES, IL-8 and XLC1 (107). The formation of these cytokines and chemokines is also elicited by interaction between NK cells and target cells. Upon target cell interaction, NK cells thus secrete IFN-γ and TNF-α as well as a battery of chemokines, including MCP-1, MIP-1α, IP-10, IL-8 and RANTES. The strength of the interaction, i.e., the type of stimulus and possible co-stimulatory factors, determines what cytokines are produced; IFN-γ requires co-stimulation of several activating receptor ligands in order to be secreted by NK cells (97). Moreover, debris from NK cell-induced killing of target cells may be taken up by APCs and cross-presented to $CD8^+$ T cells (107). Thus, NK cell recognition of target cells promotes, or maybe even initiates, an adaptive immune response.

NK cells also indirectly shape adaptive immunity via crosstalk between NK cells and DCs, which is relevant in defense against viral infections as shown in, e.g., HIV infection (116). The NK cell – DC interaction is contact-independent, where the release of TNF-α and GM-CSF regulates DC maturation, and contact-dependent, as the interaction is NKp30-dependent (117, 118). In addition, we recently demonstrated that NK cells, triggered by IL-18 and IL-1β secreted by activated neutrophils, responded vigorously to IL-12 released from DCs. The activated NK cells in turn induced DC maturation, which promoted T cell activation (**Paper SIV**). Thus, the NK cell response to signals produced by other innate cells may facilitate the development of an adaptive immune response.

Immunosuppressive cytokines secreted by other immune cells may suppress NK cell function. Two examples are transforming growth factor-β (TGF-β) and IL-10 that are released by activated APCs and dampen NK cell-mediated IFN-γ production (108). Moreover, $CD4^+$ $CD25^+$ regulatory T cells (T_{regs}) suppress NK cells via surface-bound TGF-β that down-regulates NKG2D, thereby reducing NK cell cytotoxicity and inhibiting IFN-γ secretion (119). Based on T_{reg}-mediated immunosuppression on NK

cells, strategies to inhibit the T_{reg} population have been suggested in NK cell-based immunotherapy, as discussed below.

NK CELL CYTOTOXICITY AGAINST IMMUNE CELLS

In addition to killing virus-infected and malignant cells, NK cells exert cytotoxicity towards other immune cells. NK cells thus modulate T cell-mediated anti-viral responses through cytotoxicity against activated $CD4^+$ T cells (120), kill stimulated macrophages in an NKG2D-dependent manner (121), and induce apoptosis of neutrophils via NKp46 and Fas interactions (122). The immunomodulatory role of NK cells may serve as a mechanism to terminate inflammatory responses, thereby avoiding chronic inflammation. In rheumatoid arthritis, which is an autoimmune disease characterized by chronic inflammation in the joints, the formation of neutrophil extracellular traps (NETs) by activated neutrophils has been proposed to uphold autoimmunity (123). Apoptosis of activated neutrophils may therefore be critical in the resolution of inflammation (124), and NK cell-induced apoptosis of neutrophils might constitute a mechanism to control inflammation. Thus, further insight in the regulation of NK cell-mediated elimination of inflammatory cells may pave the way for the development of new therapies in autoimmune diseases. The mechanisms and dynamics of NK cell interactions with inflammatory neutrophils was the focus in **Paper IV**, as described in detail below.

NEUTROPHILS

Neutrophils, the most abundant leukocytes in blood, are innate polymorphonuclear cells that make up a first line of defense against invading pathogens (125). Resting neutrophils survey the blood stream for signs of infection or inflammation. Upon local inflammation, epithelial cells lining the blood vessels receive signals from tissue-resident macrophages and mast cells, causing upregulation of selectins that facilitate neutrophil adhesion (126). Selectin-mediated binding of neutrophils to the vessel walls induces polarization and activation of neutrophils, and they upregulate adhesion molecules and chemotactic receptors allowing these cells to cross the epithelium and migrate towards the site of tissue injury (fig. 6). At the site of inflammation, pattern recognition receptors (PRRs) allow neutrophils to respond to invading pathogens or endogenous danger signals by sensing pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Among the PRRs is the Toll-like receptor (TLR) family, which recognizes bacteria, fungi and virus-derived PAMPs (127).

The antimicrobial activities of neutrophils include phagocytosis of pathogens and formation of NETs (127). Ingested preys are killed and digested through several

mechanisms including production of reactive oxygen species (ROS), and release of degrading enzymes and toxic substances from intracellular granules into the phagosome (125, 128). As an initial line of defense, neutrophils are one of the first cell types to arrive at inflammatory site. Despite relatively poor production of cytokines and chemokines, the early presence of neutrophils is critical for the shaping and amplification of the immune response. Activated neutrophils regulate innate and adaptive immunity by the secretion of cytokines and chemokines in a contact-independent manner and may also transport antigens to the lymph node (a process that possibly also includes antigen presentation to T cells) (125, 129, 130).

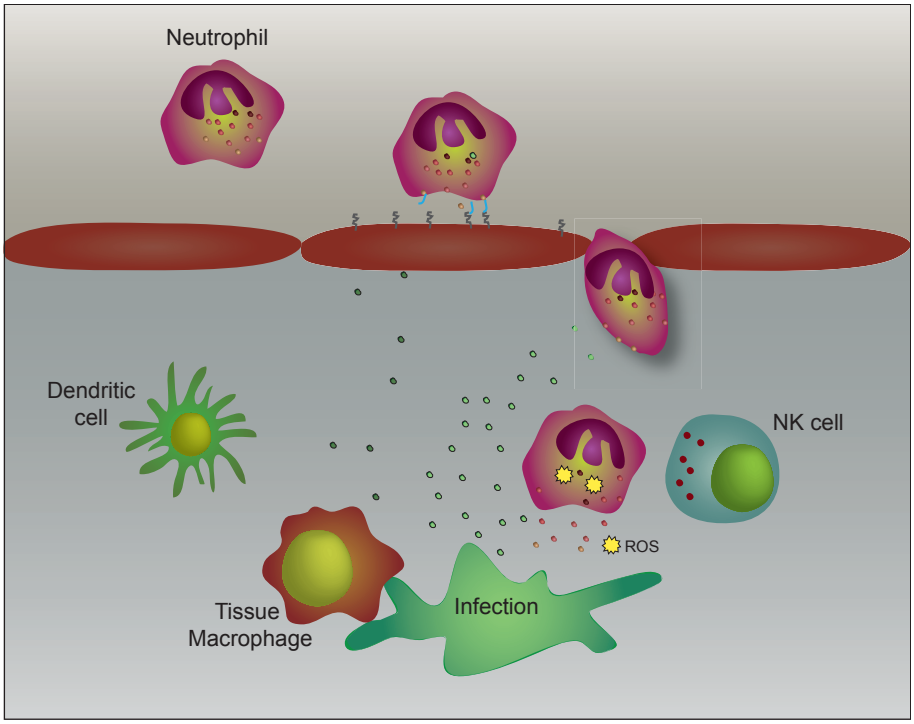

Figure 6. Neutrophil transmigration to site of infection. Inflammatory signals trigger neutrophil adhesion to epithelial cells that line the blood vessels. This allow them to cross the epithelium and migrate towards the site of inflammation, where neutrophils become further activated though recognition of PAMPs and DAMPs via their PRRs. Activated neutrophils phagocytose and digest infectious agents, and also secrete cytokines and chemokines that regulate innate and adaptive immunity.

The quick neutrophil response to invading pathogens is facilitated by the mobilization of intracellular granules containing pre-synthesized membrane proteins and soluble molecules (128, 131). Neutrophil granules are formed during neutrophil development within the bone marrow, and the granules are packed with proteins produced during certain stages of the maturation process to generate a heterogeneous granule population. Broadly, four different granule types are present in neutrophils, and these sub-populations are exocytosed in response to different stimuli; secretory vesicles and gelatinase granules are most easily mobilized to the surface, whereas the specific and azurophil granules demand additional priming in order to be mobilized (131). Azurophil, and to some extent specific granules are considered not to exocytose to the extracellular environment, but fuse with phagosomes to deliver degrading enzymes and toxic substances for digestion of ingested pathogens (125, 128). The differential propensity to exocytosis between the granule subpopulations allows for specific upregulation of proteins required for the different stages of the neutrophil response. Thus, granules containing adhesion molecules and chemotactic receptors are released first during migration, while serine-proteases are only delivered extracellularly at later stages in response to more potent stimulation (131). In this way, proteases, which are potentially toxic and tissue-destructive, are delivered at, e.g., the site of infection and not to healthy tissues.

Exocytosis of intracellular granules during neutrophil migration and activation results in phenotypic and functional changes of neutrophils, and in contrast to resting neutrophils transmigrated neutrophils are resistant to anti-apoptotic signals (132), possibly allowing for neutrophil apoptosis when the infection is cleared. Additionally, activated neutrophils modulate their expression of surface receptors. As described in **Paper IV**, neutrophil expression of ligands to NK cell receptors is altered upon activation, which results in increased susceptibility to NK cell-induced apoptosis. The apoptotic neutrophils are phagocytosed by macrophages, which induces a switch to a pro-resolution phenotype. Thus, neutrophil apoptosis promotes termination of an inflammatory response through several mechanisms; it renders neutrophils unresponsive to extracellular stimuli, arrests neutrophil secretion of cytokines, and induces inflammatory resolution responses in other immune cells. Efficient neutrophil apoptosis is, as discussed, crucial, as dysfunctional neutrophil apoptosis has been implicated in the pathophysiology of several diseases, including rheumatoid arthritis, cystic fibrosis and coronary artery disease (124).

INTERACTIONS BETWEEN NK CELLS AND NEUTROPHILS

As described above, NK cells and neutrophils modulate immune functions. Furthermore, NK cells and neutrophils modulate the functions of one another. NK cells are highly sensitive to reactive oxygen species (ROS) and neutrophil-derived ROS thus induce downregulation of activating NK cell receptors and inhibit NK cell functions including cytotoxicity and survival (130, 133-135). The suppressive effect of ROS has been implicated in inflammatory diseases as well as in cancer, where ROS derived from phagocytes at site of chronic inflammation, myeloid cells infiltrating tumors or malignant cells themselves, are proposed to dampen NK cell function. The impact of ROS-mediated NK cell inhibition, and how it may be targeted in leukemia is further discussed in the chapter "NK cells in cancer" and in **Papers I**, **II** and **III**. Neutrophils also suppress NK cell functions by the release of specific granule proteins that impede NK cell cytotoxicity, proliferation and IFN-γ production (130). Besides their suppressive actions on NK cells, molecules derived from activated neutrophils can promote NK cell activity and cytotoxicity (**Paper SIV**, 130), suggesting that neutrophils may regulate NK cells in a context-dependent manner.

Conversely, NK cells modulate neutrophil functions through multiple mechanisms. Soluble factors released by activated NK cells, including IFN-γ and GM-CSF, stimulate neutrophil survival and activation, and, although through unknown mediators, supernatants from NK cells reportedly potentiate neutrophil ROS production and phagocytosis (130). On the other hand, NK cells may induce neutrophil apoptosis in a contact-dependent manner. This interaction has been demonstrated to involve the activating NK cell receptor NKp46, presumably binding to an unknown NKp46 ligand on the surface of neutrophils (122), and most probably involving also other receptor-ligand pairs. Activated neutrophils have been demonstrated to be more sensitive to NK cell cytotoxicity (**Paper IV**), suggesting an immunomodulatory role of NK cells in terminating inflammation by inducing neutrophil apoptosis.

Altered surface expression of activating and inhibitory receptor ligands

As discussed above, granule exocytosis upon neutrophil activation mediates upregulation of receptors on the neutrophil surface and release of proteolytic enzymes, resulting in an altered phenotype of the activated cell (131). We speculated that the increased sensitivity of activated neutrophils might be explained by altered expression of neutrophil surface molecules, allowing for stronger NK cell activation upon cell-cell interaction with activated neutrophils. The results presented in **Paper IV** demonstrate that activated neutrophils are characterized by decreased expression of B7-H6, a ligand to NKp30, and HLA class I molecules (fig. 7). As described in the first chapter, ligation of HLA class I molecules to iKIRs and NKG2A delivers inhibitory signals to NK cells, which spares

healthy cells from NK cell cytotoxicity (28-31, 49). Thus, while loss of HLA class I expression, as frequently seen in viral infection (136, 137) or on solid tumor cells (138), is generally considered as an escape mechanism to avoid T cell recognition, the reduced class I expression will concomitantly render target cells more susceptible to NK cell-mediated killing. Downregulation of HLA class I molecules on inflammatory neutrophils, as reported in **Paper IV**, may thus serve as a mechanism to promote NK cell cytotoxicity. Thereby it would facilitate neutrophil apoptosis, which in turn contributes to the resolution of inflammation (schematically illustrated in figure 8).

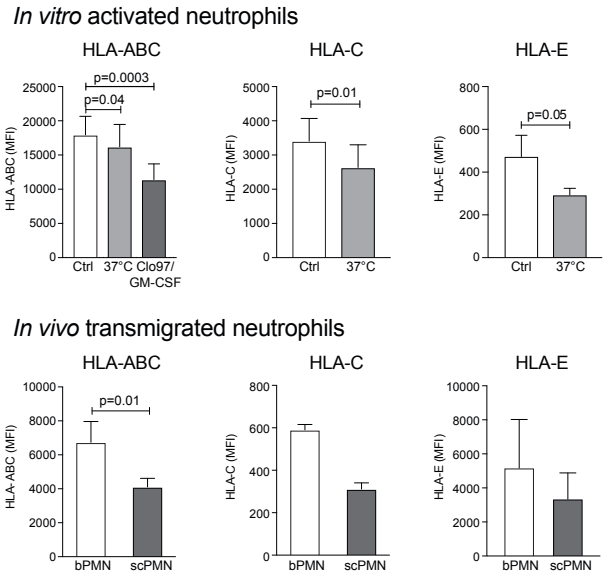

Figure 7. The expression of HLA class I is altered upon neutrophil activation. Results show the expression of HLA class I on neutrophils stimulated *in vitro* at 37°C in the presence of the TLR-agonist Clo97 and GM-SCF when indicated (n=3-7), and on *in vivo* transmigrated neutrophils collected from skin chambers (scPMN; n=2-5). Ratio paired t-test, error bars represent SEM.

Modulation of B7-H6 expression

In contrast to earlier reports, we demonstrate in **Paper IV** that neutrophils express the NKp30 ligand B7-H6 already in a resting state. On a similar note, a previous report from our group showed binding of an NKp30-Fc fusion protein to freshly isolated neutrophils (122). Shedding of B7-H6 from the surface of tumor cells has been proposed as an escape mechanism for tumor cells to avoid NK cell killing (139). The shedding may be mediated by metalloproteases, which are enzymes stored in intracellular neutrophil granules (131). As a soluble form of B7-H6 (sB7-H6) can be detected in supernatants after neutrophil stimulation with inflammatory cytokines (**Paper IV**, 140) in parallel with a decreased surface expression on neutrophils, it is conceivable that enzymatic cleavage is responsible for the reduced B7-H6 expression on activated neutrophils. Also, B7-H6 stored in intracellular organelles may be released to the extracellular space as a consequence of exocytosis. Extracellular sB7-H6 has been detected in exudates *in vivo* after the removal of transmigrated neutrophils (**Paper IV**),

as well as in serum from sepsis patients (140) and in patients diagnosed with melanoma (139), ovarian carcinoma (141) and neuroblastoma (142).

Details of the modulatory effect of sB7-H6 are yet unclear, as recombinant sB7-H6 does not alter NKp30 expression or NK cell function *in vitro* (140). However, high levels of sB7-H6 in peritoneal fluid/serum correlate with low NKp30 expression on NK cells in malignant diseases (141, 142). Moreover, B7-H6^{+} structures (possible exosomes) isolated from serum of sepsis patients impede NK cell function (140). Thus, it may be that neutrophil secretion of sB7-H6 negatively regulates NKp30-dependent NK cell functions. During an inflammatory response, NKp30-mediated killing of immature DCs by activated NK cells regulates the supply of DCs (117). Thereby, a higher degree of NK cell-induced killing of DC's will result in less DCs that activate adaptive immunity (117). Based on the data presented in **Paper IV**, we thus speculate that neutrophil secretion of sB7-H6 blocks NKp30-mediated killing of DCs, and thereby constitutes a mechanism to impede NK eradication of DCs in order to promote the development of adaptive immunity. In support of our hypothesis, it has been demonstrated that exosomes positive for BAT-3, which is another ligand to NKp30, regulates NK-DC interactions by binding to NKp30 on NK cells (143, 144).

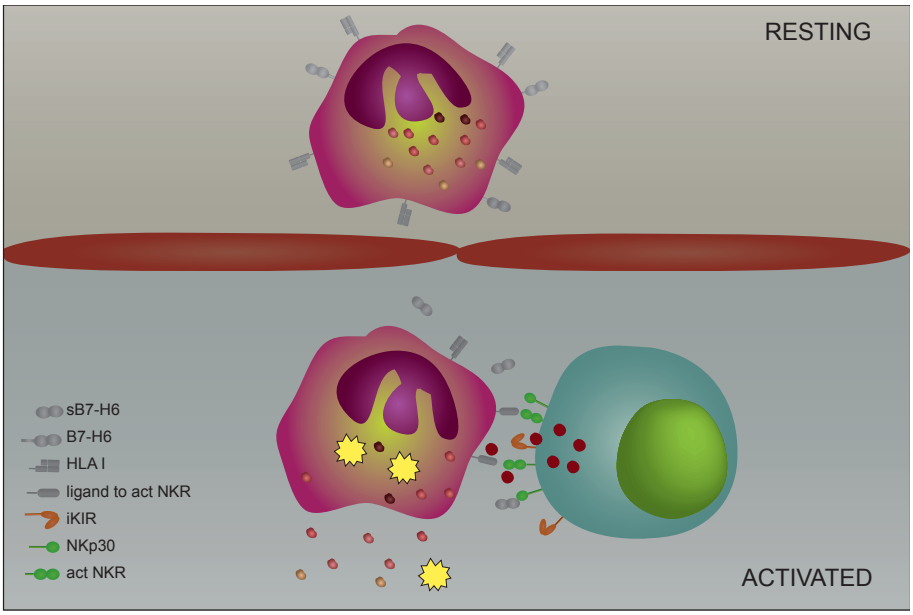

Figure 8. Altered phenotype of resting and activated neutrophils. Resting neutrophils express B7-H6 and HLA class I molecules. Upon transmigration, neutrophils modulate their expression of NK cell receptor ligands. Decreased expression of HLA class I, and thereby decreased inhibitory input to NK cells, may allow for NK cell-mediated killing of inflammatory neutrophils.

NK CELLS IN VIRAL DEFENSE

Functional NK cell responses play an essential role in the defense against viral infections, as supported by several studies demonstrating a correlation between congenital deficiency in NK cell development/function and increased susceptibility to severe virus infections (7-10). Moreover, NK cells have been identifies as pivotal effector cells in defense against several infections including cytomegalovirus (CMV), human deficiency virus-1 (HIV-1), hepatitis C virus (HCV) and influenza virus (136, 145). There are also studies that implicate NK cell-mediated lysis of papilloma virus-infected cells as important in viral control and in the development of papilloma-induced cancer (146, 147). In line with these studies, IFN-γ released by activated NK cells have been reported to promote viral defense mechanisms (148) and Th1 responses (113).

NK CELL RECOGNITION OF VIRAL INFECTION

Several phenotypic changes in virus-infected cells render them more susceptible to NK cell-mediated killing. Many virus strains downregulate MHC class I expression in order to avoid recognition by cytotoxic T cells, which leads to decreased inhibitory signaling upon interaction with NK cells (136, 137). In addition, virus-infected cells upregulate stress-ligands that bind to activating NK cells receptors. Most well described is probably virus-induced expression of ULBPs and MICA/B that are ligands to the activating NKG2D receptor (149). As for the NCRs, both NKp46 and NKp44 recognize hemagglutinins on influenza virus-infected cells (68, 69).

Further supporting a role of NK cells in virus defense are the vast numbers of escape mechanisms that viruses have developed to avoid NK cell activation. For example, during CMV infection the NKG2D ligands ULPBs and MICA/B are downregulated, which is caused by downmodulation, shut down of gene expression, and/or modified transcription (149, 150). In addition, NKp30 activation is inhibited in CMV-infected cells by the CMV tegument pp65, which upon binding to NKp30 causes dissociation of the adaptor and transduction molecule CD3ζ from NKp30 (70). CMV-infected cells also downregulate their expression of the NKp30 ligand B7-H6, apparently to escape NK cell recognition (151).

Conversely, the immune system has developed mechanisms to indirectly recognize viruses via KIR – HLA interaction affinity. Although KIR – HLA interactions are considered not to discriminate between self and non-self peptides, a model of peptide

specificity has been proposed in which viral or stress-induced epitopes, presented by HLA on infected cells, might disallow for KIR-HLA binding (136). As a result, reduced inhibitory KIR-HLA binding promotes NK cell activation and killing. On the contrary, peptide-dependent KIR – HLA binding might enable stronger interaction, as demonstrated in a recent study. In the study, weak activating KIR2DS1 – HLA-C2 interaction became stronger upon *in vitro* CMV infection, which implies that activated KIRs may recognize CMV-derived peptides in a specific manner (152). Furthermore, multiple studies have reported genetic variations of *KIR* and HLA to impact on disease progression and clearance of viral infections (153-155). One example is the HLA-B dimorphism at position -21 that has been correlated to susceptibility to HIV infection and to NK cell-mediated elimination of HIV-infected cells (156, 157); another example is recurrent respiratory papillomatosis where lack of activating KIRs was associated with a more severe course of disease (146).

In summary, there are mechanisms by which NK cells recognize viral infection, and viruses have evolved multiple escape strategies to avoid NK cell-mediated clearance. In addition to evoke an NK cell response, viral infection might affect NK cell repertoires in a longer time-frame. This effect has in particular been investigated in CMV infection, as reviewed below.

CYTOMEGALOVIRUS INFECTION

Cytomegalovirus (CMV) is a double-stranded DNA virus belonging to the family of herpesviruses (158). Different CMV strains cause infection in humans (referred to as CMV here) and mice (MCMV), and CMV infection has been extensively studied in both species. Primary CMV infection initiates viral replication in the mucosal epithelium and is often asymptomatic, but may cause infectious mononucleosis. In healthy individuals, CMV infection elicits innate, and subsequently adaptive, immune responses. However, the infection is not cleared, as the virus remains in host cells, probably of the myeloid lineage, where it establishes latent infection (158, 159). In most CMV-infected individuals, the virus remains latent throughout life, but may cause re-infection in immunocompromised subjects. Allogeneic transplantation, immunosuppressive chemotherapy and HIV infection are thus associated with CMV reactivation, at worst causing organ failure and mortality if not controlled by antiviral therapy (159). The seroprevalence of CMV (i.e., the proportion of subjects harboring IgG antibodies against CMV as a reflection of past infection) varies between ethnic groups and increases with age; in the United States 67% of the adult population are reported to be CMV seropositive (160) with 50% reported for a younger cohort (161), while the seroprevalence in Sweden is 83% in the adult population (162).

