On the role of innate cell interactions in inflammation and leukemia

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To my family

ABSTRACT

Inflammatory cells of the innate immune system launch rapid and powerful effector responses in the combat against infectious pathogens. However, these inflammatory mediators may cause excessive damage to the host and, therefore, require rigid control systems to maintain the delicate balance between prompt clearance of infections and the risk of developing immunopathology. Natural killer (NK) cells form a part of a multifaceted network of innate interactions with myeloid cells during the course of immune responses. These complex patterns of reciprocal interplay have capacity to potentiate or inactivate immunity. The first paper of this thesis describes how interaction between NK cells and neutrophils contributes to the activation of adaptive responses. In paper II, we demonstrated that NK cells negatively regulate neutrophil functions by accelerating neutrophil cell death via NKp46 and the death receptor Fas. The results presented in paper III show that leukemic myeloid cells avoid elimination by NK cells by inducing lymphocyte cell death and that inhibition of the nuclear enzyme PARP-1 restores NK cell anti-leukemic effector functions. In paper IV, the crosstalk between NK cells and myeloid cells was targeted in patients with acute myeloid leukemia who received immunotherapy with histamine dihydrochloride and low-dose interleukin-2 aiming to prevent leukemic relapse. The treatment was found to trigger expansion of NK cell subtypes in blood and to induce enhanced NK cell expression of natural cytotoxicity receptors. These features of NK cells were associated with reduced risk of relapse. In summary, this thesis work may expand the knowledge of cellular crosstalk in immunity and suggests that communication between innate immune cells may be targeted for therapeutic purposes.

Keywords: Innate immunity, neutrophils, NK cells, caspase-1, apoptosis, myeloid leukemia, ROS, immunotherapy.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Människans medfödda immunförsvar utgör en första försvarslinje mot angripande mikroorganismer. Vid upptäckt av en infekterande patogen rekryteras immunceller till infektionshärden och triggar en kaskad av inflammatoriska försvarsmekanismer. Till de medfödda immuncellerna räknas myeloiska celler, såsom neutrofiler, dendritiska celler (DC) och monocyter samt lymfocyter (naturliga mördarceller, NK-celler). Dessa immunceller ingår i ett dynamiskt samspel som kan aktivera såväl som inaktivera försvaret mot mikroorganismer.

Denna avhandling har syftat till att bidra till ökad kunskap om kommunikation mellan NK-celler och myeloiska celler och dess betydelse för försvar mot infektion och blodcancer, leukemi. Med laborativa metoder, såsom cellodling och flödescytometri, analyserades immuncellernas utseende och funktioner i blodprover från friska försökspersoner och från patienter med akut myeloisk eller kronisk myeloisk leukemi (AML respektive KML). I avhandlingens första delarbete studerades hur två celltyper, neutrofiler och NK-celler, som tidigt rekryteras till infekterad vävnad, samarbetar för att påverka utvecklingen av immunsvar. Resultaten talar för att neutrofiler reagerar på särskilda strukturer från bakterier och virus genom att producera signalerande ämnen som aktiverar NK-celler. Arbetet beskriver i detalj hur denna aktivering sker och hur aktiverade NK-celler i sin tur stimulerar det specifika immunsvaret via DC och T-celler.

Immunsystemets snabba reaktion mot patogener är nödvändig för att undvika infektioner, men inflammationssvaret kan också vara skadligt för frisk vävnad. Således måste det finnas kraftfulla system för att kontrollera och stänga av inflammationsprocessen. I delarbete två visas hur NK-celler reglerar neutrofilers funktion genom att påskynda neutrofil celldöd, vilket kan ha betydelse för att avsluta inflammatoriska reaktioner.

Det är även viktigt att hålla NK-cellers och andra lymfocyters funktion under kontroll för att skydda oss mot autoimmuna sjukdomar, som kännetecknas av att immunceller angriper frisk vävnad. Myeloiska celler använder syreradikaler för att eliminera mikroorganismer. Dessa substanser är även toxiska för NK-celler och T-celler, och hämmad produktion av syreradikaler har tidigare visats förbättra NK-cellers och T-cellers förmåga att attackera myeloiska leukemiceller. I delarbete III visas att interaktioner mellan KML- och NK-celler leder till att NK-celler elimineras. Resultaten visar att syreradikaler frisatta av leukemiska celler dödar NK-celler genom att aktivera ett DNA-reparerande enzym i

cellkärnan, PARP-1. Genom att blockera detta enzym med inhibitorer skyddades NK-celler från att dödas av syreradikalbildande KML-celler. Resultaten talar för att PARP-inhibitorer skulle kunna användas för att förbättra NK-cellers funtion vid KML.

I det fjärde delarbetet undersöktes vilka konsekvenser manipulation av samspelet mellan NK-celler och myeloiska celler får för patienter med AML. En klinisk behandlingsstudie genomfördes i vilken patienter med AML erhöll immunterapi med histamindihydroklorid och interleukin-2 för att förhindra återfall i leukemi. Resultaten visar att antalet NK-celler ökade hos patienter som erhållit immunterapi och att dessa celler uppvisade ett högre uttryck av aktiverande receptorer på cellytan. NK-cellers antal och aktiveringsgrad förutspådde vilka patienter som kunde undgå livshotande återfall i leukemi. Resultaten ger därmed stöd för teorin att NK-celler har en viktig roll för sjukdomsförloppet vid AML.

LIST OF PAPERS

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

I. **Riise, RE**; Bernson, E; Aurelius, J; Martner, A; Pesce, S; Della Chiesa, M; Marcenaro, E; Bylund, J; Hellstrand, K; Moretta, L; Moretta, A; Thorén, FB.
TLR-stimulated neutrophils instruct NK cells to trigger dendritic cell maturation and promote adaptive T cell responses.
Submitted.

II. Thorén, FB; **Riise, RE**; Ousbäck, J; Della Chiesa, M; Alsterholm, M; Marcenaro, E; Pesce, S; Prato, C; Cantoni, C; Bylund, J; Moretta, L; Moretta, A.
Human NK cells induce neutrophil apoptosis via an NKp46- and Fas-dependent mechanism.
Journal of Immunology 2012; 188: 1668-1674.

III. Aurelius, J; Martner, A; **Riise, RE**; Romero, AI; Palmqvist, L; Brune, M; Hellstrand, K; Thorén, FB.
Chronic myeloid leukemic cells trigger poly(ADP-ribose) polymerase-dependent inactivation and cell death in lymphocytes.
Journal of Leukocyte Biology 2013; 93: 155-160.

IV. Martner, A; Rydström, A; **Riise, RE**; Aurelius, J; Anderson, H; Brune, M; Foá, R; Hellstrand, K; Thorén, FB.
Role of natural killer cell subsets and natural cytotoxicity receptors for the outcome of immunotherapy in acute myeloid leukemia.
OncoImmunology 2015; 10.1080/2162402X.2015.1041701.

CONTENT

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AIF	Apoptosis-inducing factor
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
CD	Cluster of differentiation
CML	Chronic myeloid leukemia
CR	Complete remission
CTLA-4	Cytotoxic T-lymphocyte associated antigen-4
DC	Dendritic cell
FAB	French-American-British
HLA	Human leukocyte antigen
HDC	Histamine dihydrochloride
ICE	IL-1 converting enzyme
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
KIR	Killer cell immunoglobulin-like receptor
LFS	Leukemia-free survival
LPS	Lipopolysaccharide
MPS	Mononuclear phagocyte system
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	Natural cytotoxicity receptors
NK	Natural killer
OS	Overall survival
PAMP	Pathogen-associated molecular pattern
PAR	Poly(ADP-ribose)
PARP-1	Poly(ADP-ribose) polymerase-1
PD-1	Programmed death-1
PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand

1 PREFACE

The innate immune system is an evolutionary old defense strategy shared among invertebrates and vertebrates. Conserved features of pathogen recognition, the complement system and antimicrobial peptides are found in most forms of life and provide immediate defense against infections. In animals including humans, the preserved innate immune responses are strongly intertwined with the pathogen-specific and long-lasting adaptive immune system, and activation of the innate immune system is required to establish adaptive immunity. The innate cells and mediators responsible for this activation comprise a dynamic network, which has different immunological outcomes.

Within the group of innate immune cells, the role of cellular crosstalk between NK cells and myeloid cells has recently gained attention, and an increasing number of studies have demonstrated a fine-tuned and tightly regulated balance between mutual activation and selective elimination of cells. Innate immunity has also been ascribed a role in the defense against cancer cells. In myeloid leukemia, there are reports of correlations between NK cell status and prognosis to suggest that NK cells participate in defense against leukemic cells. These findings have inspired clinical trials aiming to pharmacologically boost NK cell function in leukemia for improved immune-mediated destruction of leukemic cells. In addition, innate cells may destroy leukemic cells by phagocytosis, and attempts have been made to improve elimination of leukemic cells by interference with phagocytosis-inhibitory structures expressed by leukemic cells.

This thesis addresses the functional consequences of cellular crosstalk in innate immunity with focus on communication between NK cells and myeloid cells. We have identified a novel mechanism of innate activation initiated by inflammatory neutrophils of relevance to downstream adaptive responses (Paper I), discovered mechanisms of NK cell modulation of neutrophil survival (Paper II), described a potential immune escape strategy by which malignant neutrophils trigger inhibition and apoptosis of NK cells (Paper III), and assessed the role of NK cells for outcome in patients with acute myeloid leukemia (AML) who received immunotherapy for relapse control (Paper IV).

2 INTRODUCTION

2.1 INNATE IMMUNITY

The human body is constantly exposed to microorganisms with capacity to breach the physical barriers protecting the host from its surroundings. As a result, the immune system evolved under selection pressure imposed by invading pathogens and has developed a variety of defense mechanisms[1]. The innate immune system is the first line of defense acting immediately upon encounter with an infectious agent. It is crucial for the innate recognition to detect invading pathogens. This recognition is executed by a limited set of invariant pattern recognition receptors (PRRs), present on strategic extra- and intracellular sites[2].

The PRRs identify conserved pathogen-associated molecular patterns (PAMPs). These features are essential for the survival of microbes and subject to limited variability[3]. PAMPs provide danger signals of infection or distress in tissues. Cellular PRRs are expressed by innate cells present at potential pathogen-entry sites, such as epithelial and endothelial cells. The best-characterized PRRs are the Toll-like receptors (TLRs), which are located both in surface membranes and in intracellular compartments. In humans, thirteen members of the TLR family have been identified, each responsible for recognizing different motifs of bacterial, viral or fungal origin[4]. The receptor-ligand interaction then triggers cytokine and chemokine release that causes massive infiltration of innate immune cells to the site of infection.

