Immunotherapy and immunosuppression in myeloid leukemia

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Cover illustration: Alexander Hallner

Confocal microscopy picture of an immunological synapse between an NK cell and a CMML monocyte with a nuclear stain using DAPI (blue), f-actin (red) and the NOX2 subunit gp91phox (green).
“History repeats, but science reverberates”

Siddharta Mukherjee – The Emperor of All Maladies
ABSTRACT

Acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML) are potentially life-threatening blood cancers characterized by the expansion of malignant myeloid cells in bone marrow and other organs. This thesis aimed at contributing to the understanding of the role of natural killer (NK) cells in AML and CMML with focus on the potential impact of the immunosuppression exerted by reactive oxygen species (ROS) formed by the myeloid cell NOX2 enzyme. The thesis work has comprised in vitro studies of interactions between NK cells and primary myeloid leukemic cells along with analyses of NK cell repertoires in a clinical trial using a NOX2 inhibitor, histamine dihydrochloride (HDC) in conjunction with the NK cell-activating cytokine interleukin-2 (IL-2) for the prevention of relapse of AML after the completion of chemotherapy. Paper I reports that the functions and viability of cytotoxic lymphocytes, including NK cells, were compromised by ROS produced by leukemic myeloid cells recovered from patients with CMML. The results are thus suggestive of a novel mechanism of leukemia-induced immunosuppression in this disease. Paper II analyzed aspects of myeloid cell populations in AML using blood samples from a clinical phase IV trial where AML patients (n=84) received HDC in conjunction with IL-2. The results imply that HDC may exert anti-leukemic efficacy by facilitating the maturation of myeloid cells, which impacts on the efficiency of immunotherapy with HDC/IL-2. In papers III and IV we explored the role of killer cell immunoglobulin-like receptors (KIR) for the relapse and survival of AML patients receiving HDC/IL-2. The results suggest that a subset of immature NK cells with low KIR expression may determine clinical outcome. In paper IV we further analyzed results from the above-referenced phase IV trial and observed that a past cytomegalovirus (CMV) infection predicted high relapse risk and poor survival, presumably by reducing the pool of immature NK cells. The results of paper V suggest that a dimorphism in the leader peptide of HLA-B is relevant to NK cell-mediated killing of AML cells and to the outcome of immunotherapy. In conclusion, this thesis work presents novel aspects of myeloid cell-induced immunosuppression in AML and CMML and identifies NK cell subsets of potential relevance to the benefit of immunotherapy with HDC/IL-2.

Keywords: Natural killer cells, acute myeloid leukemia, histamine dihydrochloride, immunotherapy, reactive oxygen species, chronic myelomonocytic leukemia, NK cell education, NKG2A, HLA, KIR
SAMMANFATTNING PÅ SVENSKA

Immunterapi, dvs behandling som avser att förbättra det kroppsegna försvarsmot avvikande celler, har dramatiskt förbättrat prognosen vid flera allvarliga cancerformer, och forskning som legat till grund för immunterapi vid cancer tilldelades nobelpriset i medicin 2018. Mitt avhandlingsarbete har omfattat studier av myeloid celler och natural killer celler (NK-celler), särskilt dessa försvarscellers roll vid immunterapi av myeloisk leukemi.

Akut myeloisk leukemi (AML) är den vanligaste formen av leukemi hos vuxna. Även om ungefär var tredje patient botas av cellgifter finns det stort behov av förbättrad behandling. En förklaring är att många AML-patienter återfaller i leukemi trots att den initiala behandlingen med cellgifter har varit framgångsrik. Skålet till att sjukdomen ofta återkommer anses vara att det ofta finns kvarvarande leukemiska celler som inte eliminerats av den inledande cellgiftsbehandlingen. Det är därför angeläget att nya behandlingar tas fram som förhindrar återfall, och en tänkbar strategi är att aktivera immunologisk destruktion av leukemiska celler. En befintlig strategi för detta ändamål är en kombination av histamindihydroklorid och interleukin-2 (HDC/IL-2) där IL-2 komponenten stimulerar bland annat NK-celler medan HDC samtidigt förhindrar att dessa cellers funktion undertrycks av myeloiska celler.

Det första av avhandlingens delarbeten omfattar en form av myeloisk leukemi, kronisk myelomonocytisk leukemi (KMML), som morfologiskt liknar monocytära former av AML. Arbetet visar att de leukemiska cellerna vid KMML undertrycker NK-cellers funktion och att denna immunsuppressiva mekanism kan motverkas av HDC. Vi föreslår därför att behandling med HDC/IL-2 skulle kunna vara av värde också vid KMML.

De följande delarbetena har avsett att belysa betydelsen av myeloiska celler (arbete II), omogna NK-celler (arbete III och IV) och en genetisk variant av HLA-B som reglerar NK-cellers funktion (arbete V) för överlevnad och återfallsrisk hos AML-patienter som behandlats med HDC/IL-2. Resultaten talar för att omogna NK-celler är viktiga för utfallet av sådan behandling liksom att NK-celler som kontrolleras av den inhibitoriska receptorn NKG2A har betydelse för elimination av leukemiska celler. Därmed har mitt avhandlingsarbete bidragit till förståelsen för NK-cellers roll vid AML och påvisat mätbara faktorer som skulle kunna användas för att identifiera AML-patienter som är lämpliga kandidater för immunterapi.
LIST OF PAPERS

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


   Journal of Leukocyte Biology 2017; 102:467-74

   Leukemia 2017; 31:2552-59

   Cancer Immunology Research 2018; 6:1110-19

   Submitted
Additional publications not part of this thesis:

i) **Hallner A**, Aurelius J, Thorén FB, Sander FE, Brune M, Hellstrand K, Martner A.
   Immunotherapy with histamine dihydrochloride and low-dose interleukin-2 favors sustained lymphocyte recovery in acute myeloid leukemia.
   *European Journal of Haematology 2015; 94:279-80*

   Reactive oxygen species induced by therapeutic CD20 antibodies inhibit natural killer cell-mediated antibody-dependent cellular cytotoxicity against primary CLL cells.
   *Oncotarget 2016; 7:32046-53*

   Determinants for effective ALECSAT immunotherapy treatment on autologous patient-derived glioblastoma stem cells.
   *Neoplasia 2018; 20:25-31*

iv) Aydin E, **Hallner A**, Grauers Wiktorin H, Staffas A, Hellstrand K, Martner A.
   NOX2 inhibition reduces oxidative stress and prolongs survival in murine KRAS-induced myeloproliferative disease.
   *Accepted for publication in Oncogene 2018*
CONTENT

ABBREVIATIONS ........................................................................................................ XI
PREFACE .................................................................................................................... 1
INTRODUCTION ......................................................................................................... 3
  Innate and adaptive immunity ................................................................................ 4
    Myeloid cells ........................................................................................................ 5
      Monocytes and macrophages ........................................................................... 5
      Dendritic cells ............................................................................................... 6
      Neutrophils ..................................................................................................... 7
  Lymphoid cells ...................................................................................................... 7
    B cells ............................................................................................................... 7
    T cells .............................................................................................................. 8
Natural killer cells .................................................................................................. 10
  NK cell discovery and the “missing self” hypothesis ........................................... 10
  NK cell development and receptors .................................................................. 11
  The killer cell immunoglobulin-like receptors (KIR) ........................................ 14
  NKG2A: HLA-E interactions .............................................................................. 15
  Models of NK cell education ............................................................................. 16
  “Unlicensed” NK cells ....................................................................................... 17
  NK cell target engagement and signaling pathways ....................................... 18
NK cells in disease control .................................................................................... 20
  NK cell responses in viral infections ................................................................ 20
  Impact of cytomegalovirus on NK cell repertoires ....................................... 20
  CMV in a leukemia setting ............................................................................... 22
  NK cells in malignancies ................................................................................... 23
  Chronic myelomonocytic leukemia ................................................................ 24
  Acute myeloid leukemia .................................................................................. 24
  AML and immune responses .......................................................................... 26
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
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<td>AML</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NOX2</td>
<td>NADPH oxidase type 2</td>
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<td>OS</td>
<td>Overall survival</td>
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PREFACE

In 2013 Science Magazine declared cancer immunotherapy the scientific breakthrough of the year (1). In the following years immune checkpoint inhibitors directed against CTLA-4 and PD-1, which improve T cell-mediated immunity against tumor cells, were shown to exert remarkable efficacy in solid malignancies such as malignant melanoma, renal cell carcinoma and lung cancer, thus apparently curing a significant fraction of patients with advanced cancer (2, 3). In 2018 the discoveries of CTLA-4 and PD-1 along with the identification of inhibitors of these pathways were awarded the Nobel Prize in medicine and physiology.

Hematopoietic stem cell transplantation (HSCT), an additional strategy to exploit immune-mediated elimination of malignant cells, is widely used in hematopoietic cancer. The benefit of this treatment requires a graft-versus-leukemia effect (GvL) meaning that transplanted immune cells recognize and eradicate leukemic cells (4). In addition, adoptive cell transfer, where immune cells are stimulated and/or genetically modified to recognize cancer cells and then transferred to patients (5) has attracted significant interest in recent years. For example, chimeric antigen receptor (CAR) T cells were approved in 2017 for the treatment of children and young adults with relapsed or refractory acute lymphoblastic leukemia (6).

Despite recent advances and despite the emergence of immunotherapy as a new pillar in cancer therapy, many malignancies still carry poor prognosis. Acute myeloid leukemia (AML) exemplifies that more efficacious therapy is warranted. For patients with AML, HSCT is a commonly used treatment option after initial rounds of chemotherapy, in particular for younger patients with high-risk leukemia. However, only approximately 40% of all younger patients (<60 years old) and 5-15 % of older patients are cured from AML (7). An overriding aim of this thesis was to provide a framework for novel immunotherapy for these patients with a particular focus on reducing the risk of recurrence of leukemia (relapse) after the completion of chemotherapy.

A significant part of the thesis work aimed at contributing to the understanding of the role of natural killer (NK) cells in relapse-preventive immunotherapy using histamine dihydrochloride and interleukin-2 (HDC/IL-2) in AML. I have thus aimed at defining potential biomarkers, with focus on aspects of NK cell biology, of relevance to the clinical efficacy of HDC/IL-2 in AML. The results imply that NK cell-suppressive myeloid cells and inhibitory NK cell receptors, such as killer cell immunoglobulin-like receptors (KIR) and the NKG2A/CD94...
receptor, are relevant to the clinical benefit of immunotherapy in this disease. In addition, my studies point towards a mechanism of immunosuppression in chronic myelomonocytic leukemia (CMML), where cells of the malignant clone resemble those of monocytic forms of AML, which may be targeted in immunotherapy.
INTRODUCTION

Hematopoietic stem cells (HSCs) generate and regenerate blood cells, including immune cells such as lymphoid and myeloid cells, in a process called hematopoiesis. HSCs are functionally defined by their ability to self-renew and to differentiate from pluripotent stem cells via multipotent progenitor cells into defined lineage progenitors (8, 9). The precursor of lymphoid cells is the common lymphoid progenitor (CLP) and its myeloid counterpart is the common myeloid progenitor (CMP). Together these progenitor cells yield hematopoietic cells such as red blood cells, monocytes, macrophages, neutrophils, B cells, T cells and NK cells (Figure 1) (8). The final cell products have a variable half-life but are commonly short-lived; for example, a human neutrophil has a lifespan of approximately 5 days (10). Due to the high turnover rate, hematopoiesis is required to rapidly replenish old cells, which in turn may yield somatic mutations that cause cells to transform into malignant cells (11).

