Mechanisms of leukemia-induced immunosuppression

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To my family

ABSTRACT

This thesis aimed to define the role of reactive oxygen species (ROS), produced by the NADPH oxidase of myeloid cells, in the regulation of lymphocyte function with focus on ROS-induced dysfunction of natural killer (NK) cells and T lymphocytes in myeloid leukemia. In **Paper I**, a novel mechanism is presented by which specifically activated T lymphocytes evade inactivation by ROS after antigen presentation. Antigen-presenting dendritic cells were found to induce ROS-neutralizing thiols on the surface of antigen-specific T cells, but not on T cells that lacked antigen specificity. These findings may explain why antigen-specific T cells remain viable under conditions of oxidative stress. **Paper II** shows that subsets of leukemic cells recovered from patients with acute myeloid leukemia (AML) produce and release ROS via a membrane-bound NADPH oxidase, and that ROS-producing leukemic cells initiate a PARP-1-dependent pathway of cell death (parthanatos) in NK cells and T cells. The results presented in **Paper III** demonstrate that treatment of AML patients with a NADPH oxidase inhibitor (histamine dihydrochloride) was preferentially efficacious among patients with monocytic leukemias (FAB classes M4 and M5), in which cells of the leukemic clone expressed a ROS-producing NADPH oxidase and functional histamine H_2 receptors. The results presented in **Paper IV** imply that malignant cells recovered from patients with chronic myeloid leukemia utilize the ROS/PARP-1 axis to induce NK cell parthanatos and that PARP-1 inhibition maintains functions of T cells and NK cells under conditions of oxidative stress. **Paper V** aimed to define the intracellular pathways of ROS-induced PARP-1 activation with ensuing cell death in lymphocytes. The results suggest that the mitogen-activated protein kinase ERK1/2 is involved in ROS-induced signal transduction and that ERK1/2 is activated upstream of PARP-1 in ROS-dependent lymphocyte parthanatos.

Keywords: Reactive oxygen species, NK cells, T cells, ROS, PARP-1, Acute myeloid leukemia, AML, immunosuppression, immunotherapy

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SAMMANFATTNING PÅ SVENSKA

Flera av immunsystemets komponenter kan känna igen och destruera cancerceller. Denna avhandling handlar om mekanismer som förklarar varför immunsystemets celler, särskilt natural killer (NK)-celler och T-lymfocyter, inaktiveras vid cancersjukdomar, och hur sådan kunskap kan användas för mer effektiv terapi.

Tidigare studier har visat att defekt NK- och T-cellsfunktion vid cancer ofta leder till kort överlevnad och ett snabbt sjukdomsförlopp. En av de mekanismer som anses kunna förklara varför NK-celler och T-celler är undertryckta vid cancer hänför sig till myeloiska cellers produktion av syreradikaler, som bildas av enzymet NADPH-oxidas. Syreradikaler frisätts extracellärt från t.ex. makrofager och granulocyter och är betydligt mer toxiska för NK-celler och T-celler än för cancerceller. I närvaro av radikaler inaktiveras därför NK-celler och T-celler effektivt och dör i programmerad celldöd (apoptos).

I **arbete I** visas att antigenpresenterande celler (APC) kan utrusta T-celler med tioler. Tioler är strukturer med förmåga att neutralisera syreradikaler och som därmed förhindrar att T-celler inaktiveras i vävnader med höga nivåer av syreradikaler. APC uppreglerar framförallt tioler på T-celler som är specifika för det antigen som presenteras. Detta fynd kan förklara varför antigenspecifika T-celler överlever och förblir aktiva i inflammatorisk vävnad, som ofta innehåller syreradikalbildande myeloiska celler.

Akut myeloisk leukemi (AML) är en heterogen form av blodcancer som karaktäriseras av att maligna myeloida celler ansamlas i benmärg och blod. I likhet med många andra cancersjukdomar är defekt funktion hos NK-celler och T-celler vanligt vid AML, vilket korrelerar med sämre prognos. I **arbete II** beskrivs en ny mekanism för immunsuppression vid AML. Resultaten visar att vissa typer av AML-celler (mogna monocytära celler) inducerar celldöd i NK-celler och T-celler. Dessa subtyper av AML-celler tillhör kategorierna FAB-M4 och FAB-M5 enligt French-American-British (FAB)-klassifikationen, som är ett system för indelning av AML främst baserat på de leukemiska cellernas morfologi. Vidare visas att immunsuppressionen orsakas av syreradikalproduktion via NADPH–oxidaset, som uttrycks av de maligna cellerna. För att klargöra om detta fynd kan ha klinisk signifikans genomfördes en *post hoc*-analys av resultaten från en fas III-studie av NADPH-oxidashämmaren histamin-

dihydroklorid (HDC) i kombination med den immunstimulerande cytokinen IL-2. Behandlingen ges för att stimulera NK- och T-cellers funktion (IL-2 komponenten) samtidigt som dessa celler skyddas från syreradikalinducerad apoptos (HDC komponenten). Resultaten talar för att behandling med HDC/IL-2 effektivt förebygger återfall i leukemi vid monocytär AML, dvs. FAB-M4 och FAB-M5, men inte vid FAB-M2-AML (**arbete III**). Parallellt genomfördes analyser av histaminreceptorers förekomst på maligna celler från FAB-typer av AML. Dessa analyser visade att funktionella histaminreceptorer uttrycks av FAB-M4- och FAB-M5-celler, men inte av FAB-M2-celler.

Radikalinducerad celldöd i lymfocyter har tidigare visats vara beroende av DNA-reparationsenzymet PARP-1. Genom att blockera PARP-1 kan radikalinducerad celldöd i T-celler och NK-celler förhindras. I **arbete IV** visas att CML-celler (maligna neutrofiler) inducerar celldöd i NK-celler via samma ROS/PARP-1-beroende mekanism som FAB-M4- och FAB-M5-celler. Detta arbete visar också att lymfocyter som behandlas med en PARP-1-inhibitor förblir funktionella i närvaro av toxiska syreradikaler. **Arbete V** visar att det intracellulära kinaset ERK1/2 aktiveras uppströms om PARP-1, och att lymfocyter skyddas från immunsuppressiva syreradikaler vid blockering av denna signaleringsväg.

LIST OF PAPERS

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

I. Martner, A; **Aurelius, J**; Rydström, A; Hellstrand, K; Thorén, FB. *Redox remodeling by dendritic cells protects antigen-specific T cells against oxidative stress.* J Immunol 2011;187 6243-6248.

II. **Aurelius, J**; Thorén, FB; Akhiani, A; Brune, M; Palmqvist, L; Hansson, M; Hellstrand, K; Martner, A. *Monocytic AML cells inactivate anti-leukemic lymphocytes: role of NADPH oxidase/gp91^{phox} expression and the PARP-1/PAR pathway of apoptosis.* Blood 2012; May 1. [Epub ahead of print].

III. **Aurelius, J**; Martner, A; Brune, M; Palmqvist, L; Hansson, M; Hellstrand, K; Thorén, FB. *Remission maintenance in acute myeloid leukemia: impact of functional histamine H_2 receptors expressed by leukemic cells*. Submitted 2012.

IV. **Aurelius, J**; Martner, A; Romero, AI; Riise, RE; Palmqvist, L; Brune, M; Hellstrand, K; Thorén FB. *Chronic myeloid leukemic cells trigger poly(ADP-ribose) polymerase-dependent inactivation and cell death in lymphocytes.* Submitted 2012.

V. Akhiani, AA**; Aurelius, J**; Movitz, C; Hellstrand, K; Thorén FB. *Reactive oxygen species trigger ERK pathway-dependent parthanatos in cytotoxic lymphocytes.* Submitted 2012.

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ABBREVIATIONS

AIF	Apoptosis-inducing factor
Allo-SCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
BM	Bone marrow
CD	Cluster of differentiation
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CR	Complete remission
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DLI	Donor lymphocyte Infusion
ERK	Extracellular signal-regulated protein kinase
FAB	French-American-British
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
HDC	Histamine dihydrochloride
IFN	Interferon
IMC	Immature myeloid cells
Inv(16)	Inversion(16)
KIR	Killer cell immunoglobulin-like receptor
LFS	Leukemia-free survival
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
OS	Overall survival
PAR	Poly(ADP-ribose)
PARP-1	Poly(ADP-ribose) polymerase-1
PBMC	Peripheral blood mononuclear cells
t(8;21)	Translocation(8;21)
TAM	Tumor-associated macrophage
TCR	T cell receptor

1 PREFACE AND AIM

AML is characterized by rapid expansion of myeloid cells in bone marrow and blood, and the disease is fatal if not promptly treated. At diagnosis, patients receive induction chemotherapy to eradicate leukemic cells. After the achievement of complete remission (CR), which occurs in most patients, additional courses of chemotherapy (consolidation) are administered aiming to eradicate residual leukemic cells and thus maintain CR. The long-term prognosis is, however, dismal with only 20-25% of patients remaining in life-long CR, and a significant explanation to the poor long-term prognosis is the high frequency of relapse among patients who have achieved CR [1-4].

The success of allogeneic stem cell transplantation (allo-SCT) for high-risk AML patients along with the finding that grafted T cells and NK cells eradicate leukemic cells have inspired immunotherapeutical approaches for patients who are not eligible for allo-SCT [5]. Such immunotherapy is more likely to be successful in CR, after the completion of chemotherapy, when patients may harbor a small, yet life-threatening burden of leukemic cells. The expectations of success have been high as significant evidence points towards a clinically meaningful anti-leukemic effect exerted by both of these lymphocyte subsets. [6].

A critical, and often overlooked, hurdle in the development of efficacious immunotherapy in AML is the immune dysfunction often observed in patients [7]. Immune cells in cancer patients, including those with hematological cancers, are thus frequently in a suppressed stage and numerous mechanisms explaining this functional impairment have been described. One such mechanism is the production and release of reactive oxygen species (ROS) by myeloid cells. These toxic derivatives of oxygen efficiently inactivate pivotal elements of cell-mediated immunity against leukemic cells, including NK cells and T lymphocytes.

In 2008, immunotherapy combining histamine dihydrochloride (HDC) and interleukin-2 (IL-2) was approved as relapse-preventive treatment in AML patients below 60 years of age in their first CR. The purpose of this combination treatment is to depress myeloid cell ROS production through HDC – thus protecting cytotoxic lymphocytes from inactivation - and simultaneously activate cytotoxic lymphocytes by means of IL-2 [8].

This thesis aimed to define, with a translational approach, the mechanisms and relevance of ROS-dependent immunosuppression in leukemia, including the putative role of ROS production by malignant myeloid cells. Also, the intracellular mechanisms that effectuate the toxic properties of ROS in lymphocytes have been investigated. The results point to a novel mechanism by which leukemic cells evade cell-mediated immunity and novel strategies to improve the effectiveness of cancer immunotherapy.

2 INTRODUCTION

2.1 Hematopoiesis

Hematopoiesis refers to the development of blood cells and platelets. This is a highly hierarchical process that starts with a hematopoietic stem cell (HSC) with self-renewal capacity and ends in highly differentiated cells with no self-renewal and limited proliferative capacity [9, 10]. Hematopoiesis occurs in bone marrow (BM) and the products are red blood cells (erythrocytes), platelets (thrombocytes) and white blood cells (leukocytes). Erythrocytes and thrombocytes are homogenous entities whilst leukocytes are heterogeneous comprising many cell subsets. In Figure 1, a simplified illustration of hematopoiesis is shown.