2.2 INNATE IMMUNE CELLS

2.2.1 PHAGOCYTES

In the 1880s, Elie Metchnikoff discovered and described leukocytes' ability to engulf and digest foreign particles, a process known as phagocytosis[5]. More than a hundred years later, the fundamental role of phagocytes in inflammation is well defined. The cell family originates from the myeloid lineage of hematopoiesis and is commonly divided into polymorphonuclear and mononuclear phagocytes[6].

POLYMORPHONUCLEAR PHAGOCYTES

The most abundant phagocyte in human circulation is the neutrophilic granulocyte, commonly known as the neutrophil[7]. Neutrophils are terminally

differentiated cells that maturate in the bone marrow before being release into the bloodstream[8]. Once in the circulation, neutrophils will remain dormant and undergo apoptosis if not reacting to microbial PAMPs or responding to chemotactic signals from surrounding tissues.

Pathogen recognition by resident macrophages will trigger local cytokine production (*e.g.* interleukin-1, IL-1, and tumor necrosis factor-α, TNF-α) and induce the expression of adhesion molecules on adjacent endothelial cells that line the vascular walls. The presentation of adhesion molecules enables engagement of circulating neutrophils expressing L-selectin and the interaction with the endothelial wall slows the neutrophils down to a rolling motion. Rolling along the endothelial surface facilitates tight adhesion via integrins, allowing the neutrophils to extravasate through the cellular lining into the infected tissue. Increased permeability of the endothelium will also result in an efflux of plasma proteins and antibodies, which facilitates neutrophil phagocytosis and lysis of pathogens via opsonization. The extravasation converts the neutrophils into a more responsive state mediated by local granulocyte-colony stimulating factor (G-CSF), chemokines and cytokines[9]. Furthermore, neutrophils express a broad repertoire of TLRs that enable direct interaction with the intruding microbes with ensuing triggering of antimicrobial effector functions at the infection sites[10].

FUNCTIONS

Neutrophil-mediated killing of microorganisms comprises three main effector mechanisms[8]. First, the actions of neutrophil phagocytosis are tightly connected to the generation of reactive oxygen species (ROS) inside the phagolysosome. In this manner the ingested pathogens are exposed to toxic ROS specifically produced by the leukocyte membrane enzyme NADPH oxidase (*cf.* section 2.2.2 below). Secondly, neutrophils are packed with preloaded granules that contain degrading enzymes, which upon degranulation are released into phagosomes and the extracellular space, thus eradicating surrounding microorganisms, but also potentially damaging local healthy tissue. Thirdly, neutrophils may also undergo suicidal attacks by extruding their nuclear content to form neutrophil extracellular traps (NETs) that capture and lyse nearby bacteria. The short-lived neutrophils exert their powerful effector mechanisms before entering a tightly controlled apoptosis. To minimize the risk of harming surrounding tissue by toxic neutrophil constituents, apoptotic neutrophils up-regulate structures generating eat-me signals, such as phosphatidyl serine (PS) and calreticulin, while do-not-eat-me signals, such as CD47 are downregulated[11, 12]. In this way apoptotic neutrophils are rapidly cleared by macrophages in a process known as efferocytosis[12].

In addition to the direct neutrophil-mediated killing mechanisms, neutrophil activation also results in *de novo* synthesis of anti- and pro-inflammatory cytokines that initiate and regulate subsequent immune responses. Although the biological significance of neutrophil-derived cytokines *in vivo* is not fully understood, the numerical dominance of neutrophils in the acute phase of inflammation implies that neutrophils may be an important source of immunostimulatory cytokines. The variety of cytokines generated by neutrophils indicates that these leukocytes could influence not only inflammatory responses, but also processes such as wound healing, hematopoiesis and angiogenesis[14]. Among the pro-inflammatory cytokines produced by neutrophils are members of the IL-1 family. IL-1 is an endogenous pyrogen and an important mediator in the acute phase of inflammation. The actions of IL-1 proteins are strictly controlled by a unique regulation system including a specific receptor antagonist (IL-1RA) and an activation enzyme (IL-1 converting enzyme, ICE, also known as caspase-1). Danger signals increase the transcription of inactive IL-1 precursors coinciding with the assembly and

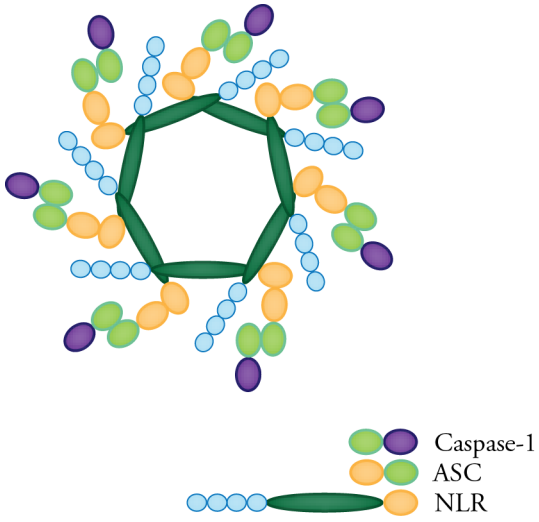

Figure 1. *Schematic illustration of the composition of the active inflammasome. Adapted from[13].*

activation of multiprotein complexes, the inflammasomes. The four known inflammasomes share the components nucleotide-binding domain leucine-rich repeat (NLR) protein, the apoptosis-associated speck-like protein (ASC) and caspase-1 (Figure 1)[13, 15]. Once assembled, the inflammasome units will activate caspase-1, which in turn proteolytically cleaves IL-1 family precursors (*e.g.* pro-IL-1β and pro-IL-18) into fully functional cytokines[16].

The fundamental role of neutrophils in host defense is evident since no genetic modification that depletes neutrophils has resulted in viable animals[17]. In the clinic, the increased susceptibility to infections in neutropenic patients is a well-known phenomenon and may rapidly become life-threatening as in neutropenic sepsis. In addition, it is becoming increasingly clear that neutrophils have the capacity to establish a significant network to regulate and guide ensuing inflammation responses.

MONONUCLEAR PHAGOCYTES

Monocytes, macrophages and dendritic cells (DCs) are collectively named the mononuclear phagocyte system (MPS)[18]. The MPS lifespan is far longer than neutrophils, which is of clinical relevance during chemotherapy and hematopoietic stem cell transplantation when neutrophil production is impaired[6]. The heterogeneous group of circulating monocytes possesses microbicidal systems equivalent to those of neutrophils and may mature into tissue macrophages or DCs when leaving the blood stream.

This classical role attributed to monocytes was recently challenged by findings showing that tissue macrophages could derive from embryonic precursors and depend on self-renewal in adult tissues[19]. The differentiated macrophages and DCs skew their effector phenotypes into becoming highly phagocytic, potent antigen presenters and less effective ROS producers[20]. Along with the ability to release immunomodulatory cyto- and chemokines, the members of MPS function as a crucial regulatory link between the innate and the adaptive immune system.

2.2.2 NAPDH OXIDASE

The phagocytes' ability to produce ROS relies on a membrane-associated enzyme named nicotinamide adenine dinucleotide phosphate (NADPH) oxidase[21]. The leukocyte NADPH oxidase consists of six subunits distributed between the cytosol; $p40^{PHOX}$, $p47^{PHOX}$, $p67^{PHOX}$ and Rac2, and the membranes; Rap1A and cytochrome b_{558} ($p22^{PHOX}$ and $gp91^{PHOX}$ complex) (Figure 2). The transmembrane component $gp91^{PHOX}$ exists in several isoforms and the particular form constituting the NADPH oxidase is named NOX2[22].

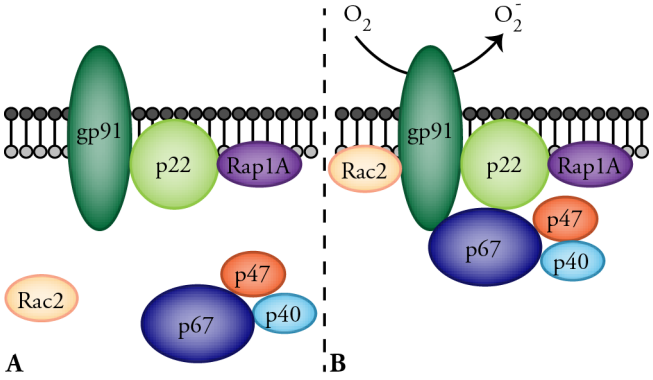

Figure 2. *The NADPH oxidase in an inactive (A) and active (B) state.*

Upon phagocyte activation the complex of cytosolic subunits is transferred to cellular membranes and assembles with the membrane components. The activated NAPDH oxidase catalyzes the reduction of oxygen into superoxide (O_2^-), by using NADPH as an electron donor[20]. Being a reactive free radical, the O_2^- generated by the enzyme serves as a starting molecule for the formation of additional reactive oxygen metabolites, such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-).

During phagocytosis the outer cell membrane is internalized as the wall of the phagolysosome, which gives the NADPH oxidase a strategic location to secrete O_2^- into the phagosome lumen. In addition, NADPH oxidase located in membranes of intracellular granules will fuse with the phagosome and further expose the engulfed microbe to ROS. The phagocyte-mediated release of extracellular ROS is not only an efficient anti-microbial strategy, but ROS are also highly suppressive to adjacent lymphocytes (*cf.* section 2.4.2 below). A functional NADPH oxidase and ensuing production of O_2^- is crucial for phagocytes' ability to clear bacterial and fungal infections, and defects in genes coding for subunits of the NADPH oxidase cause the rare inherited disorder

chronic granulomatous disease (CGD), an immunodeficiency associated with recurrent and life-threatening and recurrent infections[23].

2.2.3 DENDRITIC CELLS

Dendritic cells are stellate cells that constitute an essential link between innate and adaptive immunity. Immature DCs arise from a bone marrow progenitor shared with monocytes and macrophages and patrol both non-lymphoid and lymphoid tissues seeking out invading microbes[24]. Upon pathogen encounter, the DCs will recognize PAMPs and initiate phagocytosis of the microbes. In parallel, DCs up-regulate their expression of human leukocyte antigen (HLA) class II molecules, now loaded with antigenic fragments processed from the engulfed pathogen. Moreover, up-regulation of chemokine receptors and co-stimulatory molecules, such as the B7-family members (cluster of differentiation-80 (CD80) and CD86), and CD40 converts the DCs into fully professional antigen-presenting cells (APCs) and initiates the homing to T cell-rich areas, including lymph nodes or the spleen[25]. DCs can also be activated indirectly by cell-cell interactions and by triggering cytokines, including TNF-α and interferon (IFN), from the surrounding inflammatory environment[26]. Antigen-specific naïve T cells interact with the homecoming DCs by recognizing the peptide-HLA complex with their T cell receptor (TCR). Additional signals are generated by the binding of CD80 and CD86 to surface CD28 of naïve T cells and by DC-derived cytokines. The antigen-presentation triggers the maturation and clonal expansion of helper $CD4^+$ and cytotoxic $CD8^+$ T cells, which further enable antibody production of B cells, cell-mediated cytotoxicity and establishes immunological memory[27].