Figure 1. Simplified scheme of hematopoiesis giving rise to cell subsets discussed in this thesis.
INNATE AND ADAPTIVE IMMUNITY

The immune system comprises two major and partially overlapping components, innate and adaptive immunity. These systems complement each other in terms of recognition specificity, signaling and the timing of response where innate immunity is regarded as a first barrier against invading pathogens (12). The innate immune system encompasses many cells and tissues, including skin and epithelia. White blood cells such as monocytes, macrophages, neutrophils and dendritic cells form a vital part. These cells respond swiftly upon encounter with a pathogen and may differentiate into effector cells that eradicate microbes at an early stage.

To recognize invading pathogens, innate immune cells are equipped with a variety of pattern recognition receptors (PRRs) that are expressed on the cell surface and intracellularly. The pathogen-associated molecular patterns (PAMPs) that are recognized by PRRs include microbial structures such as LPS, peptidoglycan and bacterial DNA (12). Some PRRs also specifically recognize virus-associated molecules such as foreign double-stranded RNA produced by virus-infected cells (13). The stimulation of PRRs results in activation of innate immune cells, typically via enhanced activity of the transcription factors NF-κB or IRF3. An additional group of proteins, the damage-associated molecular patterns (DAMPs), activate innate cells to eliminate traumatized and dead cells (14). However, although the repertoire of PRRs is versatile it does not always provide complete protection and innate cells can generally not remember previous attacks. Therefore, the adaptive immune system has evolved to carry out specific and precise eradication of pathogens and to enable a renewed, faster and more efficacious attack in a process called immunological memory.

In addition to the innate immunity, which is present in most organisms, jawed vertebrates thus have evolved an adaptive immune system (15). Adaptive immunity comprises two types of lymphocytes, T cells and B cells. These cells create antigen receptors that, in contrast to PRR, are not encoded in the germline DNA but instead are unique to each antigen. This is accomplished by stochastic rearrangement of gene segments that creates B and T cells that express exclusive antigen receptors. The different combinations that are produced can vary to infinity and enable a robust probability of host defense against a high number of microbes (15). The evolvement of T cells and B cells that recognize a broad range of foreign structures may, however, be harmful when B and T cells erroneously express receptors that react with healthy cells. Under normal conditions, however, such autoreactive lymphocytes are inactivated by self-antigen contact in the bone marrow and thymus.
Innate and adaptive immunity are often described as separate entities that cover all parts of the host defense against infections or transformed cells. In the past decades this view has been modified and broadened (16). Engagement of the innate immune cells against pathogens thus leads to an inflammatory response that, in later stages, triggers the activation of adaptive immunity. It has been known since the 1960’s that activation of adaptive T cells is dependent on cells from the innate immune system, which is probably best illustrated by the requirement of antigen presentation for functions of adaptive lymphocytes.

Hence, antigen-presenting cells (APCs), out of which dendritic cells (DC) are most efficient, endocytose microbial (or other) material and present endocytosed peptides on MHC complexes I or II on their surface. After phagocytosis the APC typically migrate to secondary lymphoid organs to present the antigen. T cells with T cell receptors (TCR) recognizing the specific antigen that is presented will bind to the MHC complex and become activated. CD4+ T cells are activated in response to peptides presented on MHC I while CD8+ T cells are activated by antigens presented on MHC II. Optimal T cell activation requires secondary signals from costimulatory molecules such as CD86, and stimulatory cytokines produced by the APC.

B cell recognize antigens via their specific B cell receptor (BCR), which is a surface bound antibody. After binding to the BCR the antigen is endocytosed, digested, and peptides are presented on MHC II complexes on the cell surface (17). If the B cell presents a peptide on MHC II that is recognizable by activated CD4+ T cells, a mutual crosstalk can occur leading to the activation of both cell types and antibody production by the B cells.

Interactions with other innate cells can stimulate DC maturation to further enhance crosstalk with T cells. For example, DCs and NK cells engage in a crosstalk, in which NK cells receive activating stimuli such as IL-12, while NK cells contribute to the capacity of DCs to present antigens by producing interferon-γ (18). Other innate cells such as neutrophils have also been reported to be involved in adaptive immune response orchestration. For example, once neutrophils are recruited to the site of infection they contribute to the activation, orientation and magnitude of adaptive immunity (19, 20).

**Myeloid cells**

**MONOCYTES AND MACROPHAGES**

Monocytes represent approximately 10 % of leukocytes in human blood and form part of the innate immune system. These cells derive from bone marrow hematopoiesis with several intermediate progenitor cells such as the CMP. The
most important growth factor in the development and maturation of monocytes is macrophage colony-stimulating factor (M-CSF) as evidenced by studies showing that monocytes are markedly reduced in M-CSF-deficient mice (21). CD115 is the cell surface receptor ligated by M-CSF. Phenotypically, it has proven difficult to distinguish blood monocytes from some DC subsets as these cell types share several cell surface markers. Monocytes may be divided into three groups based on their expression of CD14 and CD16. The largest subset of monocytes (80-90%) expresses CD14 but not CD16 and are defined as “classical” monocytes. There are also two smaller monocyte subsets that are phenotypically defined as CD14+CD16+ or CD14dimCD16+ (22, 23).

Macrophages reside in many tissues and are often given specific names dependent on the harboring tissue as exemplified by microglia in the brain and spinal cord, Kupffer cells in the liver and osteoclasts in bone tissue. Tissue resident macrophages are mainly derived from yolk sac-derived erythro-myeloid progenitors (24) but may also be replenished from monocytes that extravasate from the blood stream, which means that these cell types share many surface markers (25). Macrophages may engulf and destroy microorganisms and aberrant cells by generating reactive oxygen species (ROS) and other toxic compounds. Macrophages also produce growth and migration factors and are pivotal to angiogenesis and tissue remodeling. The latter features are of significant importance in a tumor setting as macrophages are implicated in tumor progression and metastasis formation. Tumor-associated macrophages (TAMs) are healthy macrophages recruited to sites of tumor expansion and via signaling from tumor cells, TAMs may create a microenvironment that suppresses immune responses and thus may promote tumor growth by secreting growth factors and by supporting angiogenesis (26).

**DENDRITIC CELLS**

DCs are present in almost every tissue and constitute an important link between innate and adaptive immunity. DCs are often subdivided into three distinct groups, *i.e.* conventional, plasmacytoid and monocyte-derived DCs. As other cells of the innate immune system, DCs originate from bone marrow hematopoiesis and share phenotypic markers with monocytes, which can be a precursor cell although most DCs originate from the common dendritic cell precursor. Conventional DCs are specialized APCs that after sampling of antigens migrate to draining lymph nodes and present peptides to naïve T cells, which induces proliferation and T cell differentiation. The discovery of DCs was awarded with the Nobel Prize in physiology or medicine in 2011 (27, 28).

In paper II we report findings in two phenotypically different conventional DC subsets that are characterized as either CD1c+ or CD141+. Recent studies
have defined the respective functions of the CD141⁺ DCs and the larger group of CD1c⁺ DCs. CD141⁺ DCs thus express higher levels of TLR3, have a superior capacity to induce Th1 responses and excel in antigen cross-presentation (29).

**NEUTROPHILS**

Neutrophils are the most abundant circulating white blood cells. The importance of neutrophils in host defense is illustrated by the severe and often fatal infections characteristic of patients with neutrophil deficiency. Neutrophils are produced in the bone marrow and their release into the bloodstream is tightly regulated. The number of neutrophils increases rapidly and transiently upon infection. It has proven difficult to maintain viable neutrophils in experimental conditions such as cell culture or after freezing, which has hampered detailed functional studies.

However, it is clear that neutrophils kill microbes and that these cells possess a large armory of tools for this purpose. For example, neutrophils utilize reactive oxygen species (ROS) produced in a process called respiratory burst. ROS are generated either intracellularly to kill phagocytosed bacteria or extracellularly to also kill neighboring microbes. Evidence for the importance of ROS in defense against microbes has been obtained in the study of patients with chronic granulomatous disease, which is characterized by insufficiency of neutrophils and other myeloid cells to produce ROS along with recurrent bacterial and fungal infections (30). Another tool available to neutrophils is NETosis (“neutrophil extracellular traps”) where neutrophils release chromatin into the extracellular space to capture and eliminate microbes (31). In the context of cancer, several reports imply that neutrophils may facilitate tumor growth by inactivating immune cells with anti-tumor function, and several clinical studies have reported that neutrophil infiltration in solid tumors or high counts of neutrophils in the circulation are associated with poorer prognosis for survival (32, 33).

**Lymphoid cells**

**B CELLS**

B cells derive from the common lymphoid progenitor cell and undergo several maturation and checkpoint steps in the bone marrow and are subsequently released into the circulation. Early B cell development comprises the stochastic rearrangement of the light and heavy chain immunoglobulin loci that then assemble to form the B cell receptor (BCR). The BCR complex contain surface immunoglobulins that upon antigen binding signals through immunoreceptor tyrosine-based activation motifs. This process involves a rigorous checkpoint
system that inactivates B cells with the potential to produce self-reactive antibodies. Mature naïve B cells released from the bone marrow have unique B cell receptors that respond to specific antigens. Upon activation, B cells undergo further maturation and proceed either to a memory B cell or an antibody-producing plasma cell. Naïve B cells produce IgM and IgD antibodies however upon activation through CD40 via CD40L stimulation from CD4+ T cells these B cells undergo antibody class switching by changing the heavy chain part to produce IgA, IgG or IgE antibodies. This procedure preserve the antigen specificity although it enables daughter cells to produce different kinds of isotypes. The formation of antibodies against specific targets is a vital part of immune defense against foreign pathogens. In addition, the Fc part of IgG antibodies functions as an activating ligand for macrophages, cytotoxic T cells and NK cells that exert antibody-dependent cellular cytotoxicity (ADCC) (34, 35).

**T CELLS**

T cells are formed in the bone marrow although they are named after the thymus where they mature. Similar to the process described for B cells, T cells undergo several checkpoint steps during development to ensure eradication of self-reactive cells. T cells express T cell receptors (TCR) that through genetic recombination and editing recognize foreign peptides. It is estimated that a normal human T cell repertoire contains approximately $2.5 \times 10^8$ different TCRs (36). Naïve T cells migrate to secondary lymphoid organs in a process called homing. After antigen capture, APCs such as DCs migrate to the lymphoid organs to interact with and activate T cells displaying a TCR that recognizes the peptide presented on the MHC. In more specific terms CD4+ T cells recognize peptides presented on MHC class II that presents endocytosed antigens. CD8+ T cells recognizes peptides presented on MHC class I that primarily present intracellularly generated peptides. However, via cross-presentation by the APC can also present endocytosed antigens on MHC class I to CD8+ T cells. Activation of naïve T cells through TCR engagement induces proliferation of T cell clones with the same peptide specificity. Finally, the activated T cells migrate and home to the site of infection or inflammation to exert cytotoxicity. Some of the responding T cells differentiate into memory T cells to ensure a renewed T cell response in case of novel exposure to a specific antigen (37).