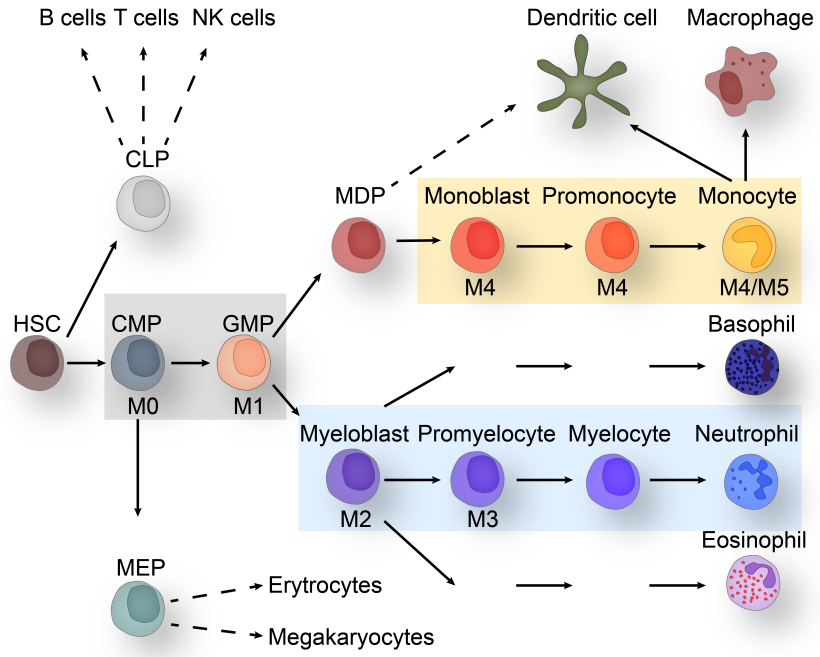

Figure 1. Schematic illustration of human hematopoiesis. Maturation stages of AML cells are indicated based on FAB classifications (M0-M5). Yellow and blue squares indicate monocytic and granulocytic differentiation, respectively. Grey square shows progenitor cells without established differentiation.

2.2 Innate and adaptive immunity

Upon confrontation, the immune system responds as a two-stage rocket involving both innate and adaptive immunity. When challenged, the innate immune cells launch an immediate response followed by a slower, pathogen-specific response by adaptive immune cells [11-13].

Innate immunity is also referred to as intrinsic or natural immunity and, as the name suggests, innate immune cells are present and functional from birth. Cellular components of innate immunity are either myeloid or lymphoid, comprising neutrophils, monocytes, dendritic cells, basophils, eosinophils and NK cells [14] and their responses are triggered through germline receptors, such as natural cytotoxicity receptors (NCRs) and toll-like receptors (TLRs), which recognize conserved structures [11, 15-17]. Innate immune responses are sometimes described as unspecific [11, 18], originating from the notion that innate immune cells do not adapt to the pathogen. This is, however, a simplistic view: receptors of innate immunity are highly specific and recent studies have highlighted the existence of memory functions in innate immunity, indicating an adaptive potential [19-21]. For example, activated NK cells may differentiate into memory NK cells and localize to the sinusoid of the liver. When the host is re-exposed to the antigen memory NK cells mediate a fast and specific response [19, 22].

The adaptive immune response is fundamentally different from that of the innate immune system and mediated by B and T cells. Adaptive immune responses are tuned to target-specific structures present on infected and transformed cells through numerous different variants of the T cell and B cell receptor [23]. Because of the need for activation and clonal expansion of specific immune cells, initial adaptive immune responses are slower [24]. However, upon re-stimulation with the same pathogen immune response will be swift as a result of adaptive memory functions [25-27].

2.2.1 NK cells

Natural killer (NK) cells are lymphoid effector cells of the innate immune system that recognize foreign and transformed cells [16, 28]. Although several investigators have claimed inventorship regarding the discovery of NK cells [29-31], Rolf Kiessling and co-workers at the Karolinska Institute were among the first to characterize these cells in 1975 [29, 30], showing that cells from mice spleen would kill leukemic cells without prior sensitization. Kiessling concluded one of the first articles

on the subject of NK cells by stating:

> *"It would thus seem fair to state that the present "spontaneous" anti-Moloney leukemia killer cell does represent a new, previously undefined cytotoxic cell. It now remains to establish what biological meaning and significance, if any, this cell type has."*

As it turned out, the killer cell did have a biological significance and even now, the full potential of the NK cell is not fully understood. Klas Kärre, then a student in Kiessling's laboratory, presented the first piece of the puzzle as the non-self hypothesis in his thesis in 1981 [32-34]. In his concluding remarks Kärre writes :

> *"…NK-mediated ("inquisitional") defence has the potential to bind and prosecute a variety of cells in the body, and will rapidly execute if the suspect fails to provide sufficient evidence for "non guilty" i.e. class I molecules of the self MHC."* [32]

This hypothesis still holds true and is, in brief, explained as a theory that NK cells will recognize absence of normal cell surface structures, i.e. non-self [34-36].

Morphologically, NK cells are large granular lymphocytes that express many lymphoid cells surface markers. NK cells are broadly defined as CD3- CD56+ lymphocytes and comprise around 5 – 15 % of peripheral blood mononuclear cells (PBMC) in healthy individuals [28, 37]. The classification as a cellular component of the innate immune system is based on the fact that NK cells lack recombination activating genes (RAG)-dependent antigen specific receptors [13, 16]. However, there are reports that NK cells share many traits of adaptive immune cells such as memory function [19, 22] and education followed by selection during development [38].

Activation

T, B and NK cells originate from a common lymphoid progenitor (CLP) cell (Figure 1) [39]. Similar to T cells, NK cells develop in the bone marrow but do not require migration to the thymus for maturation and education. Instead, these processes have been suggested to take place in secondary lymphoid tissues [28, 40]. Ligand interaction with killer cell immunoglobulin-like receptor (KIR) ligands is imperative for NK cell education [41, 42] and absence of correct KIR ligands renders NK cells hyporesponsive [43, 44]. Hyporesponsive or anergic NK cells are also

referred to as unlicensed. In contrast, engagement of several KIRs during education leads to increased responsiveness [42]. NK cells are divided in two subsets, the $CD56^{dim}$ cells, originally described primarily as a cytotoxic effector cell with potential to spontaneously lyse target cells, and the $CD56^{bright}$ cells, described as cytokine producing NK cells [45, 46]. However, the $CD56^{dim}$ cells also produce immunomodulating cytokines and chemokines, predominantly as a consequence of target cell recognition [47, 48].

After completed education and selection, $CD56^{dim}$ NK cell cytotoxicity is regulated by a system of activating and inhibitory signals. At steady state there is equilibrium between activating signals and inhibitory signals [49-52] and upon interaction with a potential target cell the balance between these signals will determine the fate of the target cell [49]. Activating signals are conveyed through an array of activating receptors, such as CD16, NKp30, NKp44, NKp46 and NKG2D among others. The most important family of inhibitory receptors is the KIR receptor family, recognizing HLA class I molecules. Transformed and infected cells modulate NK cell activity by expressing abnormal levels of ligands resulting in activation or inhibition of NK cell activity [16, 53-57].

Function

By the production and release of toxic substances, such as granzyme B and perforin, or ligation of death receptors, NK cells induce death in target cells [28, 58, 59]. Apart from being important cytotoxic cells of the innate immune system, NK cells play an important role as regulators of immune response [60, 61]. The immunoregulatory effects of NK cells are mediated through two main mechanisms, cytokine production and cytotoxicity. Cytokine production includes proinflammatory (IFN-γ, TNF-α) and immunosuppressive cytokines (IL-10), chemokines (IL-8, MIP-1α/β, RANTES), growth factors [granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF)] [12].

NK cell cytotoxicity has been ascribed the ability to shape immune responses in a variety of settings. Reported dampening of CD8 responses in LCMV-infected mice, possibly by perforin-dependent killing of cytotoxic $CD8^+$ T cells [62], selective killing of immature DCs (iDCs) via the NKp30 activating receptor [63] and terminating inflammatory reactions by triggering NKp46-dependent neutrophil apoptosis [64]. Interestingly, NK cell interactions with DCs may also result in reciprocal activation promoting DC antigen presentation and development of

adaptive T cell responses. Together, the different immune-modulating properties of NK cells are considered to constitute a bridge between innate and adaptive immunity [12, 28, 65].

2.2.2 T cells

Unlike NK cells, T cells belong to the adaptive immune system. They are produced in BM and subsequently relocalize to the thymus where education and selection ensues through a system of antigen and "self" presentation [66, 67]. T cells are divided into two main groups, T helper cells (Th) and cytotoxic T cells (CTLs). T helper cells are $CD4^+$ T cells with immune-modulating properties, shaping the immune response towards either a cellular (Th1), humoral (Th2) or Th17 response [68]. CTLs are cytotoxic $CD8^+$ T cells with ability to lyse target cells using mediators of cytotoxicity similar to those utilized by NK cells [69].

Regulatory T cells (T_{REG}) are $CD4^+$ inhibitory T cells defined by their high expression of high affinity IL-2 receptor (CD25) and transcription factor Foxp3. T_{REG}s suppress immune function by secretion of suppressive cytokines (IL-10 and TGF-β), consumption of IL-2 and expression of PD-L1 and CTLA-4 [70-72]. This cell type is highly dependent on IL-2, traditionally seen as an immunostimulatory cytokine, and will expand following IL-2 immunotherapy [73]. This fact was recently explored in patients with chronic graft-versus-host disease (GvHD), a T cell-dependent complication after hematopoietic stem cell transplantation. Administration of low-dose IL-2 reduced manifestations of GvHD, presumably by inducing expansion of T_{REGS} [74].

T cell receptor

The hallmark of the T cell is the RAG-dependent specific T cell receptor (TCR). The unique recombination feature of RAGs enables an immense number of distinct TCR specificities [23].

The T cell receptor (TCR) is a heterodimer consisting of one α-chain and one β-chain. Both chains contain a constant region and a variable region, similar to an immunoglobulin. The constant region, referred to as Cα and Cβ in α- and β-chains, respectively, is anchored to the plasma membrane while the variable region (Vα and Vβ) binds to the MHC and the antigen it presents [75].

T cell activation

MHC class I and II have similar function and present antigen to $CD8^+$ and $CD4^+$ T cells, respectively [76, 77]. MHC I is present on all nucleated cells,

displaying peptides from cytosolic and nuclear proteins that have been degraded and processed by proteasomes [77]. In this way, every MHC I-expressing cell presents "self" peptides on the cell surface that $CD8^+$ T cells monitor [76]. MHC II, on the other hand, displays peptides processed from endocytosed extracellular proteins to $CD4^+$ T cells. This process is carried out by antigen-presenting cells (APCs) such as monocyte/macrophages, B cells or dendritic cells, and the displayed proteins may originate from pathogens, toxins or foreign/transformed cells.

Binding of the TCR to a peptide, presented by MHC II on APCs, along with co-stimulatory signals, such as CD80/86 – CD28 signaling, lead to activation and expansion of T helper cells [76, 77]. MHC class I presentation of endocytosed peptides by APCs to CTLs in conjunction with co-stimulatory signals results in $CD8^+$ T cell activation [78], a process known as cross-presentation [79].

Function

The role of the $CD4^+$ T helper cell is distinct from that of the cytotoxic $CD8^+$ T cell. In brief, upon activation of $CD4^+$ T cells, the cytokine milieu regulates whether the response will be driven towards a cellular (Th_1), humoral (Th_2) or Th_{17} response [80]. The function of the T helper cell is to modulate and assist immune responses. The responsibility of activated $CD8^+$ T cells is to monitor the host and lyse cells that express antigens as determined by their TCR specificity. $CD8^+$ T cell-mediated cell lysis is, much like the NK cell, mediated through the release of perforin and granzyme B, Fas-ligand (FasL) interaction or TNF-related apoptosis inducing ligand (TRAIL) [58, 69, 81].

2.2.3 Monocytes

Monocytes are the largest leukocyte to circulate the body. They are of myeloid origin, up to roughly 20μm in diameter and comprising around 5-15% of circulating leukocytes. They have an agranular cytoplasm, kidney shaped nucleus and express myeloid surface antigens such as CD33 and CD14, among others.

Function

In circulation, monocytes are not terminally differentiated and will further differentiate into macrophages and subsets of dendritic cells upon entry into peripheral tissues [82-84]. In some tissues, macrophages are designated tissue-specific names such as Kuppfer cells

(liver), Langerhans cells (skin), microglia (CNS) or osteoclasts (bone), also known as histiocytes [11]. Monocytes and DCs originate from a common precursor, the macrophage and DC progenitor (MDP) (Figure 1).

