Immune escape in chronic leukemia

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ABSTRACT

Reactive oxygen species (ROS) are produced by myeloid cells as a mechanism of defense against infection, but also to resolve inflammation, as ROS can induce cell death in T cells and NK cells. ROS production may also be deployed as a mechanism by which myeloid cells suppress anti-leukemic lymphocytes to promote malignant progression. The aim of this thesis was to define the role of myeloid cell-derived ROS in chronic leukemias as a putative target of immunotherapy. In **paper I**, the transductional pathways leading to ROS-induced lymphocyte death were investigated and found to involve the ERK1/2 mitogen-activated protein kinase (MAPK). These results challenge the view of ROS-induced cell death being a direct consequence of ROS-inflicted DNA damage. **Papers II and III** demonstrate that anti-CD20 monoclonal antibodies (mAbs) triggered ROS production by monocytes and neutrophils, which translated into reduced NK cell-mediated antibody-dependent cytotoxicity (ADCC) towards autologous leukemic cells derived from patients with chronic lymphocytic leukemia (CLL). The anti-oxidative agent histamine dihydrochloride (HDC) was found to restore ADCC by preventing ROS formation from adjacent monocytes, suggesting that anti-oxidative therapy might increase the efficacy of therapeutic mAbs. In **paper IV**, monocytic leukemic cells obtained from patients with chronic myelomonocytic leukemia (CMML) were shown to suppress T cells and NK cells by producing ROS. HDC counter-acted the suppression of lymphocytes by preventing ROS formation, and augmented the anti-leukemic activity of NK cells. Collectively, these results suggest that myeloid cell-derived ROS may be operational in CLL and in CMML as a mechanism of immune escape and that immunotherapy by anti-oxidative intervention should be further investigated in these forms of chronic leukemia.

Keywords: Immune escape, immunotherapy, reactive oxygen species, chronic lymphocytic leukemia, chronic myelomonocytic leukemia, MAPK

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Sammanfattning på svenska

Immunsystemet är utrustat med kraftfulla mekanismer för att kunna bekämpa mikroorganismer och infekterade celler, men står under noggrann kontroll för att angrepp på frisk vävnad ska undvikas. Immunsystemet kan ofta uppfatta cancerceller som avvikande, men misslyckas trots det vanligen med att eliminera dem. En bakomliggande orsak är kroppens olika system för att hämma immunsystemet. Cancersjukdomar kan också förvärras genom att förstärka immunhämmande mekanismer. Immunterapi syftar till att öka immunologisk eliminering av cancerceller genom ökad aktivering eller minskad hämning av immunsystemet.

Fria syreradikaler kan produceras och frisättas av vissa immunceller, däribland monocyter och neutrofila granulocyter. Syreradikaler bidrar till nedbrytning av mikroorganismer, men utgör också signalämnen vid kommunikation mellan olika celler samt har en viktig roll i att dämpa inflammation. T-lymfocyter och NK-celler är lymfocyter som är viktiga vid infektioner och som har förmåga att känna igen och avdöda cancerceller. T-lymfocyter och NK-celler är känsliga för syreradikaler och dör genom reglerad celldöd vid nära kontakt med radikal-producerande celler. Således kan syreradikaler minska immunsystemets förmåga att eliminera cancerceller.

Syftet med denna avhandling har varit att studera betydelsen av syreradikalers immunhämmande effekter vid två olika typer av kronisk leukemi, samt hur läkemedel som minskar radikalfrisättning skulle kunna användas som immunterapi vid dessa sjukdomar.

Delarbete I syftade till att undersöka signalvägarna som leder till radikalorsakad celldöd. Enzymet PARP-1 finns i cellkärnan och kan aktiveras av DNA-skador. Vid normal aktivitet bidrar PARP-1 till att reparera DNA, men det har tidigare visats att radikalorsakad celldöd sker genom att PARP-1 överaktiveras. Eftersom syreradikaler kan orsaka DNA-skador har man misstänkt att överaktivering av PARP-1 varit en direkt följd av radikalorsakade DNA-skador. Det är dock inte helt kartlagt hur radikaler aktiverar PARP-1. I **delarbete I** visas att syreradikaler orsakade aktivering av det intracellulära enzymet ERK1/2 som i sin tur bidrog till att aktivera PARP-1. Genom att förhindra aktivering av ERK1/2 fann vi att lymfocyter blev mer motståndskraftiga mot radikaler. Dessa resultat tyder på ett samband mellan syreradikaler, ERK1/2 och PARP-1, vilket kan ha betydelse för immunterapier som syftar till att skydda lymfocyter från radikaler.

Patienter med kronisk lymfatisk leukemi (KLL) behandlas ofta med monoklonala antikroppar. Dessa läkemedel kan binda till leukemicellernas yta och därmed underlätta för immunceller att avdöda leukemicellerna. NK-celler bär receptorer för antikroppar (Fc-receptorer) som gör det möjligt för dem att binda till leukemiceller. Även icke-maligna radikalproducerande celler, såsom monocyter och granulocyter, uttrycker Fc-receptorer och kan således också binda till antikroppar. Inför **delarbete II och III** undersöktes hur radikalproducerande celler påverkar NK-cellers förmåga att avdöda leukemiceller från patienter med KLL med hjälp av antikroppar. Vi fann att antikroppar orsakade kraftig radikalfrisättning från monocyter och neutrofila granulocyter samt att monocyter minskade NK-cellers antikroppsmedierade avdödning av leukemiceller genom att frisätta radikaler. Antikroppar ökade också benägenheten hos monocyter och neutrofila granulocyter att hämma NK-celler genom radikalorsakad avdödning. Genom att tillsätta histamindihydroklorid (HDC), ett läkemedel som hämmar radikalproduktion, kunde NK-cellers viabilitet och förmåga att eliminera leukemiceller bevaras. Resultaten tyder på att behandling med monoklonala antikroppar skulle kunna leda till att NK-celler hämmas genom ökad radikalfrisättning, samt att läkemedel som minskar radikalfrisättning skulle kunna öka behandlingseffekten av monoklonala antikroppar vid KLL.

Kronisk myelomonocytär leukemi (KMML) är en ovanlig och allvarlig form av leukemi vid vilken en del av leukemicellerna liknar normala monocyter. I **delarbete IV** undersöktes leukemiceller från patienter med KMML med avseende på förmåga att producera immunhämmande syreradikaler. Vi fann att leukemiceller från patienter med KMML hade en hämmande effekt på NK-celler och T-lymfocyter genom att frisätta syreradikaler och därmed avdöda lymfocyterna. Vi observerade att HDC bevarade NK-cellers viabilitet och ökade deras avdödande aktivitet mot leukemiceller. Vi undersökte dessutom NK-cellers uttryck av aktiverande receptorer vid KMML och fann ett lägre uttryck av flera receptorer hos patienter än hos friska personer. Sammantaget tyder resultaten på att radikalfrisättning skulle kunna bidra till att immunsystemet förhindras att angripa leukemicellerna, samt att immunterapi med HDC bör studeras ytterligare vid KMML.

List of papers

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

I. Akhiani, A. A., O. Werlenius, J. Aurelius, C. Movitz, A. Martner, K. Hellstrand, and F. B. Thorén. 2014. Role of the ERK pathway for oxidant-induced parthanatos in human lymphocytes. *PloS one* 9: e89646

II. Werlenius, O., R. E. Riise, M. Simpanen, J. Aurelius, and F. B. Thorén. 2014. CD20 antibodies induce production and release of reactive oxygen species by neutrophils. *Blood* 123: 4001-4002

III. Werlenius, O., J. Aurelius, A. Hallner, A. A. Akhiani, M. Simpanen., A. Martner, PO. Andersson, K. Hellstrand, and F. B. Thorén. Reactive oxygen species induced by therapeutic CD20 antibodies inhibit NK cell-mediated ADCC against primary CLL cells. *Submitted*

IV. Aurelius, J., O. Werlenius, A. Hallner, R. E. Riise, L. Möllgård, M. Brune, A. Martner, F. B. Thorén, and K. Hellstrand. Immunosuppressive properties of malignant monocytes in chronic myelomonocytic leukemia: role of reactive oxygen species. *In manuscript*

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AIF	Apoptosis-inducing factor
Allo-SCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
BCR	B cell receptor
CDC	Complement-dependent cytotoxicity
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ERK	Extracellular signal-regulated protein kinase
FACS	Fluorescence-activated cell sorting
FcR	Fc-receptor
FISH	Fluorescence *in situ* hybridization
HDC	Histamine dihydrochloride
HLA	Human leukocyte antigen
IFN-γ	Interferon-γ
IL-2	Interleukin-2
KIR	Killer cell immunoglobulin-like receptor
MAb	Monoclonal antibody
MAPK	Mitogen activated kinase
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cell
MEK	Mitogen extracellular signal-regulated kinase
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	Natural cytotoxicity receptor
NK	Natural killer
PAR	Poly(ADP-ribose)
PARP-1	Poly(ADP-ribose) polymerase-1
PBMC	Peripheral blood mononuclear cell
PMN	Polymorphonuclear neutrophil
ROS	Reactive oxygen species
TCR	T cell receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell

1 Preface and aim

The immune system is essential to human life. During evolution, multiple mechanisms of recognition and elimination of invading microorganisms have accumulated to form a comprehensive and efficient defense system that protects us from infection and enables our co-existence with a plethora of potential pathogens. Although the immune system is primarily developed to overcoming infection, an increasing body of evidence supports the role of immunity in preventing and eliminating cancer cells (1, 2).

The expanding field of cancer immunotherapy aims at directing and augmenting immunologic forces against malignantly transformed cells. The efficacy of allogeneic stem cell transplantation (allo-SCT), whereby the anti-leukemic allo-reactivity of T cells and NK cells is employed, serves as an illustration of the potency of immune effector functions and remains the single treatment option with curative potential for several hematopoietic malignancies (3-5). However, the occurrence of graft-versus-host disease (GvHD), a common adverse effect of allo-SCT (6), equally clearly demonstrates the potentially devastating effects of a misdirected immune response and the need for less toxic and more specific immunotherapeutic strategies.

During the last decade, several therapies have emerged that strive to enhance the inherent anti-tumoral immune defense by targeting mechanisms of immune regulation and immunosuppression (7, 8) One such mechanism is the formation of reactive oxygen species (ROS; oxygen radicals) by myeloid cells (9) that can be targeted by histamine dihydrochloride (HDC) (10, 11), a synthetic derivative of histamine. Clinical and experimental evidence has demonstrated that HDC prevents ROS formation by healthy and malignant myeloid cells and thereby rescues lymphocytes from ROS-mediated death (9, 12-14). HDC, in combination with interleukin-2 (IL-2), is currently approved as post-consolidation maintenance therapy of acute myeloid leukemia (AML).

The main aim of this thesis was to contribute to the understanding of the role of myeloid-derived ROS for immunosuppression in two forms chronic leukemia, namely chronic lymphocytic leukemia (CLL) and chronic myelomonocytic leukemia (CMML), and to explore the rationale for counter-suppressive immunotherapy in these diseases. We also studied the intracellular signaling events leading to ROS-mediated lymphocyte death and immunosuppression.

2 Introduction

All blood cells and most cells of the immune system are formed by the bone marrow in the process of hematopoiesis. Hematopoietic cells originate from hematopoietic stem cells (HSC) with capacity of self-renewal and multipotent differentiation (15). Most blood cells have a high turnover rate, and their continuous renewal requires a highly efficient hematopoiesis throughout life. Thus, hematopoiesis is associated with a high rate of cell division, carrying a substantial risk of somatic mutations. With age, mutations are likely to accumulate in HSCs, which may result in malignant transformation and development of leukemia (16).

2.1 Innate and adaptive immunity

The immune system is conventionally divided into innate and adaptive immunity. The basis for this dichotomy is the different mechanisms for antigen specificity inherent to the two divisions.

Behind the physical and chemical barriers protecting our bodies, the innate immune system constitutes the first line of the immune defense. It is mature from birth and comprises an array of both myeloid and lymphoid cells, and also includes the complement system, a cluster of soluble proteases with microbicidal properties (17). Innate immunity responds swiftly to injury or microbial invasion. The instant recognition of foreign structures by innate immune cells is conveyed by a broad, yet limited, set of germ-line encoded receptors, collectively termed pattern recognition receptors (PRRs) (18, 19). PRRs correspond to, and recognize, microbial structures that are critical for the survival of the microorganisms, *e.g.* lipopolysaccharides (LPS), cell wall molecules or nucleic acids, which are thus unlikely to be altered or eliminated by mutation. Since many microbial patterns are shared by different classes of microorganisms, the innate mode of non-self recognition is highly sensitive despite the limited number of receptors and encoding genes (18).

In contrast, adaptive immunity, represented by T and B cells, relies on the acquisition of highly specific receptors, unique to a particular antigen. During the development of T and B cells the genes encoding their antigen receptors are subjected to stochastic rearrangements resulting in a virtually infinite repertoire of minute clones of lymphocytes, each with a unique antigen affinity (20). During a primary infection, naive clones with specific affinity for the invading pathogen are selected, activated and clonally expanded by the activity of antigen presenting cells (APCs) (21). The resulting populations of effector T cells and antibody-producing B cells are thus tailor-made for a specific pathogen.

The mounting of a primary adaptive immune response is time-consuming. Therefore, the initial phase of defense relies entirely on innate immune functions. However, once established, adaptive immunity is preserved by lingering subsets of memory T and B cells, which enable a much quicker immune response in the case of a second encounter (20).

As the understanding of the immune system has evolved, the border between innate and adaptive immunity has become less distinct (22). New roles for cell types traditionally assigned as typically innate or adaptive are frequently being described. For example, the role of neutrophils in shaping adaptive immunity is being increasingly appreciated (23, 24). Also, there is evidence to support the ability of adaptation and memory functions in NK cells (25, 26).