An adaptive immune response with expansion of CMV-specific $CD8^+$ and $CD4^+$ T cells is crucial for controlling the infection (158, 159). However, classical HLA class I molecules are downregulated in CMV infection (163) enabling CMV to evade virus-specific T-cell responses. As mentioned previously, decreased HLA class I expression may on the other hand render infected cells more susceptible to NK cells, as class I-dependent inhibitory signaling is decreased. Indeed, multiple reports of NK cell deficiencies pinpoint the role of NK cells as important effector cells in defense against herpesviruses (136, 164). The abundance of evasion strategies that CMV utilizes to avoid NK cell immunity further supports the importance of NK cells in controlling the infection. For example, CMV encodes for proteins that reduce the expression of stress ligands, including MICA/B and ULPBs that are upregulated as a result of CMV infection, thereby avoiding NK cell activation through NKG2D ligation. Moreover, CMV encodes for proteins that engage iKIRs and LILRB1, as well as the UL40 protein containing a leader sequence for HLA-E presentation; these mechanisms induce inhibitory signaling in KIR^+ and $NKG2A^+$ NK cells, respectively (136, 165). Interestingly, unlicensed NK cells have been reported as critical in protection against MCMV infection, possibly as these cells are not impeded by expression of ligands to inhibitory receptors (166).

CMV IMPACT ON THE NK CELL REPERTOIRE

CMV infection induces persistent phenotypic changes in the NK cell repertoire, characterized by an expansion of $NKG2C^+$ NK cells. The CMV-induced expanded NK cells are long-lived and apparently stable over time and have been termed adaptive, or "memory-like", NK cells (167). The term "memory" stems from the capacity of these adaptive NK cells to respond to a second infection several months after the primary infection (167). There is no precise definition of the phenotype of these CMV-induced adaptive NK cells, but reduction of the signaling proteins Syk, EAT-2 and FcRγ as well as the transcription factor PLZF are observed in expanded $NKG2C^+$ $CD56^{dim}$ NK cells after CMV infection (168, 169). Moreover, the expanded NK cell subset displays lower levels of the inhibitory receptor TIGIT and the NCRs, NKp30 and NKp46, along with increased levels of LILRB-1, KIRs (170), and CD57 (171) (fig. 9). Expression of the inhibitory PD-1 receptor, accompanied with very low NCR expression, was recently reported on mature $CD56^{dim}$ $NKG2A^-$ KIR^+ $CD57^+$ NK cells in some CMV-seropositive individuals, and the specific subset was also characterized by compromised effector functions against tumor cells (172). In addition to promoting maturation, CMV drives NK cell education, as CMV-induced adaptive cells predominantly express self-KIRs (173-175). Consistent with these observations, the results in **Paper II** imply that the frequency of mature $CD56^{dim}$ $NKG2A^-$ $NKG2C^+$ KIR^+ $CD57^+$ NK cells was higher,

in parallel with lower percentages of unlicensed NK cells, in CMV-seropositive AML patients receiving immunotherapy as compared to CMV-seronegative patients.

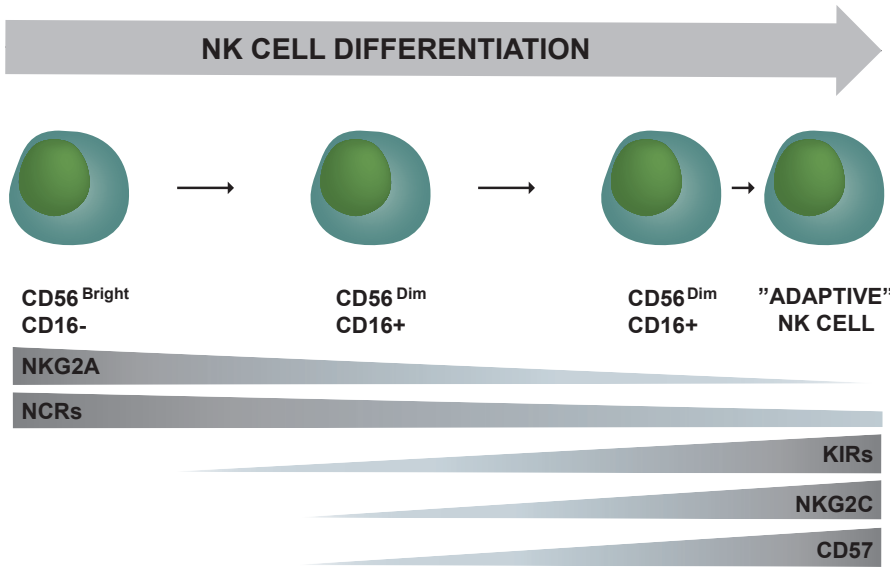

Figure 9. NK cell maturation and differentiation. $CD56^{bright}$ NK cells differentiate into $CD56^{dim}$ NK cells. During the differentiation process, NK cells lose NKG2A expression while gaining KIRs, NKG2C and CD57. CMV-driven adaptive NK cells show high expression of CD57, NKG2C and self-iKIRs, and are low in NCR expression.

The underlying mechanisms that promote expansion of memory-like NK cells in response to CMV infection are only partially understood. Induction of NKG2C is apparently driven by increased NKG2C/CD94 – HLA-E interaction, as the CMV protein UL40 allows for increased expression of HLA-E on the surface of infected cells (171, 176). In parallel, co-stimulation via adhesion receptor CD2 ligation to CD58 on infected cells contributes to activation and effector-functions of adaptive NK cells during CMV infection (177-179). CD16 binding to CMV-specific antibodies has also been implicated as a mechanism that promotes CMV-induced expansion of adaptive NK cells; despite that memory-like NK cells from CMV seropositive individuals are deficient in FcRγ and Syk, they expand in the presence of CMV-specific antibodies. Thus, CD16 signaling seems functional in memory-like $FcR\gamma^-$ Syk^- NK cells, possibly as CD16 signals via CD3ζ. Indeed, CMV-induced adaptive NK cells exhibit higher IFN-γ production to antibody-mediated stimuli compared to conventional NK cells, although there is no difference in degranulation (169).

Antibody-mediated expansion has been proposed to account for the expansion of NKG2C$^+$ NK cells observed exclusively in CMV seropositive individuals in response to other severe virus infections (180-183), where other viral infections are suggested to cause CMV reactivation that elicits production of CMV-specific antibodies (184). In addition, the cytokine milieu might be important in the generation of adaptive NK cells in CMV infection, where IL-12 has been proposed as a crucial component (185). The longevity of the pool of memory-like NK cells requires maintenance, and epigenetic modifications have been suggested to, at least in part, contribute to the long-lasting phenotypic alterations observed in CMV-induced adaptive NK cells. Epigenetic regulation has been demonstrated for FcεRγ, Syk and EAT-2 that are all downregulated in CMV-induced adaptive NK cells (168, 169).

CMV REACTIVATION IN THE TRANSPLANT SETTING

As mentioned earlier, CMV reactivation occurs mostly in immunocompromised subjects. CMV reactivation in a transplant setting is reportedly associated with elevated frequencies of adaptive NKG2A$^-$ NKG2C$^+$ self-KIR$^+$ CD57$^+$, IFN-γ producing, NK cells, with elevated levels stable up to a year after resolution of the infection (174, 175, 184). Although reactivation of CMV in transplanted patients may cause significant morbidity and mortality, several studies have correlated CMV reactivation after allogeneic transplantation (allo-SCT) in AML with lower risk of relapse (186-189), although disputed by others (190). In addition, CMV seropositive recipients tended to be more protected against relapse than those that had not experienced prior CMV infection (43, 188). However, CMV seropositivity *per se* was associated with increased relapse risk (188).

CMV-driven maturation of potent IFN-γ-producing, and possibly long-lived, NK cells has been proposed to explain the association between CMV reactivation and a favorable course of disease in allo-transplanted AML patients (43). In line with this hypothesis, a study that included several types of leukemia demonstrated high numbers of CMV-induced adaptive CD56^{dim} CD57$^+$ NKG2C$^+$ NK cells to associate with lower risk of leukemic relapse after SCT (191). However, another study reported high frequencies of terminally differentiated NK cells to correlate negatively with leukemia control (192). Another theory aiming to explain the protective effect observed after CMV reactivation in allo-SCT proposes that CMV reactivation in AML blasts may induce expression of stress ligands and thereby render the leukemic cells more susceptible to NK cell- or T cell-mediated killing (193). Still, there is no consensus regarding the impact of CMV reactivation and adaptive NK cells on allo-SCT, and the area needs to be further investigated.

With the proposed benefit of CMV-induced adaptive NK cells in combating AML, we investigated how CMV seropositivity impacted on clinical outcome of AML in a non-transplant setting. As shown in **Paper II**, we were unable to identify a benefit of CMV seropositivity in terms of clinical outcome. On the contrary, CMV seropositivity was associated with poor leukemia-free and overall survival for AML patients receiving immunotherapy for relapse prevention. As cytokine co-stimulation using IL-12 and IL-18 is insufficient to promote IFN-γ production or degranulation towards autologous $CD4^+$ T cells in adaptive NK cells from CMV seropositive individuals (168), it is tempting to speculate that the cytokine-based therapy in this setting was not sufficient to promote effector functions of CMV-induced memory-like NK cells, as further discussed in the "Results and discussion" section. Comparisons of the impact of CMV in allo-SCT and immunotherapy in AML patients for non-transplanted patients should be interpreted with caution. In the transplant setting, patients are heavily immunocompromised, which allows for reactivation of otherwise latent CMV virus, and subsequent expansion of CMV-specific memory-like NK cells. Additionally, reactivated CMV might infect AML blasts, supporting enhanced activating signaling that will not be present in latent CMV infection (193).

The memory-like property ascribed to NK cells after CMV infection has elicited significant interest. Strategies to induce memory in NK cells *in vitro*, with the aim to utilize their specific properties for therapeutic purposes in adoptive transfer, are currently underway in hematological malignancies, as reviewed in next chapter. In conclusion, not only are NK cells important in the immune defense against viral infections, but virus-induced modifications of the NK cell repertoire seem to influence NK cell effector functions towards malignant cells. Indeed, NK cells play a significant role in the elimination of malignant cells in certain cancers, as described in the next chapter.

NK CELLS IN CANCER

NK cells were first identified as lymphocytes that kill HLA-deficient tumor targets *in vitro* without prior sensitization, and have since been reported to kill a variety of tumor cells *in vitro* and in animal models *in vivo* (194). Still, the role of NK cells in immunosurveillance of cancer cells is debated, although a growing body of evidence imply a role of NK cells in both direct and indirect cancer control (11). Several studies have reported associations between low natural cytotoxic activity and high cancer risk in large cohorts of patients. The cytotoxic activity was partially determined by genes encoding NKG2D, which further supports a role for NK cells in prevention and/or control of cancer (195, 196).

NK CELL RECOGNITION OF MALIGNANT CELLS

Similar to viruses, tumor cells may escape cytotoxic T cell responses by downregulating MHC class I molecule-expression (138), which is achieved by mutations, deletions or hypermethylation in the HLA genes, loss of heterozygosity in chromosomes 6 or 15, or by transcriptional downregulation or oncogene activation (197). Although reduced MCH class I expression results in avoidance of antigen presentation, and thereby reduced activation of T cells, it will lower the inhibitory signaling delivered to NK cells upon target cell interaction, allowing for NK cell cytotoxicity (27). Thus, NK cells may act as a complement to T cells in the immunosurveillance of cancer cells. Additionally, transformed cells commonly upregulate stress ligands to activating NK cell receptors, and thereby become more susceptible to NK cell cytotoxicity. Stress-ligands commonly upregulated on malignant cells include the ULBPs, MICA and MICB, that are recognized by NKG2D, and PVR and Nectin-2 binding to DNAM-1 on NK cells (198-200). Moreover, expression of the NKp30 ligand B7-H6 has been reported on myeloid leukemia cells (64), and an NKp44 ligand has been detected on several tumor cells (66). There are also indications of expression of NKp46 ligands on tumor cells, as several cell lines display binding of soluble NKp46 fusion proteins in parallel with the finding that NKp46 blockade inhibits NK cell killing of certain tumor cell lines (201).

ESCAPE MECHANISMS OF MALIGNANT CELLS

Driven by selection pressure, evasive malignant phenotypes with a variety of defense mechanisms have evolved to avoid NK cell attack. Malignant cells may thus shed and release soluble forms of ligands to activating NK receptors, which compromises NK cell

function (202). As mentioned earlier, soluble B7-H6 has been detected in cancer patients (139, 141, 142), although its presumed immunomodulatory function remains to be elucidated. Of note, release of soluble ligands might also induce NK cell activation, as in the case of BAT-3 released by tumor cells, which triggers NK cell secretion of IFN-γ and TNF (65).

Low NK cell numbers are found in tumors, with a phenotype of mainly $CD56^{bright}$, that are more pro-angiogenic than $CD56^{dim}$ NK cells, and with high expression of inhibitory receptors. This suggests that the tumor microenvironment impairs homing of NK cells to tumor sites and alters the NK cell phenotype (138). Accumulation of myeloid-derived suppressor cells (MDSCs), driven by malignant cells, remodels the tumor micro-environment and suppresses effector T and NK cells through several mechanisms (203). Secretion of reactive oxygen or nitrogen species from MDSCs and other myeloid cells alters effector cell functions and induces apoptosis in NK cells, and MDSC-mediated depletion of essential lymphocyte metabolites arrests effector cell proliferation and signaling capacity. Moreover, the production of TGF-β by tumor cells and MDCSs inhibits NK cells by downregulating surface expression of activating receptors (204, 205). TGF-β is, together with a set of other factors, also responsible for differentiation of regulatory T cells (T_{regs}) that in turn suppress T and NK cells (206).

NK CELLS IN THE CONTROL OF MALIGNANT CELLS

NK cells have been ascribed a protective role in a broad range of malignancies. In neuroblastoma, NK cells were implicated an important role, based on studies of direct *ex vivo* NK cell cytotoxicity against malignant cells (207), and additional studies have proposed KIR/HLA genotypes and unlicensed NK cells to impact on the clinical outcome in neuroblastoma (208, 209). Moreover, *ex vivo* NK cell cytotoxicity has been demonstrated against malignant cells from patients with acute lymphoid leukemia (ALL) (210), chronic lymphocytic leukemia (CLL) (211), multiple myeloma (212), and solid tumors including gastric cancer, ovarian cancer, colon cancer and renal cell cancer (213). *In vitro* NK cell cytotoxicity against acute myeloid leukemic blasts (**Paper I**, 214) together with indirect evidence of NK cell alloreactivity after haploidentical allogeneic stem cell transplantation (allo-SCT; described further below) (215, 216) have prompted exploration of therapies enhancing NK cell reactivity in myeloid leukemia.

The seemingly exceptional position of NK cells in controlling hematological malignancies might be coupled to the immunoregulatory role of NK cells, as the malignant cells share features with immune cells. It is possible that proximity to stroma and the tumor environment of solid tumors enable for mechanisms that suppress NK cells. Interestingly, despite that the initial tumor killing capacity of NK cells was

detected in killing assays against HLA-deficient tumor cells, malignant blasts from myeloid leukemias commonly express HLA class I (217). In line with this finding, *in vitro* NK cell cytotoxicity against leukemic blasts is enhanced when there is a KIR-ligand mismatch present (214). However, NK cells reportedly kill malignant cells expressing HLA class I molecules (106, 218-220), presumably as activating signaling may override the inhibitory KIR – HLA signaling. Accordingly, we could in **Paper I** demonstrate NK cell cytotoxicity towards HLA-expressing AML blasts in an HLA-matched setting.

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by clonal expansion of abnormally or poorly differentiated cells of myeloid origin in the bone marrow, blood and other organs (221). AML is associated with several mutations, including karyotypic changes (such as gene translocations and deletions) and mutations in leukemic cells with a normal karyotype. The diagnosis is based on morphologic examination of bone marrow and blood smears, immunophenotyping and molecular analysis (221, 222). AML is classified according to the World Health Organization (WHO) classification system on the basis of genetic aberrations on leukemic cells. Previously, AML was classified using the French-American-British (FAB) system, where morphological examination stratifies AML into subtypes M0 to M7 based on the maturation stage of the leukemic cells. The FAB classification does not provide significant information of relevance to choice of treatment and prognosis with the exception of the M3 subtype (acute promyelocytic leukemia) that is treated differently than other forms of AML and also carries distinctly favorable prognosis.

The outcome of AML depends on patient-associated prognostic factors (e.g., age, health status and performance status) and disease-related prognostic factors (e.g., white cell count, genetics, prior myelodysplastic syndrome) (221, 222). Age is an independent risk factor, and only 5-15% of patients >60 years of age are cured from AML, while cure rates are higher (around 40-50%) in younger patients (221, 223). The type of genetic aberrations in leukemic cells may prognosticate outcome, and the European LeukemiaNet (ELN) classification stratifies AML by genetics into favorable, intermediate and adverse risk groups (222). For example, karyotypic abnormalities such as translocation between chromosomes 8 and 21 (t(8;21)) and translocation or inversion involving chromosome 16 ((t(16;16) or inv(16)) are associated with favorable outcome whereas t(6;9), inv(3), t(3;3) and deletions of chromosomes 5 or 7 herald poor prognosis. In normal karyotype AML, mutations of *NPM1* identifies patients with lower risk whereas mutated *FLT3*-ITD carries high risk.

Standard initial treatment for newly diagnosed AML is induction chemotherapy, comprising 3 days of anthracycline and 7 days of cytarabine (referred to as "7+3"), but the intensity and regimen may vary depending on the age and health status of the patient. Induction therapy results in complete remission (CR) in 60-85% of younger patients (<60 years) and 40-60% of older patients (>60 years), with CR defined as <5% blasts in the bone marrow and absence of circulating blasts, blasts with Auer rods or extramedullary leukemia along with the return of normal hematopoiesis (221, 222). Patients in CR are further given consolidation therapy. Most commonly, consolidation treatment encompasses additional cycles of chemotherapy (high-dose cytarabine), but may also include stem cell transplantation (SCT), either autologous (auto-SCT) or allogeneic (allo-SCT). Patients considered for allo-SCT are typically those <70 years with high-risk genetic aberrations in leukemic cells or treatment-refractory AML. Conditioning regimens, aiming to achieve myeloablation, before transplantation require that patients are expected to tolerate intense treatment, and nonmyeloablative or reduced-intensity conditioning regimens that allow allo-SCT in older patients, are now being evaluated. Together with the possibility of using donors that are not fully matched, which has evolved in recent years, these alternative conditioning regimens have allowed for a wider use of allo-SCT. Notably, however, the vast majority of AML patients are not transplanted and typically do not receive further treatment beyond the phase of consolidation chemotherapy. The high overall mortality in AML (approximately 70%) is mainly contributed by a failure to achieve CR and by relapse of leukemia after the completion of chemotherapy (222).

ALLOREACTIVE NK CELLS IN THE STEM CELL TRANSPLANTATION SETTING

Traditionally, allo-SCT in AML has been performed using either a related (usually a sibling) or an unrelated donor with a good match (10/10 or 9/10) between the donor and recipient HLA genotypes. Due to difficulties in finding siblings or other donors with a sufficient HLA match, haploidentical allo-SCT, where one haplotype is identical between the donor and recipient, is becoming more common owing to improved graft manipulation, prophylactic treatment preventing the transplanted cells from attacking the tissues of the recipient (graft versus host disease; GvHD; see below) and pre-transplantation conditioning. After infusion, donor cells engraft and reconstitute a "new" immune system in the recipient. Graft failure is a major complication in HLA-haploidentical SCT, and pre-conditioning treatment is important to allow proper engraftment (224). Successfully engrafted donor cells may then exert reactivity against the leukemic blasts, referred to as the Graft-vs-Leukemia (GvL) effect, where T cells have been identified as the main anti-leukemic effector cells. In addition to the desired GvL effect, the grafted T cells may also attack the recipient's healthy tissues, a potentially dangerous, or even lethal condition called Graft-vs-Host Disease (GvHD) (216).

Notably, donor NK cells have been suggested to decrease GvHD by contributing to eradication of APCs that otherwise initiate donor alloreactive T-cell-mediated GvHD (215). Even though GvHD may occur in a fully matched transplantation setting, the haploidentical allo-SCT increases the risk of T cell-mediated GvHD (216). In order to avoid GvHD in this setting, the graft is in some cases completely or partially depleted of T cells (222).

NK cells are the first lymphocytes to reconstitute after transplantation (43), and the reconstituted NK cells will develop in an HLA-environment represented by both the donor hematopoietic cells and the recipient stromal cells, and may therefore be educated in regards to the donor's as well as the recipient's HLA genotypes (225-227). In an HLA-haploidentical setting, NK cells have been demonstrated to engraft and give rise to NK cells expressing iKIRs that lack a matching recipient HLA molecule (216, 225, 228). As these NK cells are responsive, or licensed, in terms of the donor HLA genotype, they have been proposed to exert alloreactivity towards the recipient's leukemic blasts. Several studies have demonstrated clinical efficacy of donor/recipient KIR-ligand mismatch in the haploidentical transplantation setting, suggesting a role for alloreactive NK cells after allo-SCT (215, 216, 229, 230). However, other studies report no impact of KIR-ligand-mismatch after HLA-haploidentical SCT (231). Of note, a mere a mismatch between the donor KIR and recipient HLA has been reported to affect the transplantation outcome, as recipients that lacked HLA for a donor KIR were protected from relapse, independently of the HLA match status (232). This further supports the hypothesis that donor NK cell subsets expressing only iKIRs that lack cognate HLA on recipient's cells may attack and kill leukemic blasts. However, the question remains how long the allo-reactivity of transplanted NK cells with a KIR-ligand mismatch will last. Possibly, NK cell contact with stromal cells results in licensing of NK cells according to the recipient's HLA gene set-up, which presumably renders the allo-reactive NK cells unlicensed and hyporesponsive (226, 227).

In addition to the presence of a donor/recipient KIR-ligand mismatch, the KIR gene content of the donor cells has been reported to impact on the transplantation outcome, where donor haplotypes characterized by presence of KIR-B or aKIR genes were associated with improved clinical outcome (43, 231, 233-235). Also the HLA genotype of the recipient may impact on survival in both KIR-ligand-mismatched and -matched donor/recipient settings. Thus, presence of KIR-B or aKIRs are differentially protective in recipients with HLA-C1 present compared to recipients homozygous for HLA-C2 (236-238). Together, these data indicate that outcome of allo-SCT might depend on the activation status of the transplanted NK cells, as determined by donor KIR gene content, recipient HLA genotype, and the combination of these. Many aspects can contribute to differences observed in clinical outcome between separate studies;

preparation regimen, graft content (T cell depletion or not), graft source, inclusion criteria for patients, and the size of the alloreactive NK cell pool within the graft (111), may all affect outcome (239). Nevertheless, several studies from transplantation cohorts implicate an anti-leukemic effect of NK cells, and therapeutic strategies aiming at unleashing allo- or auto-reactivity towards malignant cells in AML are reviewed in next section.