Monocytes in circulation react to cell surface molecules on the capillary endothelium and migrate into surrounding tissues following inflammation or infection [83, 85]. In peripheral tissues, monocytes execute several tasks such as eradication of pathogens, phagocytosis and clearance of dead cells as well as regulating immune responses by secretion of anti- and pro-inflammatory compounds [83].

NADPH oxidase

A central component of the monocyte and macrophage killing mechanism is the formation and release of reactive oxygen species (ROS) in a process referred to as oxidative burst [86-88]. ROS are generated by the NADPH oxidase, a multi-unit enzyme consisting of the membrane-bound subunits gp91^{phox} and p22^{phox}, together with cytosolic p40^{phox}, p47^{phox} and p67^{phox} [89] (Figure 2). The oxidase is assembled to produce either intra- or extracellular oxygen radicals [86] and is triggered by bacterial or viral products [90-92]. Upon assembly, the NADPH oxidase generates superoxide anion ($O_2^{\bullet-}$) by transfer of electrons to 0_2 molecules[93], which is promptly converted to hydrogen peroxide (H_2O_2) and other oxidants [83, 88, 91].

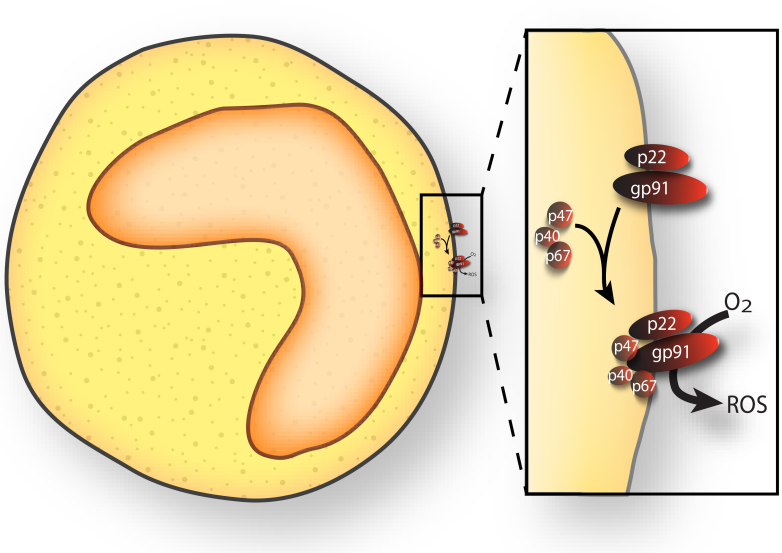

Figure 2. Membrane-bound NADPH oxidase on myeloid cell.

In addition to being pivotal mediators of bacterial killing, monocyte-derived ROS have potent immunosuppressive traits connected to lymphocyte dysfunction and cell death [94-103] as well as conditions of autoimmunity [104-107]. This will be further discussed under the section **2.5 Immunosuppression**.

2.2.4 Dendritic cells

Dendritic cells are antigen-presenting cells (APCs) of the innate immune system responsible for activating components of the immune system upon infection or malignancies [108]. In 1973, Ralph Steinman described the pivotal role of dendritic cells at a conference in Leiden [108, 109]. Steinman demonstrated that DCs were essential for CTL responses in mouse and human, establishing the DC as a fundamental component for mounting an adaptive immune response; an achievement for which he was later rewarded the Nobel price.

Function

APCs will endocytoze exogenous proteins and present peptides thereof on MHC class II molecules for recognition by $CD4^+$ T helper cells. Presentation of a peptide will lead to the proliferation and activation of antigen-specific T cells. Apart from DCs, B cells and macrophages also have capacity to present peptides on MHC II molecules [110, 111]. In addition, DCs have a unique ability to present exogenously derived peptides on MHC class I molecules to $CD8^+$ T cells by a mechanism known as cross-presentation [79]. Dendritic cells can thus present foreign peptides to both $CD4^+$ and $CD8^+$ T cells.

The principal function of DCs is to facilitate the initiation of T cell immunity. Not only do T cells need to recognize antigens that are present on a small fraction of cells located anywhere in the body, but they need to do so despite the fact that they normally circulate in peripheral blood whereas infection and malignant transformation often occur in peripheral tissues. In addition to these hurdles, MHC expression by tumors or infected cells is often scarce and co-stimulatory molecules are often down-regulated [112]. This is overcome by DCs that reside in most tissues and present captured proteins on MHC. In response to inflammatory signals and infectious agents. DCs upregulate co-stimulatory molecules and migrate to lymph nodes where they activate antigen-specific T cells. In contrast to other cells, DCs display high levels of MHC molecules on the cell surface. These traits combined allows for efficient antigen-specific T cell activation and expansion [112].

Monocyte-derived dendritic cells

For a long time, research on antigen presentation by DC was hampered by the lack of specific markers and the relative scarcity of DCs in peripheral blood. This can now be circumvented by differentiating DCs from monocyte progenitors using differentiating cytokines, primarily IL-4 and GM-CSF, occasionally alongside other stimulating agents [84, 113]. Monocyte-derived DCs (MoDCs) are potent antigen-presenting cells [84] and express cell surface markers shared by circulating dendritic cells [114].

2.3 Cell death and signaling

Programmed cell death and removal of unwanted cells is vital for the development and maintenance of tissues in the body. Numerous signaling pathways, either extrinsic or intrinsic, are involved in the events leading to cell death, each activated by distinct conditions but all eventually leading to controlled degradation of the cell without release of toxic intracellular elements. Depending on the signals triggering cell death and the pathways involved in executing it, different nomenclature is used, such as *parthanatos*, referring to PARP dependent cell death, or *extrinsic apoptosis by death receptors* which is dependent on death receptor interaction with FasL or TRAIL [115].

2.3.1 PARP-1

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear DNA binding protein involved in detection and repair of single-strand breaks (SSB) in DNA [116]. Upon detection of DNA nicks, PARP-1 attaches poly(ADP-ribose) (PAR) to nuclear proteins which will participate in DNA repair [117]. Activation of PARP-1 not only leads to DNA repair but can also result in cell death through the release of apoptosis inducing factor (AIF) from the mitochondria, which subsequently relocates to the nucleus [118]. This mode of cell death is known as parthanatos [115] and results in caspase-independent fragmentation of DNA with ensuing cell death [99, 118].

One example of parthanatos is cell death induced by monocyte-derived ROS in NK cells and T cells. Thus, upon interaction with lymphocytes, monocytes will induce cell death through an oxidative burst with ensuing activation of PARP-1, followed by loss of mitochondrial potential [99, 118, 119], translocation of AIF to the nucleus [118, 120] and ensuing DNA fragmentation and cell death [99, 121].

A recent study by Cohen-Armon et al suggests that there are alternative routes leading to PARP-1 activation. In a cell-free system, phosphorylated ERK (pERK) was shown to trigger PARP-1 activation, while PARP-1 activation was inhibited by nicked DNA [122]. This study thus suggests that PARP-1 activation may occur in the absence of massive DNA damage.

PARP inhibitors in clinical trials

PARP inhibitors are currently being explored as anti-cancer drugs [116]. One therapeutical approach is the administration of PARP inhibitors as chemosensitizers in order to increase DNA damage caused by chemotherapy or radiation treatment. This hypothesis is supported by in vivo experiments and clinical trials have shown promising results, as reviewed in [116].

The putative benefit of PARP inhibitors has been extensively explored in breast cancer susceptibility gene 1 (BRCA1) and BRCA2 mutated tumors. BRCA gene products are responsible for exact repairs of double stranded brakes (DSB). Having mutated BRCA genes results in increased risk for breast and ovarian cancers [123-125] as the result of incorrect repair of genomic DNA. The defective repair of double stranded breaks is mediated through an alternative DNA repair by non-homologous end-joining leading to surviving cells with damaged DNA [116]. When combining PARP inhibitors with DNA-damaging agents or radiation, malignant cells are flooded with DNA damage that cannot be repaired by PARP, leading to DSB upon DNA replication, which BRCA mutated cells are unable to repair. The DNA damage thereby becomes too extensive and the cell cannot survive. Inhibitors in BRCA deficient tumors have shown promising results in phase I and II clinical trials [116, 123, 124, 126].

The role for PARP inhibitors in myeloid leukemia is less clear than in BRCA mutant tumors although there are reports that PARP inhibition may be beneficial also in myeloid leukemia [127, 128]. PARP inhibitors have been shown to induce cell cycle arrest and apoptosis in AML cell lines. The reason for this is most likely the intrinsic genomic instability of AML cells that overpowers the cell when the PARP repair system is out of play [129].

2.3.2 MAPK

There are three major groups of mitogen-activated protein kinases (MAPKs); extracellular signal-regulated protein kinase (ERK), the p38

MAP kinases (p38) and c-Jun NH_2-terminal kinases (JNK) [130]. In addition, the ERK group can be divided into two groups, ERK1/2 and ERK5. MAPKs are involved in intracellular signal transduction and are activated through phosphorylation mediated by MAP kinase kinases (MKKs). This process of sequential phosphorylation is termed signaling cascade. The cascade leading to ERK-, p38- or JNK-phosphorylation can originate from a number of extra- and intracellular events. A simplified summary on MAPKs in immune cell signaling is presented in table 1 [131, 132]. MAPKs are reported to be involved in several types of cell death, both as mediators of apoptosis and preventers thereof [133].

Table 1. Overview of MAPK signaling upon extracellular stimuli.

Stimuli	Signaling cascade
Growth factors	ERK1/2, ERK5, p38, JNK
Mitogens	ERK1/2
Hormones	ERK1/2
Stress	ERK, ERK5, p38, JNK
Integrins	ERK1/2, JNK
Inflammatory cytokines	P38, JNK

2.3.3 Caspases

Many forms of cell death are mediated via activation of a group of cysteine proteases known as caspases [115]. These molecules constitute the core signaling machinery in several forms of programmed cell death but can also perform other regulating functions, not connected to apoptosis [134]. In signaling resulting in cell death, caspases are divided into (**i**) initiating caspases (caspase-2, -8, -9, -10), responsible for activation of (**ii**) executioner caspases (caspase-3, -6, -7), whom effectuate the actual cell death [135].

2.3.4 NK and T cell-initiated cell death

Upon recognition of a target cell, cytotoxic $CD8^+$ T cells and NK cells can utilize different means of target cell killing. Binding of death receptor ligands to the target cell will activate caspase 8 (or 10) through Fas-associated death domain protein (FADD) signaling. This will in turn lead to activation of the executioner caspase 3, resulting in cell death in a mitochondrial-independent manner. However, in some cell types caspase 8 mediates release of toxic substances from the mitochondrial intermembrane space which activates caspase 9 followed by caspase 3

leading to apoptosis [136, 137].
The other main mechanism of NK and T cell cytotoxicity is release of perforin and granzyme B. In essence, upon contact with the target cell perforin and granzyme B are released and perforin mediates the entry of granzyme B, which directly cleaves and activates caspase 8 and/or caspase 3 leading to apoptosis [137].

2.4 Immunity and cancer

The link between immunity and cancer is widely accepted, but the importance of the immune system in cancer surveillance is certainly under debate [138-142]. Findings nearly half a century ago that transplanted tumors were rejected even when transplanted between inbred mice [143] led to the hypothesis of immunosurveillance, put forth by both Burnet and Thomas in 1957 [144]. In 1964, Burnet expressed the theory as follows:

> *"In large, long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character."*

The hypothesis of immunosurveillance was questioned in the 1970s when immune-compromised mice, so called nude mice, failed to form more chemically induced tumors than immune competent mice, [145] nor did they develop more spontaneous tumors than wild type mice [146]. In Hanahans classic review defining the hallmarks of cancer [147] there is no mention of evading the immune system as a requisite for cancer development. When the truly immunodeficient perforin$^{-/-}$ mice strain became available, these were shown to develop more chemical induced tumors and spontaneous tumors [148-151]. These and other findings in experimental mice models demonstrated the existence of immunosurveillance *in vivo* [71, 139].