2.2 Myeloid cells

The cells of the myeloid hematopoietic lineage are highly divergent and include the granulocytes, monocytes, macrophages and dendritic cells (DCs). Together, these cells form the backbone of the innate immune system.

2.2.1 Neutrophils

Within the group of myeloid cells the neutrophilic granulocytes (polymorphonuclear neutrophils; PMNs) are the most abundant, comprising about half of all circulating leukocytes under healthy conditions. Neutrophils have an indispensible microbicidal role in the initial phase of an infectious challenge. In response to infection or stress their number can rapidly be multiplied due to mobilization of cells stored in bone marrow niches along with increased granulopoiesis (27).

Neutrophils differentiate in the bone marrow, and enter the blood stream as mature inactive cells (28). In response to inflammation, pro-inflammatory substances, *e.g.* tumor necrosis factor (TNF) and IL-1β, released by tissue macrophages, trigger neutrophil extravasation which in turn induces their activation (27). In the tissue, gradients of chemoattractant substances guide the migration of neutrophils towards the focus of infection (24, 29). There, recognition of microbes is facilitated by various surface-bound receptors, including toll-like receptors (TLR) and Fc-receptors (FcR), a process further reinforced by complement (17) and antibodies (30). The neutrophils then engulf and degrade microbes via phagocytosis, which relies on endosomal microbicidal substances, such as oxygen radicals, proteases and hypochlorous acid. As degradation takes place intracellularly, excessive leakage of reactive substances is prevented and host tissues are largely spared. Even so, during septic infections or massive local inflammation, neutrophil responses can be overwhelming and result in life-threatening immunopathology (31). Mechanisms that mediate the timely abortion of neutrophil activity are therefore of vital importance. As inflammation resolves, neutrophils thus enter apoptosis, and are cleared from the site of infection by macrophages (32). Even under resting conditions, neutrophils are only allowed to circulate for a very short period of time before being replaced by newly formed cells (33).

2.2.2 Mononuclear phagocytes

Mononuclear phagocytes constitute a prominent and heterogeneous group of innate immune cells comprised by monocytes and macrophages.

Monocytes comprise approximately 10 percent of all circulating leukocytes (34). Morphologically, they are characterized by their large size, smoothly rounded shape and unilobar nuclei. Phenotypically, monocytes are distinguished by myeloid linage markers, such as CD33. Monocytes are further divided into subsets based on their expression of CD14 and CD16/FcγRIII; the classical monocytes, comprising 90 percent of circulating monocytes, display a $CD14^{high}/CD16^{-}$ phenotype, while the non-classical subset is $CD14^{-}/CD16^{+}$ (35).

As for neutrophils, the number of monocytes may be increased in response to infection or stress, which triggers their mobilization from marginal pools (36, 37). In contrast to neutrophils, however, monocytes have maintained proliferative and differentiating capabilities after leaving the bone marrow (38). In response to inflammation, they enter the tissues where they may differentiate into macrophages or dendritic cells (34), and take part in phagocytosis, antigen-presentation as well as the resolution of the inflammatory response. Until recently, monocytes were assumed to give rise to the majority of resident tissue macrophages. However, this view has been challenged by studies suggesting that tissue macrophages stem from embryonal yolk-sac precursors (34).

Resident macrophages have a prominent role in the initiation the inflammatory response by serving as sentinels of infection and injury. Equipped with a range of PRRs they rapidly react to invading microbes, and swiftly recruit neutrophils, monocytes and other immune cells into the inflamed area by secretion of pro-inflammatory substances (39).

Moreover, monocytes, macrophages and dendritic cells (DC) posses the capability of antigen processing and presentation (33). Hence, in shaping the adaptive immune response they represent an interface between the innate and adaptive immune system. In addition, monocytes and their progeny are important sources of cytokines and chemokines with orchestrating functions in immunity, either in initiating or maintaining inflammation or contributing to its resolution (40).

2.2.3 The NADPH oxidase

A fundamental feature of myeloid cells, including neutrophils and monocytes, is the ability to produce and secrete reactive oxygen species (ROS) (41). The active production of ROS by phagocytic cells is facilitated by the leukocyte NADPH oxidase, an enzyme compiled by five subunits, of which two, $gp91^{phox}$/NOX2 and $p22^{phox}$ (phox for *ph*agocyte *ox*idase), make up the catalytic

core (42). This heterodimer, referred to as cytochrome b_{558}, is bound to the phagocyte membranes.

Under resting conditions, the enzyme is disassembled, and the remaining subunits, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, are dissolved within the cytosol. Upon activation, kinase-mediated phosphorylation of the cytosolic subunits results in assembly of the enzyme complex with ensuing catalytic activity. The activated enzyme transfers electrons from cytosolic NADPH to the opposite side of the membrane where molecular oxygen is reduced into superoxide (O_2^-). Superoxide is an instable compound that serves as the initial substrate for the formation of several other oxidants with variable reactivity and toxicity, including hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). These oxidants may be produced directly into the sealed compartment of the phagolysosome by NADPH oxidase located to the lysosomal membrane, where they participate in the controlled intracellular breakdown of microbes. Alternatively, the NADPH oxidase is assembled in the plasma membrane, giving rise to extracellular radicals, which, in addition to exerting microbicidal activity, also may participate in intercellular signaling and immune regulation (43-46).

The physiologic role of ROS is illustrated by chronic granulomatous diseases (CGD), a group of disorders characterized by a genetically dysfunctional NADPH oxidase. The incapacity of ROS production by afflicted patients is

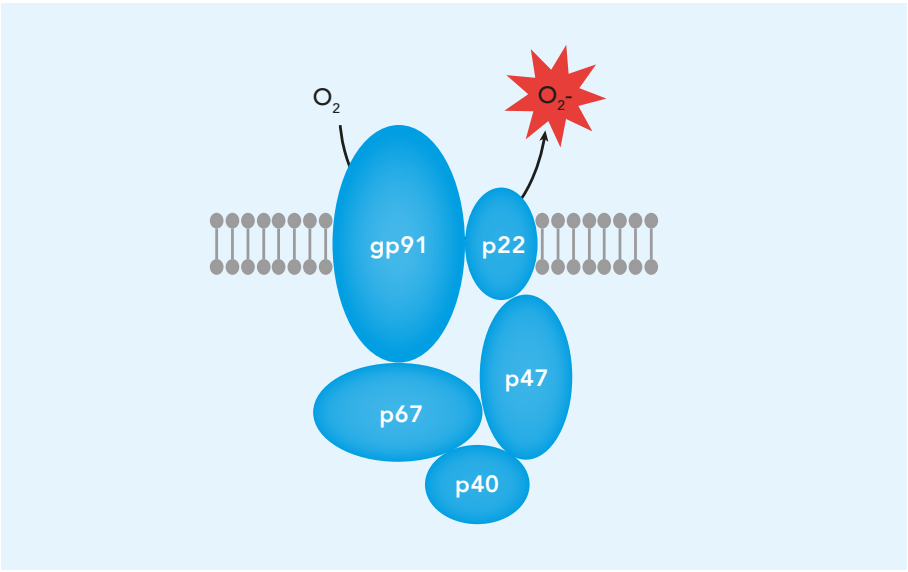

Figure 1. The active NADPH oxidase.

manifested by severe immune deficiency with recurrent bacterial and fungal infections (47). In addition, CGD is accompanied by aseptic granulomas as one manifestation of dysregulated inflammation, underscoring the role of ROS in resolving immune responses (48-50).

Notably, the NADPH oxidase is not the only inherent physiologic source of oxygen radicals. During mitochondrial cellular respiration, energy is obtained in the form of adenosine triphosphate (ATP) by a slow controlled reaction between nutrients and oxygen. This process is accompanied by a slight continuous generation of oxygen radicals (51). In addition, the constant exposure to background radiation continuously gives rise to small amounts of radicals, as when ionization of water molecules are converted into hydroxyl radicals (52).

The reactive propensity of oxygen radicals makes them potentially hazardous, as they can inflict oxidative damage upon vital cellular components, including nucleic acids. Therefore, the integrity of intracellular and tissue structures depends on anti-oxidative mechanisms, which maintain redox homeostasis and render cells and tissues tolerant to a limited burden of oxidative stress. Examples of such traits, present either intra or extracellularly, are the enzymes superoxide dismutase (SOD) and catalase (52), which degrade superoxide and H_2O_2, respectively. Another important anti-oxidative mechanism is exerted by a group of scavenging substances collectively termed thiols. Thiols contain sulfhydryl groups (-SH) that may be reversibly oxidized by the formation of disulfide bonds (-S-S-) (53, 54). Thus, upon encountering oxygen radicals, thiols can limit oxidative stress by becoming oxidized without suffering permanent damage. Immune cells differ in their level of thiol expression, and hence also in their tolerance to radicals (55). As discussed further below, some lymphocyte subsets are highly sensitive to oxidants.

2.3 Lymphoid cells

2.3.1 NK cells

NK cells are cytotoxic lymphocytes that, unlike T cells, do not require previous sensitization to recognize and kill of foreign and transformed cells (26, 56). NK cells rely on germ-line encoded receptors and are accordingly attributed to the innate immune system. Morphologically, NK cells are relatively large lymphocytes displaying granules that are pre-loaded with cytolytic granules that can be released in response to a foreign encounter (57). Phenotypically, NK cells are commonly defined as devoid of the archetypical T cell antigen CD3 and by their expression of CD56.

The expression of CD56 varies within the NK cell population, as does CD16/FcγRIII, an activating low-affinity FcR. The levels of CD56 and CD16 expression are used to define two distinct subsets of NK cells (58). Most circulating NK cells show a low ("dim") expression of CD56 and also express CD16 ($CD56^{dim}CD16^{+}$). This subset is functionally characterized by a high cytotoxic propensity and a low secretion of cytokines (58). Also, the expression of Fc-receptors renders this cytotoxic population responsive to activation by target-bound antibodies and endows them the ability to exert antibody-dependent cellular cytotoxicity (ADCC) (59). The smaller NK cell subset, with a $CD56^{bright}CD16^{-}$ phenotype, is assumed to be an immature NK cell population. This subset has poor cytolytic capacity and cannot mediate ADCC (60, 61). However, they are assumed to contribute to shaping immune responses via production and secretion of proinflammatory cytokines, mainly interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), either within the inflamed tissue or in secondary lymphoid organs (62).

Regulation

Since the discovery of NK cells, their regulation has been subjected to vigorous investigation. In the 1980s, using a murine model of lymphoma, Kärre and co-workers, demonstrated that NK cells efficiently prevented growth of malignant cells devoid of MHC class I, whereas lymphoma cells with preserved expression of MHC class I were spared (63). These findings formed the basis for the missing-self hypothesis, which predicted that NK cells are kept in check by the interaction between inhibitory receptors interacting with MHC class I (64). Thus, upon confrontation with cells missing or with down-regulated MHC class I, inhibition is lifted and activation triggered. A few years later, the discovery of the major group of NK cell inhibitory receptors, the killer immunoglobulin-like receptor (KIR) family, contributed to the fulfillment of

the missing-self hypothesis (65). The KIRs correspond to human leukocyte antigen (HLA) class I, expressed by all nucleated cells, and contribute to NK cell tolerance of autologous tissues.

While the missing-self hypothesis accounts for pivotal aspects of NK cell function it remained conceivable that NK cells, as is the case for T cells, utilize additional or supplemental mechanisms of relevance to activation and tumor cell recognition. This notion inspired the search for activating NK cell receptors, leading to the eventual discovery of the group of natural cytotoxicity receptors (NCRs) which comprise NKp46 (NCR1), NKp30 (NCR2) and NKp44 (NCR3) (66), and the activating NK cell receptors NKG2D (67), DNAM-1 (68). Some activating receptors are constitutively expressed while others are exclusively expressed upon activation (69). Moreover, a large array of additional, co-stimulatory receptors, TLRs, and cytokine receptors have been shown to contribute to the activation of NK cells (26).

Importantly, interaction via FcRs and immunoglobulin G (IgG), which bind to target cells, provides a powerful activating signal that may overcome concomitant inhibitory signaling (68). Collectively, these receptors recognize a wide range of stress- and tumor-induced ligands of host cells in addition to structures of microorganisms. So, although the "missing-self" hypothesis essentially still holds true, the prevailing view of NK cell recognition has been broadened to also include the entities of "non-self" and "altered-self" (70).

In conclusion, the cytotoxic activity of NK cells is determined by the overall concomitant input of activating and inhibitory signals. This complex arrangement of NK cell regulation reflects the biologic necessity of directing the cytotoxic action of NK cells with maximum precision, assuring efficacious attack of foreign and altered invaders while sparing the healthy cells of the host.

Cytotoxic functions

The main mode of NK cell killing is dependent on direct cell-to-cell contact. This is an active process that involves a series of sequential steps. First, contact is established between the NK cell and its target via adhesion molecules, such as lymphocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) (71). This creates a tight interface referred to as an immunological synapse (72), towards which surface molecules and intracellular granules are polarized to facilitate interactions. If the balance between activating and inhibitory signals is shifted in favor of activation, the NK cells will degranulate and release cytotoxic substances such as perforin and granzyme B, likely resulting in target cell lysis. Also, NK cells may express death receptor

ligands, *e.g.* Fas-ligand (Fas-L) and TNF-related apoptosis inducing ligand (TRAIL), which may induce an alternative, perforin-independent, pathway of apoptosis (73).

2.3.2 T cells

T cells are the key mediators of cellular adaptive immunity. T cell progenitors leave the bone morrow and migrate to the thymus where differentiation, receptor gene rearrangement and education ensue. During education, T cells are actively selected for further maturation on the basis of the affinity of their TCRs for HLA and their reluctance to bind self-antigens (74). The remaining cells, regarded as either inoperational or potentially self-reactive, are denied survival signals and enter apoptosis. Thereby, the vast majority of T cells are sacrificed, and a mere fraction allowed to leave the thymus as mature naive T cells.