T cells may be subdivided based on CD4 and CD8 expression. CD4+ T cells enhance CD8+ T cell responses, exert immunosuppression, regulate macrophage functions and help B cells to generate antibodies. The importance of CD4+ T cell function is highlighted in HIV infection where reduced CD4+ T cell numbers increases the prevalence of opportunistic infections and the risk
of malignancy. CD4+ T cells are further subdivided based on differences in cytokine secretion and expression of surface molecules. Th1 cells are the main producers of IFN-γ whereas Th2 cells instead produce IL-4, IL-5 and IL-13 as their signature cytokines (38).

Another subset of CD4+ T cells has a unique role in regulating immune responses and are denoted regulatory T cells (Tregs). Tregs are immunosuppressive cells that may be characterized by their surface expression as CD4+CD25+ and cytosolic expression of Foxp3. Maturation and accumulation of Tregs is driven by IL-2 signaling that induces Foxp3 expression in a STAT5-dependent manner. Tregs utilize several mechanisms to suppress immune responses. CD25 thus binds IL-2 with high affinity and may thereby deprive surrounding T cells from IL-2. Tregs may also suppress T cell responses by expressing CTLA-4, CD39 and CD73 (39, 40).

The second main T cell compartment, the CD8+ T cells, are specialized effector cells often referred to as cytotoxic lymphocytes (CTL). CD8+ T cells that have been activated by their specific peptide presented on MHC class I by an APC can then proceed to kill any cell type that presents the same peptide on their MHC class I. A fraction of CD8+ T cells persists as memory cells (41, 42).
NATURAL KILLER CELLS

Although regarded as part of innate immunity, NK cells are granted a separate section as these cells are in focus in several of the papers in this thesis. NK cells recognize infected or transformed cells to kill by other means than those utilized by cytotoxic T cells (“natural” cytotoxicity). Instead, NK cells carry germline-encoded receptors that regulate activation, proliferation and effector functions. These receptors typically recognize stress ligands and protein structures coupled to cellular stress. There are several similarities between NK cells and T cells in terms of cell signaling, functions and shaping immune responses. For example, NK cells secrete interleukin (IL-10) and IFN-γ, which are features shared by CD4+ T cells, and NK cells also utilize cytotoxic effector function similar to those of CD8+ T cells (43). NK cells are considered a vital part of the immune defense against viruses and malignant cells. For example, individuals with NK cell deficiency are more prone to viral and, albeit to a lesser extent, bacterial infections (44, 45). The role of NK cells for the course of cancer is well documented (46), in particular in hematological malignancies (47-49).

NK CELL DISCOVERY AND THE “MISSING SELF” HYPOTHESIS

NK cells were discovered in the mid 1970’s in studies using assays that measured the capacity of lymphocytes to spontaneously kill tumor cells in vitro. These experiments identified non-T lymphocytes (initially called “natural killer lymphocytes”) that killed malignant cells without prior sensitization (50). In the following years, NK cells in mice and humans were characterized in more detail, and the mechanism explaining how NK cells engage with their targets was further elucidated. Klas Kärre at the Karolinska Institute postulated the “missing self” hypothesis in his doctoral thesis (51). The hypothesis thus postulates that NK cells attack cells with impaired MHC class I expression but spare healthy cells with intact class I expression. The past decades have seen rapid development in the area of interactions between NK cells and their targets, and while the “missing self” hypothesis has proven largely correct it is clear that NK cells are actually more sophisticated than first appreciated.
NK CELL DEVELOPMENT AND RECEPTORS

While details of the development of murine NK cells in the bone marrow have been extensively studied, comparatively little is known about this process in humans. The development comprises five stages depending on the expression of certain cell surface markers (52) as shown in figure 2. NK cell precursors carry the CD34\(^+\)CD117\(^+\)CD123\(^+/−\) phenotype, and the first transition is a shift from CD123 to CD127 expression. The next cell in NK cell differentiation, the NK cell lineage progenitor (NKP), is characterized by the loss of CD127; this progenitor only generates functional NK cells (53). The ensuing stages comprise the most studied NK cell compartments. Stage four is thus characterized by the loss of CD34 and the acquisition of CD94; these cells are referred to as CD56\(^{bright}\) NK cells. In the final stage of NK cell development, there is a gradual loss of CD94 along with the acquisition of CD16. This group of NK cells is commonly known as CD56\(^{dim}\) NK cells (52).

Figure 2. NK cell maturation scheme with defining markers.
Despite being postulated already in the “missing self” hypothesis it took almost a decade before the inhibitory receptors responsible for sensing MHC class I molecules were fully identified. In the early and mid-1990’s several investigators reported the presence of killer cell immunoglobulin-like inhibitory receptors (KIR) on NK cells in mice and humans (54-57). One of the missing pieces of the hypothesis was that the lack of MHC class I expression was insufficient for NK cell target eradication. However, several research groups described activating NK cell receptors that were incorporated into the theory of NK cell activation. The modified hypothesis thus stated that NK cells attack cells that lack MHC class I molecules and simultaneously express stress ligands that ligate activating receptors such as NKp30, NKp44, NKp46 and NKG2D on the NK cells (Figure 3) (58-60).
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</tr>
<tr>
<td>NKp30</td>
<td>B7-H6, BAG-6</td>
</tr>
<tr>
<td>NKp44</td>
<td>PCNA</td>
</tr>
<tr>
<td>NKp46</td>
<td>Viral HA</td>
</tr>
<tr>
<td>NKp80</td>
<td>AICL</td>
</tr>
<tr>
<td>NKG2D</td>
<td>MICA/B, ULBP-1-6</td>
</tr>
<tr>
<td>DNAM-1</td>
<td>Nectin-2, PVR</td>
</tr>
<tr>
<td>2B4</td>
<td>CD48</td>
</tr>
<tr>
<td>NTB-A</td>
<td>NTB-A</td>
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<tr>
<td>CD16</td>
<td>IgG</td>
</tr>
<tr>
<td>LFA-1</td>
<td>ICAM-1</td>
</tr>
</tbody>
</table>

**Figure 3. NK cell receptors and their ligands.** Inhibiting interactions are marked with a red box and activating interactions are marked with green.
**THE KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS (KIR)**

The genes encoding KIR are located on chromosome 19 within the leukocyte receptor complex. Inhibitory KIRs are necessary for proper NK cell education and maturation, but certain KIRs may also activate NK cells. The nomenclature of KIR receptors distinguishes inhibitory (e.g. 2DL1) with long cytoplasmic tails from the activating receptors (e.g. 2DS1) with short cytoplasmic tails. The major ligands for inhibitory KIRs are four epitopes of HLA-A, -B and –C, where KIR3DL1 recognizes Bw4 epitopes on HLA-A and –B, KIR2DL2/L3 recognizes the HLA-C1 epitope and KIR2DL1 the HLA-C2 epitope. KIR receptor genes and HLA genes are encoded by different chromosomes. Each individual may therefore carry 1 up to all 4 epitopes that serve as KIR ligands.

The degree of education and inhibitory input received from KIRs will therefore vary between individuals depending on their HLA allotypes. The term education is defined as the process in which NK cells gain reactivity and will be further explained in a later section. From an evolutionary perspective the KIR system is a recent NK regulatory mechanism and directed towards a more specific way of NK cell education with specified HLA ligands (61). Additionally, a dimorphism in HLA-Bw4 at position 80 affects the strength of interaction with KIR3DL1. Presence of isoleucine (80I) yields higher binding affinity than presence of threonine (80T). The dimorphism modulates the inhibitory regulation of NK cells and has been proposed to affect the outcome of allogeneic bone marrow transplantation and immunotherapy (62, 63).

There are 13 genes encoded by the KIR locus and 3 additional genes that are usually not transcribed and hence lack proper nomenclature. The KIR locus and haplotypes are highly polymorphic and vary in terms of number and binding affinity between individuals, as previously exemplified with position 80 in 3DL1. Only 2DL4 and 3DL2 occur in all haplotypes. KIR genes are often grouped into haplotypes A and B (64). The group A haplotype contains fewer genes than group B and comprises a battery of inhibitory receptors but only one activating receptor (2DS4). Also, group A haplotypes usually encompass the 2DL3-2DL1 inhibitory receptor combination. Group B haplotypes commonly encode the 2DL2 inhibitory receptor in addition to a range of activating receptors such as 3DS1, 2DS1-3 and 2DS5. The frequencies of group A and B haplotypes are almost even in the population and presents an interesting possibility for analysis of the impact of these haplotypes on differences in NK cell function among individuals (65). Interestingly allo-transplantation studies have suggested that NK cells from KIR B donors have better outcome in terms of event-free survival (66).
NKG2A: HLA-E INTERACTIONS

The inhibitory receptor NKG2A/CD94 is a heterodimer of the subunits CD94 and NKG2A, henceforth referred to as NKG2A. The ligand for NKG2A is the evolutionarily oldest class I HLA isotype HLA-E. This receptor-ligand interaction educates, in a process explained in detail in upcoming sections, the largest number of NK cells (61). HLA-E displays peptides derived from the leader sequence of other MHC class I complexes. This means that expression of HLA-E on the cell surface serves as a marker of the total MHC class I expression in the cell. CD94 can also form a complex with NKG2C, which is an activating receptor that recognizes HLA-E although with 6-fold lower affinity than NKG2A. There are also data supporting that NKG2C acts in a more peptide-specific manner than NKG2A sensing viral peptides displayed on HLA-E, which has been ascribed a role in defense against viral infections (67, 68).

As mentioned above, HLA-E presents leader peptides from other HLA class I sequences. Thus, in the absence of the leader peptide HLA-E does not properly fold and does not reach the cell surface (Figure 4). Peptides are constantly provided by HLA-A and HLA-C. However due to a polymorphism only some HLA-B alleles contribute to leader peptides that can be efficiently presented. At position 2 in the leader peptide of HLA-B corresponding to position -21 of HLA-B there is either a methionine (-21M) or a threonine (-21T). HLA-A, -C and the -B alleles carrying a methionine at this position in the leader peptide and can hence bind to HLA-E for proper folding and expression. The other HLA-B alleles carrying a threonine will produce leader peptides that do not bind efficiently to HLA-E (69).

Figure 4. HLA-E folding upon HLA-B leader peptide binding.
Genetic screening has shown that -21M HLA-B alleles are rarely found in haplotypes carrying genes encoding ligands for 3DL1 (HLA-Bw4) or 2DL1 (HLA-C2) but rather together with the weaker 2DL2/L3 ligand HLA-C1. This polymorphism impacts significantly on NK cell regulation. The -21M haplotype will provide NKG2A ligands to a larger extent and -21T haplotypes supply more KIR ligands. It was demonstrated in an ADCC setting that individuals grouped into having at least one -21M (M/x) harbor NKG2A+ NK cells that are better educated and more functional compared with individuals having two copies of -21T (T/T) (70). Little is known about the clinical implications of the -21 polymorphism, as further discussed in Paper V. However there is a proposed connection between -21T and NK cell-mediated control of viral load in HIV infection (71, 72).