2.2.4 INNATE LYMPHOID CELLS

In addition to the myeloid leukocytes, the innate immune system consists of a newly defined family of innate lymphoid cells (ILCs) that has been the focus of intense investigation in recent years[28]. The ILC members are classified based on their cytokine profile and characteristic helper-like or cytotoxic functions (Table 1)[29].

Group	Members	Signature cytokines	Cytotoxic capacity
ILC1	NK cells	IFN-γ, TNF-α	+++
	ILC1	IFN-γ, TNF-α	+
ILC2	ILC2	IL-4, IL-5, IL-13	-
ILC3	ILC3	IL-17, IL-22	-

Table 1. Classification of ILCs based on functional characteristic features.

While subsets of ILCs, such as cytotoxic NK cells, have been known for decades, the identification of helper-like ILC1, ILC2 and ILC3 has revealed a large family with activity mirroring differentiated T cells and with designated roles in wound healing, protection against infections and immunosurveillance[29, 30].

NK CELLS

In 1975, a novel cytolytic effector lymphocyte was identified by Rolf Kiessling and co-workers[31]. In contrast to cytotoxic T cells, these lymphocytes were shown to lyse leukemic cells without prior immunization or previous exposure to their targets and were therefore given the name natural killer cells. Forty years later, extensive research has increased our understanding of the mechanisms involved in NK cell recognition and killing of target cells. Recent findings also imply that NK cells possess some characteristic features of adaptive immunity, including transformation into antigen-specific memory cells[32, 33].

DEFINITION

Human NK cells represent 5-15% of circulating lymphocytes, but constitute a larger proportion of lymphocytes in specific tissues, such as the liver and the uterus[34]. Traditionally, NK cells are phenotypically defined by the lack of the T cell marker CD3 and by the expression of the neural adhesion molecule CD56, with unknown NK cell function. The surface expression of CD56, together with the Fc-receptor CD16 (FcγRIII), divides NK cells into two distinct subsets; $CD56^{dim}CD16^{+}$ cytotoxic NK cells and $CD56^{bright}CD16^{-}$ cytokine-producing NK cells. The latter subtype is considered as a precursor of $CD56^{dim}$ cells and rather than contributing to natural cytotoxicity, this minor population efficiently produces pro-inflammatory cytokines. Differential expression of chemokine receptors by $CD56^{bright}$ and $CD56^{dim}$ cells suggests different homing locations for the NK cell subtypes[35]. Resting $CD56^{bright}$ cells in peripheral blood express receptors for homing to secondary lymphoid tissues, such as CCR7 and L-selectin[36]. Hence, $CD56^{bright}$ NK cells appear uniquely equipped to traffic sites of developing adaptive responses to establish a dynamic interaction with T cells and APCs through IFN-γ secretion. On the contrary, circulating $CCR7^{-}CD56^{dim}$ cells patrol non-lymphoid sites and change their homing receptor repertoire during inflammatory responses, hence promoting NK cell migration to regional lymph nodes.

Several NK cell effector functions are shared with cytotoxic T cells, and the signaling pathways used by the T cell receptor are overlapping those deployed by NK cell-activating receptors[37]. Despite these similarities, NK cells lack the genes responsible for somatic recombination of antigen specific receptors found in T cells and B cells. Instead, NK cells utilize clusters of conserved molecules, such as TLRs, to detect microbes and highly polymorphic receptors for the

recognition of target cells. This variation in individual germline genes is unusual and equips NK cells with a dynamic and specialized recognition system.

RECOGNITION

In the 1980s, pivotal experiments performed by Klas Kärre and coworkers revealed that NK cells identify and eliminate target cells that fail to express HLA class I molecules on their surface[38, 39]. The understanding that reduced or lost "self" identification as a consequence of tumor transformation or viral infection was critical for NK cell-recognition was termed the "missing-self" hypothesis[39]. For the "missing-self" theory to be consistent NK cells should possess a detection system that negatively feeds back upon proper interaction with healthy cells. Subsequent studies have clarified that NK cells recognize HLA class I molecules via inhibitory surface receptors[40, 41] and, thus, failed interactions of these inhibitory receptors with HLA class I molecules may trigger NK cell activation and cytotoxicity. Further research addressing NK cell inhibitory receptors has identified the killer cell immunoglobulin-like receptor (KIR) family and the lectin-like CD94/NKG2A receptor as prominent actors in the NK cell regulation by HLA class I[34].

Later discoveries presented a more complex view of NK cell recognition, specifically dependent on the net balance of signals from activating and inhibitory surface NK cell receptors (Figure 3). Since the identification of NK cell activation receptors, one particular family collectively named natural cytotoxicity receptors (NCRs) have been thoroughly investigated. The NCRs comprise NKp46, NKp44 and NKp30 and the surface density of NCRs correlate with the degree of NK cell cytolytic activity[42]. NKp46 and NKp30 are constitutively expressed on NK cells, while NKp44 is exclusively present on activated cells. Although the cellular ligands of NKp46 have not been fully identified, the recognition of viral products, specifically hemagglutinin on influenza-infected cells, by NKp46 is reportedly critical for the control of viral infections[43]. Two ligands for NKp44 were recently identified in various healthy tissues (MLL5) and on a broad range of tumor cell lines and primary tumors (NKp44L)[44]. B7-H6 is another newly identified NCR ligand, which is recognized by NKp30 and a member of the B7 family of immunoreceptors[45]. Initially, expression of B7-H6 was considered to be strictly limited to malignant cells. However, Matta *et al.* revealed that surface-bound and soluble forms of B7-H6 is also induced by healthy neutrophils and monocytes upon stimulation, implicating that B7-H6 is involved in tumor surveillance as well as in the regulation of the immune response[45].

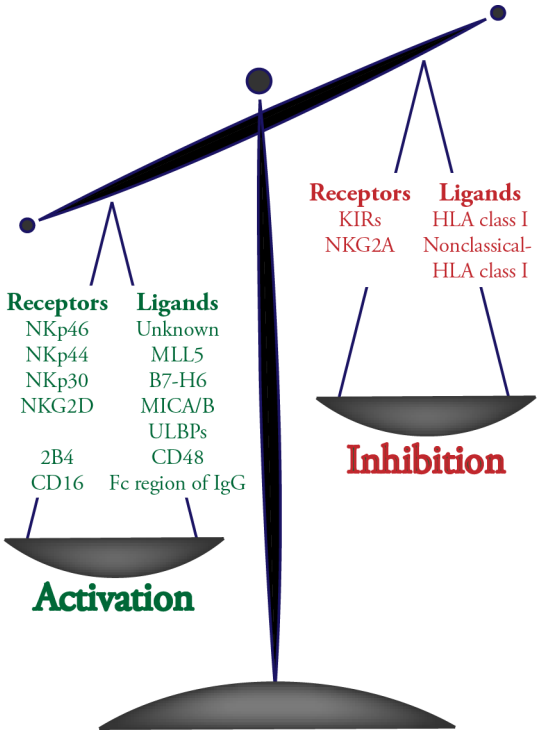

Figure 3. *The balance of signals in NK cell recognition by activating and inhibitory receptors and their corresponding ligands.*

The NCR family of receptors appears to act synergistically or complementary with other NK cell activation receptors, in particular 2B4, NKG2D and CD16. 2B4 was previously described as a co-receptor strictly dependent on crosslinking to NKp46[42] but emerging knowledge has instead unraveled its dependency on interaction with intracellular signaling adaptor proteins along with dual functions of 2B4 in both NK cell and T cell responses[46]. NKG2D detects "altered-self" by monitoring up-regulated stress-ligands, such as MHC class I chain-related genes (MICA and MICB) and ULBP proteins, on damaged host cells[47]. The stress-induced self-recognition by NKG2D overcomes the inhibitory signals provided by the interaction via HLA class I and activates the NK cell. In genetic knockout models, NKG2D-deficiency results in higher incidence and accelerated progression of spontaneous cancers[48]. Additionally, the genome of cytomegalovirus (CMV) encodes proteins that prevent up-regulation of NKG2D ligands on virus-infected cells, which has been proposed as a strategy to avoid recognition and eradication by NK cells[47]. The NK cell activating receptor CD16 defines the highly cytotoxic $CD56^{dim}$ population, which utilizes CD16 to induce antibody-dependent cellular cytotoxicity (ADCC) by binding to the Fc portion of IgG antibodies that opsonize the

target. The crosslinking of antibodies to CD16 induces a powerful activation signal, which can overcome NK cell tolerance to healthy or transformed cells[49]. The ADCC reaction is utilized in the treatment of cancer, in particular in lymphoma and lymphatic leukemia, where patients receive antibodies to trigger ADCC against tumor-specific antigens for elimination of malignant cells.

FUNCTIONS

NK cells are widely considered to have important roles in the defense against a variety of tumor cells and virus-infected cells[50]. Although NK cells display spontaneous cytotoxicity, the NK cell effector functions can be further potentiated by a wide range of immunostimulatory agents. NK cell activation is thus achieved by chemokines[35] and cytokines released by innate cells, such as IL-2, IL-12, IL-15, IL-18 and type I IFNs (IFN-α and –β). These cytokines modulate NK cell functions in different ways; IL-2 and IL-15 induce proliferation and stimulate cytotoxicity; IL-12 and IFNs enhance cytotoxicity, while IL-18 may induce an IFN-γ-producing "helper" phenotype with a receptor repertoire that indicates capacity to home to secondary lymph nodes[51].

Along with being potent producers of pro-inflammatory IFN-γ and TNF-α, NK cells may also produce an array of other cytokines, including immunosuppressive IL-10 and growth factors such as GM-CSF, thus reflecting the diverse immunoregulatory role of NK cells[32]. NK cell-derived cytokines are reportedly of importance for induction of protective immunity by DCs during vaccination[52] and in promoting the generation of antigen-specific inflammatory T cells[53]. Interestingly, during early phases of pregnancy a unique subset of NK cells predominate the maternal decidua. Current evidence suggests that the decidual NK cells play a major role in promoting uterine vascular remodeling critical for efficient blood flow through the placenta, and that a dysfunctional decidual NK cell activation correlates with obstetrical complications[54].

NK cell cytotoxicity is executed by either of two pathways that require direct contact between NK cells and target cells. The first pathway involves the engagement of apoptosis-inducing ligands to death receptors, such as Fas and TNF-related apoptosis-inducing ligand (TRAIL)-receptors, resulting in classical apoptosis of the target. In the second pathway, the cellular interaction triggers the NK cells to form immune synapses with the target cell and release of cytotoxic granules containing proteases, known as granzymes, and perforin[55]. The exact mechanism of action of perforin has been the subject of debate and two different mechanisms have been proposed.