There are two main subsets of T cells; the $CD4^+$ T helper cells (Th) and the $CD8^+$ cytotoxic T cells (CTL). As their name implies, the T helper cells have assisting and orchestrating roles in immunity. The TCR of Th interacts with HLA class II and antigen peptides displayed by APCs. Depending on the type of infection specialized Th subgroups with different cytokine profiles help skew the immune response in a favorable direction (75). For instance, Th17 cells contribute in recruiting neutrophils to an infected tissue by initiating a cascade leading to the secretion of attracting cytokines. The Th2 subset are engaged in B cell development and the formation of antibody responses, while Th1 cells produce interferon-γ (IFN-γ), which, among other things, stimulates the activity of NK cells and enhances the killing capacity of phagocytes (76).

The TCR of CTLs enables interaction with APCs and all nucleated cells via MHC class I. Upon antigen presentation by an APC, naive $CD8^+$ T cell are activated and stimulated to proliferate, forming an expanded clone of antigen specific effector CTLs (77). Effector CTLs circulate in blood and tissues, monitoring cells for their cognate peptide in conjunction with HLA class I. Upon confrontation, the CTL will recognize the cell as potentially infected or altered. Cytotoxic activity ensues in a mode similar to NK cell killing, *i.e.* via release of perforin, granzyme B or by displaying death receptor ligands (72).

2.3.3 B cells

B cells are dedicated to the formation of the humoral immune defense by producing and secreting antibodies. Antibodies are formed in response to an infectious encounter in a delayed process, similar to the mounting of the T cell response. Therefore, antibodies may contribute to finalize the defense of a primary infection, but have a more significant role in immunologic memory (78).

B cell development commences in the bone marrow and involves the rearrangement of genes encoding the heavy and light immunoglobulin (Ig) chains, which subsequently assemble to form the B cell receptor (BCR) and, eventually, soluble antibodies (76). Antibody development is subjected to rigorous quality control, involving a series of checkpoints where functionality of individual Ig chains and the expression of the BCR are assessed. As the stochastic gene rearrangement may result in a virtually infinite number of Ig, most B cells fail in this process and are sorted out, analogous to the deletion of dysfunctional or self-reactive T cells. Immature B cells enter the circulation and undergo maturation in secondary lymphoid organs. Maturation renders the naive B cells responsive to an antigen encounter, upon which clonal expansion and differentiation into antibody secreting cells ensues. Following a process of affinity maturation, B cells with confirmed utility as producers of high-affinity Ig may further differentiate into memory B cells or antibody-producing plasma cells. Throughout the maturation of the B cell immune response, the antibodies formed are refined towards a more avid antigen affinity (76, 78).

Antibodies exert several effector mechanisms. Their role may be to neutralize a foreign structure, *e.g.* bacterial toxins or adhesion molecules of importance for microbial virulence, by concealing it. Also, antibodies provide a link between foreign cells and other mechanisms of immune elimination. By binding to antigens on target cells, antibodies serve as markers that facilitate the activation of immune cells and complement, thereby conveying various modes of target cell killing (79). Immunoglobulin G (IgG), the most abundant antibody isotope in the circulation, has potent capacity to interact with immune effector cell by ligation of FcRs, which may result in target cell elimination by ADCC or antibody-dependent cellular phagocytosis (ADCP) (68, 80, 81).

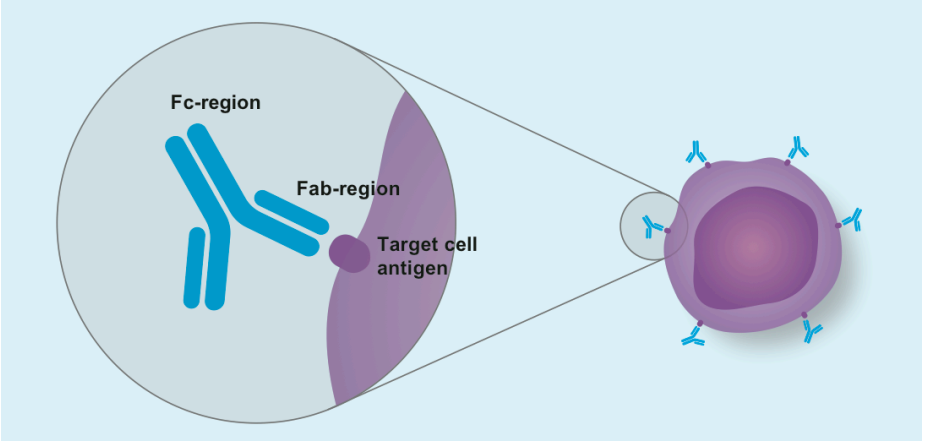

Figure 2. IgG antibodies bound to a target cell via surface antigens. The antibody-binding region (Fab) is variable, while the Fc-region is constant.

2.4 Cell death and signaling

The current notion of cell death stipulates two principal modes, accidental and regulated (82). Accidental cell death (ACD) refers to the uncontrolled death that that may occur in response to extreme physical or chemical insults such as direct trauma, burns or non-physiological pH levels. Although ACD may be operational in some infectious and malignant conditions, it is considered a physiologically and therapeutically rare event, and will not be further discussed here.

Regulated cell death (RCD), where programmed cell death (PCD; apoptosis) constitutes one entity, is a controlled, genetically encoded process that concerns a multitude of physiologic aspects of multicellular organisms. Embryologic development, tissue homeostasis, formation and maturation of the immune system as well as inherent mechanisms preventing malignancy are examples of vital processes that rely on the ability of individual cells to die in a regulated way. Accordingly, an array of genes, molecular structures, and signaling pathways are involved in RCD, and several different entities of RCD have been described (83). As research has advanced, the taxonomic definitions of the different entities of cell death have changed. However, all modes of RCD result in the enzyme-regulated controlled degradation of the cell in a process that generally comprises chromatin condensation, nuclear fragmentation and membrane permeabilization with minimal concomitant effects on surrounding cells.

2.4.1 Apoptosis

Apoptosis is the dominant entity of RCD (83). Apoptosis can be induced either by the extrinsic or intrinsic pathway. Extrinsic apoptosis refers to externally initiated signaling, transferred via death receptors displayed on the cell surface. Examples of such ligand/receptor pairs are Fas-ligand (FasL)/Fas (73, 84), TNFα/TNFα receptor 1 (TNFR1) and TRAIL/TRAIL receptor (TRAILR) (85). Death receptors trigger the pro-apoptotic caspase protease cascade (86). When the net sum of apoptosis-initiating input overcomes balancing forces, the activation of downstream executioner caspases results in irreversible cell death.

The intrinsic pathway of apoptosis is ignited by adverse events inside the cell, *e.g.* irreparable DNA damage or excessive intracellular ROS formation (83). Intrinsic pro-apoptotic signaling will converge in the mitochondria where they may add to cause mitochondrial outer membrane permeabilization (MOMP) and loss of mitochondrial transmembrane polarization ($\Delta\psi_m$). These events

translate into mitochondrial leakage of various pro-apoptotic proteins that can either promote the caspase route to apoptosis, or enter the nucleus to induce DNA fragmentation and cell death in a caspase-independent fashion (83).

2.4.2 Parthanatos

DNA damage constitutes a threat to cell functionality and may lead to malignant transformation. As DNA replication is inevitably accompanied by faults, it is consistently under surveillance of a wide set of nuclear enzymes that sense DNA damage. However, as excessive damage may be irreparable, the survival of the host may require the cell to convert from striving to protect the integrity of the genome to instead surrender and die. This requirement is reflected by the versatility of the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 detects DNA-breaks and catalyzes the formation of poly(ADP-ribose) (PAR) and the activation of nuclear DNA repair enzymes by poly ADP-ribosylation (87). Under normal cellular conditions with low-grade DNA damage, the low levels of PAR produced are confined to the nucleus. However, during excessive DNA damage PARP-1 activity is accordingly increased. This leads to PAR accumulation, allowing PAR to leave the nucleus (88). In the cytosol, PAR will induce MOMP with ensuing mitochondrial release of apoptosis-inducing factor (AIF). In turn, AIF will translocate to the nucleus and cause DNA fragmentation (89) and thus induce RCD independently of caspases (90).

Notably, the substrate for PARP-1 is NAD^+, a co-enzyme necessary for upholding redox balance and for generation of ATP. Therefore, a rise in PARP-1 activity also contributes to depletion of the cellular supplies of NAD^+ and ATP. Thus, cellular starvation and accumulation of intracellular ROS is accompanied by PARP-1 over-activity and was previously believed to be the mechanism responsible for PARP-induced cell death (91). However, later reports have revealed PARP-initiated cell death to be operational also in the absence of depletion of energy supplies (87). Furthermore, experiments in a system free of NAD^+, in which AIF was blocked, the PARP-1 mediated death was abrogated, thus demonstrating AIF to be critical for PARP-1 induced cell death (88, 90). In conclusion, the prevailing concept of PARP-mediated, caspase-independent, AIF-dependent cell death is regarded as a specific entity of RCD, and has been termed *parthanatos*, from *Thanatos,* the personification of death in Greek mythology (87).

Parthanatos has been shown to be involved in oxidant-mediated lymphocyte death (13, 90, 92). T cells and NK cells are sensitive to exposure of myeloid cells with capacity to produce extracellular oxygen radicals via NADPH-oxidase

(9, 93-95) (refer to section 2.2.3). Co-culture of lymphocytes with myeloid cells was shown to result in ROS-dependent lymphocyte death involving activation of PARP-1, mitochondrial release of AIF and DNA fragmentation (90, 96). However, the specific events leading to PARP-1 activation after ROS-exposure have remained unknown.

Physiologically, myeloid ROS-mediated suppression of lymphocytes has been ascribed a prominent role in regulating autoimmunity as studies indicate that animals displaying deficient NADPH-oxidase are prone to develop autoimmune arthritis and multiple-sclerosis-like neurologic disease (97, 98). In humans, parthanatic lymphocyte death has been predominantly associated with immune suppression induced by several forms of myeloid leukemic cells (refer to section 2.5.1) (13, 92). In addition, PARP-1 dependent cell death has been attributed a role in the pathophysiology of ischemic and degenerative neurologic diseases and in myocardial infarction (99-102).

2.4.3 MAP kinases

In addition to DNA damage, activation of PARP-1 has been suggested to occur via an alternative pathway, involving the extracellular signal-regulated kinase (ERK) (103). ERK belongs to the family of mitogen-activated protein kinases (MAPKs) that, in addition to ERK 1 and 2 (ERK1/2), encompass the c-Jun N-terminal kinases (JNK1-3), the p38 MAPKs (p38α-δ) and ERK5 (104). Together, MAPKs participate in multiple signaling pathways and networks in response to growth factors, mitogens, stress and inflammation. The outcomes of their signaling transductions are heterogeneous and situation-dependent, and include cell proliferation and death, and thus have implications in many pathologic conditions, including cancer (104, 105).

2.5 Immune surveillance

The concept of immune surveillance refers to the hypothesis that the immune system can detect and eliminate malignant transformation at a preclinical stage and thus prevent the formation of overt cancer (1). This theory was originally proposed already at the beginning of the 20th century by Paul Ehrlich (106), but was officially launched in the late 1950s by Burnet and Thomas following the recent appreciation of cellular immunology and antigen recognition by lymphocytes (107, 108). Initially though, the hypothesis did not properly fulfill its experimental predictions. The insufficiently immune-deficient mouse models available at the time failed to develop more spontaneous and carcinogen-induced tumors, other than virally induced tumors, than did immunocompetent mice, leading to a recession for the hypothesis. However, a revival of the immunosurveillance theory came in the 1990s along with methods of efficient gene silencing using knockout mice and inhibitory monoclonal antibodies (109). Since then, numerous studies have demonstrated that mice with severe immune deficiencies are indeed more prone to tumor development than animals with intact immune systems. Murine studies using knockout models or blocking antibodies for mechanisms necessary to mount specific responses by T cells, NK cells and NKT cells have demonstrated distinct roles in tumor immunosurveillance for these lymphocyte subsets (110-113). Further supporting a role of cytotoxic effector lymphocytes in anti-tumoral immunity are studies showing that perforin-deficient mice have increased susceptible to tumors (114-116).

The theory of immune surveillance has gained further momentum from transplantation studies in mice, assessing the immunogenicity of tumors developed under different immunologic pressures. In essence, these studies have shown that tumors that develop in immunocompetent mice are more aggressive than those that evolve in immune deficient animals. (1). These and other findings have supported the formation of the immunoediting theory, which states that immunity promotes immune escape and tumor progression by selecting clones with poor immunogenicity or immunosuppressive traits for survival in a Darwinian manner (117).

Although the abundance of murine studies provides proof-of-principle and suggests a role for immunity in prevention and formation of human cancer, the importance of immune surveillance in human cancer has remained a matter of debate. The opponents of the immune surveillance theory sometimes argue that earlier epidemiologic reports merely demonstrated increased frequencies of cancers of viral origin in immunosuppressed individuals (118). However, these

studies suffered from some relevant limitations. Firstly, the pharmacological immunosuppression used in the prevention of organ-rejection is not associated with immune deficiencies as grave as those of murine knockouts (1), and are mainly suppressed in their T and B cells compartments. Hence, residual effector functions including NK cell activity may serve as protection from tumor formation. Secondly, the increased morbidity of organ-transplanted patients, accompanied by their shorter life span, is likely to confound any increased susceptibility to cancer development.

Nevertheless, several later follow-up studies of organ-transplanted patients have shown that this patient category is indeed at higher risk of developing malignant diseases, including forms of non-viral origin (119-125). Consistent with these epidemiological findings are results from histopathologic studies demonstrating a beneficial correlation between prognosis and the occurrence of tumor infiltrating lymphocytes (TILs) in several solid cancers (126-133).