IMMUNITY IN AML

Many patients diagnosed with AML are not eligible for transplantation due to disease-related factors, high age or health issues. As allo-SCT currently is the only consensually curative treatment for AML patients beyond induction and consolidation chemotherapy, there is significant need for other non-transplant treatment strategies for AML patients in the post-consolidation phase (222). Also in the non-transplant setting, NK cell function is implicated in clinical outcome, presumably by exerting cytotoxicity against leukemic cells. In non-transplanted AML, downregulation of the NCRs, NKp46 and NKp30, has been observed, with an NCR^{dull} phenotype correlating with poor *in vitro* killing capacity and poor clinical prognosis (12, 13). A poor clinical outcome has also recently been correlated with NK cell repertoires characterized by a low maturation profile ($CD56^{bright}$ KIR^{-} $CD57^{-}$) (240).

Several NK cell-related immune escape mechanisms in AML have been observed, including AML blast downregulation or shedding of ligands to activating NK cell receptors, which result in impaired NK cell recognition and cytotoxicity (241). AML patients commonly harbor low numbers of NK cells with a deficient cytotoxic capacity, suggesting that immunosuppressive mechanisms depressing NK cell function may be operable (in addition to the immunosuppression likely achieved by the previous chemotherapy). One of these mechanisms is the production of reactive oxygen species (ROS) by both non-malignant and malignant myeloid cells, which has been demonstrated to reduce NK cell functionality and causing them to undergo apoptosis (134, 242-245). Upregulation of the kinase GSK3-β in NK cells in AML patients is another mechanism proposed to contribute to NK cell dysfunction in AML (which will be mentioned later in this chapter) (246). Additionally, AML cells secrete other immunosuppressive factors including TGF-β and a soluble form of the IL-2 receptor, which may inhibit NK cell proliferation and cytotoxicity (241).

NK CELL-BASED IMMUNOTHERAPY IN AML

With the seemingly important role of intact NK cell function in AML, and based on the multiple evasion mechanisms present in AML, many immunotherapeutic approaches are currently being under development with the aim to utilize NK cells for improved elimination of leukemic blasts.

Inhibitory blockade

As AML blasts express MHC class I, one approach to increase the NK cell activity is to therapeutically target inhibitory signaling. Administration of a monoclonal antibody directed towards KIR2DL1 and KIR2DL2/L3 (IPH2101; Lirilumab), was proven safe with efficacious blocking of KIR, in a phase I trial enrolling AML patients in CR1 (247). IPH2101 was also well tolerated in combination with the approved drug lenalidomide in phase I trial in multiple myeloma (248). Moreover, an antibody targeting NKG2A (IPH2201; monalizumab) is currently being evaluated for targeting inhibitory interactions in several HLA-E expressing tumor types with limited reported toxicity of the drug (249). As AML blasts express HLA-E, and since NKG2A expression is commonly upregulated on AML NK cells, blocking NKG2A has been suggested as an approach to increase NK cell-mediated cytotoxicity towards AML cells (250). Nevertheless, the responsiveness of an NK cell is tuned by the strength of the inhibitory input (57). Thus, a potential caveat with blocking mAbs targeting inhibitory receptors is that NK cells might become hyporesponsive in the absence of inhibitory signaling. This can be compared to the situation after HLA-haploidentical SCT, where donor NK cells may be educated with respect to recipient HLA repertoire expressed on stromal cells (226, 227), and thereby lose their alloreactivity.

BiKEs and TriKEs

Another approach to facilitate enhanced interaction and response between NK cells and AML cells is the use of bispecific killer engagers (BiKEs) or trispecific killer engagers (TriKEs). These reagents are smaller than conventional antibodies and combine variable portions of antibodies directed towards antigens that are expressed on leukemic blasts, and CD16 on NK cells (251, 252). For AML therapy, a BiKE directed against CD33 has been developed. In addition to CD33, TriKEs have been produced either directed against CD123, or containing an IL-15 linker that co-stimulates NK cells (251-253). A therapy based on BiKEs or TriKEs has evoked interest as it can be provided as an off-the-shelf product have shown promising data, and a clinical phase I trial are currently under way to further evaluate the potential use of a TriKE in AML and MDS patients (251, 252).

Engineered NK cells

Another potential off-the-shelf strategy is to use engineered NK cell lines. Activated NK cells (aNKs) are derived from the NK-92 cell line, and a phase I trial that administered aNKs to patients with refractory renal cell carcinoma and melanoma reported infusions of these cells to be safe (254). High-affinity NK cells (haNKs) are modified aNKs that express high-affinity CD16 and produce IL-2. Used together with antibody therapy, haNKs are proposed to improve the antibody-induced ADCC response (255). The use of engineered T cells transduced with chimeric antigen receptors (CARs) directed against tumor antigens, CAR-T cells, has emerged in recent years, hitherto mostly for B cell malignancies. NK cell therapies based on the same concept, CAR-NK cells, are currently under investigation. As for haNKs, most CAR-NK cells are engineered NK-92 cells. CAR-transduced NK cells directed against CD19 have been reported to kill malignant B cells, and CAR-NK cells directed against CD33 for AML therapy are under investigation (256, 257).

Additional means to achieve NK cell-mediated eradication of malignant cells comprise the targeting of NK cell inhibitory (PD-1, TIGIT) or activating receptors (e.g., NKG2D), as well as pathways regulating NK cell cytotoxicity and maturation (258).

Cytokine-based immunotherapy

IL-2 is a T cell-derived cytokine that efficiently stimulates cytolytic and proliferative activity of NK cells and promotes expansion and differentiation of effector $CD8^+$ T cells (259-261). In 1992, IL-2 was approved by the FDA as monotherapy in renal cell carcinoma and metastatic melanoma (262). Remarkably, 5-10% of patients with metastatic melanoma or renal cell carcinoma experience complete regression of metastatic disease after IL-2 therapy, and approximately 70% of these patients were cured from cancer (263). In AML, six studies with a total of >1,500 patients have evaluated IL-2 as post-consolidation immunotherapy for the prevention of relapse, but with disappointing results as IL-2 therapy failed to significantly reduce relapse rate or improve survival in any of the studies (264-269). A meta-analysis supported the inefficiency in IL-2 to prevent relapse for AML patients in remission (270).

A reason for the inefficiency of IL-2 to mount a powerful anti-leukemic response may be that several immunosuppressive pathways are operable in AML. One of these is production of ROS by myeloid cells, which suppress NK cell functions and induce NK cell apoptosis (243). One clue to understanding why IL-2 did not prove effective came with the finding that addition of monocytes to co-cultures of IL-2 stimulated NK cells and AML blasts abolished the NK cell-mediated cytotoxicity towards AML blasts (271, 272). Monocyte-derived ROS were demonstrated to be the cause of this effect, causing

ROS-sensitive NK cells to undergo apoptosis (243). In 1996, Brune *et al.* demonstrated that histamine synergized with IL-2 to promote NK cell-mediated killing of AML blasts in the presence of ROS-producing monocytes (273). Histamine acts by ligating H2-histamine receptors on myeloid cells, leading to reduced or inhibited production of oxygen radicals by the myeloid NADPH oxidase (NOX2) (242). These findings translated into the development of an immunotherapeutic regimen combining low dose IL-2 and histamine dihydrochloride (HDC; a histamine salt). HDC/IL-2 was first tested in malignant melanoma and renal cell carcinoma, and later administered to AML patients in the post-consolidation phase in a phase I/II trial (271), suggesting that the regimen was safe and feasible. In a randomized phase III trial, patients treated with HDC/IL-2 displayed significantly improved leukemia-free survival compared to the control arm (274). In agreement with early-phase studies, the treatment was tolerable and devoid of severe toxicity were observed (274), which contrasts with studies using high-dose IL-2 regimens (275). HDC/IL-2 administered in the postconsolidation phase, is approved in the EU for relapse prevention in AML patients (reviewed in, 276).

Histamine dihydrochloride and IL-2 immunotherapy in AML

Immunotherapy with HDC/IL-2 affects the immune system in multiple ways. Firstly, IL-2 and HDC have individual effects including the above-mentioned lymphocyte stimulation exerted by IL-2 (259-261), and the reduced HDC-induced reduction of NOX2-derived ROS caused by activation of H2 receptors on myeloid cells (242). Secondly, the addition of HDC might also potentiate the efficacy of IL-2 therapy, as observed in melanoma patients with liver metastasis (277, 278) or in patients with renal cell carcinoma (279), who were treated with either HDC/IL-2 in combination or with IL-2 alone.

A phase IV trial (the Re:Mission trial, NCT01347996, see BOX), designed to assess immunomodulatory effects of HDC/IL-2, was administered to AML patients in CR1. The immunotherapy induced expansion of both $CD56^{bright}$ and $CD16^{+}$ NK cells, along with upregulation of NK cell NCR expression (280). High blood counts of $CD56^{bright}$ NK cells before treatment start and an NCR^{high} phenotype predicted leukemia-free and overall survival (LFS and OS, respectively) (280, 281), implying that the expansion and activation of NK cells is clinically relevant. In further support for a role for NK cells in AML during HDC/IL-2 immunotherapy, a low HLA-ABC expression in myeloid cells, indicating low-grade inhibitory signaling to NK cells, was associated with favorable clinical outcome in the Re:Mission trial (**Paper SIII**). In **Paper I**, high proportions of unlicensed NK cells were found to predict LFS. Moreover, an additional effect was observed in patients with high proportions of unlicensed NK cells and an NCR^{high} phenotype. Thus, NK cells characterized by both low inhibition (i.e., $iKIR^{-}$ $NKG2A^{-}$

unlicensed NK cells) and high activation (high NCR expression) were protective. CMV-driven education of NK cells was reported in **Paper II,** where AML patients seropositive for CMV harbored NK cell repertoires characterized by high inhibitory input through expression of inhibitory KIRs to self-HLA. In agreement with the protective effect of low inhibitory receptor expression to self-HLA in **Paper I**, CMV seropositivity impacted negatively on outcome (**Paper II**). In **Paper III**, we reported that a polymorphism at the -21 position of *HLA-B*, which determines whether NK cells are licensed via NKG2A or KIR, affected clinical outcome, as patients with a genotype that predict NK cell-licensing via NKG2A showed improved LFS. Together, these results indicate that NK cell repertoires characterized by low inhibitory input are relevant to the clinical outcome and also that IL-2/HDC induces effector functions in NK cells that receive low inhibitory input from AML blasts (a subject that will be discussed further in the "Results and discussion" section).

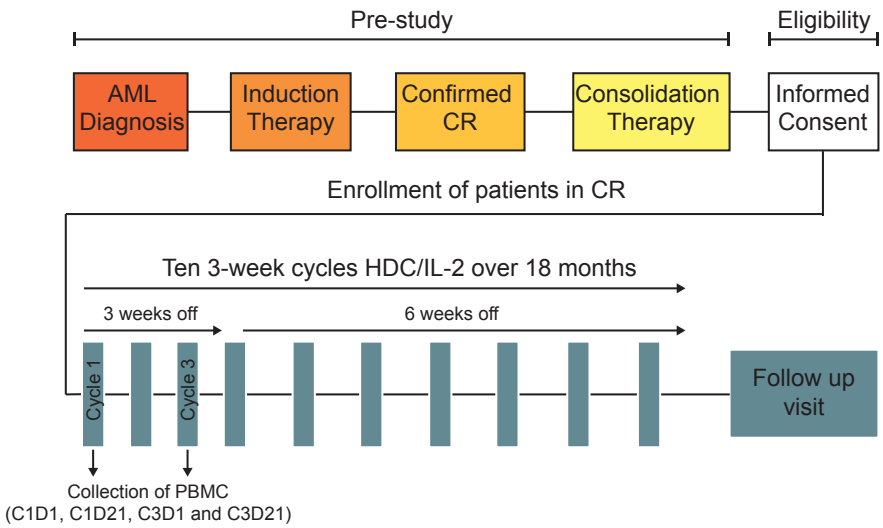

Figure 10. Overview of the Re:Mission trial. Patients diagnosed with AML were enrolled in the phase IV Re:Mission trial after they had achieved complete remission (CR) and received consolidation therapy. HDC/IL-2 was administered in ten 3-week cycles with three (first 3 cycles) or six (following cycles) weeks off in between. Samples were collected at onset and after cycle 1 and 3. Data obtained from analysis of these samples are presented in **Paper I-III**.

BOX: Histamine and IL-2 immunotherapy in AML – the Re:Mission trial

In order to gain a deeper understanding on the immunomodulatory properties of the treatment, a phase IV clinical trial was conducted. The "Re:Mission trial" (NCT01347996) enrolled 84 AML patients (FAB-M3 excluded) in first complete remission (CR1), from 20 centers in Europe. Patients received ten 3-week cycles of HDC (0.5 mg)/IL-2 (16,400 U/kg) with 3 (first three cycles) or 6 (cycles 4-10) weeks rest periods (fig. 10). Both drugs were delivered subcutaneously and could be administered by the patients themselves at home without supervision. Patients were followed for at least two years for leukemia-free survival (LFS) and overall survival (OS). PBMCs were collected at onset and end of cycle 1 and 3, and were shipped to our lab at Sahlgrenska Cancer Center, which served as the central laboratory for immunological assessments. Multicolor flow cytometry panels were designed to measure immunological parameters on subsets of NK cells, T cells and myeloid cells (**Paper I-III**, and **SI-SIV**, 280, 281). In addition, genotyping was performed to determine KIR and HLA genotypes. Presented data in **Papers I-III** concerning post-consolidation immunotherapy of HDC/IL-2 are all derived from samples analyzed within the Re:Mission trial.

IL-15-based immunotherapy

The IL-2 receptor consists of three subunits, or "chains"; the α, β and γ chains (261). The α chain is also called CD25 and binds IL-2 with low affinity, while a complex of β and γ binds IL-2 with intermediate affinity. For high-affinity IL-2 binding, all three chains need to be engaged. The β component of the IL-2 receptor, IL-2Rβ, is essential also for IL-15, and the effects of IL-15 resemble those of IL-2, with IL-15 promoting NK cell development, differentiation and survival (282). *In vitro* assays have demonstrated IL-15-stimulated NK cells to harbor higher amounts of granzyme B, perforin and CD107a. IL-15 stimulation also induced increased expression of LFA-1 with a more open conformation after co-incubation with target cells, leading to enhanced inside-out signaling. In a recent study, it was suggested that IL-15 stimulation *in vitro* primarily primes cytotoxic responses in $CD56^{bright}$ NK cells, supporting the notion that cytotoxic effector functions can be exerted by both mature $CD56^{dim}$ NK cells, but also by the more immature $CD56^{bright}$ NK cells (112).

In order to avoid toxicity associated with high-dose IL-2 and expansion of regulatory T cells (T_{regs}) observed in low-dose IL-2-based regimens (the issue of T_{reg} expansion during IL-2 therapy will be further discussed below), replacement of IL-2 with IL-15 has been proposed for cytokine-based cancer immunotherapy (230, 283). A clinical study where IL-15 was administered to patients with melanoma or renal cell carcinoma reported substantial effects on NK and T cell expansion (284). Endogenously produced IL-15 is mainly presented to NK cells in a membrane-bound form where it is associated with IL-15Rα (258). As α chains contribute to the binding affinity both when present on the cell presenting IL-15, or the cell binding to IL-15, this presentation allow for high affinity binding of IL-15. Soluble IL-15, which is not associated with IL-15Rα, will therefore induce weaker responses than membrane-bound IL-15 (258). Approaches to enhance binding affinity of therapeutic IL-15 to NK cell receptors by the use of engineered IL-15 agonist complexes (e.g., the super agonist complex ALT-803), are currently being evaluated in clinical trials (258).

Adoptive transfer of NK cells

In an attempt to make use of NK cell alloreactivity, adoptive transfer of KIR/HLA-mismatched NK cells is being evaluated. Several clinical studies have demonstrated adoptive transfer of NK cells to be safe, with transient donor NK cell engraftment and expansion (285, 286). Adoptively transferred NK cells do not permanently engraft unless patients receive intense conditioning prior to the cell transfer. To stimulate *in vivo* expansion of the transferred NK cells, IL-2 has been administered to patients after adoptive transfer (285, 286). Lately, a shift from IL-2 to IL-15 in this setting has been suggested in order to avoid expansion of T_{regs} (230, 283).

Cytokine-induced memory-like NK cells

Prior to an adoptive transfer, NK cells are enriched *ex vivo* using different strategies including CD3 depletion and/or CD56 enrichment, and may also be further expanded before infusion through cytokine stimulation or co-culturing with feeder cells (230). Expansion of NK cells *in vitro* may also be performed in presence of a combination of IL-12, IL-15 and IL-18, followed by incubation in low-dose IL-15 for 1-3 weeks. This regimen was reported to generate NK cells that respond vigorously to a second stimulus by increased IFN-γ production, and that possess increased proliferative capacity compared to conventional NK cells weeks after the first cytokine exposure (4). Due to their ability to respond to re-stimulation, these NK cells have been named cytokine-induced memory-like (CIML) NK cells. In contrast to the memory-like NK subset found after *in vivo* CMV infection, the repertoire of cytokine-induced memory-like NK cells mainly comprises $CD56^{bright}$ NK cells. Further, while the CIML cells are high in NKG2A, NKG2D and NCR expression, their expression of CD57 is decreased, while the NKG2C and KIR expression pattern remains largely unchanged (4, 287). Additionally, in contrast to CMV-induced memory-like NK cells, no differences in FcRγ or Syk expression have been observed between CIML and conventional NK cells (288).

Despite the increased IFN-γ response, CIML NK cells do not degranulate more vigorously than control NK cells stimulated with IL-15. However, CIML NK cells display increased expression of high affinity IL-2Rαβγ, and stimulation with IL-2 renders CIML NK cells more cytotoxic than conventional NK cells against *in vitro* tumor cell lines (289). Adoptive transfer of CIML NK cells together with repeated cytokine infusion *in vivo* (low-dose IL-2 or IL-15) is currently evaluated as treatment strategies in hematological malignancies (283, 287, 289, 290). A recent phase I trial (NCT01898793) demonstrated substantial proliferation and expansion of *in vivo*-transplanted allogeneic CIML NK cells to AML patients (287). Interestingly, the authors demonstrated that not only the licensed NK cell subset responded to cytokine stimulation, but also the unlicensed CIML NK cells were functional and responded to tumor targets or ligation of activating receptors (288). This indicates that cytokine stimulation may render unlicensed NK cells functional and responsive. The results further imply that the anti-leukemic effector function of adoptively transferred CIML NK cells might, at least in part, be exerted by unlicensed NK cells that will not be inhibited by HLA molecules expressed on the leukemic blasts.

In contrast to HLA haploidentical allo-SCT, where engrafted NK cells may be educated by HLA molecules present on grafted cells (225), adoptively transferred NK cells will presumably be educated by the recipient's HLA molecules. Thus, there is a risk that the allo-reactivity based on KIR/HLA mismatch is lost, although this possibility should be further studied. Based on the results in **Paper I**, where we observed a benefit of high frequencies of unlicensed NK cells on clinical outcome during HDC/IL-2 AML immunotherapy, it may be speculated that also unlicensed CIML NK cells are endowed with anti-leukemic effector function.

CMV-induced adaptive NK cells

Another approach currently investigated for adoptive NK cell transfer is to take advantage of the anti-tumor properties ascribed to CMV-induced adaptive NK cells. One example is a study that aims to utilize NK cells from CMV seropositive individuals expanded in an environment where the kinase GKS3 is inhibited. GKS3 is upregulated in AML, and inhibition of GSK3 in healthy donor NK cells enhanced their cytotoxicity against AML blasts (246). Moreover, GLS3 inhibition in IL-15-activated NK cells from CMV-seropositive donors led to NK cell maturation, induced expression of CD57 and several transcription factors, and increased secretion of TNF-α and IFN-γ (246, 291). With this background, an ongoing clinical trial (FATE NK-100) will assess the efficacy of adoptive transfer of those highly mature NK cells, expanded during GKS3 inhibition, to patients with refractory or relapsed AML (283, 291).

Albeit promising, no significant clinical long-term benefit of adoptive transfer of NK cells has been demonstrated. However, a fraction of patients acquire complete remission, which may make them eligible for subsequent allo-SCT. Thus, adoptive transfer of NK cells may be used as a bridging therapy allowing more patients to undergo allo-SCT (230, 287).

RESULTS AND DISCUSSION

A main goal of this thesis work was to contribute to the understanding of the role of NK cells in leukemia and inflammation with emphasis on the potential impact of inhibitory interactions between NK cells and HLA class I molecules expressed by host cells. NK cell cytotoxicity against malignant cells was originally described as a consequence of "missing self", where NK cells could attack cancer cells that lack MHC class I on their surface (16). Accordingly, solid tumors commonly downregulate HLA class I and thus become susceptible to NK cell cytotoxicity. By contrast, in accordance with previous reports, we have observed that malignant blasts in both AML and chronic myeloid leukemia (CML) present HLA class I expression at levels comparable with healthy cells (**Paper I** and unpublished, 217)**.** The state of the art in this research area is that inhibitory input tunes the functional response of NK cells such that those NK cells that receive a high amount of inhibitory signaling will possess a larger cytotoxic potential (57). Thus, upon an encounter with an HLA-deficient cell, NK cells that express multiple inhibitory receptors will be the cells that display most missing self-recognition, and hence the most efficient killers.

However, in a normal HLA environment, these NK cells will also be the most inhibited subset. It seems paradoxical that a body of evidence points towards a key role for NK cells in myeloid leukemia, where the malignant blasts express HLA and thus may put the missing self-recognition out of play. With intact expression of HLA class I on malignant myeloid cells, an NK cell must override or avoid the inhibitory signaling input to exert cytotoxicity. According to the current dogma, NK cells that lack inhibitory receptors to self-HLA molecules will not receive any inhibitory input and will consequently be hyporesponsive. This may be a way to avoid NK cell autoreactive reactions. (56). However, ample evidence suggests that these unlicensed NK cells can become responsive upon *in vitro* cytokine stimulation (**Paper I**, 55, 56, 62, 209, 288). It is thus conceivable that NK cells that express few or no inhibitory receptors may have higher capacity to kill HLA class I-expressing malignant cells under certain conditions.