MODELS OF NK CELL EDUCATION

The mechanisms that regulate NK cells and the order in which these cells gain and retain cytotoxic capacity have been extensively studied. Two co-existing models have been proposed to explain how NK cells become educated. The licensing/arming model postulates that NK cells, upon receiving inhibitory signaling, undergo a “licensing program” where molecular pathways and receptors are mobilized to create an educated and cytotoxic NK cell (73, 74). In support for this theory, the deletion of SHP-1, a key mediator of inhibitory signaling, results in accumulation of immature NK cells, although the mechanisms explaining how the inhibitory signaling functions in order to educate NK cells remain unknown (75). The second model, the disarming model, postulates that all NK cells initially are highly reactive but then lose their cytotoxicity due to chronic activation if they do not receive inhibitory input (76). The idea is that inhibitory signals preserve NK cell function and their reactive potential. The model is supported by the finding that KIR2DS1+ NK cells in homozygous HLA-C2 individuals are hyporesponsive, which most likely is due to a large input of activating stimuli (77). An interesting recent study fits into both proposed models by showing that NK cells expressing iKIRs with the cognate HLA ligand present store larger cytotoxic granules than uneducated NK cells (78).

There are also models that focus on the level of education rather than the pathway of achieving education. The rheostat model thus states that the cytotoxicity of each NK cell is set based upon its capability to sense MHC class I molecules. This means that NK cells with many inhibitory receptors maintain a higher cytotoxic capacity (79). The rheostat model is challenged by the finding that KIR2DL1 and KIR2DL3 were found to be equally educating despite differences in binding affinities (80). The second model of NK cell cytotoxic maintenance is similar to the rheostat model but introduces the
possibility of fast tuning of NK cell function. The tuning model thus postulates
that NK cell education is an ever-changing process and dependent upon the
inhibitory input to each NK cell receives in their current environment. The
model proposes that NK cells may transit from being highly educated, and thus
highly cytotoxic, into less educated cells depending on the HLA environment
(81). Notably, the models presented are not necessarily contradictory to each
other, and the education of NK cells and the tuning of their function might
comprise a combination of these theories.

In summary, education is a description of the process through which an NK
cell becomes programmed for reactivity and does not per se take into account
how reactivity is acquired or retained (Figure 5). Education is tightly connected
to expression of inhibitory receptors. NK cells are thus considered educated
when they express either NKG2A or inhibitory KIRs (iKIR) that correspond
to a host specific HLA ligand (82). In the maturation of NK cells NKG2A is
the first receptor to be expressed and usually the only inhibitory receptor
present on CD56<sup>bright</sup> NK cells. As NK cells transit into CD56<sup>dim</sup> cells they
gradually lose NKG2A and acquire iKIRs in a partly stochastic manner (83).

**Figure 5. NK cell interactions.** Schematic drawing of NK cell outcomes in different
settings to illustrate that educated NK cells have lower threshold for activation.
Adapted from (82).

**“UNLICENSED” NK CELLS**
Some individuals will not have all KIR ligands present in their HLA
haplotypes and hence harbor a subset of NK cells expressing only the iKIR
receptor with no matching ligand. According to the models presented in the
previous section, these NK cells will remain uneducated and are commonly referred to as unlicensed NK cells. A significant proportion of NK cells that expresses only one KIR have remaining NKG2A expression distorting the definition. In our lab we utilize the term self-iKIR (S-iKIR) expressing NK cells and nonself-iKIR (NS-iKIR) expressing NK cells disregarding NKG2A positivity. The unlicensed NK cells are hyporesponsive in their normal state since these cells may damage healthy cells due to the lack of proper inhibition.

There are, however, several studies reporting the importance of unlicensed NK cells in settings under conditions of immune activation, such as in autologous transplantation or viral infections (84, 85). There are also reports suggesting that unlicensed NK cells become activated and cytotoxic upon cytokine stimulation (86, 87). These findings thus imply that a small proportion of NK cells could, in an immune response phase, become highly activated and avoid inhibition. Aspects on the potential impact of unlicensed NK cells in cancer immunotherapy are presented in papers III - IV.

**NK CELL TARGET ENGAGEMENT AND SIGNALING PATHWAYS**

NK cell engagement and formation of a lytic synapse with a target cell can be divided into three main stages: the recognition and initiation stage, the effector stage and the termination stage (88). It is not known exactly which molecules and surface receptors that are responsible for each step when NK cells recognize and initiate contact with a target cell. Tethering receptors such as CD62L and adhesion receptors such as CD2 and LFA-1 are, however, likely to play major roles. The signal triggered by LFA-1 in combination with a co-activation signal such as from CD2 results in firm adhesion to the target cell (89). After the cell contact has been initialized additional signaling receptors trigger F-actin reorganization, phosphate generation and tyrosine kinase activation (90, 91). At this point of synapse formation it is likely decided whether or not the NK cell will mount a cytotoxic response by degranulating its cytotoxic granules. This signaling may derive from immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are largely depending on SHP-1 downstream signaling (92). A dominating inhibitory signal will quickly disrupt further conjugate formation and F-actin reorganization. CD16 may exemplify how activating signaling results in NK cell cytotoxicity. Activation of CD16 thus results in Src kinase phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3ζ and FceRγ, which in turn activate SYK and ZAP70 for further downstream signaling. A crucial effector molecule downstream of activating receptors is Vav1, which also mediates key interactions between inhibitory and activating signals. SHP-1 is activated by inhibitory receptors and uses Vav1 as a substrate, which leads to dephosphorylation of Vav1 and disrupted NK cell activation (93, 94).
After completion of the initiation stage NK cells transit into an effector phase that culminates in the release of cytotoxic granules towards the target cell surface. In this process, F-actin will continue to reorganize in the cytosol and granules will polarize towards a structure formed by microtubule called the microtubule organizing center (MTOC). Studies have shown that movement of granules and MTOC towards and into the synapse is carried out in a dynein-dependent fashion (95). Another key component in the exocytosis of granules is the release of Ca\(^{2+}\) from storages in the endoplasmic reticulum. Ca\(^{2+}\)-flux can be used to monitor early activation of NK cells and has been employed in studies of co-activated receptors on NK cells (96, 97).

The lytic granules share features of secretory lysosomes (98). They thus contain lysosomal enzymes along with proteins only found in lytic granules. These proteins comprise perforin, granzymes, Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL), all of which participate in NK cell-mediated lysis of target cells. The lysosome-associated membrane protein (LAMP)-1 (CD107a) is a lytic granule protein widely used as a marker of NK cell degranulation (97). Recent findings suggest that CD107a is not a lytic protein but rather protects NK cells from degranulation-associated suicide (99).

Apart from releasing toxic components towards target cells through exocytosis of granules, activation of NK cells also triggers production of NK cell-derived cytokines and chemokines. This is a slower process than degranulation and chemokines are secreted several hours after activation. Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) are often measured, in conjunction with CD107a, to reflect this phase of NK cell activation. IFN-γ exerts several important immune functions such as immunoregulation and anti-tumor properties, TNF-α serves as a chemoattractant for neutrophils and stimulates phagocytosis (97).

After the release of lytic granule the NK cells initiate the termination stage of the encounter. Only minor changes in NK cell signaling and reorganization occur while the target cell starts undergoing apoptosis. When detachment progresses NK cells may shed activating receptors to reduce stimulatory input as described for CD16 and NKG2D (100, 101). The exact kinetics of NK cell detachment is not fully understood although this process is observed to be faster when killing occurs (102).
NK CELLS IN DISEASE CONTROL

NK CELL RESPONSES IN VIRAL INFECTIONS
Functional NK cells play a vital role in the host defense against invading viruses and other microbes. This claim is supported by observations made in individuals with a deficient NK cell repertoire. Thus, increased susceptibility to infections was found to correlate with reduced NK cell dysfunctionality (44, 45, 103). In particular, NK cells have been ascribed a role in defense against infections with human immunodeficiency virus (HIV), hepatitis C virus (HCV) and cytomegalovirus (CMV) (104-106).

Virus-infected cells undergo several modifications that can be detected by NK cells, including down-regulation of MHC class I on the cell surface to avoid T cell recognition. In this scenario NK cells will be the optimal effector cell reacting towards a target cell with reduced expression of inhibitory ligands. Furthermore, infected cells increase their expression of stress- and virus-induced ligands that engage activating receptors on NK cells (107). The most common stress ligands in viral infections are ULBPs and MICA/B that ligate the activating receptor NKG2D. Notably, some viruses are reported to avoid NKG2D-induced activation by downregulating ligands on infected cells through transcription of viral proteins that ligate NKG2D (108).

In HIV infection, NK cell cytotoxicity controls viral load and certain KIR and HLA alleles are reportedly beneficial in controlling the infection. Individuals carrying a Bw4 motif were thus found to better control viremia and showed slower disease progression (109). Subsequent studies showed that disease progression was slower also in individuals carrying the high-affinity ligand Bw4-801 along with the KIR3DS1 receptor (110). This implies that KIR3DS1+ NK cells receive high affinity activating signaling and hence efficiently remove virus-infected cells. The same group observed improved control of HIV in subjects carrying low-expressed alleles of KIR3DL1, which implies a favorable impact also of reduced inhibitory input to NK cells (111).

IMPACT OF CYTOMEGALOVIRUS ON NK CELL REPERTOIRES
Human cytomegalovirus (CMV) is a large double-stranded DNA virus belonging to the family of herpesviruses. It is highly prevalent in humans and >80 % of individuals will become infected in their lifetime, mostly in childhood or adolescence. In a screening performed in our laboratory in search for CMV seronegative blood donors for the studies in paper IV we found that only approximately 10 % were seronegative. CMV infection is often relatively harmless, in particular in childhood infections. In teenagers and younger adults, a primary infection may cause symptoms of mononucleosis. CMV is a
source of morbidity and mortality in immunocompromised individuals, such as transplant recipients undergoing extensive immunosuppressive therapy, where CMV can reactivate from a latent infection. A primary or reactivated CMV infection during pregnancy may cause congenital infection and birth defects. After the primary infection, latent CMV persists in stroma cells and monocyte precursors, and immune surveillance control is needed to prevent reactivation (107).

CMV utilizes similar mechanisms of immune escape pattern as many other viruses. Peptide-specific T cell responses are key components in combating CMV, and downregulation of HLA class I molecules is thus commonly observed in CMV-infected cells (112). Since downregulation of classical MHC molecules facilitates NK cell cytotoxicity, CMV has developed several defense mechanisms to also avoid NK cell recognition. For example CMV expresses a MHC homolog, UL18, which interacts with the NK cell receptor LIR-1 and inhibits cytotoxicity (113). To further avoid destruction of infected cells, CMV has developed strategies to lower the expression of NKG2D ligands. Thus, CMV encodes proteins targeting the ligands MICA, MICB and several ULBP variants. In addition, CMV encodes mRNA sequences that inhibit gene translation of the ligand MICB (114). A third strategy is to enhance HLA-E in order to inhibit NK cells through enhanced interaction with NKG2A. A CMV protein, UL-40, binds to HLA-E and increases its surface expression. In this manner CMV-infected cells can make use of the low HLA-ABC expression to avoid T cell recognition and still inhibit NK cells through maintained or increased HLA-E expression (115).

In spite of the defense mechanism developed by CMV, there are additional means to control the infection. The NKG2C receptor is a heterodimer complex together with CD94 and serves as an activating receptor that is specifically employed to combat CMV infection. However, NKG2C+ NK cells also exemplify that CMV infection makes a dramatic imprint on immunity. The impact of CMV infection on immunity was observed in immune screenings in identical twins showing that 50 % of all measured immune parameters differed between seronegative and seropositive individuals (116). NK cell subsets differed significantly between seropositive and seronegative subjects. The major difference was observed in the NKG2C+ subsets that constituted 2-3 % of NK cells in seronegative subjects and approximately 25 % in seropositive subjects (68).