The effect of immunity on malignant cells is a dual sword. The malignant cells may be completely eradicated, but if the immune system fails to kill all cells, the selective pressure exerted by the immune system may contribute to the evolution of less immunogenic malignant cells, a process known as immunoediting. The model of immunoediting was

originally divided into three separate stages; **(i)** elimination, **(ii)** equilibrium and **(iii)** escape. However, these stages do not necessarily come in sequential order. After successful cancer treatment, patients can be considered to be in a state of equilibrium that is either tilted towards elimination, i.e. cure, or escape, i.e. relapse in disease. The efficacy of immunotherapeutical approaches indicate that is indeed takes place in humans.

(i) Elimination

NK cells and T cells harbor potential to eradicate transformed cells. As mentioned earlier, T cells eradicate cells that present abnormal peptides on MHC I whilst NK cells on the other hand, will recognize cells with down-modulated MHC I or up-regulated ligands for activating NK cell receptors. Together, these two effector cells can detect a wide range of cellular alterations that may arise in cancer cells, enabling cancer eradication before the development of overt cancer. In support of this are reports that immunosuppressed patients have higher incidence of many forms of cancer [152-154], representing a form of proof of principle. Of note is that this is true also for cancers not caused by transforming viral infections [139, 155]. As mentioned earlier, elimination not only occurs in newly developed cancers but also when immune cells and cancer cells are in equilibrium.

(ii) Equilibrium

When immune cells or cancer therapy fail to completely eradicate tumor cells a situation of equilibrium can arise, where immune cells keeps malignant expansion under control. An elegant proof of principle was presented in 2007 when Koebel *et al.* [156] injected mice with a chemical carcinogen and monitored the development of tumors. Mice that were tumor-free at day 200 were divided into two groups and injected with T cell, IFN-γ and/or IL-12 depleting antibodies or control IgG. Around 50% of immunodeficient mice developed tumors compared to less than 10% in control group, indicating that there was a dormant tumor controlled by the immune system. Interestingly, NK or NKT cell depletion did not affect tumor development [142, 156]. In line with these findings is a report showing that immunoediting of chemically induced sarcomas require expression tumor-specific antigens, pointing towards T cell-mediated immunoediting [157].

iii) Escape

If elimination of cancer cells fails and equilibrium is broken, transformed cells will expand, resulting in overt cancer. In the case of immune

evasion, the immune system has selected the least immunogenic and/or most aggressive clone through Darwinian selection [158, 159], potentially leading to an aggravated course of disease. Immune escape may be caused by either loss of immunogenicity, increased expansion through additional mutations or acquiring of immunosuppressive traits, as described below.

2.5 Immunosuppression

There are several mechanism of immunosuppression described in patients burdened by cancer [140, 160, 161]. These can be categorized into two main subgroups; effects that are indirectly caused by malignant cells, *i.e.* recruitment or expansion of suppressive cells that are not themselves malignant, and immunosuppressive effects that are directly caused by the *de facto* malignant cells [160].

One mechanism of immunosuppression in cancer is mediated by immature myeloid cells (IMCs) that have been activated to become myeloid-derived suppressor cells (MDSCs). These cells were first describe more than 20 years ago in tumor-bearing mice [162] and have since then been described in several types of cancers in both humans and mice [160, 163]. In healthy individuals IMCs constitute around 0.5% of circulating cells, develop in the bone marrow and readily differentiate into mature myeloid cells [164]. However, in many forms of cancer, MDSCs accumulate as a result of a block in myeloid differentiation and up-regulate immunosuppressive machineries such as arginase activity, nitric oxide synthase (NOS) activity and ROS production [165]. In a recent publication, MDSCs were found to expand and promote carcinogenesis in histidine decarboxylase deficient mice [166], pointing towards a role for histamine in myeloid maturation.

MDSCs are not a homogenous population but rather a combination of cells from different myeloid lineages, both monocytic and neutrophilic, along with precursors thereof [163, 164]. In humans, phenotypic definitions of MDSCs are not completely clear but are often defined as either $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$ or $CD11b^{+}CD14^{-}CD33^{+}$ [160].

Another mechanism of indirect immune suppression in cancer is the recruitment of macrophages to the tumor microenvironment. Tumor-associated macrophages (TAMs) are differentiated, mature macrophages [167] that promote cancer progression by aiding angiogenesis [168] and metastasis [169] and by inhibiting T cells and NK cells. TAMs inhibit

lymphocytes by secreting immunosuppressive molecules prostaglandin E2, transforming growth factor beta, nitric oxide (NO) and ROS [102, 170, 171].

As mentioned earlier, T_{REG}s have potent immunoregulatory effects, exerted via numerous mechanisms, among them interaction between CTLA-4 on T_{REG}s and CD80 and CD86 on DCs, which interferes with DC/T cell interaction. Lack of CTLA-4 on T_{REG} increases autoimmunity, as well as anti-tumor activity verifying their importance as regulators of immunity [140, 172]. This has recently been tested clinically with CTLA-4 blocking antibodies in metastatic melanoma, which improved event-free survival [173]. However, the CTLA-4 blocking antibody may also have exerted its effect on CTLA-4 expressing effector T cells.

Transformed cells can directly suppress immune cells in a variety of ways. Malignant cells may elude Fas-mediated cell death, also referred to as extrinsic apoptosis [115] by secreting soluble Fas (sFas), or counterattack cytotoxic T cells by up-regulating FasL to induce extrinsic apoptosis in CTLs [174, 175].

The other cytotoxic effector cell of the immune system, the NK cell, can be inhibited in a similar way. By secreting soluble MHC class I-related chain A (sMICA), tumor cells can trigger internalization of the MICA receptor NKG2D, leading to reduced NKG2D on the surface of NK cells and down-regulated NKG2D-mediated cytotoxicity [174, 176, 177].

Solid and hematological tumors can secrete numerous soluble factors that hinder efficacious immune responses. IL-10, IDO, Galectin-1 and TGF-β have all been reported to modulate immunity [71, 178, 179].

This thesis will focus on ROS-mediated immune suppression and the mechanisms mediating ROS-induced immunosuppression.

Reactive oxygen species and immunosuppression

As mentioned above, immune suppression by means of production and release of ROS is one of numerous immunosuppressive mechanisms in cancer. Through the NADPH oxidase, myeloid cells possess machinery to eliminate not only pathogens but also adjacent lymphocytes by producing ROS. *In vitro* interactions between healthy monocytes and lymphocytes lead to inactivation and subsequent apoptosis in NK cells and T cells [94, 99, 180]. Monocytes share this ability of lymphocyte inactivation with neutrophils [181, 182] and CML cells [183].

Using this mechanism, ROS producing myeloid cells function as a constitutive break or dampener of immune responses, designed to keep immunity in balance. This is an appealing theory as mice lacking NADPH oxidase are predisposed for autoimmune disease [104, 105, 184]. In humans, lack of functional NADPH oxidase is known to cause chronic granulomatous disease (CGD), which is associated with not only risk of severe infections but also non-infectious granulomas [86, 185, 186]. Together these findings point towards a fundamental role for ROS in immune regulation [106, 107].

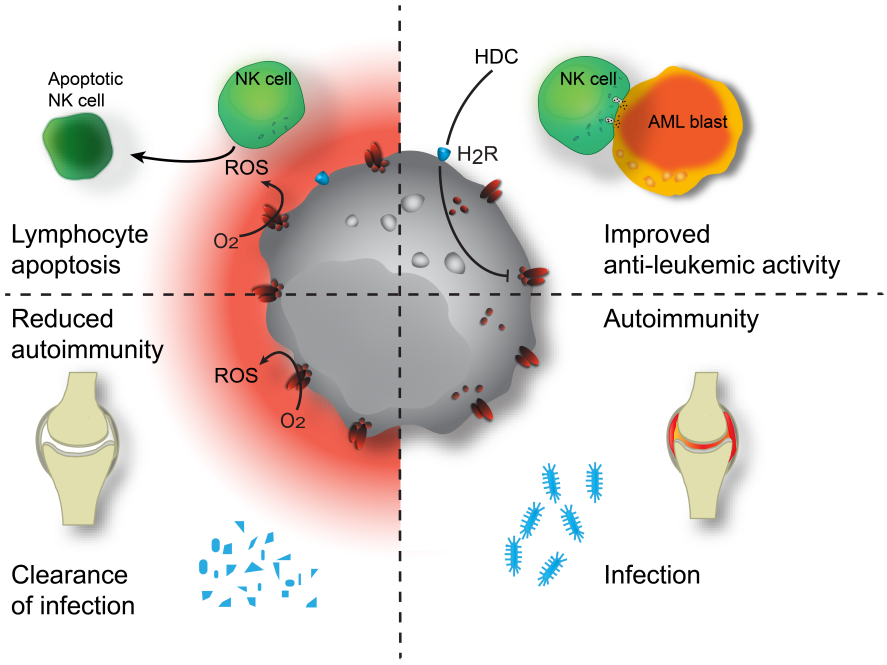

Figure 3. Illustration of ROS-mediated immunosuppression. A functional NADPH oxidase can cause cell death in lymphocytes, potentially detrimental in malignant conditions (top left quadrant), clearance of infection, vital for host defense against microbials and reduced autoimmunity (bottom left quadrant). Inhibiting ROS-production with HDC maintain lymphocyte function (top right quadrant). However, patients lacking functional NADPH oxidase have increased risk of autoimmunity and infection.

Similar machinery for immune suppression is found in both MDSCs and TAMs [102, 187] and ROS production by myeloid cells has been shown to influence the progression of several autoimmune diseases [188, 189].

Thiols and oxidative stress

There are a number of compounds on the cell surface with anti-oxidative effects; among them are glutathione, cysteine and thioredoxin. The common element for these compounds is the presence of high levels of sulfhydryl groups, also termed thiols, with capacity to neutralize ROS and thus protect cells against oxidants. In brief, the thiol/thioredoxin ROS-reducing apparatus works as follows: sulfhydryl groups (-SH), on molecules such as glutathione and cysteine, can neutralize toxic ROS by formation of disulfide bonds upon oxidation (R-S-S-R). The disulfide bonds are then reduced by thioredoxin or other enzymes, enabling thiols to resume ROS degradation. Thioredoxin is subsequent reduced by thioredoxin reductase [190].

An increased level of oxidative stress characterizes the inflammatory environment seen in infection and tumors [191]. To survive and function in this environment, effector lymphocytes need mechanisms to escape ROS-mediated inactivation. Thiols expressed by T cells promote T cell function such as proliferation and reactivity [192] and thiol expression by T cells may be induced by APCs [193-195]. APCs have been proposed to take up oxidized cystine, reduce them intracellularly to cysteine, and release cysteine to the microenvironment during antigen presentation [193]. T cell redox levels are critical for maintaining T cell function and maintaining immune homeostasis. This is exemplified by the fact that T cells from mice with reduced capacity to produce ROS, due to mutation of the NADPH oxidase, express increased levels of cell surface and intracellular thiols [192], show impaired clearing of autoreactive T cells and develop autoimmunity to a higher degree [106].

2.6 AML

The reported annual incidence of AML in Western Europe, USA and Australia ranges between 2.5 and 3.5 cases / 100.000 [4, 196-198]. AML is primarily a disease of the elderly – the median age at onset is approximately 72 years in Sweden and among adults the incidence in Sweden is reportedly close to 5 cases / 100,000 [199]. According to the Swedish AML registry, 981 cases of AML were reported from 2007 to 2009 [200].

AML is characterized by clonal expansion of malignant hematopoietic cells arising from a genetically altered leukemic stem cell (LSC) or progenitor cell [197, 201, 202]. These genetic aberrations lead to defective differentiation, resistance to apoptosis and/or excessive proliferation of leukemic cells [4]. Being a heterogeneous disease, AML can involve one or more myeloid differentiation paths and several stages of maturation resulting in many subtypes of disease; these are summarized in table 2. Development of leukemia is analogous to normal hematopoiesis and leukemic cells can often be described based on their corresponding cell type in normal hematopoiesis as depicted in Figure 1 [203].

2.6.1 Diagnosis and prognosis

Diagnosis of AML is most often based on cell counts and morphology of BM aspirates; however, diagnosis can also be established based on examination of peripheral blood (PB) [200]. Cell counts should include all nucleated cells (myeloblasts, monoblasts, promonocytes, promyelocytes, myelocytes, metamyelocytes, band neutrophils, segmented neutrophils, eosinophils, basophils, monocytes, lymphocytes, plasma cells, erythroid precursors and mast cells [198]. As a complement to cell counts, cytogenetics, fluorescent *in situ* hybridization (FISH) and mutational PCR analysis are used to verify diagnosis [198, 201, 204].

