Targeting NOX2 in cancer

Ebru Aydin

Sahlgrenska Cancer Center,
Institute of Biomedicine, Sahlgrenska Academy

UNIVERSITY OF GOTHENBURG
Gothenburg 2018
ABSTRACT

Reactive oxygen species (ROS) are short-lived, toxic derivatives of oxygen that are produced during mitochondrial respiration and by NADPH oxidases (NOX). By enzymatically generating ROS, the myeloid cell NOX2 plays a critical role in defense against bacteria and other microorganisms. The NOX2-derived ROS have also been ascribed immunosuppressive properties and may damage DNA to induce mutagenesis, but details regarding the role of NOX2 and ROS for the initiation and progression of cancer are partly unexplored. This thesis work utilized genetic and pharmacological tools including transgenic mice, genetically modified cells and pharmacological NOX2 inhibitors to further define the role of NOX2 in cancer. The results presented in paper I implied that a NOX2 inhibitor, histamine dihydrochloride (HDC), promotes the development of monocyte-derived, antigen-presenting dendritic cells to control the in vivo growth of a murine lymphoma (EL-4). Paper II was designed to elucidate the impact of NOX2 on the process of metastasis. The results suggested that extracellularly released NOX2-derived ROS from myeloid cells may dampen natural killer (NK) cell-mediated defense against murine melanoma cells to promote hematogenous metastasis. Paper III aimed at defining the role of NOX2 in a mouse model of chronic myeloid leukemia (CML). It was observed that genetic ablation of NOX2 delayed the in vivo expansion of leukemic cells carrying the BCR-ABL1 mutation. In paper IV, it is shown that genetic and pharmacological inhibition of NOX2 delayed the development of myeloproliferation in a murine model of Kras-induced myeloid leukemia and, also, that inhibition of NOX2 function may confer protection against oxidative stress and DNA damage in cells of the leukemic clone. In summary, these studies identify NOX2 as a conceivable target in cancer therapy.

Keywords: Reactive oxygen species, cancer, immunotherapy, histamine, NOX2, NK cells, melanoma, metastasis, KRAS, leukemia, MPD, AML, CML
LIST OF PAPERS

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


IV. Aydin E, Hallner A, Wiktorin HG, Hellstrand K, Martner A. NOX2 inhibition reduces oxidative stress and prolongs survival in murine KRAS-induced myeloproliferative disease (Submitted)
# TABLE OF CONTENTS

**ABSTRACT** .................................................................................................................. iii

**LIST OF PAPERS** ........................................................................................................ iv

**TABLE OF CONTENTS** .................................................................................................. v

**ABBREVIATIONS** .......................................................................................................... vi

**INTRODUCTION** ......................................................................................................... 1

1.1 Malignant cells ........................................................................................................... 1

1.2 The tumor microenvironment .................................................................................. 2

1.3 Immune-mediated control of malignant cells ....................................................... 3

1.3.1 Myeloid cells ....................................................................................................... 5

1.3.2 Lymphoid cells ..................................................................................................... 7

1.4 Immunotherapy ......................................................................................................... 10

1.4.1 Stimulation of host immune responses ............................................................. 10

1.4.2 Passive immunotherapies .................................................................................... 13

1.5 Redox characterization of TME .............................................................................. 14

1.5.1 Cellular sources of ROS .................................................................................. 14

1.5.2 Control of redox homeostasis ......................................................................... 18

1.5.3 ROS and cancer .................................................................................................. 19

1.5.4 ROS-related cancer therapies .......................................................................... 20

1.6 Tumor models used in this study .......................................................................... 21

1.6.1 Melanoma ......................................................................................................... 21

1.6.2 Hematopoietic cancer ....................................................................................... 22

**AIM** ............................................................................................................................. 25

**MATERIALS & METHODS** ....................................................................................... 26

**RESULTS & DISCUSSION** ......................................................................................... 35

Paper I ............................................................................................................................... 35

Paper II ............................................................................................................................. 38

Paper III ........................................................................................................................... 41

Paper IV ............................................................................................................................ 43

**CONCLUDING REMARKS** ....................................................................................... 47

**ACKNOWLEDGEMENTS** ............................................................................................. 49

**REFERENCES** .............................................................................................................. 52
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CMML</td>
<td>Chronic myelomonocytic leukemia</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>HDC</td>
<td>Histamine dihydrochloride</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer cell immunoglobulin-like receptor</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MPD</td>
<td>Myeloproliferative disease</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NMH</td>
<td>N-methyl histamine</td>
</tr>
<tr>
<td>NOX2</td>
<td>NADPH oxidase type 2</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
INTRODUCTION

1.1 Malignant cells

Cancers are mostly derived from somatic changes in a single clone of cells (1, 2). The genetic changes that entail malignant transformation comprise two principal categories, gain-of-function and loss-of-function events. The concept of gain-of-function mutations in cancer refers to an altered gene product that acquires a new or abnormal function. A proto-oncogene is a normal gene involved in the regulation of cellular events with the possibility of becoming an oncogene with ensuing development of malignant cells. Gain-of-function mutations are dominant, i.e. the mutation of a proto-oncogene in one allele is sufficient to initiate cancer. A proto-oncogene may transform to an oncogene by acquiring a point mutation, a chromosomal translocation or a localized duplication or deletion of a gene segment. *RAS, MYC, ABL* and *WNT* are examples of proto-oncogenes that are commonly mutated in human cancer cells (3).

Loss-of-function mutations in tumor suppressor genes are additional contributors in malignant transformation. Tumor suppressor genes encode proteins that inhibit cell proliferation and metastasis or promote apoptosis; these proteins may influence proliferation by modulating intracellular signaling (PTEN), influence checkpoint control of cell proliferation (retinoblastoma protein, p16), inhibit metastasis (BRMS1), promote apoptosis (TP53), or regulate DNA repair (TP53, BRCA) (4-7). Loss-of-function mutations in tumor suppressor genes are mostly recessive as one copy of a tumor suppressor gene is sufficient to halt cell proliferation.