Adaptive memory-like NK cells were first introduced by the work of Lewis Lanier and colleagues who described rapid expansion of NK cells upon CMV infection in mice (117). The NKG2C+ cells respond better in terms of IFN-γ
production in seropositive donors, which implies specific functionality (118). These cells are phenotypically characterized by expression of NKG2C, CD2 and CD57 but show reduced expression of classical activating receptors such as NKp30 and NKp46. The adaptive NK cell seem to be dependent on antibody-binding of opsonized-CMV infected cells through CD16 which trigger response and expansion (119). The exact mechanism how expansion of adaptive NK cells is carried out is not known although there are reports suggesting a major role for CD2 co-stimulation in adaptive NK cell responses (120). Consistent with recent findings the results in paper IV show that the frequency of mature NKG2C⁺KIR⁺CD57⁺ NK cells were higher in seropositive patient which also correlated to fewer unlicensed NK cells suggesting a more mature phenotype in that patient group.

**CMV IN A LEUKEMIA SETTING**

The preferred treatment option for high-risk AML patients is allogeneic hematopoietic stem cell transplantation (HSCT). The intensive immunosuppression associated with transplantation may cause reactivation of CMV, which is a cause of morbidity and mortality. However, patients with CMV reactivation after transplantation had a more mature NK cell signature profile 3-6 months after transplantation (121) and several reports showed that the improved establishment of mature NK cells after CMV reactivation was associated with reduced relapse risk (122, 123). In non-transplanted AML patients, little is known about the impact of CMV infection on relapse risk. **Paper IV** addresses this question in non-transplanted patients receiving immunotherapy for relapse prevention.
NK CELLS IN MALIGNANCIES

The precise role of NK cells in neoplastic disease remains a matter of debate (48, 124). A prospective study showed that high cytotoxic activity of NK cells was associated with reduced cancer incidence (46). In a follow-up study, these authors demonstrated that haplotypes of NKG2D were associated with cancer risk further supporting NK cell involvement in the control of malignant cells (125).

The immune defense against arising cancer cells likely involves complementary actions of innate and adaptive immunity in a process called immunosurveillance. In order to mount a specific T cell response DCs or other APCs need to present peptides in secondary lymphoid organs. A recent study suggests NK cell involvement in this process where NK cells kills a transformed cell, which triggers IFN-γ production that stimulates DCs to migrate to the lymph nodes after antigen uptake, thus evoking a T cell response (126). Then CD8+ T cells can exert anti-tumor effects through peptide recognition on MHC molecules (127, 128). Due to the selective pressure from T and NK cell-mediated surveillance, cancer cells need to evolve means to avoid detection. Such alteration of cancer cells during pressure from immunity is denoted “immunoediting” and comprises mutations and phenotypic alterations, including modification of MHC expression (129) (130, 131).

The expression of stress-induced ligands such as the NKG2D ligands MICA, MICB and ULBPs along with DNAM-1 ligands PVR and nectin-2 (132, 133) is commonly up-regulated on cancer cells. Also, the B7-H6 ligand for NKp30 is expressed by myeloid leukemic cells (134). To avoid elimination by NK cells, cancer cells were shown to shed activating ligands such as the NKp30 ligand B7-H6 or NKG2D ligands (135-137). The shedding of ligands to the surrounding is believed to exhaust NK cells by constant activation in the absence of target cells.

The tumor microenvironment is of relevance to NK cell-mediated control of cancer cells. Presence of NK cells in the tumor microenvironment correlates with favorable outcome in some cancer types, but in many solid cancers the number of NK cells is in fact low (138). Myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) are frequently present in tumor tissue; these cells suppress functions of T cells and NK cells by, e.g., producing cytokines that are counterproductive to immune activation, expression of immunosuppressive receptors such as PD-1 or by producing immunosuppressive extracellular reactive oxygen species causing (139-141).
CHRONIC MYELOMONOCYTIC LEUKEMIA

Chronic myelomonocytic leukemia (CMML) is a hematopoietic malignancy with dismal prognosis. It is a highly heterogeneous malignancy with features of both myelodysplastic and myeloproliferative features (142). Patients commonly present with anemia, thrombocytopenia or infections due to neutropenia. The persistence of leukemic monocytes in peripheral blood is one of the diagnose criteria (143). The median age at diagnosis is 70 years with an incidence of 0.5-1 /100,000. CMML typically progresses slowly but a large group of patients transform into secondary AML. The only therapy with curative potential is allo-SCT although this is rarely a viable option in elderly patients (144). Patients are commonly treated with hypomethylating agents such as azacitidine and decitabine. However these regimens have not been fully evaluated in randomized trials for this indication (145, 146). For the majority of patients CMML is regarded as incurable and the median survival ranges from 2-3 years (147, 148).

Immunity in CMML is not well described in the literature. Carlsten and colleagues reported that bone marrow NK cells in MDS patients show decreased expression of DNAM-1 and NKG2D. A decrease in NCR expression correlated to bone marrow blast counts (149). In paper I we report similar findings regarding NK cell NCR expression in a cohort of CMML patients and suggest a role for immunotherapy. Our laboratory is currently involved in an ongoing phase I/II trial for CMML patients to investigate the feasibility and efficacy of immunotherapy with HDC/IL-2 in CMML (NMDSG14A).

ACUTE MYELOID LEUKEMIA

In terms of incidence, AML is the most common form of acute leukemia in adults with 3 – 5 cases new cases 100 000 individuals/year in most western countries. The incidence is 1.3/100 000 in individuals below 65 years old and 12.2/100 000 in those above 65 (150, 151). AML is thus primarily a disease of the elderly but also constitutes approximately 20% of acute leukemia in children. The diagnosis is based on cell counts and morphology of blood and bone marrow samples in combination with immunophenotyping and mutational analysis of leukemic cells (152). AML was previously categorized using the French-American-British (FAB) classification that primarily takes the stage of maturation of leukemic cells into account. The FAB classification system thus divides AML into subtypes M0-M7 where, for example, M4-M5 represents myelomonocytic or monocytic AML. The WHO system, which classifies AML based on genetic aberrations in leukemic cells, is nowadays the most widely used classification system (143, 153).
AML prognosis is dependent on host factors (age, health and performance status) and disease factors (genetics aberrations in leukemic cells, cell count and prior hematopoietic disease). High age is correlated with poor outcome and only between 5-15 % of patients above 60 years old are cured compared with 35-40 % in younger patients. The most common mutations are NPM1, DNMT3A and FLT3. Based on the mutation status and cytogenetics AML patients are grouped into risk groups (favorable, intermediate or adverse). Cytogenetic alterations such as translocations are present in 60 % of AML cases and is referred to as an aberrant karyotype. One common example of an aberrant karyotype is the translocation between chromosome 8 and 21 (t(8;21)) which is favorable in terms of relapse risk and survival (7). In approximately 40% of AML cases, however, chromosomes are morphologically intact (‘normal karyotype AML). Instead, these cases are characterized by distinct DNA mutations in the leukemic cells. Mutations in FLT3, for example, are associated with adverse risk, while AML with NPM1 mutations have a favorable risk profile (154).

The standard AML therapy has not significantly changed in the last decades. Patients will normally receive induction chemotherapy (mostly high-dose cytarabine and an anthracycline) aiming to attain microscopic resolution of leukemia and recovery of normal hematopoiesis (complete remission (CR)). CR is achieved in approximately 70 % of patients below 60 years and 40-60 % in patients above 60. After CR, patients are intended to undergo 2-4 rounds of consolidation chemotherapy, mostly high-dose cytarabine alone or supplemented with an anthracycline; this therapy is, however, often adjusted based on the health and age of the patient (7).

After the completion of chemotherapy, there is a high risk (60-80 %) of life-threatening relapses of AML. Patients may be candidates for an allogeneic stem cell transplant that provides protection against relapse by exerting graft-versus-leukemia (GVL). Allogeneic transplantation may be offered to patients below 70 years old with high-risk and, occasionally, intermediate risk AML. The graft-versus-leukemia effect is mediated by donor T cells and NK cells, but there is also a high incidence of graft-versus-host disease (GVHD) reflecting that transplanted cells may also attack healthy tissue. A mild GVHD reduces the relapse risk, most likely because the GVHD is paralleled by GVL (155). However, most AML patients are not eligible for transplantation due to age, lack of donors, intercurrent disease or favorable genetic changes in leukemic cells, where transplantation is not beneficial. For patients who are not candidates for transplantation, there is a high need for improved therapy, in particular to reduce the risk of relapse (156).
AML AND IMMUNE RESPONSES
The involvement of T cells and NK cells in eradicating leukemic cells is showcased in the transplant setting where anti-leukemic properties of the transplanted cells are relevant to the prevention of relapse. While transplanted T cells may be considered the dominant anti-leukemic cell, there are studies suggesting that NK cells may also participate. For example, by creating a mismatch in KIR receptor-ligand pairs between the donor and the recipient, donor NK cells will avoid inhibition by KIR/HLA interaction. This strategy of transplantation has yielded improved leukemia-free survival in some studies, although disputed by others (157-159).

NK cells have been ascribed a protective role also in non-transplanted AML patients. For example, downregulated surface expression of activating NK cell receptors such as NKp30, NKp44, NKp46 and DNAM-1 (160-162) is associated with poor survival outcome. Notably, NK cells regain NCR expression after cytokine stimulation \textit{in vitro} and \textit{in vivo} thus implying that immunotherapeutic strategies may improve NK cell functionality for relapse control (163).

AML cells may avoid NK cell recognition through several mechanisms. Downregulation and shedding of NKG2D ligands may impair NK cell-mediated lysis of leukemic cells (164), and leukemic cells may produce immunosuppressive mediators. Subsets of myeloid leukemias may thus release reactive oxygen species (ROS) produced by the myeloid cell NADPH oxidase (NOX2). ROS exposure induces apoptosis in NK cells, in particular in the CD16$^+$ subset, and reduces NK cell receptors and cytotoxicity in remaining cells (165-170). This mechanism is addressed in paper \textit{II} where expression of histamine type 2 receptors (H$_2$R), which regulate NOX2 activity in AML cells, was found to correlate with favorable outcome in patients receiving immunotherapy with HDC/IL-2.

NK cell immunotherapy in leukemia

HDC/IL-2
HDC/IL-2 is not merely an NK cell immunotherapy as it also targets myeloid cells and T cells. The IL-2 component activates NK cells and T cells to proliferation and enhanced cytotoxicity (171-173). The HDC component targets myeloid cells to induce maturation and to inhibit ROS formation via H$_2$R signaling (167, 174, 175). Immunotherapy with HDC/IL-2 has been evaluated in clinical trials in renal cell carcinoma and metastatic melanoma (176-178). A phase III trial conducted in 320 AML patients in remission compared treatment with HDC/IL-2 with standard of care and showed
significantly improved leukemia-free survival in the HDC/IL-2 arm. These results formed the basis for the approval of HDC/IL-2 for relapse prevention in Europe and Israel (179). IL-2 as a monotherapy have been tested extensively in AML but without any significant benefit. In a meta-analysis evaluating several trials using IL-2 as remission maintenance in AML no significant improvement of leukemia-free or overall survival was observed (180).