Collectively, a substantial amount of evidence from different areas of research thus supports a role of immunity in cancer development. This notion was recently highlighted by the fact that evasion of immune destruction was recognized as an emerging hallmark of cancer (118).

2.6 Immune escape

As noted, recognition by T cells relies on the expression of specific tumor antigens via HLA class I. Thus, as malignant cells are not foreign to the host, but represent *"altered-self"*, an insufficient expression of foreign antigens and a preserved expression of self-antigens may render malignant cells difficult for the immune system to recognize. This obstacle of tumor recognition is likely to apply to several, it not all, human cancers. In addition, consistent with the immunoediting theory, malignant cells deploy several other traits by which they circumvent immune recognition and destruction.

The expression pattern of ligands for immune cell receptors is commonly deviant in cancer cells as to promote immune tolerance. One way to evade T cell recognition is down-regulation of HLA class I (134-141). Thereby, tumor-antigens are withheld from identification by T cells. However, this is likely to result in increased susceptibility to NK cell cytotoxicity, in accordance with the "missing self" hypothesis (refer to section 2.3.1).

Conversely, up-regulation of ligands for inhibitory T and NK cell receptors may also induce immune tolerance. A group of surface molecules that are currently attracting significant clinical interest are the receptor-ligand pairs referred to as immune checkpoints. These are co-inhibitory pathways, which down-tune the activity of CTLs, and other immune effectors (142). For example, CD80 and CD86 are ligands for the inhibitory cytotoxic T lymphocyte-associated protein 4 (CTLA-4), expressed by activated T cells, and the programmed death receptor ligands 1 and 2 (PD-L1/2) ligate the PD1 receptor of activated T cells, NK cells and NKT cells (142, 143). The expression of CD80/CD86 and PD-L1/2 is enhanced on tumor cells in various malignancies, resulting in reduced lymphocyte activity. Inhibition of these inhibitory pathways is successfully being exploited for immunotherapeutic purposes (refer to section 2.6.2) (142). Other examples of aberrant expression by malignant cells that impede immune mechanisms are ligands for death receptors, such as TRAIL and FasL with capacity to induce apoptosis in immune effector cells (84).

Tumor cells also commonly produce and secrete substances, including anti-inflammatory cytokines such as transforming growth factor beta (TGF-β) (144), IL-10 and others (145), which exert a range of immunosuppressive activities, including interference with CTL and NK cell cytotoxicity (146). Cancer-induced immunosuppression may be directly inflicted by the malignant clone or

by immunosuppressive third-party immune populations, derived either from the lymphoid or the myeloid lineage.

An extensively studied entity of tumor-promoting immune cells is the $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs). Tregs are a subset of T helper cells with a physiologic role in prevention of autoimmune T cell responses (147, 148) and can be recruited towards the tumor in response to TGF-β, IL-10 or other anti-inflammatory signals. In the tumor microenvironment, Tregs further facilitate tumor progression by various inhibitory actions on T and NK cells (149). For example, they neutralize IL-2 and constitute an additional source of TGF-β and IL-10. In addition, Tregs act on APCs by reducing their expression of co-stimulatory molecules.

Another group of immune cells commonly associated with malignant progression is the heterogeneous entity of myeloid immunosuppressive cells. Immature myeloid cells as well as monocytes, macrophages, dendritic cells and granulocytes can be exploited in tumor advancement to exert pro-malignant, anti-inflammatory tasks. In fact, their promotion of tumor development stretches beyond immune suppression, as they also may contribute to tumor angiogenesis and metastasis (150).

The entity of myeloid cells currently receiving the most attention in the context of cancer is the myeloid-derived suppressor cells (MDSC) (151). MDCS were originally described in mice, but their expansion has also been demonstrated in several types of human cancer and correlate with inferior prognosis (152). MDSCs arise as a result of tumor interference with the maturation of healthy myeloid cells. This interference results in the expansion of subsets of myeloid cells with immature phenotypes, which may share characteristics with normal monocytes or neutrophils. In comparison to healthy myeloid cells, MDSCs are endowed with an exaggerated armamentarium of bactericidal and immunoregulatory traits, and have been shown to subvert tumoricidal immune functions through various mechanisms including production of oxygen and nitrogen radicals and secretion of IL-10 (151, 153). Another strategy of MDSC immunosuppression is to deny the anti-tumoral immune cells important nutrients, as illustrated by their expression of arginase 1 (ARG-1) and inducible nitric oxide synthase (iNOS) (151). As both enzymes use L-arginine as their substrate, they deprive L-arginine supplies and thereby restrict T and NK cell responses (154). Similarly, expression and activity of indoleamine 2.3-dioxygensase (IDO), an enzyme that consumes tryptophan and produces noxious metabolites, contributes to lymphocyte suppression (154, 155).

2.6.1 Reactive oxygen species

As noted above, the NADPH oxidase is expressed by many types of myeloid cells, including healthy monocytes, neutrophils, MDSCs and several entities of leukemic myeloid cells, which endows them the capacity of ROS production (9, 13, 14, 156). In vitro, myeloid cells from healthy individuals and leukemic cells from patients with chronic myeloid leukemia (CML) and AML have been demonstrated to induce ROS-mediated PARP-1 dependent cell death (*parthanatos*; refer to section 2.4.2) in T cells and NK cells (13, 92). Since immature myeloid cells do not express a functional NADPH oxidase, the immunosuppressive status of leukemic cells depend on their differentiation stage. Hence, in AML, the predominantly immunosuppressive subtypes are those with monocytic differentiation (12, 13).

Experimental studies have also demonstrated that myeloid-derived ROS induce functional impairments in T cells and NK cells also following non-lethal exposure. For example Romero *et al.* showed that NK cells exposed to ROS down-regulate the activating receptors NKp46 and NKG2D (157), and a study by Kono and co-workers showed that myeloid-derived ROS induced down-regulation of the ζ (zeta)-subunit of the T cell receptor and the FcγRIII/CD16, structures critical for T cell recognition and NK cell mediated ADCC (158). Moreover, several murine studies have suggested a role for myeloid-derived ROS as instrumental for induction of CTL tolerance by tumor-employed myeloid cells (159, 160).

2.7 Immunotherapy

Immunotherapy refers to the employment of immune effector functions in the treatment of cancer. The concept originated with allogeneic stem cell transplantations (Allo-SCT) more than 50 years ago (161), but since the introduction of monoclonal antibodies (mAbs) in the late 1990s the field of immunotherapy has expanded enormously. Here, a brief overview is provided of some immunotherapies of relevance to this thesis.

2.7.1 Allogeneic stem cell transplantation

The efficacy of allo-SCT relies on two mechanisms. Firstly, the stem cell graft provides rescue from bone marrow toxicity, and thus allows the administration of myeloablative doses of conditioning treatment prior to transplantation. This aims at reducing the malignant burden to minimal levels. In addition, the conditioning regimen serves to suppress the recipient's immune system and thus allow engraftment. For this purpose however, reduced intensity conditioning therapy (RICT) is sufficient, and has expanded the use of allo-SCT to older patients. Secondly, allo-SCT employs allo-reactive immune responses of the donor-derived immune system to eradicate residual leukemia. Although the recipient and the donor are matched for HLA compatibility, differences in the expression of other allogeneic structures will differ, and thus enable recognition and killing of residual malignant cells by donor derived lymphocytes (161). T cells have been shown to be the key mediators of the graft-versus-leukemia (GvL) effect (162), but a prominent role of NK cells in GvL has also been demonstrated (163). Allo-SCT is a potentially curative treatment option for several malignancies, including CLL (3) and CMML (4, 5, 164), but its wide-spread use is limited due to the accompanied risks of mortality and severe immunopathology in the form of graft-versus-host disease (GvHD) (165).

2.7.2 Monoclonal antibodies

The technique to produce monoclonal antibodies (mAbs) was developed in the 1970s by Georges J.F. Köhler and César Milstein (166). Briefly, the method involves the fusion of murine antibody-producing B cells, derived from immunized animals, with myeloma cells, thus forming cell lines termed hybridomas, *i.e.* monoclonal cells producing identical antibodies (167). The murine mAbs obtained by this procedure came to revolutionize diagnostics and biomedical research by its utility in an array of laboratory applications including immunohistochemistry and flow cytometry. However, early attempts to use

murine antibodies for therapeutic purposes were thwarted by the immunogenicity and side effects of the mAbs (168).

With the development of refined genetic engineering and recombinant technology came the ability to generate chimeric, *i.e.* partly humanized, mAbs with tolerable side effects and longer half-lives (169). The first mAb to be marketed for treatment of malignant disease was rituximab, which was approved by regulatory authorities in the US in 1997 and in the EU in 1998 for the treatment of non-Hodgkin lymphomas. Rituximab is a chimeric IgG1 antibody recognizing the B lineage marker CD20 (170). The exclusive expression of the CD20 antigen by B cells, and the fact that its' expression is commonly preserved in B cell malignancies, makes CD20 an attractive target for therapies directed targeting B cell populations. Indeed, rituximab has contributed to the treatment of numerous B cell malignancies including CLL, for which it mainly has come to serve as an addition to chemotherapeutic regimens (171-173). However, as single agent treatment, rituximab has limited efficacy for most B cell malignancies including CLL, with low complete response rates, and short response durations (174-176).

As CD20 antigen is expressed on all B cells, treatment with rituximab and other CD20 mAbs, results in depletion of non-malignant B cells. Therefore, rituximab has become a valued treatment option for autoimmune diseases, including rheumatoid arthritis (177), and hematologic autoimmune disorders (178) for which B cells have a prominent role. Following the success of rituximab, other CD20 mAbs, with refined properties, have emerged. Ofatumumab, a further humanized antibody with affinity for another epitope of the CD20 antigen, has shown promising efficacy in treatment of CLL (179, 180). The most recently approved CD20 mAb was obinutuzumab, with an Fc-portion engineered for higher FcR affinity. In a recent large randomized trial, obinutuzumab demonstrated superiority over rituximab in conjunction with chlorambucil (181).

The elimination of malignant cells by mAbs is presumed to rely on several mechanisms, including direct induction of apoptosis, complement-dependent cytotoxicity (CDC) and employment of cytotoxic immune cells expressing Fc receptors (182). NK cells, macrophages, monocytes and neutrophils all carry Fc-receptors and have been attributed roles in mediation of malignant cell elimination either by ADCC or ADCP (24, 183-186). However, investigational approaches aiming to dissect the individual contributions of different effector mechanisms *in vivo* have not been conclusive. The most extensively studied effector cells *in vitro* are the NK cells, of which numerous studies have

demonstrated potent cytotoxic capacity via antibody-dependent cellular cytotoxicity (ADCC) (80, 187, 188). NK cells attach to the Fc portion of IgG1 mAbs via the activating Fc-receptor CD16/FcγRIII expressed on their cell surface. Although concomitant inhibitory receptor signaling, *e.g.* by KIRs, may limit ADCC, IgG binding to CD16 functions as the single most activating signal for NK cells, and has the potential to override concomitant inhibitory signaling (188). More recent CD20 mAbs are constructed to augment the degree of cell-mediated elimination. One example is obinutuzumab, for which the Fc-portion has been modified for increased FcR-affinity, and thus exerts ADCC more efficiently than rituximab (189).

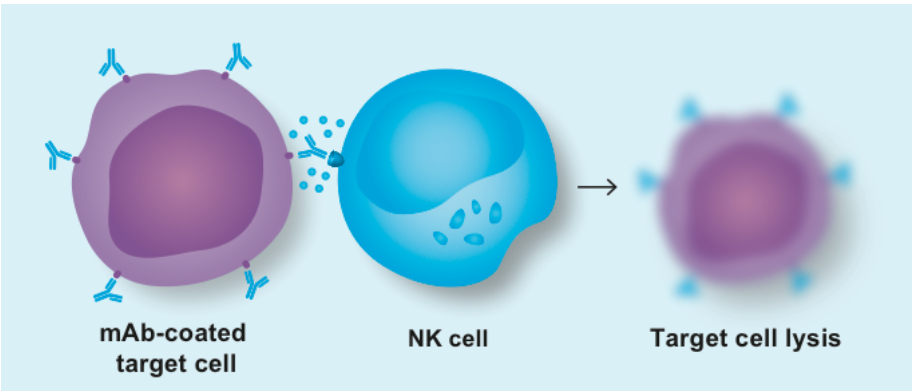

Figure 3. NK cells are key mediators of antibody-dependent cellular cytotoxicity (ADCC).

In recent years, several mAbs with avidity for other antigens have been developed. However, in addition to mAbs with affinity for tumor-associated antigens (TAAs), *i.e.* structures displayed by the malignant cells *per se*, some mAbs are developed aiming to inhibit mechanisms that promote tumor progression. For this purpose, antibodies with neutralizing properties are desired, which induce minimal activation of FcR-carrying immune cells. Therefore, inhibitory mAbs are preferentially constructed using IgG subtypes with less affinity for activating FcRs, such as IgG2 or IgG4 (169).

2.7.3 Immune checkpoint blockade

Recently, the group of newly developed mAbs directed at immunoregulatory pathways, or immune checkpoints, have come to attract considerable interest. The first immune checkpoint to be targeted was the cytotoxic T lymphocyte antigen 4 (CTLA 4). CTLA-4 is an inhibitory receptor, expressed by activated T cells, that interacts with the CD80/86 surface molecules of APCs and tumor cells. This interaction represents a mechanism by which the activity of CTLs is restricted, thus contributing to T cell tolerance and protection form autoimmunity (190), but may also facilitate tumor immune evasion (191). Ipilimumab, an antagonizing mAb against CTLA-4, has shown efficacy in several forms of solid cancer including malignant melanoma, renal cell cancer, prostate cancer and ovarian cancer, and was approved in 2011 by the US Food and Drug Administration (FDA) and in 2012 by the European Medicines Agency EMA) for the treatment of advanced malignant melanoma (192).