Classical NK cell activation assays typically use cell lines, such as the HLA-deficient myeloid leukemic K562 cell line, as target cells. Although degranulation assays against HLA-deficient cell lines constitute an important model system for studies on NK cell cytotoxicity, the lack of HLA signaling disregards the important contribution of interactions between inhibitory receptors and HLA. In order to study NK cell cytotoxicity in the context of HLA-mediated signaling, we performed degranulation

assays using HLA-matched AML blasts as target cells (presented in **Paper I**). As expected, both S-iKIR$^+$ and NKG2A$^-$ NS-iKIR$^+$ NK cells responded poorly without prior stimulation; S-iKIR$^+$ NK cells because they were inhibited by signaling via ligation of HLA class I, and NKG2A$^-$ NS-iKIR$^+$ NK cells because of their hyporesponsive state. However, upon IL-2 stimulation, NKG2A$^-$ NS-iKIR$^+$ NK cells gained functionality, as illustrated by increased degranulation towards AML blasts (**Paper I**). This increased responsiveness might be explained by the reported IL-2-induced replenishment of the granular stock of perforin and granzyme B (292), and convergence of lytic granules (293).

A majority of all individuals have a genetic discordance between *KIR* and *HLA*, and one may ask why this discordance has not disappeared during evolution. Clinical observations imply an anti-malignant function mediated by unlicensed NK cells. In neuroblastoma and follicular lymphoma, patients who received treatment with a monoclonal antibody (mAb) targeting the malignant cells were protected if they carried a missing ligand genotype (that is, presence of a *KIR* lacking its cognate *HLA*) (209, 294). A similar observation was made after auto-SCT followed by mAb therapy in patients with neuroblastoma (208). These studies did not involve cytokines that activate unlicensed cells; instead, the data supports the interpretation that unlicensed NK cells became activated by mAb-mediated CD16 ligation. Accordingly, *in vitro* experiments show that while ADCC of S-iKIR-expressing subsets is blocked, NS-iKIR subsets may become the most efficient NK cell effector population (295).

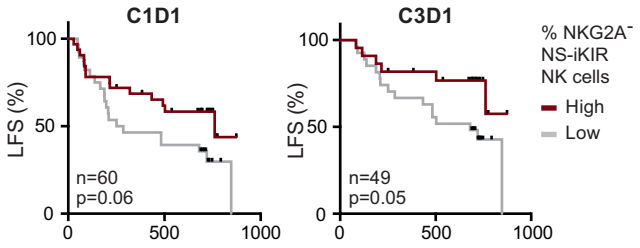

Figure 11. Impact of unlicensed NK cells on clinical outcome in the Re:Mission trial. Leukemia-free survival (LFS) for patients dichotomized into high or low frequency of unlicensed NKG2A$^-$ NS-iKIR NK cells at treatment start (C1D1) and at onset of cycle 3 (C3D1).

With this background, we speculated that HDC/IL-2 immunotherapy administrated to AML patients in the Re:Mission trial might activate unlicensed NK cells, and that this may impact on clinical outcome. Among the trial patients with a missing ligand genotype, the frequency of unlicensed NKG2A$^-$ NS-iKIR NK cells varied considerably. We therefore investigated how the frequency of NKG2A$^-$ NS-iKIR NK cells impacted on outcome, and observed that a high proportion of NKG2A$^-$ NS-iKIR NK cells was associated with reduced incidence of relapse (**Paper I**, fig. 11). Presumably, HLA class I expressed on AML blasts inhibits activation of S-iKIR NK cells, while IL-2 stimulated NS-iKIR NK cells, that are not inhibited by HLA-mediated inhibition, might be able to exert cytotoxicity against the leukemic cells. With the observed benefit of high counts of unlicensed NK cells, it was unexpected that a missing ligand genotype *per se* was not associated with improved LFS (**Paper I**). However, it seems reasonable to assume that in order to benefit from a missing ligand genotype, the patient must have functional NK cells; and secondly, the missing ligand benefit can arguably only be beneficial if the patient has a substantial number of unlicensed NK cells. Accordingly, our continued studies demonstrated that a missing ligand genotype was exclusively protective in the group of patients with high NCR expression (fig. 12, un-published data). Moreover, CMV-driven differentiation of the NK cell repertoire, which concomitantly leads to depletion of unlicensed NK cells, correlated with poor prognosis for patients seropositive for CMV, and a missing ligand genotype was protective only in patients who had not experienced CMV infection (**Paper II**, fig. 13). The importance of activation in parallel with lower inhibition was further highlighted by the findings that high expression of NKp46 and presence of KIR-B genes (encoding activating KIRs) were only associated with LFS in patients with a missing ligand genotype (**Paper I**).

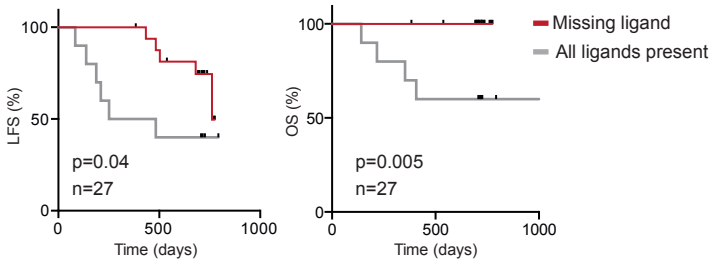

Figure 12. Impact of a missing ligand genotype for AML patients with functional NK cells. Leukemia-free and overall survival (LFS) for the patients in the Re:Mission trial with above-median expression of NKp46. Patients were dichotomized based on their KIR and HLA genotype, with either a missing ligand genotype (allowing for presence of unlicensed NK cells) or all ligands present.

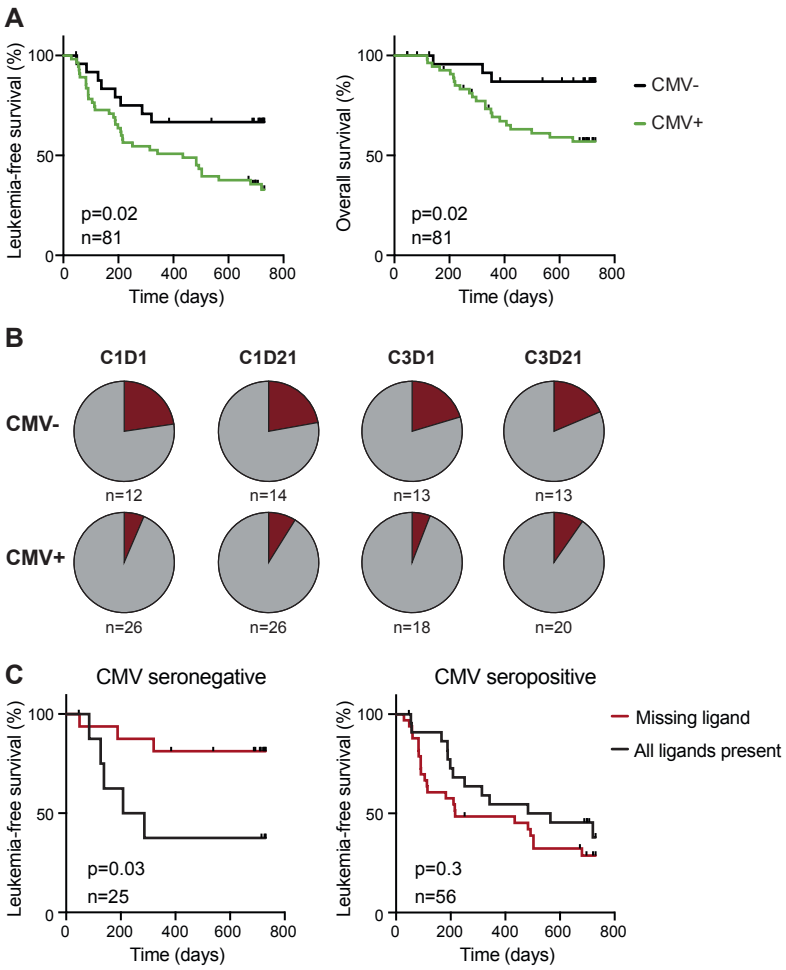

Figure 13. Impact of CMV seropositivity on outcome in the Re:Mission trial. A) Leukemia-free and overall survival for patients within the Re:Mission trial seropositive (CMV+) or seronegative (CMV-) for CMV-IgG. B) Pie charts illustrate median frequencies of unlicensed NKG2A^{-} NS-iKIR CD56^{dim} NK cells at specified time points in seropositive or seronegative patients. C) Leukemia-free survival for AML patients with a missing ligand or all ligands present genotype.

The hypothesis that unlicensed cells act as effector cells upon stimulation is to a large extent based on correlative data comparing *KIR* and *HLA* genotypes with clinical outcome. However, even though a missing ligand genotype allows for presence of NS-iKIR^{+} NK cells, the model does not take into account the more conserved HLA-E – NKG2A interactions. When we instead investigated how the frequency of NKG2A$^{+/-}$ NS-iKIR NK cells impacted on outcome in the Re:Mission trial, a similar protection pattern was observed for this subpopulation as for the NKG2A^{-} NS-iKIR NK cells reported in **Paper I** (fig 14, un-published data). Thus, the impact of NS-iKIR NK cells was equally evident when NKG2A expression was disregarded. We can therefore not determine whether it is IL-2-activation of hyporesponsive NKG2A^{-} NS-iKIR NK cells that is responsible for the protective effect of NS-iKIR NK cells, or if the effect is caused by IL-2 enabling licensed NKG2A^{+} NS-iKIR NK cells to override the inhibitory NKG2A-signal. Nevertheless, these data suggest that NK cells that lack S-iKIRs receive lower inhibition and hence can eradicate the HLA-expressing AML-blasts after IL-2 activation.

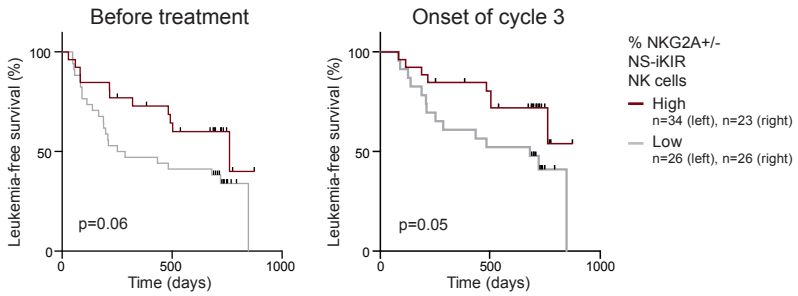

Figure 14. Impact of non-self inhibitory KIR (NS-iKIR) NK cells on clinical outcome in the Re:Mission trial. Panels show leukemia-free survival for AML patients receiving HDC/IL-2 immunotherapy. Patients were dichotomized based on high or low frequency of NKG2A$^{+/-}$ NS-iKIR NK cells according to receiver-operating characteristics (ROC) curves and Youden index (AUROC and 95% CI: 0.600, 0.452-0.747 before treatment, and 0.623, 0.464-0.782 at onset of third treatment cycle). Outcomes were analyzed using the log-rank test.

Elevated levels of NKG2A^{+} NK cells are commonly found in AML patients (296), and we observed further induction of NKG2A upon HDC/IL-2 treatment within the Re:Mission study (**Paper II**). This incited us to investigate the role of NKG2A for the outcome of the immunotherapy. Expression of the NKG2A ligand, HLA-E, is dependent on the presence of leader peptides from the classical HLA class I molecules. As mentioned in the first chapter, the expression level of HLA-E is to a certain extent regulated by a dimorphism in -21 position of HLA-B, with a methionine allowing for

functional presentation of HLA-E, while a threonine residue does not (48). HLA-B -21T is commonly associated with the strong KIR ligands, Bw4$^+$ HLA-B and HLA-C2, while -21M commonly associates with the weaker HLA-C1 (50). Thus, individuals homozygous for -21T at HLA-B (T/T) harbor NK cells that are primarily licensed by iKIRs, while NKG2A dominates in individuals with an M/x genotype (50). The clinical consequences of this dimorphism are largely unexplored. In the Re:Mission trial, an M/x genotype was associated with relapse control and prolonged survival, while corresponding analysis in a cohort of allotransplanted AML patients did not reveal any survival benefit of either an M/x or T/T genotype (**Paper III**, fig. 15). Thus, despite the high levels of NKG2A-expression in the Re:Mission trial, presence of NK cells controlled via NKG2A interactions impacted beneficially on the clinical outcome.

These data indicate that NK cell repertoires that are mainly licensed via NKG2A might exert a stronger anti-leukemic response after IL-2 stimulation compared to repertoires biased towards KIR-dependent licensing. With the observed elevated levels of NKG2A expression in the Re:Mission trial, it is tempting to speculate that HDC/IL-2 therapy could be combined with therapeutic antibodies targeting NKG2A, such as monalizumab. On the other hand, we did not observe any negative impact of high NKG2A expression or high frequency of NKG2A$^+$ NK cells in the trial. Thus, another possibility is that NKG2A-mediated signaling can be more easily overridden than corresponding KIR-mediated signaling, which would offer an explanation why the M/x genotype was protective during HDC/IL-2 AML therapy. Accordingly, multiple studies report that NKG2A-mediated inhibition may be overridden by activating stimuli, either by signaling via KIR2DS1 – HLA-C2 (297) or by co-engagement of NKG2D and MICA/B (26). In the presence of IL-2, we observed that NKG2A$^+$ iKIR$^-$ NK cells degranulated significantly more than NKG2A$^-$ S-iKIR$^+$ NK cells towards HLA-matched AML blasts (**Paper III**, fig. 15). This finding suggests that NK cells receive a stronger inhibitory input via their iKIR compared to NKG2A in the presence of cognate HLA molecules, and that this difference renders iKIR$^+$ less cytotoxic towards AML blasts after cytokine stimulation. As strong inhibitory input also tunes the responsiveness of NK cells, the hypothesis of a stronger interaction mediated by iKIRs as compared to NKG2A is supported by the lower responsiveness of NKG2A$^+$ KIR$^-$ NK cells compared to NKG2A$^-$ KIR$^+$ NK cells in assays towards HLA-deficient target cells (298).

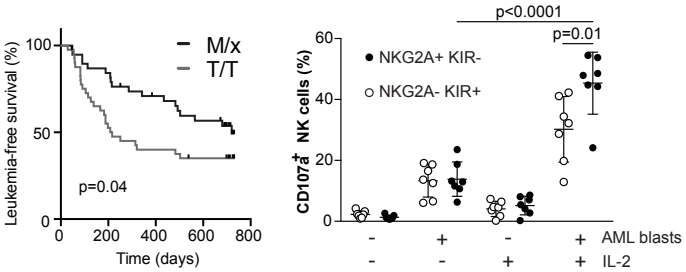

Figure 15. Impact of NK cell licensing via NKG2A or inhibitory KIRs. Left: Impact of the HLA-B -21 dimorphism on clinical outcome. Leukemia-free survival for AML patients receiving HDC/IL-2 therapy dichotomized based on their HLA-B -21 genotype; M/x vs. T/T (n=80). Right: Frequency of CD107a+ NKG2A$^-$ self-KIR$^+$ (open circles) or NKG2A$^+$ KIR$^-$ (filled circles) NK cells that were stimulated with 500 U/ml IL-2 overnight or not stimulated (ctrl) and exposed to C1/C1 matched AML blasts (n=7; one-way ANOVA and Bonferroni's multiple comparison test).

The protective impact observed for an M/x genotype (**Paper III**), together with the association between high levels of NS-iKIR$^+$ NK cells and favorable clinical outcome regardless of NKG2A expression (**Paper I**, fig. 14), suggests the presence of an inhibitory signaling hierarchy. NKG2A and iKIRs both signal via ITIMs, and it is intriguing that there seems to be a difference in their inhibitory potential. While NKG2A is a type II membrane protein, with its C terminal exposed to the exterior, and the N-terminal located at the cytoplasmic side of the membrane, type I membrane iKIRs are organized in the opposite direction (299). Hence, the two ITIMs in the intracellular portion of the receptors will be oriented in opposite directions, with the N terminal ITIM located proximally in KIRs and distally for NKG2A in relation to the membrane (300). This results in a difference in the ITIM-mediated binding to the two SH2 domains of SHP-1/2 on NKG2A and iKIR (see fig. 16). Even though docking of only one SH2 is capable of activating SHP-1/2, simultaneous engagement of both domains promotes a more potent activation (300). Moreover, the N terminal ITIM has been reported to play the most important role in both KIR and NKG2A signaling (300, 301). It is possible that the opposite orientation of the ITIMs results in different capacity of NKG2A and iKIRs to signal via the N terminal ITIM, or to engage both SH2 domains of SHP-1/2 simultaneously, which ultimately may lead to a disparate potency to transduce an inhibitory signal. Thus, NK cells expressing NKG2A may receive a lower level of inhibitory input compared to NK cells licensed via S-iKIRs. In a missing-self setting, the NKG2A$^-$ S-iKIR$^+$ NK cells will accordingly respond stronger than NKG2A$^+$ iKIR$^-$ NK cells. However, in presence of IL-2 stimulation, the magnitude of the activating input is increased, and as the weaker inhibitory signal received by NKG2A$^+$ S-KIR$^-$ NK cells may be more easily overridden, these NK cells will exert stronger degranulation responses against HLA-matched malignant cells (illustrated in figure 18).

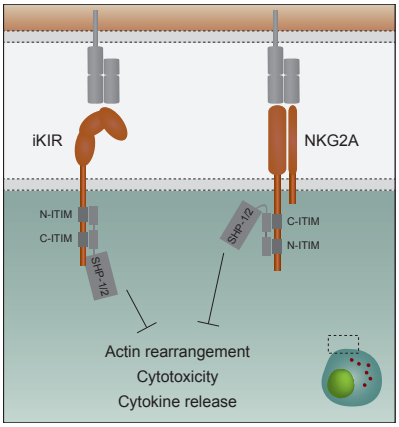

Figure 16. Signal transduction by NKG2A and inhibitory KIRs. NKG2A and KIR have their ITIMs oriented in opposite directions, with the N-terminal membrane-proximal in iKIRs and membrane-distal in NKG2A. Thus, the binding of SHP-1/2, via the two SH-2 domains, will differ in these two inhibitory receptors. SHP-1/2 transduces the inhibitory signal further in the intracellular signaling cascade, ultimately resulting in inhibition of NK cell effector functions.

In parallel with reports implicating unlicensed NK cells as important effector cells in combating malignancies, a population of CMV-induced adaptive NK cells has been proposed to be protective in AML (191). Patients seropositive for CMV harbor a larger fraction of adaptive $CD56^{dim}$ NK cells characterized by high expression of NKG2C, CD57 and self-KIRs (**Paper II**, 171:Beziat, 2013 #54, 174). It may seem paradoxical that the proposed effector cells would be on the one side immature, hyporesponsive and unlicensed, and on the other side highly mature with high expression of S-iKIRs. However, the potential cytotoxicity of different NK cell phenotypes may be context-dependent. Within the Re:Mission trial, there was no correlation between the abundance of highly mature $CD56^{dim}$ $NKG2A^{-}$ KIR^{+} $NKG2C^{+}$ $CD57^{+}$ NK cells and clinical outcome (**Paper II**). It is tempting to speculate that these otherwise protective NK cells lose relative importance during cytokine-based immunotherapy. Mature $CD57^{+}$ $CD56^{dim}$ NK cells reportedly express lower levels of IL-2Rβ (60), and CMV-induced adaptive NK cells were less responsive to cytokine stimulation, including IL-2 and IL-15 (168, 172, 302). Hence, in the context of cytokine-based immunotherapy, the CMV-induced adaptive NK cells might not be the main NK cell subpopulation that becomes activated. Instead, other normally hyporesponsive, low-grade-inhibited NK cells may become pivotal effector cells. However, among the CMV-seropositive group of patients, who harbor low levels of these autoreactive NK cells, a high proportion of $NKG2C^{+}$ NK cells after the first treatment cycle was indeed associated with lower relapse rate (fig. 17, un-published data). It is thus possible that patients that lack low-grade-inhibited S-iKIR NK cells benefit from presence of more mature NK cells with high expression of the activating NKG2C receptor.

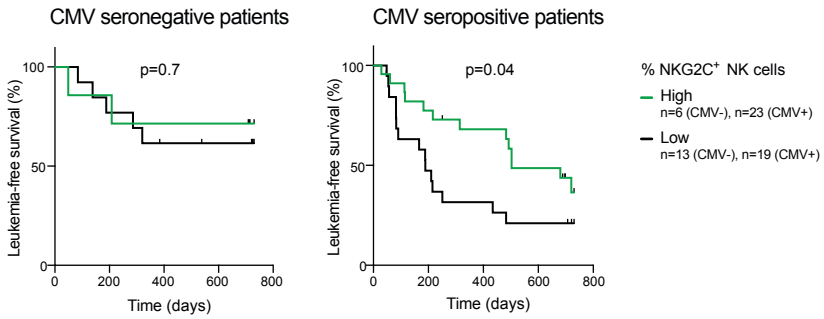

Figure 17. Impact of NKG2C$^+$ NK cells on clinical outcome. Graphs show leukemia-free survival after first cycle of HDC/IL-2 treatment for AML patients in the Re:Mission trial. Patients were dichotomized based on high or low proportion of NKG2C$^+$ NK cells among the mature CD56^{dim} NKG2A$^-$ NK cells. Outcomes were compared using the log-rank test.

It should be noted that there is no consensus phenotype of adaptive NK cells, and the phenotypic analysis in **Paper II** did not include expression of TIGIT, Siglec-7 or PD-1, or abundance of FcεRγ, EAT-2, SYK or PLZF, all reported to be altered in adaptive NK cells from CMV seropositive individuals (168, 172, 175, 303). Therefore, it cannot be excluded that presence of specific adaptive NK cells could associate with a beneficial outcome not detected in the analysis.

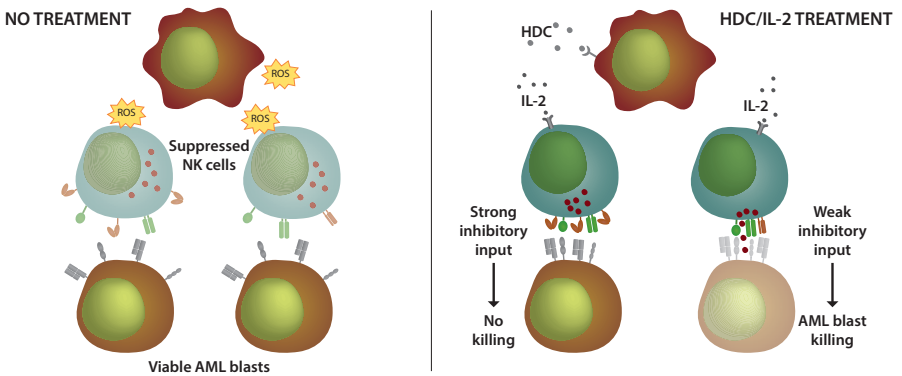

Figure 18. HDC/IL-2-induced NK cell-mediated killing of AML cells. Reactive oxygen species (ROS) produced by myeloid cells suppress NK cell functions and the NK cells cannot respond to AML blasts. Treatment with histamine dihydrochloride (HDC) inhibits formation of ROS, and allows IL-2-stimulation of NK cells. Upon interaction with AML blasts, NK cells that receive activating input in parallel with low inhibitory input may mediate cytotoxicity against the leukemic blasts.