In a follow-up phase IV trial (NCT01347996, the Re:Mission trial) 84 patients were scheduled to receive HDC/IL-2. Peripheral blood was sampled at the start and end of cycles 1 and 3, see figure 6. The treatment was found to expand NK cell counts in all maturation stages and also induced upregulation of NCRs, which correlated with improved clinical outcome. Additionally a high number of CD56<sup>bright</sup> cells at the onset of treatment predicted reduced relapse risk and/or improved overall survival (163, 181). These results support that NK cell participate in the control of leukemic cells in AML and also that NK cell activation is feasible for relapse control. Further results from the Re:Mission phase IV trial in combination with in vitro data are presented in papers II, III, IV, V.

![Figure 6. Overview of the Re:Mission phase IV trial in AML. Samples were collected at the start and end of cycles 1 and 3.](Image)

**CHECKPOINT INHIBITION**

The success of checkpoint inhibitors targeting inhibitory receptors in T cells in, e.g, melanoma and lung cancer suggests the possibility of targeting similar NK cell-inhibitory pathways in leukemia (182). Lirilumab is a therapeutic monoclonal antibody against KIR2DL-1,2,3 that blocks the interaction of
inhibitory KIRs with the corresponding HLA. Initial results in vitro and in mouse models indicated improved NK cell function using lirilumab as the single agent or in combination with other immunostimulatory compounds (183). Unexpectedly, early trial results implied that treatment with lirilumab reduced rather than enhanced NK cell functionality. Infusion of lirilumab thus decreased the expression of KIR2D on NK cells, and the lack of inhibitory input resulted in hyporesponsive cells (184). A recent trial in elderly AML patients in remission did not demonstrate improved leukemia-free survival compared with controls (185). While the detailed mechanisms remain to be defined, it is conceivable that blockade of inhibitory KIR may also impact negatively on NK cell education, thus reducing NK cell function. An additional antibody, monalizumab, targets NKG2A and thus blocks the NK cell-inhibitory interaction between NKG2A and HLA-E. Monalizumab has been evaluated in early clinical trials in head and neck cancer showing encouraging safety data and promising early responses (186) but clinical results in AML are not yet available.

ADOPTIVE TRANSFER
The concept of adoptive transfer is based on using endogenous or allogeneic immune effector cells, including NK cells that are stimulated and expanded ex vivo and then infused to patients. Cytokines such as IL-2 are often administered to ensure viability and function of the infused cells (187). A multitude of cell types and cytokine combinations have been evaluated for adoptive transfer in AML, but no trial results based on randomized comparisons are yet available. The field of adoptive transfer is largely founded on pioneering studies by Miller and co-workers where haploidentical NK cells were infused after both transplantation and in a non-transplant setting (188, 189). A study treating patients with high-risk myelodysplastic syndrome with infusions of IL-2-activated haploidentical NK cells reported tolerance and activity against leukemic cells (190). Another study reported NK cell anti-tumor activity using IL-2-expanded NK cells in conjunction with an IgG1-antibody inducing ADCC towards gastric or colorectal cancer (191). In other trials a cytokine combination of IL-12/15/18 was employed to expand NK cells ex vivo creating a NK cell subset defined as cytokine-induced memory-like NK cells. These cells showed improved interferon-γ production and increased cytotoxicity against HLA-expressing AML blasts despite being KIR+, thus suggesting that the cytokine activation overrides inhibitory signals. Infusion of these NK cells into AML patients with active disease showed promising results with five responders out of nine patients with residual leukemia, including four complete remissions (192).
CHIMERIC ANTIGEN RECEPTOR NK CELLS

Chimeric antigen receptor (CAR) T cells against CD19 expressed by B cells were recently introduced in the treatment of B cell malignancies, and a similar strategy is currently evaluated using NK cells as the cytotoxic effector cells. CAR NK cell therapy could potentially reduce the side-effects of CAR T cells and may also be used as an “off-the-shelf” therapy. In CAR T cell therapy, the construct contains three components: one extracellular region recognizing a target ligand (e.g. CD19), one linker region that provides receptor stability and flexibility and one intracellular signaling region that activates the effector cell upon target ligand binding. Several strategies are employed to create optimal intracellular signaling; the most recent generation of CARs commonly utilize CD3ζ together with CD28 (193). A phase I/II trial (NCT03056339) currently evaluates a CAR NK cell construct in relapsed or refractory B cell lymphoma or leukemia. The CAR construct is designed to recognize CD19 together with an intracellular IL-15-producing construct in cord blood-derived NK cells (194). Another in vitro study of CAR NK cell constructs suggests that redirected primary NK cells may override inhibitory NKG2A signaling (195).

ANTIBODIES, BIKES AND TRIKES

CD33 is a conceivable target in AML as this antigen is commonly expressed by AML cells. Single-agent antibodies against CD33 have not proven efficacious in AML. Gemtuzumab-ozogamicin (GO) is an antibody against CD33 conjugated with calicheamicin, an anthracycline. GO does not mediate ADCC and may be viewed as a method to deliver chemotherapy directly to leukemic cells rather than an immunotherapy. GO is approved as induction therapy in older AML patients (196) (197) but does not reduce the incidence of relapse in the post-chemotherapy phase of AML (198).

Apart from GO, no monoclonal antibodies against structures expressed by leukemic cells are approved for use in AML. Many leukemic antigens are shared with healthy hematopoietic progenitor cells and may thus entail toxicity, and the variability of antigen expression within the leukemic clone poses an additional challenge. Antibodies against CD123 are currently evaluated in AML (199) along with antibodies against IL1RAP, which is highly expressed on immature AML cells and hence could offer a more specific target towards leukemic stem cells (200).

Another emerging concept is bispecific killer cell engagers (BiKEs) and trispecific killer cell engagers (TriKEs), which comprise engineered antibody fragments with multiple signaling. A BiKE contains two antibody fragments, one of which is typically CD16 aiming to mediate ADCC (201). The construct was further developed by the addition of an IL-15 crosslinker that promotes
NK cell expansion, thus creating a TriKE. A clinical trial will commence in late 2018 to evaluate the CD16/CD33/IL-15 TriKE in CD33\(^+\) myeloid malignancies (202, 203).
AIMS

The main objective of this thesis was to describe aspects of immunity during HDC/IL-2 immunotherapy and to identify predictive and/or prognostic markers with a focus on the role and impact of NK cells. The specific aims of each paper are listed below.

**Paper I** aimed to describe mechanism of immune evasion in CMML with focus on ROS production from malignant myeloid cells.

**Paper II** aimed to assess the dynamics of myeloid cell populations during immunotherapy with HDC/IL-2.

**Paper III** aimed to define the impact of KIR/HLA interactions and NK cell education for the outcome of immunotherapy with HDC/IL-2.

**Paper IV** aimed to investigate the impact of a past CMV infection on clinical outcome in patients receiving HDC/IL-2.

**Paper V** aimed to define the role and impact of the HLA-B -21 dimorphism on clinical outcome during HDC/IL-2 treatment.
PATIENTS and METHODS

PATIENTS

In **paper I** data are presented from a cohort of CMML patients seen at the university hospitals in Lund and Gothenburg, where leukemic cells and peripheral blood cells were recovered. The study aimed at determining the potential immunosuppressive properties of leukemic CMML cells and to define strategies to overcome the immunosuppression. **Papers II – V** present results from the Re:Mission trial (NCT01347996), which was an open-label single-arm phase IV study that enrolled 84 (age 18-79) patients in first CR with de novo or secondary AML. Patients received 10 cycles of HDC (0.5 mg by subcutaneous injection twice daily) and human recombinant IL-2 (16 400 IU/kg by subcutaneous injection twice daily). The scheduled treatment duration was 18 months and patients were followed for up to 2 years after enrollment. The primary objectives were to monitor minimal residual disease (MRD) during the course of treatment and to monitor quantitative and qualitative pharmacodynamics effects with focus on the phenotype and function of NK cells, T cells and myeloid cells. All patients gave written informed consent prior to enrollment and the trial was approved by the Ethical committees in all participating regions. **Papers III – V** also include experiments using samples of peripheral blood from healthy donors. In the latter studies we utilized two sample cohorts of PBMC collected for HLA allele typing and CMV serostatus. All buffy coats were obtained from the component laboratory at the Sahlgrenska hospital.

Methods

**FLOW CYTOMETRY**

Flow cytometry is a powerful experimental tool that enables the simultaneous analysis of extracellular and intracellular components in individual cells. From a specific sample it is possible to obtain information of the cell size, granularity and phenotype. Flow cytometry analysis is the experimental basis in 4 papers in this thesis and was employed in studies of immune phenotypes, NK cell degranulation, NK cell cytokine production and target cell death. In addition, several experiments comprised fluorescence-activated cell sorting (FACS) in which specific cell subsets are isolated based primarily on cell surface markers that are stained with fluorescent antibodies.
NK CELL EFFECTOR FUNCTIONS
Most assays of NK cell cytotoxicity presented in this thesis monitored degranulation responses in specific NK cell subsets. This assay was preferred since assays of target cell death only provide information of the function of NK cells as a group. In addition to degranulation some experiments also included intracellular staining of the cytokines interferon-γ and tumor necrosis factor-α aiming to identify cytokine-producing NK cells.

In a commonly employed experimental set-up, unstimulated or IL 2-activated purified NK cells were co-incubated with target cells such as leukemic blasts from newly diagnosed AML patients or leukemic cell lines. Cells were incubated for 4 hours in the presence of a CD107a antibody in degranulation assays and for 5 hours with the addition of protein transport inhibitors after 1 hour in cytokine activation assays. After incubation cells were stained with NK cell identification markers (such as CD56 or NKp46) and other markers defining NK cell subsets or maturation stage. In IFN-γ and TNF-α assays, cells were permeabilized prior to intracellular staining. After staining, the samples were analyzed on a 5-laser BD LSRFortessa flow cytometer.

GENOTYPING USING PCR
DNA from patients for genotyping assays were purified using a Roche MagNAPure 96 instrument for clinical trial patient samples and for healthy donors using the Qiagen DNEasy Blood & Tissue kit followed by quantity and purity check on a Nanodrop machine. Several PCR-based methods were used for the characterization of aspects of NK cell KIR and HLA content as well as donor HLA-B and HLA-C content.

Presence of HLA ligands for NK cell KIR receptors was determined using the Olerup SSP KIR HLA ligand kit followed by electrophoresis to verify specific amplification. Determination of KIR genotypes was performed using the One Lambda KIR SSO Genotyping Test and HLA-B and –C allele characterization was performed with the One Lambda LABType SSO Class I Locus Typing Test. The kits from One Lambda involve PCR amplification followed by bead conjugation and analysis on a Bio-Plex 200. Final data analysis was performed using the HLA Fusion software.
STATISTICAL ANALYSIS
Student’s $t$-test compares the mean of two datasets and can be used to determine whether or not they are significantly different. The $t$-test requires normal distribution of groups and equal variance. Paired and unpaired two-sided student’s $t$-tests are used in several papers when two groups were compared.

The One-way ANOVA test compares the means of two or more samples based on a single input variable. The ANOVA may be followed by multiple comparison test such as Bonferroni’s or Tukey’s multiple comparison tests.