Another immune checkpoint is the interaction between the programmed death 1 (PD-1) receptor and its ligands PD-L1/2. Like CTLA-4, PD-1 serves as an inhibitory receptor for T cells, but PD1 is also expressed by NK cells, NKT cells, B cells and monocytes (193). Under physiologic conditions, PD-L1 is mainly found on APCs, while PD-L2 is expressed by various tissues, and thus apparently has a role in inducing self-tolerance by down-tuning immune activity in the periphery. However, PD-L1 is commonly expressed by tumor cells, and may thus serve as an immune escape mechanism (142). Inhibition of the PD-1 checkpoint has also demonstrated significant clinical efficacy, with responses seen in various advanced solid cancers and in Hodgkin's lymphoma (192). Currently, PD-1 antagonists nivolumab and pembrolizumab are approved for use by the FDA and EMA for treatment of advanced melanoma. In the US, nivolumab also recently received approved for non-small cell lung cancer.

As CTLA-4 and PD-1 represent different inhibitory pathways, attempts have been made to combine treatments for the two checkpoints. Encouraged by results obtained in mice, suggesting a synergistic effect of double blockade (194), the combination of ipilimumab and nivolumab has been evaluated in advanced melanoma with impressive results. A phase I study demonstrated a response rate of 53 percent achieving at least 80 percent tumor regression and a two-year survival rate of 79 percent (195), and two recently published randomized trials, comparing combined ipilimumab with nivolumab with single-agent treatment, demonstrated superiority for the combination regimen, which induced response rates of 51 to 61 percent (196, 197). However, checkpoint blockade is associated with significant rates of severe immunopathology, in particular treatments combining different inhibitors.

2.7.4 Histamine dihydrochloride and interleukin-2

The cytokine interleukin-2 (IL-2) was discovered during the 1970s as a lymphocyte-derived substance that enabled long-term *ex vivo* cultures of lymphocytes (198). Later studies revealed that the predominant physiologic source of IL-2 are T cells, and that it stimulates the proliferation and differentiation of several lymphocytic subsets, including CTLs and NK cells (199). Recombinant high-dose IL-2 was explored as immunotherapy for treatment of several types of advanced malignant disease. Malignant melanoma and renal cell cancer displayed particular responsiveness to the treatment with small, but significant rates of complete and sometimes durable responses (200). Given the relapse-preventive efficacy of allo-SCT in hematologic malignancies, treatment with IL-2, also reliant on T and NK cell activity, has been extensively evaluated for prevention of relapse of AML. The results have been disappointing however, as six trials have failed to demonstrate any benefit from IL-2 as single-agent therapy for this disease (201-206).

In 2006, Brune and co-workers reported the results from a randomized phase III trial demonstrating that the combinatory regimen of low-dose IL-2 and histamine dihydrochloride (HDC), administered as maintenance treatment in complete remission (CR), prevented relapse of AML (10). The rationale for supplementing IL-2 with HDC was based on the hypothesis that myeloid-derived ROS might be operative in AML, and thus prevent the immunostimulatory effects of IL-2 (207). The hypothesis was supported by previous studies by Hellstrand and co-workers showing that myeloid cells constrain the anti-leukemic activity of NK cells by production and secretion of ROS, and that HDC restored NK cell cytotoxicity (94, 208, 209). HDC prevented the production of ROS via interaction with histamine H_2 receptors (H_2Rs) expressed by myeloid cells, thus reducing the activity of the phagocyte NADPH oxidase. Importantly, a synergistic effect of IL-2 and HDC had been demonstrated *in vitro*, as the stimulatory effect of IL-2 on NK cells was abrogated in the presence of myeloid cells, and the addition of HDC to IL-2 counter-acted myeloid immunosuppression and restored NK cell cytotoxicity (210).

Further support for combining IL-2 and HDC in AML was provided by functional and phenotypic studies, showing significant impairments in the NK cell repertoire of activating receptors (211, 212). *In vitro*, myeloid cells had been shown to down-regulate the activating NK cell receptors NKG2D in the absence, but not in the presence of HDC (157, 213).

Recently, two reports by Aurelius *et al.* further elucidated the role of ROS as a reversible mechanism of immune escape in AML (12, 92). Functional and phenotypic analyses demonstrated that the leukemic cell subsets with monocytic differentiation (M4/M5) exerted ROS-mediated immune suppression *per se* (13). ROS-production by monocytic leukemic cells was prevented by HDC. In contrast, the immature blast populations of any AML subtype were incapable of ROS production. Consistent with these findings, a *post hoc* analysis of the original phase III trial revealed that the relapse-preventive efficacy of HDC and IL-2 was restricted to monocytic subtypes of AML, *i.e.* to patients harboring an immunosuppressive leukemic clone (12).

Apart from it's proven efficacy in AML, the combination of HDC and IL-2 has also been evaluated as treatment for solid cancers for which IL-2 alone has previously shown efficacy. For patients with malignant melanoma with liver metastases, HDC was shown to prolong over-all survival compared to IL-2 alone (214) and to promote an anti-tumoral T cell response (215). In renal cell cancer, HDC and IL-2 were evaluated in two randomized phase II trials comparing combination treatment with IL-2 alone. One trial demonstrated a significant benefit for the combination regarding 1-year survival, and time to progression, whereas the other study failed to show any difference (216)

2.8 Chronic lymphocytic leukemia

Chronic lymphocytic leukemia is a disease characterized by a slow accumulation of malignant, highly differentiated B cells in the bone marrow, blood and in secondary lymphoid organs (217). CLL is the most common leukemia in western countries with an annual incidence of 4-5/100000 and a male to female predominance of close to 2:1. The median age at diagnosis is approximately 70 years and the prevalence increases dramatically with age (218). Typically, the malignant clone in CLL is homologous, displaying mature lymphocytic morphologic features and a preserved expression of the B lineage markers CD19, CD20, CD23 with additional expression of CD5 (217).

2.8.1 Pathogenesis and diagnosis

CLL can be considered as a leukemic form of lymphoma, although a proportion of patients lack lymph node engagement. The diagnostic criteria for CLL include the persistent presence of phenotypically distinctive clonal B cells of a minimum of $5x10^9$ cells/L in the peripheral blood. Therefore, the diagnosis can generally be established by means of a blood count, a differential count and immunophenotyping of peripheral blood by flow cytometry (219). In case the criterion of $5x10^9$ cells/L is not fulfilled, the diagnosis of monoclonal B lymphocytosis (MBL), a preclinical state of CLL, may be made (220, 221). Notably, MBL is not always associated with CLL transformation, but is accompanied by a risk of CLL progression of 1-2 percent per year (221). The solitary nodal manifestation of CLL, without bone marrow engagement and leukemia, is termed small cell lymphocytic lymphoma (SLL) and is clinically considered equivalent to CLL (219).

For a significant proportion of patients the diagnosis of CLL is the result of a blood count taken for other reasons. Indeed, the proportion of incidentally diagnosed patients has increased along with the development and accessibility of automated cell counting and flow cytometry.

Although still a matter of debate, the notion that CLL arises from pre-malignant hematopoietic stem cells (HSC) is gaining support (222). In other forms of cancer, including myeloid malignancies and acute lymphocytic leukemia (ALL), several lines of evidence imply that leukemogenesis occurs through the sequential acquisition of mutations commencing in HSCs (223-228), but this chain of events remains unproven for the development in B cell neoplasms (222). Recently, however, by using a xenotransplantation engraftment model, Kikushige and co-workers demonstrated that HSCs derived from patients with CLL gave rise to hematopoiesis biased towards the

B lineage with development of CLL-like clonal populations (229). Also, by using sequencing methodology, Damm *et al.* were able to detect an increased frequency of acquired mutations, including oncogenes of purported relevance for CLL pathogenesis, in sorted multipotent hematopoietic progenitor cells derived from CLL patients (230). These studies suggest the involvement of, and possible requirement for, the multi-step acquisition of pre-leukemic mutations of the HSC compartment in the pathogenesis of CLL.

2.8.2 Prognosis

For the vast majority of patients, CLL is an incurable disease. Thus, a long-standing paradigm has been not to initiate treatment unless called upon by signs of disease progression (219). This concept still applies, as earlier studies have failed to demonstrate a clinical benefit from preemptive treatment (231, 232). Also, approximately one third of all patients never acquire symptoms, and hence never require treatment. In these cases the clinical approach is watch-and-wait. According to current guidelines, treatment should be considered in case of symptoms or signs as (219):

- Nightly sweats
- Weight loss
- Fever
- Cytopenias due to bone marrow failure
- Splenomegaly
- Lymph node enlargement
- Rapid increase in lymphocyte counts

The clinical course of CLL is highly heterogeneous. Since the 1970s, the staging systems developed by Rai (233) and Binet (234) are widely used for CLL management and risk stratification. Based on a physical examination and a blood count, they provide an accessible tool to aid the decision of when to initiate treatment along with a rough estimation of the prognosis.

During the past decades, several prognostic biomarkers have emerged. These include serum markers such as β_2-microglobulin (β_2m) and serum thymidine kinase (sTK), phenotypic features as CD38 and the zeta chain associated protein kinase 70 (ZAP70), the elevation or increased expression of, respectively, predict inferior prognosis (235). Also, the mutational rearrangement status of the immunoglobulin heavy chain (IgVH) gene locus divides CLL patients into two distinct prognostic groups, with a low mutational level heralding poor prognosis (236). A clinically widespread prognostic assay is the fluorescence *in situ* hybridization (FISH)-based panel of molecular

cytogenetic aberrations that includes the genetic aberrations del(11q), trisomy 12, del(13q) and del(17p) (237).

However, the clinical utility of the vast majority of available staging systems and biomarkers is limited due to their poor capacity to predict treatment outcome. In fact, the only validated predictive biomarkers with significance for treatment outcome are the del(17)q and mutation of the TP53 locus (238). The presence of either aberration signals the loss of p53 function, a tumor suppressor protein critical for induction of cell death. In CLL, a deficient p53 is associated with dismal prognosis and inferior responsiveness to standard treatment regimens (238). Importantly, patients with loss of p53 function should be considered for allo-SCT since several studies have shown that allo-SCT may allow long-term survival also for this high-risk patient category (239).

2.8.3 Treatment

Several aspects, including age, expected life span and the comorbidities of the individual patient are considered when choosing the appropriate treatment. For younger patients the objective is to maximize the depth and duration of the treatment response, and thus prolong survival (240). Accordingly, a relatively high level of toxicity is acceptable. However, for older patients, or those with significant comorbidities, a minimum of toxicity is tolerated. Therefore, the aim of the treatment may be restricted to achieve symptom control and accepting a residual leukemic burden.

The treatment of CLL has evolved greatly in recent years, progress that has translated into improved clinical outcome (241). Randomized trials have demonstrated that chemoimmunotherapy, *i.e.* the combination of chemotherapy with mAbs, improves outcome in terms of response rates, progression-free survival and overall survival compared with chemotherapy alone (172, 242). The current standard of care for younger patients, excluding those carrying del(17p), is the combination of fludarabine, cyclophosphamide and rituximab (FC-R). Other combinatory regimens, including rituximab/ bendamustine are at hand when relapse occurs. For the youngest patients, and patients with particularly high-risk disease, allo-SCT should be considered (239, 240).

For the frail patients, the treatment options are fewer. The alkylating agent chlorambucil (Clb) is still widely used for these patients due to its tolerability, low price and easy oral administration. However, responses are typically shallow and short lasting. A recent phase III trial comparing Clb in combination with ofatumumab or rituximab to Clb as single-agent, demonstrated superior

efficacy of the combination regimens, but at the expense of increased significant toxicity (181). Hence, this combination is of limited use for treating the most fragile patients, yet also likely to be rejected for the more fit patients due to the availability of other, more efficacious options.

Newer drugs have recently been introduced, some of which are first-in-class small molecule drugs targeting the BCR signaling pathway. The Bruton's tyrosine kinase (BTK) inhibitor ibrutinib (243) and the phosphatidylinositide 3-kinase (PI3K) inhibitor idelalisib have shown impressive anti-leukemic efficacy along with tolerable side effects, also for more frail patients (244). Importantly, these drugs induce leukemic cell apoptosis in a p53 independent fashion and have indeed demonstrated efficacy also for patients with defective p53 function (239). These drugs are currently primarily used as first-line therapy for patients with p53 dysfunction and for relapsed patients, but their clinical utility is likely to increase as the result of ongoing and future studies.

2.8.4 CLL and immunity

CLL is associated with pronounced immunodeficiency, involving hypogammaglobulinemia and increased susceptibility to infections (245). There is also evidence of various malfunctions within the T cell and NK cell compartments, such as defective immunological synapse formation (246) and increased expression of inhibitory receptors, including CTLA-4, PD1 (247) and KIRs (248). Also, the NK cell population of CLL patients has been shown to display impaired cytotoxic activity (249), and a decreased expression of activating NK cell receptors reportedly correlates with anemia and a high lymphocyte count (250).

2.9 Chronic myelomonocytic leukemia

Chronic myelomonocytic leukemia (CMML) is a morphologically highly heterogeneous malignancy with overlapping myelodysplastic and myeloproliferative features (217). The persistent presence of clonal monocytes in the peripheral blood is a diagnostic criterion that is generally accompanied by variable degrees of myelodysplasia and myeloproliferation (217). The disease is rare, with an annual incidence below 1/100000 (251), but with markedly increasing incidence among the elderly. The median age at diagnosis is approximately 70 years, with a male to female predominance of 2:1.

Due to its biologic heterogeneity and features shared with other myeloid entities, the classification of CMML has historically been a matter of debate (252). Previously, CMML was classified among the myelodysplastic syndromes (MDS) (253), and the FAB classification system from 1994 made a distinction between the myelodysplastic (MD) and the myeloproliferative (MP) forms of CMML (254). The current classification by the World Health Organization (WHO) places CMML in the relatively new group of myelodysplastic/ myeloproliferative disorders (217).

