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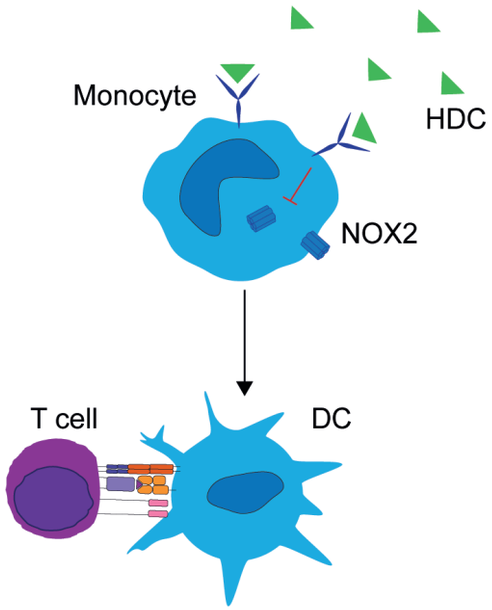

Redox reactions in cancer: impact and regulation

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The reduction-oxidation (redox) reaction involves a change in the oxidation state of molecules where a molecule that donates an electron is oxidized and a molecule that accepts an electron is reduced. The NADPH oxidase of myeloid cells, NOX2, is a major source of oxidants in the form of reactive oxygen species (ROS), which are short-lived oxygen derivatives. NOX2-derived ROS have been ascribed a pivotal role in the elimination of pathogens and may be toxic also to host cells and tissues. ROS may also act as signaling molecules and thus regulate biological processes such as cell cycle proliferation, differentiation, cell death, blood vessel formation, and immunity. The purpose of this thesis was to contribute to the understanding of the impact and regulation of redox reactions in cancer with focus on the role of NOX2. The studies have comprised cells and animals that were genetically or pharmacologically deprived of NOX2 activity, and attempts were made to define the significance of the findings in a clinical setting. The results presented in **paper I** imply that ROS may inhibit the maturation of monocytes into antigen-presenting dendritic cells, which may favor tumor growth *in vivo*. **Paper II** reports that treatment of mice with the NOX2 inhibitor histamine dihydrochloride (HDC) resulted in reduced expansion and reduced immunosuppressive activity of myeloid-derived suppressor cells. Treatment of mice with HDC also improved the efficacy of checkpoint inhibitors to reduce the growth of murine lymphoma and colon cancer. The results of **paper III** suggest that HDC, by targeting NOX2-derived ROS, promotes the differentiation of acute myeloid leukemia (AML) cells *in vitro* and *in vivo*, thus implying that the intrinsic formation of ROS by AML cells contributes to their malignant features. In **paper IV** it is reported that functional NOX2 is relevant to the induction of chronic myeloid leukemia by murine *BCR-ABL1*$^{+}$ cells. In conclusion, these results support that NOX2 is a conceivable therapeutic target in cancer.

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ABSTRACT

The reduction-oxidation (redox) reaction involves a change in the oxidation state of molecules where a molecule that donates an electron is oxidized and a molecule that accepts an electron is reduced. The NADPH oxidase of myeloid cells, NOX2, is a major source of oxidants in the form of reactive oxygen species (ROS), which are short-lived oxygen derivatives. NOX2-derived ROS have been ascribed a pivotal role in the elimination of pathogens and may be toxic also to host cells and tissues. ROS may also act as signaling molecules and thus regulate biological processes such as cell cycle proliferation, differentiation, cell death, blood vessel formation, and immunity. The purpose of this thesis was to contribute to the understanding of the impact and regulation of redox reactions in cancer with focus on the role of NOX2. The studies have comprised cells and animals that were genetically or pharmacologically deprived of NOX2 activity, and attempts were made to define the significance of the findings in a clinical setting. The results presented in **paper I** imply that ROS may inhibit the maturation of monocytes into antigen-presenting dendritic cells, which may favor tumor growth *in vivo*. **Paper II** reports that treatment of mice with the NOX2 inhibitor histamine dihydrochloride (HDC) resulted in reduced expansion and reduced immunosuppressive activity of myeloid-derived suppressor cells. Treatment of mice with HDC also improved the efficacy of checkpoint inhibitors to reduce the growth of murine lymphoma and colon cancer. The results of **paper III** suggest that HDC, by targeting NOX2-derived ROS, promotes the differentiation of acute myeloid leukemia (AML) cells *in vitro* and *in vivo*, thus implying that the intrinsic formation of ROS by AML cells contributes to their malignant features. In **paper IV** it is reported that functional NOX2 is relevant to the induction of chronic myeloid leukemia by murine *BCR-ABL1*$^{+}$ cells. In conclusion, these results support that NOX2 is a conceivable therapeutic target in cancer.

Keywords: Cancer, immunotherapy, reactive oxygen species, NOX2, histamine dihydrochloride, myeloid-derived suppressor cells, checkpoint inhibition, acute myeloid leukemia, chronic myeloid leukemia

SAMMANFATTNING PÅ SVENSKA

Mer än var tredje person kommer under sin livstid att drabbas av cancer och cancer orsakar cirka 20 % av alla dödsfall i västvärlden. Traditionell cancerbehandling såsom kirurgi, cellgifter och strålning, har gjorts mer effektiv under de senaste årtiondena, vilket har bidragit till att prognosen har förbättrats vid flera cancerformer. Därtill har nyare behandlingar introducerats såsom att hämma cancercellers felreglerade proteiner eller att eliminera tillväxtfaktorer som cancerceller behöver för överlevnad. Denna avhandling handlar i första hand om immunoterapi, som är en sammanfattande benämning för behandlingar som avser att stimulera kroppens immunsystem till att avlägsna cancerceller.

Immunsystemet omfattar celler med förmåga att eliminera cancerceller. Flera metoder har föreslagits för att farmakologiskt aktivera de immunceller som tycks vara mest effektiva mot cancerceller, d.v.s. cytotoxiska T-celler och natural killer (NK)-celler. Dessa cellers funktion är dock ofta undertryckt i tumörvävnad, p.g.a. hämmande signaler från cancerceller eller från tumörinfiltrerande myeloida celler. För att immunförsvaret mot cancerceller ska kunna aktiveras är det av vikt att identifiera och motverka dessa hämmande signaler.

En sådan signal förmedlas av reaktiva syreradikaler (ROS) producerade av myeloiska celler, såsom makrofager och granulocyter, som ofta infiltrerar tumörvävnad. Om ROS bildas i en tumör kan T- och NK-celler inaktiveras och därmed riskerar behandling som aktiverar dessa celler att bli mindre effektiv eller verkningslös. Därtill kan vissa cancerceller, t.ex. vid akut och kronisk myeloisk leukemi (AML och KML), själva producera ROS vilket kan medföra att dessa celler undgår att elimineras av T- och NK-celler. Om ROS-produktion i tumörer hämmas kan därför immunstimulerande behandling vid cancer bli mer effektiv. ROS är därtill viktiga signalmolekyler i celler och modulerar proteiners funktion, men kan också skada DNA-molekyler så att mutationer uppkommer.

Mitt avhandlingsarbete har syftat till att bidra till kunskap om hur ROS som bildas av ett enzym i myeloiska celler, NOX2, påverkar cancerutveckling. Jag har använt cancerceller och försöksdjur som modifierats genetiskt för att undertrycka NOX2 samt substanser som hämmar NOX2-aktivitet. Resultaten talar för i) att NOX2 har betydelse för dendritiska cellers funktion, som i sin tur är avgörande för T-cellers funktion, ii) att NOX2-aktivitet undertrycker T-cellsfunktion i tumörvävnad, iii) att inhibition av NOX2 underlättar leukemicellers differentiering och därmed motverkar leukemi i försöksdjur, iv) att funktionellt NOX2 påskyndar utveckling av KML och v) att farmakologisk inhibition av NOX2 hämmar tumörtillväxt och därtill gör modern immunoterapi mer effektiv.

LIST OF PAPERS

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

I. Martner A, **Grauers Wiktorin H**, Lenox B, Ewald Sander F, Aydin E, Aurelius J, Thorén FB, Ståhlberg A, Hermodsson S, Hellstrand K. Histamine promotes the development of monocyte-derived dendritic cells and reduces tumor growth by targeting the myeloid NADPH oxidase.
J Immunol 2015;194(10):5014-5021

II. **Grauers Wiktorin H**, Nilsson MS, Kiffin R, Ewald Sander F, Lenox B, Rydström A, Hellstrand K, Martner A. Histamine targets myeloid-derived suppressor cells and improves the anti-tumor efficacy of PD-1/PD-L1 checkpoint blockade.
Accepted for publication in Cancer Immunology Immunotherapy 2018

III. Kiffin R, **Grauers Wiktorin H**, Nilsson MS, Aurelius J, Aydin E, Lenox B, Nilsson JA, Ståhlberg A, Thorén FB, Hellstrand K, Martner A. Anti-leukemic properties of histamine in monocytic leukemia: The role of NOX2.
Front Oncol 2018;8(JUN):218

IV. **Grauers Wiktorin H**, Nilsson T, Aydin E, Hellstrand K, Palmqvist L, Martner A. Role of NOX2 for leukaemic expansion in a murine model of BCR-ABL1^{+} leukaemia.
Br J Haematol 2018;182(2):290-294

Additional publications not part of this thesis:

SI **Grauers Wiktorin H**, Nilsson T, Jansson A, Palmqvist L, Martner A. Mutated NPM1 in combination with overexpression of Meis1 or Hoxa9 is not sufficient to induce acute myeloid leukemia.
Exp Hematol Oncol 2016;5(1):25

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

CONTENT

ABBREVIATIONS

AML	Acute myeloid leukemia
APC	Antigen-presenting cell
BM	Bone marrow
CML	Chronic myeloid leukemia
CR	Complete remission
DC	Dendritic cell
HDC	Histamine dihydrochloride
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
KIR	Killer cell immunoglobulin-like receptor
KO	Knock-out
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NK cell	Natural killer cell
NOX2	NADPH oxidase type 2
ROS	Reactive oxygen species
TCR	T cell receptor
TIL	Tumor-infiltrating lymphocyte
TME	Tumor microenvironment
WT	Wild-type

INTRODUCTION

CANCER

There exist more than 100 forms of cancer originating from various tissues and cell types, all with distinct characteristics. The transformation of a healthy cell to a malignant cell encompasses the acquisition of several features referred to as the "hallmarks of cancer" (1). These include sustained proliferation, resistance against apoptosis and immune cell destruction, invasion of adjacent tissues and the capacity to spread to other organs by metastasis, enhanced angiogenesis, deregulation of cellular energetics, and genomic instability (2). The characteristics of leukemias, cancers of blood-forming cells, differ from those of solid tumors in that leukemic cells rarely infiltrate tissues or form solid tumors in distant organs.

The past decades have seen significant progress in the understanding of how mutations give rise to malignant tumors. In 1976, Peter Nowell forwarded the clonal evolution theory proposing that cancer development is a multistep process initiated by a primary mutation in a single cell followed by the acquisition of additional alterations in daughter cells resulting in a clonal disorder where the daughter cells are no longer genetically identical (3). While most genetic variants will be deleterious to the survival of the mutated cells, certain genetic alterations may provide potentially malignant cells with advantageous genotypes and phenotypes and following natural selection, the fittest subclones will prevail and expand (4). The occurrence of alterations in genes encoding proteins involved in the DNA repair system will result in genomic instability, escalating the rate of mutation in the cells (5). Sequential rounds of gained alterations and selection thus result in disease progression and the development of malignant tumors (4).

In recent years, a hierarchy of malignant cells within a tumor has been proposed, leading to a new, or supplementary, theory referred to as the cancer stem cell (CSC) model. This theory is founded on the assumption that normal developmental programs are still valid during cancer progression and that the initial genetic alteration, providing a survival advantage, occurs in or gives rise to a cell with indefinite self-renewal capacity, i.e. a stem cell (6). Much of the current understanding of cancer evolution originates from advances in gene sequencing demonstrating the coexistence of malignant clones with diverse self-renewing capacity within tumors (7). Additionally, the development of fluorescence-activated cell sorting (FACS) has allowed for the isolation of phenotypically distinct malignant cells.

Much of the early work demonstrating a hierarchical architecture of the malignant cells was performed in studies of malignant cells from patients with acute myeloid leukemia (AML). Phenotypically different AML cells were isolated and the initial results implied that only the $CD34^+CD38^-$ leukemic cells, referred to as the leukemic-initiating cells (L-ICs), were capable of proliferation, differentiation, and unlimited self-renewal. The L-ICs initiated disease when transplanted to immunodeficient mice, and the evolving malignant cells comprised L-ICs as well as more differentiated leukemic cells, thus resembling those recovered from the patient from whom the cells originated (8). More recent experiments imply that also $CD34^+CD38^+$ leukemic cells may possess disease-initiating potential upon transplantation to immunodeficient mice under certain conditions, but the L-ICs are more rare among the $CD34^+CD38^+$ cells than in the $CD34^+CD38^-$ population (9, 10). Since the identification of L-ICs in leukemias, malignant cells from various cancer types have been transplanted to immunodeficient mice leading to the documentation of tumor-initiating cells (T-ICs) in solid malignancies such as $CD133^+$ cells in breast (11), brain (12), and colon cancer (13) and $CD44^+$ cells in head and neck cancer (14). Collectively, these studies support that several malignancies are hierarchically organized where only rare cells have cancer-initiating and propagating capacity.

The seemingly consensual view is that the course of cancer is best described as a combination of the CSC model and the clonal evolution theory, which is sometimes referred to as the mixed model (Figure 1). Studies in leukemia have revealed that several subclones of L-ICs coexist and develop through clonal evolution and natural selection (15, 16). It has been suggested that early in the tumor development, the hierarchical model may best describe features of the tumor whereas upon disease progression, the T-ICs acquire and accumulate mutations in a process similar to that proposed in the clonal evolution theory. Hence, subclones of both T-ICs and non-T-ICs coexist and evolve in parallel (6, 15, 16). The subclones may present with distinct driver mutations resulting in different capacities to e.g. multiply, form metastases, or induce angiogenesis. In addition, the localization of a tumor cell within the tumor microenvironment (TME) may have impact on its phenotype and stemness by altering what signaling molecules, such as nitric oxide (NO) and Wnt ligands, that the cancer cell is exposed to (17, 18).

The increased understanding of the genetic and phenotypic diversity within tumors has unraveled differences in treatment responses between CSCs and the bulk of malignant cells. CSCs have, similar to healthy stem cells, been proposed to be quiescent and thus show reduced sensitivity to treatments that target proliferation such as chemotherapeutic agents (19). CSCs may also express drug efflux pumps, anti-apoptotic proteins, or have an efficient reactive oxygen species (ROS) scavenging system allowing for tolerance to increasing ROS levels in response to radiation and chemotherapy (20-22). Due to their tolerance to

traditional therapies and their potential to initiate disease, CSCs have been proposed as a principal cause of disease recurrence. In recent years, cancer immunotherapy has emerged as an additional option in cancer therapy that may fundamentally change the concept of cancer treatment allowing for selective targeting of CSCs (23, 24).

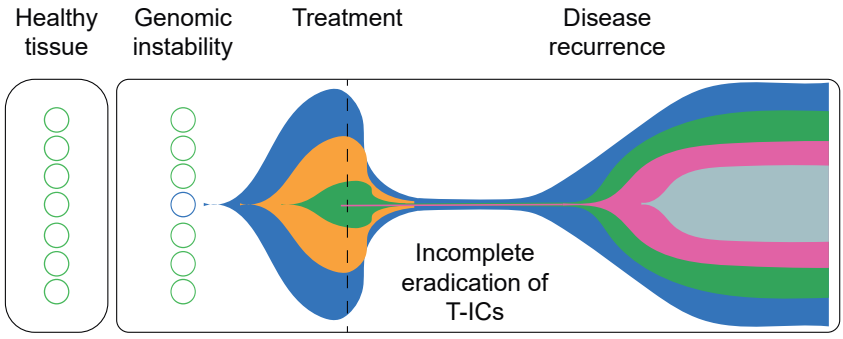

Figure 1. Mixed model of cancer progression. The initial alteration, resulting in a proliferative advantage of the transformed cell, occurs in a cell with self-renewal potential. Additional genetic changes result in increased genomic instability and the generation of malignant subclones that further promote cancer progression. At the time of diagnosis, different subclones have evolved in a process involving selective pressure and natural selection. Treatment results in eradication of the bulk of tumor cells but a few T-ICs persist, which later cause disease recurrence and the regeneration of previously eradicated as well as new malignant clones.

ACUTE MYELOID LEUKEMIA

Genetic alterations in myeloid cells may initiate the development of AML where immature myeloid cells accumulate in the bone marrow (BM) and in peripheral tissues. AML is genetically heterogenous and the leukemic cells may harbor an abnormal karyotype because of e.g. gene translocations and deletions but may also carry a normal karyotype with genetic mutations as drivers of the disease (25). These genetic alterations provide a proliferative advantage to the leukemic cells and cause a block in myeloid differentiation. Depending on the genetic alteration, the block in differentiation may occur at different stages of hematopoiesis leading to different maturity and disease characteristics of the predominant leukemic clone (26).

Traditionally, the French-American-British (FAB) classification system has been employed to classify AML based on the phenotypic characteristics of the predominant malignant clone, resulting in eight subtypes of AML referred to as M0-M7 (27). In recent years, the World Health Organization (WHO) classification system, which is based on the specific genetic abnormalities of the leukemic cells, has largely replaced the FAB classification system. The WHO classification system has proven to better prognosticate disease outcome, since

specific genetic alterations often are associated with prognosis (28). Hence, the genomic landscape of the leukemic cells is taken into consideration in the decision to proceed to allogeneic hematopoietic stem cell transplantation (allo-HSCT) after initial treatment with high doses of chemotherapy. Allo-HSCT is reportedly only beneficial in intermediate- and high-risk leukemia with, for example, FMS-like tyrosine kinase-3 internal tandem duplications (FLT3-ITD) mutation, *KMT2A* rearrangement, mutated *TP53*, or *RUNX1* mutations (25, 29).

The treatment of AML typically includes cycles of high doses of chemotherapy. Chemotherapy given at diagnosis is denoted induction therapy and aims at achieving complete remission (CR), defined as the disappearance of leukemic cells and reestablished normal hematopoiesis (25, 26). Approximately 70-80 % of patients <60 years old and 45-60 % of older patients will attain CR after induction therapy (30). However, the vast majority of patients will experience relapse of AML in the absence of further treatment. Therefore, induction therapy is followed by a consolidation phase of chemotherapy aiming at eradicating residual and non-detectable leukemic cells to reduce the risk of relapse (31). Allo-HSCT may follow consolidation therapy in intermediate- and high-risk group patients to further increase the likelihood of disease-free survival (25). Despite the aggressive treatment in AML the prognosis is relatively poor with 40 % of younger (<60 years) and 10 % of older patients being long-term survivors (30).

Several targeted therapies aiming at improving survival in AML are currently being evaluated, including FLT3 inhibitors (32), inhibitors of isocitrate dehydrogenase (33), and bi-specific antibodies directed towards AML-specific antigens (34). Midostaurin and enasidenib, which target FLT3 and isocitrate dehydrogenase-2, respectively, were recently approved by the US Food and Drug Administration. Targeted therapy is also available for a subgroup of AML, acute promyelocytic leukemia (APL), which is characterized by a fusion protein between retinoic acid receptor α (RARα) and promyelocytic leukemia protein (PML) resulting from a translocation between chromosome 15 and 17. The RARα-PML fusion protein interferes with retinoic acid signaling, resulting in altered gene transcription and a blocked differentiation. Treatment with all-trans retinoic acid (ATRA) has dramatically improved prognosis in this group of patients with cure rates exceeding 80 %. In recent years, the addition of arsenic trioxide has been shown to further enhance the efficacy of ATRA in APL (35).