In humans, most cancers arise from epithelial cells (carcinoma) that line the cavities and surfaces of blood vessels, channels and organs. Under the epithelial lining, there is a layer of supporting connective tissue, the stroma, and these two layers are separated by a basement membrane (or basal lamina). The basement membrane is a form of extracellular matrix (ECM) and provides a structural scaffold to the tissue. In addition to carcinomas, cancer may also arise from non-epithelial cells to form sarcomas, neuroectodermal tumors and hematopoietic cancers.
1.2 The tumor microenvironment

The tumor microenvironment (TME) may comprise fibroblasts, immune cells, signaling molecules, extracellular matrix (ECM) and vasculature-associated cells (8, 9) (Figure 1).

Figure 1. Constituents of the tumor microenvironment.
A widely held view is that malignant cells orchestrate the host tissue to facilitate tumor progression and, also, that tumor growth may be targeted by compounds that affect the TME (9). For tumors to exceed a diameter of 2 mm, new blood vessels must form to supply tumor cells with oxygen and nutrients. The process of angiogenesis within tumors is often dysregulated leading to a leaky vasculature with a chaotic organization (10), which impacts on tumor progression by several means, including by contributing to hypoxia. Hence, a prominent difference between the tumor microenvironment and the surrounding, non-malignant tissue is a reduced oxygen level. In addition to inefficient oxygen delivery due to an inadequate vascular network, hypoxia is also caused by increased oxygen consumption incurred by the rapidly dividing cancer cells (11, 12). Hypoxia may also contribute to epithelial-mesenchymal transition, which favors tumor invasiveness and metastasis (13).

An additional feature of the tumor microenvironment, acidity, is closely related to hypoxia. While cancer cells maintain their pH, the TME is mostly acidic. A major reason for acidification in the TME is the lactate produced during anaerobic glycolysis, but acidity is also contributed by CO₂ from the pentose phosphate pathway and proton pumps (14).

In addition to stimulating angiogenesis, malignant cells may regulate the recruitment of immune cells, including inflammatory cells, to the TME by producing cytokines and growth factors that affect the endothelium and facilitate chemotaxis (15). Tumor-derived soluble mediators may also exert systemic effects to favor the formation of pre-metastatic niches in distant organs (10, 16). For example, tumor-derived VEGF and TNF have been shown to enhance the recruitment of CD11b⁺ myeloid cells to the lungs of tumor-bearing mice, which creates a niche favoring metastasis (10, 16, 17).

### 1.3 Immune-mediated control of malignant cells

The immune system comprises two major parts, innate and adaptive immunity, based on the differences in timing, specificity and intensity of immune reactions. The innate immune system consists of barriers, cells and mediators that provide a first line of defense. The principal components of innate immunity are physical barriers, natural killer (NK) cells, phagocytes, proteins of the complement systems and cytokines (18). The immune
reactions elicited by innate immunity occur promptly, and their magnitude of intensity is unchanged over time, as innate immunity typically does not encompass aspects of immunological memory. Innate immune cells recognize foreign structures via a broad, yet limited, set of pattern recognition receptors (PRRs). The structures that bind to PRRs, and thus stimulate innate immune responses, comprise pathogen-associated microbial patterns (PAMPs) and damage associated molecular patterns (DAMPs). PAMPs are mostly unique to microbes and include dsRNA in replicating viruses, lipopolysaccharide (LPS) in Gram-negative bacteria and bacteria-specific unmethylated CpG DNA sequences. In addition to recognizing microbial antigens, innate immune cells may also identify stressed or injured host cells as these cells may express heat-shock proteins and abnormal membranes that may be denoted as DAMPs (19).

Adaptive immunity, which is more diverse and specific, is mediated by T cells, B cells and their secreted products including antibodies (20). These lymphocytes arise from stem cells in the bone marrow and go through several stages of maturation. Somatic hypermutation and genetic recombination of antigen receptor gene segments during lymphocyte development form the basis for immune diversity. T and B cells are endowed with memory, meaning that adaptive immune responses evolve to become more effective over time.

Immunity is, however, a network of innate and adaptive responses rather than two mutually exclusive systems. Dendritic cells (DC), which are antigen-presenting innate immune cells, serve as a bridge between innate and adaptive immunity as these cells present antigens and activate T cells. Also, inflammatory mediators \textit{i.e.} cytokines, chemokines and growth factors, contribute in the crosstalk between innate and adaptive immunity (21).

The components of innate and adaptive immunity may recognize and eliminate several types of cancer cells. The evidence for a role of immune surveillance in cancer stems from mouse tumor models, where targeted gene deletions or neutralizing antibodies removing specific components of innate or adaptive immunity results in enhanced tumor growth (22-25). In support for a surveillance role of immunity in human cancer, several studies imply that patients with severe immunosuppression, induced by \textit{e.g.} hereditary immunodeficiency, HIV infection or organ transplantation, are at higher risk
of cancer development. A meta-analysis thus suggested that HIV-infected patients as well as organ transplant recipients frequently develop cancers, in particular those associated with infectious agents such as human herpesvirus type 8 (HHV-8)-related Kaposi’s sarcoma (3,600 times higher incidence) or EBV-related lymphoma (80 times higher incidence). A weak but significant association between immunodeficiency and cancer has been reported also for tumors of non-viral origin. Patients undergoing heart transplantation were thus found to be 25 times more likely to develop lung carcinomas and 2-4 times more likely to develop melanoma or sarcoma (26).