The traditional view forwards that perforin generates pores in the plasma membrane and create a channel, which enables granzyme entry into the intracellular space. Alternatively, perforin has been proposed to cause transient pores in the membrane that trigger repair mechanisms and subsequent endocytosis of the damaged membrane. Inside the endosome, perforin may create pores in the endosomal membrane, thus releasing the cargo of granzymes into the cytosol and programmed cell death is initiated[56].

The effector functions of NK cells have been thoroughly evaluated in the context of controlling viral infections[57]. Immunodeficient mice specifically lacking NK cells are highly susceptible for a wide range of virus infections, including murine cytomegalovirus (MCMV), influenza virus and herpes simplex virus (HSV). Also, recent studies imply that expression of viral HLA class I-like proteins by infected host cells is a microbial strategy to avoid NK cell eradication[58, 59] and that IL-18 is an important mediator for sufficient NK cell-responses during MCMV infection[60]. In humans, increased sensitivity to infections of HSV, Epstein-Barr virus (EBV) and HCMV are linked to impaired NK cell activity.

NK cells were first identified for their ability to kill tumor cells and their contribution in controlling tumor growth and metastasis in animal models is well established [61, 62]. Veillette described that NK cells appear to be particularly prone to exert cytotoxicity against hematopoietic malignant cells[63]. In line with these findings, the adoptive transfer of allogeneic NK cells resulted in clearance of leukemic cells in patients with AML[64, 65]. Moreover, NK cells are reportedly critical to the therapeutic benefit of anti-CD20 monoclonal antibody (rituximab)-mediated tumor clearance in chronic lymphocytic leukemia (CLL)[66]. Additional clinical observations in support for a protective role of NK cells in human cancer comprise an 11-year follow-up study in patients demonstrating a link between low NK cell cytotoxicity and increased cancer risk[67]. Furthermore, accumulation of tumor-infiltrating NK cells was reported to correlate with favorable outcome in patients with gastric and colorectal carcinomas and lung cancer[68-70]. Single agent NK cell-activating immunotherapy with, e.g., IL-2 or IFN-α is approved in renal cell carcinoma and melanoma, but the moderate clinical efficacy and the toxicity of these cytokines have limited their wide-spread use. The combinational therapy of low-dose IL-2 and histamine dihydrochloride (HDC) was recently approved as immunotherapy in AML[71]. Other novel strategies, such as the use of bispecific or trispecific killer engagers to simultaneously target CD16 on NK cells and tumor antigens are under investigation[72]. Given the role of NK cells in host defense against cancer, new approaches are likely to develop for more efficacious NK cell-based and targeted immunotherapies.

NK cells modulate the responses of surrounding immune cells during inflammation. As previously discussed, this regulation is mediated by the secretion of pro- and anti-inflammatory proteins in combination with selective cellular cytotoxicity against adjacent innate and adaptive leukocytes. For innate cells, the complex network of interactions involves dynamic cellular crosstalk and has multiple immunological outcomes. NK cells and neutrophils both accumulate during the early phases of inflammation and therefore have the potential to enter into a reciprocal interplay. In support for such interactions, neutropenic mice were reportedly unable to clear bacterial infection due to lack of NK cell-derived IFN-γ and subsequent lack of DC-mediated IL-12[73]. Furthermore, NK cell functions and development were severely impaired in mice lacking neutrophils and in neutropenic patients[74]. The lack of peripheral neutrophils correlated with hyporesponsive NK cells, despite the presence of neutrophils in bone marrow, which may indicate that neutrophil-mediated NK cell regulation depends on close proximity between the innate cells throughout NK cell maturation.

In recent years, studies addressing interactions between NK cells and DCs have unraveled the phenomenon of DC editing. Mature DCs are the main APCs for initiating adaptive responses, while immature DCs induce immunological tolerance[75]. NK cells have the ability to discriminate between the mature and immature DCs by triggering apoptosis of the latter[76, 77], due to their low expression of HLA class I molecules[78] and CD1 proteins[79]. The NK cell killing of DCs is executed by interaction of the activating receptor NKp30 to an unidentified ligand expressed by DCs[80], but unknown additional factors have also been ascribed important roles independently of NCRs[81]. Alongside the NK cell-generated IFN-γ and TNF-α, the selective elimination process by NK cells promotes the development of maturing DCs and may thereby affect the balance between immunity and tolerance. In the reverse interaction, knockdown of NKp46 (but not NKp30 or NKG2D) using small interfering RNA (siRNA) prevented NK cell IFN-γ production stimulated by DCs[81]. In addition, DC-derived cytokines, including IL-12, IL-15 and IL-18, potentiate NK cell cytotoxicity, IFN-γ release and proliferation. In a study by Borg *et al.* formation of stimulatory synapses between NK cells and DCs was found to allow secretion of pre-stored IL-12 by DCs to enable directed NK cell activation[82].

The role of NK cells for the maturation of DCs may represent a key mechanism to bridge the NK cell response to induction of T cell functions. NK cells may further directly influence T cell responses by promoting T cell polarization via pro-inflammatory cytokines[83] or by specifically eliminating activated CD4 T cells to avoid autoimmunity in chronic viral infection[84]. Emerging studies have also demonstrated a bidirectional activation pathway comprising NK cells

and monocytes resulting in the release of IFN-γ and TNF by respective cell types[85, 86]. Moreover, NK cells have been shown to eradicate activated macrophages during virus infection[87] or eliminate lipopolysaccharide (LPS)-overstimulated macrophages *in vitro* to constrain immunopathology[88].

2.3 CELL DEATH

Programmed cell death is an evolutionary conserved and finely orchestrated process for maintaining cell and tissue homeostasis. It is also a vital component for the elimination of infected, transformed or damaged cells by the immune system. Dysregulation of the controlling mechanisms of cell survival and death are potentially detrimental for the host and the balance needs to be strictly controlled.

CLASSICAL APOPTOSIS

The initiation of cell apoptosis originates from external signals or internal cellular stress[89]. Stimulation of death receptors by ligands, such as FasL and TNF, triggers the *external pathway* with caspase-8 activation and subsequent cleavage of downstream effector caspases. The *intrinsic pathway* is initiated by signals of intracellular stress or damaged mitochondria resulting in leakage of cytochrome c into the cytoplasm. Cytochrome c binds and facilitates the creation of the multiprotein complex apoptosome. The assembly of apoptosomes generates active caspase-9, which in turn initiates an effector caspase cascade. The end result of the caspase cascades in both the extrinsic and intrinsic pathways is the activation of caspase-3. Along with exerting effector activities in the cytosol, this effector caspase is translocated into the nucleus and initiates DNA fragmentation, with detrimental effects on the cell: the nuclear envelope is disassembled, the cytoskeleton collapses and the cell shrinks and condenses. Importantly, during this suicidal program the cell display markers that alert surrounding phagocytes to digest the corpse before any leakage of its contents occurs.

CASPASE-INDEPENDENT CELL DEATH

Human cells are not dependent on the actions of caspases to undergo cellular suicide. Instead, caspase-inhibition may skew the cell death program into alternative pathways of cellular suicide, such as autophagy and necrosis, with different morphological outcomes[90].

PARTHANATOS AND PARP-1

Parthanatos is a caspase-independent mechanism of cell death that involves the DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1)[91]. The abundant nuclear PARP-1 senses DNA nicks and breaks, and the binding results in increased PARP-1 activity. In the presence of massive DNA damage, PARP-1 is over-activated and causes excessive production of PAR and subsequent energy depletion. Leakage of PAR into the cytosol generates loss of mitochondrial membrane potential (Ψ_m) with ensuing release of apoptosis-inducing factor

(AIF) from mitochondria. AIF migrates into the nucleus and induces DNA fragmentation and cell death.

Due to the significant role in maintenance of genomic integrity, PARP-1 has become an attractive target in chemotherapy. Thus, PARP-1 inhibitors render malignant cells more sensitive to DNA damaging chemotherapeutic agents, such as cisplatin, or irradiation[92]. Moreover, in BRCA1 and BRCA2 positive tumors the compromised ability to repair DNA breaks renders the malignant cells highly susceptible to inhibition of PARP-1 and enables selective killing of tumor cells[93, 94].

2.4 IMMUNITY AND CANCER

The presence of leukocytes within tumors was one of the first indications of a possible link between the immune system and cancer. Progress in immunology has advanced our understanding of the antitumor mechanisms and a role for inflammation in tumor development is now generally accepted.

2.4.1 IMMUNOEDITING

The concept of cancer immunoediting embraces the immune systems dual roles during tumorgenesis in both conveying protection against cancer development and in facilitating tumor progression[94]. This dynamic process is divided into three distinct phases:

- *Elimination*
- *Equilibrium*
- *Escape*

The *elimination* phase essentially refers to cancer immunosurveillance, in which innate and adaptive immune cells and complement factors detect and destroy developing tumors. Numerous models of immunodeficient mice show that a dysfunctional immune system and cancer development are closely connected[94, 95]. According to these studies, the removal of innate[96] or adaptive lymphocytes[97] results in increased susceptibility to spontaneous and induced tumors. Furthermore, knockout models in which experimental animals lack functional IFN-γ, perforin or TRAIL showed higher incidence and more aggressive forms of transplanted or chemically induced cancer[98, 99], indicating the importance of intact pro-inflammatory effector functions in tumor immunity. In line with these findings, the presence of tumor-infiltrating lymphocytes (TILs) strongly correlates with patient survival in melanoma, ovarian, colon and lung cancer[94]. In addition, the incidence of several cancers may be linked to immunosuppression after transplantation in humans[100].

Cancer immunosurveillance is currently considered to function as an effective extrinsic tumor-suppressor system and avoiding immunosurveillance is one of Hanahan and Weinberg's set of hallmarks of cancer[101]. However, the heterogeneous population of tumor cells may acquire means of avoiding the immunosurveillance and persist in a dynamic balance of *equilibrium*. The *equilibrium* is probably the longest of the three phases, as estimated by Loeb *et al.*: many solid tumors can persist for 20 years between initial carcinogen exposure and clinical detection[102]. In experimental models, Koebel *et al.* gained support for the existence of an *equilibrium* phase by showing that silencing of CD4/CD8/IFN-γ gives rise to late-forming tumors[103]. During

this period, the possible outcomes for the cancer cells are eventual elimination by the immune system, permanent tumor dormancy in equilibrium or *escape* from antitumor immunity. Tumor cells that survive and escape the immunosurveillance may have acquired genetic changes or may progress opportunistically upon immune system failure due to aging, disease or pharmacological immunosuppression. These cells are capable of growing in an immunologically unrestricted manner and may transcend into a clinically detectable disease. In these cases, the immune system has not only failed to eliminate or control transformed cells, but it may also have promoted tumor progression by selecting more aggressive variants. Accordingly, Shankaran *et al.* demonstrated that tumors developed under selective pressure in immunocompetent hosts are less immunogenic than tumors formed in an immunodeficient environment[97]. In the latter case, 40% of tumors derived in immunodeficient mice were rejected when transplanted into wild-type animals, while tumors developed in immunocompetent hosts spread progressively after transplantation.