Additionally, immunotherapies aiming at stimulating the anti-leukemic efficacy of cytotoxic natural killer (NK) cells and T cells have been evaluated in AML, including NK cell infusions (36), stimulatory cytokines such as interleukin 2 (IL-2), and the combination of IL-2 with histamine dihydrochloride (HDC) that reduces the production of immunosuppressive ROS (37, 38). HDC/IL-2 is approved as post-consolidation immunotherapy in Europe and remains the only

non-transplant therapy that has reduced the risk of relapse in the post-chemotherapy phase of AML in a phase III trial (37). The results of post-hoc analyses imply that treatment with HDC/IL-2 improves leukemia-free survival in AML patients harboring leukemic cells that express functional histamine H_2 receptors (H_2R) and the ROS-forming myeloid nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) (FAB-M4 and -M5 AML) (39). In these patients, the treatment may act by inhibiting the formation of leukemia-derived ROS via H_2R expressed by leukemic cells, thus protecting immune cells from ROS-induced toxicity in the leukemic microenvironment (37, 39).

CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is, in contrast to AML, a genetically and morphologically homogeneous disease where >98 % of patients harbor mature granulocytes carrying a reciprocal chromosomal translocation between chromosome 9 and 22, referred to as the Philadelphia chromosome (40-42). The Philadelphia chromosome results in the formation of an oncogene on chromosome 22 known as breakpoint region protein-abelson murine leukemia viral oncogene homolog 1 (*BCR-ABL1)*, and the reciprocal gene *ABL1-BCR* on chromosome 9. Both genes may be transcriptionally active but only *BCR-ABL1* contributes to the initiation and maintenance of leukemia (41, 43). Expression of *BCR-ABL1* results in a constitutively active ABL1 tyrosine kinase that promotes the survival of the *BCR-ABL1*$^+$ cells (44). Although the malignant cells are granulocytes, the *BCR-ABL1* translocation may be found in a variety of cell types in the periphery suggesting that CML, as proposed by the CSC model, is a stem cell disease arising from the acquisition of the *BCR-ABL1* translocation in a hematopoietic stem or early progenitor cell (45).

If untreated, CML progresses through a chronic phase (ongoing for several years), an accelerated phase (typically lasting 4-6 months), and a final stage resembling acute leukemia (blast crisis). Most patients are diagnosed in the chronic phase by the detection of mature *BCR-ABL1*$^+$ granulocytes in blood. Upon disease progression, the *BCR-ABL1*$^+$ leukemic progenitor cells acquire additional mutations that may result in rapid accumulation of malignant blasts in blood and BM. The blast crisis in CML is characterized by a differentiation arrest and a disease more akin to that of AML and thus requires induction and consolidation chemotherapy. However, the blast crisis may also be dominated by lymphocytic leukemic cells (46, 47).

In the past, CML was a disease with dismal long-term prognosis, but the introduction of the first-generation tyrosine kinase inhibitor (TKI), imatinib, revolutionized the treatment and dramatically improved prognosis (48). The life-expectancy of patients with CML is nowadays close to that of the general population provided that TKI treatment is initiated in the chronic phase of the

disease (48, 49). Imatinib competes with adenosine triphosphate (ATP) for a binding site on ABL1 and thereby efficiently inactivates the kinase activity of the oncoprotein (50). The early treatment response to imatinib is optimally monitored by measuring *BCR-ABL1* transcripts in blood after treatment initiation and are important indicators of prognosis (51). Imatinib has since 2002 been the standard of care and first-line therapy in CML, but imatinib intolerance or resistance have led to the development and approval of second- and third-generation TKIs such as nilotinib, dasatinib, bosotinib, and ponatinib (52).

The second- and third-generation TKIs are more potent with a shorter time to reach early and optimal molecular responses along with lower risk of disease progression. However, with these more potent inhibitors, tolerability and toxicity have become increasingly problematic (53). Thus, despite a reduction in CML-related deaths, the overall survival after treatment with second- or third-generation TKIs remains the same as that in patients receiving imatinib, likely due to increased non-CML related mortality (54). Imatinib hence remains the first-line treatment of CML but the new inhibitors are valuable tools for high-risk group patients and for patients that progress despite imatinib treatment.

Over 90 point mutations in the kinase domain of ABL1 have been identified leading to resistance to imatinib. In this regard, the T315I mutation is the most frequent and also results to resistance to nilotinib, dasatinib, and bosotinib. Ponatinib was developed to retain TKI activity in the presence of the T315I mutation. For patients progressing on imatinib it is therefore important to identify kinase mutations in order to select for optimal TKI substitutes. Ponatinib is also efficacious in heavily pretreated patients who have failed to achieve major molecular responses after receiving first- and second-generation TKIs (55).

Despite the efficacy of TKIs in eradication of the bulk of the malignant cells, the L-ICs in CML are seemingly not dependent on the expression of *BCR-ABL1* for their survival and thus prevail in most patients (56, 57). Therefore, TKI therapy has been considered to be life-long with high societal costs and significant morbidity. In recent years, the possibility to safely discontinue TKI therapy has been increasingly explored and a significant fraction of patients will remain in leukemia-free remission after TKI discontinuation. The time on TKI before treatment discontinuation and the depth of molecular response at TKI withdrawal are predictive for disease-free survival (58). TKI discontinuation has not been associated with major safety concerns as relapsing patients almost invariably regain major molecular response after restarting TKI therapy (59, 60). Several investigators are currently aiming at identifying strategies that increase the fraction of patients who will remain leukemia-free after TKI withdrawal, including attempts to eliminate L-ICs before TKI cessation (61).

THE IMMUNE SYSTEM

The immune cells that contribute to the defense against pathogens and transformed cells may be divided into innate immune cells, with germ-line encoded receptors recognizing a fixed set of antigens, and adaptive immune cells, with a broader antigen-specificity due to genetic recombination of the receptors in individual somatic cells. Upon infection, innate tissue-resident macrophages sense and respond to a pathogen following the secretion of cytokines that recruit additional phagocytes to the site of infection. Phagocytes, foremost dendritic cells (DCs), are additionally responsible to evoke an adaptive immune response. After antigen uptake, DCs thus migrate to the closest secondary lymphoid organ where the antigens are presented to induce the activation of T cells and B cells, recognizing the same antigens, in a highly controlled process.

NEUTROPHILS

Neutrophils, or polymorphonuclear granulocytes, are the most abundant leukocytes in blood and an important effector cell of innate immunity (62). Upon infection, tissue-resident macrophages will secrete factors that stimulate epithelial cells in the vessel walls to upregulate adhesion molecules allowing for neutrophil extravasation, in addition to chemotactic factors that will guide neutrophils to the site of infection (63). Neutrophils are hence one of the first cell types mobilized at the site of infection. The expression of a variety of pattern recognition receptors, with specificity for molecules common for many pathogens, allows neutrophils to respond to invading microorganisms via activation of among other transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB signaling activates the neutrophils to secrete pro-inflammatory cytokines, chemokines, and adhesion molecules that orchestrate the immune response (64). The main effector function of neutrophils is phagocytosis by which they engulf pathogens for internal degradation in a process highly dependent on the expression of the ROS-generating oxidase NOX2 in the phagosomal membranes. Cell membrane-bound NOX2 also allows for extracellular release of ROS and degradation of surrounding pathogens. Neutrophils can also eject networks of DNA and bactericidal proteins to trap a pathogen in a process called neutrophil extracellular traps (63, 64).

MONOCYTES

Monocytes develop from BM precursors when exposed to macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (65, 66) and are subdivided into classical and alternative monocytes (67). Both subtypes express CD11b in humans and mice whereas only human monocytes carry CD14 (68). Classical and alternative monocytes show different surface expression profiles. In humans, classical monocytes are defined as $CD14^{high}CD16^{-}$ cells whereas alternative monocytes are $CD14^{dim}CD16^{high}$ (67). The expression level of Ly6C is used to distinguish classical and alternative monocytes in mice where classical monocytes show a high expression of Ly6C whereas alternative monocytes are $Ly6C^{dim}$ (69).

At homeostasis, monocytes are found in the blood but the largest pool of monocytes resides in the BM and spleen (70). Distinct mechanisms are involved in the recruitment of monocytes to the periphery during inflammation. The chemokine (C-C motif) ligand 2 and 7 (CCL2 and CCL7, respectively), which are strongly upregulated in inflammation, are involved in the recruitment of classical monocytes from the BM to the periphery (71). Upon further stimulation, e.g. induced by infection, classical monocytes migrate from the blood stream to infectious tissues where they differentiate into macrophages or, in certain settings, into monocyte-derived DCs (72, 73).

Alternative monocytes are believed to have a more patrolling role characterized by crawling along the surface of small vessels to sample their surroundings to identify dying or infected cells and effectuate their removal (73). The alternative monocytes have also been suggested to contribute in defense against cancer metastasis (74). In mice, the alternative monocytes express CX_3-chemocine receptor 1 and their recruitment thus relies on the secretion of chemokine (C-X3-C motif) ligand 1 (75). Functional aspects of alternative monocytes have mainly been studied in mice and the functionality of these cells may differ in humans. However, in both mice and human, alternative monocytes may differentiate from classical monocytes (73).

MACROPHAGES

Macrophages are large phagocytic cells that reside in essentially all tissues throughout the body. Depending on the tissue in which these cells reside they gain distinct functions with individual names such as the Kupffer cells in the liver and the microglial cells in the nervous system. The main function of macrophages is to rapidly mobilize an immune response towards an invading pathogen by recruiting cells of both innate and adaptive immunity to the site of infection. Macrophages express a battery of surface receptors recognizing structures of potential pathogens such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptor, and, in humans, the lipopolysaccharide

receptor CD14 (76). Upon interaction of a receptor with its ligand a signal cascade results in macrophage activation mainly through activation of NF-κB with ensuing secretion of cytokines that recruit additional effector cells, including neutrophils, to the site of infection (77). Macrophages also play a crucial role in the host's early attempt to eradicate a pathogen owing to their capacity to exert phagocytosis where pathogens are engulfed and exposed to toxic, lysosomal ROS formed by NOX2. The mannose receptor CD206 and scavenger receptors are involved in the initiation of phagocytosis. Later in the inflammatory process macrophages may also aid in activation of adaptive immunity through antigen presentation to T cells (76).

Macrophages are, similarly to monocytes, divided into distinct populations referred to as M1, classically activated, and M2, alternatively activated macrophages. The M2 macrophages are further divided into M2a, M2b, and M2c subtypes. M1 macrophages are activated by interferon-γ (IFN-γ) mainly produced by T helper 1 (T_H1) lymphocytes and NK cells. Exposure of macrophages to IFN-γ enhances their release of ROS (78). In addition to ROS production, inducible nitric oxide synthase (iNOS) that forms NO is an important mediator of M1 immunity (77). M2 macrophages have instead been ascribed a role in defense against parasites and contribute to debris scavenging, angiogenesis, wound healing, and downregulation of immune responses, in part by producing IL-10 (77). Activation of M2 macrophages is achieved mainly by IL-4 (79), IL-13 (80), and IL-10 signaling (81). While details of the respective functions of M1 and M2 macrophages remain to be defined, M2-mediated immune responses are considered more immunosuppressive whereas M1 immunity is considered immune-activating.

Traditionally, macrophages were believed to derive from BM- or blood monocytes (82). In recent years, evidence has however been presented supporting that tissue-resident macrophages may also be yolk sac-derived or stem from embryonic progenitors that entered tissues before birth, where they retain the pool of tissue-resident macrophages throughout adulthood (83). While embryonic progenitor cells are important for the maintenance of tissue-resident macrophages at steady state, blood-derived monocytes are considered to be the precursors of macrophage expansion in inflammation (84).

DENDRITIC CELLS

DCs, macrophages, and B cells are referred to as professional antigen-presenting cells (APCs). Among those, DCs are the most efficacious APC and hence provide an important bridge between innate and adaptive immunity. Similar to macrophages, DCs express a range of receptors for pathogen recognition and are specialized in the uptake, processing, and presentation of antigens on major histocompatibility complex (MHC) class I and II (76). DCs may be divided into plasmocytoid DCs (pDCs) and conventional DCs (cDCs) which can be further categorized as cDC1 and cDC2 populations (85, 86). The pDC, cDC1, and cDC2 populations have distinct characteristics and phenotypes that are highlighted in Figure 2.

At steady state, pDCs are circulating cells with a poor capacity to present antigen but acquire a DC-like morphology along with the ability to process and present antigen upon viral stimulation. These DCs can also produce high levels of type I interferons (IFN-$\alpha\beta$) that exert antiviral activity and may activate NK cells and macrophages (87). pDCs are also present as resident DCs in lymphoid organs.

The cDC1 and cDC2 populations may be either migratory or lymphoid tissue-resident. The migratory cDCs patrol peripheral tissue and sample their surrounding for antigens to be presented to T cells after migration to the nearest lymph node. Upon encountering a microbial antigen, cDCs become activated resulting in the upregulation of co-stimulatory molecules, to enhance their T cell-activating properties, and an increased expression of C-C chemokine receptor type 7 (CCR7), which enhances the capacity of the cDC to migrate to lymph nodes. In this process, cDCs elaborate dendrites that allow for more efficient interactions with T cells. Activation of cDCs also comprises a reduction in their capacity to engulf, process, and present additional antigens. Once in the lymph node, the cDCs localize to the cortical T cell area and secret CCL18 to attract T cells. cDCs can produce a wide range of cytokines including type III interferons (IFN-λ), IL-12, IL-23, IL-10, and transforming growth factor-β (TGF-β) that direct polarization of helper T cell populations (76, 85, 86, 88).

Lymphoid tissue-resident cDCs are sentinels of the lymphoid organs and are restricted to a specific lymphoid organ throughout their life span. Whereas migratory cDCs are mature upon arrival to a lymphoid organ, lymphoid tissue-resident cDCs reside in the lymphoid tissues in an immature state and their maturation is initiated only after antigen uptake in the lymphoid organs (89). Lymphoid tissue-resident cDCs are essential during infections that infect DCs themselves such as influenza virus. Under such circumstances, the migratory cDCs carrying the antigen to the lymph node may be killed by the infection. The antigen can thus be transferred to a healthy lymphoid tissue-resident cDC that

has a retained antigen-presenting machinery allowing for proper T cell activation (76, 86, 89).

In an inflammatory setting, a supplementary entity of DCs is believed to be generated from monocytes that enter the site of inflammation and differentiate into inflammatory monocyte-derived DCs (85, 86). Inflammatory DCs, or monocyte-derived DCs, are suggested to participate in the innate immune defense and T cell activation. These cells have been suggested to reinforce and replace the function of cDCs during uncontrolled infections (69, 90).

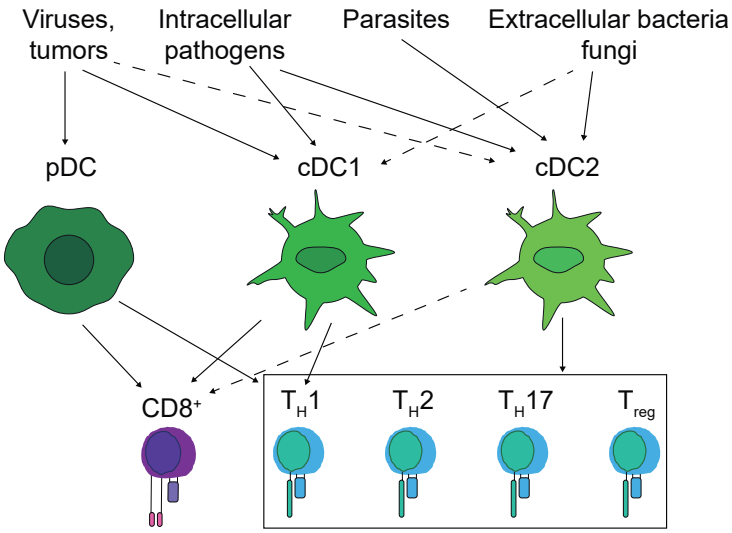

Figure 2. DC subsets. DCs are classified into three main populations namely plasmacytoid DCs (pDCs), conventional DC1s (cDC1s), and conventional DC2s (cDC2s). The subpopulations are specialized on shaping T cell immunity in response to different types of infections resulting in the activation of cytotoxic $CD8^+$ T cells ($CD8^+$), $CD4^+$ helper T cells (T_H1, T_H2, T_H17), or regulatory T cells (T_{regs}).

ANTIGEN PRESENTATION

APCs are defined by their ability to present antigens on MHC class I and class II. In general, intracellular antigens are presented on MHC class I to $CD8^+$ T cells, while MHC class II presents extracellular antigens to $CD4^+$ T cells. Whereas all nucleated cells present intracellular peptides on MHC class I, only professional APCs are capable of also presenting extracellular antigens on MHC I. Additionally, presentation of peptides on MHC class II is also restricted to professional APCs.

Presentation on MHC class II involves the uptake of extracellular antigens through phagocytosis to endocytic vesicles. The endosomes will fuse with lysosomes generating endolysosomes in which the phagocytosed material is degraded into peptides. Newly synthesized MHC class II cargo vesicles fuse with the endolysosomes allowing the degraded peptides to attach to MHC class II. Complexes with MHC class II molecules and adherent peptides later translocate to the cell membrane where the MHC class II and associated peptide is presented to $CD4^+$ T cells to evoke adaptive immunity (76, 91-93).

All nucleated cells present their intracellular protein content on MHC class I, which is an essential mechanism for the identification of infected or transformed cells by activated cytotoxic T cells. The normal mode of presentation of peptides on MHC class I involves the degradation of endogenous proteins by cytosolic and nuclear proteasomes. The peptides produced during this process are translocated to the endoplasmic reticulum where they bind to the peptide-binding groove of MHC class I molecules following transport through Golgi to the cell membrane for activation of $CD8^+$ T cells. For the activation of naïve $CD8^+$ T cells the presentation of peptides on MHC class I needs to be accompanied by costimulation, which only professional APCs are capable of providing.

Furthermore, as stated above, certain types of APCs, foremost cDC1s, have the ability to process phagocytosed antigens and load peptides derived from the exogenous proteins on MHC class I molecules. This process of antigen presentation is termed cross-presentation. Whereas the processes resulting in the presentation of endogenous peptides on MHC class I and exogenous peptides on MHC class II are well established, the underlying mechanism for cross-presentation is less well understood. Cross-presentation has been suggested to occur either via a vacuolar pathway or a cytosolic pathway or a combination of both these pathways. The initial step in cross-presentation is endocytosis of exogenous antigens to a phagocytic vesicle. Thereafter antigens are either degraded and bound to MHC I within the phagolysosome (vacuolar pathway) or exported to the cytoplasm before being degraded by proteasomes and loaded on MHC I (cytosolic pathway) (76, 91, 93-95).

Peptides that are to be cross-presented on MHC class I need to be protected from complete lysosomal degradation. NOX2 is recruited to the DCs early phagosomes and produce low levels of ROS in the lysosomal lumen that consumes protons and thus leads to alkalization of the lysosome. The reduced pH in the lysosomes inhibits lysosomal proteases which reduce degradation of exogenous antigens, and thus enhances cross-presentation (96-98).

T CELLS

Immature T cells, which are formed in the BM from lymphoid progenitors, complete their maturation to become naïve T cells in the thymus. The T cell progenitors first lose their stem cell markers, but do not express the T cell markers CD4 or CD8 and are thus referred to as double-negative thymocytes (99). The double-negative thymocytes will initiate the rearrangement of the genes encoding for the α, β, γ, and δ subunits of the T cell receptor (TCR). Rearrangement of the α- and γ-chains involves variable, joining, and constant regions whereas the β- and δ-chains also include diversity gene segments. During gene rearrangement these segments are recombined and addition, deletion, or substitution of nucleotides occurs. In this fashion, the relatively small set of genes encoding TCR segments generates a highly diverse pool of T cells (100), each with an unique antigen specificity. Gene rearrangement commits the T cell to either an α:β or a γ:δ T cell where the most frequent outcome is the generation of an α:β T cells, at this stage, expressing both CD4 and CD8 and are thus referred to as a double-positive thymocytes (76, 101, 102).

Once the TCR is in place, the T cell is subjected to positive selection where only T cells that recognize self-MHC class I or II molecules receive survival signals allowing them to continue their development (76, 103). Following positive selection, which also commits the T cells to the $CD4^+$ or $CD8^+$ linage, DCs mediate a process in which T cells recognizing self-antigens are eradicated referred to as negative selection (104, 105). Cells that have completed positive and negative selection are denoted naïve and are small non-dividing cells that circulate between the blood, the lymph, and the secondary lymphoid organs (106, 107). In the secondary lymphoid organs the naïve T cell will roll alongside DCs to seek for the peptide:MHC complex corresponding to its TCR. If the naïve T cell does not encounter a cognate peptide:MHC it will continue circulating in an inactive state, which can endure for years. In the opposite scenario, the naïve T cell finds its complementary peptide:MHC complex and is retained in the node until it has become fully activated.