2.9.1 Pathogenesis and diagnosis

CMML has convincingly been shown to arise from hematopoietic stem cells (224, 255-257) that undergo clonal or oligoclonal evolution and expansion (258). Detectable genomic aberrations are common but no pathognomonic mutational markers have been defined (259).

The diagnosis of CMML requires a bone marrow smear along with peripheral blood and differential counts. Immunophenotyping is not required but is commonly part of the initial workup. CD33, CD13 and CD14 are commonly expressed surface markers (259). The occurrence of a $CD34^+$ blast population of up to 19 percent in in the bone marrow is consistent with CMML diagnosis, while a blast fraction of 20 percent or more by defines AML, reflecting the close biologic relationship between CMML and AML. The frequency of blasts is also used to divide CMML into two categories; less than 5 percent blasts in the peripheral blood or 10 percent in the bone marrow defines CMML-1, while blast fractions exceeding these cut-off values define CMML-2 (217).

2.9.2 Prognosis

The prognosis of CMML is generally poor with median overall survival times ranging between 20 and 31 months from diagnosis (260, 261). Large variations

occur though, and some patients live for many years with no need for intervention (259). Transformation into secondary AML, with poor prospects of long-term survival, occurs in approximately one third of all patients (261, 262). Among identified risk factors for poor survival, the blast count is the most important, as it is strongly associated with AML development.

2.9.3 Treatment

Few efficacious treatment options exist for patients with CMML. Patients with myeloproliferation are commonly treated with the oral cytostatic agent hydroxyurea to suppress leukocyte counts and ease symptoms (263). The only treatment with curative potential is allo-SCT, conveying long-term disease-free survival for fractions between 29 and 40 percent in recently published retrospective studies of selected younger patients (4, 5, 164). However, as most patients are elderly, allo-SCT is usually not a realistic option. The hypomethylating agents azacitidine and decitabine have demonstrated clinical efficacy with reported over-all response rates of 43-51 percent (264, 265) and 25-58 percent (266, 267), respectively. However, hypomethylating agents have not been evaluated in randomized trials for treatment of CMML. Therefore, their impact on survival has not been evaluated.

2.9.4 CMML and immunity

Data on the functionality of anti-leukemic immune cells is scarce for CMML specifically, but one study by Marcondes *et al.* reported that NK cells derived from patients with CMML were inferior to those of healthy control regarding cytotoxicity (268). Likewise, Carlsten and co-workers demonstrated that NK cells derived from patients with MDS and CMML were impaired regarding cytotoxicity towards $CD34^+$ blasts, and displayed inferior expression levels of the NK cell activating receptors DNAM-1 and NKG2D (269).

3 Patients and methods

3.1 Patients

For papers II-IV patient blood samples were obtained and used for experimental purposes. The acquisition of patient samples was approved by the Ethical Review Board of Gothenburg and conducted after informed consent. Most patients enrolled were seen at the Hematology Section of Sahlgrenska University Hospital, and some patients were recruited by the physicians involved in the studies included in this thesis. Patients for whom the treating physician is the recruiting scientist calls for particular ethical consideration, as the invitation to participate in a research project may be perceived as a stressful and demanding request. Therefore, in asking for patient participation, voluntariness has been particularly emphasized, and the fact underscored that declining would not affect medical care.

Recruitment of patients by the treating physician also requires caution regarding the risk of a biased patient selection. For reasons of accessibility, in the papers II and III, which concern CLL, all patients were asymptomatic, Binet stage A and not undergoing treatment at the time of participation.

For paper IV, regarding CMML, a more heterogeneous cohort was recruited, including patients without symptoms and no concomitant treatment, patients with ongoing treatment with hydroxyurea and one patient who had relapsed following allo-SCT.

3.2 Methods

3.2.1 Assessment of ROS

The method for assessing NADPH oxidase activity in myeloid cells by chemiluminescense was developed by Dahlgren and co-workers more than three decades ago. By measuring the light emission following addition of a chemiluminescent reagent to myeloid cells in the presence of horseradish peroxidase (HRP), the production of superoxide by the NADPH oxidase can be dynamically monitored, and the total ROS production calculated as the area under the curve (AUC). By using the hydrophilic luminescent reagent isoluminol, which is incapable of penetrating the cell membrane, exclusively extracellular ROS are measured (156).

3.2.2 Flow cytometry

Flow cytometry enables high-throughput analyses of a multitude of cellular properties, including information of size, granular complexity and phenotypic expression of extra- and intracellular structures. In this thesis, flow cytometry was used for assessment of lymphocyte death, cytotoxicity assays, immunophenotyping and functional assessments by quantification of intracellular enzymes and proteins.

Furthermore, fluorescence-activated cell sorting (FACS), a technique by which isolation of highly purified cell subsets can be obtained, was utilized for papers III and IV.

3.2.3 Lymphocyte cell death

Lymphocyte suppression by myeloid cells was assessed as the proportion of apoptotic lymphocytes after co-cultures with myeloid cells at various ratios. To distinguish between apoptotic and viable cells, the samples were stained with an amine-reactive dye, which is impermeable to the intact membranes of live cells, but enters and stains the intracellular structures of permeabilized, apoptotic cells. The samples were then analyzed by flow cytometry. Although cell surface proteins will also react with the dye, the difference in staining intensity between dead and viable cells is distinct.

This method thus requires staining at a time point sufficiently late for membrane integrity to be lost. Thus, staining and analysis was performed after 16-18 hours of incubation. One limitation of this method is that cells that have

undergone complete disintegration will be lost to analysis. Thus, the risk of underestimating the number of dead cells should be considered.

3.2.4 Cytotoxicity assays

In cytotoxicity experiments, target cell death is the primary endpoint. For this thesis, several different ADCC assays were performed, investigating the efficacy of NK cell-mediated killing of various malignant cells, including the 721.221 B lineage lymphoblastoid cell line (270) and primary leukemic cells derived from patients with CLL and CMML. To enable cell death assessment by flow cytometry, the target cells were labeled with a fluorescent dye prior to incubation. After four hours, the incubation was aborted, and the samples subjected to LIVE/DEAD staining. Thus, this method was used to assess the proportion of target cells with lysed cell membranes. In some experiments, NK cell degranulation was assessed along with target cell lysis.

A key issue with cytotoxicity assays is determining the appropriate time for the cell death read-out. As membrane permeabilization is a late apoptotic event, four-hour cytotoxicity assays were consistently used. Longer incubation times may result in increased target cell killing, but are likely to result in lysed cells being fragmentized, and therefore lost to analysis. Hence, lengthier assays would carry the risk of underestimating cell death by this method.

The expression of CD107a on the cell surface is a marker for degranulation of cytotoxic granules, an event that correlates with target cell death (271). By measuring the NK cell expression of CD107a by flow cytometry thus allows for assessment of NK cell cytotoxic activity. This may be an advantage if this is the primary study objective. However, as degranulation does not necessarily translate into target cell lysis, the method should be used as a complement to other cytotoxicity assays, and not a replacement.

3.2.5 General considerations

This thesis is based on results obtained *in vitro*. Experimental laboratory research may be of significant scientific value, as laboratory conditions provide the possibility to investigate biologic processes under highly controlled circumstances. However, the physiologic and clinical relevance of any *in vitro* experimental approach can righteously be questioned, and may require validation by studies in animals, or ideally, in humans.

4 Results and discussion

4.1 Role of MAPKs in lymphocyte death

Oxidant-induced lymphocyte cell death by myeloid cells has been proposed to occur via a caspase-independent pathway involving the nuclear DNA repair enzyme PARP-1 (90). In response to excessive DNA damage, PARP-1 is over-activated, resulting in the translocation of PAR into the cytosol followed by mitochondrial AIF release and regulated cell death (272) (refer to section 2.4.2). As oxygen radicals are known to inflict DNA damage (41) a causal relationship between ROS, DNA damage and the PAR/AIF axis has been assumed, but the events linking ROS and DNA damage in parthanatic cell death are incompletely understood.

Oxidants have important roles as signaling molecules and have been shown to affect pathways involving MAP kinase signaling (43). In cell-free experiments, Cohen-Armon *et al.* showed that activation of ERK1/2, a member of the MAPK family, resulted in PARP-1 activation independently of DNA damage, thus suggesting a link between the MAPKs and PARP-1 activation (273). With this background, we aimed to evaluate whether ROS-induced lymphocyte death results from MAPK signaling rather than excessive DNA-damage.

First, to investigate the roles of different MAPKs in lymphocyte death, we specifically inhibited MEK1/2, p38 and JNK before exposing lymphocytes to H_2O_2. We found that inhibition of MEK1/2, the enzyme responsible for activating ERK1/2, significantly preserved lymphocyte viability, while inhibition of p38 or JNK did not. In co-culture experiments with lymphocytes and ROS-producing monocytes, inhibition of MEK1/2 markedly upheld lymphocyte viability, corroborating the results obtained with H_2O_2 (figure 4). These findings thus supported a role of the MEK/ERK pathway in the chain of events leading from ROS to lymphocyte death. We therefore sought to determine whether lymphocyte exposure to ROS resulted in ERK1/2 activation. By using flow cytometry following intracellular staining for activated, *i.e.* phosphorylated ERK1/2 (pERK), a significant, transient induction of pERK was registered in response to H_2O_2 exposure. The results were confirmed by western blot methodology.

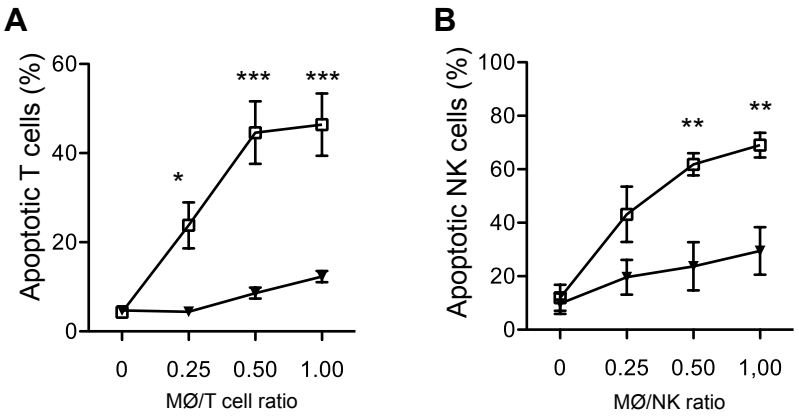

Figure 4. Inhibition of the ERK1/2 pathway protects lymphocytes from myeloid cell-induced death. T cells (A) or NK cells (B) were co-cultured with monocytes overnight in presence or absence of the MEK-inhibitor PD98059 (filled triangles) or control (DMSO, open squares).

In the following two series of experiments, we explored the relationship between ERK1/2 and PARP-1 by exposing lymphocytes to ROS in presence or absence of inhibitors of either MEK1/2 or PARP-1. In line with our prior observations, we found that inhibition of either enzyme prevented the accumulation of intracellular PAR, as determined by flow cytometry, thus underscoring a role of ERK1/2 in parthanatos. Importantly though, inhibition of MEK1/2, but not PARP-1, prevented ERK1/2 activation, suggesting ERK1/2 being upstream of PARP-1 in the signal transduction leading from ROS to PARP-1 (figure 5).

We also determined the functionality of NK cells rescued from ROS induced death by inhibition of MEK1/2. For this purpose we exposed NK cells to monocytes overnight in presence or absence of a MEK inhibitor. Subsequently, the NK cells were assessed for cytotoxicity as determined by killing of the B lymphoblastic cell line 721.221 (270) in presence of rituximab. These experiments confirmed that the NK cells rescued by the MEK1/2 inhibitor were functional in terms of cytotoxicity.

Taken together, the results of **paper I** suggest that the MEK/ERK signaling pathway is involved in ROS-mediated parthanatos of lymphocytes, and that activation of ERK occurs prior to PARP-1 activation in response to ROS. These findings thus challenge the view of ROS-induced PARP-1 activation as being a direct consequence of excessive DNA damage. Instead, the results underscore the role of ROS as signaling substances affecting pathways of vital importance for cell survival.

The strategy to protect lymphocytes from cell death by preventing ROS production has demonstrated clinical efficacy for treatment of AML (10). A conceivable alternative approach to preserve lymphocyte viability is to increase lymphocyte resistance to ROS by intervention with transductional pathways conveying ROS-mediated death. The results displayed in **paper I** indicate that preventing ERK1/2 activation, by inhibiting MEK1/2 a feasible approach to uphold lymphocyte viability in the presence of ROS-producing myeloid cells. Inhibition of MEK1/2 is being explored therapeutically, and has demonstrated efficacy as treatment of melanoma (274). Our results imply that a contributing mechanism of action for MEK inhibitors may be to uphold lymphocyte capacity to withstand myeloid-derived ROS.

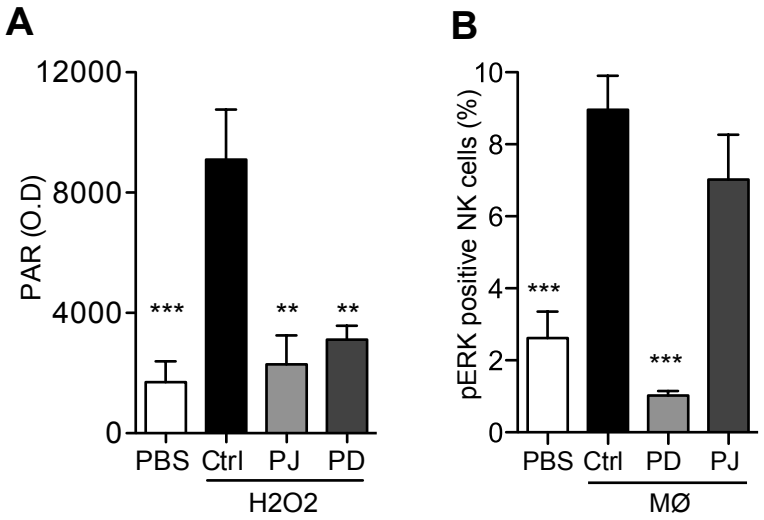

Figure 5. ERK is upstream of PARP-1 in ROS-induced lymphocyte death. (A) Accumulation of PAR was prevented by inhibition of either PARP-1 (PJ34) or the ERK pathway (PD98059). (B) Inhibition of MEK/ERK, but not PARP-1, prevented ERK phosphorylation in response to ROS.