Along with clonal heterogeneity and genomic instability of cancer cells, the different tumor escape strategies involve inducing tolerance and evading immune recognition by both innate and adaptive immunity. Down-regulation of HLA class I molecules, tumor-specific antigens and insensitivity to IFNs prevents T cell-mediated elimination and loss of NKG2D-ligands in combination with the secretion of factors impairing DC differentiation enables resistance to innate defense mechanisms[104, 105]. Moreover, there is evidence to suggest that tumor cells overproduce immunosuppressive factors to establish a less inflammatory environment to facilitate tumor cell growth[99].

2.4.2 IMMUNOSUPPRESSION

In the cellular microenvironment malignant cells employ a wide range of suppressive strategies, which may act in concert to counteract efficient immune responses. These mechanisms comprise both effects directly exerted by the tumor cells or indirect effects, such as recruitment and accumulation of immunosuppressive non-malignant cells.

Cancer cells display multiple mechanisms to escape immunosurveillance, involving release of immunosuppressive and pro-apoptotic mediators or mediating negative costimulatory signals[106]. Tumor expression of surface CTLA-4 ligand and programmed death receptor ligand 1 (PD-L1) effectively interrupts antitumor responses, while TRAIL, FasL and soluble indoleamine

2,3-dioxygenase (IDO) trigger lymphocyte apoptotic pathways upon cellular interactions. The immunoregulatory enzyme IDO catalyzes the oxidative breakdown of tryptophan, which is an essential amino acid for protein synthesis and metabolic functions in cells[107]. The depletion of tryptophan, along with accumulation of toxic catabolites, inactivates effector T cells and DCs in the tumor microenvironment[108]. Overproduction of tumor-derived anti-inflammatory cytokines, such as IL-10 and TGF-β, has been shown to impair DC maturation and dampen cytotoxic T cell responses. Interestingly, work by Thomas and Massagué showed that immunosuppressive TGF-β specifically inhibits the expression of cytotoxic gene products, such as perforin, granzymes, FasL and IFN-γ, which are crucial mediators in cytotoxic T cell and NK cell-mediated killing[109]. Elevated levels of soluble MICA in sera were reported in patients with various malignancies and could be a result of MICA shedding from the tumor surface[110]. Moreover, regulatory T cells (Tregs) are present at high numbers in multiple forms of human cancers[106] and create a favorable environment for tumor escape by exerting suppressive mechanisms, involving negative costimulatory signals via CTLA-4 and production of anti-inflammatory cytokines[111].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immune cells that play a critical role in pathological conditions, including cancer[108]. These suppressive immature cells are generated in the bone marrow and differentiate into mature macrophages, granulocytes and DCs in healthy individuals. In contrast, in murine tumor models and cancer patients MDSCs are activated and infiltrate lymphoid organs and tumors in response to growth factors and cytokines. MDSCs utilize a variety of mechanisms to suppress tumor immunity, including L-arginine deprivation[108]. By containing high intracellular levels of enzymes responsible for degradation of L-arginine, *i.e.* arginase and iNOS, the induction of MDSC leads to shortage of L-arginine. This, in turn, is associated with impaired T cell functions and T cell cycle arrest and, thus, results in ineffective tumor immunity. One particular member of the MDSC family is tumor-associated macrophages (TAMs), the functions of which are shaped by locally produced signals upon recruitment to tumor sites[112]. The abundance of TAMs has been linked to specific pathological features of cancers, for example metastasis, invasiveness, immunosuppression and poor response to therapy, thus supporting a tumor-promoting role for TAMs[113].

Furthermore, a common but not exclusive characteristic of MDSCs is their substantial production of ROS. The trait of forming these efficient immunosuppressive mediators is shared with malignant and non-malignant myeloid cells and may causes harmful effects on cells, such as DNA damage, lipid peroxidation, protein oxidation and inactivation of enzymes. *In vitro*

studies also demonstrate that exogenous hydrogen peroxide and oxygen radicals derived from leukemic monocytes inactivate lymphocytes and induce lymphocyte apoptosis[114, 115].

2.4.3 LEUKEMIA

Leukemias are malignancies of the hematopoietic system, originating from progenitor cells residing in the bone marrow. The accumulation of malignant cells in the bone marrow commonly causes insufficient hematopoiesis, including granulocytopenia, thrombocytopenia or anemia. Depending on the lineage of the transformed stem cell, leukemia is classified into the myeloid or lymphatic group. This thesis discusses the two most common forms of myeloid leukemia.

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with expansion of abnormal hematopoietic progenitor cells in the bone marrow and other organs[116]. These leukemic cells almost invariably carry detectable chromosomal aberrations or other genetic abnormalities of relevance to the development of leukemia[117]. Additionally, the leukemic cells vary in their degree of differentiation, morphological appearance and reactivity to histological dyes. These feature was previously used for classification by the French-American-British (FAB) system[118]. However, the FAB classification has been replaced by the World Health Organization (WHO) classification based on genetic abnormalities of the leukemic cells, which provide information about prognosis and guidance in the choice of therapy[119].

Standard regimens for treatment of AML include two phases of chemotherapy, *i.e.* induction chemotherapy followed by several courses of consolidation. The induction chemotherapy aims to reduce the total leukemic cell population with chemotherapeutic drugs such as cytarabine and daunorubicin. By definition, the patient enters complete remission (CR) if the malignant population decreases to below 5% of nucleated cells the bone marrow with concurrent recovery of neutrophils and platelets[116]. However, it is generally considered that a significant proportion of leukemic cells escape the initial chemotherapy and will cause relapse if no further post-remission therapy is administered. The main options for consolidation therapy are additional treatments with high-dose cytarabine and daunorubicin, alone or followed by allogeneic stem cell transplantation. Successful consolidation and post-consolidation therapy may result in sustained CR and long-time survival. The majority of AML patients attain CR after induction chemotherapy, but despite the ensuing phases of

therapy the relapse rate is in the range of 50-70% within five years[120] and there is a need for new therapeutic strategies, maintaining remission, in AML.

ROLE FOR IMMUNITY

The role of neutrophils in AML is evident during chemotherapy, which rapidly depletes neutrophils[116]. Chemotherapy-induced neutropenia increases the risk of infection and contributes to mortality in AML patients[121]. One approach to overcome the chemotherapy-induced neutropenia may be to stimulate and accelerate the production of granulocytes in the bone marrow by colony-stimulating factors (CSFs). While the evaluation of controlled trials regarding the potential benefit of administration of G- and GM-CSF after induction therapy suggested a shortened neutrophil recovery time and a tendency toward higher incidence of CR[122], a more recent meta-analysis including over 5,000 AML patients treated with CSFs during and following chemotherapy showed no impact on infection rate in this patient group[123].

Moreover, there is clear evidence to suggest that AML cells are susceptible targets for cytotoxic lymphocytes. The most convincing data on the susceptibility of AML cells to immune attacks originate from studies of patients undergoing allogeneic stem cell transplantations, where functions of NK and T cells are critical for the graft-versus-leukemia effect, which in turn is critical for a favorable outcome[124]. The leukemic cells reportedly express variable levels of MIC-A/B and HLA class I molecules, making them variably susceptible to T and NK cell functions, respectively[124]. In line with these findings, Fauriat *et al*. demonstrated that NK cells from a majority of AML patients exert impaired cytolytic functions as a result of NCR down-regulation, correlating with poor survival[125].

CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by accumulation of transformed myeloid progenitor cells at various stages of differentiation[126]. In most cases (approximately 85%), the patients are diagnosed in the chronic phase with risk of progression into an accelerated phase and blast crisis. The hallmark and diagnostic criteria of CML is the chromosomal translocation in which parts of chromosome 9 and 22 switch places generating the so-called Philadelphia chromosome *t*(9;22)(q34;q11)(Figure 4).

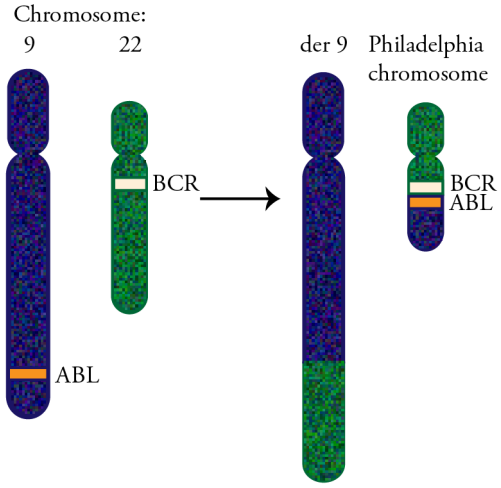

Figure 4. *Reciprocal chromosomal translocation results in generation of the Philadelphia chromosome and the elongated chromosome 9 (derivative chromosome or der 9).*

The translocation results in the abnormal fusion of the *BCR* and *ABL* genes. The BCR-ABL gene product possesses increased tyrosine kinase activity that drives the expansion of the malignant clone. Given the critical role of BCR-ABL kinase activity, tyrosine kinase inhibitors (TKIs) have become standard treatment of chronic phase CML[127]. The TKIs compete with the phosphorylating entity ATP by binding and blocking the active site on the kinase, causing a conformational change of the enzyme. This targeted therapy efficiently reduces the tumor burden in most cases of CML. Since the approval of the first TKI drug imatinib in 2001, CML has transformed from being a fatal leukemia into a controllable chronic disease. However, TKI resistance and intolerance are emerging issues and discontinuation of TKI treatment without relapse of CML is possible only in subsets of patients[128].

ROLE FOR IMMUNITY

TKI therapy is considered life-long treatment in patients with CML, due to high risk of relapse after discontinuation, which is presumably caused by reactivation of latent CML stem cells[129]. However, accumulating evidence indicates that the immune status of patients receiving imatinib is relevant for predicting and preventing relapse. In a recent study, a fraction of patients remained relapse-free after stopping imatinib-treatment despite the presence of residual leukemic cells[130]. This suggests that other anti-leukemic mediators are controlling residual CML cells in the absence of imatinib. The suggested immunosurveillance in CML patients seems partly dependent on NK cell effector functions, as higher levels of functional NK cells correlate with reduced

risk of relapse after TKI discontinuation[131]. In accordance with these reports, NK cells of newly diagnosed CML patients are reduced in number and show functional abnormalities[127, 132]. Moreover, the expression of NKG2D is down-regulated on NK cells in CML, which could potentially impair NK cell recognition of leukemic cells[133]. These results suggest that intact immune functions are important for the safe discontinuation of imatinib and provide a rationale for NK cell-based immunotherapy in CML.