Activation of a naïve T cell includes the interaction between the cognate TCR and a peptide:MHC complex, CD4 or CD8 with MHC class II or I, and costimulation via for example CD80 and CD86 expressed by the APCs and CD28 expressed by the naïve T cell (108). In addition, cytokines produced by the APCs are important to modulate T cell activation and direct T cell polarization. These APC-derived signals result in the activation of several aspects of T cells, including the formation of IL-2, which triggers T cell proliferation and thus promotes clonal expansion of the selected T cell (109-111). Once the T cells have undergone complete activation they will leave the secondary lymphoid organ and migrate via the blood to the site of infection to combat the pathogen. T helper 2 (T_H2) cells that may aid in B cell immunoglobulin class switching can however remain in the secondary

lymphoid organs allowing them to interact with passing B cells. The development and activation of T cells is depicted in Figure 3.

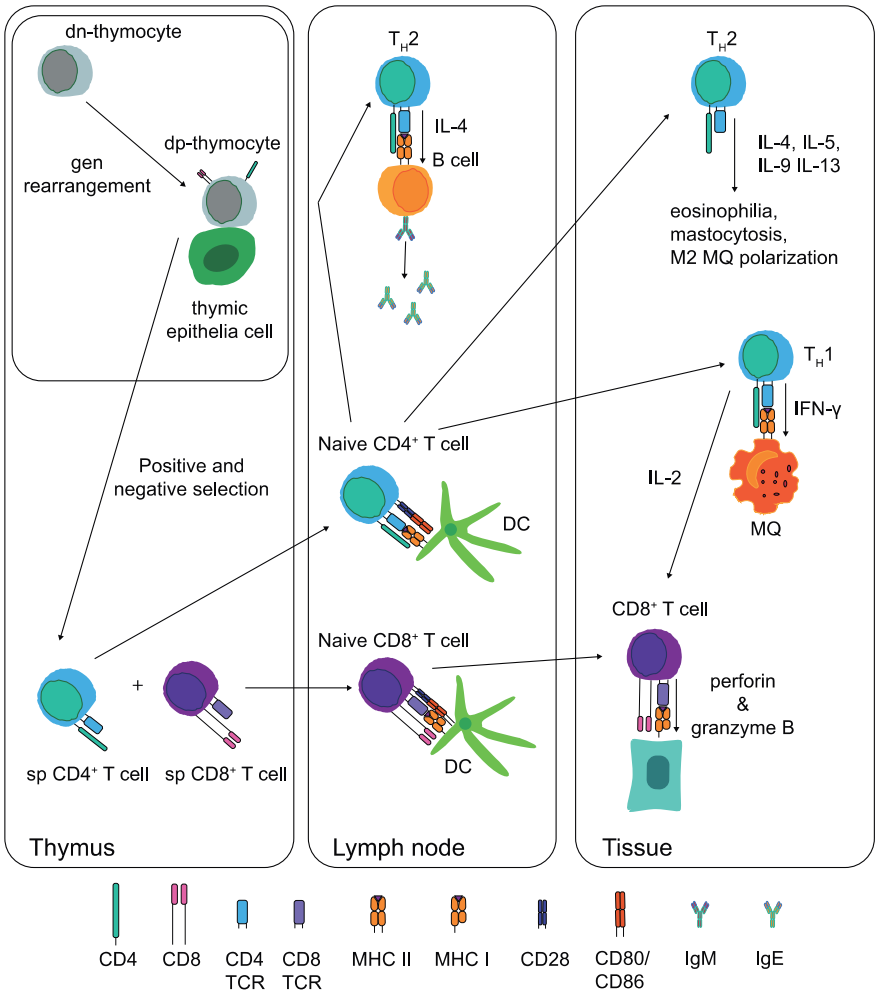

Figure 3. T cell development, activation, and function. The early T cell development takes place in the thymus where double negative thymocytes (dn-thymocytes) through a process involving gene rearrangement and positive and negative selection generates single positive (sp) $CD4^+$ or $CD8^+$ T cells. The naïve T cells are released from the thymus and will circulate between blood, lymphatic vessels, and secondary lymphoid tissues, until encountering an APC presenting its cognate peptide. The activated T cells mediate various effector functions. T_H2 cells can induce IgE antibody switching in B cells, stimulate eosinophils and mast cells, or polarize macrophages (MQ) to the M2 lineage. T_H1 cells produce IFN-γ that enhances expression of MHC, enhances macrophage activation and M1 polarization, and induces IgG2a and IgG3 antibody class-switch in B cells. Activated $CD8^+$ T cells can kill virus-infected or malignant cells.

$CD4^+$ T CELLS

$CD4^+$ T cells act either by down-regulating immune responses, as for the regulatory T cells, or by boosting the activity of other immune cells, as for helper T cells such as T_H1, T_H2, or T_H17 $CD4^+$ T cells. T_H1 T cells are important in the defense against infectious agents that reside in the phagosomes of macrophages. The T_H1 T cells produce IFN-γ, tumor necrosis factor (TNF), and IL-2. In particular IFN-γ is known to have antiviral, immunoregulatory, and anti-tumor properties, via induction of MHC molecules on APCs and normal cells. Additionally, IFN-γ increases activation and differentiation of macrophages and also induces IgG2a and IgG3 antibody switch in B cells (76, 112). The functions of T_H2 cells include activation of M2 macrophages, eosinophils, mast cells, and antibody isotype switching to IgE through the production of IL-4, IL-5, IL-9, and IL-13 (113). The T_H17 cells produce and secrete IL-17 and are believed to be pro-inflammatory by helping epithelial cells and fibroblasts to recruit inflammatory cells to the site of infection (76, 112, 114).

The main function of regulatory $CD4^+$ T cells (T_{regs}) is to dampen immune responses by suppressing $CD4^+$ and $CD8^+$ T cells. T_{regs} produce large quantities of immunosuppressive and anti-inflammatory cytokines such as IL-4, IL-10, and TNF-β, but their immunosuppressive features are also dependent on the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) (76, 115, 116).

$CD8^+$ T CELLS

Activated $CD8^+$ T cells show increased expression of adhesion molecules that facilitate their interactions with target cells. Hence, activated, but not resting, $CD8^+$ T cells form an immunological synapse when encountering cells presenting their matching peptide in the context of MHC class I even in the absence of costimulation (117). The activation of effector $CD8^+$ T cells results in the production of IFN-λ and cytotoxins, such as perforin and granzyme B, that are packed into lytic granules. Once the $CD8^+$ T cell releases its lytic granules in the synaptic cleft, perforin will create pores in the target cell membrane allowing granzyme B to translocate into the cytosol where it will induces apoptosis by cleaving caspases (118, 119). $CD8^+$ T cells are serial killers and may proceed to kill additional cells when new lytic granules have been synthesized.

NK CELLS

Natural killer (NK) cells are derived from BM precursors and are phenotypically defined as cells lacking the expression of CD3 while expressing CD56. Subdivision of NK cells is based on the expression level of CD56 and CD16. The immature $CD56^{bright}CD16^{dim}$ NK cells are considered precursors of the more differentiated $CD56^{dim}CD16^{bright}$ NK cells, which are endowed with higher cytotoxic capacity (120). NK cells are innate lymphocytes that identify and eradicate transformed or virus-infected cells but are also an important source of cytokines and chemokines. A target cell is recognized by a NK cell by its ligand expression profile where a variety of ligands either impede or stimulate the cytotoxicity of the NK cell. Thus, when a NK cell interacts with a potential target cell the balance between inhibitory and activating stimuli will determine the outcome of the interaction. Consequently, a target cell expressing more activating than inhibitory ligands will trigger the NK cell to form an immunological synapse with the target cell into which the NK cell releases its granules. Similar to $CD8^+$ T cells, NK cell-derived granule contain perforin and granzyme B that induce an apoptotic cascade in the target cell (121). NK cells may also express membrane-bound death-ligands, such as TNF-related apoptosis-inducing ligands (TRAILs) and Fas ligands, which may induce caspase-dependent apoptosis of target cell (122). In contrast to T cells, NK cells do not require any prior sensitization and may mobilize a rapid immune response upon encountering infected or transformed cells, dependent on their ligand expression profile.

While downregulation of MHC class I on aberrant cells is a mechanism by which an infected or a malignant cell may avoid $CD8^+$ T cell-mediated immunity, a reduced expression of MHC class I renders a target cell more susceptible for NK cell identification, which is referred to as the "missing-self" model (123). The expression of MHC class I provides an inhibitory signal to NK cells expressing NKG2A and certain types of killer-cell immunoglobulin-like receptors (KIRs). The ability of NK cells to reject MHC class I-deficient cells thus provides a second level of protection against virus-infected and transformed cells in case of inadequate eradication by $CD8^+$ T cells. The "missing-self" model does nevertheless not explain why autologous MHC class I-deficient cells, such as erythrocytes, are not depleted by NK cells. However, a reduced expression of inhibitory receptors is not sufficient to promote NK cell activation. Instead, the target cell also needs to express activating ligands, such as stress ligands that are frequently upregulated on virally infected and malignant cells or proteins associated with viral and bacterial infections or tumor cells. These ligands engage activating receptors, such as NKG2D or natural cytotoxicity receptors (including NKp46 and NKp30), on NK cells. Thus, the balance between activating and inhibiting stimuli received by the NK cell from the target cell determines the outcome of the interaction (124-126).

Additionally, CD16 which is an Fc receptor allows $CD16^+$ NK cells to attach to the constant region of antibodies. Target cells coated with antibodies may thus trigger NK cell activation in a process denoted antibody-dependent cellular cytotoxicity (ADCC).

B CELLS

B cells develop from precursor cells in the BM where also positive and negative selection of the B cell receptor occurs. The final stages of maturation, to become naïve BM cells, occurs in the secondary lymphoid organs. Naïve BM cells circulate between blood and lymphoid organs and are responsive to antigens binding to their B cell receptor. Upon encountering with an antigen recognized by the cognate B cell receptor the B cell becomes activated and with help from B follicular helper T cells and follicular DCs the B cells differentiates into antibody producing plasma cells (127). Activated B cells secrete antibodies and are referred to as plasma cells. The synthesized antibodies may neutralize bacterial toxins or opsonize bacteria to stimulate ADCC and thereby aid in elimination of pathogens (76).

REACTIVE OXYGEN SPECIES

ROS are oxygen-derived chemicals that oxidize other compounds (i.e. "steal electrons"). ROS include radicals, with unpaired valence electrons, like superoxide ($O_2^{\bullet-}$) and hydroxyl anion ($\bullet OH$), and non-radicals with similar oxidizing capacity, such as hydrogen peroxide (H_2O_2), hypochlorous acid, ozone, and singlet oxygen (128, 129). The non-radical H_2O_2 may be converted to hydroxyl radicals in the presence of ferrous ions in a process referred to as the Fenton reaction. Another highly reactive oxidant, peroxynitrite, is formed in a reaction between superoxide and nitric oxide synthase (NOS)-derived NO (130). ROS are produced in response to exogenous or endogenous stimuli. ROS may thus be generated from tobacco, smoke, pollutants, ionizing radiation, formed as bi-products during oxidative respiration, or specifically by radical-generating enzymes such as the NADPH oxidases (NOXs) and xanthine oxidase.

ROS have multiple physiological and non-physiological functions within cells. In phagocytes, NOX2-derived ROS contribute to eradication of pathogens within the phagolysosome. Accordingly, patients with genetic *NOX2* deficiency are at high risk of developing recurrent bacterial and fungal infections (131). ROS may also serve as signaling molecules by turning on or off the activity of various proteins in redox reactions. An advantage of ROS as regulatory molecules is their short half-life time and that some ROS, such as H_2O_2, can diffuse across biological membranes allowing for the control of protein functions intra- and extracellularly. ROS act as regulatory molecules by reversibly oxidizing cysteine

residues within proteins. At physiological pH, cysteine residues exist as thiol anions ($Cys\text{-}S^-$) that are easily oxidized into Cys-OH with a resulting allosteric change of the protein structure and an altered function. Cys-OH is reduced to its original form by disulfide reductases resulting in restored protein function. A setting in which the ROS levels exceed the antioxidative defense system is referred to as oxidative stress. During oxidative stress, the redox balance is tilted towards more of the oxidized variants of cysteines and ROS may thus inflict permanent tissue damage by oxidizing proteins, lipids, carbohydrates, or nucleic acids (130, 132). Several cellular systems exist with the purpose of neutralizing radicals and non-radicals, thus protecting cells from ROS-induced damage. The most powerful enzymatic scavengers of ROS include superoxide dismutase, catalase, peroxiredoxins, glutathione peroxidase-1, and thioredoxin (133).

MITOCHONDRIAL ROS

The mitochondria are a main source of cellular ROS. The mitochondrion is a double-membrane organelle localized in the cytosol of cells and is responsible for generating energy in the form of ATP through the oxidation of carbohydrates and fatty acids in a process denoted oxidative phosphorylation. During this process, electrons pass through the electron transport chain via redox reactions while releasing energy in the form of ATP (134). The final electron acceptor is molecular oxygen, most of which is converted into water. However, superoxide is produced as a bi-product due to incomplete reduction of oxygen to water. Accordingly, mitochondria harbor the highest levels of antioxidants in a cell to protect from superoxide produced during ATP generation.

NOX-DERIVED ROS

Seven NADPH oxidases have been identified: NOX1-5 and DUOX1-2. The NOX family of oxidases share the capacity to transfer electrons across biological membranes to generate ROS but differ in distribution between cell types and in subcellular localization (135). NOX1 is most highly expressed in the colon, NOX2 in phagocytes, NOX3 in the inner ear and in fetal tissues, NOX4 in the kidney, NOX5 in lymphoid tissues and testis, and DUOX1-2 in the thyroid tissue and in the gastrointestinal tract.

NOX2 consists of membrane-bound ($gp91^{phox}$, also referred to as NOX2, and $p22^{phox}$) and cytosolic ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac) subunits where the membrane-bound subunits encompass the catalytic function of the oxidase. $gp91^{phox}$ and $p22^{phox}$ constitutively interact to form a membrane-bound complex referred to as flavocytochrome b_{558} but the activation of the oxidase requires translocation of the cytosolic subunits to the membrane. Activation of NOX2 may be induced by growth factors, cytokines, or interactions involving pathogen-associated molecular patterns, damage-associated molecular patterns, or bacterial peptides that result in the phosphorylation of $p47^{phox}$. At rest, $p40^{phox}$ and $p67^{phox}$

are generally associated in the cytosol. The phosphorylation of p47^{phox} increases its ability to assemble with p67^{phox} forming a trimeric cytosolic protein complex as well as causing a conformational change of p47^{phox} allowing it to interact with membrane-bound p22^{phox}. Thus, p47^{phox} recruits the cytosolic subunits of NOX2 to the membrane. Once the subunits of the oxidase have assembled at the membrane, NOX2 becomes functional and gains its ROS-generating capacity involving the ability to transfer one electron from NADPH in the cytosol to oxygen on the external side of the membrane generating superoxide as depicted in Figure 4 (136). NOX2 may be expressed in the membrane of phagosomes or lysosomes thus generating intraphagosomal or intralysosomal ROS, or on the plasma membrane to generate extracellular ROS.

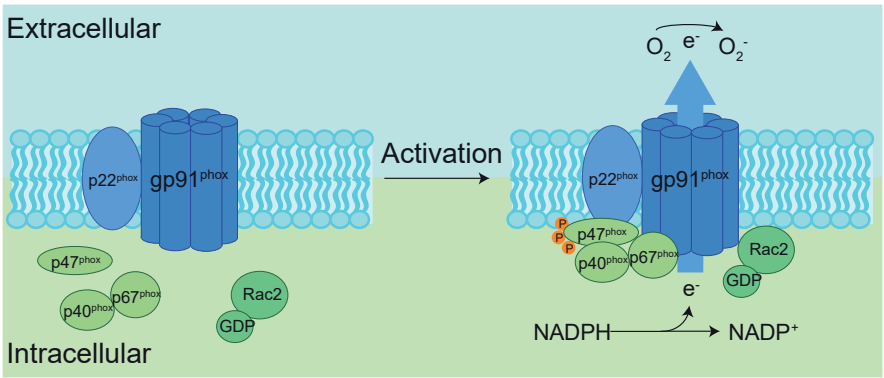

Figure 4. NOX2 activation and ROS production. Upon assembly of the membrane bound and cytosolic subunits, NOX2 becomes active and capable of ROS formation.

THE DUAL ROLE OF ROS IN CANCER

ROS have been proposed to affect the initiation and progression of cancer on multiple levels. The arguably most established mechanism is that ROS may damage DNA with ensuing mutations and risk of cancer initiation (137-140). ROS have also been proposed to impact on established cancer cells by modulating the function of protein phosphatases (PTPs) and protein kinase C (PKC) through oxidation. PTPs and PKC are enzymes involved in post-translational modification of proteins. In an oxidized form, PTPs become inactivated whereas oxidation of PKC renders it active. ROS have been ascribed a role in increasing survival and preventing apoptosis of pancreatic cancer cells (141-143) and non-small cell lung cancer cells (144, 145) through regulating PTPs. Increased ROS levels in these cells resulted in inactivation of PTPs with a resulting sustained activity of Janus kinase 2 (JAK2). Active JAK2 leads to phosphorylation of signal transducer and activators (STATs) resulting in activated transcription of anti-apoptotic proteins (143). ROS may also influence the tissue-invasive properties of cancer cells by modulating the activity of mitogen-activated protein kinase via oxidation of PTPs and PKC (146-148). Moreover, NOX-derived ROS have also

been linked to a sustained cytoskeleton (149), increased proliferation, and prevented apoptosis of endothelial cells which are crucial features for successful angiogenesis (149, 150). ROS have additionally been implicated in signaling of the powerful pro-angiogenic factor, vascular endothelial growth factor (VEGF) (151-153).

The purported role of ROS in tumorigenesis and cancer progression has inspired the evaluation of antioxidant strategies for cancer prevention. These strategies include antioxidant diets (154, 155) and the administration of ROS scavengers (156). Whereas some of these studies support that certain antioxidants may reduce the risk of cancer development (154-156), there are also opposing results. In a Finnish trial, approximately 30,000 male smokers between 50 and 69 years were randomized to receive alpha-tocopherol, beta-carotene, a combination of alpha-tocopherol and beta-carotene, or placebo. At five-year follow-up, the administration of beta-carotene was associated with a significantly higher incidence of lung cancer (157). Similar results were obtained in the CARET study that was prematurely stopped due to an increased incidence of lung cancer in participants supplemented with beta-carotene and alpha-tocopherol (158).

While the reason for these partly contradictory results is incompletely understood, it is reasonable to assume that the pleiotropic actions of ROS may preclude a meaningful assessment of the clinical impact of systemic antioxidant supplementation. It is thus likely that the evaluation of ROS-modulating therapies in cancer should take the type of tumor, the phase of tumor development, the immune mechanisms that are relevant in controlling a specific tumor, the method of ROS modulation and, in particular, the source of ROS into account.

ROS IN MYELOID LEUKEMIAS

Genetic abnormalities including *BCR-ABL1*, FLT3-ITD, NRAS/BCL2 that are commonly observed in AML, CML, and myelodysplastic syndromes have been associated with enhanced cellular levels of ROS, as well as with accumulated DNA damage (138, 159, 160). As discussed above, increased ROS levels in malignant cells have been suggested to contribute to malignant cell proliferation, survival, genomic instability, and migration implying that oxidative stress in transformed cells may promote disease progression.

ROS IN AML

One of the most common genetic alterations observed in AML is the FLT3-ITD mutation where FLT3 is constitutively active in leukemic cells with resulting downstream signaling involving the activation of STAT5. Cells transfected with FLT3-ITD and primary AML cells carrying FLT3-ITD show increased ROS levels that appear to be regulated through STAT5 signaling and Rac1 activation providing a possible mechanism for ROS generation. Using FLT3-ITD inhibitors it was shown that the induction of ROS was inhibited once signaling downstream FLT3-ITD was neutralized (159). It was suggested that the distinctively poor prognosis of patients with FLT3-ITD$^+$ AML may be a result of FLT3-ITD-induced ROS levels with a resulting genomic instability that drives the emergence of additional genetic alterations.