1.3.1 Myeloid cells

Neutrophils

Neutrophils, also called polymorphonuclear leukocytes, constitute the dominant population of nucleated cells in blood and form part of innate immunity (27). These cells are produced in the bone marrow and leave this tissue in a fully differentiated and functional form (28). The major effector function utilized by neutrophils is phagocytosis, which is strongly connected to the production of ROS inside lysosomes; neutrophils thus engulf microorganisms, which triggers ROS formation from the myeloid NOX2 enzyme with ensuing elimination of microbes, in particular bacteria. To lyse surrounding bacteria, neutrophils may release ROS into the extracellular space or form neutrophil extracellular traps (NETs), which are ejected networks of extracellular fibers primarily composed of DNA and bactericidal proteins.

Monocytes and macrophages

Monocytes are mononuclear cells that are produced in bone marrow from myeloid progenitors and released into the blood stream (29). When monocytes enter tissues they mature and differentiate into macrophages or, in certain settings, into monocyte-derived DCs (30). Macrophages residing in tissues often have distinct functions and display organ-specific patterns of surface receptors. These cells are given specific names in the organs where they reside such as microglial cells in the nervous system, Kupffer cells in the liver and alveolar macrophages in lungs. Similar to neutrophils, a primary function of monocyte/macrophages is to identify, ingest and destroy
microbes; they thus utilize phagocytosis and ROS formation in lysosomes as a principal mechanism of anti-microbial defense. Compared to neutrophils, macrophages respond more vigorously by cytokine production when encountering microbes or aberrant cells, and the mediators produced serve, in part, to facilitate the recruitment of additional immune cells.

In the TME, macrophages are often classified into two subsets i.e. M1 (classically activated by for example IFN-γ) and M2 (alternatively activated by for example IL-4). M1 macrophages produce high levels of IL-12 and other pro-inflammatory mediators and are assumed to facilitate Th1-type T cell responses (cf. below). In certain forms of cancer, overrepresentation of M1-type macrophages in the TME heralds favorable prognosis (31, 32). The M2 subset is assumed to participate in tissue repair and in down-regulating immune responses by virtue of IL-10 production. Presence of M2 macrophages in the TME may promote tumor growth (33, 34); however, the subdivision of macrophages into these distinct populations is complicated by the finding that tumor-resident macrophages may simultaneously display characteristics of M1 and M2 cells (35-38).

**Myeloid-derived suppressor cells**

In several neoplastic diseases immature myeloid cells are expanded and activated to inhibit T cell responses (39). These myeloid-derived suppressor cells (MDSCs) may accumulate in the TME and in the periphery, and their expansion/activation is likely driven by tumor-derived soluble factors (40). MDSCs are not homogenous, but comprise subpopulations with granulocytic or monocytic features that differ in suppressive function (40). Several potential mechanisms for MDSC-mediated T cell suppression have been described, including enhanced production of ROS, arginase, nitric oxide (NO), TGF-β and IL-10 (41-46). The expansion of MDSCs is evident in patients with most histiotypes of cancer, as well as in practically all murine tumor models (45, 47-49). The presence of MDSCs in cancer patients correlates with a more aggressive course of disease and a poor prognosis (50-53).
Dendritic cells

APCs capture antigens and present these foreign structures to lymphocytes in addition to producing cytokines that ensure lymphocyte activation. Macrophages and B cells are endowed with antigen-presenting function, but DCs are considered the dominant APC in terms of induction of T cell responses, in particular from naïve T cells. DCs are derived from hematopoietic bone marrow progenitor cells and are present in a wide range of tissues, in particular those that are in contact with the external environment such as the skin and the epithelial barriers of nose, lungs, stomach and intestines. Immature DCs are, by virtue of their long cytoplasmic projections, endowed with high endocytic activity and sample the surrounding environment for foreign antigens. DCs are activated by danger signals via pattern recognition receptors such as toll-like receptors (TLRs). DCs process antigens for display on their surface MHC I and MHC II molecules and traffic to lymph nodes where they encounter T cells. Upon activation by danger signals, DCs also upregulate their expression of co-stimulatory molecules, such as CD86 and CD40, that enhances their capability to activate T cells. DCs have crucial anti-tumor activities that involve sampling tumor antigens and displaying them to cytotoxic T cells. Several groups have reported functionally deficient DCs in animal models of cancer and in cancer patients (54-56) and many of the functional deficiencies in DCs may be attributable to weak co-stimulatory molecule expression (57-59).

1.3.2 Lymphoid cells

B cells

B cells are antibody-producing cells that are generated and partially undergo maturation in bone marrow to mediate the humoral part of adaptive immunity (60). Once B cells leave the bone marrow, they are diverted to secondary lymphoid organs for further differentiation. When a B cell encounters an antigen that binds to its cognate B cell receptor the antigen will be internalized, processed and presented on MHC II molecules. If CD4+ helper T cells with the correct specificity are present, these cells will provide signals to initiate an activation process resulting in the formation of specific antibodies (61).
T cells

T cells are produced in the bone marrow from lymphoid progenitors and are named after the thymus, the organ where they mature. Developing T cells undergo several checkpoints to ensure that only cells with adequate T cell receptors (TCR) will complete maturation. Lymphocytes that express receptors with too low or too high affinity for self-MHC molecules are eliminated during positive and negative selection rounds. T cells recognize a broad range of antigens by virtue of rearrangement of their antigen receptor genes during lymphocyte development (62). Naïve T lymphocytes traffic to secondary lymphoid organs in search for their specific antigen presented on MHC by APCs. When T cells encounter the specific antigen this results in expansion of T cells with that particular specificity and the differentiation into effector and memory T cells.