FAB classification

Up until approximately 2005, the French-American-British (FAB)-classification was the main basis for classifying AML. FAB-classes (M0-M7) are defined based on the maturation stage and differentiation path of the malignant clone [205] and do not take into account chromosomal or sub-chromosomal aberrations [204]. Thus, the FAB-classification is solely based on cell counts and morphological and phenotypical assessment of maturation and differentiation of the leukemic cells. The AML FAB-classes are summarized in Table 2.

WHO classification

The third edition of the WHO classification of tumors of hematopoietic and lymphoid tissues was published in 2008 [198]. In this WHO classification account has been taken to different genetic mutations known to cause or drive AML [204, 206]. Some of these mutations have been classified as separate entities leading to a more complex but also more informative grouping compared to the FAB classification. The

acute myeloid leukemias, as defined by the WHO classification, are outlined in Table 2.

For the diagnosis of AML, the WHO classification requires that 20% or more BM or PB cells are myeloblasts, and/or monoblasts/promonocytes and/or megakaryoblasts [197]. This was adjusted from 30%, which was the blast count required according to the FAB classification [204, 205]. In the WHO classification, the requisite for 20% blasts is abolished for AML with the presence of as translocation (8;21)(q22;q22), inversion (16)(p13q22), t(16;16)(p13;q22) and t(15;17) (q22;q12). These entities are always to be considered and treated as AML, regardless of blast count [197, 204].

Table 2. Different subtypes of acute myeloid leukemia according to the FAB and WHO classifications.

FAB-classification	WHO classification
AML with minimal differentiation (M0)	AML with t(8;21)
AML without maturation (M1)	AML with inv(16) or t(16;16)
AML with maturation (M2)	Acute promyelocytic leukemia (APL) with (15;17)
Acute promyelocytic leukemia (APL) (M3)	AML with t(9;11)
Acute myelomonocytic leukemia (M4)	AML with (6;9)
Acute monocytic/monoblastic leukemia (M5)	AML with inv(3) or t(3;3)
	AML with mutated NPM1
Acute erythroid leukemia (M6)	AML with mutated CEBPA
Acute megakaryoblastic leukemia (M7)	AML, not otherwise specified. Patients categorized in this group will be further classified according to FAB

Phenotype and properties

Both FAB and WHO classifications are to a large extent based on the maturation of the malignant clone. This also implies that the malignant cells have different phenotypical and functional properties. In this thesis focus is on functional traits of leukemic cells rather than molecular markers and therefore the FAB-classification will be used when describing AML samples. In brief, AML subclasses can be defined phenotypically as follows [198, 205]:

FAB-M0 Blasts are small and minimally differentiated, frequently express early myeloid markers such as CD34, CD38 and HLA-DR. Markers associated with neutrophilic or monocytic differentiation (CD14, CD15, CD64) are missing.

FAB-M1 Blast population is characterized as immature and blasts constitute more than 90% of non-erythroid cells. Some morphological maturation towards both monocytes and neutrophils can be seen. Approximately 70% of cases are $CD34^+$ whilst there are no markers of maturation such as CD14 or CD15 on blasts.

FAB-M2 Defined as presence of more than 20% blasts as well as >10% maturing cells of neutrophil lineage (promyelocytes, myelocytes and mature neutrophils). Monocytic cells are below 20% of non-erythroid cells. Blast normally express CD33 and often CD34. Monocytic markers are routinely missing. t(8;21) accumulate in FAB-M2 AML [197].

FAB-M3 Also known as acute promyelocytic leukemia (APL), this is a distinct entity that is successfully treated with all-*trans* retinoic acid (ATRA) [207]. t(15;17) is required for APL diagnosis. Because of the specific treatment of APL, it will not be discussed further in this thesis.

FAB-M4 Acute myelomonocytic leukemia is distinguished by maturation along both neutrophilic and monocytic lineages. A least 20% of cells should be monocytes and their precursors and neutrophils and their precursors should constitute more than 20%. Most cases comprise an immature blast population expressing CD34. Inv(16) and t(16;16) are most often classified as FAB-M4 AML [197].

FAB-M5 Acute monoblastic and monocytic leukemia are leukemias with monocytic differentiation. In monoblastic leukemia the majority of the leukemic clone are monoblasts and in monocytic leukemia, the majority are promonocytes. Cells frequently express myeloid and monocytic markers such as CD33 and CD14.

FAB-M6 Acute erythroid leukemia comprises, in most cases, both erythroid and myeloid precursors. Because of more than 50% erythroid cells in this subtype of AML, it will not be further discussed in this thesis.

FAB-M7 Acute megakaryoblastic leukemia originates in a megakaryocyte progenitor. Megakaryocytes are cells responsible for platelet production and since their differentiation diverges from other myeloid lineages at an early stage it will not be discussed further.

Survival

For younger patients (<60 years), treatment and overall survival of AML patients has improved significantly over the last decades [2, 4]. However, the overall prognosis is still poor with a 5-year survival of around 20% [1, 4]. A comparison between 5-year overall survival between 1984 and 2004 in the US show that among younger patients (15-34 years old) 5-year survival increased from 17.4% to 52.3%, however, this positive trend is to a large extent limited to the youngest patients [1, 4]. In patients between 55-64 years of age survival increased from 6.7% to 19.9% [1]. For older patients, no improvement of survival was observed. Of the 981 patients diagnosed with AML in Sweden between 2007 and 2009 2-year survival was 26%. In patients <60 years of age 2-year overall survival was 52% [200].

AML is categorized as favorable, intermediate and unfavorable risk based on cytogenetic traits of the malignant clone [4, 208]. Worth mentioning is that inv(16), t(16;16) and t(8;21), also denominated core-binding factor (CBF) leukemias, are associated with a relatively favorable prognosis [209] whilst normal karyotype is associated with intermediate risk, together with certain genetic mutations e.g. t(9;11), del(5q), -Y. Included in unfavorable cytogenetics are inv(3) or t(3;3), t(6;9), -7 [208]. Together with age, cytogenetics is the most important prognostic factor. Five-year cumulative incidence of relapse in CR for the three risk groups are 51%, 67% and 92% respectively [208], demonstrating the need for more efficacious relapse-preventing strategies.

On the molecular level, mutations can be of vital importance. The CBF-leukemias [209] have an increased relapse risk if combined with *KIT* mutations. In patients with normal karyotype, *FLT3-ITD* mutations have unfavorable impact on prognosis whilst *CEBPA* and *NPM1* (if not

accompanied by *FLT3-ITD* mutation) entail favorable outcome [2]. These examples serve to show the complexity of AML diagnostic and prognostic factors.

Immunity and AML

AML is a disease in which host immunity is considered to play an important role. The capacity of immune cells to target AML cells is most evident in allogeneic stem cell transplantation (allo-SCT) where it has been shown that depletion of T cells from the graft, or using twin grafts, significantly decreases the efficacy of allo-SCT by increasing risk of relapse [210, 211]. Also, by donor lymphocyte infusions (DLIs) after allo-SCT relapse, an anti-leukemic effect can be achieved with complete remissions in 29% of cases [5]. The graft versus leukemia (GvL) effect of DLI is, for unknown reasons, more pronounced in other leukemias, such as chronic myeloid leukemia (CML) where 76% of patients receiving DLIs achieve CR [5].

More indirect evidence of immune participation in AML progression is provided by studies of functionality of immune cells in AML. AML cells can regulate and evade the immune system, and thus avoid destruction, in several ways. By decreased expression of MHC molecules as well as decreased expression of co-stimulatory molecules, AML cells circumvent specific cytotoxicity exerted by T cells [5, 212]. Secretion of immunosuppressive cytokines and induction of CD3ζ down-regulation also result in impaired T cell responses [178]. Interestingly, CD3ζ is also down-regulated by exogenous ROS [102] and by macrophage-induced oxidative stress [103]. Deficiencies in NK cell function are also common in AML where natural cytotoxicity receptors (NCRs) are frequently down-regulated, correlating with poor survival [212, 213].

In a study by Gale and Opelz [154], the risk of developing AML was investigated in immunosuppressed patients by analyzing data from kidney transplant recipients (n=217,219) and heart transplant recipients (n=31,005). Transplant recipients are treated with immunosuppressive drugs to avoid graft rejection, resulting in compromised immunity for long periods of time. The analysis revealed that immunosuppressed patients indeed showed an increased risk of developing AML. These data favor the hypothesis that there is ongoing immunosurveillance in humans and also confirms an immune component in AML eradication, indicating once again that immunotherapy may be a feasible approach in AML therapy.

2.6.2 Induction and consolidation treatment

The first objective in the treatment of AML is complete remission, which is attained in 60-80% of patients receiving one or two full-dose induction chemotherapy courses. [201, 214, 215]. In Sweden, the recommended induction regimen consists of 3 days daunorubicin alongside 5 days of cytarabine. Daunorubicin is an anthracycline inhibiting DNA and RNA synthesis whereas cytarabine (Ara-C) causes cell cycle arrest in S-phase by being incorporated in DNA during replication, leading to defective DNA and subsequent killing of the leukemic cell [216]. Induction treatment causes grave cytopenia and is associated with 5-15% mortality mainly due to infections and organ toxicity and multiple erythrocyte and platelet transfusions and anti-microbial treatment are needed [197].

The major challenge in AML treatment is to prevent relapse in patients in CR by eliminating residual leukemic cells. This can be accomplished by the administration of additional 1-3 chemotherapy consolidation courses, which are essential for curing AML [4, 217]. Typically, consolidation courses include daunorubicin and cytarabine at similar or higher doses than in the induction regimen.

2.6.3 Post-consolidation treatment

Induction treatment will in general yield good results, with approximately 80% of younger patients (<60 years) achieving CR [2]. However, a majority of patients will relapse after the consolidation therapy, and the 1-year survival after first relapse is approximately 30% [218]. Therefore, after one or two consolidation courses, allo-SCT is considered in patients with intermediate or high-risk disease.

Stem cell transplantation

Allogeneic stem cell transplantation was originally employed as a mean to replenish patients with stem cells in order to enable higher doses of chemotherapy [219]. It soon became evident that immunocompetent cells in the grafts also exercised an anti-leukemic effect independent of the chemotherapy. This activity is known as the graft-versus-leukemia (GvL) effect [219, 220]. As mentioned, T cells are mainly responsible for the GvL effect, as shown by the fact that depletion of T cells from grafts result in higher relapse rates [210, 219].

The most common adverse effects of allo-SCT are risk of infections and severe graft versus host disease (GvHD). The most important risk factor

for GvHD is HLA-mismatch between patient and donor. Severe acute GvHD carries high mortality, whereas chronic GvHD is associated with risk of opportunistic infections caused by virus or fungi. In addition, chronic GvHD results in symptoms resembling autoimmune diseases such as keratoconjunctivitis sicca, or sclerodermia causing reduced quality of life. Notably, mild chronic GvHD is associated with a reduced risk of relapse, thus demonstrating a significant GvL-effect. [219].

In recent years, more attention has been given to NK cells in the transplantation setting. The use of T cell-depleted grafts from haploidentical donors, i.e. donors with one identical HLA-haplotype and one fully mismatched, has been reported to yield favorable results without severe GvHD, given that there is KIR/HLA mismatch between graft and host [221-224].

In Sweden, between 2007 and 2009, around 30% of patients in CR1 below 65 years of age were allografted [200], indicating that for remaining patients no further treatment options are available after consolidation chemotherapy, and a majority of these patients will relapse. This has called for development of new relapse-preventive strategies.

Remission maintenance by immunotherapy

Immunotherapies aiming at remission maintenance aim to mimic the anti-leukemic effect of allo-SCT by activating T and NK cells to become reactive against residual malignant cells. The immunostimulatory cytokine interleukin-2 (IL-2) has been deployed in several phase III trials as a mean to activate cytotoxic lymphocytes. Results are, however, disappointing [225-230]. Other immunotherapeutic approaches such as peptide vaccinations [231-233], whole cell vaccines [234] and dendritic cell based vaccines [235] have shown encouraging results but are still under development and evaluation.