2.4.4 IMMUNOTHERAPY

Therapeutical approaches designed to elicit, enhance or dampen immune responses to treat diseases are collectively named immunotherapies. The use of immunomodulators and cell-based immunotherapies is advancing in a variety of fields, including autoimmune diseases, allergy and cancer. Immunosuppression induced by malignant cells, by the tumor microenvironment or by chemotherapy is subject to intense investigation. Breaking immune tolerance using so called checkpoint inhibitors has recently proven to be a powerful approach to gain control over tumors that escape immunosurveillance. Thus, checkpoint inhibitors such as ipilimumab (anti-cytotoxic T-lymphocyte associated antigen-4; anti-CTLA-4) and nivolumab (anti-programmed death-1; anti-PD-1) have shown remarkable clinical efficacy in patients with advanced melanoma and in treatment-refractory lymphoma[134-136].

In hematological malignancies, the anti-CD20 antibody rituximab (MabThera®) is considered the standard of care in treating B cell leukemias and lymphomas[137]. The binding of rituximab to CD20-expressing B cells triggers multiple effects including complement-dependent cytotoxicity (CDC) along with ADCC exerted by NK cells and myeloid cells. In CML, the implementation of targeting leukemia-specific antigens have expanded beyond the oncoprotein BCR-ABL, which effectively targeted by TKIs, to focus on recently identified tumor-specific antigens. For example, Wilms tumor antigen 1 (WT1) and the preferentially expressed antigen of melanoma (PRAME) are promising targets due to their overexpression in leukemic stem cells[138]. Immunotherapeutic strategies in AML aim to prevent relapse by mimicking the beneficial graft-versus-leukemia observed after allogeneic stem cell transplantation[139]. Attempts to activate anti-leukemic responses with immunostimulatory mediators, such as IL-2 and IL-15, in the post-chemotherapy phase have proven challenging. Multiple trials using monotherapy with IL-2 have reported lack of clinical benefit in preventing relapse or prolonging CR in AML patients[140-143]. However, new approaches

with modified versions of IL-2 are being developed and one particular IL-2 variant appears to be highly potent in expanding cytotoxic T cells and improving subsequent anti-tumor efficacy[144].

IL-2/HISTAMINE DIHYDROCHLORIDE

In 2008, a combinational immunotherapy comprising histamine dihydrochloride (HDC) and low-dose IL-2 was approved within the EU for use in AML as post-consolidation treatment to prevent leukemic relapse for patients in CR. The efficacy of this treatment was demonstrated in a randomized phase III study comprising 320 adult patients receiving either ten 21-day cycles of HDC/IL-2 twice daily or no further treatment (standard of care)[71]. HDC/IL-2 treated patients showed significantly improved leukemia-free survival over control patients. Subsequent retrospective studies suggested that the clinical benefit of HDC/IL-2 was pronounced in myelomonocytic/monocytic forms of AML[115, 145]. Leukemic cells from the myelomonocytic/monocytic subclasses of AML were found to express functional histamine receptors and produced immunosuppressive ROS that triggered NK cell-apoptosis, which may explain the clinical efficacy in these forms of AML.

In 2009, a phase IV trial (Re:Mission NCT01347996) was initiated enrolling 84 patients recruited from 20 European centers. This study address the immune status of HDC/IL-2 treated AML patients in CR, with the primary endpoint of evaluating the functional and phenotypic properties of T and NK cells in addition to assessing their role for treatment outcome in terms of relapse risk. An interim analysis report of this trial is presented in paper IV of this thesis.

3 AIM

The overall aim of this thesis was to identify novel mechanisms in the dynamic interplay between NK cells and myeloid cells during immune responses and in leukemia.

The specific aims are:

PAPER I
To elucidate the role of neutrophils in initiating and regulating adaptive immune responses.

PAPER II
To investigate whether NK cells affect neutrophil functions during acute inflammation.

PAPER III
To assess the immunosuppressive capacity of malignant CML cells on lymphocytes.

PAPER IV
To evaluate the impact of immunotherapy on the T and NK cell compartments in patients with AML and to determine the role of these cells for relapse risk.

4 PATIENTS AND METHODS

This section presents the main *in vitro* systems used and general information regarding the patient material in paper III and IV. Detailed descriptions are found in each of the papers.

PATIENTS, TREATMENT AND SAMPLING

The leukemic patients included in paper III, were diagnosed as *BCR-ABL*-positive CML in chronic phase, treated at Sahlgrenska University Hospital. All gave written consent. Peripheral blood was obtained from newly diagnosed patients before receiving chemotherapy or TKI treatment. All samples included in the study were freshly isolated and analyzed within two days, due to phenotypical and functional changes when freezing CML cells.

In the clinical phase IV trial presented in paper IV, AML patients in first CR were recruited from 20 European centers. All patients were adults and fulfilled the inclusion criteria of achieving CR and completing consolidation chemotherapy no more than 6 and 3 months, respectively, prior to enrollment.

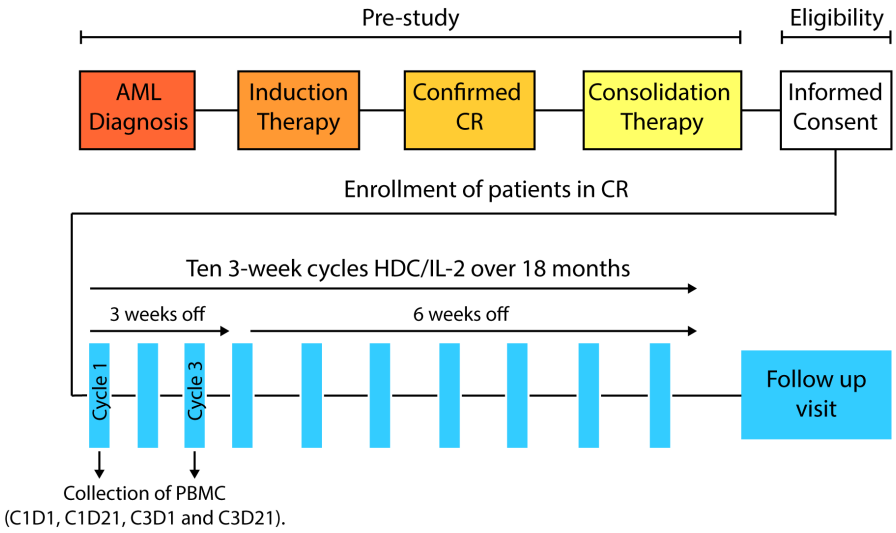

Figure 5. *Re:Mission trial overview. After induction and consolidation chemotherapy, AML patients in first CR were evaluated and eligible patients received ten 3-week cycles of HDC/IL-2 over 18 months. PBMC were collected before and after treatment cycles 1 and 3 (Paper IV).*

Patients received cycles of HDC/IL-2 subcutaneous twice daily and peripheral blood was collected before and after treatment cycle 1 and 3 (Figure 5). Samples of peripheral blood mononuclear cells (PBMC) were isolated and frozen at the collaborating centers and stored in liquid nitrogen before shipment to our laboratory. Thawed patient samples were stained with a viability marker and fluorochrome-conjugated antibodies in panels and analyzed by flow cytometry.

FLOW CYTOMETRY

Flow cytometry is a technique used for determining the origin, nature and behavior of cells by categorizing cell populations of defined parameters[146]. Commonly, the analysis of cell size, granularity and complexity are studied in relation to expression of cell surface and intracellular antigens.

In principle, particles or cells in suspension flow in a single cell stream through multiple laser beams. This generates a light scatter, which is filtered and detected by optical detectors. The direction of the light scatter, forward or side, will generate information on the morphological properties of the cell. The photodetectors will measure the amount of photons reaching the detector and convert this to a numerical value, which is displayed as a point on an analysis plot. Additional to the information about the morphological appearance, a wide range of fluorescent probes is utilized to estimate other cellular parameters. These probes are conjugated to ligands and antibodies specific for surface, cytosolic and nuclear antigens and the emitted fluorescent signal correlates with the cell-markers' density and distribution. Many fluorescent dyes can be combined in multicolor panels, which enables analyzes of several cellular antigens simultaneously.

Flow cytometry is routinely used in clinical laboratories to diagnose and characterize hematological malignancies[147]. In this thesis, phenotyping by flow cytometry has been performed in all four papers.

CELL DEATH

Analyses of cell death were performed by using several markers specific for different stages of apoptosis. As presented in paper II, during early apoptosis the plasma membrane loses asymmetry, causing PS to be exposed and detected extracellularly by Annexin-V. Another feature of early apoptosis is the depolarization of mitochondrial membrane potential (Ψ_m). To identify lymphocytes with disrupted Ψ_m the MitoProbe™ JC-1 (Molecular Probes®) was used. In viable lymphocytes with unaffected Ψ_m, JC-1 accumulates in the mitochondria and form aggregates, emitting red fluorescent light, detectable by flow cytometry. Upon entering early apoptosis, the alteration in Ψ_m results in release of JC-1 monomers, which is detected as increase in green fluorescence.

The ratio between the red and green fluorescence determine the state of Ψ_m in lymphocytes.

In later apoptotic phases, the cellular membranes are compromised and thereby permeable to the DNA-stain To-Pro-3. In paper III, apoptotic cells were determined by detection of free intra- and extracellular amines by a dye named ViVid. Notably, amine-reactive ViVid will bind to extracellular amines on viable cells as well, but with a less intensity than dying cells with porous membranes.

CELL PROLIFERATION

The binding of intracellular amines was further utilized in paper I, for analyzing cell proliferation by CellTrace™Violet (Invitrogen™). This probe diffuses into the cells, binds covalently to free amines and the color intensity decreases for every new generation of dividing cells.

TRANSMIGRATION OF LEUKOCYTES

In paper II, an *in vivo* skin chamber model is used for investigating infiltrating leukocytes from blood to tissue. Blisters are induced on the forearm by applying negative pressure. When the epidermis is separated from dermis a fluid-filled blister is formed and an acute inflammatory response is triggered with accumulation of migrating leukocytes[148]. This is a reliable model for studying leukocyte trafficking in a setting of sterile inflammation in healthy volunteers.

5 RESULTS AND DISCUSSION

PAPER I

Neutrophils have for long been considered as solitary killers with limited interactions with other leukocytes. However, in recent years a few studies have addressed and identified a potential reciprocal activation between neutrophils and NK cells[73, 149, 150]. In paper I, we sought to characterize the interaction between these cells and investigate if it impacted on the adaptive immune responses. In brief, we showed that TLR-stimulated neutrophils produce chemokines that attract and activate NK cells *in vitro*. In co-culture assays, neutrophil-conditioned NK cells exhibited increased cytokine production, cytotoxicity and enhanced responsiveness to exogenous or DC-produced IL-12.