The majority of T cells in blood are so called ‘αβ T cells’ that express α and β TCR, whereas ‘δγ T cells’ constitute a smaller fraction of unconventional T cells. The major T cell populations are the CD4+ T cells and the CD8+ T cells. CD4+ T cells are helper cells that recognize peptides presented on MHC class II molecules on APCs. The activated CD4+ T cells exert their action by secreting cytokines that serve to help, or regulate, responses of other immune cells. Depending on the APC and the microenvironment where antigen presentation takes place, the T cells may become polarized into Th1, Th2, Th17 or regulatory T cells, which secrete different patterns of cytokines and have distinct effects on immune responses. Th1 cells are assumed to participate in defense against cancer cells by producing the signature cytokine IFN-γ that, in turn, activates IL-12 production from innate immune cells to stimulate the tumor-killing capacity of CD8+ T cells and NK cells (63-66).

CD8+ T cells, also called cytotoxic lymphocytes (CTLs), recognize antigens presented on MHC class I. Naïve CD8+ T cells only become activated when their specific antigen is presented on MHC I by an APC, but all nucleated cells express MHC I and expose cytosolic antigens on this receptor. After activation, the effector CD8+ T cells therefore can detect and kill any cell that presents aberrant peptides on MHC I (61).
NK cells

NK cells are a subset of lymphocytes that kill target cells, including malignant cells, in the absence of additional activation (‘natural’ cytotoxicity) (67). NK cells are derived from bone marrow precursors and constitute 5-20% of the mononuclear cells in human blood. Previously, the anti-tumor activity exerted by these cells was believed to be only a background activity in cytotoxicity assays, but NK cells are now recognized as a morphological and phenotypic entity with an undoubted role in defense against viral infections and cancer cells. NK cells are phenotypically heterogeneous and conventionally distinguished by their absence of the T cell marker CD3 and expression of the neural adhesion molecule CD56. Immature NK cells carry a CD56brightCD16dim phenotype and down-modulate CD56 expression along with increasing CD16 expression as they undergo maturation. The CD56brightCD16dim NK cells constitute approximately 10% of the NK cell population and usually produce more cytokines, in particular IFN-γ. CD56dimCD16bright NK cells are mostly endowed with higher cytotoxic activity than their CD56brightCD16dim precursors.

The cytotoxicity of NK cells relies on complex interactions between inhibitory and activating NK cell receptors that ligate distinct structures on target cells. Whether or not an NK cell proceeds to kill a target cell is the result of a balance between these activating and inhibitory receptors. To ensure self-tolerance, inhibitory killer immunoglobulin-like receptors (KIRs) on NK cells interact with host cell MHC class I molecules. Virus-infected cells or malignant cells may reduce the level of MHC class I expression to avoid T cell-mediated immunity, but the reduced expression of HLA class I may render these target cells susceptible to elimination by NK cells. When inhibitory receptors, including KIRs and NKG2A, recognize HLA class I molecules, a signaling cascade comprising the phosphorylation of immune-receptor tyrosine-based inhibitory motifs (ITIMs) is initiated and the activation of NK cell halts. However, absence of MHC class I molecules on target cells is insufficient to induce NK cell activation as NK cells also require signals via the NK cell-activating receptors, including NKp30, NKp46 and NKG2D. The ligands for these activating receptors include stress ligands that may be expressed by tumor cells. NK cells may also attach the Fc portion of antibodies to exert antibody-dependent cellular cytotoxicity (ADCC) against aberrant cells, including antibody-coated tumor cells.
NK cells exert effector functions by two principal mechanisms that require direct contact with target cells. NK cells may thus release granules that contain granzyme and perforin; to release these cytotoxic molecules (degranulation), an immunological synapse with the target cell is formed followed by reorganization of the NK cell cytoskeleton. Thereby, NK cells may fuse their granules with the cellular membrane of the target cell. Perforins create pores and facilitate the entry of granzymes, which are apoptosis-inducing serine proteases, into the target cell (68). A second mechanism of NK cell-mediated killing is based on death receptor pathways such as TRAIL or FasL.

In addition to exerting cytolytic activity against aberrant cells, NK cells modulate other aspects of immunity by producing and releasing cytokines. NK cells thus produce cytokines (TNF-α), growth factors (GM-CSF, G-CSF, IL-3) and chemokines (MCP-1, RANTES, IL-8). NK cells are a major source of IFN-γ that induces and regulates antiviral, antibacterial and antitumor responses. NK cell activity and/or expansion is, in turn, stimulated by cytokines such as IL-2, IL-18, IL-21, IL-15 and macrophage-derived IL-12.

**1.4 Immunotherapy**

Traditional cancer therapy comprises the use of drugs that kill dividing cells or inhibit cell division. These cytotoxic or cytostatic drugs are devoid of specificity and exert effects also on normal dividing cells. In recent years immunotherapy, defined as treatment comprising the induction or reinforcement of an immune response, has gained momentum in cancer treatment. Two major, and partly overlapping, types of immunotherapies have emerged over the last decades: strategies to achieve stimulation of host immunity and passive immunotherapy.

**1.4.1 Stimulation of host immune responses**

**Induction of tumor-specific T cells**

As tumor cells are derived from host cells they mostly express a limited number of antigens that may be recognized as foreign by antigen-specific T cells. These immunogenic antigens include tumor-specific antigens that are generated via mutations or by oncogenic viruses. Viral proteins, products of
mutated genes, abnormal forms of surface glycoproteins and glycolipids hence are conceivable non-self targets for host immunity. In addition, cancer cells may overexpress tumor-associated antigens that rather than being unique to the tumor cells show an altered pattern of expression. The identification of tumor-specific or tumor-associated antigens has attracted renewed interest in recent years after the successful introduction of efficacious immunotherapies, including checkpoint inhibitors (see below). A commonly applied method of identifying tumor antigens is denoted ‘reverse immunology’ that involves a computer-based screening of sequences of selected proteins for peptides with high-affinity binding to different HLA molecules (69). These tumor antigens are used as components of tumor vaccines (70). Additionally, antibodies and effector T cells may be generated against the particular antigens (71).