4.2 CD20 antibodies trigger ROS production

As noted previously, the addition of CD20 mAbs to chemotherapy has improved the outcome for patients with CLL (240, 241), but the efficacy of rituximab as single agent is limited (174-176). Several lines of evidence have demonstrated that immune cells carrying FcRs contribute to the efficacy of mAbs by their ability to exert ADCC or ADCP (68, 80, 81, 275). However, the distinct roles of individual immune cells for mAb efficacy are incompletely defined. NK cell cytotoxicity and viability have been shown to be affected by myeloid cells in a ROS-dependent fashion (9), which seem to suppress NK cell cytotoxicity rather than add to target cell killing (208, 276). Therefore, we hypothesized that myeloid cells might limit the efficacy of CD20 mAbs in CLL by restricting the cytotoxic activity of NK cells by ROS-production. We addressed this hypothesis by assessing the effects of myeloid cell-derived ROS on NK cell functionality and viability in the presence of therapeutic mAbs. The results, presented in **papers II and III**, were partly obtained concomitantly.

We commenced by investigating the impact of monocytes on NK cell mediated ADCC using NK cell and cells derived from healthy blood donors. In a series of experiments using NK cells against the B lymphoblastic cell line 721.221 (270) and rituximab as the linking antibody, we observed that ADCC was largely abrogated in the presence of monocytes. The inhibitory effect was partly reversed by the ROS scavenger catalase, or by prevention of ROS formation by HDC, thus suggesting monocyte-derived ROS as the main mechanism of inhibition. These findings incited us to investigate whether the results could be reproduced using CLL cells as target cells and patient-derived NK cells and monocytes. By using fluorescence-activated cell sorting (FACS) we isolated NK cells and monocytes from PBMCs derived from CLL patients. From the same PBMC fraction, leukemic cells were isolated by immunomagnetic depletion, thus allowing a cytotoxicity assay using autologous cells. CLL cell lysis was assessed by four-hour assays in the presence or absence of rituximab and the

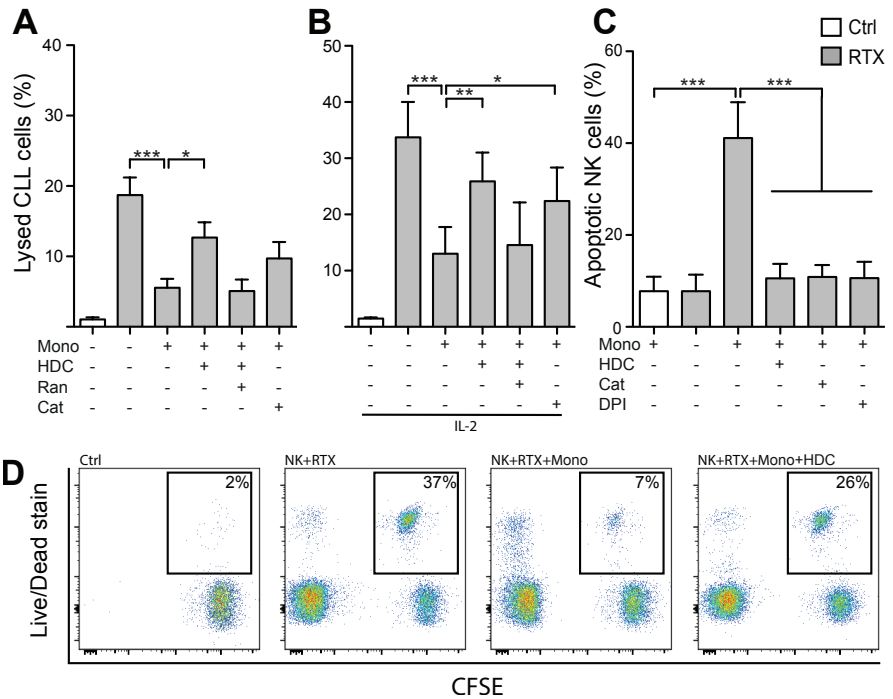

Figure 6. Monocytes impede NK cell-mediated ADCC against primary CLL cells by ROS production. (A) NK cell-mediated ADCC) of autologous leukemic cells by rituximab (RTX) in the presence or absence of monocytes, HDC, catalase (Cat), H_2R-antagonist ranitidine (Ran) and IL-2 (B). (C) NK cell death after overnight incubation with monocytes in the presence of immobilized rituximab and the anti-oxidative compounds HDC, catalase and DPI. (D) Representative FACS dot-plot of the readout for dead, CFSE-labeled CLL cells by live/dead staining.

NK cell activating cytokine IL-2. The results from these experiments showed that the presence of monocytes strongly reduced ADCC, both in the presence and absence of IL-2, thus confirming the findings obtained using the 721.221 cell line. HDC and catalase significantly upheld ADCC, demonstrating inhibition to be mainly ROS-mediated (figure 6A and B).

Golay *et al.* have shown that CD20 mAbs vary regarding their propensity to induce activation of neutrophils (277). For example, these authors demonstrated that obinutuzumab, which was recently approved for use in CLL, was capable of more efficiently activating neutrophils than rituximab in an FcR-dependent fashion. It was claimed that activation was triggered without concomitant ROS production. However, the authors had used a FACS-based assay, measuring intracellular ROS. This incited us to assess neutrophil ROS production in response to CD20 mAbs by using isoluminol-enhanced chemiluminescense, a sensitive method for assessment of extracellular ROS (156). Indeed, we found that neutrophils responded to mAbs by substantial release of ROS. With the aim of investigating the immunosuppressive properties of mAb-exposed neutrophils, we proceeded by performing co-culture experiments with neutrophils and NK cells in presence of rituximab and ofatumumab. These experiments revealed that neutrophils exposed to CD20 mAbs induced significant ROS-dependent NK cell death, while unexposed neutrophils did not (figure 7).

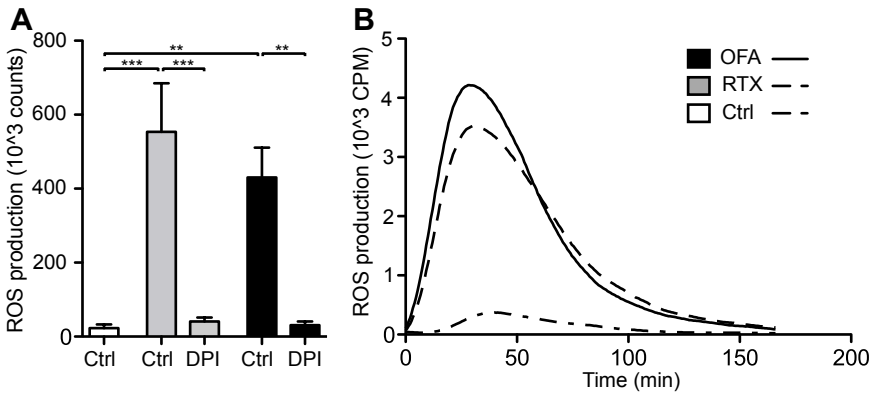

Figure 7. CD20 mAbs trigger ROS-production in neutrophils. (A) Extracellular ROS-production from neutrophils derived from patients with CLL in response to immobilized, plate-bound rituximab (RTX) and ofatumumab (OFA) in the presence or absence of the NADPH oxidase inhibitor DPI.

The findings that mAbs triggered ROS by neutrophils were consistent with the results that monocytes were found to impede ADCC in a ROS-dependent fashion. Therefore, we assessed the effect of mAbs on monocyte ROS production, and similarly found a marked ROS-release in response to mAbs. Co-culture experiments with monocytes and NK cells revealed that mAb-induced ROS production by monocytes translated into immune suppression in terms of augmented ROS-dependent NK cell death in the presence of mAbs. These immunosuppressive events were significantly prevented by the presence of HDC (figure 6C).

To investigate the mechanism of ROS-induction by CD20 mAbs, $F(ab')_2$-fragments, *i.e.* antibody fragments devoid of the Fc-region, were obtained by pepsin digestion (278), and used as control reagent in ROS-assessment and apoptosis experiments. OFA-derived $F(ab')_2$ fragments were found not to induce ROS-release, suggesting that CD20-mAb ROS induction to be FcR-mediated (figure 8).

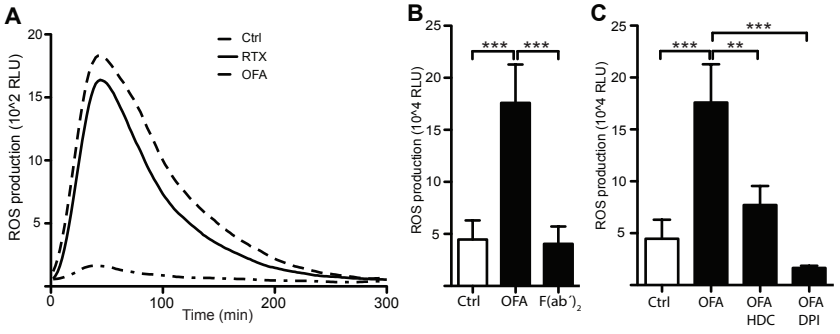

Figure 8. MAb-induced ROS production by monocytes is dependent on Fc-receptors and preventable by anti-oxidative agents. (A-C) Extracellular ROS-production by monocytes in response CD20-mAbs. (B) ROS production is triggered by OFA, but not OFA-derived $F(ab')_2$ fragments, suggesting that ROS production is FcR-dependent. (C) ROS production is prevented by NADPH oxidase inhibitors HDC or DPI.

Taken together, the results of **papers II** and **III** imply that therapy with CD20 mAbs contributes to an oxidative immunosuppressive environment, which may affect NK cell function and viability. Thereby, the full anti-leukemic potential of mAbs may by limited. HDC and other ROS-inhibitors reduced immunosuppression by preventing ROS formation in the presence of CD20 mAbs. These results suggest that the efficacy of CD20 mAbs might increase with the addition of anti-oxidative therapy, and that this immunotherapeutic strategy should be further investigated.

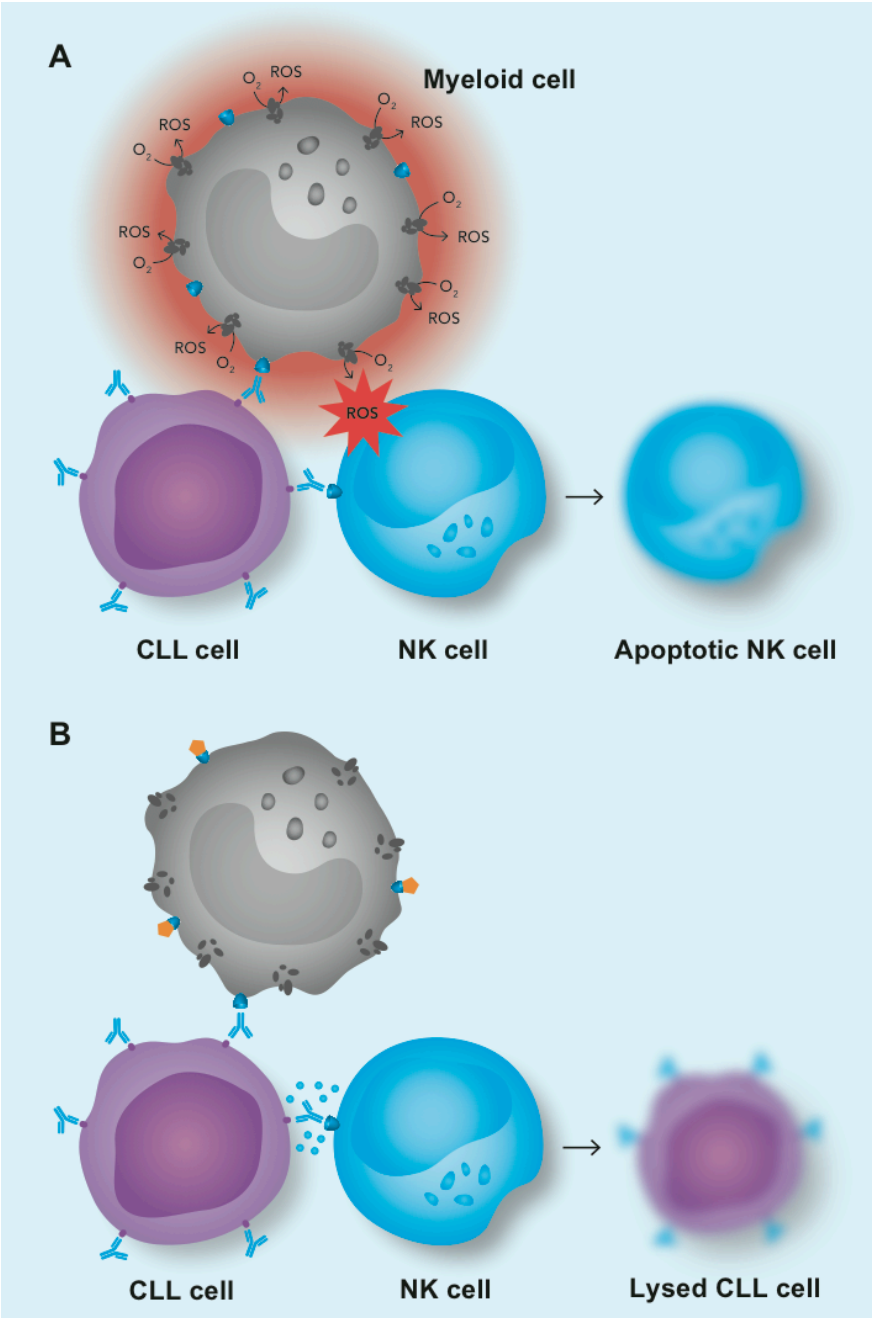

Figure 9. Proposed mechanism of mAb-induced immunosuppression. (A) In the presence of myeloid cells, mAbs trigger ROS-production resulting in NK cell death. (B) ADCC is preserved by preventing ROS production by HDC.