Impact of regulatory T cells during HDC/IL-2 immunotherapy

A potential caveat with IL-2-based immunotherapy is the induced proliferation and expansion of regulatory T cells (T_{reg}), as these cells express the high-affinity IL-2 receptor (304, 305). T_{regs} suppress cytotoxic functions of lymphocytes, among them NK cell cytokine production and cytotoxicity (306, 307). In fact, IL-2-driven T_{reg} expansion is evaluated as a strategy to decrease acute GvHD or chronic GvHD after allo-SCT (305, 308-310). In accordance with these studies, we observed that HDC/IL-2 induced profound T_{reg} expansion in the Re:Mission trial (**Paper SII**). Surprisingly, no association was observed between clinical outcome and the number of T_{regs} at treatment start, after three weeks of therapy, or the magnitude of the T_{reg} expansion during those three weeks. Hence, T_{reg} numbers after three weeks of treatment probably reflects a patient's capacity to mount a treatment response rather than immune system inhibition. A possible explanation to the failure of T_{regs} to negatively impact on outcome was offered by the finding that the expanded T_{reg} population collapsed to baseline levels already one week after the end of the first treatment cycle, while NK cell levels remained elevated (fig. 19) (**Paper SII**, 280, 281). The T_{reg} induction was also significantly lower during later treatment cycles, possibly due to exhaustion of the supply of T_{regs}. However, we did observe that a high reduction of T_{regs} between the first and later cycles correlated with improved clinical outcome (**Paper II**). Collectively, these results suggest that T_{regs} may negatively impact on the efficiency of HDC/IL-2 immunotherapy, but that the benefit of IL-2-induced NK cell expansion dominates.

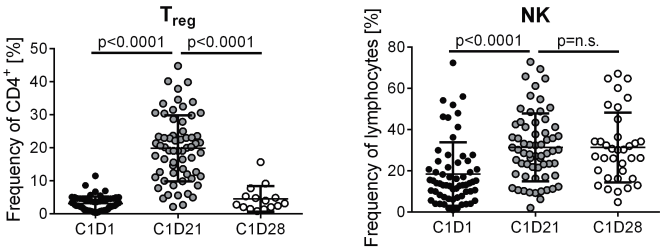

Figure 19. HDC/IL-2-induced expansion of T_{regs} and NK cells. Graphs show frequency of T_{regs} and NK cells before HDC/IL-2 treatment start (C1D1), after the first treatment cycle (C1D21) and one week after the end of the first treatment cycle (C1D28).

There is a possibility that combining HDC/IL-2 with therapeutic approaches that target T_{regs} might further improve the efficacy of this immunotherapy. Several strategies to avoid T_{reg} induction or deplete the T_{reg} population have been proposed. Approaches to inhibit T_{regs} include blocking CTLA-4, or targeting T_{reg} proliferation and function using anti-cancer drugs (306). Moreover, conjugating IL-2 to diphtheria toxin (IL2DT) has been tested as a strategy to deplete CD25-expressing cells before adoptive NK cell-transfer in AML (311). Although this strategy resulted in enhanced expansion of donor NK cells, it may be detrimental in a non-transplant setting, as it would also target $CD25^{+}$ $CD56^{bright}$ NK cells. Instead, an alternative approach would be to replace IL-2 with a lymphocyte-stimulating cytokine that does not stimulate T_{regs}, such as a modified version of IL-2, an IL-2 "superkine", with increased binding affinity to IL-2Rβ but less effect on T_{regs}. As discussed above, an additional alternative would be to combine HDC with IL-15 or engineered IL-15 agonist complexes that do not trigger T_{regs} (258).

CONCLUDING REMARKS

The detailed analyses of the immunological parameters within the Re:Mission study have revealed a set of biomarkers and other factors that seem to impact on clinical outcome. These include age, the number of induction cycles acquired to achieve CR, HLA class I expression in the myeloid compartment, expression of NKp46, presence of S-iKIR$^-$ NK cells, CMV serostatus, genotype of KIR/HLA and -21 HLA-B, and, as not mentioned in this thesis, transition of effector memory T cells (T_{EM}) to effector T cells (T_{eff}). While the observed associations between immune status and outcome should be confirmed in future studies, the results point to biomarkers that may be useful in prognosticating outcome, and in the selection of optimal therapy. The Re:Mission trial was a phase IV trial, and this did not include a control arm of patients who did not receive HDC/IL-2 therapy. It is conceivable that some biomarkers are predictive specifically for the outcome of the immunotherapy, whereas other biomarkers may prognosticate clinical outcome also in untreated patients (fig. 20). Yet another group of biomarkers may be both prognostic and predictive. For example, high numbers of CD56^{bright} NK cells at treatment start were associated with increased LFS in the trial. High numbers of these cells may reflect bone marrow recovery, which presumably is beneficial for all patients. On the other hand, immature CD56^{bright} NK cells express CD25 and hence respond vigorously to IL-2. Thus, pre-treatment levels of CD56^{bright} NK cells will most likely also affect the treatment response. Another example is the phenomenon of CD8 T cell transition; in the trial, HDC/IL-2 immunotherapy induced a shift in the CD8$^+$ T cell population in a fraction of patients, which resulted in decreased percentage of effector memory T cells and increased percentage of effector T cells (**Paper SI**). Patients displaying such a shift showed superior LFS and OS. Although the shift is clearly therapy-dependent, it cannot be excluded that the altered distribution of T cell subsets may reflect inherent immunity that determines favorable prognosis also in patients that do not receive immunotherapy.

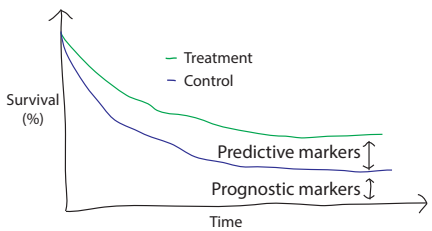

Figure 20. Prognostic and predictive markers. Prognostic factors provide information of outcome in untreated individuals, whereas predictive factors may predict the outcome for individuals after treatment.

Another limitation to the conclusions of the Re:Mission trial is that the trial design did not allow for analyses of the contribution by the respective components of the HDC/IL-2 regimen for the observed, and potentially clinically relevant, immunomodulation. While monotherapy with IL-2 reportedly does not prevent relapse in AML, it seems probable that many aspects of immune modulation noted during therapy, including NK cell expansion in blood and induction of NK cell NCR expression, are indeed explained by the IL-2 component. It may thus be hypothesized that the addition of HDC to a regimen of IL-2 unleashes NK cell and T cell functions, including their inducibility by IL-2, in the malignant microenvironment by targeting a pivotal mechanism of immuno-suppression.

CMV seropositivity did not affect the outcome in a control cohort of non-transplanted AML patients in CR. Thus the negative impact of CMV seropositivity observed in the Re:Mission trial (**Paper II**) is likely restricted to patients receiving immunotherapies that activate normally hypo-responsive autoreactive NK cell populations. Accordingly, the positive impact of a genetic KIR/HLA discordance, predicting presence of NK cells characterized by low inhibitory input, in malignancies has been observed in settings where the treatment has disturbed the homeostasis of the immune system (by either cytokines, therapeutic antibodies or autologous transplantation) (**Paper I-III**, 208, 209, 294). These findings suggest that HDC/IL-2 is responsible for the improved clinical outcome observed for patients harboring NK cell repertoires characterized by low-grade inhibition.

Based on our data implicating NK cells and T cells as anti-leukemic effector cells during HDC/IL-2 therapy in AML, the question emerges whether the two populations independently contribute to the anti-leukemic effect. Analysis of the impact of T_{EM}-T_{eff} transition and NKp46^{high} on clinical outcome revealed an additive effect when patients were both transition-positive and harbored NK cells with high NKp46 expression (**Paper SI**). Moreover, while the contribution of a high NKp46 expression impacted on leukemic relapse only for patients with a missing ligand genotype but not for patients with all ligands present, a T cell-transition was associated with reduced relapse in both patient groups (**Paper I**). Together, these findings suggest that NK cells as well as cytotoxic T cells exert anti-leukemic action during HDC/IL-2 therapy in AML.

ACKNOWLEDGEMENT

You are many that I want to thank for making this thesis possible;

First of all, the biggest thanks to my main supervisor **Fredrik Bergh Thorén**. I am grateful that you gave me the chance to work in your group, and for introducing me to the world of immunology and NK cells. It has been a great adventure to work with you, at all times ready for discussion providing enthusiastic and intellectual input. I admire your ability to always strive for improvement, which without a doubt has helped me to reach a higher level. Also, thanks for bringing us to the top of an active volcano during one of our conference trips, it was great fun!

Karin Christenson, co-supervisor. Thanks for sharing your expertise in neutrophil biology. We have had some extensive lab-sessions together, and it has been great to work together with you. Thanks for your care and support, and also for being an excellent room mate during our conferences in Italy!

Johan Bylund, co-supervisor. Thanks for your support during this time, and for providing me with perspectives from outside the "NK-bubble".

Thanks to **Kristoffer Hellstrand** for your generous supervision (without actually being my supervisor) and for your excellent input on papers and this thesis. And thanks for the bringing the Friday feeling with your guitar and song. **Anna Martner**, thank you for all valuable input in my projects, and for sharing your inspirational competence.

Many thanks to my fellow PhD-student colleagues: **Hanna** for inspiring me with your genuine "all-in"-mentality and engagement in both research and everyday issues, and for your fantastic support during the writing of this thesis. **Alexander** for topnotch collaboration in the lab and on our papers, and for sharing your skills in developing and trying out new techniques and assays. **Ebru** for travelling the PhD-student-time together, and for your care and kindness. **Junko** for your hugs. **Malin N** for creating a friendly atmosphere in the lab with your positive attitude. **Brwa** for taking on the PhD-student role in the Thorén group with great enthusiasm. **Belson** for contributing to making the a great place to work.

Also many thanks to all other lab members: **Johan A**, for always being helpful and creating a nice atmosphere in the lab. **Ali** for being a great room companion and

tolerating my mess during intense writing periods. **Roberta** for providing the most friendly environment. **Anna R** for sharing your desk and positive energy, and for great co-work during the labs in the Re:Mission project. And to the newcomers, **Annika** and **Anna S** for joining the lab with energy and enthusiasm. **Malin P** for your work on the neutrophil paper. And thanks to former lab members: **Rebecca** for introducing me in the lab and sharing both your knowledge and projects with me. Also thanks for being an outstanding travel partner! **Frida** for sharing all your knowledge in multicolor flow cytometry, for great collaboration in the Re:Mission project, and for some fun days in Montreal. **Olle** for creating a nice atmosphere in the lab and for generously contributing with your expertise on hematology. **Lotta M** for your excellent pedagogical skills that has not only made it enjoyable to teach in your courses, but that has also provided me with much new knowledge. I would also like to thank all students that have worked in the lab during these years, it has always been fun to follow your progress and you have contributed with enthusiasm and new energy in the lab.

Thanks to **Patric** and **Julie** for first introducing me to research and the academia during my work in the Biological Physics group at Chalmers.

To the **BT-06** class at the Chalmers biotechnology program, thanks for filling that time with memories and experiences, and for making it a bit less tough. Special thanks to **Eva**, **Mona** and **Erik** for your friendship, and to **Leif**, **David** and **Emma** for nice and valuable lunch-discussions.

I would also like to thank my friends, in particular **Helena** and **Lotta**, who have accompanied me since early childhood, and who has provided great support, care and encouragement during the PhD-journey.

Endless thanks to my mom, **Jenny**, and dad, **Christer**, who have supported and encouraged me in my dreams and ideas throughout life. My sister and friend **Sigrid**, I am so grateful for having you in my life, knowing that you're always only a phone-call away (even though our different choices of careers have resulted in somewhat different working hours ;)). Thank you **Farmor May** for all your love, you are an inspiration with your never-ending curiosity. Also, thanks to my extended family for you care and support.

David, you wrote in your thesis that you would "pay back the duties". You have done so much more than that, with your fantastic support, loving care and never-ending understanding. This journey would not have been the same without you and I look soo much forward to be mr and mrs PhD, and have time to realize our dreams. I love you.

BIBLIOGRAPHY

1. Goodman, L. S., and M. M. Wintrobe. 1946. Nitrogen mustard therapy; use of methyl-bis (beta-chloroethyl) amine hydrochloride and tris (beta-chloroethyl) amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. *JAMA* 132: 126-132.
2. Couzin-Frankel, J. 2013. Breakthrough of the year 2013. Cancer immunotherapy. *Science* 342: 1432-1433.
3. Cerwenka, A., and L. L. Lanier. 2016. Natural killer cell memory in infection, inflammation and cancer. *Nat. Rev. Immunol.* 16: 112-123.
4. Romee, R., S. E. Schneider, J. W. Leong, J. M. Chase, C. R. Keppel, R. P. Sullivan, M. A. Cooper, and T. A. Fehniger. 2012. Cytokine activation induces human memory-like NK cells. *Blood* 120: 4751-4760.
5. Logie, C., and H. G. Stunnenberg. 2016. Epigenetic memory: A macrophage perspective. *Semin. Immunol.* 28: 359-367.
6. Orange, J. S. 2008. Formation and function of the lytic NK-cell immunological synapse. *Nat. Rev. Immunol.* 8: 713-725.
7. Biron, C. A., K. S. Byron, and J. L. Sullivan. 1989. Severe Herpesvirus Infections in an Adolescent without Natural Killer Cells. *N. Engl. J. Med.* 320: 1731-1735.
8. de Vries, E., H. R. Koene, J. M. Vossen, J. W. Gratama, A. E. von dem Borne, J. L. Waaijer, A. Haraldsson, M. de Haas, and M. J. van Tol. 1996. Identification of an unusual Fc gamma receptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. *Blood* 88: 3022-3027.
9. Jawahar, S., C. Moody, M. Chan, R. Finberg, R. Geha, and T. Chatila. 1996. Natural Killer (NK) cell deficiency associated with an epitope-deficient Fc receptor type IIIA (CD16-II). *Clin. Exp. Immunol.* 103: 408-413.
10. Mace, E. M., V. Bigley, J. T. Gunesch, I. K. Chinn, L. S. Angelo, M. A. Care, S. Maisuria, M. D. Keller, S. Togi, L. B. Watkin, D. F. LaRosa, S. N. Jhangiani, D. M. Muzny, A. Stray-Pedersen, Z. Coban Akdemir, J. B. Smith, M. Hernandez-Sanabria, D. T. Le, G. D. Hogg, T. N. Cao, A. G. Freud, E. P. Szymanski, S. Savic, M. Collin, A. J. Cant, R. A. Gibbs, S. M. Holland, M. A. Caligiuri, K. Ozato, S. Paust, G. M. Doody, J. R. Lupski, and J. S. Orange. 2017. Biallelic mutations in IRF8 impair human NK cell maturation and function. *J. Clin. Invest.* 127: 306-320.
11. Malmberg, K. J., M. Carlsten, A. Bjorklund, E. Sohlberg, Y. T. Bryceson, and H. G. Ljunggren. 2017. Natural killer cell-mediated immunosurveillance of human cancer. *Semin. Immunol.*
12. Costello, R. T., S. Sivori, E. Marcenaro, M. Lafage-Pochitaloff, M.-J. Mozziconacci, D. Reviron, J.-A. Gastaut, D. Pende, D. Olive, and A. Moretta. 2002. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. *Blood* 99: 3661-3667.

13. Fauriat, C., S. Just-Landi, F. Mallet, C. Arnoulet, D. Sainty, D. Olive, and R. T. Costello. 2007. Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. *Blood* 109: 323-330.
14. Kiessling, R., E. Klein, and H. Wigzell. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* 5: 112-117.
15. Herberman, R. B., M. E. Nunn, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer* 16: 216-229.
16. Kärre, K. 1981. On the immunobiology of natural killer cells. *Doctoral thesis, Karolinska Institute*.
17. Kärre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2 deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319: 675-678.
18. Ljunggren, H.-G., and K. Kärre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* 11: 237-244.
19. Kärre, K. 2008. Natural killer cell recognition of missing self. *Nat. Immunol.* 9: 477-480.
20. Ljunggren, H. G., and K. Kärre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *The Journal of Experimental Medicine* 162: 1745-1759.
21. Karlhofer, F. M., R. K. Ribaudo, and W. M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358: 66-70.
22. Moretta, A., M. Vitale, S. Sivori, C. Bottino, L. Morelli, R. Augugliaro, M. Barbaresi, D. Pende, E. Ciccone, M. Lopez-Botet, and L. Moretta. 1994. Human natural killer cell receptors for HLA-class I molecules. Evidence that the Kp43 (CD94) molecule functions as receptor for HLA-B alleles. *J. Exp. Med.* 180: 545-555.
23. Wagtmann, N., R. Biassoni, C. Cantoni, S. Verdiani, M. S. Malnati, M. Vitale, C. Bottino, L. Moretta, A. Moretta, and E. O. Long. 1995. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* 2: 439-449.
24. Sivori, S., M. Vitale, L. Morelli, L. Sanseverino, R. Augugliaro, C. Bottino, L. Moretta, and A. Moretta. 1997. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *The Journal of Experimental Medicine* 186: 1129-1136.
25. Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta, and A. Moretta. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *The Journal of Experimental Medicine* 187: 2065-2072.
26. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727-729.
27. Long, E. O., H. S. Kim, D. Liu, M. E. Peterson, and S. Rajagopalan. 2013. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu. Rev. Immunol.* 31: 227-258.

28. Moretta, A., G. Tambussi, C. Bottino, G. Tripodi, A. Merli, E. Ciccone, G. Pantaleo, and L. Moretta. 1990. A novel surface antigen expressed by a subset of human CD3- CD16+ natural killer cells. Role in cell activation and regulation of cytolytic function. *J. Exp. Med.* 171: 695-714.
29. Moretta, A., C. Bottino, D. Pende, G. Tripodi, G. Tambussi, O. Viale, A. M. Orengo, M. Barbaresi, A. Merli, E. Ciccone, and L. Moretta. 1990. Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J. Exp. Med.* 172: 1589-1598.
30. Moretta, A., M. Vitale, C. Bottino, A. M. Orengo, L. Morelli, R. Augugliaro, M. Barbaresi, E. Ciccone, and L. Moretta. 1993. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J. Exp. Med.* 178: 597-604.
31. Litwin, V., J. Gumperz, P. Parham, J. H. Phillips, and L. L. Lanier. 1994. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J. Exp. Med.* 180: 537-543.
32. Vilches, C., and P. Parham. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu. Rev. Immunol.* 20: 217-251.
33. Rajagopalan, S., and E. O. Long. 2012. KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front. Immunol.* 3: 258.
34. Parham, P., and A. Moffett. 2013. Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nat. Rev. Immunol.* 13: 133-144.
35. Ahlenstiel, G., M. P. Martin, X. Gao, M. Carrington, and B. Rehermann. 2008. Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. *J. Clin. Invest.* 118: 1017-1026.
36. Sim, M. J. W., S. A. Malaker, A. Khan, J. M. Stowell, J. Shabanowitz, M. E. Peterson, S. Rajagopalan, D. F. Hunt, D. M. Altmann, E. O. Long, and R. J. Boyton. 2017. Canonical and Cross-reactive Binding of NK Cell Inhibitory Receptors to HLA-C Allotypes Is Dictated by Peptides Bound to HLA-C. *Front. Immunol.* 8: 193.
37. Stewart, C. A., F. Laugier-Anfossi, F. Vély, X. Saulquin, J. Riedmuller, A. Tisserant, L. Gauthier, F. Romagne, G. Ferracci, F. A. Arosa, A. Moretta, P. D. Sun, S. Ugolini, and E. Vivier. 2005. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proceedings of the National Academy of Sciences* 102: 13224-13229.
38. Cella, M., A. Longo, G. B. Ferrara, J. L. Strominger, and M. Colonna. 1994. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *The Journal of Experimental Medicine* 180: 1235-1242.
39. Marra, J., J. Greene, J. Hwang, J. Du, L. Damon, T. Martin, and J. M. Venstrom. 2015. KIR and HLA genotypes predictive of low-affinity interactions are associated with lower relapse in autologous hematopoietic cell transplantation for acute myeloid leukemia. *J. Immunol.* 194: 4222-4230.
40. Brooks, A. G., J. C. Boyington, and P. D. Sun. 2000. Natural killer cell recognition of HLA class I molecules. *Rev. Immunogenet.* 2: 433-448.

41. Parham, P., P. J. Norman, L. Abi-Rached, and L. A. Guethlein. 2012. Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 367: 800-811.
42. IPD-KIR. 2017. IPD - the Immuno Polymorphism Database. EMBL-EBI - The Eurpean Bioinformatics Institute, https://www.ebi.ac.uk/ipd/.
43. Foley, B., M. Felices, F. Cichocki, S. Cooley, M. R. Verneris, and J. S. Miller. 2014. The biology of NK cells and their receptors affects clinical outcomes after hematopoietic cell transplantation (HCT). *Immunol. Rev.* 258: 45-63.
44. Borges, L., and D. Cosman. 2000. LIRs/ILTs/MIRs, inhibitory and stimulatory Ig-superfamily receptors expressed in myeloid and lymphoid cells. *Cytokine Growth Factor Rev.* 11: 209-217.
45. Colonna, M., F. Navarro, T. Bellon, M. Llano, P. Garcia, J. Samaridis, L. Angman, M. Cella, and M. Lopez-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *The Journal of Experimental Medicine* 186: 1809-1818.
46. Stanietsky, N., H. Simic, J. Arapovic, A. Toporik, O. Levy, A. Novik, Z. Levine, M. Beiman, L. Dassa, H. Achdout, N. Stern-Ginossar, P. Tsukerman, S. Jonjic, and O. Mandelboim. 2009. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* 106: 117858-117863.
47. Braud, V. M., D. S. Allan, C. O'Callaghan, K. Söderström, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, L. L. Lanier, and A. J. McMichael. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795-799.
48. Lee, N., M. Llano, M. Carretaro, A. Ishitani, F. Navarro, M. Lopez-Botet, and D. Geraghty. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci* 95: 5199-5204.
49. Brooks, A. G., P. E. Posch, C. J. Scorzelli, F. Borrego, and J. E. Coligan. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *The Journal of Experimental Medicine* 185: 795-800.
50. Horowitz, A., Z. Djaoud, N. Nemat-Gorgani, J. Blokhuis, H. G. Hilton, V. Béziat, K. J. Malmberg, P. J. Norman, L. A. Guethlein, and P. Parham. 2016. Class I HLA haplotypes form two schools that educate NK cells in different ways. *Science Immunology* 1: eeag1672.
51. Yunis, E. J., V. Romero, F. Diaz-Giffero, J. Zuniga, and P. Koka. 2007. Natural Killer Cell Receptor NKG2A/HLA-E Interaction Dependent Differential Thymopoiesis of Hematopoietic Progenitor Cells Influences the Outcome of HIV Infection. *Journal of Stem Cells* 2: 237-248.
52. Long, E. O. 2008. Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunol. Rev.* 224: 70-84.
53. Burshtyn, D. N., and E. O. Long. 1997. Regulation through inhibitory receptors: Lessons from natural killer cells. *Trends Cell Biol.* 7: 473-479.
54. Faure, M., D. F. Barber, S. M. Takahashi, T. Jin, and E. O. Long. 2003. Spontaneous Clustering and Tyrosine Phosphorylation of NK Cell Inhibitory Receptor Induced by Ligand Binding. *The Journal of Immunology* 170: 6107-6114.

55. Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709-713.
56. Yokoyama, W. M., and S. Kim. 2006. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunol. Rev.* 214: 143-154.
57. Brodin, P., T. Lakshmikanth, S. Johansson, K. Karre, and P. Hoglund. 2009. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* 113: 2434-2441.
58. Wende, H., M. Colonna, A. Ziegler, and A. Volz. 1999. Organization of the leukocyte receptor cluster (LRC) on human chromosome 19q13.4. *Mammalian Genome: Official Journal of the International Mammalian Genome Society* 10: 154-160.
59. Parham, P. 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat. Rev. Immunol.* 5: 201-214.
60. Bjorkstrom, N. K., P. Riese, F. Heuts, S. Andersson, C. Fauriat, M. A. Ivarsson, A. T. Bjorklund, M. Flodstrom-Tullberg, J. Michaelsson, M. E. Rottenberg, C. A. Guzman, H. G. Ljunggren, and K. J. Malmberg. 2010. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* 116: 3853-3864.
61. Malmberg, J. A. 2016. Conference presentation at the *16th Annual meeting of the Society for Natural Immunity*, Taormina, Italy.
62. Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105: 4416-4423.
63. Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 1999. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *The Journal of Experimental Medicine* 190: 1505-1516.
64. Brandt, C. S., M. Baratin, E. C. Yi, J. Kennedy, Z. Gao, B. Fox, B. Haldeman, C. D. Ostrander, T. Kaifu, C. Chabannon, A. Moretta, R. West, W. Xu, E. Vivier, and S. D. Levin. 2009. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J. Exp. Med.* 206: 1495-1503.
65. Pogge von Strandmann, E., V. R. Simhadri, B. von Tresckow, S. Sasse, K. S. Reiners, H. P. Hansen, A. Rothe, B. Boll, V. L. Simhadri, P. Borchmann, P. J. McKinnon, M. Hallek, and A. Engert. 2007. Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity* 27: 965-974.
66. Baychelier, F., A. Sennepin, M. Ermonval, K. Dorgham, P. Debre, and V. Vieillard. 2013. Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. *Blood* 122: 2935-2942.
67. Rosental, B., M. Brusilovsky, U. Hadad, D. Oz, M. Y. Appel, F. Afergan, R. Yossef, L. A. Rosenberg, A. Aharoni, A. Cerwenka, K. S. Campbell, A. Braiman, and A. Porgador. 2011. Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. *J. Immunol.* 187: 5693-5702.

68. Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409: 1055-1060.
69. Arnon, T. I., M. Lev, G. Katz, Y. Chernobrov, A. Porgador, and O. Mandelboim. 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* 31: 2680-2689.
70. Arnon, T. I., H. Achdout, O. Levi, G. Markel, N. Saleh, G. Katz, R. Gazit, T. Gonen-Gross, J. Hanna, E. Nahari, A. Porgador, A. Honigman, B. Plachter, D. Mevorach, D. G. Wolf, and O. Mandelboim. 2005. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat. Immunol.* 6: 515-523.
71. Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 285: 730-732.
72. Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein, and T. Spies. 1999. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc. Natl. Acad. Sci. U. S. A.* 96: 6879-6884.
73. Cosman, D., J. Müllberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N. J. Chaplupny. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14: 123-133.
74. Del Zotto, G., E. Marcenaro, P. Vacca, S. Sivori, D. Pende, M. Della Chiesa, F. Moretta, T. Ingegnere, M. C. Mingari, A. Moretta, and L. Moretta. 2017. Markers and function of human NK cells in normal and pathological conditions. *Cytometry Part B: Clinical Cytometry*.
75. Davis, S. J., and P. A. van der Merwe. 1996. The structure and ligand interactions of CD2: implications for T-cell function. *Immunol. Today* 17: 177-187.
76. Moretta, L., E. Montaldo, P. Vacca, G. Del Zotto, F. Moretta, P. Merli, F. Locatelli, and M. C. Mingari. 2014. Human natural killer cells: origin, receptors, function, and clinical applications. *Int. Arch. Allergy Immunol.* 164: 253-264.
77. Ivarsson, M. A., J. Michaelsson, and C. Fauriat. 2014. Activating killer cell Ig-like receptors in health and disease. *Front. Immunol.* 5: 184.
78. Vyas, Y. M., K. M. Mehta, M. Morgan, H. Maniar, L. Butros, S. Jung, J. K. Burkhardt, and B. Dupont. 2001. Spatial Organization of Signal Transduction Molecules in the NK Cell Immune Synapses During MHC Class I-Regulated Noncytolytic and Cytolytic Interactions. *The Journal of Immunology* 167: 4358-4367.
79. Davis, D. M., I. Chiu, M. Fassett, G. B. Cohen, O. Mandelboim, and J. L. Strominger. 1999. The human natural killer cell immune synapse. *Proceedings of the National Academy of Sciences* 96: 15062-15067.
80. Orange, J. S., K. E. Harris, M. M. Andzelm, M. M. Valter, R. S. Geha, and J. L. Strominger. 2003. The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proceedings of the National Academy of Sciences* 100: 14151-14156.
81. Schmidt, R. E., G. Bartley, H. Levine, S. F. Schlossman, and J. Ritz. 1985. Functional characterization of LFA-1 antigens in the interaction of human NK clones and target cells. *J. Immunol.* 135: 1020-1025.

82. Barber, D. F., M. Faure, and E. O. Long. 2004. LFA-1 Contributes an Early Signal for NK Cell Cytotoxicity. *The Journal of Immunology* 173: 3653-3659.
83. Bryceson, Y. T., H. G. Ljunggren, and E. O. Long. 2009. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. *Blood* 114: 2657-2666.
84. Kwon, H. J., N. Kim, and H. S. Kim. 2017. Molecular checkpoints controlling natural killer cell activation and their modulation for cancer immunotherapy. *Exp. Mol. Med.* 49: e311.
85. Riteau, B., D. F. Barber, and E. O. Long. 2003. Vav1 phosphorylation is induced by beta2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization. *J. Exp. Med.* 198: 469-474.
86. Krzewski, K., and J. E. Coligan. 2012. Human NK cell lytic granules and regulation of their exocytosis. *Front. Immunol.* 3: 335.
87. Atkinson, E. A., J. M. Gerrard, G. E. Hildes, and A. H. Greenberg. 1990. Studies of the mechanism of natural killer (NK) degranulation and cytotoxicity. *J. Leukoc. Biol.* 47: 39-48.
88. Maghazachi, A. A. 2005. Insights into seven and single transmembrane-spanning domain receptors and their signaling pathways in human natural killer cells. *Pharmacol. Rev.* 57: 339-357.
89. Hsu, H. T., E. M. Mace, A. F. Carisey, D. I. Viswanath, A. E. Christakou, M. Wiklund, B. Onfelt, and J. S. Orange. 2016. NK cells converge lytic granules to promote cytotoxicity and prevent bystander killing. *J. Cell Biol.* 215: 875-889.
90. Peterson, M. E., and E. O. Long. 2008. Inhibitory receptor signaling via tyrosine phosphorylation of the adaptor Crk. *Immunity* 29: 578-588.
91. Topham, N. J., and E. W. Hewitt. 2009. Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology* 128: 7-15.
92. Lopez, J. A., O. Susanto , M. R. Jenkins, N. Lukoyanova, V. R. Sutton, R. H. P. Law, A. Johnston, C. H. Bird, P. I. Bird, J. C. Whisstock, J. A. Trapani, H. R. Saibil, and I. Voskoboinik. 2013. Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood* 121: 2659-2668.
93. Trapani, J. A., and V. R. Sutton. 2003. Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Curr. Opin. Immunol.* 15: 533-543.
94. Lettau, M., H. Schmidt, D. Kabelitz, and O. Janssen. 2007. Secretory lysosomes and their cargo in T and NK cells. *Immunol. Lett.* 108: 10-19.
95. Warren, H. S., and M. J. Smyth. 1999. NK cells and apoptosis. *Immunol. Cell Biol.* 77: 64-75.
96. Krzewski, K., A. Gil-Krzewska, V. Nguyen, G. Peruzzi, and J. E. Coligan. 2013. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* 121: 4672-4683.
97. Fauriat, C., E. O. Long, H. G. Ljunggren, and Y. T. Bryceson. 2010. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 115: 2167-2176.
98. Grzywacz, B., N. Kataria, and M. R. Verneris. 2007. CD56(dim)CD16(+) NK cells downregulate CD16 following target cell induced activation of matrix metalloproteinases. *Leukemia* 21: 356-359; author reply 359.

99. Bryceson, Y. T., M. E. March, D. F. Barber, H. G. Ljunggren, and E. O. Long. 2005. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J. Exp. Med.* 202: 1001-1012.
100. Bryceson, Y. T., M. E. March, H. G. Ljunggren, and E. O. Long. 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 107: 159-166.
101. Stebbins, C. C., C. Watzl, D. D. Billadeau, P. J. Leibson, D. N. Burshtyn, and E. O. Long. 2003. Vav1 Dephosphorylation by the Tyrosine Phosphatase SHP-1 as a Mechanism for Inhibition of Cellular Cytotoxicity. *Mol. Cell. Biol.* 23: 6291-6299.
102. Matalon, O., S. Fried, A. Ben-Shmuel, M. H. Pauker, N. Joseph, D. Keizer, M. Piterburg, and M. Barda-Saad. 2016. Dephosphorylation of the adaptor LAT and phospholipase C-γ by SHP-1 inhibits natural killer cell cytotoxicity. *Science Signaling* 9: ra54.
103. Abeyweera, T. P., E. Merino, and M. Huse. 2011. Inhibitory signaling blocks activating receptor clustering and induces cytoskeletal retraction in natural killer cells. *J. Cell Biol.* 192: 675-690.
104. Bernson, E., A. Hallner, F. E. Sander, O. Wilsson, O. Werlenius, A. Rydstrom, R. Kiffin, M. Brune, R. Foa, J. Aurelius, A. Martner, K. Hellstrand, and F. B. Thoren. 2017. Impact of killer-immunoglobulin-like receptor and human leukocyte antigen genotypes on the efficacy of immunotherapy in acute myeloid leukemia. *Leukemia* E-pub ahead of print.
105. Vivier, E., J. A. Nunès, and F. Vély. 2005. Natural killer cell signaling pathways. *Science* 306: 1517-1519.
106. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proceedings of the National Academy of Sciences* 98: 11521-11526.
107. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science* 331: 44-49.
108. Caligiuri, M. A. 2008. Human natural killer cells. *Blood* 112: 461-469.
109. Bjorkstrom, N. K., H. G. Ljunggren, and J. Michaelsson. 2016. Emerging insights into natural killer cells in human peripheral tissues. *Nat. Rev. Immunol.* 16: 310-320.
110. Gaynor, L. M., and F. Colucci. 2017. Uterine Natural Killer Cells: Functional Distinctions and Influence on Pregnancy in Humans and Mice. *Front. Immunol.* 8: 467.
111. Fauriat, C., S. Andersson, A. T. Bjorklund, M. Carlsten, M. Schaffer, N. K. Bjorkstrom, B. C. Baumann, J. Michaelsson, H. G. Ljunggren, and K. J. Malmberg. 2008. Estimation of the Size of the Alloreactive NK Cell Repertoire: Studies in Individuals Homozygous for the Group A KIR Haplotype. *The Journal of Immunology* 181: 6010-6019.
112. Wagner, J. A., M. Rosario, R. Romee, M. M. Berrien-Elliott, S. E. Schneider, J. W. Leong, R. P. Sullivan, B. A. Jewell, M. Becker-Hapak, T. Schappe, S. Abdel-Latif, A. R. Ireland, D. Jaishankar, J. A. King, R. Vij, D. Clement, J. Goodridge, K. J. Malmberg, H. C. Wong, and T. A. Fehniger. 2017. CD56bright NK cells exhibit potent antitumor responses following IL-15 priming. *J. Clin. Invest.*
113. Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat. Immunol.* 5: 1260-1265.

114. Orange, J. S., B. Wang, C. Terhorst, and C. A. Biron. 1995. Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *The Journal of Experimental Medicine* 182: 1045-1056.
115. Aydin, E., J. Johansson, F. H. Nazir, K. Hellstrand, and A. Martner. 2017. Role of NOX2-Derived Reactive Oxygen Species in NK Cell-Mediated Control of Murine Melanoma Metastasis. *Cancer Immunol Res* 5: 804-811.
116. Altfeld, M., L. Fadda, D. Frleta, and N. Bhardwaj. 2011. DCs and NK cells: critical effectors in the immune response to HIV-1. *Nat. Rev. Immunol.* 11: 176-186.
117. Ferlazzo, G., M. L. Tsang, L. Moretta, G. Melioli, R. Steinman, M., and C. Münz. 2002. Human Dendritic Cells Activate Resting Natural Killer (NK) Cells and Are Recognized via the NKp30 Receptor by Activated NK Cells. *J. Exp. Med.* 195: 343-351.
118. Vitale, M., M. Della Chiesa, S. Carlomagno, D. Pende, M. Arico, L. Moretta, and A. Moretta. 2005. NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor. *Blood* 106: 566-571.
119. Ghiringhelli, F., C. Ménard, F. Martin, and L. Zitvogel. 2006. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. *Immunol. Rev.* 214: 229-238.
120. Waggoner, S. N., M. Cornberg, L. K. Selin, and R. M. Welsh. 2011. Natural killer cells act as rheostats modulating antiviral T cells. *Nature* 481: 394-398.
121. Nedvetzki, S., S. Sowinski, R. A. Eagle, J. Harris, F. Vély, D. Pende, J. Trowsdale, E. Vivier, S. Gordon, and D. M. Davis. 2007. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 109: 3776-3785.
122. Thoren, F. B., R. E. Riise, J. Ousback, M. Della Chiesa, M. Alsterholm, E. Marcenaro, S. Pesce, C. Prato, C. Cantoni, J. Bylund, L. Moretta, and A. Moretta. 2012. Human NK Cells induce neutrophil apoptosis via an NKp46- and Fas-dependent mechanism. *J. Immunol.* 188: 1668-1674.
123. Angelotti, F., A. Parma, G. Cafaro, R. Capecchi, A. Alunno, and I. Puxxeddu. 2017. One year in review 2017: pathogenesis of rheumatoid arthritis. *Clin. Exp. Rheumatol.* 35: 368-378.
124. El Kebir, D., and J. G. Filep. 2010. Role of neutrophil apoptosis in the resolution of inflammation. *ScientificWorldJournal* 10: 1731-1748.
125. Mocsai, A. 2013. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J. Exp. Med.* 210: 1283-1299.
126. Nauseef, W. M., and N. Borregaard. 2014. Neutrophils at work. *Nat. Immunol.* 15: 602-611.
127. Thomas, C. J., and K. Schroder. 2013. Pattern recognition receptor function in neutrophils. *Trends Immunol.* 34: 317-328.
128. Borregaard, N., and J. B. Cowland. 1997. Granules of the Human Neutrophilic Polymorphonuclear Leukocyte. *Blood* 89: 3503-3521.
129. Tecchio, C., and M. A. Cassatella. 2016. Neutrophil-derived chemokines on the road to immunity. *Semin. Immunol.* 28: 119-128.
130. Costantini, C., and M. A. Cassatella. 2011. The defensive alliance between neutrophils and NK cells as a novel arm of innate immunity. *J. Leukoc. Biol.* 89: 221-233.

131. Borregaard, N., O. E. Sorensen, and K. Theilgaard-Monch. 2007. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* 28: 340-345.
132. Christenson, K., L. Bjorkman, J. Karlsson, M. Sundqvist, C. Movitz, D. P. Speert, C. Dahlgren, and J. Bylund. 2011. In vivo-transmigrated human neutrophils are resistant to antiapoptotic stimulation. *J. Leukoc. Biol.* 90: 1055-1063.
133. Romero, A. I., F. B. Thoren, M. Brune, and K. Hellstrand. 2006. NKp46 and NKG2D receptor expression in NK cells with CD56dim and CD56bright phenotype: regulation by histamine and reactive oxygen species. *Br. J. Haematol.* 132: 91-98.
134. Mellqvist, U. H., M. Hansson, M. Brune, C. Dahlgren, S. Hermodsson, and K. Hellstrand. 2000. Natural killer cell dysfunction and apoptosis induced by chronic myelogenous leukemia cells: role of reactive oxygen species and regulation by histamine. *Blood* 96: 1961-1968.
135. Thoren, F. B., A. I. Romero, S. Hermodsson, and K. Hellstrand. 2007. The CD16–/CD56bright Subset of NK Cells Is Resistant to Oxidant-Induced Cell Death. *The Journal of Immunology* 179: 781-785.
136. Jost, S., and M. Altfeld. 2013. Control of human viral infections by natural killer cells. *Annu. Rev. Immunol.* 31: 163-194.
137. Korner, C., C. R. Simoneau, P. Schommers, M. Granoff, M. Ziegler, A. Holzemer, S. Lunemann, J. Chukwukelu, B. Corleis, V. Naranbhai, D. S. Kwon, E. P. Scully, S. Jost, F. Kirchhoff, M. Carrington, and M. Altfeld. 2017. HIV-1-Mediated Downmodulation of HLA-C Impacts Target Cell Recognition and Antiviral Activity of NK Cells. *Cell Host Microbe* 22: 111-119 e114.
138. Garrido, F., F. Perea, M. Bernal, A. Sanchez-Palencia, N. Aptsiauri, and F. Ruiz-Cabello. 2017. The Escape of Cancer from T Cell-Mediated Immune Surveillance: HLA Class I Loss and Tumor Tissue Architecture. *Vaccines (Basel)* 5.
139. Schlecker, E., N. Fiegler, A. Arnold, P. Altevogt, S. Rose-John, G. Moldenhauer, A. Sucker, A. Paschen, E. P. von Strandmann, S. Textor, and A. Cerwenka. 2014. Metalloprotease-mediated tumor cell shedding of B7-H6, the ligand of the natural killer cell-activating receptor NKp30. *Cancer Res.* 74: 3429-3440.
140. Matta, J., M. Baratin, L. Chiche, J. M. Forel, C. Cognet, G. Thomas, C. Farnarier, C. Piperoglou, L. Papazian, D. Chaussabel, S. Ugolini, F. Vely, and E. Vivier. 2013. Induction of B7-H6, a ligand for the natural killer cell-activating receptor NKp30, in inflammatory conditions. *Blood* 122: 394-404.
141. Pesce, S., G. Tabellini, C. Cantoni, O. Patrizi, D. Coltrini, F. Rampinelli, J. Matta, E. Vivier, A. Moretta, S. Parolini, and E. Marcenaro. 2015. B7-H6-mediated downregulation of NKp30 in NK cells contributes to ovarian carcinoma immune escape. *Oncoimmunology* 4: e1001224.
142. Semeraro, M., S. Rusakiewicz, V. Minard-Colin, N. F. Delahaye, D. Enot, F. Vély, A. Marabelle, B. Papoular, C. Piperoglou, M. Ponzoni, P. Perri, A. Tchirkov, J. Matta, V. Lapierre, T. Shekarian, S. Valsesia-Wittmann, F. Commo, N. Prada, V. Poirer-Colame, B. Bressac, S. Cotteret, L. Brugieres, F. Farace, N. Chaput, G. Kroemer, D. Valteau-Couanet, and L. Zitvogel. 2015. Clinical impact of the NKp30/B7-H6 axis in high-risk neuroblastoma patients. *Sci. Transl. Med.* 7: 283ra255.

143. Simhadri, V. R., K. S. Reiners, H. P. Hansen, D. Topolar, V. L. Simhadri, K. Nohroudi, T. A. Kufer, A. Engert, and E. Pogge von Strandmann. 2008. Dendritic cells release HLA-B-associated transcript-3 positive exosomes to regulate natural killer function. *PLoS One* 3: e3377.
144. Binici, J., and J. Koch. 2014. BAG-6, a jack of all trades in health and disease. *Cell. Mol. Life Sci.* 71: 1829-1837.
145. Pollmann, J., A. Rölle, M. Hofmann, and A. Cerwenka. 2017. Hepatitis C Virus and Human Cytomegalovirus-Natural Killer Cell Subsets in Persistent Viral Infections. *Front. Immunol.* 8: 566.
146. Bonagura, V. R., Z. Du, E. Ashouri, L. Luo, L. J. Hatam, J. A. DeVoti, D. W. Rosenthal, B. M. Steinberg, A. L. Abramson, D. W. Gjertson, E. F. Reed, and R. Rajalingam. 2010. Activating killer cell immunoglobulin-like receptors 3DS1 and 2DS1 protect against developing the severe form of recurrent respiratory papillomatosis. *Hum. Immunol.* 71: 212-219.
147. Espinoza, J. L., V. H. Nguyen, H. Ichimura, T. T. Pham, C. H. Nguyen, T. V. Pham, M. I. Elbadry, K. Yoshioka, J. Tanaka, L. Q. Trung, A. Takami, and S. Nakao. 2016. A functional polymorphism in the NKG2D gene modulates NK-cell cytotoxicity and is associated with susceptibility to Human Papilloma Virus-related cancers. *Sci. Rep.* 6: 39231.
148. Biron, C. A., and L. Brossay. 2001. NK cells and NKT cells in innate defense against viral infections. *Curr. Opin. Immunol.* 13: 458-464.
149. Sun, J. C., and L. L. Lanier. 2009. The Natural Selection of Herpesviruses and Virus-Specific NK Cell Receptors. *Viruses* 1: 362.
150. Ma, Y., X. Li, and E. Kuang. 2016. Viral Evasion of Natural Killer Cell Activation. *Viruses* 8: 95.
151. Charpak-Amikam, Y., T. Kubsch, E. Seidel, E. Oiknine-Djian, N. Cavaletto, R. Yamin, D. Schmiedel, D. Wolf, G. Gribaudo, M. Messerle, L. Cicin-Sain, and O. Mandelboim. 2017. Human cytomegalovirus escapes immune recognition by NK cells through the downregulation of B7-H6 by the viral genes US18 and US20. *Sci. Rep.* 7: 8661.
152. van der Ploeg, K., C. Chang, M. A. Ivarsson, A. Moffett, M. R. Wills, and J. Trowsdale. 2017. Modulation of Human Leukocyte Antigen-C by Human Cytomegalovirus Stimulates KIR2DS1 Recognition by Natural Killer Cells. *Front. Immunol.* 8: 298.
153. Khakoo, S. I., C. L. Thio, M. P. Martin, C. R. Brooks, X. Gao, J. Astemborski, J. Cheng, J. J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A. M. Little, G. J. Alexander, M. E. Cramp, S. J. O'Brien, W. M. C. Rosenberg, D. L. Thomas, and M. Carrington. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305: 872-874.
154. Bernard, N. F. 2017. Natural killer (NK) cell receptor-HLA ligand genotype combinations associated with protection from HIV infection: investigation of how protective genotypes influence anti HIV NK cell functions. *AIDS Res. Ther.* 14: 38.
155. McLaren, P. J., and M. Carrington. 2015. The impact of host genetic variation on infection with HIV-1. *Nat. Immunol.* 16: 577-583.
156. Merino, A. M., W. Song, D. He, J. Mulenga, S. Allen, E. Hunter, J. Tang, and R. A. Kaslow. 2012. HLA-B signal peptide polymorphism influences the rate of HIV-1 acquisition but not viral load. *J. Infect. Dis.* 205: 1797-1805.