The logrank test, which is also called the Mantel-Cox test, compares the survival distribution of two groups. In this thesis the logrank test was employed to compare survival or relapse between patient groups with e.g. variable receptor intensity, KIR content or HLA-B polymorphisms. In most calculations, the comparison was made by dichotomizing these variables by the median followed by analysis of clinical outcome.
RESULTS

**Paper I – NOX2-dependent immunosuppression in chronic myelomonocytic leukemia**

In paper I we aimed to describe mechanisms of immune evasion in CMML with focus on the role of NOX2-derived ROS produced by cells of the malignant clone. Previous studies have suggested that the clinical efficacy of HDC/IL-2 is pronounced in AML patients with monocytic differentiation where leukemic cells produce ROS via NOX2, which in turn is regulated by histamine acting via H₂R (204). In a first set of experiments, we determined the expression of H₂R and NOX2 (gp91phox) on monocytes from CMML patients. CD14⁺ monocytes displayed high levels of both H₂R and the NOX2 subunit gp91phox (Figure 7A). We tested the functionality of these cells in a ROS production assay and observed that CMML monocytes produced high levels of ROS and that agonistic ligation of H₂R inhibited ROS formation (Figure 7B-C).

![Figure 7](image)

**Figure 7. CMML patients harbor monocytes with ROS-producing capacity.** A) Expression of the NOX2 subunit gp91phox and H₂R on CD34⁺ and CD14⁺ cells from patients with CMML (n = 11) and healthy donors (n = 10). B) ROS production, as measured by chemoluminescence, by CD14⁺ CMML monocytes following fMLF stimulation in the presence or absence of HDC, the H₂R antagonist ranitidine, or a chemical control to ranitidine AH202399AA. C) ROS production from CMML monocytes in response to fMLF in the presence or absence of HDC (n = 11).

We next performed co-incubation assays aiming to determine the function and viability of NK cells exposed to CMML cells. In these experiments, we utilized an anti-CD33 antibody (lintuzumab) to opsonize CMML cells and to achieve antibody-dependent killing of the leukemic cells. Addition of lintuzumab increased the amount of CD107a⁺ (degranulating) NK cells, which were
further significantly increased by the addition of HDC (Figure 8A). Degranulation was assayed after 4 hours of co-culture. Following overnight co-culture of NK cells and CMML monocytes the amount of apoptotic NK cells was measured. A majority of NK cells displayed features of end-stage apoptosis measured as compromised membrane integrity upon co-incubation. The addition of HDC or other ROS inhibitors or scavengers rescued a significant proportion of NK cells (Figure 8B). These results support the hypothesis that the malignant myeloid cells exert immunosuppression against cytotoxic lymphocytes by producing ROS.

Finally we investigated the NCR expression of NK cells recovered from CMML patients and age-matched healthy donors. We found that CMML patients harbored NK cells that were deficient in several NCRs. The reduced NCR expression was correlated to the presence of blasts in peripheral blood. Patients with a higher proportion of blasts thus showed lower expression density of NKp30, NKp80 and 2B4 (Figure 9A). In addition, patients with a high proportion of blasts had a lower frequency of circulating NK cells expressing NKp30, NKp46, NKp80, DNAM-1 and 2B4 (Figure 9B).

In summary, the results of paper I suggest that CMML cells exert immunosuppression towards NK cells by producing ROS, and that the leukemia-induced immunosuppression is targeted by HDC, acting via H2R. Additionally, CMML patients harbor functionally defect NK cells that correlated with presence of immature leukemic cells in blood.
Figure 9. NK cell NCR expression in CMML patients. A) Median fluorescence intensity (MFI) of expression of the activating receptors NKp30, 2B4 and NKp80 on NK cells from healthy donors (H.D \( n = 10 \)) or CMML patients divided into patients with < 2 % peripheral blasts \( (n = 5) \) or > 2 % peripheral blasts \( (n = 5) \). B) Percentage of NK cells positive for the specified NCRs in healthy donors and CMML patients.
Immunotherapy and immunosuppression in myeloid leukemia

**Paper II – Dynamics of myeloid cell populations during relapse-preventive immunotherapy in acute myeloid leukemia**

In recent years, endogenous histamine has been ascribed a role in myeloid cell maturation. A study of mice with complete genetic histamine deficiency in peripheral tissues reported that the lack of histamine entailed accumulation of immature myeloid cells and, also, that histamine-deficient mice were highly prone to develop malignant tumors (205). Others report that histamine promotes the differentiation of monocytes into antigen-presenting dendritic cells in vitro and in vivo (170, 175). With this background, the studies presented in **paper II** assessed myeloid cell populations during the course of treatment with HDC/IL-2 in the above-referenced Re:Mission trial.

We utilized flow cytometry for the analysis of peripheral blood samples drawn at the start and end of treatment cycles 1 and 3. Within each 3-week treatment cycle the counts of white blood cells increased although some myeloid cell compartments decreased. Eosinophils showed the highest expansion while counts of monocytes and neutrophils were reduced in the first cycle (Figure 10A-B).

![Figure 10. Strong monocyte reduction during cycle 1 predicts LFS. A) Counts of WBC and B) monocytes in the blood of AML patients during HDC/IL-2 treatment cycles. C-D) Patients were dichotomized by the median reduction of monocytes during the first HDC/IL-2 treatment cycle and C) leukemia free survival (LFS) or D) overall survival (OS) for the two patient groups were compared.](image-url)
Patients were dichotomized by the median reduction of monocytes during the first HDC/IL-2 treatment cycle into a low/no reduction group and a high reduction group. A strong reduction in monocyte counts during cycle 1 significantly predicted LFS (Figure 10C-D). Since HDC signals through H2R we investigated the expression level (MFI) of H2R on classical CD14++ monocytes and non-classical CD14+CD16+ monocytes during treatment cycles. We observed an increase of H2R receptor expression on classical monocytes that persisted from the start of treatment to the start of cycle 3. H2R expression also increased during both treatment cycles (Figure 11A). Patients were dichotomized based on H2R expression on classical monocytes on cycle 1 day 21 into below or above median expression. High H2R expression on day 21 correlated with significantly improved LFS and OS for classical as well as CD16+ monocytes (Figure 11B-C).

Figure 11. H2R expression on monocytes impacts on outcome during immunotherapy. A) H2R expression (MFI) on classical monocytes in AML patients during HDC/IL-2 cycle 1 and 3. B) LFS and C) OS for patients dichotomized by the median H2R expression on classical monocytes at C1D21.
It was also observed that the expression of HLA-ABC increased during treatment cycles in all myeloid subsets (Figure 12A). A low HLA-ABC expression on monocytes predicted significantly increased LFS during the course of treatment (Figure 12B-C).

In summary, paper II described the dynamics of myeloid cells during treatment with HDC/IL-2. During treatment, we observed a reduction of monocytes in blood, along with increased H₂R expression. These features were associated with favorable outcome.

**Figure 12.** HLA-ABC expression on monocytes predicts LFS. A) HLA-ABC expression (MFI) on classical monocytes from AML patients during HDC/IL-2 treatment cycles 1 and 3. B) LFS and C) OS of patients dichotomized by the median HLA-ABC expression on classical monocytes at onset of immunotherapy.
Paper III – Impact of killer-immunoglobulin-like receptor and human leukocyte antigen genotypes on the efficacy of immunotherapy in acute myeloid leukemia

In paper III we aimed to describe the impact of a missing ligand genotype in the context of relapse-preventive treatment with HDC/IL-2. We characterized the Re:Mission trial cohort for KIR and HLA genotypes and categorized patients based on missing ligands versus all ligands present and based on KIR content into belonging to the mostly inhibiting KIR A/A genotype or to the more activating KIR B/x genotype. Having a missing ligand or the B/x genotype did not per se impact on LFS in the trial.

Earlier studies report that up-regulation of NK cell NCRs such as NKp30 and NKp46 is relevant to the clinical efficacy of HDC/IL-2 (163, 181). Therefore we investigated how the benefit of NCR expression correlated with a missing ligand profile and KIR genotypes. Patients were grouped into missing ligand or all ligands present and then dichotomized based on the mean expression of NKp46 into a high and a low NKp46 group. We observed that only patients with a missing ligand profile benefited significantly from a high NKp46 expression, whereas there was no difference in the all ligands present group (Figure 13A-B). A similar pattern was found when patients were grouped into KIR A/A or KIR B/x genotype with a significant benefit of high NKp46 expression in the KIR B/x group (Figure 13C-D). These results imply that in order to benefit from a high NCR expression, suggesting functional NK cells, a mismatch in the KIR-HLA repertoire or having a higher number of activating KIRs is of significant importance.

Figure 13. Clinical benefit of high NCR expression in patients with an activating NK cell genetic profile. Leukemia-free survival was analyzed for AML patients treated with HDC/IL-2 who were dichotomized based on above or below median expression of NKp46 on CD16+ NK cells on C1D21 with A) missing ligand genotype, B) all ligands present, C) KIR A/A genotype or D) KIR B/x genotype.
In individuals with a missing ligand genotype a subset of NK cells will lack inhibitory KIR ligand referred to as unlicensed NK cells or non-self iKIR (NS-iKIR) NK cells. This NK cell subset is at resting conditions regarded to be hyporesponsive but studies suggest that cytokine stimulation of these cells can evoke cytotoxicity towards HLA-deficient target cells (73). To clarify the capacity of IL-2 stimulated NS-iKIR NK cells to kill target cells, we performed assays using either an HLA-deficient cell line or using AML blasts that were fully matched regarding KIR ligands. In line with previous findings, we observed that unstimulated unlicensed NK cells responded poorly towards K562 cells and primary AML blasts. Also, unlicensed NK cells responded poorly towards K562 cells in comparison with licensed NK cells expressing a single KIR (Figure 14A). The addition of IL-2 largely abolished the difference observed between unlicensed and licensed NK cells. Notably, in the AML blast setting IL-2 stimulated unlicensed NK cells responded significantly more than unstimulated cells and also showed equal responses compared with the licensed subset (Figure 14B).

**Figure 14. In vitro responses of unlicensed NK cells.** Percentage of unstimulated or IL-2 stimulated NS-iKIR or S-iKIR NK cells that degranulated towards A) K562 cells ($n=6$) or B) HLA matched AML blasts ($n=7$).
**Paper IV – Cytomegalovirus serostatus affects autoreactive NK cells and outcomes of IL2-based immunotherapy in acute myeloid leukemia**

The connection between CMV infection and induction as well as maintenance of adaptive-like NK cells expressing NKG2C is well established. Expansion of these adaptive cells has been suggested to favorably affect the outcome of AML after allogeneic stem cell transplantation (206, 207). One of the proposed mechanisms is that CMV reactivation drives maturation of NK cells after transplantation resulting in a mature NK cell phenotype, which would benefit a GvL effect. However the impact of a past CMV infection in AML patients not receiving transplantation has not been previously characterized.

We determined CMV IgG serostatus (as a marker of a past infection) in 81 patients from the Re:Mission phase IV trial and in 58 healthy donors. As expected, CMV+ patients showed significantly higher frequency of mature KIR+NKG2C+CD57+ NK cells in blood at treatment start with a similar trend at the start of cycle 3 (Figure 15A). Analysis of NK cell compartments showed that CMV+ AML patients had an NK cell repertoire skewed towards differentiated cells before and during treatment. Analysis of the impact of CMV serostatus on LFS and OS in the HDC/IL-2 treated patients showed that CMV− patients had significantly better survival than CMV+ patients (Figure 15B-C). This association was not observed in a cohort of AML patients that did not receive immunotherapy.