In 2010, Rubnitz *et al*. [236] treated pediatric AML patients in remission with haploidentical NK cell infusions without concomitant transplantation. All patients remained in CR but the effect of the NK cell infusion is unclear. Similar treatment has also been tried in elderly patients with promising results [237]. These regimens were well tolerated and show that cellular therapy utilizing alloreactive NK cells may be utilized in post-consolidation immunotherapy.

HDC/IL-2 immunotherapy

In 2006 Brune *et al.* showed that combination therapy of histamine dihydrochloride (HDC) and IL-2 prevents relapse in AML patients in CR [8]. The aim of adding HDC to IL-2 was prevent ROS-dependent, myeloid cell-induced immunosuppression [90, 98, 180, 181, 238]. HDC suppresses or inhibits ROS production by normal myeloid cells, acting via the histamine H_2-receptor. The use of HDC was further bolstered by the findings that T cells and NK cells frequently are in a persistent state of suppression and inactivation in AML [212, 213, 239], and that similar inactivation is observed *in vitro* when exposing lymphocyte to oxidative stress. Romero *et al*. [181] showed that NK cells, when exposed to ROS secreting monocytes, down-regulated the activating NK cell receptors NKp46 and NKG2D, which was reversed by the addition of HDC to the culture. Activating receptors on NK cells are known to play a role in leukemia and impacts prognosis [212, 213] and in-vitro killing of malignant cells [12, 16, 50].

HDC/IL-2 regimens have been deployed in a number of different disorders in which suppression through myeloid cells is thought or known to be of importance [240]. In 2002 and 2005, HDC/IL-2 were evaluated as immunotherapy for melanoma patients [241-243]. These trials showed promising results, demonstrating that HDC/IL-2 promotes immune responses more efficient than IL-2 alone. In renal cell carcinoma HDC/IL-2 administration has been shown to increase numbers of intratumoral lymphocytes whilst intratumoral myeloid cells remained constant. In contrast, IL-2 alone increased circulating macrophages [244].

Reviews by Romero *et al*. [245], Martner *et al*. [229] and commentary by Thorén *et al*. [240] summarizes preclinical and clinical data supporting the use of HDC/IL2 in AML.

Phase III trial of HDC/IL-2

Between 1998 and 2000, an international randomized phase III trial was performed, enrolling a total of 320 adult patients with acute myeloid leukemia in complete remission [8]. Patients were randomized after the completion of all chemotherapy into a group of HDC/IL-2 for 18 months or a control group receiving standard-of-care, i.e. no treatment. In line with previous phase I/II trials [246, 247] HDC/IL-2 was well tolerated and no severe adverse events were reported [8, 248]. Primary efficacy of treatment was measured as duration of LFS. Secondary end-points included LFS in first CR (CR1) as well as subsequent CR (CR>1) and

overall survival (OS). The trial showed a marked increase in leukemia free survival in the treated group, an effect that was even more pronounced in the CR1 group [8]. No significant efficacy was observed in elderly patients (>60 years old) or in patients who were in second or subsequent CR at inclusion.

This trial together with preclinical studies led to the approval of HDC/IL-2 immunotherapy as remission maintenance treatment for patients in CR1 under the age of 60 in Europe and Israel.

3 PATIENTS AND METHODS

The patients and methods used in **papers I-V** are described in detail in the corresponding articles and only when there is need for special consideration will they be discussed in this section.

3.1 Patients

Phase III trial

320 patients were recruited from 100 centers in 10 countries after completion of induction and consolidation treatment and randomized into either treatment (HDC/IL-2) or standard-of-care. Patients were instructed on how to administer HDC 0.5mg, subcutaneous (s.c), twice daily) and IL-2 (16.400 U/kg, s.c twice daily) in their homes. Treatment comprised 10 consecutive 3-week cycles with 3 or 6 weeks off-treatment periods in-between over 18 months. Patients were followed for at least 36 months; patients lost to follow up were censored at last known date. FAB-classification was carried out at diagnosis at participating centers [8].

The *post hoc* analyses presented in **paper III** included 145 patients in CR1 under the age of 60 and diagnosed with FAB-M0, M1, M2, M4, M5, M6 or M7. AML-M3 (APL) patients were excluded based on the fact that they were initially not intended for enrollment and their treatment is fundamentally different.

Newly diagnosed patients

In **paper II** and **III**, bone marrow or peripheral blood specimens from 26 newly diagnosed, untreated patients were obtained from Sahlgrenska University hospital or Lund University hospital. Nine fresh samples and 17 frozen samples were analyzed. Patients were categorized based on FAB classification (6 FAB-M1, 8 FAB-M2 and 12 FAB-M4/M5). Patient characteristics are outlined in table 3.

Pat	FAB	Age	Sex	Sample	Cytogenetics	Molecular genetics
1	M1	77	M	PBMC	C.K.	N.A
2	M2	62	F	PBMC	N.K.	FLT3-ITD$^+$ NPM1$^+$
3	M2	42	F	PBMC	N.K.	FLT3-ITD$^+$ NPM1$^+$
4	M2	53	M	PBMC	N.K.	N.D.
5	M4	61	M	PBMC	Inv (16)	N.D.
6	M4	23	M	PBMC	t(16;16), -21q	N.D.
7	M4	77	M	PBMC	N.K.	FLT3-ITD$^+$
8	M4	60	F	PBMC	N.K.	NPM1$^+$
9	M4	48	F	PBMC	N.K.	NPM1$^+$
10	M5	67	F	PBMC	46,XX,t(9;11) (p21;q23)	N.D.
11	M1	67	M	BM	N.K.	N.A.
12	M1	21	F	BM	46,XX,i(17q)	N.A.
13	M1	63	F	BM	C.K.	N.A.
14	M1	69	F	BM	C.K.	N.A.
15	M1	63	F	BM	N.K.	N.A.
16	M2	65	M	BM	N.K	N.A.
17	M2	66	M	BM	N.A.	N.A.
18	M2	65	M	BM	N.K.	CEBPA$^+$
19	M2	39	M	BM	46,XX del7, t(8;21)	N.D.
20	M2	75	M	BM	N.K.	N.D.
21	M4	73	M	BM	C.K.	N.A.
22	M4	64	M	BM	N.K.	N.A.
23	M4	69	M	BM	N.K.	N.A.
24	M4	77	F	BM		N.D.
25	M4	77	F	BM	N.K.	FLT3-ITD$^+$ NPM1$^+$
26	M5b	34	F	BM	N.K.	N.A.

Table 3. Patient characteristics of enrolled, newly diagnosed patients.

N.A. -Not Available; **C.K.** -Complex Karyotype; **N.K.** -Normal Karyotype; **N.D.** -None Detected.

3.2 Methods.

Superantigen-based assay of T cell activation

Superantigens are compounds causing oligoclonal activation of T cells. Staphylococcal enterotoxin A (SEA) and SEB are *staphylococcus aureus*-derived superantigens that stimulate T cells by binding laterally to MHC II and TCR, creating a pseudospecific DC/T cell interaction [249]. In this way T cells and DCs are brought together, enabling signaling through the TCR as well as co-stimulatory molecules. SEA and SEB are not unspecific as these compounds only bind to certain Vβ-regions on the TCR; thus SEA stimulates T cells carrying Vβ16 and Vβ22 and SEB stimulates Vβ17 cells, among others. Instead of using normal antigens for T cell stimulation, which would stimulate <1% of T cells, stimulation with SEA or SEA can stimulate up to 20% of T cells. The binding of staphylococcal superantigens to MHC/TCR is schematically shown in Figure 4.

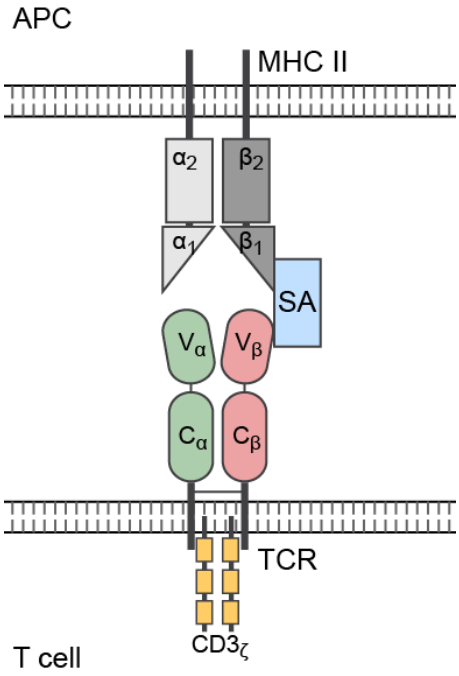

Figure 4. Schematic illustration of MHC II/TCR interaction mediated by lateral binding of superantigen (SA)

Flow cytometry

Flow cytometry analysis is a powerful tool used in a vast number of different experimental settings. This method is especially useful in the field of immunology because of the single cell state of immune cells. A flow cytometry analysis can generate large amount of data and enables analysis of several (exceeding 20) parameters simultaneously [250]. However, there is call for precaution when evaluating data generated by flow cytometry. The bottom line is that data generated by flow cytometry are dependent on the researcher's interpretation of the sample. What you see is based not only on the actual properties of the sample but also on the staining of the sample, the daily state of the flow cytometer and the gating that determines what is shown in the plots. This calls for careful analysis and proper setup in order to avoid errors and false data. There are no definite rights but there are many pitfalls on handling data, and the need for normalization etc. should be considered carefully in each study.

Flow cytometry-based techniques

Phenotyping by flow cytometry is standard procedure in many laboratories and most researchers can readily interpret information presented as dot plots. This thesis presents phenotypical data comprising, at most, seven fluorochromes and two light-scatter properties analyzed simultaneously (**paper II** and **III**). An important factor in maintaining the quality of such experiments is to properly compensate fluorochromes [251].

Cell death can be measured in several ways. In the publications included in this thesis two methods have been used; an amino-reactive dye, referred to as ViViD [252] and the DNA stain To-Pro. ViVid binds to amines, both extracellularly and intracellularly, on live and dead cells. However, dead cells with compromised cell membranes will stain more intensely due to the abundance of amines in the cytoplasm as compared to amines bound extracellularly. To-Pro binds to DNA and will mainly stain cells that have compromised cell membranes, allowing the dye to enter the nucleus.

Cell sorting using a fluorescence-activated cell sorter (FACS) is a convenient and efficient way to acquire highly purified populations based on phenotypical properties. In general, FACS sorting is not based on negative selection, which is a potential weakness compared to magnet-based negative selection.

Co-culture experiments

In this thesis, results based on co-culture experiments are presented in all five papers. Co-culture experiments can sometimes be judged as un-physiologic and thereby questioned. Objections are often based on the notion that the conditions observed in the laboratory would never or rarely occur in the body. This is most likely true but the argument is still flawed. *In vitro* experiments only serves to create conditions under which certain processes can be observed; the main conclusions are seldom the conditions themselves. One example of this is DC/T cell co-culture from **paper I**. In these experiments we used DC : T cell ratio 1:3, not because this is a ratio we believe is most comparable to *in vivo* scenarios but because under these conditions thiol-upregulation can be analyzed *in vitro*.

Freezing and thawing of samples

AML patients commence treatment with shortest possible delay meaning that predictability of sample arrival was sometimes short, leading to the need to freeze samples. Freezing of samples is precarious as functional traits such as ROS production capacities are sometimes affected.

In **papers I, II, and III** frozen cells are used. By using thawing protocols as described in **paper II** high viability and function of leukocytes is achieved. However, some traits can be altered upon freezing. Bone marrows and peripheral blood that were frozen at other sites had higher median fluorescence intensity (MFI) with regard to $gp91^{phox}$ than similar samples frozen on site. Samples frozen on site displayed no differences in MFI when comparing before and after freezing nor between BM and PBMC. Normalizing MFI values against an internal lymphocyte population, known to be $gp91^{phox}$ negative, circumvented this issue.