157. Merino, A. M., S. Sabbaj, J. Easlick, P. Goepfert, R. A. Kaslow, and J. Tang. 2013. Dimorphic HLA-B signal peptides differentially influence HLA-E- and natural killer cell-mediated cytolysis of HIV-1-infected target cells. *Clin. Exp. Immunol.* 174: 414-423.
158. La Rosa, C., and D. J. Diamond. 2012. The immune response to human CMV. *Future Virol.* 7: 279-293.
159. Marchesi, F., F. Pimpinelli, F. Ensoli, and A. Mengarelli. 2017. Cytomegalovirus infection in hematologic malignancy settings other than the allogeneic transplant. *Hematol. Oncol.*: E-pub ahead of print.
160. Simanek, A. M., J. B. Dowd, G. Pawelec, D. Melzer, A. Dutta, and A. E. Aiello. 2011. Seropositivity to cytomegalovirus, inflammation, all-cause and cardiovascular disease-related mortality in the United States. *PLoS One* 6: e16103.
161. Bate, S. L., S. C. Dollard, and M. J. Cannon. 2010. Cytomegalovirus seroprevalence in the United States: the national health and nutrition examination surveys, 1988-2004. *Clin. Infect. Dis.* 50: 1439-1447.
162. Olsson, J., E. Kok, R. Adolfsson, H. Lovheim, and F. Elgh. 2017. Herpes virus seroepidemiology in the adult Swedish population. *Immun. Ageing* 14: 10.
163. Noriega, V., V. Redmann, T. Gardner, and D. Tortorella. 2012. Diverse immune evasion strategies by human cytomegalovirus. *Immunol. Res.* 54: 140-151.
164. Vivier, E., E. Tomasello, M. Baratin, T. Walzer, and S. Ugolini. 2008. Functions of natural killer cells. *Nat. Immunol.* 9: 503-510.
165. Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, B. P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. J. McMichael, and G. W. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 287: 1031-1033.
166. Orr, M. T., W. J. Murphy, and L. L. Lanier. 2010. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat. Immunol.* 11: 321-327.
167. Cichocki, F., J. S. Miller, S. K. Anderson, and Y. T. Bryceson. 2013. Epigenetic regulation of NK cell differentiation and effector functions. *Front. Immunol.* 4: 55.
168. Schlums, H., F. Cichocki, B. Tesi, J. Theorell, V. Beziat, T. D. Holmes, H. Han, S. C. Chiang, B. Foley, K. Mattsson, S. Larsson, M. Schaffer, K. J. Malmberg, H. G. Ljunggren, J. S. Miller, and Y. T. Bryceson. 2015. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity* 42: 443-456.
169. Lee, J., T. Zhang, I. Hwang, A. Kim, L. Nitschke, M. Kim, J. M. Scott, Y. Kamimura, L. L. Lanier, and S. Kim. 2015. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. *Immunity* 42: 431-442.
170. Guma, M., A. Angulo, C. Vilches, N. Gómez-Lozano, N. Malats, and M. López-Botet. 2004. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* 104: 3664-3671.
171. Lopez-Verges, S., J. M. Milush, B. S. SChwartz, M. J. Pando, J. Jarjoura, V. A. York, J. P. Houchins, S. Miller, S.-M. Kang, P. J. Norris, D. F. Nixon, and L. L. Lanier. 2011. Expansion of a unique CD57+NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proceedings of the National Academy of Sciences* 108: 14725-14732.

172. Pesce, S., M. Greppi, G. Tabellini, F. Rampinelli, S. Parolini, D. Olive, L. Moretta, A. Moretta, and E. Marcenaro. 2017. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. *J. Allergy Clin. Immunol.* 139: 335-346 e333.
173. Beziat, V., L. L. Liu, J. A. Malmberg, M. A. Ivarsson, E. Sohlerg, A. T. Björklund, C. Retiere, E. Sverremark-Ekström, J. Traherne, P. Ljungman, M. Schaffer, D. A. Price, J. Trowsdale, J. Michaelsson, H. G. Ljunggren, and K. J. Malmberg. 2013. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood* 121: 2678-2688.
174. Foley, B., S. Cooley, M. R. Verneris, M. Pitt, J. Curtsinger, X. Luo, S. Lopez-Verges, L. L. Lanier, D. Weisdorf, and J. S. Miller. 2012. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood* 119: 2665-2674.
175. Della Chiesa, M., M. Falco, M. Podestà, F. Locatelli, L. Moretta, F. Frassoni, and A. Moretta. 2012. Phenotypic and functional heterogeneity of human NK cells developing after umbilical cord blood transplantation: a role for human cytomegalovirus? *Blood* 119: 399-410.
176. Rolle, A., J. Pollmann, E. M. Ewen, V. T. Le, A. Halenius, H. Hengel, and A. Cerwenka. 2014. IL-12-producing monocytes and HLA-E control HCMV-driven NKG2C+ NK cell expansion. *J. Clin. Invest.* 124: 5305-5316.
177. Rolle, A., A. Halenius, E. M. Ewen, A. Cerwenka, H. Hengel, and F. Momburg. 2016. CD2-CD58 interactions are pivotal for the activation and function of adaptive natural killer cells in human cytomegalovirus infection. *Eur. J. Immunol.* 46: 2420-2425.
178. Wagner, J. A., and T. A. Fehniger. 2016. Human Adaptive Natural Killer Cells: Beyond NKG2C. *Trends Immunol.* 37: 351-353.
179. Liu, L. L., J. Landskron, E. H. Ask, M. Enqvist, E. Sohlberg, J. A. Traherne, Q. Hammer, J. P. Goodridge, S. Larsson, J. Jayaraman, V. Y. S. Oei, M. Schaffer, K. Tasken, H. G. Ljunggren, C. Romagnani, J. Trowsdale, K. J. Malmberg, and V. Beziat. 2016. Critical Role of CD2 Co-stimulation in Adaptive Natural Killer Cell Responses Revealed in NKG2C-Deficient Humans. *Cell Rep* 15: 1088-1099.
180. Bjorkstrom, N. K., T. Lindgren, M. Stoltz, C. Fauriat, M. Braun, M. Evander, J. Michaelsson, K. J. Malmberg, J. Klingstrom, C. Ahlm, and H. G. Ljunggren. 2011. Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J. Exp. Med.* 208: 13-21.
181. Beziat, V., O. Dalgard, T. Asselah, P. Halfon, P. Bedossa, A. Boudifa, B. Hervier, I. Theodorou, M. Martinot, P. Debre, N. K. Bjorkstrom, K. J. Malmberg, P. Marcellin, and V. Vieillard. 2012. CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *Eur. J. Immunol.* 42: 447-457.
182. Guma, M., C. Cabrera, I. AErkizia, M. Bofill, B. Clotet, L. Ruiz, and M. Lopez-Botet. 2006. Human cytomegalovirus infection is associated with increased proportions of NK cells that express the CD94/NKG2C receptor in aviremic HIV-1-positive patients. *The Journal of Infectious Diseases* 194: 38-41.
183. Petitdemange, C., P. Becquart, N. Wauquier, V. Beziat, P. Debre, E. M. Leroy, and V. Vieillard. 2011. Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. *PLoS Pathog.* 7: e1002268.

184. Foley, B., S. Cooley, M. R. Verneris, J. Curtsinger, X. Luo, E. K. Waller, C. Anasetti, D. Weisdorf, and J. S. Miller. 2012. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J. Immunol.* 189: 5082-5088.
185. Sun, J. C., S. Madera, N. A. Bezman, J. N. Beilke, M. H. Kaplan, and L. L. Lanier. 2012. Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J. Exp. Med.* 209: 947-954.
186. Lönnqvist, B., O. Ringdén, P. Ljungman, B. Wahren, and G. Gahrton. 1986. Reduced risk of recurrent leukaemia in bone marrow transplant recipients after cytomegalovirus infection. *Br. J. Haematol.* 63: 671-679.
187. Elmaagacli, A. H., N. K. Steckel, M. Koldehoff, Y. Hegerfeldt, R. Trenschel, M. Ditschkowski, S. Christoph, T. Gromke, L. Kordelas, H. D. Ottinger, R. S. Ross, P. A. Horn, S. Schnittger, and D. W. Beelen. 2011. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood* 118: 1402-1412.
188. Green, M. L., W. M. Leisenring, H. Xie, R. B. Walter, M. Mielcarek, B. M. Sandmeier, S. R. Riddell, and M. Boeckh. 2013. CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood* 122: 1316-1324.
189. Takenaka, K., T. Nishida, Y. Asano-Mori, K. Oshima, K. Ohashi, T. Mori, H. Kanamori, K. Miyamura, C. Kato, N. Kobayashi, N. Uchida, H. Nakamae, T. Ichinohe, Y. Morishima, R. Suzuki, T. Yamaguchi, and T. Fukuda. 2015. Cytomegalovirus Reactivation after Allogeneic Hematopoietic Stem Cell Transplantation is Associated with a Reduced Risk of Relapse in Patients with Acute Myeloid Leukemia Who Survived to Day 100 after Transplantation: The Japan Society for Hematopoietic Cell Transplantation Transplantation-related Complication Working Group. *Biol. Blood Marrow Transplant.* 21: 2008-2016.
190. Teira, P., M. Battiwalla, M. Ramanathan, A. J. Barrett, K. W. Ahn, M. Chen, J. S. Green, A. Saad, J. H. Antin, B. N. Savani, H. M. Lazarus, M. Seftel, W. Saber, D. Marks, M. Aljurf, M. Norkin, J. R. Wingard, C. A. Lindemans, M. Boeckh, M. L. Riches, and J. J. Auletta. 2016. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood* 127: 2427-2438.
191. Cichocki, F., S. Cooley, Z. Davis, T. E. DeFor, H. Schlums, B. Zhang, C. G. Brunstein, B. R. Blazar, J. Wagner, D. J. Diamond, M. R. Verneris, Y. T. Bryceson, D. J. Weisdorf, and J. S. Miller. 2016. CD56dimCD57+NKG2C+ NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. *Leukemia* 30: 456-463.
192. Bjorklund, A. T., T. Clancy, J. P. Goodridge, V. Beziat, M. Schaffer, E. Hovig, H. G. Ljunggren, P. T. Ljungman, and K. J. Malmberg. 2016. Naive Donor NK Cell Repertoires Associated with Less Leukemia Relapse after Allogeneic Hematopoietic Stem Cell Transplantation. *J. Immunol.* 196: 1400-1411.
193. Barrett, A. J. 2011. CMV: when bad viruses turn good. *Blood* 118: 1193-1194.
194. Smyth, M. J., Y. Hayakawa, K. Takeda, and H. Yagita. 2002. New aspects of natural-killer-cell surveillance and therapy of cancer. *Nature Reviews Cancer* 2: 850-861.
195. Imai, K., S. Matsuyama, S. Miyake, K. Suga, and K. Nakachi. 2000. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *The Lancet* 356: 1795-1799.

196. Hayashi, T., K. Imai, Y. Morishita, I. Hayashi, Y. Kusunoki, and K. Nakachi. 2006. Identification of the NKG2D haplotypes associated with natural cytotoxic activity of peripheral blood lymphocytes and cancer immunosurveillance. *Cancer Res.* 66: 563-570.
197. Garrido, F., T. Cabrera, and N. Aptsiauri. 2010. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy. *Int. J. Cancer* 127: 249-256.
198. Raulet, D. H., S. Gasser, B. G. Gowen, W. Deng, and H. Jung. 2013. Regulation of ligands for the NKG2D activating receptor. *Annu. Rev. Immunol.* 31: 413-441.
199. Lanier, L. L. 2005. NK cell recognition. *Annu. Rev. Immunol.* 23: 225-274.
200. Arnon, T. I., G. Markel, and O. Mandelboim. 2006. Tumor and viral recognition by natural killer cells receptors. *Semin. Cancer Biol.* 16: 348-358.
201. Pessino, A., S. Sivori, C. Bottino, A. Malaspina, L. Morelli, L. Moretta, R. Biassoni, and A. Moretta. 1998. Molecular cloning of NKp46 A novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J. Exp. Med.* 188: 953-960.
202. Groh, V., J. Wu, C. Yee, and T. Spies. 2002. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* 419: 734-738.
203. Marvel, D., and D. I. Gabrilovich. 2015. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. *J. Clin. Invest.* 125: 3356-3364.
204. Castriconi, R., C. Cantoni, M. Della Chiesa, M. Vitale, E. Marcenaro, R. Conte, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2003. Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* 100: 4120-4125.
205. Lee, J. C., K. M. Lee, D. W. Kim, and D. S. Heo. 2004. Elevated TGF- 1 Secretion and Down-Modulation of NKG2D Underlies Impaired NK Cytotoxicity in Cancer Patients. *The Journal of Immunology* 172: 7335-7340.
206. De Sanctis, F., S. Solito, S. Ugel, B. Molon, V. Bronte, and I. Marigo. 2016. MDSCs in cancer: Conceiving new prognostic and therapeutic targets. *Biochim. Biophys. Acta* 1865: 35-48.
207. Castriconi, R., A. Dondero, M. V. Corrias, E. Lanino, D. Pende, L. Moretta, C. Bottino, and A. Moretta. 2004. Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer Res.* 64: 9180-9184.
208. Venstrom, J. M., J. Zheng, N. Noor, K. E. Danis, A. W. Yeh, I. Y. Cheung, B. Dupont, R. J. O'Reilly, N. K. Cheung, and K. C. Hsu. 2009. KIR and HLA genotypes are associated with disease progression and survival following autologous hematopoietic stem cell transplantation for high-risk neuroblastoma. *Clin. Cancer Res.* 15: 7330-7334.
209. Tarek, N., J. B. Le Luduec, M. M. Gallagher, J. Zheng, J. M. Venstrom, E. Chamberlain, S. Modak, G. Heller, B. Dupont, N. K. Cheung, and K. C. Hsu. 2012. Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *J. Clin. Invest.* 122: 3260-3270.
210. Torelli, G. F., A. Guarini, R. Maggio, C. Alfieri, A. Vitale, and R. Foa. 2005. Expansion of natural killer cells with lytic activity against autologous blasts from adult and pediatric acute lymphoid leukemia patients in complete hematologic remission. *Haematologia (Budap.)* 90: 785-792.

211. Werlenius, O., J. Aurelius, A. Hallner, A. A. Akhiani, M. Simpanen, A. Martner, P.-O. Andersson, K. Hellstrand, and F. B. Thoren. 2016. Reactive oxygen species induced by therapeutic CD20 antibodies inhibit natural killer cell-mediated antibody-dependent cellular cytotoxicity against primary CLL cells. *Oncotarget* 7: 32046-32053.
212. Frohn, C., M. Höppner, P. Schlenke, H. Kirchner, P. Koritke, and J. Luhm. 2002. Anti-myeloma activity of natural killer lymphocytes. *Br. J. Haematol.* 119: 660-664.
213. Re, F., C. Staudacher, L. Zamai, V. Vecchio, and M. Bregni. 2006. Killer cell Ig-like receptors ligand-mismatched, alloreactive natural killer cells lyse primary solid tumors. *Cancer* 107: 640-648.
214. Diermayr, S., H. Himmelreich, B. Durovic, A. Mathys-Schneeberger, U. Siegler, U. Langenkamp, J. Hofsteenge, A. Gratwohl, A. Tichelli, M. Paluszewska, W. Wiktor-Jedrzejczak, C. P. Kalberer, and A. Wodnar-Filipowicz. 2008. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. *Blood* 111: 1428-1436.
215. Ruggeri, L., M. Capanni, E. Urbani, K. Perruccio, W. D. Shlomchik, A. Tosti, S. Posati, D. Rogaia, F. Frassoni, F. Aversa, M. F. Martelli, and A. Velardi. 2002. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295: 2097-2100.
216. Ruggeri, L., F. Aversa, M. F. Martelli, and A. Velardi. 2006. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. *Immunol. Rev.* 214: 202-218.
217. Brouwer, R. E., P. van der Heiden, G. M. T. Schreuder, A. Mulder, G. Datema, J. D. H. Anholts, R. Willemze, F. H. J. Claas, and J. H. F. Falkenburg. 2002. Loss or downregulation of HLA class I expression at the allelic level in acute leukemia is infrequent but functionally relevant, and can be restored by interferon. *Hum. Immunol.* 63: 200-210.
218. Leiden, J. M., B. A. Karpinski, L. Gottschalk, and J. Kornbluth. 1989. Susceptibility to natural killer cell-mediated cytolysis is independent of the level of target cell class I HLA expression. *J. Immunol.* 142: 2140-2147.
219. Pena, J., C. Alonso, R. Solana, R. Serrano, J. Carracedo, and R. Ramirez. 1990. Natural killer susceptibility is independent of HLA class I antigen expression on cell lines obtained from human solid tumors. *Eur. J. Immunol.* 20: 2445-2448.
220. Litwin, V., J. Gumperz, P. Parham, J. H. Phillips, and L. L. Lanier. 1993. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type. *The Journal of Experimental Medicine* 178: 1321-1336.
221. Döhner, H., D. J. Weisdorf, and C. D. Bloomfield. 2015. Acute Myeloid Leukemia. *N. Engl. J. Med.* 373: 1136-1152.
222. Döhner, H., E. Estey, D. Grimwade, S. Amadori, F. R. Appelbaum, T. Büchner, H. Dombret, B. L. Ebert, P. Fenaux, R. A. Larson, R. L. Levine, F. Lo-Coco, T. Naoe, D. Niederweiser, G. J. Ossenkoppele, M. Sanz, J. Sierra, M. S. Tallman, H.-F. Tien, A. H. Wei, B. Löwenberg, and C. D. Bloomfield. 2017. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* 129: 424-447.

223. Regionalt cancercentrum Syd. 2016. Akut myeloisk leukemi - Nationellt vårdprogram. Svensk föreing för hematologi. 1-133.
224. Fuchs, E. J., X. J. Huang, and J. S. Miller. 2010. HLA-haploidentical stem cell transplantation for hematologic malignancies. *Biol. Blood Marrow Transplant.* 16: S57-63.
225. Pende, D., S. Marcenaro, M. Falco, S. Martini, M. E. Bernardo, D. Montagna, E. Romeo, C. Cognet, M. Martinetti, R. Maccario, M. C. Mingari, E. Vivier, L. Moretta, F. Locatelli, and A. Moretta. 2009. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* 113: 3119-3129.
226. Boudreau, J. E., X. R. Liu, Z. Zhao, A. Zhang, L. D. Shultz, D. L. Greiner, B. Dupont, and K. C. Hsu. 2016. Cell-Extrinsic MHC Class I Molecule Engagement Augments Human NK Cell Education Programmed by Cell-Intrinsic MHC Class I. *Immunity* 45: 280-291.
227. Ebihara, T., A. H. Jonsson, and W. Yokoyama. 2013. Natural killer cell licensing in mice with inducible expression of MHC class I. *Proceedings of the National Academy of Sciences* 110: E4232-4237.
228. Ruggeri, L., A. Mancusi, M. Capanni, E. Urbani, A. Carotti, T. Aloisi, M. Stern, D. Pende, K. Perruccio, E. Burchielli, F. Topini, E. Bianchi, F. Aversa, M. F. Martelli, and A. Velardi. 2007. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* 110: 433-440.
229. Velardi, A., L. Ruggeri, A. Mancusi, F. Aversa, and F. T. Christiansen. 2009. Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a tool for immunotherapy of leukemia. *Curr. Opin. Immunol.* 21: 525-530.
230. Handgretinger, R., P. Lang, and M. C. Andre. 2016. Exploitation of natural killer cells for the treatment of acute leukemia. *Blood* 127: 3341-3349.
231. Symons, H. J., M. S. Leffell, N. D. Rossiter, M. Zahurak, R. J. Jones, and E. J. Fuchs. 2010. Improved survival with inhibitory killer immunoglobulin receptor (KIR) gene mismatches and KIR haplotype B donors after nonmyeloablative, HLA-haploidentical bone marrow transplantation. *Biol. Blood Marrow Transplant.* 16: 533-542.
232. Miller, J. S., S. Cooley, P. Parham, S. S. Farag, M. R. Verneris, K. L. McQueen, L. A. Guethlein, E. Trachtenberg, M. Haagenson, M. M. Horowitz, J. P. Klein, and D. J. Weisdorf. 2007. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood* 109: 5058-5061.
233. Cooley, S., E. Trachtenberg, T. L. Bergemann, K. Saeteurn, J. Klein, C. T. Le, S. G. Marsh, L. A. Guethlein, P. Parham, J. S. Miller, and D. J. Weisdorf. 2009. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood* 113: 726-732.
234. Cooley, S., D. J. Weisdorf, L. A. Guethlein, J. P. Klein, T. Wang, C. T. Le, S. G. Marsh, D. Geraghty, S. Spellman, M. D. Haagenson, M. Ladner, E. Trachtenberg, P. Parham, and J. S. Miller. 2010. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood* 116: 2411-2419.