It has additionally been suggested that AML cells may hijack and take advantage of ROS-regulated signaling pathways. NOX2-derived ROS have thus been proposed to provide pro-survival stimuli in the M07e AML cell line (161). The survival of primary AML cells also reportedly relies on NOX2-derived superoxide that facilitates energy transfer from BM stromal cells to the leukemic cells (162). There are however also conflicting reports suggesting that the induction of ROS triggers differentiation of AML cells (163).

Monocytic AML cells have additionally been shown to produce immunosuppressive NOX2-derived extracellular ROS that trigger dysfunction in adjacent lymphocytes, a feature shared with healthy monocytes. The NOX2 inhibitor HDC has been shown to protect NK cells from this mechanism of leukemia-induced immunosuppression, which may contribute to the clinical benefited of HDC-based therapy for relapse prevention in AML (37, 164). Moreover we present results in **paper III** implying that the inhibition of NOX2-derived ROS, using HDC, stimulates the maturation of AML cells. Neutralization of ROS may thus target several aspects of pro-tumorigenic events orchestrated by ROS such as triggering the differentiation of AML cells, disrupting pro-survival signaling pathways, reducing the genomic instability believed to promote disease progression, and facilitating immune-mediated clearance of leukemic cells.

ROS IN CML

ROS have been implicated in the progression of CML from chronic phase through accelerated phase to blast crisis by increasing the genomic instability in the leukemic cells with a resulting increase in the mutational rate. In support of this assumption, presence of the *BCR-ABL1* translocation is associated with enhanced ROS levels in mature and immature cells (137, 138, 165). The degree of oxidative stress further increases during the accelerated phase and may predict disease progression (166). Disease progression is also associated with a depletion of non-enzymatic antioxidants that may further contribute to elevated ROS levels

(167). Diminished activity of glutathione S-transferase π, an enzyme involved in the protection against DNA oxidation, is associated with increased risk of developing CML at an early age and a reduced responsiveness to TKI therapy (168). In agreement with these reports, the results of **paper IV** imply that murine *Nøx2*-deficient *BCR-ABL1*$^+$ cells caused a less severe leukemia upon transplantation to mice as compared to *Nøx2*-sufficient counterparts.

In further support for oxidative stress as contributor to the genomic instability observed in CML, CD34$^+$ cells transduced to express *BCR-ABL1*$^+$ reportedly display approximately three to eight times more oxidized nucleobases compared with non-transformed cells (169). The ROS-induced DNA mutations encoded clinically relevant amino acid substitutes known to cause imatinib resistance. Also, treatment with ROS scavengers such as *N*-acetyl-L-cysteine (NAC) and vitamin E was found to decrease the mutagenesis rate and the frequency of imatinib resistance in transformed cells (165). Similar results were observed in a murine model of chronic phase CML where ROS-induced DNA damage in leukemic stem cells caused TKI-resistant mutations such as the gatekeeper mutation T315I (137). Furthermore, despite TKI therapy the enhanced ROS levels in the leukemic stem and progenitor cells remain elevated (137, 170-172). Thus, ROS may contribute to the genomic instability observed in CML cells and to TKI resistance. Treatment with the antioxidant NAC in conjunction with TKI have additionally also been shown to render *BCR-ABL1*$^+$ cells more vulnerable to TKI therapy by promoting TKI-induced apoptosis suggesting that the enhanced ROS levels may also make the cells less sensitive to the therapy (173).

Treatment discontinuation of TKI therapy has been evaluated in patients in deep molecular remission. An interim analysis of the European Stop Tyrosine Kinase Inhibitor Study revealed that 53 % of patients who had been treated with TKIs for < 8 years and 74 % of patients treated with TKIs for > 8 years remained in treatment-free remission six months after TKI cessation (174). The results also showed that patients who remained in remission had significantly higher counts of NK cell with cytotoxic phenotype at enrolment compared with patients experiencing leukemic relapse (175). The authors concluded that cytotoxic NK cells may be of value for successful TKI discontinuation. Human and murine *BCR-ABL1*$^+$ CML cells reportedly express functional NOX2 (176, 177) and produce NOX2-derived immunosuppressive ROS (176). Hence, it may be speculated that the targeting of NOX2-derived ROS, produced by the leukemic cells, may protect NK cells from ROS-induced dysfunction in CML.

TUMOR MICROENVIRONMENT

The TME is a complex environment comprised not only of malignant cells but also of stromal cells, cancer-associated fibroblasts, lymphocytes, and epithelial cells lining the tumor vasculature. The tumor-infiltrating cells as well as the extracellular matrix that embed the tumor and the physiological condition within the tumor mass, such as oxygen supply and acidification, are factors orchestrated by the malignant cells that will influence tumor progression.

NEOVASCULARIZATION AND ACIDIFICATION OF THE TME

The oxygen tension in the TME is typically reduced resulting in a state of chronic hypoxia, which is likely explained by the enhanced proliferation of malignant cells resulting in shortage of nutrients and oxygen. The hypoxic environment stimulates the malignant cells to secrete factors such as VEGF that triggers branching of preexisting vessels to initiate the formation of new vessels (178). The quickly increasing demand of vasculature by the growing tumor results in the generation of dysfunctional vessels with a fluctuating blood flow and intermittent oxygen availability, which is referred to as cycling hypoxia (179-181). Chronic and cycling hypoxia exert effects on the TME, many of which are mediated via the transcription family of hypoxia-inducible factors (HIFs) (178, 182). Hence, HIFs are the main mediators of cellular adaptation to hypoxia. In normoxic tissues, oxygen sensors, such as propyl hydroxylases, will induce the degradation of one of the HIF subunits, HIF-α, by the proteasome. In hypoxic environments the oxygen sensors lose the ability to induce HIF-α degradation and as a consequence it will translocate to the nucleus where it dimerizes with HIF-β subunits to initiate the transcription of HIF-target genes (178, 183). In this manner, the activation of HIF by hypoxia influences cancer by i) promoting tumor growth by inducing neovascularization via VEGF (184), ii) increasing cell survival via growth factors and inhibition of proapoptotic pathways (185), iii) contributing to metastasis by modulating cell adhesion molecules and mobility through the regulation of epithelial-to-mesenchymal transition (186, 187), and iv) conferring resistance to chemotherapy by inducing quiescence of malignant cells (178). Hypoxia also regulates several signaling pathways, enhances ROS generation, and inhibits homologous recombination and mismatch repair, thus increasing genomic instability (178). The expression of HIF-α in primary tumors and metastasis is associated with adverse prognosis and more aggressive diseases (188). Despite these observations, the anti-neoplastic efficacy of HIF inhibitors in humans remains to be established.

The malignant transformation of a cell typically involves a metabolic reprogramming from oxidative phosphorylation to aerobic glycolysis. The hypoxic milieu in the TME favors anaerobic glycolysis over aerobic energy

metabolism leading to the generation of lactic acid and acidification of the surrounding tissue (189). The generation of lactate inhibits the ubiquitination and the proteasomal degradation of HIF-α and hence the acidification and the hypoxia promote one another (190). The resulting acidification of the TME influences on various tumor-infiltrating cells and may results in immunosuppression. A reduced pH has thus been shown to diminish the cytotoxicity of both $CD8^+$ T cells (191) and NK cells (192). In addition to suppressing cytotoxic lymphocytes with anti-tumor functions, the acidification recruits myeloid cells that may differentiate into immunosuppressive myeloid-derived suppressor cells (192) or M2-like tumor-associated macrophages (TAMs) (193). HIF-1α activation has also been shown to enhance the expression of the T_{reg} specific transcription factor forkhead box P3 (194).

TUMOR INFILTRATING IMMUNE CELLS

The presence of DCs, T cells, and NK cells within or adjacent to tumors is associated with favorable outcomes in several forms of cancer (195). However, immune activation within the tumor may result in the development of chronic inflammation that promotes exhaustion of the tumor-infiltrating lymphocytes (TILs) and the recruitment of immunosuppressive immune cells, such as myeloid-derived suppressor cells, TAMs, and T_{regs} (196, 197). Together with the acidic and hypoxic conditions already manifested in the TME the milieu may rapidly shift into a hostile environment for anti-tumor immune cells with ensuing immune escape.

NEUTROPHILS AND MONOCYTES IN CANCER – MYELOID-DERIVED SUPPRESSOR CELLS

Patients with cancer often present with elevated numbers of a heterogeneous population of immature myeloid cells (IMCs) in peripheral tissues and in the TME. While IMCs likely exert multiple functions, they have been shown to efficiently suppress functions of cytotoxic lymphocytes (38, 198-202). Initially, the IMCs were characterized based on their lack of expression of markers for B cells, T cells, NK cells, and macrophages. There was however no consensus regarding the nomenclature and the cells have during the years been referred to as IMCs, myeloid suppressor cells, and $GR1^+$ cells since GR1 distinguishes these cells in mice. In 2007, Gabrilovich and co-workers proposed the term myeloid-derived suppressor cells (MDSCs) to encompass myeloid cells that dampen cellular immunity and the term have since been used to refer to this heterogenous population of cells (203). Cells with a phenotype similar to MDSCs are generated also during acute inflammation, but under these circumstances the cells are generally not highly immunosuppressive, but rather contribute in tissue repair, in wound healing, and to homeostasis (204).

The chronic inflammation characteristic of the TME triggers myelopoiesis and consequently IMCs are released from the BM and accumulate in the periphery and in the TME. Tumor cell-derived cytokines, such as GM-CSF and IL-6, promote the expansion of IMCs (205-210) while factors secreted by tumor stromal cells and activated T cells, including IFN-γ, ligands for TLRs, IL-4, IL-13, and TGF-β, induce the activation of IMCs into MDSCs (204, 211-213). Hence the generation of MDSCs is sometimes referred to as a "two-signal model" and illustrates that tumors may highjack preexisting biological mechanisms to recruit cells that promote their progression. The generation of MDSCs in cancer is depicted in Figure 5.

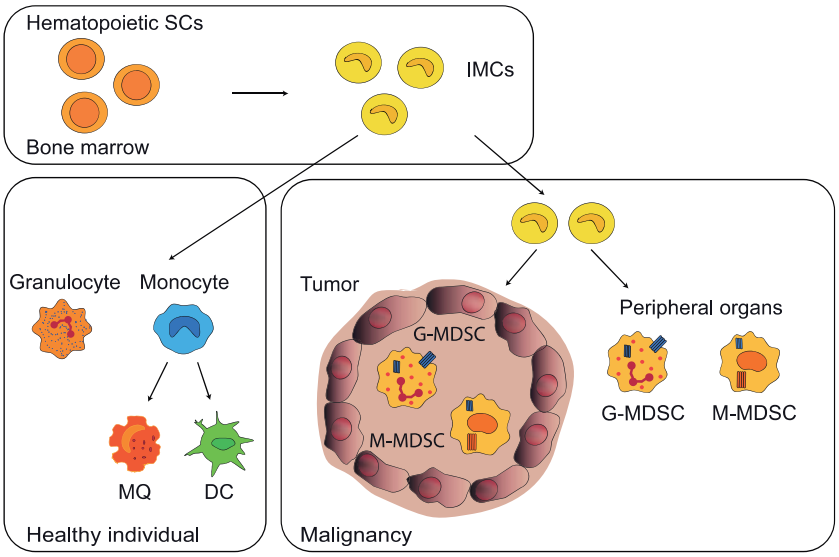

Figure 5. Myeloid differentiation in health and in cancer. In healthy individuals, IMCs rapidly differentiate into mature myeloid cells upon migration from the BM to the periphery. Cancer patients often present with impaired myeloid differentiation where IMCs accumulate in the periphery in response to tumor-derived growth factors. Additional factors mainly produced by activated T cells and stromal cells in the TME, activate IMCs to become MDSCs that are divided into granulocytic- or monocytic MDSCs (G- and M-MDSCs, respectively).

MDSCs are currently acknowledged as key players in immunosuppression and immune escape in cancer and positively correlate with the tumor burden and the stage of disease (214-217). In addition, there is a negative correlation between MDSCs and immune responses in cancer therapy (218) and the targeting of MDSCs in mice have been shown to improve immune responses and to reduce tumor growth rate (219, 220). A compelling body of evidence hence suggests that the accumulation of MDSCs in cancer is a disadvantageous event and a possible therapeutic target.

Characteristics of MDSCs

MDSCs are not a unique subset of cells but a population of immunosuppressive cells that differ in morphology, phenotype, and suppressive effector functions. These cells are often divided into monocytic- and granulocytic-MDSCs (M- and G- or PMN-MDSCs, respectively, Figure 6). In mice the phenotypic identification of MDSCs, M-, and G-MDSCs is relatively straightforward. Murine MDSCs are characterized by their expression of CD11b and GR1. The expression level of the GR1 epitopes Ly6C and Ly6G is employed to distinguish M-MDSCs from G-MDSCs, where M-MDSCs are $CD11b^{+}GR1^{+}Ly6C^{high}Ly6G^{-}$ and the G-MDSCs are $CD11b^{+}GR1^{+}Ly6C^{low}Ly6G^{+}$. No human marker corresponding to GR1 in mice has been identified and hence the phenotype of human MDSCs is under debate. However, the currently most used definition of human M-MDSCs is $CD33^{+}CD11b^{+}CD14^{+}HLA\text{-}DR^{low}$ cells whereas G-MDSCs are defined as $CD33^{+}CD11b^{+}CD15^{+}CD66b^{+}HLA\text{-}DR^{-}$ cells. In addition, the G-MDSCs are found in the peripheral blood mononuclear cell fraction upon density gradient centrifugation in contrast to neutrophils that are pelleted out over Ficoll-Paque (221, 222). G-MDSCs are, similarly to normal neutrophils, sensitive to cryopreservation and can therefore mainly be studied using fresh samples. Hence, many clinical studies are restricted to examining the impact of M-MDSCs for, e.g., the course of disease. The phenotypic definition of MDSCs is however not sufficient to classify a cell as a MDSC, as an additional requirement is that the cell is capable of immunosuppression. The characterization of MDSCs hence involves phenotypical as well as functional assessment.

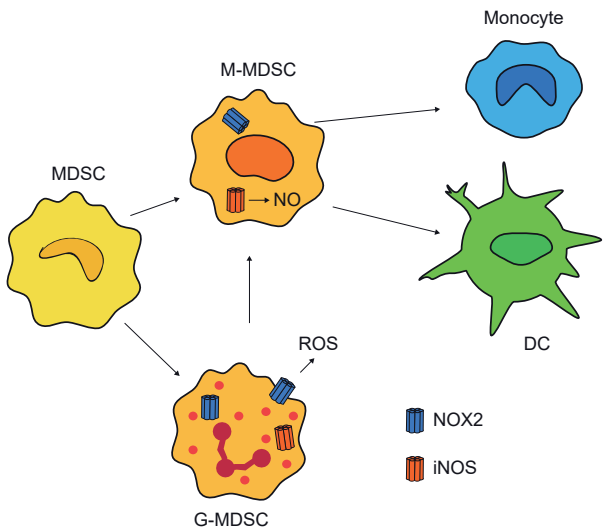

Figure 6. G- and M-MDSCs. MDSCs are categorized as G- or M-MDSCs. G-MDSCs may further differentiate into M-MDSCs that in turn may be triggered to differentiate into monocytes or DCs. MDSCs express NOX2 and iNOS by which they generate immunosuppressive ROS and NO triggering dysfunction in adjacent lymphocytes.

Expansion and activation of MDSCs

Cytokines like GM-CSF and IL-6 drive the expansion of MDSCs via stimulating phosphorylation of the transcription factor STAT3 in myeloid cells. The activation of STAT3 in IMCs has been shown to increase the survival and proliferation of myeloid cells and to block their differentiation with ensuing expansion of incompletely differentiated myeloid cells with characteristics similar to those of MDSCs (211). Support for a role of STAT3 in the expansion of MDSCs is also provided by studies reporting that MDSCs from tumor-bearing mice show upregulated expression of STAT3 (223) and that *Stat3*-deficeint mice show reduced expansion of MDSCs (224).

The activation of MDSCs is mainly dependent on STAT1 and STAT6 (211). IFN-γ, IL-4, and IL-13 are assumed to be involved in triggering the activation of STAT1 and STAT6, inducing the generation of MDSCs. Signaling via the IFN-γ receptor or the IL-4 receptor α results in downstream activation of STAT1 and STAT6, respectively, following upregulation of arginase 1 and iNOS, which are two of the main immunosuppressive mechanisms utilized by MDSCs (225, 226). The STAT1-involvement in MDSC activation is supported by results showing that *Stat1* knockout mice are incapable of arginase 1 and iNOS upregulation and do not inhibit T cell responses (227).

Main suppressive mechanisms of MDSCs

Arginase 1 and iNOS

L-arginine is essential for proper function of effector T cells and thus crucial for anti-tumor T cell responses. Shortage of L-arginine in the microenvironment results in T cell dysfunction because of reduced expression of the CD3ζ-chain and reduced T cell proliferation. MDSCs have been shown to express the two enzymes arginase 1 and iNOS that uses L-arginine as a substrate (211, 228). Arginase 1 converts L-arginine into urea and L-ornithine whereas iNOS uses L-arginine in the generation of NO and L-cirtrulline (229). Because of the high enzymatic activity of arginase 1 and iNOS by intratumoral MDSCs, L-arginine is depleted from the TME resulting in dysfunctional tumor-infiltrating T cells. In addition, NO exerts immunosuppression by inhibiting JAK3 and STAT5 signaling in T cells and by preventing MHC class II expression (230, 231).

ROS

Similar to normal phagocytes, MDSCs express the myeloid ROS-generating enzyme NOX2. STAT3, which is activated during cancer, is ascribed responsible for the enhanced NOX2-derived ROS-generating properties of MDSCs (213). Myeloid cells from *Nox2* knockout mice fail to upregulate ROS production during tumor growth supporting that the increased ROS formation observed by MDSCs is dependent on a functional NOX2 (232). The importance of NOX2-derived ROS as a promotor of tumor growth is somewhat controversial. In certain

experimental tumor models of melanoma and Lewis lung carcinoma there is a reduced tumor growth in *Nox2*-deficient mice (220, 233). In contrast, *Nox2* knockout mice do not show reduced sensitivity to sarcoma, lymphoma, and prostate cancer growth (232-235).

While the importance of NOX2 for solid tumor growth may vary, NOX2-derived ROS may have a greater impact on promoting metastasis formation. Hence, intravenously injection of B16-F10 melanoma cells or mouse mammary cancer cells consistently gave rise to less metastases in mice lacking NOX2 (220, 236). Furthermore, in a study by Okada et al, no difference on primary tumor growth was observed between wild type and *Nox2* knockout mice for two murine cancer cell lines, whereas *Nox2* knockout mice exhibited reduced spontaneous metastasis formation with fewer metastatic nodules in both models. The effect was linked to NOX2-derived ROS-induced expression of Thymosin β4 that enhanced the motility of the tumor cells (237).

Extracellular release of NOX2-derived ROS may trigger dysfunction and apoptosis in adjacent T cells and NK cells (38, 235, 238, 239). MDSC-derived ROS have also been shown to inhibit antigen-specific $CD8^+$ T cell responses (235). The effect has been linked to nitration of the TCR that was found to occur when ROS reacted with NO to form peroxynitrite during direct MDSC-T cell interactions. It has been proposed that nitration of the TCR results in a conformational change of the receptor resulting in less efficient interaction with and killing of tumor cells presenting the cognate peptide (240). Myeloid cell-derived ROS purportedly also impedes the effector function of NK cells (38). In accordance with these findings, genetic and pharmacological disruption of NOX2 resulted in reduced tumor growth in a murine model of metastatic melanoma. The effects of NOX2 inhibition were lost upon depletion of $GR1^+$ cells or NK cells, thus implying that NOX2-mediated NK cell suppression may affect metastasis (220).

Upregulation of NOX2 further seems to be important for proper generation of MDSCs since both tumor-bearing *Stat3* and *Nox2* knockout mice present with a more mature myeloid population and fewer MDSCs than wild type mice (232, 241). These results are coherent with the observed effect of ATRA, which has been shown to upregulate the levels of the antioxidant enzyme glutathione synthase with a subsequent neutralization of ROS with ensuing differentiation of MDSCs and DCs from breast cancer, colorectal cancer, and renal cell carcinoma patients (242-245). ATRA was further shown to target MDSCs in murine tumor models as well as in patients with metastatic renal cell carcinoma (246, 247). The role of ROS as inhibitor of maturation is further supported by results suggesting that the antioxidant NAC triggers differentiation of MDSCs (248).