**Cytokines in cancer therapy**

IFN-α, an antiviral cytokine produced mainly by leukocytes, has been used in therapy of human neoplasms for several decades. Treatment with IFN-α may be of benefit to patients with melanoma (72, 73), renal cell carcinoma, chronic myeloid leukemia and multiple myeloma, but its use is declining in favor of targeted therapy and modern immunotherapy. The anti-neoplastic action of IFN-α is likely multi-faceted and may comprise anti-proliferative effects, enhanced antigen presentation and stimulation of innate immunity, including NK cells.

IL-2, a T cell-derived cytokine that efficiently activates NK cell cytotoxic functions in addition to inducing NK cells and T cells to proliferation, is approved for use in patients with metastatic melanoma and renal cell carcinoma. IL-2 therapy is, however, only efficacious in a small proportion of patients and also entails accumulation of regulatory T cells (Treg) that may dampen T cell-mediated antitumor functions (74, 75). IL-15 is an additional immune-activating cytokine that is currently undergoing evaluating in several types of cancer (76, 77); IL-15 is structurally similar to IL-2 and activates NK cells and T cells but is devoid of a preferential expansion of Tregs (78). The current development in this area of immunotherapy comprises a multitude of strategies to improve and sharpen the anti-tumor efficiency of these cytokines, used alone or in combination with other immunotherapies.
**Checkpoint inhibitors**

In recent years, strategies to block T cell-inhibitory pathways have gained attention as a strategy to treat cancer. Antibodies that block these inhibitory pathways (‘checkpoint inhibitors’) represent a major advancement in cancer therapy.

B7 molecules on the surface of DCs pair with CD28 on T cells to facilitate T cell-mediated immunity. However, B7 molecules may also ligate CTLA-4 that instead inhibits T cell activity (79). Anti-CTLA-4 antibodies prevent CTLA-4 mediated downregulation of T cell activity (80, 81). PD-1, an additional receptor expressed by activated T cells, inhibits T cell activities when binding to the ligands PD-L1 or PD-L2 that may be expressed by cancer cells or by tumor-infiltrating myeloid cells (82). Hence antibodies blocking the interaction between PD-1 and PD-L1/L2 enhance T cell functionality (83, 84).

CTLA-4, PD-1 and other ‘checkpoint’ molecules likely function to avoid T cell-mediated injury to healthy tissue during immune activation. In the setting of cancer, however, this type of inhibition may also prevent T cell-mediated killing of tumor cells. Additionally, tumor cells may over-express for example PD-L1 and PD-L2 to avoid immune destruction. Strategies to target immune checkpoints using monoclonal antibodies against CTLA-4, PD-1 and, PD-L1 have markedly improved the prognosis in patients with metastatic melanoma, Hodgkin’s lymphoma, non-small cell lung cancer and advanced urothelial cancer (80, 81, 85-87). Immune checkpoint inhibitors are currently evaluated also in other forms of cancer. In coherence with its proposed mechanism of action, checkpoint inhibition may trigger side-effects related to autoimmunity. A further limitation is that this therapy is likely inefficacious unless antigen-specific T cells are present in the TME. Therefore, checkpoint inhibitors are evaluated in combination with several other immunotherapies, including those that facilitate T cell entry into tumors.

**Targeting immunosuppressive myeloid cells**

The TME is, as discussed above, often infiltrated by immunosuppressive myeloid cells, including TAMs and MDSCs. Strategies targeting myeloid
cells include preventing the recruitment of myeloid cells into the TME, reducing their suppressive properties, converting M2 macrophages into M1 macrophages and inducing the maturation of MDSCs into mature myeloid cells (88).

Monocytes express the chemokine receptor CCR2 and are recruited to inflammatory tissues in response to its ligand CCL2. CCL2 is produced by tumor cells and tumor-infiltrating myeloid cells, and high CCL2 levels may herald poor prognosis in cancer (89-91). However, clinical trials involving inhibition of CCL2 have thus far yielded discouraging results (92, 93). Additionally, colony-stimulating factors (CSF1) drive the differentiation of hematopoietic cells into monocytes/macrophages and also enhance the proliferation and survival of these cells. Its receptor, CSF1R, is a tyrosine kinase receptor and the ligation of this receptor stimulates several pathways including RAS. Blocking of the CSF1-CSFR1 axis by antibodies or kinase inhibitors has been evaluated in early clinical trials; the results suggest that this strategy may reduce the recruitment of TAM and MDSC into the TME along with enhancing intratumoral CD8⁺ T cell infiltration (94, 95).

The immunosuppressive actions of MDSCs may also be targeted by agents that promote the differentiation of these cells. All-trans retinoic acid (ATRA, an active metabolite of vitamin A) efficiently induces differentiation of MDSCs into DCs and has been evaluated in clinical trials in cancer in combination with DC vaccines and CAR-T-therapy (96). TAM and MDSC additionally produce immunosuppressive NOX2-derived ROS (41, 45) as discussed in more detail below.

1.4.2 Passive immunotherapies

Adoptive cellular therapies

Adoptive cellular therapy in cancer refers to the transfer of cultured immune cells to a tumor-bearing host to boost immunity. For example, lymphokine-activated killer (LAK) cells, referring to lymphocytes that have acquired high cytotoxicity upon in vitro-culture with IL-2, have been employed in melanoma, renal cell carcinoma and other forms of cancer. While the use of LAK cells has not gained wide-spread acceptance, more recent approaches
are evaluated including the adoptive transfer of NK cells, tumor-infiltrating lymphocytes or T cells with defined antigen specificity.

Chimeric receptor antigen (CAR)-T cell therapy comprises the harvesting of patient T cells followed by genetic engineering of these cells to express surface antibody-like receptors coupled to an intracellular machinery of T cell receptor-related signal transduction. After expansion of CAR-T cells in vitro, these engineered cells are adoptively transferred to the patient. CAR-T cells that recognize CD19 of B cells were recently introduced in the treatment of B cell leukemia and lymphoma (96).