4 RESULTS

Paper I

T cells are pivotal players in the defense against malignant cells, both solid and hematopoietic [172]. Alleviating T cell suppression in cancer has been exploited therapeutically as exemplified by the recently developed treatment of metastatic melanoma with anti-CTLA4 antibodies aiming to alleviate T cell inactivation induced by T_{REG}s [173]. T cells appear, however, to be exposed to myeloid cell-derived ROS in the malignant microenvironment [102, 103], and understanding the mechanisms by which T cells avoid ROS-induced inhibition may help to design more efficacious immunotherapeutic regimens,

In **paper I** we explored the ability of DCs not only to activate T cells through antigen presentation and co-stimulation but also their ability to equip T cells with anti-oxidative structures required to maintain their functionality and viability in an inflammatory microenvironment with oxidative stress. Previous work from our group showed that DCs protect NK cells and T cells from ROS-induced cell death by inducing and/or transferring cell surface thiols to lymphocytes and thereby enabling them to neutralize oxygen radicals [194]. In **paper I**, we expand these findings using a superantigen based assay, as explained in **3.2 Methods**, and demonstrate that DCs preferentially convey protection to T cells through a specific interaction that results in expression of anti-oxidative thiols by antigen-specific T cells and an increased resistance to oxidative stress.

In brief, monocyte-derived DCs, pulsed for 2h with SEA or SEB, were cocultured overnight with $CD3^+$ T cells with ensuing measurement of cell surface and intracellular thiols on T cell subsets. This analysis revealed that superantigen-pulsed DCs only induced thiols on T cells with V_β chain-specificity corresponding to the superantigen presented by DC (Figure 5). This effect was not seen when the superantigen was presented to T cells by MHC II on B cells, even though both DCs and B cells induced similar levels of CD69 expression on antigen-specific T cells. By FACS sorting the antigen-specific thiol-positive T cells, using the surrogate marker CD69, we found that T cells with high expression of cell surface thiols after DC interaction showed an elevated capacity to neutralize ROS and to survive in the presence of toxic levels of exogenously added H_2O_2 (Figure 5).

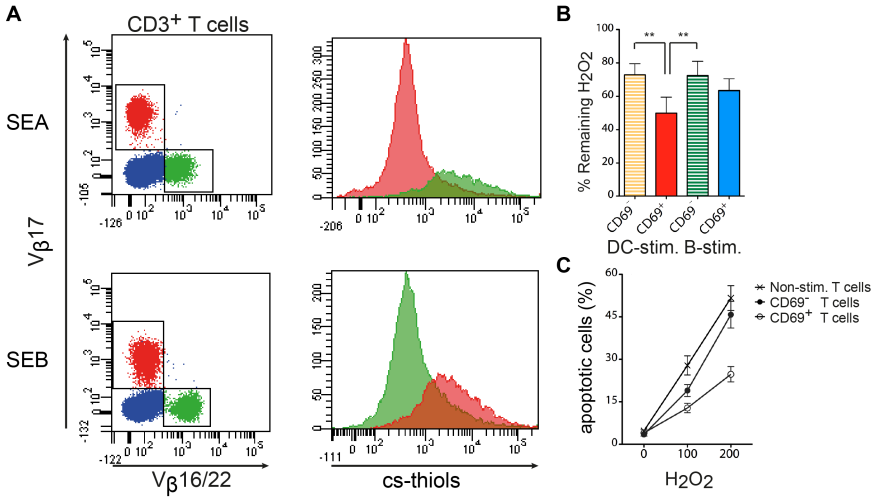

Figure 5. (A) DCs pulsed with SEA and SEB induce upregulation of cell surface (cs) thiols on $V_\beta16/22$ and $V_\beta17$, respectively. (B) $CD69^+$ T cells, i.e. with upregulated thiols, have superior H_2O_2 neutralizing capacities as measured by PHPA-oxidation assay. (C) T cells with increased cs-thiols show increased resistance to H_2O_2-induced apoptosis.

Papers II and III

In **paper II** we sought to investigate the ability of AML cells to produce immunosuppressive ROS. We first observed that BM cells from monocytic AML, i.e. FAB-M4 and FAB-M5, possessed the ability to produce high levels of oxygen radicals. Detailed phenotypical analysis of blood and BM samples disclosed that only a certain subtype of AML cells expressed the NADPH oxidase, as measured by the membrane-bound subunit gp91^{phox}. NADPH oxidase positive malignant cells were of mature CD33^{+}CD14^{+} monocytic phenotype and only seen in FAB-M4 and FAB-M5 leukemia. FACS sorting of these mature AML cells revealed that they were indeed responsible for ROS production whilst immature cells, which are phenotypically more immature, did not produce ROS. In line with these findings, only mature FAB-M4 and FAB-M5 AML cells were able to induce PARP-1-dependent parthanatos in T cells and NK cells (Figure 6). Of note is that out of six sorted mature populations from AML-M4 and AML-M5, five were confirmed malignant by either FISH or mutational PCR analysis. The finding that monocytic AML cells expressed gp91^{phox} was further supported by microarray data from 207 patients at diagnosis deposited at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), accession number GSE1159. [253] (Figure 6). To our knowledge this is the first report of direct ROS-dependent immunosuppression exerted by malignant AML cells.

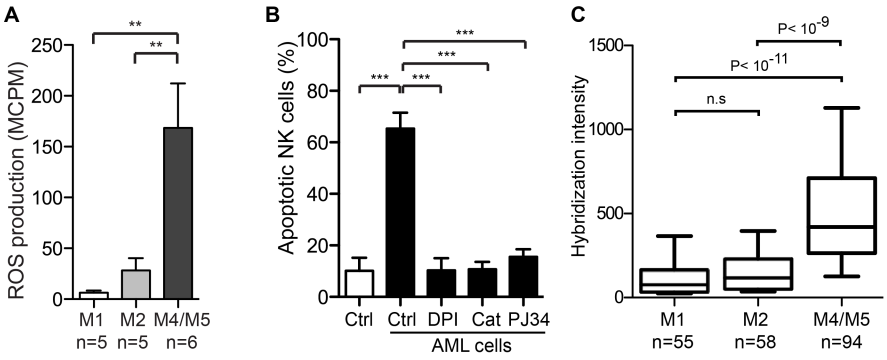

Figure 6. (A) ROS production in unsorted BM cells upon stimulation with PMA. The ability to produce ROS is restricted to the gp91^{phox} expressing subsets FAB-M4 and FAB-M5. (B) Mature leukemic cells induce NK cell apoptosis. Apoptosis is abolished by the presence of NADPH oxidase inhibitor DPI, ROS scavenger catalase and PARP-1 inhibitor PJ34. Panle C shows gp91^{phox} mRNA levels in untreated AML patients.

These findings enticed us to re-analyze the results of HDC/IL-2 phase III trial from 2006 [8]. Since the proposed mechanism of HDC is to reduce the formation of NADPH oxidase-derived ROS and thus preserve anti-leukemic immunity, we asked whether the clinical benefit of treatment may differ in subtypes of AML that express or do not express the NADPH oxidase. In the original article the FAB class sub-analysis comprised favorable and unfavorable FAB-classes, i.e. FAB-M2/M3/M4 (favorable) against FAB-M0/M1/M5/M6 (unfavorable). As reported in the original publication, the efficacy of treatment did not differ between these patient groups. When performing *post hoc* analysis of 145 patients under the age of 60 in first remission, i.e. patients for whom treatment is currently approved, we found that HDC/IL-2 immunotherapy showed preferential effect in monocytic AML. HDC/IL-2 was thus highly efficacious in AML-M4 and AML-M5 (3 year LFS 62.5%) but completely inefficient in AML-M2 (3 year LFS 35.7%) (Table 4 and Figure 7) An additional analysis of non-M2 AML showed a clear benefit of treatment within this subgroup (Table 4).

Table 4. Treatment benefit in non-M2 AML

FAB	LFS 3 years Ctrl	LFS 3 years HDC/IL-2	Hazard ratio	95% CI	*P*-value (Log-Rank)
CR1, <60 years					
M0/M1, n=41	11.1%	39.1%	0.51	0.24 - 1.05	n.s. (.16)
M2, n=41	39.7%	35.7%	1.14	0.49 - 2.63	n.s. (.65)
M4/M5, n=58	26.9%	62.5%	0.37	0.18 - 0.75	* (0.017)
Non-M2, n=104 (M0,1,4,5,6,7)	21.7%	52.5%	0.43	0.26 - 0.71	** (0.0089)

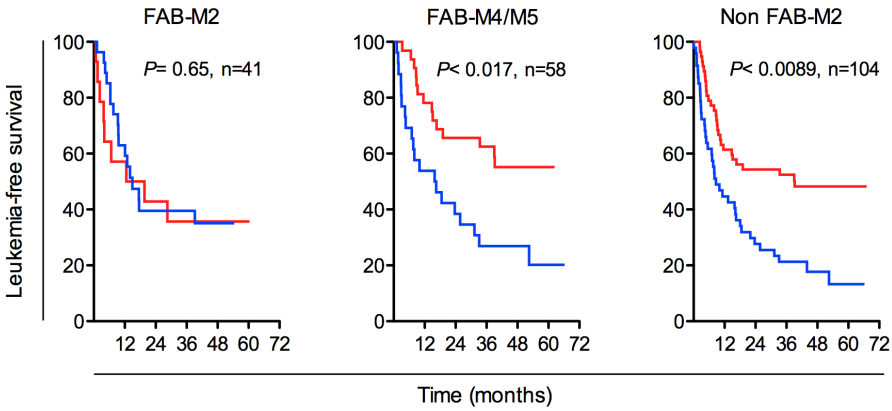

Figure 7. Kaplan-Meier plots of LFS for patients in CR1 (<60 years old) receiving post-consolidation immunotherapy with HDC/IL-2 (red line) or standard-of-care (no treatment, blue line). FAB classification was performed at each participating center at diagnosis, and the FAB classes were grouped as AML with maturation (FAB-M2) and monocytic forms of AML (FAB-M4 and FAB-M5). Non-M2 AML refers to all patients classified as FAB classes M0, M1, M4, M5, M6, or M7. All patients were followed for LFS for at least 3 years (median follow-up 47 months) and all results were analyzed according to ITT.

In parallel we showed that the mature $CD33^{+}CD14^{+}$ monocytic AML cells from AML-M4 and AML-M5 patients expressed functional histamine H_2 receptors that inhibited ROS formation and release when activated by HDC. In contrast, histamine H_2 receptors were rarely seen on AML-M1 and AML-M2 cells (Figure 8).

In summary, these results imply that in monocytic AML, there is a subset of AML cells capable of direct ROS-mediated lymphocyte suppression that express functional histamine H_2 receptors and thus respond to HDC. AML patients that harbor these cells respond favorably to HDC/IL-2 immunotherapy. It must be emphasized, however, that the clinical results presented in **paper III** should be validated in prospective studies.

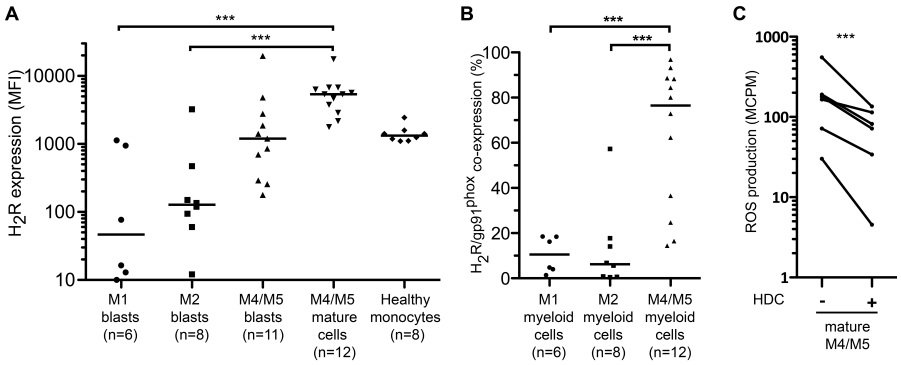

Figure 8 (A) H2Rs are primarily expressed by myeloid cells in M4 and M5 AML and above all by mature $CD33^{+}CD14^{+}$ myeloid cells. (B) Myeloid cells co-expressing NADPH oxidase and H_2R are primarily seen in FAB-M4/M5 AML. Panel C shows reduction in ROS production in sorted mature FAB-M4/M5 cells.