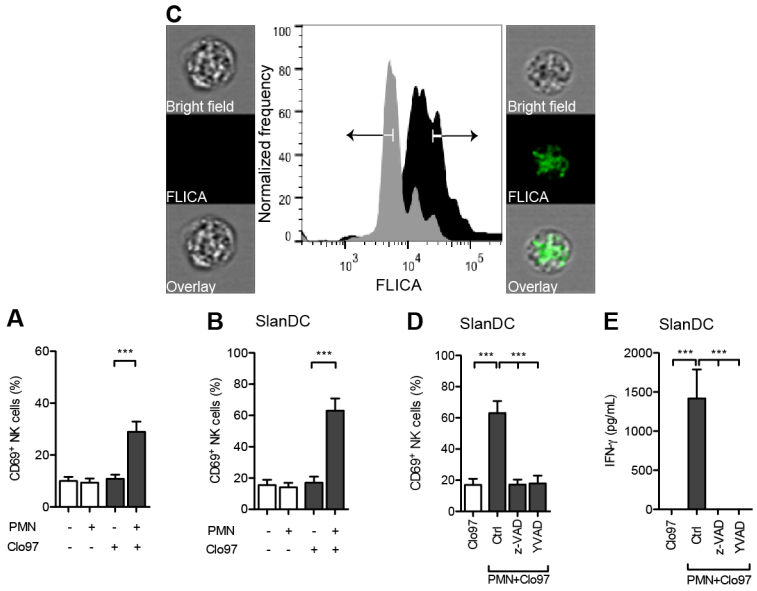

Figure 6. *PMN conditioning of NK cells is highly dependent on caspases. TLR-stimulated PMNs trigger NK cell expression of activation marker CD69 (A) after 24 hours and potentiate subsequent interaction between NK cells and SlanDCs (B). TLR-agonist trigger up-regulation of active caspases (C) and blockade of pan-caspases (z-VAD) and specifically caspase-1 (YVAD) in PMN-NK cell co-cultures completely inhibited the PMN-mediated NK cell CD69 expression (D) and IFN-γ release (E).*

The neutrophil activation of NK cells was abolished in the presence of caspase-1 inhibitors (Figure 6) and more specifically by natural antagonists for IL-1 and IL-18.

Interestingly, we discovered that neutrophil elastase cleaved IL-18 to fragments that remained capable of activating NK cells. A similar phenomenon has been described for the third IL-1 family member IL-33, which upon neutrophil-mediated protease cleavage generates forms exerting a ten-fold higher activity compared to full-length IL-33[151]. In a previous study, exogenous IL-18 was shown to promote NK cells to acquire "helper" functions, such as, producing high amounts of IFN-γ upon exposure to additional pro-inflammatory signals[51]. We further strengthened this concept by showing that neutrophil-conditioned NK cells promoted maturation of DCs and enhanced their capacity to trigger ensuing T cell proliferation and IFN-γ production.

Collectively, this study suggests that neutrophils play a far more sophisticated role in initiating and orchestrating immune responses than previously appreciated. However, it is also conceivable that dysregulation of such interactions may be associated with considerable immunopathology. Accordingly, Costantini *et al*. published data showing co-localization of neutrophils and NK cells in inflamed lesions from patients with autoimmune psoriasis and Crohn's disease[152]. Thus, pharmacological manipulation of this crosstalk may be beneficial both in cases where enhanced immune responses are desired, and in autoimmune diseases where immunity needs to be suppressed.

PAPER II

The potent effector functions by neutrophils are crucial for host defense during acute inflammation. At the site of infection rapid accumulation of activated neutrophils calls for a controlled and well-timed apoptosis program to avoid extensive damage of healthy tissues. Neutrophil-derived chemokines attract NK cells to the site of infection and promote NK cell-mediated immunomodulation, including cytotoxicity against other innate immune cells[35]. Hence, in the second paper, we explored whether NK cells could influence the regulation of neutrophil actions and survival. In co-culture experiments, NK cells triggered neutrophil apoptosis via contact-dependent mechanisms.

This finding was challenging a previous report by Bhatnagar *et al*. suggesting that supernatants from stimulated NK cell prolonged the survival and promoted activation of neutrophils[149]. However, in our hands survival signals from pro-inflammatory cytokines were overridden by the pro-apoptotic effect of interacting NK cells. Importantly, the apoptotic neutrophils remained intact implying that NK cells induced a controlled apoptosis rather than neutrophilic lysis (Figure 7). Our studies show that NK-neutrophil interaction resulted in engagement of the Fas pathway and subsequent activation of caspase-dependent neutrophil apoptosis.

The NK cell capacity to accelerate neutrophil apoptosis was not shared with other lymphocytes and was significantly inhibited by blocking of NKp46. Accordingly, neutrophils displayed a hitherto unknown ligand to NKp46, and to some extent a ligand to NKp30. Since the publication of Paper II, Matta *et al.* demonstrated that the NKp30 ligand, B7-H6, is induced on inflammatory neutrophils[45].

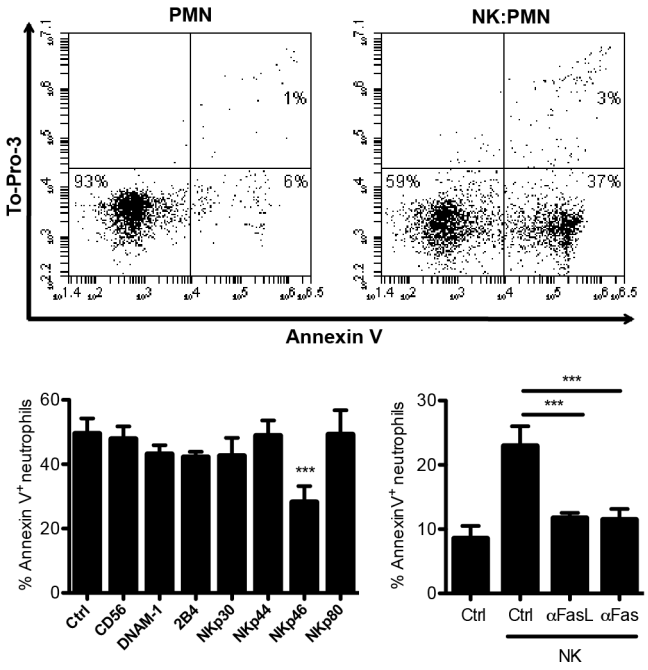

Figure 7. *Interactions with NK cells trigger neutrophil apoptosis (upper panel). The NK cell-induced neutrophil cell death was prevented with blocking antibodies against NK cell activating receptor NKp46 (lower, left) and the Fas pathway (lower, right). Adapted from[153].*

Taken together, we show that NK cells accelerate neutrophil apoptosis and that it involved NKp46 interactions with a neutrophil ligand and activation of the Fas pathway. These findings expand the immunoregulatory role by NK cells and may implicate a novel function in control and resolution of acute inflammation.

PAPER III

Malignant neutrophilic cells show susceptibility to immune-mediated destruction by cytotoxic NK cells and T cells. However, CML is associated with quantitative and functional NK cell deficiency[127], which may affect the course of disease and the response to antileukemic treatment. In paper III we elucidated the immunosuppressive mechanisms of malignant CML cells. Results show that leukemic neutrophils isolated from BCR-ABL$^+$ CML patients express a functional ROS-forming NADPH oxidase. Furthermore, CML cells triggered massive NK cell death at low cell-cell ratios, which was prevented in the presence of ROS-scavenging catalase. These findings are in line with reports of cytotoxic NK cells showing high sensitivity to exogenous[154] or myeloid cell-derived oxygen radicals[155, 156]. In additional experiments, we observed that NK cells exposed to CML cells attained cell death features, such as depolarized Ψ_m and accumulation of AIF in the nucleus, and were efficiently rescued from CML-induced cell death by the PARP-1 inhibitor PJ-34. These results imply that CML-generated ROS trigger NK cell parthanatos as a possible mechanism for CML cells to avoid elimination by cytotoxic lymphocytes.

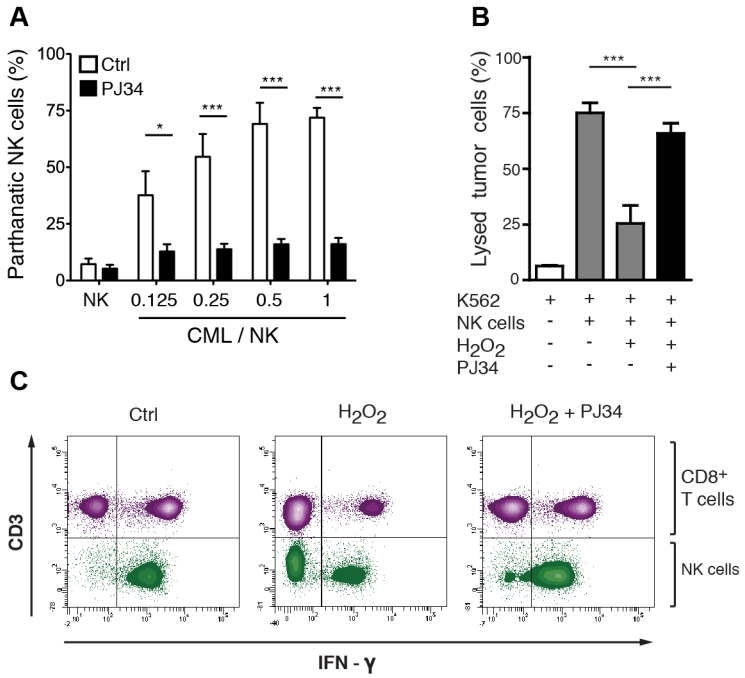

Figure 8. *CML-mediated killing of NK cells is prevented by PARP-1 inhibitor (A). After H_2O_2 treatment, the PARP-1 inhibited NK cells retained their cytotoxic capacity against target cell K562 (B). Moreover, PJ-34 pre-treated cytotoxic T cells and NK cells showed preserved up-regulation of intracellular IFN-γ during oxidative stress (C). Adapted from[157].*

In addition, the finding that PARP-1 inhibitors protect lymphocytes from ROS-mediated cell death suggests a potential use of them as immunotherapeutic agents. However, for the rescued lymphocytes to exert antileukemic actions, the surviving leukocytes must maintain their immunological effector functions. We therefore investigated the functional status of lymphocytes after oxidative stress in the presence of a PARP-1 inhibitor (Figure 8). In cytotoxicity assays, the NK cell-mediated degranulation and killing of CML cell line K562 was sustained by PARP-1 inhibition after exposure to H_2O_2. Moreover, similar results of intact lymphocyte functions were obtained in analyses of lymphocyte proliferation and IFN-γ production as response to IL-2 or upon stimulation with PMA/Ionomycin, respectively. A drawback with the potential use of PARP-1 inhibitors in immunotherapeutic regimens is their role in DNA repair, as interfering with DNA repair pathways may compromise the genomic integrity and DNA transcription of cells[158]. With this in mind, several clinical trials have addressed PARP-1 inhibition in combinational trials with cytotoxic agents, aiming to enhance DNA damage and cell death in malignant cells[159, 160].