**Anti-tumor antibodies**

Antibodies against tumor cell antigens, in particular B cell epitopes such as CD20, have been widely used in B cell malignancies for more than two decades; these antibodies are assumed to act by facilitating NK cell-mediated ADCC or by directly killing malignant cells by inducing apoptosis or by complement activation (97).

A new generation of engineered anti-tumor antibodies, or antibody fragments, aims to simultaneously recognize epitopes on tumor cells and CD3 on T cells, thereby guiding T cells to tumor cells. The first bispecific T cell engager (BiTE) approved for clinical use was blinatumomab with one arm attaching CD3 and the other arm binding to CD19 for recognition of malignant B cells (98, 99). A trifunctional bispecific antibody, catumaxomab, has also been approved for use in cancer. This antibody links three cell types: one arm attaches the EpCAM antigen, which is expressed by epithelial tumors, the other arm binds to CD3 on T cells, and a Fc domain ligate NK cells or macrophages via Fc receptors (100).

**1.5 Redox characteristics of the TME**

**1.5.1 Cellular sources of ROS**

Reactive oxygen species (ROS) are short-lived oxygen-derived compounds that are formed as bi-products during metabolism and in a regulated fashion by cellular enzymes. Oxygen radicals contain a single unpaired electron in their outermost orbit, which makes them highly reactive towards a variety of
molecules, including proteins, lipids, carbohydrates and nucleic acids. ROS refer both to oxygen radicals, such as superoxide anion \((\text{O}_2^\cdot\)) and hydroxyl radicals \((\cdot\text{OH})\), and to non-radicals (including hydrogen peroxide \((\text{H}_2\text{O}_2)\) that share the oxidizing capacity of radicals and may be converted into radicals (101).

The intracellular levels of ROS affect cellular redox signaling and homeostasis, while ROS released to the surrounding may also affect adjacent cells. During environmental stress and in cancer, the levels of ROS in cells and tissues may increase dramatically, with significant consequences for the survival and function of cells. Conditions where the biological systems that detoxify ROS are insufficient are referred to as “oxidative stress” with potentially irreversible damage to target molecules and cells.

**Figure 2.** Cellular sources of ROS.

Within the TME, several cell types contribute to ROS formation, including cancer cells, MDSCs and TAMs. There are exogenous inducers and endogenous sources of ROS. The exogenous inducers include smoke, pollutants, UV-radiation and \(\gamma\)-radiation. Endogenous ROS are formed during metabolism and by cellular enzymes. All cells generate superoxide and hydrogen peroxide as bi-products during ATP generation in mitochondria (see below). The major producers of enzymatic ROS are the nicotinamide
adenine dinucleotide phosphate (NADPH) oxidases (NOX) (102). The isotypes of NOX differ in expression levels and tissue distribution, as discussed in detail below. Other enzymatic sources of endogenous ROS include the xanthine oxidase and a variety of oxidoreductases, often localized within peroxisomes (103) (Figure 2). Furthermore, nitric oxide (NO), which is produced by nitric oxide synthase (NOS), may react with superoxide to form peroxynitrite, a highly reactive oxidant. Iron ions play a role in redox homeostasis by mechanisms including catalyzing the formation of free radicals from hydrogen peroxide in the Fenton reaction.

**Mitochondrial sources of ROS**

Mitochondrial ROS are generated as a byproduct of oxidative phosphorylation, i.e. the metabolic pathway that all cells utilize to oxidize nutrients to generate energy in the form of ATP. During oxidative phosphorylation, electrons are passed via the electron transport chain in the mitochondrial inner membrane. The electrons participate in several oxidation and reduction reactions, and the last electron acceptor in this chain is molecular oxygen. The majority of oxygen is then reduced to produce water. However, approximately 0.1-2% of electrons passing through the chain are incompletely reduced and give rise to superoxide (104) which is electrophilic and therefore cannot pass through the outer mitochondrial membrane. It may, however, be dismutated into $\text{H}_2\text{O}_2$ that freely passes across biological membranes. Superoxide dismutase (SOD) catalyzes the process of dismutation of superoxide into $\text{H}_2\text{O}_2$ and oxygen.

**NOX**

NOX is a family of membrane-bound enzymes whose only known function is to generate ROS. NOX produce superoxide or $\text{H}_2\text{O}_2$ by one-electron or two-electron reduction of molecular oxygen (102). Seven structurally conserved isoforms of NOX, i.e. NOX1-5 and DUOX1-2 that differ regarding distribution between cell types and in their subcellular location, have been identified; for many years, however, only the NOX2 isoform was known to
exist. NOX2 is expressed by phagocytes and is responsible for the ‘respiratory burst’ (referring to the formation of ROS as a strategy to combat microbes) that is induced when phagocytic myeloid cells come into contact with microbes. Upon activation, the components of NOX2 assemble at the phagosome membrane or at the plasma membrane to generate intracellular or extracellular superoxide that is further metabolized to other ROS species (105, 106). The physiological role of NOX2-derived ROS is illustrated by a rare genetic disorder, chronic granulomatous disease (CGD), which is characterized by dysfunctional NOX2; these patients present with recurrent bacterial and fungal infections (107, 108) thus highlighting the impact of NOX2 in microbial defense.

NOX2 consists of membrane-bound and cytosolic subunits. The membrane-bound subunits are gp91phox (or NOX2) and p22phox (cytochrome b558) and make up the catalytic core of the enzyme. The cytosolic subunits comprise p67phox, p47phox, p40phox and the small GTPase Rac. In its inactive form, the membrane-bound and cytosolic subunits are separated but upon activation, elicited by e.g. growth factors, cytokines or interactions involving PAMPs and DAMPs, a functional superoxide-generating complex is formed (Figure 3).

Figure 3. Components and activation of NADPH oxidases.