Papers IV and V

The DNA repair enzyme PARP-1 was recently shown to be involved in lymphocyte cell death after exposure to ROS [99]. In **papers IV** and **V** we pursue these findings further in order to investigate if inhibition of the PARP-1/AIF signaling pathway is a feasible way to maintain lymphocyte function under conditions of oxidative stress and in order to elucidate what intracellular signaling pathways that may be involved in lymphocyte parthanatos.

CML cells have previously been demonstrated to induce ROS-dependent cell death in NK cells, however the signaling pathway responsible for mediating cell death has remained unknown. To elucidate CML-mediated cell death further, we did phenotypical analyses of primary CML cells showing that these expressed the NADPH oxidase subunit $gp91^{phox}$ and produced high levels of oxygen radicals. In addition, we performed co-culture experiments with NK cells and CML cells showing that CML cell-mediated immunosuppression is mediated through PARP-1 signaling. When treated with the PARP-1 inhibitor PJ34, lymphocytes were not only rescued from parthanatos triggered by ROS-producing CML cells, but also remained functional as assessed by cytotoxicity, IFN-γ production and proliferation in the presence of exogenous H_2O_2. Results from **paper IV** are summarized in Figure 9.

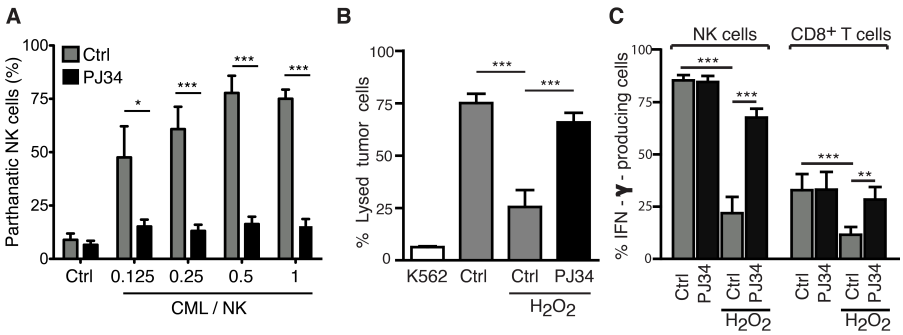

Figure 9. (A) Malignant neutrophils (CML cells) trigger apoptosis in NK cells (grey bars) in a PARP-1 -dependent manner, as shown by the PARP-1 inhibitor PJ34 (black bars). Panel B shows that PJ34 maintains NK cell cytotoxicity against K562 target cells in presence of H_2O_2. (C) NK cell and T cell IFN-γ-production is preserved by PARP-1 inhibition in cultures with exogenous H_2O_2.

Oxygen radicals trigger caspase-independent cell death through PARP-1 [99] and inhibiting this enzyme in lymphocytes upholds viability and function (**Paper IV**). However, the signals leading to PARP-1 activation in lymphocytes after ROS exposure have remained unclear. One possibility is that ROS cause DNA damage with subsequent PARP-1 activation, but ROS are also potent signaling molecules [254], which may lead to PARP-1 activation. Involvement of extracellular signal-regulated kinase 1/2 (ERK1/2) has been reported in oxidant induced cell death in other tissues [255-257] and pERK was recently reported to trigger PARP activation in a cell-free system [122]. In **paper V** we investigated the involvement of the MEK/ERK signaling pathway in PARP-1-dependent lymphocyte parthanatos.

Results in **paper V** imply that inhibition of MEK1/2, responsible for ERK1/2 activation, rescues lymphocytes from ROS-induced parthanatos. Also, inhibition of MEK1/2 prevented formation of phosphorylated ERK (pERK) as well as accumulation of poly(ADP-ribose) (PAR) after ROS exposure. In contrast, inhibition of PARP-1 prevented accumulation of PAR but did not affect pERK formation (Figure 10), implying that ERK is upstream of PARP-1 in ROS-induced parthanatos signaling.

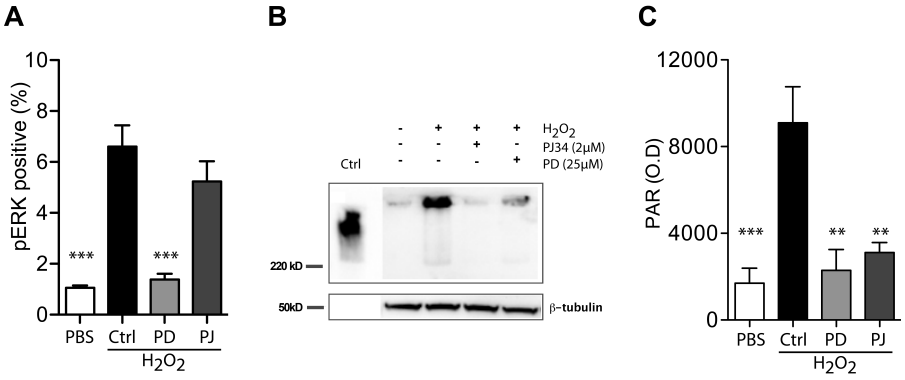

Figure 10. (A) ERK-phosphorylation is inhibited by the MEK-inhibitor PD98059 but not PARP-inhibitor PJ34. (B) Representative western blot analysis of PAR accumulation. (C) PAR accumulation is prevented by both MEK1/2-inhibitor and PARP-inhibitor, thus establishing that ERK1/2 phosphorylation is upstream of PARP activation.

5 DISCUSSION AND CONCLUSIONS

T cells and NK cells are essential components of cell-mediated immunity directed against solid tumor cells and leukemic cells. In myeloid leukemia there are a number of tumor-associated antigens (TAAs) towards which T cells of the host can elicit a specific immune response [232, 258]. NK cells exert anti-leukemic activity mediated not through specific receptors but rather by identifying "missing self" or increased activating ligands [12, 28, 34, 50]. However, functions of NK cells and T cells are frequently impaired in AML patients, which has implications for the course of disease and for attempts to activate cellular immunity for therapeutic purposes.

ROS-mediated immune suppression leads to dysfunction and parthanatos in lymphocytes, a process that can be prevented by inhibiting PARP-1 (**paper II** and **IV**) or by interfering with the ERK1/2 signaling pathway (**paper V**). While the results in **papers II** and **IV** would point to the possibility of using PARP-1 inhibitors as adjuncts in immunotherapy, it should be cautioned that systemic interference of a DNA repair enzyme may be harmful. PARP-1 inhibition should thus most likely be approached cautiously if the sole purpose is to maintain lymphocyte function. However, in the event of using PARP-1 inhibitors as chemosensitizers with the goal of flooding malignant cells with DNA damage [116], maintenance of lymphocyte function may be a favorable contributing factor.

pERK-mediated activation of PARP-1, upon exposure to ROS (**paper V**) opens the possibility to target this pathway when exploring means to protect lymphocyte function. Interestingly, when PARP-1 is exposed to recombinant pERK in a cell-free system, PARP-1 activation ensues. This activation can be prevented by nicked DNA indicating that damaged DNA and pERK compete for a shared binding site on PARP-1 and, more importantly, that interference with the ERK pathway would not impair DNA repair function since nicked DNA has higher affinity for the shared binding site [122]. The finding that ERK is phosphorylated after ROS exposure, leading to PARP-1 activation, along with the finding that PARP-1 inhibition maintains lymphocyte function, suggests that DNA damage may not be the primary event leading to parthanatos in lymphocytes after ROS exposure.

Early relapse in AML correlates with poor survival due to a resumed expansion of residual malignant cells after treatment termination [218, 259]. In addition, the proliferating cells are generated from the most drug-resilient clone, which may explain why additional CRs are harder to achieve. Late relapse in AML is perhaps more difficult to explain. Very late relapse in AML (>5 years after CR) are rare and it is unclear if these are true relapse or *de novo* AML [260]. More interesting are patients that do not relapse immediately but still relapse some time later (approximately 1-2 years after achieving CR). It is tempting to speculate that these cases are examples of disrupted equilibrium between the immune system and the malignant cells, with relapse being the effect of subsequent immune escape (see section **2.4 Immunity and Cancer**). AML is in general seen as a monoclonal disease where the malignant clone evolves in a Darwinian manner [261, 262] with mutations and adaptations shaping the malignant clone. Upon relapse in CR, one of two things have occurred; either the malignant stem cell or progenitor cell has acquired additional mutations allowing it to expand more rapidly or the malignant clone, already having potential for expansion, has acquired a feature allowing it to evade immunity. Either way, the time point for immunotherapy is in CR when tumor burden is low and disease is still in a state resembling equilibrium.

Immune-mediated clearance of residual leukemic cells during, for example, graft-versus-leukemia is most likely to take place in BM, but the circumstances under which this occurs are open to speculation. It is unlikely that the absolute number of anti-leukemic cells is the most important factor for eradication of the leukemic clone. Instead, a more likely scenario is that an individual immune cell encounters a residual AML cell and that this interaction, or lack thereof, determines if the leukemic cell is lysed.

As one factor influencing the outcome of such an interaction, we propose that individual leukemic cells of FAB-M4 and M5 AML can surround themselves with an immunosuppressive cloud of reactive oxygen species (figure 3). This immunoevasive mechanism would protect the individual AML cell from lysis by triggering lymphocyte parthanatos, via pERK and PARP-1 (**paper II**, **IV** and **V**), and possibly creating a hostile microenvironment, thus protecting adjacent AML cells. This hypothesis would help to explain the preferential effect of HDC/IL2 immunotherapy in monocytic forms of AML (**paper II** and **III**) where AML cells comprise a $gp91^{phox+}$ monocytic population with suppressive traits that expresses functional histamine H_2 receptors. In further support of this theory are

reports that AML-M4 and AML-M5 patents have inferior prognosis after allo-SCT [263-266]. In this context, it is of interest that T cells activated by specific interactions with DCs are equipped with both extra- and intracellular thiols, potentially enabling T cells to endure AML cell-generated oxidative stress (**paper I**). In addition, DCs have been shown to induce thiol up-regulation in NK cells generating similar resistance to ROS mediated cell death [194]. These findings may explain how T cells and NK cells can remain functional in environments with high levels of oxidative stress, such as tumor sites or inflammation and might influence the ability of myeloid cells, such as MDSCs or ROS producing AML cells, to suppress T cells. In cancer immunotherapy it may be beneficial to ensure that regimens of T cell activation also involves an up-regulation of thiols in order to achieve optimal efficacy.

Another factor in HDC/IL-2 immunotherapy is the IL-2 component of this regimen. As mentioned earlier, IL-2 promotes expansion of T_{REG}s, which may dampen efficacy of anti-neoplastic T cells [72, 267]. However, IL-2 also promotes NK cell expansion and in patients treated with HDC/IL-2, T_{REG} and NK cell expansion is seen concurrently (Rydström A, personal communication). These findings may, speculatively, call for the development of a modified IL-2 molecule with increased binding to low-affinity IL-2 receptors, resulting in expansion of cytotoxic T cells but not T_{REG}s [268]. An alternative approach would be to combine HDC with another stimulatory cytokine, such as IL-15 or IL-21, which promotes NK cell expansion without concomitant T_{REG} induction [269, 270].

Novel insights into the mechanism of HDC/IL-2 immunotherapy may assist in anticipating which subgroups of AML patients benefit from treatment with HDC/IL-2. One such indication could be following allo-SCT for patients diagnosed with FAB-M4 or FAB-M5 AML or in combination with adoptive transfer of NK cells to AML patients, in particular if the malignant clone express $gp91^{phox}$ and histamine H_2 receptors, indicative for lymphocyte suppression. Another such setting is chronic myelomonocytic leukemia (CMML), a disease in many ways comparable to acute myelomonocytic leukemia. Similar to AML-M4, CMML encompass a suppressive monocytic population, expressing both H_2R and $gp91^{phox}$ (unpublished data).

In conclusion, the translational work presented in this thesis – ranging from studies of molecular mechanisms to the evaluation of clinical samples and clinical trials – may help to further improve the effectiveness of immunotherapy in leukemia.

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