4.3 Role of ROS in CMML

Monocytic subtypes of AML (M4/M5) have been reported to respond favorably to HDC/IL-2 immunotherapy compared to other AML subtypes (12). This fact has been attributed to the immunosuppressive properties demonstrated for monocytic leukemic cell subsets by their ability to produce immunosuppressive extracellular radicals, and the responsiveness of these subsets to HDC, that reduces ROS formation (12, 13). CMML shares many features with monocytic AML, including the occurrence of monocytic leukemic differentiation (217). The aim of **paper IV** was to investigate the role of ROS as a putative mechanism of immune escape in CMML.

First, by using flow cytometry to characterize the leukemic subsets of CMML, we found that the $CD33^+/CD14^+$ mature monocytic population, but not the $CD33^+/CD34^+$ blasts, co-expressed the NADPH oxidase and H_2Rs, thus suggesting that the monocytic clone is capable of ROS production and might respond to HDC. These findings were confirmed by ROS measurements using chemiluminescense, as monocytes were found to produce significant amounts of extracellular ROS upon activation with *N*-formylmethionyl-leucyl-phenyl-alanine (fMLF), in the absence but not in the presence of HDC (figure 10).

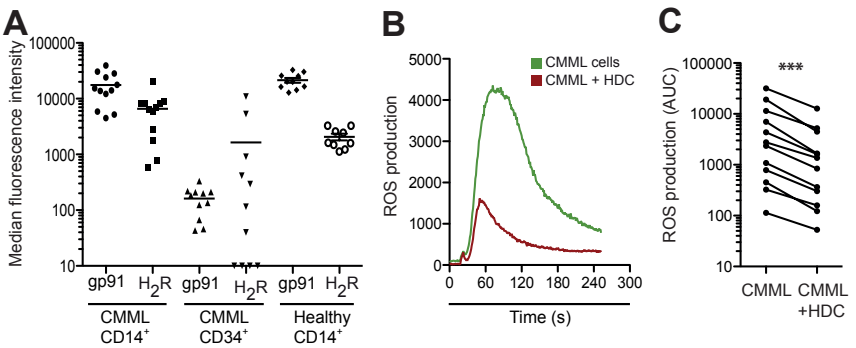

Figure 10. (A) Expression of the NADPH oxidase subunit $gp91^{phox}$ and histamine 2-receptors (H_2Rs) by leukemic cells subsets from patients with CMML compared to monocytes from healthy controls. (B and C) ROS production by CMML-derived monocytes response to fMLF in presence and absence of HDC.

Next, the immunosuppressive features of CMML-derived monocytes were assessed in co-cultures with different lymphocyte subsets. These leukemic cells were found to induce substantial PARP-1 dependent cell death in NK cells, $CD4^+$ and $CD8^+$ T cells. Cell death was ROS-mediated as the ROS scavenging enzyme catalase or prevention of ROS formation by HDC or the NADPH oxidase inhibitor DPI prevented death in all lymphocyte subsets (figure 11). Together, these observations imply that the monocytic leukemic subset may suppress anti-leukemic lymphocytes via extracellular ROS release, and that leukemia-induced ROS formation may be a mechanism of immune escape in CMML.

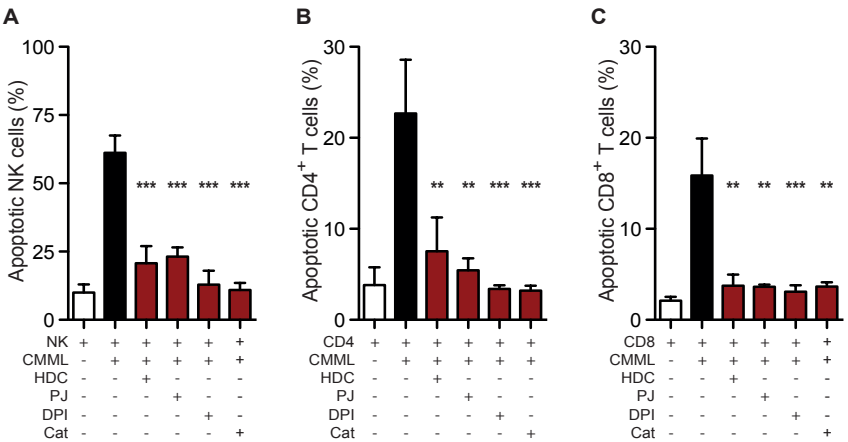

Figure 11. CMML cells induce ROS-mediated, PARP-1-dependent cell death in lymphocytes. Lymphocytes were incubated overnight with monocytes derived from patients with CMML in the presence or absence of HDC, PARP-1 inhibitor PJ34, DPI or catalase.

To clarify whether anti-oxidative intervention may augment the anti-leukemic immune efficacy of cytotoxic effector cells, we performed cytotoxicity experiments using NK cells as effector cells and monocytic leukemic cells as target cells. The anti-CD33 mAb lintuzumab was used as the linking mAb to trigger NK cell-mediated ADCC. These experiments showed that the anti-leukemic activity of IL-2-stimulated NK cells was significantly augmented in the presence of HDC, as measured by degranulation. However, the increase in NK cell degranulation merely translated into a non-significant trend regarding target cell lysis. This may be explained by a low number of experiments, but raises the question whether these leukemic cells may be resistant to NK cell cytotoxicity. It is also conceivable that the apoptotic process is particularly slow for these cells, and that a method for assessing earlier apoptotic events, *e.g.* loss

of mitochondrial membrane potential or by staining with Annexin V, would have yielded more pronounced results. These issues are to be further investigated.

Oxygen radicals have been demonstrated to inflict down-regulation of activating NK cell receptors (157). Moreover, reduced expression of activating receptors was previously demonstrated in NK cells derived from patients with AML (211, 212) and MDS (269). This incited us to assess the expression of activating NK cell receptors of patients with CMML. By immunophenotyping of patient samples along with samples from age- and sex- matched control individuals, we observed that the patient samples displayed significant impairments in their expression of NKp30, NKp80 and NKp46 with a similar, yet non-significant, trend for NKp46. Also, the frequency of NK cells expressing NKp30, NKp46 and NKp80 was found to be lower in patients than in healthy subjects. Notably, by culturing NK cells derived from CMML patients with IL-2, the NCRs NKp30 and NKp46 were up-regulated, implying that the NK cell compartment in CMML is not permanently hampered and that immunostimulatory intervention may restore leukemia-associated immunodeficiencies.

Taken together, our results demonstrate that patients with CMML harbor a leukemic cell subset with pronounced immunosuppressive potential towards anti-leukemic lymphocytes. Immune suppression was ROS-mediated as demonstrated by the preventive effect of HDC and other ROS-inhibitors. These findings suggest that anti-oxidative immunotherapy may be efficacious in CMML, as previously demonstrated for AML (10, 12).

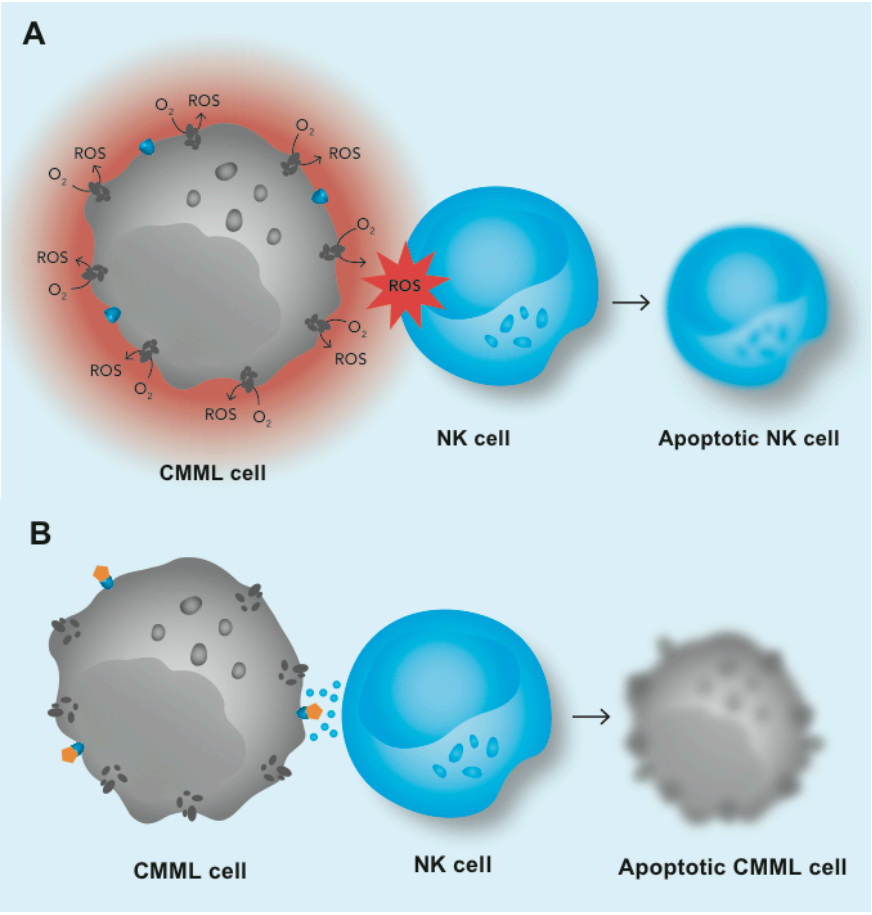

Figure 12. Proposed mechanism of immune escape in CMML. (A) Leukemia-derived ROS induce lymphocyte death. (B) Anti-leukemic lymphocytes are preserved by anti-oxidative intervention with HDC.

5 Concluding remarks

Physiologic systems are commonly governed by multiple pathways, which preserve homeostasis and functionality. In immunity, this notion is illustrated by the multitude of regulatory functions that prevent autoimmunity and excessive inflammation. Moreover, clonal evolution, driven by the selective pressure of the surrounding environment, promotes the survival of clones with low immunogenicity and immunosuppressive traits. Consequently, the immune system almost invariably fails to mount an immune response sufficient to reject clinically overt cancer.

The strategy to target cancer-related immune tolerance has gained momentum in recent years. Treatment with HDC, aiming at protecting NK cells and T cells from oxidative inhibition, has been introduced in AML therapy (10) and antibodies, such as ipilimumab and nivolumab, targeting immunosuppressive pathways of relevance to T and NK cell function, have shown significant efficacy in advanced solid cancer (196, 197). These findings provide an incentive to further explore the immunosuppressive mechanisms of relevance to cancer development. Importantly, the fact that mechanisms of immune evasion are not specific to a certain disease encourages the investigation of immunotherapies in multiple malignancies.

Oxidants mediate suppression of lymphocytes, and can be targeted by the NADPH oxidase inhibitor HDC (11). As shown in paper I, we found a prominent role of the MEK/ERK pathway in the transductional events resulting in ROS-induced lymphocyte death. These results imply that inhibition of these signaling events is a conceivable alternative strategy to uphold lymphocyte viability in an oxidative, immunosuppressive environment.

We also found that immunotherapy with anti-CD20 mAbs induced ROS production by neutrophils and monocytes, which translated into immuno–suppressive events including interference with NK cell-mediated ADCC against primary CLL cells. The observation that HDC restored NK cell cytotoxicity suggests that CD20 mAbs and HDC, or other strategies to reduce antibody-induced ROS production, is an immunotherapeutic combination for CLL and other B cell malignancies that merits further investigation.

Our findings are consistent with a mainly immunosuppressive role for monocytes and neutrophils in the context of mAb-based immunotherapy. It should be emphasized, however, that myeloid cells have been shown to

contribute to mAb-mediated elimination via ADCP and ADCC (182), processes that partly rely on ROS-production. For HDC to augment the clinical efficacy of mAbs it is thus required that the alleviation of ROS production results in an increment in NK cell ADCC that exceeds the potential inhibitory effects on leukemic cell killing by myeloid cells. This issue should be considered in further investigating mAbs and anti-oxidative therapy as a putative immunotherapeutic combination.

We investigated the role of oxygen radicals as an immunosuppressive mechanism in CMML, a disease for which the presence of a monocytic leukemic population is a defining criterion (217). We found that the monocytic leukemic cells produced substantial amounts of ROS and significantly suppressed lymphocytic subsets in a ROS-dependent fashion. The addition of HDC reduced ROS production and maintained lymphocyte viability in the presence of leukemic cells. These observations suggest that leukemia-induced ROS production may serve as a mechanism of immune escape that promotes progression of CMML. Furthermore, our results demonstrate that ROS formation by malignant cells is a feature that is shared between CMML and monocytic forms of AML, in which immunotherapy with HDC and IL-2 has demonstrated particular clinical efficacy (12, 13). Therefore, we hypothesize that HDC and IL-2 may be efficacious also in CMML. To address this hypothesis, an exploratory clinical trial with HDC and IL-2 for the treatment of CMML has been initiated (Appendix).

In AML, the HDC/IL-2 regimen has demonstrated relapse-preventive potential, but has not been evaluated as treatment of manifest leukemia. However, CMML commonly advances slowly, which may allow for an anti-leukemic immune response to be mounted, and for immunotherapy to be efficacious also in situations with a significant clonal burden.

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