In addition to participating in defense against invading microorganisms, NOX2-derived ROS, as well as ROS generated from other sources, have been implicated in modulating redox-sensitive signaling pathways, for example by oxidizing tyrosine phosphatases with ensuing effects on cell differentiation, proliferation and survival. Extracellularly released NOX2-derived ROS have
also been shown to inactivate and induce apoptosis of lymphocytes, including NK cells and T cells (109-113).

Myeloid cells, including monocytes, macrophages, neutrophils, MDSCs and TAM, express the highest levels of NOX2. However, other cells may express low levels of NOX2 in addition to low levels of other NOX isoforms (102). Furthermore, myeloid cells have been shown to express other isoforms of NOX such as NOX1 and NOX4 (114). Various NOX isoforms may also be expressed by tumor cells (115, 116). While ROS from all cellular sources participate in redox regulation, specific physiological and pathophysiological functions have also been ascribed to some of the NOX enzymes, as summarized in Table 1.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tissue</th>
<th>Major Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOX1</td>
<td>Colon, uterus, prostate</td>
<td>Redox regulation</td>
</tr>
<tr>
<td>NOX2</td>
<td>Myeloid cells</td>
<td>Host defense, redox regulation</td>
</tr>
<tr>
<td>NOX3</td>
<td>Inner ear, fetal tissue</td>
<td>Otoconia synthesis, redox regulation</td>
</tr>
<tr>
<td>NOX4</td>
<td>Kidney</td>
<td>Oxygen sensing, redox regulation</td>
</tr>
<tr>
<td>NOX5</td>
<td>Lymphoid tissue, testis</td>
<td>Redox regulation</td>
</tr>
<tr>
<td>DUOX1-2</td>
<td>Thyroid, lung, GI tract</td>
<td>Hormone synthesis, redox regulation</td>
</tr>
</tbody>
</table>

Table 1. Tissue distribution of NOX enzymes.

1.5.2 Control of redox homeostasis

Uncontrolled ROS production may damage several cellular structures by disrupting nucleic acids, lipids and proteins. Cells have thus developed
systems to protect themselves from ROS-induced toxicity. The enzymatic scavengers of ROS, including SOD, catalases (CAT), glutathione peroxidase-1 (GPx-1), peroxiredoxins (Prx) and thioredoxin reductase (Trx), are assumed to provide the most efficient protection of cells from oxidative damage (8). While $O_2^{•−}$ may spontaneously dismutate to $H_2O_2$ this reaction is significantly accelerated by SOD. CATs, on the other hand, metabolize $H_2O_2$ into water and $O_2$ whereas Gpx-1 detoxifies $H_2O_2$ by oxidation of reduced glutathione (GSH) to oxidized (GSSG). Intracellular GSH levels are regulated by glutathione reductase (GR) (Figure 4).

Figure 4. Enzymatic mediators of redox homeostasis.

Also non-enzymatic scavengers of ROS exist, including naturally occurring metabolites and vitamins (including vitamin E), as well as molecules that act as chelators of iron, to prevent catalyzing the production of the hydroxyl radical (8, 117).

1.5.3 ROS and cancer

Tumor cells often produce excessive amounts of ROS due to enhanced respiration resulting from by rapidly proliferating cells and/or as the result of dysregulated formation of NOX-derived ROS. Hence, oxidative stress may
be closely associated with carcinogenesis and many cancer-related events, such as cell proliferation, invasion and metastasis are under redox regulation.

For example, redox signaling comprises intracellular pathways that overlap with the growth factor receptor (GFR) signaling that controls cell proliferation, and cancer cells may utilize this mechanism to sustain growth. Growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), activate tyrosine kinases whose phosphorylation triggers signaling pathways including PI3K-AKT and RAS-MEK-ERK (118, 119), (120). These pathways are key regulators of cell proliferation and cell survival, and stimulation via the PI3K and RAS pathways has also been shown to trigger NOX1- and NOX2-derived ROS production (121, 122). The ROS thus generated are believed to affect redox-sensitive sites of protein tyrosine phosphatases (PTPs) to inactivate these enzymes. Since the PTPs serve to remove phosphate groups from proteins, and thereby negatively regulate for example the PI3K-AKT and RAS-MEK-ERK pathways, their inactivation reinforces signaling triggered via tyrosine kinases. By inactivating PTPs, ROS may establish a positive feedback loop to promote activation of additional NOX-derived ROS and enhanced downstream signaling events (123, 124).

ROS may also damage DNA, typically by oxidizing deoxyguanosine to 8-oxo-2-deoxyguanosine (8-OHdG). 8-OHdG may pair with adenine instead of cytosine leading to mutations that may serve to activate oncogenes, inhibit tumor-suppressor genes or disrupt DNA repair, which may promote cancer development. Additionally, NOX2-derived ROS have, as mentioned above, been shown to exert significant immunosuppression when released into the extracellular space. While the contribution of ROS and NOX2 in defined forms of cancer remains to established, it is conceivable that the impact of the NOX2/ROS axis for induction and progression of cancer may be more pronounced in primary or metastatic tumors that contain infiltrating NOX2+ cells, in cancers related to chronic inflammation or infection and in myeloid malignancies, where the malignant cell clone comprises NOX2+ cells.

1.5.4 ROS-related cancer therapies

While low ROS levels in cells are required to maintain proliferation, high ROS levels are toxic to cells. Several chemotherapeutics, as well as
radiotherapy, trigger excessive ROS production in cancer cells, leading to ROS-dependent apoptosis (125-127). Also, strategies to inhibit ROS have been evaluated in cancer therapy, including trials utilizing ROS scavengers such as vitamin E or N-acetyl cysteine (NAC). These trials, as well as animal experiment comprising the administration of ROS scavengers in cancer treatment, have shown mixed results (128-131).