In conclusion, this study shows that malignant neutrophils form immunosuppressive ROS, which induce extensive lymphocyte cell death. The CML-mediated lymphocyte suppression was suppressed by PARP-1 inhibition and viable T cells and NK cells maintained their antileukemic cytotoxicity, cytokine production and proliferation despite the oxidative stress. These findings implicate a rationale for targeting the parthanatos pathway in immunotherapy.

PAPER IV

As previously mentioned, AML is the most common acute leukemia in adults, and the incidence increases with age. Despite improved treatments with chemotherapy, long-term remission is only being achieved in <50% of patients and generally not durable due to high incidence of relapse. Thus, there is a need for new treatments to prevent relapse and a promising approach is to augment antitumor responses in these patients.

The combinational immunotherapy with HDC/IL-2 has been shown to prolong LFS after 3 years compared to untreated control, but the immunological mechanisms involved have not been defined. In the forth paper of this thesis, the immune status of patients treated with HDC/IL-2 was evaluated with focus on T and NK cell responses. The administration of HDC/IL-2 profoundly enhanced absolute NK cell counts in peripheral blood after 21-days of treatment in cycle 1 and 3. This increase was observed in both $CD56^{dim}$ and $CD56^{bright}$ phenotypes of NK cells and the levels of cytotoxic $CD56^{dim}$ cells remained elevated between the treatment cycles. In contrast, the $CD56^{bright}$ NK cells returned to base line levels between cycles and re-accumulated during the next treatment cycle. Moreover, expression of activating receptor NKp30 was significantly induced on both NK cell subsets, while only $CD56^{dim}$ cells showed elevated expression of NKp46 at the end of the first treatment cycle.

To investigate if these elevated NK cell numbers and NCR expression affected the leukemia-free survival (LFS) or overall survival (OS), the patients were dichotomized based on median cell counts or receptor MFI and analyzed using logrank tests. Before treatment in the first cycle, high levels of $CD56^{bright}$ cells positively correlated with both LFS and OS, but not after three weeks of therapy. The importance of these immature NK cells might reflect the capacity of HDC/IL-2 to initially activate and expand precursor $CD56^{bright}$ cells followed by differentiation into cytotoxic $CD56^{dim}$ NK cells. Interestingly, a significant induction of $CD56^{dim}$ cells between the treatment cycles was observed in patients with sustained remission high induction of $CD56^{dim}$ cells between treatment cycles was associated with a positive trend towards favorable outcome.

High absolute numbers of $CD56^{dim}$ NK cells did not impact on outcome in patients with AML. Instead, elevated surface expression of NKp46, both pre- and post-treatment in cycle 1, on these cells correlated with higher LFS and OS (Figure 9). A positive trend was also observed for high NKp30 expression before and after the first cycle treatment and a similar tendency towards favorable LFS and OS for patients expressing high NKp30 and NKp46 on $CD56^{bright}$ cells. These data are the first to demonstrate an impact of NCRs on survival in AML

patients in remission and confirm previous reports showing the importance of NCRs for favorable disease outcome in AML[125].

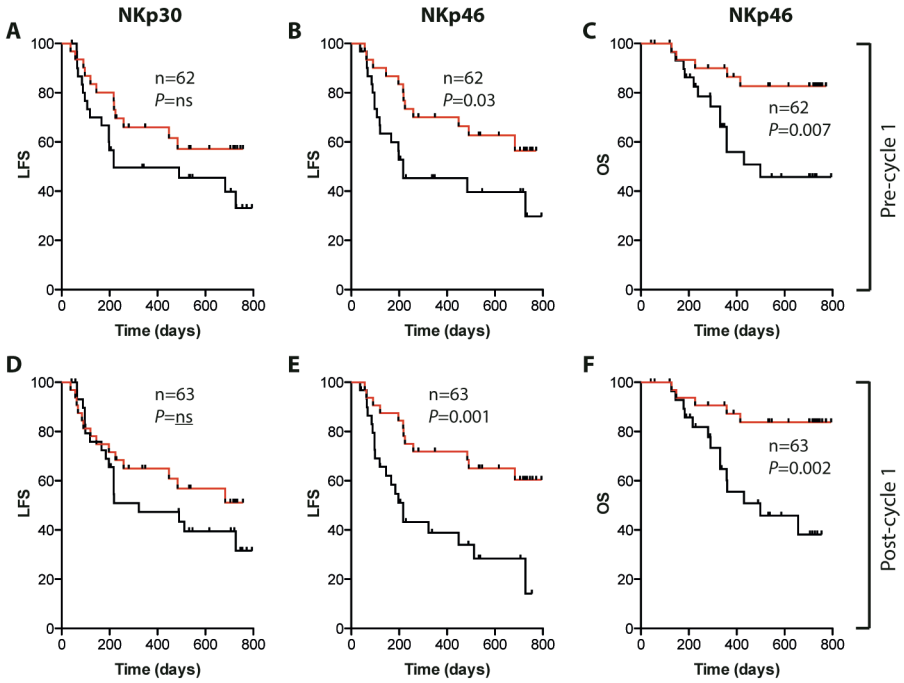

Figure 9. *NCR expression of CD56^{dim} NK cells has an impact on LFS and OS. The patients were dichotomized based on high (red) or low (**black**) median expression of NKp30 (A and D) or NKp46 (B, C, E and F) before and after HDC/IL-2 treatment.*

Collectively, this study shows that responsiveness of NK cell subsets play a role in the efficacy of immunotherapy with HDC/IL-2 in AML. The treatment induced expansion of CD56^{dim} and CD56^{bright} NK cells in peripheral blood with increased surface expression of activating NKp30 and NKp46. Moreover, high cell counts of CD56^{birght} cells and high expression of NKp30 and NKp46 on CD56^{dim} cells was associated with favorable LFS and OS. Our data suggests that therapeutical regimens targeting NK cell functions could potentially improve anti-leukemic effects in cancer patients and that NCR expression may be a valid biomarker for evaluating immunotherapy in AML.

6 CONCLUDING REMARKS

Innate immune cells are equipped with a battery of receptors designed to detect infectious agents. Trough a sophisticated innate crosstalk, the immune response is first amplified to enable clearance of the infection, but at the same time tightly regulated to avoid excessive collateral damage to host tissues. This thesis comprises mechanistic studies of the initial response to pathogens. Thus, in Paper I of this thesis, it is shown how TLR stimulation affects innate interactions between inflammatory neutrophils and NK cells. The crosstalk involved cytokines of the IL-1 family and enhanced the cytotoxic effector functions of NK cells but also rendered NK cells capable of promoting DC maturation, which in turn stimulated the adaptive immune response. The study highlights the role of neutrophils in immunity. On a per-cell basis, neutrophils may not be potent producers of cytokines, but given their relative abundance in peripheral blood and their prompt response to inflammatory cues, their contribution is likely to be important in the earliest phase of the immune response.

It is conceivable that stimulation of these innate interactions would be beneficial in cancer to boost the anti-tumor immune response. In fact, such a strategy was introduced already in the end of the 1800s, when Dr. William Coley inoculated cancer patients with streptococci[161]. On the other hand, in inflammatory conditions, such as Crohn's disease, in which neutrophils and NK cells reportedly co-localize in inflammatory loci[152], it may be beneficial to target these interactions with inhibitory agents, such as IL-1RA (Anakinra) or IL-18BP.

NK cells and neutrophils also regulate each other. Several reports have demonstrated the suppressive role of ROS derived from neutrophils and other myeloid cells on NK cell function and viability[115, 155, 162] On the other hand, in the second paper of this thesis, it is shown that NK cells can also accelerate neutrophil apoptosis in a cell-cell contact-dependent manner. The mechanisms involved were NKp46 engagement to an unidentified ligand expressed by neutrophils and the Fas apoptosis pathway. This novel neutrophil regulation by NK cells is a possible mechanism for restraining neutrophil actions at the inflammatory site, which may protect the surrounding tissue from excessive damage by toxic neutrophil-derived substances. These two papers collectively illustrate the complex interplay between neutrophils and NK cells; depending on the conditions, the crosstalk can either result in mutual activation or the demise of either one of the cells.

In myeloid leukemias, intact regulatory functions, such as ROS production, enable leukemic cells to escape NK cell surveillance. The generation of lymphocyte-suppressive ROS by malignant cells from patients with CML induced massive T and NK cell death, which was mediated by enzymatic actions of the nuclear protein PARP-1. Pharmacological inhibition of PARP-1 rescued cytotoxic lymphocytes from ROS-induced parthanatos. Importantly, surviving T and NK cells displayed sustained effector functions, which suggests that PARP inhibition could be a viable strategy to protect immune cells from leukemia-induced immunosuppression. However, given the crucial role of PARP-1 in DNA repair, therapeutical inhibition of PARP-1 may compromise the genomic integrity. With this in mind, targeting other factors involved in the PARP/AIF pathway, such as PAR, would be a safer option. Future studies are warranted to explore the potential of using PARP-1 inhibitors as immunotherapeutic regimens in combination with immunostimulatory agents to maintain and enhance the functions of cytotoxic lymphocytes, which is reportedly important for favorable disease outcome in CML.

In the clinical phase IV trial, eighty-four patients with AML in CR was treated with post-consolidation HDC/IL-2 immunotherapy and monitored for NK cell responsiveness. As presented in Paper IV, the combination of pro-inflammatory IL-2 and antioxidative HDC increased both $CD56^{dim}$ and $CD56^{bright}$ absolute numbers and effector functions in patients with AML and correlates with favorable outcome.

Five clinical trials evaluating monotherapy with IL-2 in AML have collectively demonstrated that IL-2 on its own is not efficient in preventing relapse or prolonging CR in AML [140-143, 163]. These results suggest that the addition of HDC is crucial for efficacy in AML. A direct head to head comparison of IL-2 and IL-2/HDC has not been performed in AML. However, in melanoma patients, the addition of HDC potentiates the immunostimulatory effects of IL-2 [164]. Futhermore, in a study by Aurelius *et al. in vitro* experiments showed that HDC effectively reduced ROS-production and significantly lowered NK cell apoptosis mediated by isolated AML cells [145]. A recent study has also demonstrated that HDC promote differentiation of DCs in vivo and lower the tumor-burden in NOX2-sufficient mice, but not in NOX-deficient, $gp91^{-/-}$ mice [165].

NK cells are reportedly important in other types of myeloid leukemia, such as CML. CML is generally well controlled by TKIs, but this therapy is life-long and associated with resistance and toxicity. As mentioned above, in a fraction of patients with undetectable disease, attempts have been made to discontinue with TKI therapy. These studies suggest that patients with high NK cell function are

more prone to remain in remission. It is thus conceivable that NK cell-stimulatory immunotherapy, such as HDC/IL-2, given before TKI discontinuation may increase the chances of maintained remission.

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