Further, albeit indirect, support that the increased ROS levels in MDSCs are of relevance to maintain an immature phenotype of MDSCs stems from experiments performed in histidine decarboxylase-deficient mice. Histidine decarboxylase is highly expressed by myeloid cells and MDSCs and is responsible for the synthesis of histamine, a known NOX2 inhibitor. Histidine decarboxylase-deficient mice show an increased susceptibility to colon and skin cancer and reduced growth of glioma. Tumor-bearing histidine decarboxylase-deficient mice presented with increased amounts of MDSCs along with diminished $CD8^+$ T cells effector functions (249, 250). Exogenous administration of histamine to histidine decarboxylase-deficient tumor-bearing mice induced maturation of myeloid cells and suppressed the growth of tumor allografts (249). MDSCs from tumor-bearing mice additionally showed reduced histidine decarboxylase activity compared with cells isolated from non-tumor-bearing mice and an inverse correlation between histidine decarboxylase activity and the immunosuppressive properties of MDSCs was also observed (250). The potential importance of histamine in cancer prevention is also highlighted by epidemiological studies suggesting that allergens, likely driving the endogenous production of histamine, may be protective against glioma and leukemia (251, 252). An inverse correlation between risk of cancer development and atopy has been reported for colorectal cancer and childhood leukemia whereas studies in other malignancies did not show this association (253). While further studies are needed in this area, the results are compatible with the view that presence of an endogenous NOX2 inhibitor (histamine) in tissues may control tumor growth by targeting MDSCs, which is supported by our findings in **paper I**, **paper II**, and **paper III** suggesting a pro-differentiating effect of HDC on myeloid cells.

MACROPHAGES IN CANCER

Macrophages, similar to MDSCs, frequently accumulate in the TME during tumor progression (tumor-associated macrophages, TAMs) (254). CCL2 have been shown to promote the recruitment of monocytes to the tumor area whereas cytokines that are present in the TME, such as IL-4, IL-10, and IL-13, may induce the differentiation of monocytes into M2-like macrophages (254-257). M2 TAMs are assumed to promote tumor progression and may herald adverse prognosis in cancer (258). M1 macrophages may instead kill tumor cells by releasing NO and by producing TNF (259). Redirection of M2 macrophages into M1 macrophages by *in vivo* administration of anti-IL-10 has been shown to synergize with CCL16 and TLR stimulation to promote tumor rejection (257). The subdivision of TAMs into the M1 and M2 phenotypes is however challenged by reports describing multiple TAM phenotypes and TAMs presenting with M1 as well as M2 characteristics (260, 261).

DENDRITIC CELLS IN CANCER

The mobilization of a robust anti-tumor immune response is dependent on the activation of cDCs. Studies in murine models imply that the primary function of cDCs in cancer is to endocytose dead neoplastic cells or cellular debris and process and present the associated tumor antigens to T cells (262, 263). The ability of the cDC1s to cross-present tumor antigens is essential for activation of anti-tumor cytotoxic T cells as suggested, for example, by the finding that *Batf3*-deficient mice, which do not generate cDC1s, cannot reject highly immunogenic cancer cells. In addition, these mice are poor responders to immunotherapy with programmed cell death protein 1 (PD-1) neutralizing antibodies (264, 265).

Several studies support that DCs are dysfunctional in cancer patients, which may partly explain the state of immunosuppression characteristic of many cancer forms (266, 267). DCs from cancer patients accordingly express reduced levels of costimulatory molecules such as CD80 and CD86 (268). In addition, tumor cells may promote the maturation of DCs to express OX40 ligand favoring the development of T_H2 immune responses. These cells secrete IL-4 and IL-13 that may prevent tumor cell apoptosis and stimulate immunosuppressive TAMs. Presence of intratumoral T_H2 cells has been reported to promote breast cancer growth and also correlate with adverse prognosis in patients with pancreatic cancer (269, 270). Numerous strategies, including DC vaccines and the administration of cytokines known to stimulate DCs such as GM-CSF, have been proposed to boost cellular immunity in cancer (262, 268).

T CELLS IN CANCER

MHC class I-mediated recognition of malignant cells requires that the MHC class I-presented peptide on the tumor cell is a tumor antigen. There are two major forms of tumor antigens, tumor-associated antigens and tumor-specific antigens. The tumor-specific antigens are expressed solely by the malignant cells and originate from mutated proteins. The tumor-associated antigens instead arise from an altered expression profile of the transformed cells resulting in increased expression of proteins that normally are dimly expressed or only expressed in specific tissues (271). The tumor-associated antigens include cancer-germline genes, differentiation antigens, overexpressed antigens, and viral antigens.

Depending on the mutational burden, a tumor may be more or less immunogenic since tumors expressing a high number of neoantigens are likely to present more mutated peptides that may be recognized by T cells. Melanoma is associated with a high mutational frequency whereas patients with AML typically present with fewer non-synonymous mutations (272). The importance of the number of mutations for the ability of the immune system to evoke an anti-tumor immune response is underlined by results suggesting that there is a positive correlation

between the clinical efficacy of checkpoint inhibitors in cancer and the tumor mutational burden (273).

The importance of T cell-mediated anti-tumor immunity in cancer is highlighted by results obtained in, inter alia, melanoma (274), non-small cell lung cancer (275), breast cancer (276, 277), and ovarian cancer (278) showing that infiltration of tumors with anti-tumor T cells heralds favorable prognoses. In addition, it has been reported that a high $CD8^+$ T cell to T_{reg} ratio is associated with a more favorable clinical outcome in cancer, thus suggesting that T_{regs} may negatively impact on the cytotoxic function of $CD8^+$ T cells (278-280). Many efforts to boost T cell immunity in cancer have focused on triggering $CD8^+$ T cell responses, but $CD4^+$ T_H1 cells are also endowed with anti-tumor effector functions, in particular by providing help during the priming of $CD8^+$ T cells. Studies in mice show that depletion of $CD4^+$ T cells reduces the efficacy of tumor vaccines and immunotherapy using IL-7 (281, 282). A protective role of T_H1 $CD4^+$ T cells in anti-tumor defense was further supported by experiments using adoptive transfer of melanoma-reactive T_H1 $CD4^+$ T cells to tumor-bearing mice (283, 284).

T cell exhaustion in cancer

T cell-mediated immune responses in cancer follow the same biological traits as those observed during infections with viruses or intracellular bacteria. Since these pathways are likely evolutionary developed mainly to combat acute infections various immunosuppressive mechanisms eventually evolve to prevent prolonged immune effector function and autoimmunity and to aid in wound healing and restoration of tissue homeostasis (285). Consequently, tumor-infiltrating T cells are often subjected to exhaustion. Complete elimination of a tumor would however likely require extended immunity.

The immunosuppressive environment in the TME, including hypoxia, neovascularization, and immunosuppressive cells such as MDSCs and TAMs, has been discussed in previous sections but additional mechanisms are of relevance to T cell dysfunction and exhaustion. One intrinsic driver of T cell exhaustion is the persistent exposure to cognate antigen in the TME resulting in upregulated expression of immune checkpoint inhibitors on T cells (286, 287). The expression of PD-1 on T cells with a concomitant expression of programmed death-ligand 1 or 2 (PD-L1/L2) in the TME has emerged as a critical aspect of cancer-related T cell dysfunction. PD-1 expression on tumor-infiltrating T cells is thus considered a hallmark of T cells with reduced effector functions and is often associated with the expression of additional checkpoints. These include lymphocyte-activation gene 3 (LAG3), CTLA-4, T cell immunoglobulin and mucin domain-2 protein, along with T cell immunoglobulin and ITIM domain that all contribute in reducing T cell effector functions (286-288). The importance of checkpoints in controlling T cell functions is underscored by the clinical success of using PD-1, PD-L1, and CTLA-4 neutralizing antibodies in cancer immunotherapy (289-292).

The T cells propagating the TME are not only $CD8^+$ and T_H1 $CD4^+$ T cells. Regulatory T cells, inhibiting immune responses, may represent a substantial part of the $CD4^+$ T cell pool within the TME. In mouse models, the depletion of T_{regs} has been shown to improve anti-tumor immunity (293). While the detailed mechanisms involved remain to be defined, T_{regs} are known to reduce the expression of costimulatory molecules by APCs (116) and to produce immunosuppressive cytokines including TNF-β and IL-10 resulting in impaired T cell activation, survival, and expansion (285).

NK CELLS IN CANCER

Animal models have revealed that depletion of NK cells or transplantation of tumor cells into NK cell-deficient mice results in elevated tumor growth and, in particular, increased formation of metastases (220, 294-296). Similar results were obtained when mice lacking NK cell effector molecules such as perforin or IFN-γ were challenged with tumor cells (220, 297). In humans the infiltration of NK cells into solid tumors is mostly modest but the presence of tumor-infiltrating NK cells may herald favorable prognosis (298-300). Additionally, patients harboring circulating NK cells with intermediate or high cytotoxic function showed reduce cancer risk in a 11-year-follow up study, which supports a role for NK cells in the early control of transformed cells (301).

The killing of MHC class I-expressing transformed cells by cytotoxic T cells results in a selective pressure that favors the survival of malignant cells with reduced expression of MHC class I. The downregulation of MHC class I can be the result of mutations, deletions or hypermethylation in human leukocyte antigen (HLA)-genes, loss of heterozygosity in chromosome 6 or 15, or by transcriptional downregulation of HLA-related genes (302). The reduced expression of HLA molecules however renders the malignant cells more susceptible to NK cell cytotoxicity since NK cells are inhibited upon encountering cells expressing HLA class I molecules. Functions of NK cells against transformed cells are also promoted by the expression of stress ligands, including ULBPs and MHC class I chain related A and B (MICA and MICB, respectively), on tumor cells (303-305). It has also been reported that tumor cells express ligands for natural cytotoxicity receptors, such as ligands for NKp30 and NKp44, contributing to anti-tumor NK cell activity (306, 307).

NK cells are considered to be less efficacious in controlling established solid tumors than in the control of early malignant cells, possibly as a consequence of NK cell immune escape. The suppressive microenvironment in the TME, including the presence of immunosuppressive cells and cytokines, has been suggested to diminish NK cell-mediated immunity through reduced expression of NK cell activating receptors (308, 309). Additionally, tumor cells may shed and

release soluble MIC ligands that bind to NKG2D with ensuing inhibition of NK cell function (310).

NK cells however appear to play a critical role in the control of hematological malignancies. One can speculate that the importance of NK cells in hematological malignancies stems from a less immunosuppressive environment compared with that observed within solid tumors. On the other hand malignant blasts reportedly frequently express HLA class I molecules to inhibit NK cell cytotoxicity via ligating inhibitory KIRs on NK cells (311). The importance of KIR signaling is highlighted by results showing that the NK cell cytotoxicity against malignant myeloid cells is enhanced when there is a KIR-ligand mismatch between NK cells and target cell *in vitro* (312). In further support of the importance of inhibitory KIR-signaling for NK cell cytotoxicity, infusion of donor NK cells with a mismatched HLA-KIR profile between donor and recipient has been suggested to be protective against leukemic relapse in AML and myelodysplastic syndrome compared with a matched regimen (313-315). These results imply that antibodies neutralizing KIR-signaling, blocking the engagement of inhibitory KIRs with cognate ligands, may have a clinical value analogous to checkpoint inhibitors designed to target T cell dysfunction. One such antibody, IPH2101 that targets KIR2D, has been evaluated and shown efficacy in preclinical models of multiple myeloma and lymphoma (316-318). In addition, AML patients with NK cells at early stages of maturation show a poorer clinical outcome than those harboring mature and functional NK cells (319). The expression of natural cytotoxicity receptors also influences on the prognosis of AML as patients with a low expression of NKp30 and NKp46 at diagnosis display reduced survival rates (320).

Remission maintenance therapy with HDC and low-dose IL-2 has been shown to reduce the risk of relapse in the post-chemotherapy phase of AML (37, 39, 321). Serial analyses of the immune phenotype of patients undergoing HDC/IL-2 therapy have revealed that the NK cell repertoire of a patient impacts on outcome where, among other factors, expression of the NK cell-activating receptors NKp46 and NKp30 is associated with favorable prognosis (164, 322, 323). These results thus provide additional support for the relevance of NK cell function in determining the outcome of leukemia.

As in AML, dysfunctional NK cells are found in human CML before and on TKI treatment as well as in mice carrying *BCR-ABL1*$^+$ cells (324). In addition, it has been reported that the number of NK cells in patients with CML gradually decreases as patients progress from chronic to accelerated phase (325). Patients with CML present with granulocytic *BCR-ABL1*$^+$ CML cells with characteristics of MDSCs which are endowed with potent immunosuppressive features (176, 326-328). The presence of M-MDSC-like cells has also been observed in CML patients (329) and the levels of both G- and M-MDSCs are reportedly elevated in

patients at the time of diagnosis (327, 329). However, it remains uncertain whether TKI treatment restores the MDSC-levels to that of healthy controls (326, 327, 329). It may thus be speculated that a remaining pool of MDSCs sustained in a fraction of patients also after treatment with TKIs may explain the quantitative and functional NK cell defects observed in CML. As discussed in detail previsouly, recent results imply that the NK cell status in CML influences the likelihood of successful TKI discontinuation (330). Means to restore the functionality of NK cells, possibly through targeting the remaining pool of MDSCs, is thus a conceivable strategy to improve the likelihood of sustained leukemia-free survival after cessation of TKIs.

B CELLS IN CANCER

Certain B cells are capable of producing autoantibodies that are directed against self-antigens and hence may cause autoimmune diseases. Despite that the formation of autoantibodies is considered an undesired event, autoantibodies with specificity for neoplastic cells, "tumor-associated autoantibodies", may participate in controlling malignant growth and may also be useful as diagnostic markers. While understanding the role of B cells in cancer requires further study, it has been proposed that B cells may mount and modulate pro- as well as anti-tumor innate and T cell responses within the TME (331, 332).

IMMUNOTHERAPIES

Cancer immunotherapy, aiming at inducing or reinforcing immune responses against malignant cells, has emerged as a viable cancer treatment. The prototypic immunotherapy is allogeneic bone marrow transplantation (allo-BMT), which is commonly used in leukemia and lymphoma, where the hematopoietic cells of a patient are replaced by cells from a matched donor aiming at achieving a graft-versus-leukemia reaction to eliminate malignant cells. For example, allo-BMT is practiced in younger patients with intermediate- or high-risk AML (29). The lack of matched donors, the age barrier, the treatment-related mortality and morbidity along with the lack of clinical benefit of allo-BMT in most forms of solid cancer has inspired the development of alternative strategies aiming at ameliorating tumor immunity.

The early development in this area was focused on the activation of cytotoxic lymphocytes and included the administration of IFN-α (333, 334), a pleiotropic NK cell-activating cytokine, and IL-2 that promotes NK and T cell cytotoxicity and proliferation (335-337). Both of these compounds have been used clinically, mainly in metastatic melanoma and metastatic renal cell carcinoma. In AML, the only cytokine-based therapy that has proven clinically efficacious in a controlled phase III trial is HDC/IL-2 that reduces the risk of leukemic relapse in the post-

chemotherapy phase of AML (37, 321). In recent years, "checkpoint inhibitors" have been successfully implemented in the treatment of patients with advanced or metastatic cancer. Checkpoint inhibitor therapy, which aims at countering T cell suppression in the TME, is currently approved in several forms of cancer and the list of cancers in which such therapy is significantly clinically beneficial is rapidly growing (338-340).

IMMUNOSTIMULATORY CYTOKINES

Cytokines are growth factors that allow for communication between immune cells to coordinate immune responses and are mainly produced by innate and adaptive immune cells. The outcome of an interaction between a cytokine receptor and a cytokine is determined by the local concentration of the cytokine, the expression profile of the cytokine receptor as well as what response a cytokine triggers in the affected cell (341). Cytokines may either be immune activating or immunosuppressive. Cytokine-based cancer therapies thus include strategies that inhibit immunosuppressive cytokines as well as the administration of immune-activating cytokines. Immunosuppressive cytokines include IL-10 and TGF-β whereas IL-2, IL-15, and IFNs are examples of immune activating cytokines. However, cytokines are in general pleiotropic and may trigger dissimilar outcomes in different cells as exemplified by IL-2 that triggers the activation of the effector T cell compartment and the T regulatory compartment simultaneously, potentially leading to concomitant immune activation and inhibition (342).

The importance of cytokines in the control of tumor cells is highlighted by the results of murine studies demonstrating that mice genetically deficient in IFN-γ receptors or proteins essential for downstream IFN signaling more frequently develop spontaneous and chemically induced cancer (297, 343). It has further been shown that cytokines and lymphocytes collaborate to protect against the development of carcinogen-induced sarcomas and spontaneous epithelial carcinomas, but simultaneously select for tumor cells with reduced immunogenicity (343). In line with these murine studies, adjuvant IFN-α has been used in the treatment of stage III melanoma and was previously used as frontline therapy for CML whereas IL-2 has shown efficacy in the treatment of metastatic melanoma and renal cell carcinoma (333, 344-347).

The response to IFN-α is likely multi-faceted involving enhanced expression of MHC class I on tumor cells and induction of DC maturation, as well as activation of T cell and NK cell effector functions (348-351). The mechanism of anti-tumor action of IFN-α is hence complex but its ability to induce MHC class I expression on melanoma cells has been forwarded as a pivotal aspect of efficacy. The hypothesis is bolstered by the clinical effectiveness of IFN-α in hairy cell leukemia where malignant cells carry mutations in the *BRAF* gene as also reported for

melanoma cells (352, 353). In melanoma, *BRAF* mutations reportedly result in reduced expression of tumor-associated antigens (354). A similar effect on *BRAF*-mutated hairy cell leukemia cells would, in part, explain why two seemingly different disease respond to IFN-α therapy.

IL-2 is predominantly produced by T_H1 $CD4^+$ T cells and stimulates the proliferation of NK cells and T cells along with efficiently boosting NK cell cytotoxicity. In 1984 a heavily pretreated female with metastatic melanoma achieved long-lasting CR in response to high-dose IL-2 demonstrating that T and NK cell stimulating therapies could result in complete eradication of advanced cancers (355). However, only < 10 % of patients with advanced melanoma or renal cell carcinoma achieve CR upon high-dose IL-2 therapy (356, 357). The effect of IL-2 on T_{regs} and its ability to trigger activation-induced immune cell death may dampen the antitumor immune functions (358). Thus, cytokines that selectively activate effector T cells and NK cells without inducing T_{regs} may show superior antitumor activity. The current development in this area comprises the evaluation of modified variants of IL-2 to reduce off-target immunosuppression and IL-15 that shares the immune activation achieved by IL-2 but with reduced T_{reg} expansion (359-363).

ADOPTIVE CELL THERAPY

Adoptive cell therapy (ACT) refers to the infusion of *in vitro*-expanded lymphocytes. Allo-BMT is the most robust form of ACT and an effective therapeutic option in leukemias as previously discussed. For ACT in solid malignancies the lymphocytes, most often autologous T cells or NK cells, are usually isolated from peripheral blood, but occasionally also from tumor draining lymph nodes or tumor tissue. This strategy may circumvent potential inhibitory mechanisms manifested in the host such as immunosuppression and tolerance to tumor antigens. The isolated lymphocytes are cultured in a cocktail of growth factors aiming at expanding the antitumor immune cells and to reverse potential functional impairment. To increase the likelihood of sustained function and survival of the infused cells lymphodepletion often precedes infusion aiming at reducing immunosuppressive host populations such as MDSCs and T_{regs}. Patients receiving ACT also frequently receive cytokines to further support the viability and functionality of the infused cells (364).