235. Verheyden, S., R. Schots, W. Duquet, and C. Demanet. 2005. A defined donor activating natural killer cell receptor genotype protects against leukemic relapse after related HLA-identical hematopoietic stem cell transplantation. *Leukemia* 19: 1446-1451.
236. Cooley, S., D. J. Weisdorf, L. A. Guethlein, J. P. Klein, T. Wang, S. G. Marsh, S. Spellman, M. D. Haagenson, K. Saeturn, M. Ladner, E. Trachtenberg, P. Parham, and J. S. Miller. 2014. Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J. Immunol.* 192: 4592-4600.
237. Venstrom, J. M., G. Pittari, T. A. Gooley, J. H. Chewning, S. Spellman, M. Haagenson, M. M. Gallagher, M. Malkki, E. Petersdorf, B. Dupont, and K. C. Hsu. 2012. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *N. Engl. J. Med.* 367: 805-816.
238. Stringaris, K., and A. J. Barrett. 2017. The importance of natural killer cell killer immunoglobulin-like receptor-mismatch in transplant outcomes. *Curr. Opin. Hematol.*
239. Curti, A., L. Ruggeri, S. Parisi, A. Bontadini, E. Dan, M. R. Motta, S. Rizzi, S. Trabanelli, D. Ocadlikova, M. Lecciso, V. Giudice, F. Fruet, E. Urbani, C. Papayannidis, G. Martinelli, G. Bandini, F. Bonifazi, R. E. Lewis, M. Cavo, A. Velardi, and R. M. Lemoli. 2016. Larger Size of Donor Alloreactive NK Cell Repertoire Correlates with Better Response to NK Cell Immunotherapy in Elderly Acute Myeloid Leukemia Patients. *Clin. Cancer Res.* 22: 1914-1921.
240. Chretien, A.-S., C. Fauriat, F. Orlanducci, C. Galseran, J. Rey, G. Bouvier Borg, E. Gautherot, S. Granjeaud, J.-F. Hamel-Broza, C. Demerle, N. Ifrah, C. Lacombe, P. Cornillet-Lefebvre, J. Delaunay, A. Toubert, E. Gregori, H. Luche, M. Malissen, C. Arnoulet, J. A. Nunes, N. Vey, and D. Olive. 2017. Natural Killer Defective Maturation Is Associated with Adverse Clinical Outcome in Patients with Acute Myeloid Leukemia. *Front. Immunol.* 8: 573.
241. Lion, E., Y. Willemen, Z. N. Berneman, V. F. Van Tendeloo, and E. L. Smits. 2012. Natural killer cell immune escape in acute myeloid leukemia. *Leukemia* 26: 2019-2026.
242. Hellstrand, K., A. Asea, C. Dahlgren, and S. Hermodsson. 1994. Histaminergic regulation of NK cells. Role of monocyte-derived reactive oxygen metabolites. *J. Immunol.* 153: 4940-4947.
243. Hansson, M., A. Asea, U. Ersson, S. Hermodsson, and K. Hellstrand. 1996. Induction of apoptosis in NK cells by monocyte-derived reactive oxygen metabolites. *J. Immunol.* 156: 42-47.
244. Aurelius, J., F. B. Thoren, A. A. Akhiani, M. Brune, L. Palmqvist, M. Hansson, K. Hellstrand, and A. Martner. 2012. Monocytic AML cells inactivate antileukemic lymphocytes: role of NADPH oxidase/gp91(phox) expression and the PARP-1/PAR pathway of apoptosis. *Blood* 119: 5832-5837.
245. Aurelius, J., A. Martner, R. E. Riise, A. I. Romero, L. Palmqvist, M. Brune, K. Hellstrand, and F. B. Thoren. 2013. Chronic myeloid leukemic cells trigger poly(ADP-ribose) polymerase-dependent inactivation and cell death in lymphocytes. *J. Leukoc. Biol.* 93: 155-160.
246. Parameswaran, R., P. Ramakrishnan, S. A. Moreton, Z. Xia, Y. Hou, D. A. Lee, K. Gupta, M. deLima, R. C. Beck, and D. N. Wald. 2016. Repression of GSK3 restores NK cell cytotoxicity in AML patients. *Nat Commun* 7: 11154.

247. Vey, N., J.-H. Bourhis, N. Boissel, D. Bordessoule, T. Prebet, A. Charbonnier, A. Etienne, P. Andre, F. Romagne, D. Benson, H. Dombret, and D. Olive. 2012. A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission. *Blood* 120: 4317-4323.
248. Benson, D. M., Jr., A. D. Cohen, S. Jagannath, N. C. Munshi, G. Spitzer, C. C. Hofmeister, Y. A. Efebera, P. Andre, R. Zerbib, and M. A. Caligiuri. 2015. A Phase I Trial of the Anti-KIR Antibody IPH2101 and Lenalidomide in Patients with Relapsed/Refractory Multiple Myeloma. *Clin. Cancer Res.* 21: 4055-4061.
249. Seymour, L., A. Tinker, H. Hirte, N. Wagtmann, and P. Dodion. 2015. O3.2 * Phase I and dose ranging, phase II studies with IPH2201, a humanized monoclonal antibody targeting HLA-E receptor CD94/NKG2A. *Ann. Oncol.* 26: ii3-ii3.
250. Nguyen, S., N. Dhedin, J.-P. Vernant, M. Kuentz, A. Al Jijakli, N. Rouas-Freiss, E. D. Carosella, A. Boudifa, P. Debre, and V. Vieillard. 2005. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood* 105: 4135-4142.
251. Felices, M., T. R. Lenvik, Z. B. Davis, J. S. Miller, and D. A. Vallera. 2016. Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells. *Methods Mol. Biol.* 1441: 333-346.
252. Davis, Z. B., D. A. Vallera, J. S. Miller, and M. Felices. 2017. Natural killer cells unleashed: Checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. *Semin. Immunol.*
253. Vallera, D. A., M. Felices, R. McElmurry, V. McCullar, X. Zhou, J. U. Schmohl, B. Zhang, A. J. Lenvik, A. Panoskaltsis-Mortari, M. R. Verneris, J. Tolar, S. Cooley, D. J. Weisdorf, B. R. Blazar, and J. S. Miller. 2016. IL15 Trispecific Killer Engagers (TriKE) Make Natural Killer Cells Specific to CD33+ Targets While Also Inducing Persistence, In Vivo Expansion, and Enhanced Function. *Clin. Cancer Res.* 22: 3440-3450.
254. Arai, S., R. Meagher, M. Swearingen, H. Myint, E. Rich, J. Martinson, and H. Klingemann. 2008. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. *Cytotherapy* 10: 625-632.
255. Boissel, L., H. Klingemann, K. Campbell, K. Nichols, F. Toneguzzo, P. Marcus, B. Williams, A. Keating, and P. Soon-Shiong. 2016. An 'off the shelf,' GMP-grade, IL-2-independent NK cell line expressing the high-affinity Fc-receptor to augment antibody therapeutics. [abstract]. In *Proceedings of the 107th Annual Meeting of the American Association for Cancer Research*. AACR; Cancer Res 2016, New Orleans, LA. Philipelpdia (PA). Abstract nr 2302.
256. Imai, C., S. Iwamoto, and D. Campana. 2005. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* 106: 376-383.
257. Martin-Antonio, B., G. Sune, L. Perez-Amill, M. Castella, and A. Urbano-Ispizua. 2017. Natural Killer Cells: Angels and Devils for Immunotherapy. *Int J Mol Sci* 18.
258. Muntasell, A., M. C. Ochoa, L. Cordeiro, P. Berraondo, A. Lopez-Diaz de Cerio, M. Cabo, M. Lopez-Botet, and I. Melero. 2017. Targeting NK-cell checkpoints for cancer immunotherapy. *Curr. Opin. Immunol.* 45: 73-81.
259. Henney, C. S., K. Kuribayashi, D. E. Kern, and S. Gillis. 1981. Interleukin-2 augments natural killer cell activity. *Nature* 291: 335-338.

260. Kim, H. P., J. Imbert, and W. J. Leonard. 2006. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. *Cytokine Growth Factor Rev.* 17: 349-366.
261. Liao, W., J. X. Lin, and W. J. Leonard. 2011. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Curr. Opin. Immunol.* 23: 598-604.
262. Dutcher, J. P. 2002. Current status of interleukin-2 therapy for metastatic renal cell carcinoma and metastatic melanoma. *Oncology* 16: 4-10.
263. Rosenberg, S. A. 2012. Raising the Bar: The Curative Potential of Human Cancer Immunotherapy. *Sci. Transl. Med.* 4: 127ps128.
264. Baer, M. R., S. L. George, M. A. Caligiuri, B. L. Sanford, S. M. Bothun, K. Mrozek, J. E. Kolitz, B. L. Powell, J. O. Moore, R. M. Stone, J. Anastasi, C. D. Bloomfield, and R. A. Larson. 2008. Low-dose interleukin-2 immunotherapy does not improve outcome of patients age 60 years and older with acute myeloid leukemia in first complete remission: Cancer and Leukemia Group B Study 9720. *J. Clin. Oncol.* 26: 4934-4939.
265. Blaise, D., M. Attal, J. Reiffers, M. Michallet, C. Bellanger, J. L. Pico, A. M. Stoppa, C. Payen, G. Marit, R. Bouabdallah, J. J. Sotto, J. F. Rossi, M. Brandley, T. Hercend, and D. Maraninchi. 2000. Randomized study of recombinant interleukin-2 after autologous bone marrow transplantation for acute leukemia in first complete remission. *Eur. Cytokine Netw.* 11: 91-98.
266. Kolitz, J. E., V. Hars, D. J. Deangelo, S. L. Allen, T. C. Shea, R. Vij, E. Hoke, C. D. Bloomfield, M. A. Caligiuri, and S. L. George. 2007. Phase III trial of immunotherapy with recombinant interleukin-2 (rIL-2) versus observation in patients < 60 years with acute myeloid leukemia (AML) in first remission (CR1): preliminary results from Cancer and Leukemia GroupB (CALGB) 19808. *ASH Annual Meeting Abstracts* 110: 157.
267. Lange, B. J., F. O. Smith, J. Feusner, D. R. Barnard, P. Dinndorf, S. Feig, N. A. Heerema, C. Arndt, R. J. Arceci, N. Seibel, M. Weiman, K. Dusenbery, K. Shannon, S. Luna-Fineman, R. B. Gerbing, and T. A. Alonzo. 2008. Outcomes in CCG-2961, a children's oncology group phase 3 trial for untreated pediatric acute myeloid leukemia: a report from the children's oncology group. *Blood* 111: 1044-1053.
268. Pautas, C., F. Merabet, X. Thomas, E. Raffoux, C. Gardin, S. Corm, J. H. Bourhis, O. Reman, P. Turlure, N. Contentin, T. de Revel, P. Rousselot, C. Preudhomme, D. Bordessoule, P. Fenaux, C. Terre, M. Michallet, H. Dombret, S. Chevret, and S. Castaigne. 2010. Randomized study of intensified anthracycline doses for induction and recombinant interleukin-2 for maintenance in patients with acute myeloid leukemia age 50 to 70 years: results of the ALFA-9801 study. *J. Clin. Oncol.* 28: 808-814.
269. Lotzova, E., C. A. Savary, and R. B. Herberman. 1987. Induction of NK cell activity against fresh human leukemia in culture with interleukin 2. *J. Immunol.* 138: 2718-2727.
270. Berry, S. M., K. R. Broglio, and D. A. Berry. 2011. Addressing the incremental benefit of histamine dihydrochloride when added to interleukin-2 in treating acute myeloid leukemia: a Bayesian meta-analysis. *Cancer Invest.* 29: 293-299.
271. Brune, M., and K. Hellstrand. 1996. Remission maintenance therapy with histamine and interleukin-2 in acute myelogenous leukaemia. *Br. J. Haematol.* 92: 620-626.
272. Asea, A. 1995. Role of histamine in the regulation of natural killer cells. *Thesis, University of Gothenburg*.

273. Brune, M., M. Hansson, U. H. Mellqvist, S. Hermodsson, and K. Hellstrand. 1996. NK cell-mediated killing of AML blasts: role of histamine, monocytes and reactive oxygen metabolites. *Eur. J. Haematol.* 57: 312-319.
274. Brune, M., S. Castaigne, J. Catalano, K. Gehlsen, A. D. Ho, W. K. Hofmann, D. E. Hogge, B. Nilsson, R. Or, A. I. Romero, J. M. Rowe, B. Simonsson, R. Spearing, E. A. Stadtmauer, J. Szer, E. Wallhult, and K. Hellstrand. 2006. Improved leukemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukemia: results of a randomized phase 3 trial. *Blood* 108: 88-96.
275. Schwartz, R. N., L. Stover, and J. P. Dutcher. 2002. Managing toxicities of high-dose interleukin-2. *Oncology* 11 Suppl 13: 11-20.
276. Martner, A., F. B. Thoren, J. Aurelius, and K. Hellstrand. 2013. Immunotherapeutic strategies for relapse control in acute myeloid leukemia. *Blood Rev.* 27: 209-216.
277. Asemissen, A. M., C. Scheibenbogen, A. Letsch, K. Hellstrand, F. B. Thoren, K. Gehlsen, A. Schmittel, E. Thiel, and U. Keilholz. 2005. Addition of histamine to interleukin 2 treatment augments type 1 T-cell responses in patients with melanoma in vivo: immunologic results from a randomized clinical trial of interleukin 2 with or without histamine (MP 104). *Clin. Cancer Res.* 11: 290-297.
278. Agarwala, S. S., J. Glaspy, S. J. O'Day, M. Mitchell, J. Gutheil, E. Whitman, R. Gonzalez, E. Hersh, L. Feun, R. Belt, F. Meyskens, K. Hellstrand, D. Wood, J. M. Kirkwood, K. R. Gehlsen, and P. Naredi. 2002. Results from a randomized phase III study comparing combined treatment with histamine dihydrochloride plus interleukin-2 versus interleukin-2 alone in patients with metastatic melanoma. *J. Clin. Oncol.* 20: 125-133.
279. Donskov, F., M. Middleton, K. Fode, P. Meldgaard, W. Mansoor, J. Lawrance, N. Thatcher, H. Nellemann, and H. von der Maase. 2005. Two randomised phase II trials of subcutaneous interleukin-2 and histamine dihydrochloride in patients with metastatic renal cell carcinoma. *Br. J. Cancer* 93: 757-762.
280. Martner, A., A. Rydstrom, R. E. Riise, J. Aurelius, H. Anderson, M. Brune, R. Foa, K. Hellstrand, and F. B. F. T. R. M. S. G. Thoren. 2015. Role of natural killer cell subsets and natural cytotoxicity receptors for the outcome of immunotherapy in acute myeloid leukemia. *Oncoimmunology* 5: e1041701.
281. Martner, A., A. Rydstrom, R. E. Riise, J. Aurelius, M. Brune, R. Foa, K. Hellstrand, and F. B. Thoren. 2015. NK cell expression of natural cytotoxicity receptors may determine relapse risk in older AML patients undergoing immunotherapy for remission maintenance. *Oncotarget* 6: 42569-42574.
282. Huntington, N. D. 2014. The unconventional expression of IL-15 and its role in NK cell homeostasis. *Immunol. Cell Biol.* 92: 210-213.
283. Colucci, F., and J. Traherne. 2017. KIR on the cusp of modern immunogenetics. *Immunology*.
284. Conlon, K. C., E. Lugli, H. C. Welles, S. A. Rosenberg, A. T. Fojo, J. C. Morris, T. A. Fleisher, S. P. Dubois, L. P. Perera, D. M. Stewart, C. K. Goldman, B. R. Bryant, J. M. Decker, J. Chen, T. A. Worthy, W. D. Figg, Sr., C. J. Peer, M. C. Sneller, H. C. Lane, J. L. Yovandich, S. P. Creekmore, M. Roederer, and T. A. Waldmann. 2015. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J. Clin. Oncol.* 33: 74-82.

285. Miller, J. S., Y. Soignier, A. Panoskaltsis-Mortari, S. A. McNearney, G. H. Yun, S. K. Fautsch, D. McKenna, C. Le, T. E. Defor, L. J. Burns, P. J. Orchard, B. R. Blazar, J. E. Wagner, A. Slungaard, D. J. Weisdorf, I. J. Okazaki, and P. B. McGlave. 2005. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* 105: 3051-3057.
286. Rubnitz, J. E., H. Inaba, R. C. Ribeiro, S. Pounds, B. Rooney, T. Bell, C. H. Pui, and W. Leung. 2010. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *J. Clin. Oncol.* 28: 955-959.
287. Romee, R., M. Rosario, M. M. Berrien-Elliott, J. A. Wagner, B. A. Jewell, T. Schappe, J. W. Leong, S. Abdel-Latif, S. E. Schneider, S. Willey, C. C. Neal, L. Yu, S. T. Oh, Y. S. Lee, A. Mulder, F. Claas, M. A. Cooper, and T. A. Fehniger. 2016. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci. Transl. Med.* 8: 537ra123.
288. Wagner, J. A., M. M. Berrien-Elliott, M. Rosario, J. W. Leong, B. A. Jewell, T. Schappe, S. Abdel-Latif, and T. A. Fehniger. 2017. Cytokine-Induced Memory-Like Differentiation Enhances Unlicensed Natural Killer Cell Antileukemia and FcgammaRIIIa-Triggered Responses. *Biol. Blood Marrow Transplant.* 23: 398-404.
289. Leong, J. W., J. M. Chase, R. Romee, S. E. Schneider, R. P. Sullivan, M. A. Cooper, and T. A. Fehniger. 2014. Preactivation with IL-12, IL-15, and IL-18 induces CD25 and a functional high-affinity IL-2 receptor on human cytokine-induced memory-like natural killer cells. *Biol. Blood Marrow Transplant.* 20: 463-473.
290. Berrien-Elliott, M. M., J. A. Wagner, and T. A. Fehniger. 2015. Human Cytokine-Induced Memory-Like Natural Killer Cells. *J. Innate Immun.* 7: 563-571.
291. FateTherapeutics. 2017. FATE NK-100: A novel NK cell-mediated cancer therapy. http://fatetherapeutics.com/wp-content/uploads/2017/06/2017-AACR-FATE-NK100-Poster.pdf.
292. Bhat, R., and C. Watzl. 2007. Serial killing of tumor cells by human natural killer cells--enhancement by therapeutic antibodies. *PLoS One* 2: e326.
293. James, A. M., H. T. Hsu, P. Dongre, G. Uzel, E. M. Mace, P. Banerjee, and J. S. Orange. 2013. Rapid activation receptor- or IL-2-induced lytic granule convergence in human natural killer cells requires Src, but not downstream signaling. *Blood* 121: 2627-2637.
294. Du, J., S. Lopez-Verges, B. N. Pitcher, J. Johnson, S. H. Jung, L. Zhou, K. Hsu, M. S. Czuczman, B. Cheson, L. Kaplan, L. L. Lanier, and J. M. Venstrom. 2014. CALGB 150905 (Alliance): rituximab broadens the antilymphoma response by activating unlicensed NK cells. *Cancer Immunol Res* 2: 878-889.
295. Terszowski, G., C. Klein, and M. Stern. 2014. KIR/HLA interactions negatively affect rituximab- but not GA101 (obinutuzumab)-induced antibody-dependent cellular cytotoxicity. *J. Immunol.* 192: 5618-5624.
296. Sandoval-Borrego, D., M. C. Moreno-Lafont, E. A. Vazquez-Sanchez, A. Gutierrez-Hoya, R. Lopez-Santiago, L. A. Montiel-Cervantes, M. Ramirez-Saldana, and J. Vela-Ojeda. 2016. Overexpression of CD158 and NKG2A Inhibitory Receptors and Underexpression of NKG2D and NKp46 Activating Receptors on NK Cells in Acute Myeloid Leukemia. *Arch. Med. Res.* 47: 55-64.

297. Foley, B., D. De Santis, L. Lathbury, F. Christiansen, and C. Witt. 2008. KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2-negative individuals. *Int. Immunol.* 20: 555-563.
298. Yu, J., G. Heller, J. Chewning, S. Kim, W. M. Yokoyama, and K. C. Hsu. 2007. Hierarchy of the Human Natural Killer Cell Response Is Determined by Class and Quantity of Inhibitory Receptors for Self-HLA-B and HLA-C Ligands. *The Journal of Immunology* 179: 5977-5989.
299. Houchins, J. P., T. Yabe, C. McSherry, and F. H. Bach. 1991. DNA Sequence Analysis of NKG2, a Family of Related cDNA Clones Encoding Type II Integral Membrane Proteins on Human Natural Killer Cells. *J. Exp. Med.* 173: 1017-1020.
300. Kabat, J., F. Borrego, A. Brooks, and J. E. Coligan. 2002. Role That Each NKG2A Immunoreceptor Tyrosine-Based Inhibitory Motif Plays in Mediating the Human CD94/NKG2A Inhibitory Signal. *The Journal of Immunology* 169: 1948-1958.
301. Brunhns, P., P. Marchetti, W. H. Fridman, E. Vivier, and M. Daëron. 1999. Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. *J. Immunol.* 162: 3168-3175.
302. Min-Oo, G., and L. L. Lanier. 2014. Cytomegalovirus generates long-lived antigen-specific NK cells with diminished bystander activation to heterologous infection. *J. Exp. Med.* 211: 2669-2680.
303. Sarhan, D., F. Cichocki, B. Zhang, A. Yingst, S. R. Spellman, S. Cooley, M. R. Verneris, B. R. Blazar, and J. S. Miller. 2016. Adaptive NK Cells with Low TIGIT Expression Are Inherently Resistant to Myeloid-Derived Suppressor Cells. *Cancer Res.* 76: 5696-5706.
304. Gasteiger, G., and W. Kastenmuller. 2012. Foxp3+ Regulatory T-cells and IL-2: The Moirai of T-cell Fates? *Front. Immunol.* 3: 179.
305. Koreth, J., K. Matsuoka, H. T. Kim, S. M. McDonough, B. Bindra, E. P. Alyea, 3rd, P. Armand, C. Cutler, V. T. Ho, N. S. Treister, D. C. Bienfang, S. Prasad, D. Tzachanis, R. M. Joyce, D. E. Avigan, J. H. Antin, J. Ritz, and R. J. Soiffer. 2011. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N. Engl. J. Med.* 365: 2055-2066.
306. Pedroza-Pacheco, I., A. Madrigal, and A. Saudemont. 2013. Interaction between natural killer cells and regulatory T cells: perspectives for immunotherapy. *Cell. Mol. Immunol.* 10: 222-229.
307. Wing, K., Y. Onishi, P. Prieto-Tarin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322: 271-275.
308. Kim, N., Y. W. Jeon, Y. S. Nam, J. Y. Lim, K. I. Im, E. S. Lee, and S. G. Cho. 2016. Therapeutic potential of low-dose IL-2 in a chronic GVHD patient by in vivo expansion of regulatory T cells. *Cytokine* 78: 22-26.
309. Koreth, J., H. T. Kim, K. T. Jones, P. B. Lange, C. G. Reynolds, M. J. Chammas, K. Dusenbury, J. N. Whangbo, S., E. P. Alyea, P. Armand, C. Cutler, V. T. Ho, Y.-B. Chen, D. Avigan, B. R. Blazar, J. H. Antin, J. Ritz, and R. J. Soiffer. 2016. Efficacy, durability, and response predictors of low-dose interleukin-2 therapy for chronic graft-versus-host disease. *Blood* 128: 130-137.

310. Kennedy-Nasser, A. A., S. Ku, P. Castillo-Caro, Y. Hazrat, M. F. Wu, H. Liu, J. Melenhorst, A. J. Barrett, S. Ito, A. Foster, B. Savoldo, E. Yvon, G. Carrum, C. A. Ramos, R. A. Krance, K. Leung, H. E. Heslop, M. K. Brenner, and C. M. Bollard. 2014. Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity. *Clin. Cancer Res.* 20: 2215-2225.
311. Bachanova, V., S. Cooley, T. E. DeFor, M. R. Verneris, B. Zhang, D. H. McKenna, J. Curtsinger, A. Panoskaltsis-Mortari, D. Lewis, K. Hippen, P. McGlave, D. J. Weisdorf, B. R. Blazar, and J. S. Miller. 2014. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood* 123: 3855-3863.