![Figure 15. Impact of CMV serostatus on NK cell maturation and patient survival during HDC/IL-2 treatment. A) Frequency of adaptive-like NK cells in CMV− and CMV+ AML patients at the start of HDC/IL-2 treatment cycles 1 and 3. B) LFS and C) OS for AML patients in the Re:Mission trial stratified by the CMV serostatus.](image)
There were no differences in the outcome of patients having low or high numbers of adaptive-like NKG2C+ NK cells. In paper III we reported significantly improved survival in patients with functional NK cells and a missing ligand genotype providing unlicensed NK cells. When patients were divided into 4 groups based on CMV serostatus and KIR/HLA concordance we observed that only CMV− patients had a significant benefit of a missing ligand genotype. These findings suggests that the pro-differentiating effect of CMV skews the NK cell repertoire towards a more mature phenotype and thus depletes the subset of unlicensed cells that may comprise an important effector population during HDC/IL-2 immunotherapy. We also performed a series of in vitro experiments using NK cells from healthy donors (Figure 16A). We observed that unlicensed NK cells degranulated significantly more efficiently than licensed NK cells towards HLA-matched AML blasts. There was no differences in the response from unlicensed NK cells when comparing CMV− and CMV+ donors. However, the proportion of unlicensed NK cells among all degranulating NK cells was significantly larger in CMV− donors (Figure 16B-D).

Figure 16. Degranulation response of NK cell subsets towards cell lines and AML blasts. A) Frequency of CD107a+ NK cells in degranulation assays towards Ab-coated Daudi cells (n = 10), K562 cells (n = 10) or AML blasts (n = 10 for unstimulated and (n = 14) for IL-2 stimulated experiments). B) Degranulation responses towards Ab-coated Daudi cells by NS-iKIR NK cells from CMV− and CMV+ donors. C-D) Percentage of CD107a+ NS-iKIR NK cells among all degranulating NK cells from CMV− and CMV+ donors towards Ab-coated Daudi cells (C) or AML blasts (D).
In summary, the studies presented in paper III and paper IV suggest a role for unlicensed, functional NK cells, thus expressing few KIRs, for the outcome of immunotherapy with HDC/IL-2. Our study also implies that a past CMV infection may yield an NK cell profile that is more mature and less prone to respond to the immunotherapy.
Paper V – The HLA-B -21 dimorphism impacts on NK cell education and clinical outcome of immunotherapy in acute myeloid leukemia

The previous papers have suggested a beneficial role for NK cells expressing few KIRs for the outcome of immunotherapy, in particular in patients with a missing ligand genotype. NK cell maturation comprises a gradual loss of NKG2A expression with increasing KIR number expression. In healthy individuals approximately 40–60 % of NK cells express NKG2A. In paper V we aimed to describe the role of the inhibitory receptor NKG2A and the connection to the HLA-B -21 polymorphism that has been shown to skew NK cell education towards a dependence on either NKG2A (M/x) or KIR (T/T) interactions.

We observed that AML patients receiving HDC/IL-2 had elevated frequencies of NKG2A⁺ NK cells compared with healthy donors. NK cell repertoires were gated based on the number of KIRs expressed by NK cells along with presence or absence of NKG2A expression (Figure 17A). Treatment of patients with HDC/IL-2 further increased the frequency of NKG2A⁺ NK cells. This observation was reproduced in vitro using NK cells that were exposed to IL-2 for 3 days (Figure 17B-C). The induction of NKG2A by IL-2 was further supported by increased transcript levels of NKG2A in NK cells (Figure 17D). In addition, IL-2 was found to induce increased expression of cytotoxic mediators such as granzyme B, in particular in NKG2A⁺ subsets (Figure 17E-F).
Figure 17. Expression of NKG2A in NK cells exposed to IL-2.

A) Frequency of NK cells expressing KIRs and NKG2A in AML patients before HDC/IL-2 treatment start (n = 64) and in healthy donors (n = 24). Frequency of NKG2A⁺ NK cells in NK cell subsets expressing 0, 1, 2 or 3 KIRs in B) AML patients (n = 54) before or after HDC/IL-2 treatment cycle 1 and C) unstimulated and in vitro IL-2 stimulated healthy donor cells (n = 6). D) Fold increase in mRNA expression of NKG2A in healthy donor NK cells after in vitro stimulation with IL-2. E) Granzyme B expression (MFI) in unstimulated and in vitro IL-2 stimulated NK cell subsets from healthy donors (n = 24). F) Percent increase of granzyme B expression in unstimulated and in vitro IL-2 stimulated NK cells from healthy donors (n = 24).
The functional effect of NKG2A and HLA-E interactions have mostly been studied using the 721.221-AEH cell line transfected to express supraphysiological levels of HLA-E. To address the impact of NKG2A in a more physiologically relevant setting, we took advantage of the T2 cell line transfected with a soluble HLA-E construct that only expresses surface HLA-E when provided with suitable peptides. In IL-2 stimulated NK cells we observed a significant inhibition of degranulation in NKG2A+ NK cells towards 721.221-AEH. However, no significant inhibition was observed using the T2 cells expressing lower levels of HLA-E (Figure 18A-B). Since HLA-E levels on AML blasts is even lower than in the peptide-stimulated T2 cells we hypothesized that IL-2 stimulated NKG2A+ NK cells would respond towards primary AML blasts. Indeed, IL-2 stimulated NKG2A+ NK cells responded significantly better than IL-2 stimulated KIR+ NK cells towards HLA-matched AML blasts (Figure 18C). These results suggest that NKG2A signaling is more prone to be overridden in cytokine stimulated NK cells.

Next we investigated the impact of HLA-B polymorphism in a cytotoxic assay against K562 cells with or without IL-2 stimulation. In short our experiments showed that individuals with HLA-B-21M (M/x) have NKG2A+ NK cells that are better educated and more functional compared to individuals having two copies of -21T. We genotyped 24 healthy donors and grouped them into M/x or T/T genotypes. Overall, NK cells from M/x donors degranulated...
significantly more than those from T/T donors and contained a significantly larger proportion of IFN-\(\gamma\) producing cells (Figure 19A). When NK cells were gated into subgroups based on NKG2A and KIR expression, we observed that the NKG2A\(^+\) compartment in M/x donors responded significantly better than corresponding cells in T/T donors. These results imply that NKG2A\(^+\) NK cells are better educated in M/x donors. To elucidate the role in a setting where both KIR and NKG2A ligands were present, we performed a similar set of experiments against fully KIR-ligand-matched AML blasts. Again, NK cells from M/x donors degranulated significantly more and when the cells were divided into subsets it was shown that the NKG2A\(^+\) NK cell subsets responded more vigorously in M/x donors (Figure 19B).

**Figure 19. Education assay based on HLA-B -21.** A) Frequency of responding NK cells towards K562 cells in terms of CD107a, IFN-\(\gamma\) and TNF-\(\alpha\) positivity in unstimulated and IL-2 stimulated NK cells from donors with an M/x (red, \(n = 11\)) or T/T (white, \(n = 13\)) genotype. B) Frequency of degranulating CD107a\(^+\) NK cells towards HLA-matched CD34\(^+\) AML blasts using unstimulated and IL-2 stimulated NK cells from M/x (\(n = 11\)) or T/T (\(n = 10\)) donors.
Finally, we investigated if the HLA-B polymorphism of the AML patients in the Re:Mission trial impacted on the outcome of HDC/IL-2 treatment. Patients with the M/x genotype had significantly improved LFS and OS compared with T/T patients (Figure 20A-B).

**Figure 20. Impact of HLA-B -21 genotype on outcome of AML patients.** A) Leukemia-free and B) overall survival for AML patients with an M/x genotype (n = 38) or T/T genotype (n = 42) receiving HDC/IL-2 treatment. Five patients (6%) had a genotype corresponding to M/M, 33 patients (41%) were M/T genotype and 42 patients (53%) were T/T.

In conclusion, the results presented in paper V suggest that patients with an M/x genotype giving rise to higher levels of leader peptides of relevance of NK cell inhibition show better outcome of HDC/IL-2 immunotherapy. The results suggest that having NK cells that depend on education through NKG2A might be beneficial for patients undergoing IL-2 based immunotherapy since cytokine stimulation may enable NK cells to override NKG2A-mediated inhibitory signaling.
CONCLUDING REMARKS

The overall aim of this thesis was to explore the immunological changes occurring during HDC/IL-2 immunotherapy and to identify predictive and/or prognostic markers. **Paper I** and **paper II** mainly encompass effects of the HDC component of this regimen with focus on myeloid cells in terms of immunosuppression and myeloid cell maturation.

In **paper I**, the results imply that the function and viability of cytotoxic lymphocytes is impaired by immunosuppressive ROS produced by malignant myeloid cells recovered from CMML patients. NK cell deficiency in myeloid malignancies is well described although the mechanisms involved remains to be fully understood (149, 208, 209). Our results suggest that NK cells and CD8$^+$ T cells in CMML display weak antileukemic function, possibly due to ROS production causing lymphocyte apoptosis and downregulation of NK cell NCRs. The results also suggest that additional strategies targeting ROS production from CMML cells are conceivable in the treatment of this disease.

Treatment of AML patients with HDC/IL-2 was developed to target the NOX2-dependent formation of immunosuppressive ROS (HDC component) in conjunction with the activation of anti-leukemic effector cells (IL-2 component). **Paper II** sheds light on this mechanism but also addresses effects of the HDC component on myeloid cell maturation. HDC has been shown to induce DC maturation (175) and a recently published study reported that HDC also impact on the maturation of myeloid leukemic cell lines via triggering alterations in intracellular ROS levels (170). In line with these results, we observed induction of HLA-DR, CD86, and CD40 in monocytes and dendritic cells in patients receiving HDC/IL-2. In addition, expression of HLA-ABC increased in all myeloid populations during therapy. Collectively, these findings support that the anti-leukemic efficacy of immunotherapy may comprise direct effects on the maturation status of myeloid cell populations and that these effects may be mediated by the HDC component of the regimen.

The main conclusion from **paper III** is that functional NK cells, in terms of NCR expression and expression of KIRs and their ligands, are relevant to the outcome of immunotherapy with HDC/IL-2. The results suggest that non-self-KIR expressing NK cells with anti-leukemic efficacy may be activated during immunotherapy, and thus that further strategies to unleash the cytotoxicity of these cells should be considered in future immunotherapy protocols. These findings are further expanded in **paper IV**, where a past CMV infection was shown to herald poor clinical outcome of immunotherapy, presumably by...
Immunotheraphy and immunosuppression in myeloid leukemia

depleting a pool of immature NK cells. In line with these findings, the results in paper V suggest that improved NK cell education from NKG2A due to a polymorphism in HLA-B impacted positively on the efficiency of immunotherapy.

Papers III – V thus point towards a role for immature NK cells during HDC/IL-2 immunotherapy. It seems beneficial to avoid strong KIR inhibition exerted by for example KIR2DL1 and instead depend on early NKG2A education. Our results also suggests a differential impact of HLA-E levels on AML cells in comparison with levels seen in solid cancer. Overall, our findings support the hypothesis that NK cells that have not reached the final stage of maturation are relevant to the benefit of HDC/IL-2 in AML.

The hypothesis-generating nature of these findings should be emphasized. Also, the studies aiming to determine which aspects of immunity are relevant to the clinical benefit of HDC/IL-2 do not allow for identification of the active component of the regime in terms of immunoregulation. In addition, our studies in patients were restricted to analyses performed using peripheral blood and it is conceivable, albeit impractical, that a clearer picture would emerge in studies of immune reactivity in bone marrow. With these limitations, our studies point to a role for immature, less inhibited NK cells for the clinical efficacy of HDC/IL-2 in AML.
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