Although results of randomized comparisons that evaluate the clinical efficacy of ACT are not available, a lymphodepleting conditioning regimen in conjunction with the infusion of TILs together with supplementation of IL-2 has shown promising results in the treatment of metastatic (phase IV) melanoma (365, 366). ACT is however not widely used for the treatment of solid malignancies, partly due to the high technical requirements needed to establish a functional infusion product. Additionally, TIL therapy has shown limited clinical efficacy in solid

malignancies besides melanoma. The high mutational burden in melanoma, which is associated with the presence of multiple tumor antigens has been suggested to explain why melanoma patients may be more likely to respond to ACT compared with patients with other forms of cancer (364). A limiting factor of ACT in solid malignancies stems from a defective homing of the infused cells to the tumors. Means to improve lymphocyte infiltration to the tumor site have thus been evaluated aiming to improve the effectiveness of the therapy. The *ex vivo* expansion of human NK cells results in increased expression of the chemokine receptor CXCR3 which is associated with improved intratumoral infiltration of NK cells and reduced tumor growth of CXCL10-positive tumors in a murine model of melanoma (367). Additionally, transduction of NK cells to express the chemokine receptors CXCR2 is proposed as a strategy to improve homing of NK cells to tumors and thus the efficacy of NK cell ACT (368).

ENGINEERED T CELLS

Attempts are being made to broaden the use of ACT by engineering T cells, which could result in a "off-the-shelf" *in vivo* allogenic product accessible for a larger proportion of patients. Two main types of engineered T cells are currently available and referred to as TCR-engineered T cells and chimeric antigen receptor (CAR) T cells. The TCR-engineered T cells are modified to express a TCR recognizing an HLA tumor-derived peptide complex. The TCR-engineered T cells would thus be accessible for any patient harboring tumor cells that present the cognate HLA and target antigen. The use of TCR-engineered T cells has so far been limited, mostly due to significant off target effects with destruction of healthy tissues also expressing the tumor-associated antigen (369). The identification of more selective tumor antigens, unique for the tumor tissues, is hence desirable. An additional challenge for TCR-engineered T cell therapy is that it requires tumor cells to carry a functional antigen processing machinery and that antigens are presented on MHC. Hence, the commonly observed downregulation and impairment of MHC presentation, whereby the malignant cells circumvent $CD8^+$ T cell immunity, will negatively influence on the anti-tumor efficacy of this therapy.

The second type of engineered T cells, CAR T cells, do not require MHC presentation by the target cells as they carry an Ig variable domain from an antibody fused to the constant domain of a TCR (370). A CAR T cell thus encompasses an antibody that may recognizes any extracellular structure expressed by a cell, independently of MHC presentation, as well as the cytotoxic features of a $CD8^+$ T cell. A limitation in CAR T cell therapy is however that only extracellularly expressed antigens can be detected. CAR T cells have hitherto mainly been developed for the recognition of CD19 on malignant B cells (in particular in B cell acute lymphoblastic leukemia) with initial CR rates ranging from 70 to 90 % in clinical trials (371). A major challenge to increase the efficacy of CAR T cell therapy is to improve the infiltration and survival of the infused

cells in solid tumors and, also, to develop strategies that take the phenotypic diversity of cancer cells into account.

ANTIBODIES IN CANCER IMMUNOTHERAPY

Monoclonal antibodies are commonly used in cancer therapy. They may be designed to i) inhibit signaling molecules of relevance to tumor expansion such as anti-VEGF antibodies, ii) neutralize checkpoint inhibition such as anti-PD-1 antibodies, iii) induce ADCC by binding to cell surface markers expressed by the malignant cells such as CD19, and iv) to kill tumor cells directly or via complement dependent lysis. The inability of monoclonal antibodies to aid an immune response involving cytotoxic T cells has inspired the development of engineered antibodies with multiple antigen specificities, referred to as bispecific antibodies (BsAbs) (372). BsAbs most often consist of two or three Fab fragments linked together to form a construct that can recognize structures on different cells and hence provides a bridge between diverse cell types. BsAbs are either IgG-like with an intact Fc domain or non-IgG-like that lack the Fc region. IgG-like constructs resemble conventional antibodies in structure and have a longer serum half-life due to the intact FcRn region that mediates recycling of antibodies by reducing lysosomal degradation in endothelial cells. The non-IgG-like constructs typically only consist of divergent Fab fragments linked to each other resulting in smaller molecules with better tissue penetration but with reduced serum half-life, partly due to a non-intact FcRn region (373).

Several types of BsAbs have been generated where the bispecific T cell engagers (BiTEs), bispecific killer cell engagers (BiKEs), and the trispecific antibodies (TrAbs) are the most studied. The most common design of BiTEs and BiKEs is one Fab fragment recognizing CD3 on T cells or CD16 on NK cells fused with another Fab fragment recognizing a structure expressed by the malignant cells. Thereby, BiTEs and BiKEs induce T cell- or NK cell-mediated cytotoxicity against the cell that the BsAb attaches to.

Two BsAbs, catumaxomab and blinatumomab, are approved for use in cancer therapy. Catumaxomab links three cell types: one Fab fragment of the antibody binds to EpCAM expressed by epithelial malignant cells and the other Fab fragment to CD3 on T cells. The third arm is an Fc domain potentially linking Fc receptor-expressing cells with cytotoxic function, such as NK cells or macrophages, to the tumor cells. Treatment with catumaxomab thus aims at inducing T cell cytotoxicity, phagocytosis, and ADCC against tumor cells (374) and is currently used in $EpCAM^+$ cancers such as gastric, ovarian, and breast cancer (375, 376). The second approved BsAb, blinatumomab, is a BiTE with two Fab fragments recognizing CD3 and CD19, respectively, and is hence a non-IgG-like-BsAb. Blinatumuab is approved in the treatment of B cell precursor acute

lymphoblastic leukemia (377). Numerous BsAbs are currently being evaluated in solid and hematopoietic malignancies.

CHECKPOINT INHIBITORS

Checkpoint inhibitors are antibodies designed to reduce T cell suppression via the PD-1, the CTLA-4, or related inhibitory pathways. The currently available antibodies targeting the PD-1- or the CTLA-4-pathways are approved in several forms of cancer. Expression of CTLA-4 is induced on conventional T cells following their activation. It binds to CD80 and CD86 on APCs with a higher affinity than the stimulatory receptor CD28 and transmits inhibitory signals to the T cell to prevent excessive activation. In addition, CTLA-4 is constitutively expressed by regulatory T cells, where it contributes to its immunosuppressive functions, possibly by capturing and removing costimulatory CD80 and CD86 from the surface of APCs (116, 378). The rationale of using anti-CTLA-4 antibodies is thus to enhance activation of anti-tumor T cells that may contribute in tumor eradication. The first anti-CTLA-4 antibody, ipilimumab, has been shown to induce tumor regression and improve overall survival in patients with metastatic melanoma (338). The clinical use of ipilimumab may, however, be associated with adverse immune related side-effects likely explained by enhanced T cell activation with ensuing autoimmunity.

Whereas anti-CTLA-4 therapy enhances T cell activation during priming, blockade of the PD-1 pathway is assumed to foremost restore functions of memory T cell populations. PD-1 is expressed by activated T cells and its ligands, PD-L1 or PD-L2, are expressed by target cells such as malignant cells but may also be expressed by cells in the TME such as MDSCs and other myeloid cells. Under normal conditions the PD-1-pathway serves to dampen ongoing effector immune responses to restore homeostasis. The expression of PD-L1/L2 in the TME does however dampen anti-tumor immunity (369) and tumor cells are assumed to highjack this pathway since increased PD-L1/L2 expression renders effector T cells inactive. The first trials showing clinical efficacy of PD-1 blockade were performed in metastatic melanoma, where approximately 30 % of patients experienced durable tumor regression and markedly prolonged survival (339). Antibodies neutralizing the PD-1-pathway are currently approved for use in patients with melanoma, non-small cell lung cancer, lymphoma, renal cell carcinoma, and urothelial cancer (369). Patients receiving anti-PD-1 therapy are less prone to develop autoimmunity than those receiving anti-CTLA-4, possibly because the targeting of PD-1 preferentially restores functions of previously activated T cells.

CANCER VACCINES

Several approaches to develop cancer vaccines have been explored including non-targeted peptides or proteins, antigens coupled to anti-DC-antibodies, and infusion of *ex vivo* loaded DCs. Non-targeted vaccines are typically synthetic peptides that may be infused in combination with adjuvants to achieve optimal DC activation (379). Several vaccines have been shown to increase the number of peptide-specific T cells but the impact on the clinical course of cancer has been modest (380-382). The lack of clinical efficacy despite T cell activation has been suggested to be explained by $CD4^+$ T cell responses skewed to secrete type II cytokines rather than type I cytokines such as IFN-γ (382). Combining peptide vaccines with IFN-γ has been shown to optimize T cell polarization with significantly more IFN-γ producing antigen-specific T cells (383). Additionally, recent technical development has resulted in personalized cancer vaccines where the peptides correspond to patient-specific neoantigens (379). In addition, substances limiting the attraction of T_{regs} may be added to hinder immunosuppression (384). Another approach where GM-CSF is used to boost DC activation is the cancer cell-based vaccine GVAX where irradiated tumor cells are modified to express GM-CSF aiming to enhance DC recruitment and activation (385). *In vivo* targeting of DCs can also be achieved by the conjugation of antibodies directed against DC surface molecules to the tumor antigens (386-388). Several adjuvants are additionally evaluated aiming at reducing activation of T_{regs} and to achieve a more robust induction of anti-tumor T cells (388, 389).

An additional DC vaccine approach is the *ex vivo* generation and loading of DCs which are later infused to the patient. Sipuleucel-T is such a cellular product approved by the US Food and Drug Administration used for the treatment of metastatic prostate cancer. The treatment involves culturing antigen-presenting cells in the presence of a fusion protein composed of a neoantigen and GM-CSF and has been shown to prolong the survival of prostate cancer patients with four months (390). Patient-derived AML cells have also been fused with autologous DCs creating a broad range of neoantigens in the context of DC-derived MHC presentation. AML patients in CR were infused with the vaccine to target residual leukemic cells. The approach resulted in induced expansion of leukemic-specific T cells and entailed long CR duration in a smaller study (391).

Several single agent cancer vaccines boost immune rejection of tumor cells but fail to demonstrate pro-longed efficacy in late-stage clinical studies. Thus, combinations of different immunotherapies, including those targeting immunosuppression, may be necessary for durable responses to cancer vaccines.

TARGETING MDSCS

Various strategies have been explored to target MDSCs pharmacologically. Cancer chemotherapies such as gemcitabine and 5-flurouacil reportedly result in a reduction of MDSCs in murine cancer models, which may contribute to its therapeutic effects (392, 393). The prostaglandin E2 and cyclooxygenase-2 (COX-2) pathway have been suggested important for the induction of MDSCs (394, 395). Thus, inhibition of the expansion of MDSCs has been explored via targeting COX-2 (394, 396, 397). Additionally, the inhibition of the CSF-1 receptor, that growth factors driving myelopoiesis act via, have further been explored in an attempt to prevent the expansion of MDSCs. The infiltration of CSF-1R positive myeloid cells reportedly predicts poor survival in patients with neuroblastoma and the addition of a CSF-1R antagonist dampens tumor cell-induced generation of human MDSCs *in vitro* and reduces the formation of neuroblastoma tumors in a transgenic mouse model. Additionally, combining CSF-1R blockade with PD-1/PD-L1 checkpoint inhibition enabled superior tumor control over monotherapy with either treatment (398).

Approaches to stimulate MDSC differentiation into less suppressive myeloid cells include stimulation with ATRA and NOX2 inhibition (242-248) and are in agreement with the results from **paper II**. The effect of ATRA has been assessed in renal cell carcinoma and non-small cell lung cancer and was found to dramatically reduce MDSC numbers and improve the effects of immunotherapies using IL-2 and DC vaccines against p53 (247, 399). The efficacy of the combination of ATRA and ipilimumab is currently evaluated in stage IV melanoma (ClinicalTrials.gov identifier: NCT02403778).

Targeting the immunosuppressive mechanisms utilized by MDSCs is another explored therapeutic strategy. A phosphodiesterase-5 inhibitor, tadalafil, reportedly targeted MDSCs and restored T cell function in a phase II clinical trial in patients with head and neck squamous cell carcinoma (400). Tadalafil was further tested as palliative treatment in metastatic melanoma with promising results. The treatment reduced the formation of NO by MDSCs in the majority of responding patients whereas no such effect was observed in patients progressing on tadalafil (401). Production of NO, ROS, and peroxynitrate results in nitration of the TCR and is one of the most potent immunosuppressive features of MDSCs. Drugs like nitroasparins that reduce oxidation is hence a promising approach to limit MDSC-mediated T cell suppression. Oral administration of a nitroasparin, NCX 4016, to tumor-bearing mice has been shown to reduce immunosuppression without affecting the numbers of suppressive myeloid cells thus suggesting the NCX 4016 influences on events downstream of myeloid cell activation. In further analysis NCX 4016 was found to reduce the enzyme activity of arginase 1 and iNOS in $CD11b^+$ splenocytes from tumor-bearing mice to levels comparable with the same cell fraction isolated from tumor-free hosts (402).

AIMS

The overall aim of this thesis work was to further define the role of NOX2-derived ROS for myeloid cell function and differentiation in cancer. The specific aims for each paper are listed below.

Paper I aimed to define the effect of NOX2-inhibition, using HDC, on the development of monocyte-derived DC *in vitro* and to study the impact of this mechanism on solid tumor growth *in vivo*.

Paper II aimed to clarify how NOX2 inhibition, using HDC, affected MDSCs *in vitro* and *in vivo*.

Paper III aimed to determine effects of NOX2 inhibition on differentiation of AML cells *in vitro* and *in vivo*.

Paper IV aimed at assessing the importance of NOX2-derive ROS for the initiation and progression of CML *in vivo*.

MATERIALS AND METHODS

ANIMAL MODELS

The animal experiments performed in this study were approved by the Research Animal Ethics Committee in Gothenburg. Mice were maintained under pathogen-free conditions with unlimited supply of food and water according to the guidelines issued by the University of Gothenburg. The studies have been conducted in the following strains of mice: C57BL/6J, B6.129S6-*Cybb*^{tm1Dim} lacking the gp91^{phox} subunit of NOX2 (*Nox2*-deficient) and are thus incapable of NOX2-derived ROS formation, BALB/c, NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac (NOG) deficient of NK and T cells, and Rag2/OT-1 with T cells specific for the peptide SIINFEKL.

SOLID TUMOR MODELS

This thesis confers studies examining the role of NOX2-derived ROS for the *in vivo* tumor growth of murine EL-4 lymphoma, 4T1 mammary carcinoma, and MC-38 colon adenocarcinoma (presented in **paper I** and **paper II**). These tumor cell lines were chosen since they are known to induce a pronounced expansion of MDSCs in tumors and in the periphery. The EL-4 cell line was originally established by the induction of lymphoma in a C57BL/6N mouse by treatment with the mutagen 9,10-dimethly-1,2-benzanthracene. The EL-4 cells line is one of the most commonly used cell lines for studies of MDSCs during tumor growth in mice. The 4T1 tumor cells were originally isolated from a mammary tumor that was spontaneous arising in BALB/cfC3H mice. The MC-38 cells originate from a chemically induced grade II colon cancer in a female C57BL/6 mouse.

Solid EL-4, 4T1, and MC-38 tumors were established in *Nox2*-competent (wild type, WT) or *Nox2*-deficient (*Nox2* knockout, KO) mice by subcutaneous injections of tumor cells. Mice were treated with 1000-1500µg HDC three times weekly starting one day before tumor cell inoculation and/or 100-240µg anti-PD-1/anti-PD-L1 antibodies three, six, and ten days post tumor cell injection. Tumors were measured three times per week and mice were scarified once tumors of untreated controls reached a median size of 1.5cm in diameter. The set-up of the solid tumor models is depicted in Figure 7.

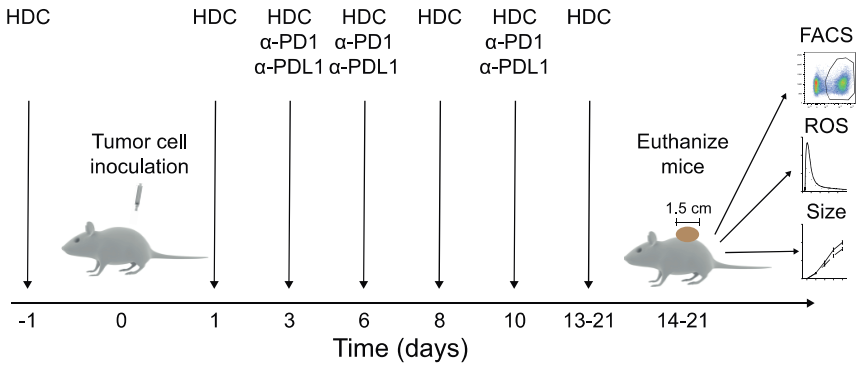

Figure 7. Experimental set-up of solid tumor models. Mice received intraperitoneal injections of 1000-1500μg HDC thrice weekly starting one day before tumor cell inoculation. Three, six, and ten days post tumor cell inoculation mice received intraperitoneal injections of 100-240μg anti-PD-1 (α-PD1) and anti-PD-L1 (α-PDL1). Mice were euthanized once tumors of control mice reached a median size of 1.5cm in diameter. Single cell suspensions from spleens and tumors were analyzed for ROS production, content of myeloid and lymphoid cell populations, and immunosuppressive features.

AML MODEL

In **paper III** the human AML cell line PLB-985 was used to study the effect of NOX2 inhibition on AML cell differentiation. The PLB-985 cell line was isolated from a 38-year-old woman in 1985 with myelomonocytic AML. By *in vitro* manipulation, a *NOX2* knockout clone of the PLB-985 cells has been generated (403). Experiments utilizing WT and *NOX2* KO PLB-985 cells allows for identification of the NOX2 specific targeting mechanisms of HDC. To determine *in vivo* effects of HDC on leukemic cells capable (*NOX2* WT) and incapable (*NOX2* KO) of NOX2-derived ROS-formation, 2 x 10^6 cells were transplanted to sublethally irradiated immunodeficient NOG mice by intravenous injections. Starting two weeks post transplantation, mice received 1500μg HDC thrice weekly administrated via intraperitoneal injections. Mice were euthanized once presenting with disease symptoms. Figure 8 shows the experimental design of this study.

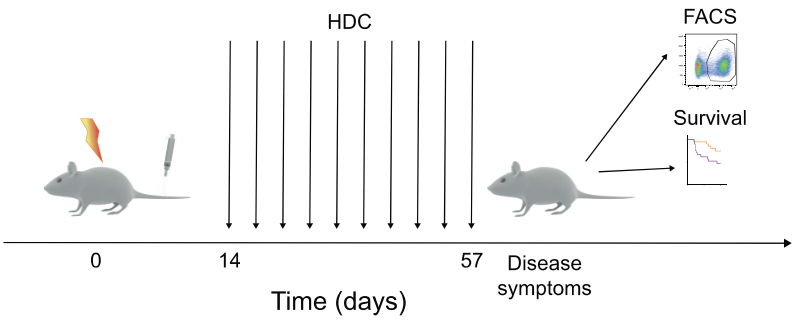

Figure 8. AML disease model. Sublethally irradiated NOG mice were transplanted with WT or *NOX2* KO PLB-985 cells. Two weeks post-transplantation, mice received intraperitoneal injections of 1500μg HDC thrice weekly. Mice were euthanized once showing disease symptoms. The frequency of human cells in BM of diseased mice was examined using flow cytometry.

The AML cell line OCI-AML3 was also utilized in **paper III**. This cell line was established from the blood of a 57-year old man diagnosed with M4 AML in the 1980s. The OCI-AML3 cells carry one of the most frequently occurring genetic alterations observed in AML, i.e. mutated nucleophosmin. These cells are thus commonly used to study the characteristics of AML carrying nucleophosmin mutations.

BCR-ABL1 MODEL

In **paper IV** the importance of a functional NOX2 was examined for the ability of *BCR-ABL1*$^+$ cells to initiate a CML-like disease in an *in vivo* model. Murine *BCR-ABL1*$^+$ cells were generated by transduction of 5-flurouacil-treated BM cells isolated from female C57BL/6J WT or *Nox2* KO mice. Transduced cells were expanded and *BCR-ABL1*$^+$ cells (reflected by the reporter protein green fluorescence protein) were isolated using flow cytometry. The following day, 1 x 10^5 *BCR-ABL1*$^+$ WT or *Nox2* KO cells were transplanted to lethally irradiated female WT C57BL/6J mice together with 2 x 10^6 rescue BM cells. Disease initiation and progression was monitored by contentious blood draws and mice were euthanized upon disease symptoms or when presenting with elevated white blood cell counts. The *in vivo* CML model is depicted in Figure 9.

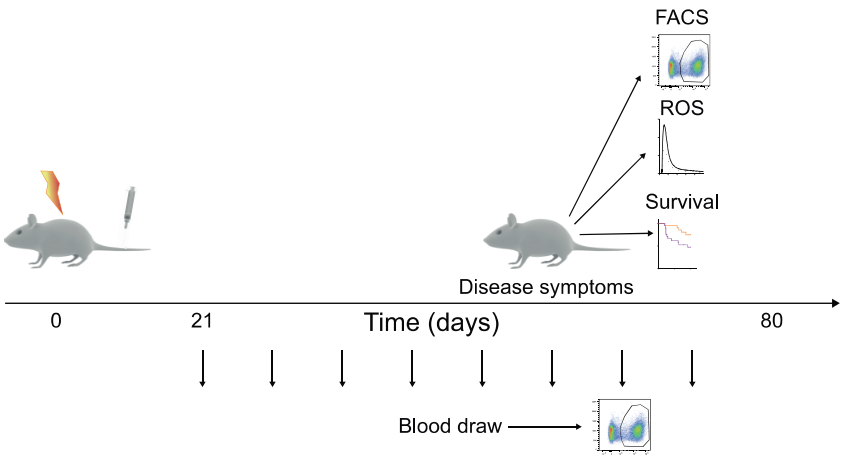

Figure 9. CML disease model. Lethally irradiated mice were transplanted with 100 000 WT or *Nox2* KO *BCR-ABL1*$^{+}$ cells together with 2 million rescue BM cells. Blood was collected every other week and analyzed for the presence of *BCR-ABL1*$^{+}$ cells, reflected by positivity for green fluorescence protein, by flow cytometry. Mice were sacrificed when showing signs of disease upon which single cell suspensions from liver, spleen, blood, and BM were analyzed for ROS production and leukemic cell frequency.

RE:MISSION TRAIL

In **paper II** and **paper III** samples from patients receiving remission maintenance therapy with HDC and IL-2 were studied. The samples were obtained from a phase IV trial (Re:Mission trial, NCT01347996) which enrolled 84 AML patients in first CR from 20 centers in Europe. The trial was approved by the Regional ethical review board in Gothenburg and all procedures of this study were performed in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants before enrollment.

Patients received ten three-week cycles of HDC/IL-2 with three to six weeks of resting period in between cycles. The treatments included 0.5mg histamine and 1μg/kg body weight IL-2 administrated by the patients themselves at home by subcutaneously injections twice daily. Blood was collected before and after cycle one and three and shipped to the Tumor Immunology lab at the Sahlgrenska Cancer Center that served as the central laboratory for immunological assessments. Patients were followed for at least two years for leukemia-free and overall survival (Figure 10).

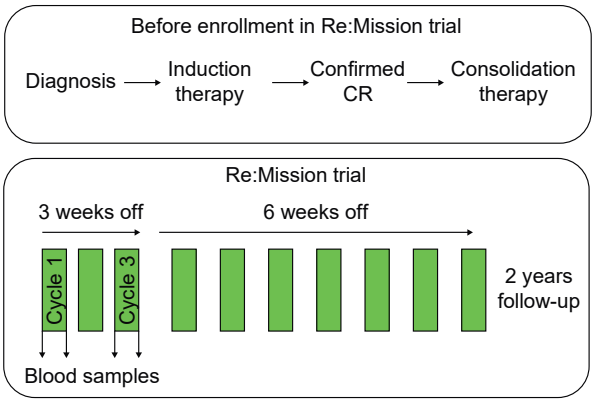

Figure 10. Design of Re:Mission trial. AML patients in CR were enrolled after the completion of chemotherapy. Patients received ten three-week cycles of HDC/IL-2 therapy and blood was collected at the beginning and end of cycle one and three.

STATISTICS

STUDENT´S T-TEST

Two-tailed student´s *t*-test was used to determine if the means of two data sets were significantly different. The test requires that each data set is normally distributed. The Wilcoxon matched-pairs signed rank test was used for paired analyses.

ANALYSIS OF VARIANCE

Analysis of variance (ANOVA) was employed to determine if the means of more than two groups differed significantly. When more than two comparisons were made the risk of false positive significances increases, which the ANOVA takes into account. We have used one-way ANOVA to compare the means between more than two groups and two-way ANOVA to determine how a response is affected by two factors such as time and treatment.

For multiple comparisons Bonferroni, Holm-Sidak, and Tukey´s methods were used. The Bonferroni or Holm-Sidak methods compare the difference between selected means whereas Tukey´s method compares the means of each group with the mean of every other group.

LOGRANK TEST

The logrank test was employed to compare differences in survival distribution between two groups. It is a nonparametric test that does not require a normal

distribution. The test is constructed by adding the observed and expected number of events at each time point for each of the study groups.

ADDITIONAL TECHNIQUES

For a detailed description of standard techniques, such as flow cytometry, ROS measurement, and cell culturing, the reader is referred to the materials and methods section of the respective papers.

RESULTS

PAPER I

In **paper I** we aimed to study the effect of NOX2 inhibition, using HDC, on monocyte differentiation to DCs. Impaired generation of sufficient numbers of functional DCs is a commonly observed event in cancer (266, 267). Since DCs are essential for proper activation and propagation of T cells, including anti-tumor T cells, promoting the generation and function of DCs may result in improved anti-tumor immunity. The initial experiments in **paper I** assessed if HDC may synergize with GM-CSF and IL-4 in promoting the maturation of DCs from human monocytes. We observed that the addition of HDC to cell cultures resulted in more elongated cells, which is characteristic for DCs. Using FACS we demonstrated that DCs generated in the presence of HDC expressed increased levels of HLA-DR (Figure 11a) and the costimulatory molecules CD86 (Figure 11b) along with CD40 and thus displayed a phenotype associated with professional APCs.

We next examined if the activated phenotype of HDC-generated DCs resulted in improved functionality. Compared with control DCs, HDC-generated DCs were significantly better at activating $CD4^+$ T and $CD8^+$ T cells, as determined by enhanced proliferation and production of IFN-γ and IL-4. To assess if the HDC-induced expression of CD86 contributed to the observed induction of DCs, CD86 neutralizing antibodies were added to the cultures. This resulted in a significant reduction of T cell proliferation suggesting that the HDC-mediated induction of CD86 on DCs contributed to the improved functionality.

HDC reduces NOX2-derived ROS production in human myeloid cells (38). In a next series of experiments, we aimed to assess if this mechanism may explain the pro-differentiating properties of HDC on myeloid cells. Human DCs generated in the presence of HDC contained reduced levels of intracellular ROS, as determined by DCFDA staining. The extracellular ROS scavenger catalase did not mimic the effect of HDC on DC maturation. To further define the role of NOX2-derived ROS for HDC-induced myeloid maturation, we utilized WT and *NOX2* KO variants of the human myelomonocytic PLB-985 cell line and observed that only the *NOX2*-sufficient PLB-985 cells responded to HDC therapy by differentiation (Figure 11c). We thus concluded that the pro-differentiating effect of HDC was reliant on the expression of functional NOX2. We also observed that the pro-differentiating effects of HDC were mediated by H_2R signaling, since HDC did not promote differentiation of DCs or PLB-985 WT cells in the presence of the H_2R antagonist ranitidine (Figure 11c).

To explore if a similar pro-differentiating effect of HDC occurred *in vivo*, naïve mice were treated with HDC followed by analysis of DCs in spleen and in the peritoneal cavity. *In vivo* treatment with HDC did not impact on the number of DCs, nor their expression of MHC class II or CD86 in this setting. We hypothesized that the pro-differentiating effects of HDC might be more pronounced in an environment with a higher redox state, such as in the TME. We therefore treated EL-4 tumor-bearing mice with HDC and observed a significant increment in the number of intratumoral DCs (Figure 11d) along with a reduced tumor growth rate. A positive correlation between the number of intratumoral DCs and $CD8^+$ T cells also supported that DCs favor anti-tumor immune responses. To determine if the *in vivo* effect of HDC was dependent on myeloid cells expressing functional NOX2, we repeated these experiments in *Nox2* KO mice. In this setting, where the myeloid cells were unable to generate NOX2-derived ROS, HDC did not enhance DC infiltration into tumors (Figure 11e) and did not reduce tumor growth *in vivo*.

In conclusion, the results of **paper I** imply i) that HDC stimulates DC maturation by inhibiting NOX2-derived ROS formation and ii) that HDC controls growth of EL-4 tumors in a NOX2-dependent manner.

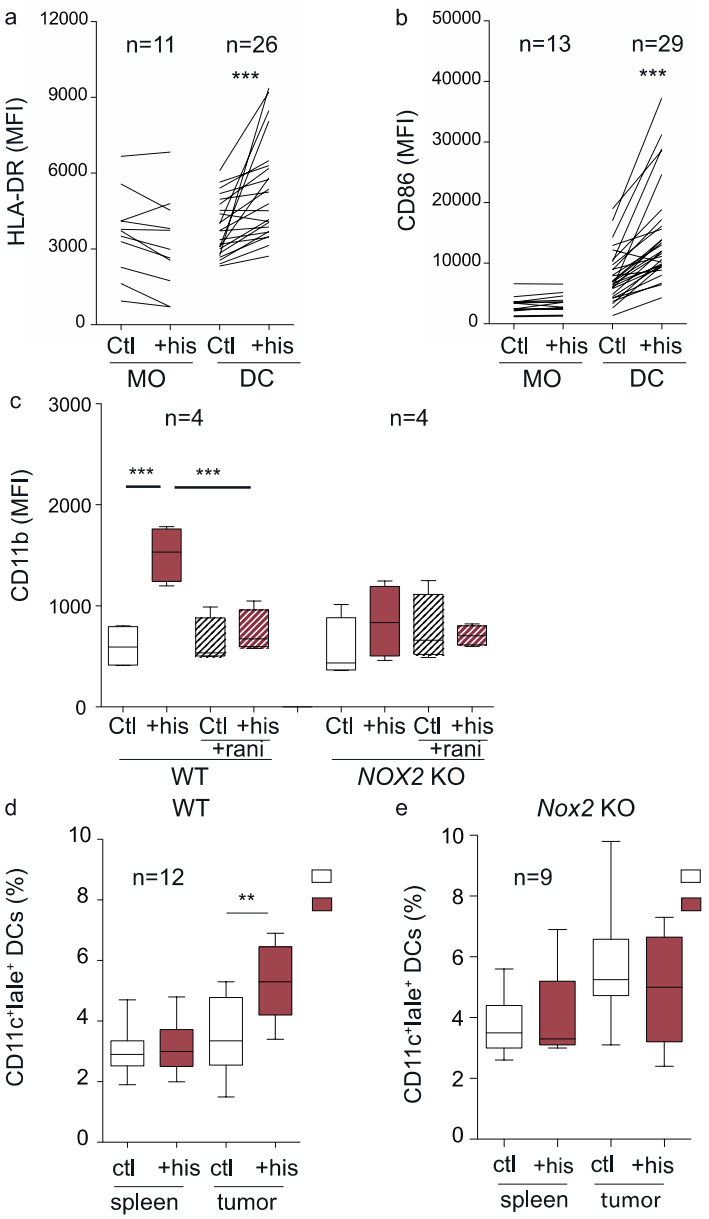

Figure 11. Results paper I. (**a-b**) Expression of (**a**) HLA-DR and (**b**) CD86 on monocytes (MO) and monocyte-derived DCs (DC) cultured in the absence (Ctrl) or presence of HDC (his). (**c**) Expression of CD11b on WT and *NOX2* KO PLB-985-cells in the absence (Ctrl) or presence of HDC (his) and ranitidine (rani). (**d-e**) Frequency of $CD11c^{+}IaIe^{+}$ DCs in spleen and tumor of EL-4 tumor-bearing (**d**) WT and (**e**) *Nox2* KO mice. **p<0.01, ***p<0.001.

PAPER II

Paper II aimed to further elaborate the findings from **paper I** of the pro-differentiating effects of HDC on myeloid cells, with specific focus on MDSCs. In cancer, MDSCs show compromised differentiation and produce excessive amounts of ROS, which may impair anti-tumor immunity. We hypothesized that HDC might reverse these features of MDSCs. In a first series of experiments we confirmed and extended the *in vivo* findings from **paper I** showing that HDC-treatment of EL-4 tumor-bearing mice resulted in reduced tumor growth in *Nox2*-sufficient but not in *Nox2*-deficient mice (Figure 12a-b). We also showed that HDC weakly but significantly reduced the *in vivo* growth of 4T1 mammary carcinoma with a similar trend observed in MC-38 colon carcinoma. The depletion of MDSCs using GR1-neutralizing antibodies abrogated the anti-tumor efficacy of HDC suggesting that HDC may act by targeting GR1-expressing MDSCs.

Further analyses of the tumor-bearing mice revealed that *in vivo* treatment with HDC resulted in reduced accumulation of MDSCs in EL-4 tumors and a less pronounced expansion of splenocytes (which were dominated by MDSCs) in 4T1 tumor-bearing mice. The frequency of intratumoral MDSCs in EL-4 tumor-bearing mice and the number of splenocytes in 4T1 tumor-bearing mice correlated positively with tumor progression in control mice. We did not observe such correlations in HDC-treated mice, suggesting that the myeloid cells of treated mice may be less immunosuppressive and thus less prone to influence tumor growth. We thus examined if leukocytes from tumors and spleens of HDC-treated EL-4 and 4T1 tumor-bearing mice showed reduced ROS-forming capacity. Treatment with HDC *in vivo* resulted in reduced ROS formation from myeloid cells of tumor-bearing mice *ex vivo*. Furthermore, spleen $GR1^+$ cells of HDC-treated EL-4-bearing mice were less suppressive towards $CD8^+$ T cells compared with MDSCs isolated from control EL-4 bearing mice (Figure 12c). In line with these results, a higher fraction of tumor-infiltrating T cells showed an effector phenotype in HDC-treated mice suggesting that the treatment resulted in a less immunosuppressive TME, favoring T cell effector responses.

Effects of HDC on MDSC generation were also assessed in human monocytic cultures. Human monocytes were incubated in medium supplemented with IL-6 and GM-CSF, which resulted in the generation of cells with a MDSC-like phenotype characterized by increased ROS formation and reduced expression of HLA-DR. In line with the results in mice, the addition of HDC to these cultures limited the generation of MDSC-like cells. We also examined the effect of HDC therapy on human $CD14^+HLA\text{-}DR^{low}$ MDSCs (M-MDSCs) in a phase IV clinical trial in AML where patients received cycles of HDC/IL-2 treatment. We found that treatment with HDC/IL-2 significantly reduced the frequency and numbers

of M-MDSCs (Figure 12d) and that patients with an above median reduction of M-MDSC numbers during the first cycles showed improved leukemia-free survival (Figure 12e).

Our results implying that HDC targets MDSCs, both by inducing their maturation and by limiting their immunosuppressive functions, incited us to investigate if HDC may improve the response of tumor-bearing mice to PD-1/PD-L1 blockade *in vivo*. We thus combined HDC treatment with antibodies against PD-1 and PD-L1 and observed that the combined therapy was superior in reducing tumor growth compared with monotherapy with either HDC or PD-1/PD-L1 checkpoint inhibition alone in EL-4 and MC-38 tumor-bearing mice (Figure 12f).

Paper II thus extended the findings reported in **paper I** to show that HDC-treatment i) results in reduced accumulation of MDSCs with less immunosuppressive features, ii) enhances the effector phenotype feature of tumor infiltrating $CD8^+$ T cells, and iii) improved the anti-tumor efficacy of checkpoint inhibition.

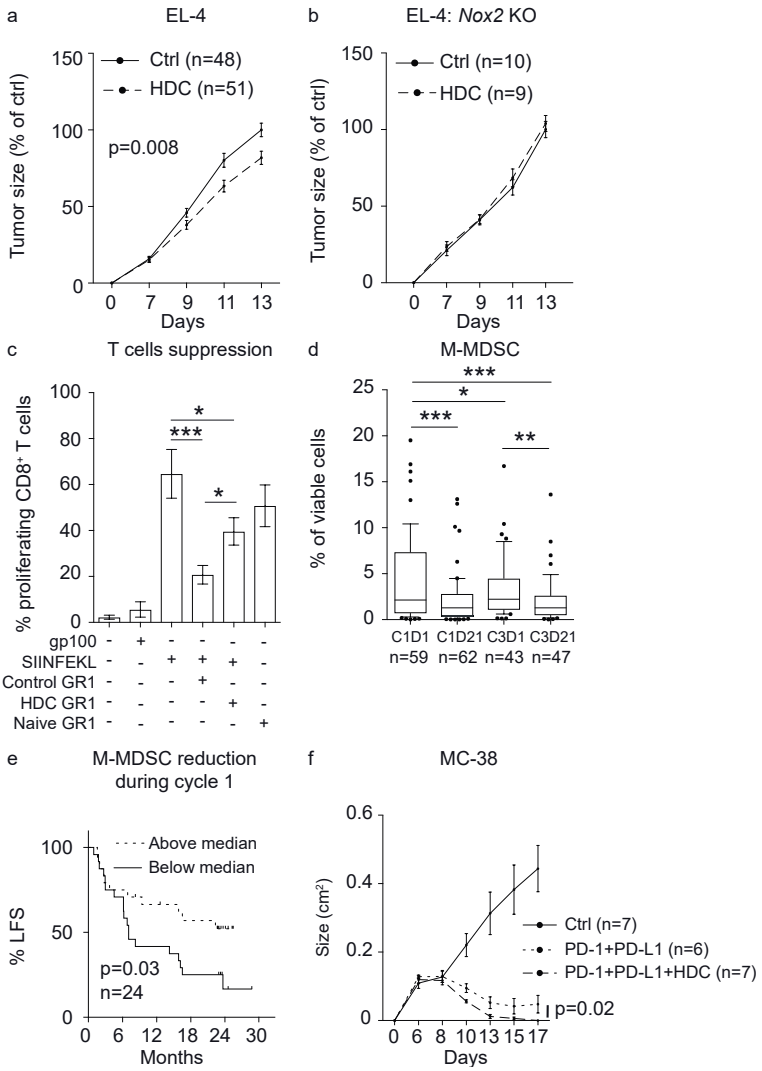

Figure 12. Results paper II. (**a-b**) EL-4 tumor growth in WT (**a**) and *Nox2* KO (**b**) mice. Untreated (solid line), HDC-treated (dashed line). (**c**) Percentage of proliferating $CD8^+$ T cells in the absence of stimuli (n=3), in response to a control peptide (gp100, n=3) or in response to the OT-1 specific peptide (SIINFEKL, n=3). In specified wells, $GR1^+$ cells that had been isolated from control (n=5) or HDC-treated (n=6) EL-4-bearing mice or $GR1^+$ cells isolated from tumor-free mice (n=2) were present at a 1:1 ratio with the SIINFEKL stimulated OT-1 splenocytes during the course of proliferation. (**d**) Frequency of M-MDSCs before (cycle 1, day 1; C1D1) and after the first treatment cycle (cycle 1, day 21; C1D21) and at the beginning (cycle 3, day 1; C3D1) and end (cycle 3, day 21; C3D21) of the third treatment cycle. (**e**) Impact of M-MDSC reduction on leukemia free-survival (LFS) in patients undergoing HDC/IL-2 therapy. Patients were dichotomized by the median reduction of M-MDSC counts during the first treatment cycle (n=24 for each group). (**g**) MC-38 tumor growth in control (solid line), anti-PD-1/anti-PD-L1-treated (dotted line), and HDC/anti-PD-1/anti-PD-L1-treated (dashed line) mice. *p<0.05, **p<0.01, ***p<0.001.

PAPER III

The aim of **paper III** was to test if HDC, in addition to inducing differentiation of DCs and MDSCs, may also promotes differentiation of AML blasts that express NOX2 and H_2R. Previous studies have shown that AML cells of the FAB-classes M4 (myelomonocytic) and M5 (monocytic) frequently express both NOX2 and H_2R (39), which was confirmed in this study.

The addition of HDC to the NOX2- and H_2R-positive myelomonocytic AML cell lines, PLB-985 and OCI-AML3, triggered increased expression of the myeloid maturation markers CD11b (Figure 13a), CD14, formyl peptide receptor 1 and 2 (FPR1 and FPR2). HDC did however not alter differentiation of *NOX2* KO PLB-985 cells (Figure 13a) while DMSO, a non-specific inducer of maturation, was equally efficacious in promoting maturation of *NOX2* WT and KO cells (Figure 13b). In agreement, treatment of primary human AML cells expressing NOX2 and H_2R (i.e. FAB-M4/M5 AML cells) with HDC increased the expression of HLA-DR (Figure 13c), FPR1, and FPR2. No such upregulation was observed in AML cells lacking the expression of NOX2 and H_2R (i.e. FAB-M0/M1/M2 AML cells) (Figure 13c).

The only known biological role of NOX2 is to generate ROS. As expected, only WT, but not *NOX2* KO PLB-985 cells, produced ROS in response to the NOX2-stimulus fMLF. HDC inhibited the ROS production from WT PLB-985 cells. Further analysis showed that *NOX2* KO cells also contained significantly less cytoplasmic ROS and produced lower levels of mitochondrial ROS (Figure 13d). These data suggest that not only are NOX2-derived ROS reduced in *NOX2* KO cells, but there is an interplay, or feed-forward mechanism, between different sources of ROS. To further illuminate the role of ROS for myeloid differentiation WT and *NOX2* KO PLB-985 cells were treated with the ROS scavengers luminol (that scavenges intra- and extracellular ROS) or isoluminol (that only scavenges extracellular ROS). Luminol triggered a pronounced differentiation of both WT and *NOX2* KO PLB-985 cells, while the effect was modest for isoluminol (Figure 13e). These results indicate that intracellular ROS in leukemic cells impede myeloid maturation and that other sources of ROS, in addition to NOX2, contribute to the effect.

To determine NOX2-dependent down-stream effects of HDC on AML cells, WT and *NOX2* KO PLB-985 cells were treated with HDC for two days and then subjected to microarray analysis. We found that a fraction of genes was regulated by HDC in WT cells, while virtually no genes were significantly regulated by HDC in *NOX2* KO cells. Among genes specifically regulated by HDC in WT cells were genes involved in cell cycle progression. We thus performed experiments to

elucidate cell cycling effects of HDC and found that HDC blocked S-phase entry of WT but not of *NOX2* KO PLB-985 cells.

Effects of HDC on AML cells *in vivo* were further investigated in a xenograft model where immunodeficient NOG mice were transplanted with WT or *NOX2* KO PLB-985 cells. HDC-treated mice transplanted with WT PLB-985 AML cells survived slightly longer than untreated control mice and presented with significantly fewer $CD45^+$ AML cells in BM at the time of sacrifice (Figure 13f). In contrast, HDC treatment did not affect leukemic expansion in mice transplanted with *NOX2* KO PLB-985 AML cells (Figure 13f). The results of **paper III** thus support that HDC exerts direct effects on $NOX2^+$ monocytic leukemic cells.

In summary, the results of **paper III** imply that i) HDC exerts pro-differentiating effects on monocytic AML cells, ii) reduces leukemic expansion *in vivo* by targeting NOX2, and iii) HDC-mediated reduction of intracellular ROS facilitates the maturation of monocytic leukemic cells.

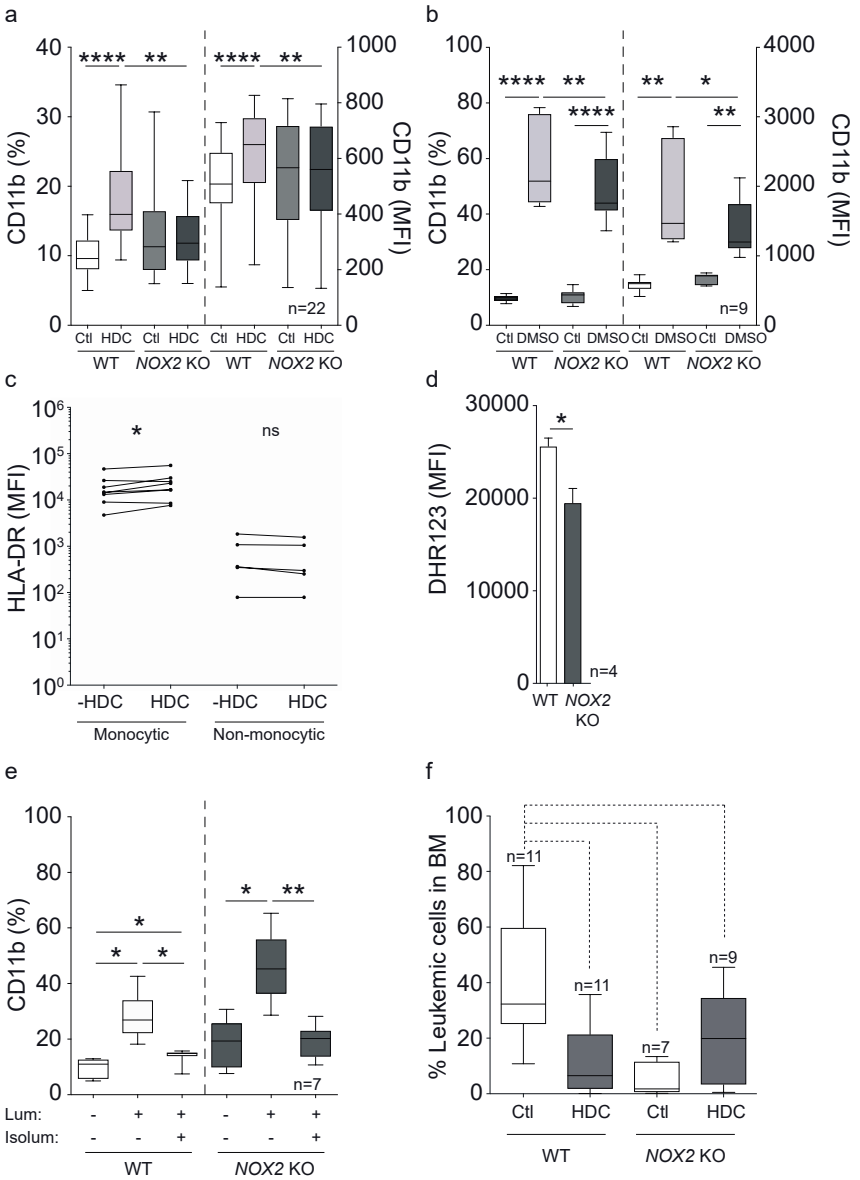

Figure 13. Results paper III. (**a-b**) Expression of CD11b+ on WT and *NOX2* KO PLB-985 cells after (**a**) HDC exposure or (**b**) DMSO exposure. (**c**) Expression of HLA-DR of monocytic and non-monocytic primary AML cells after stimulation with HDC. (**d**) Levels of mitochondrial ROS in WT and *NOX2* KO PLB-985 cells. (**e**) Frequency of CD11b+ WT and *NOX2* KO PLB-985 cells after culture with luminol (Lum) or isoluminol (Isolum). (**f**) Percentage of leukemic cells at the time of sacrifice in control (Ctl) and HDC-treated (HDC) mice transplanted with WT or *NOX2* KO PLB-985 cells. *p<0.05, **p<0.01, ***p<0.001.

PAPER IV

CML is initiated and sustained by the *BCR-ABL1* fusion gene. *BCR-ABL1* expression has been shown to result in elevated intracellular ROS levels in transformed cells but little is known regarding the role of NOX2-derived ROS for *BCR-ABL1*-induced leukemogenesis. The aim of **paper IV** was to investigate if NOX2-derived ROS contributed to the disease-initiating properties of *BCR-ABL1*$^+$ cells *in vitro* and *in vivo*. Colony-forming unit assay of murine *BCR-ABL1*$^+$ *Nox2* WT and KO cells revealed that *Nox2* deficiency reduced the serial colony formation of *BCR-ABL1*$^+$ cells suggesting that NOX2 impacts on the proliferation and differentiation of these cells (Figure 14a).

In an *in vivo* setting, we observed that transplantation of *Nox2* WT *BCR-ABL1*$^+$ cells to irradiated mice resulted in a more aggressive leukemia. Hence, mice transplanted with *Nox2* WT *BCR-ABL1*$^+$ cells showed a more rapid increment of leukemic cells in blood (Figure 14b) and also had a shorted lifespan than mice transplanted with *BCR-ABL1*$^+$ *Nox2* KO cells (Figure 14c). Analysis of BM cells of diseased mice indicated that the *BCR-ABL1*$^+$ *Nox2* KO cells tended to be more mature, with a higher fraction of $CD11b^+GR1^+$ cells and fewer $CD34^+$ immature cells.

In conclusion, the results presented in **paper IV** suggest that i) NOX2 contributes to the initiation of *BCR-ABL1* leukemia and ii) that further studies are warranted to define if NOX2-targeting therapies may be of value in *BCR-ABL1*$^+$ leukemia.

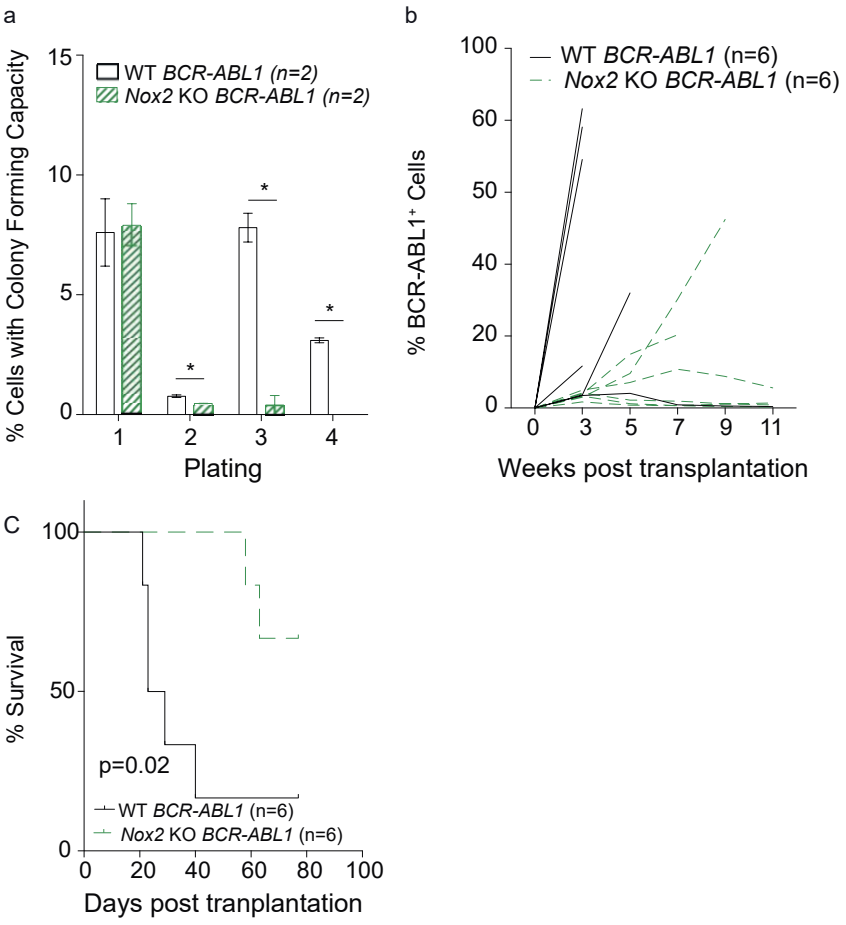

Figure 14. Results paper IV. (**a**) Colony forming capacity of WT and *Nox2* KO *BCR-ABL1*$^+$ cells. (**b**) Frequency of *BCR-ABL1*$^+$ WT and *Nox2* KO *BCR-ABL1*$^+$ cells in blood of transplanted mice. (**c**) Survival of mice transplanted with WT and *Nox2* KO *BCR-ABL1*$^+$ cells. *p<0.05.

DISCUSSION

This thesis aimed at contributing to the understanding of the role of ROS in cancer and to shed light on the possibility of targeting NOX2 for therapeutic purposes. A recurring finding in my studies was that NOX2 is a negative regulator of the differentiation of normal and leukemic myeloid cells, which may be reverted by genetic or pharmacological targeting of NOX2.

It has previously been shown that MDSCs from tumor-bearing, *Nox2*-deficient mice more readily differentiate into mature macrophages and DCs compared with WT counterparts *ex vivo* (232) implying that the induction of NOX2-derived ROS in MDSCs is a mechanism by which these cells maintain an immature phenotype. Furthermore, ATRA has been shown to trigger the generation of DC from monocytes (244) and to induce the maturation of MDSCs into DCs or macrophages by a mechanism involving upregulation of the antioxidant glutathione synthase, that in turn reduces the intracellular ROS levels in myeloid cells (248). A similar effect on differentiation was observed when MDSCs were subjected to the antioxidant NAC (248).

Our results confirm and extend these previous findings by showing that the cytokine-induced generation of monocyte-derived DCs was favored by NOX2-derived ROS inhibition using HDC. Similarly, HDC limited the cytokine-induced generation of human MDSCs *in vitro*. A pro-differentiating effect of HDC was also observed *in vivo* since treatment with HDC reduced the accumulation of intratumoral MDSCs and increased the infiltration of DCs in tumor-bearing mice. Furthermore, pharmacological inhibition of NOX2-derived ROS reduced tumor growth *in vivo* in several experimental models of solid cancer.

We also determined effects of NOX2-inhibition on malignant NOX2-expressing myeloid cells and observed that HDC stimulated the maturation of H_2R- and NOX2-expressing AML cells *in vitro* and *in vivo* and also reduced the expansion of leukemia. Similarly, transplantation of mice with *NOX2* KO OCI-AML3 resulted in reduced expansion of leukemia and prolonged survival compared with WT counterparts in a xenograft AML model (162). We additionally investigated effects of genetic ablation of *Nox2* on *BCR-ABL1*$^+$ murine cells and found that *Nox2*-depleted cells showed reduced colony formation, which is indicative of a more mature phenotype of these cells. In line with these results, we observed a prolonged survival of mice transplanted with *Nox2* KO *BCR-ABL1*$^+$ cells vs. corresponding WT cells. These results thus suggest that pharmacological inhibition or genetic ablation of *NOX2* impacts on the differentiation of non-malignant and malignant myeloid cells and may thus reduce the growth of solid tumors as well as the expansion of NOX2-expressing leukemic cells.

The HDC-induced myeloid differentiation seems to rely on the ability of HDC to limit the generation of intracellular ROS rather than inhibiting the extracellular release of NOX2-derived ROS. This assumption is based on results showing that only scavengers of intracellular ROS mimicked the pro-differentiating effect of HDC. However, the addition of an intracellular scavenger of ROS to *NOX2* KO PLB-985 cells promoted differentiation suggesting that sources of ROS other than NOX2 contributed to the impaired differentiation of these cells. Experiments using the H_2R antagonist ranitidine showed that the pro-differentiating effect of HDC was mediated by H_2R. We also observed that only NOX2-expressing myeloid cells differentiated in response to HDC and additionally that HDC did not affect EL-4 tumor growth in *Nox2*-deficient mice or in mice depleted of $GR1^+$ myeloid cells. Similarly, HDC therapy did not alter leukemic cell differentiation or progression in NOG mice transplanted with *NOX2* KO monocytic PLB-985 cells. Based on these results we propose that HDC facilitates the differentiation of myeloid cells by ligating H_2R to inhibit the formation of intracellular NOX2-derived ROS. This assumption is, in part, supported by studies showing that overexpression of H_2R in the monocytic U937 cell line or exposure of these cells to specific H_2R agonists results in terminal differentiation (404, 405).

Treatment of AML patients with HDC has been proposed to facilitate immune-mediated clearance of leukemic cells by targeting immunosuppressive ROS (39, 198, 202). Since MDSCs may exert NOX2-dependent, ROS-mediated immunosuppression we speculated that HDC, in addition to promoting the maturation of MDSCs, may also render these cells less immunosuppressive. We thus examined the *ex vivo* formation of ROS from leukocytes from control and HDC-treated mice and observed that cells isolated from HDC-treated mice produced significantly less ROS compared with cells from non-treated mice. The reduced formation of ROS translated into cells with reduced ability to suppress antigen-induced T cell proliferation. In line with the *ex vivo* results, we observed that HDC treatment triggered an increased frequency if $CD8^+$ T cells with an effector phenotype within tumors suggesting that reduced ROS levels in the TME promoted anti-tumor immunity.

The anti-tumor efficacy of antibodies targeting the PD-1-pathway has been reported to rely on pre-existing cytotoxic $CD8^+$ T cells (406) and anti-CSF-1 therapy, that limits the expansion of MDSCs, was previously shown to synergize with antibodies targeting the PD-1-pathway in reducing the growth of murine neuroblastoma (398). We observed that treatment of mice carrying EL-4 lymphoma or MC-38 colon carcinoma with HDC potentiated the anti-tumor efficacy of checkpoint inhibitors to the PD-1 pathway. In summary, the results suggest that targeting of NOX2 using HDC is conceivable in cancers where myeloid cells, such as MDSCs, generate immunosuppressive ROS. Our findings

also point to the possibility of adding HDC, or other NOX2-inhibitors, in cancer therapy using immune checkpoint inhibitors.

CONCLUDING REMARKS

Our results imply that NOX2-derived ROS exert multiple functions in different malignancies and that the targeting of NOX2-derived ROS may have clinical implications. Whereas the results in **paper I** and **paper II** support that the inhibition of NOX-derived ROS using HDC promotes the differentiation of immature immunosuppressive cells and thus stimulates the generation of DCs in solid tumors, the results from **paper III** and **paper IV** imply that NOX2 inhibition may also directly target malignant myeloid cells.

In the attempts to define the impact of NOX2 on tumor growth *in vivo* we utilized several murine tumor models, all of which have significant limitations. The cell lines used to generate solid tumors were not orthotopically implanted and may not appropriately reflect the course of disease in these malignancies. We opted for this approach as the heterotopic implantation of EL-4 lymphoma, 4T1 mammary carcinoma, and MC-38 colon cancer cells results in a pronounced expansion of MDSCs. It thus remains to be established whether our results are relevant to the course of lymphoma, breast cancer, and colon cancer. The results may instead reflect the effects of NOX2 inhibition in cancer characterized by MDSCs expansion. In the CML model the transplantation of *BCR-ABL1*$^{+}$ cells to mice resulted in vigorous myeloproliferation and a rapid onset of leukemia. The disease that develops in these mice hence resembles the acute phase of CML rather than the chronic phase of the disease.

In all parts of this thesis we have utilized *Nox2*-deficient mice or *NOX2*-deficient human cell lines to decipher effects of HDC in the context of NOX2 inhibition. The overriding conclusion is that treatment with HDC does not affect tumor growth and does not regulate malignant cell differentiation when NOX2 is genetically depleted. A limitation to these models is that *NOX2* KO cells and *Nox2*-deficient mice may express features unrelated to NOX2 deficiency to compensate for the inability to induce NOX2-derived ROS. Notably, however, a recent study utilized mice that were genetically deprived of any one of the five major NOX2 subunits. The authors observed reduced tumor growth and/or metastasis regardless of which NOX2 subunit that was deleted, thus reducing the likelihood that phenotypes unrelated to NOX2 depletion may have biased the interpretation of the results (236).

The effects of HDC on MDSCs were also evaluated in the Re:Mission trial. This was a phase IV clinical trial where all patients received HDC/IL-2. We therefore cannot exclude that the IL-2 component of this regimen may have contributed to the observed effects on MDSCs. However, a study in renal cell carcinoma showed that combined therapy with ATRA and IL-2 reduced the number of MDSCs whereas monotherapy with IL-2 did not impact on MDSC numbers (247). An

additional reservation is that all patients included in the trial had received high doses of chemotherapy before inclusion. The observed effects on myeloid populations could hence reflect the re-establishment of normal hematopoiesis.

The results from all *in vitro* and *in vivo* models however support that HDC reduces tumor cell growth by i) targeting immunosuppressive ROS and/or ii) by stimulating myeloid cell differentiation. With the above-mentioned reservations, NOX2 inhibition is a conceivable target for improved cancer therapy.

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