The only approved ROS-targeting therapy is the NOX2-inhibitor HDC that is approved for use, in conjunction with low-dose IL-2, within the EU to prevent relapse of leukemia in the post-chemotherapy phase of AML. In vitro studies support that HDC promotes cellular immunity by protecting subsets of cytotoxic lymphocytes from ROS-induced inactivation (132-134), but details regarding the impact of the NOX2/ROS axis for the observed anti-leukemic action of HDC-based therapy in vivo remain to be established.

1.6 Tumor models used in this study

1.6.1 Melanoma

In paper II, we have utilized murine melanoma cells (B16F10) in the study of the impact of immunity, in particular aspects of tumor-related immunosuppression, on the process of melanoma metastasis.

Melanomas originate from the pigment-producing melanocytes in the basal layer of the epidermis. The incidence of melanoma has increased over the past three decades (135-137). In Europe, the highest incidence rates is reported in Scandinavia (15 cases/100,000 inhabitants/year) (138). Primary melanomas (stage I-II) may metastasize to local lymph nodes (stage III) or to distant organs such as subcutaneous tissue, liver, lungs, bones and brain (stage IV). While a significant fraction of patients with stage III melanoma are cured by surgical removal of afflicted lymph nodes, patients with stage IV disease have a median survival of 6 to 10 months with few long-term survivors.

The past decade has seen the introduction of inhibitors of B-RAF, based on a high rate of BRAF mutations that promote the growth of human melanomas. B-RAF inhibitors may produce dramatic reduction of metastatic disease but
the responses are typically short-lasting. In recent years, immunotherapy has gained significant momentum in the treatment of melanoma. As reviewed above, previous studies utilized e.g. high-dose IL-2 or IFN-α for activation of anti-neoplastic lymphocytes such as NK cells or cytotoxic T cells. While such therapy was occasionally associated with significant reduction of the tumor burden among patients with stage IV disease, the benefit in terms of overall survival remains uncertain (139, 140). However, a study in patients with stage III melanoma showed that leukocyte-derived (‘natural’) IFN-α, thus encompassing multiple subtypes of human IFN-α, markedly improved long-term survival (72, 141).

A recent trend in melanoma therapy is to take mechanisms of cancer-related immunosuppression into account. The growth and spread of melanomas and other malignant cells are thus promoted by multiple pathways of immunosuppression, including CTLA-4, which inhibits T cell function, and PD-1, which is expressed by T cells and targets ligands (PD-L1 and PD-L2) expressed by malignant cells or tumor-infiltrating myeloid cells with ensuing T cell suppression. Ibilimumab, an antibody that blocks the CTLA-4, and antibodies against PD-1 or PD-L1 have markedly improved the treatment of stage IV melanoma and serve as inspirational examples spurring further development in cancer immunotherapy (80).

1.6.2 Hematopoietic cancer

Cancer of hematopoietic cells is broadly divided into leukemia, originating from myeloid cells or lymphocytes, and lymphoma, where lymphocytes of B cell or T cell origin accumulate in lymph nodes and other tissues. Depending on the course of disease, leukemia is denoted acute or chronic thus forming the major subgroup of AML, ALL, CML and CLL. In this thesis work, we have utilized murine in vivo models of T cell lymphoma (paper I), CML (Paper III) and RAS-related myeloid leukemia largely resembling human AML (paper IV).

Acute myeloid leukemia is the most common form of myeloid leukemia in adults and affects approximately 350 patients in Sweden per year. At diagnosis, AML patients receive induction chemotherapy, mainly cytarabine and daunorubicin, followed by consolidation chemotherapy to eliminate residual leukemic cells (142). The chemotherapy typically reduces the
malignant clone to microscopic undetectable levels, along with the return of normal hematopoiesis (complete remission, CR). However, approximately 70% of patients achieving CR will experience relapse of leukemia, mostly within 2 years, with poor prospects of long-term survival (143). A minority of patients may receive allogeneic transplants in the post-remission phase. This strategy efficiently reduces the risk of relapse by inducing T cell-mediated elimination of leukemic cells (‘graft-vs.-leukemia’) but for the majority of AML patients few treatment options are available beyond the phase of chemotherapy.

AML is assumed to arise from several types of genetic aberrations, including translocation, deletion or duplication of gene segments or distinct mutations (e.g. NPM1mut or FLT3mut). At present, targeted therapies are only available for patients with FLT3mut AML and in promyelocytic AML (APL) where a translocation involving the retinoic acid receptor-α gene dictates responsiveness to all-trans retinoic acid (ATRA).

AML patients may harbor NK cells and cytotoxic T cells endowed with anti-leukemic function. Thus, immunotherapy in the post-remission phase aiming at activating these aspects of cell-mediated immunity may be useful in preventing relapse, and several such immunostimulatory strategies are currently being evaluated (144). Immunotherapy with the NOX2-inhibitor histamine dihydrochloride in conjunction with low-dose IL-2 (HDC/IL-2) is approved for relapse prevention in AML throughout the EU (133), and several recent studies suggest that the benefit of this therapy stems from activation of cell-mediated immunity (109-111, 145).

*CChronic myeloid leukemia* is characterized by abnormal proliferation and accumulation of mature granulocytic cells in the bone marrow. In CML, juxtapositioning of ABL1 on chromosome 9 and BCR on chromosome 22 results in constitutive expression of the ABL tyrosine kinase that maintains proliferation and survival of mutated cells. Since the beginning of the early 2000’s, patients with CML are treated with BCR/ABL1-specific tyrosine kinase inhibitors (TKIs, including imatinib and related compounds) that mostly efficiently reduce the malignant clone to microscopically undetectable levels by targeting the ABL tyrosine kinase formed as the result of the t(9;22) translocation.
The introduction of specific TKIs has dramatically improved the long-term survival in CML and is arguably, together with the use of ATRA in APL, the hitherto most successful examples of targeted cancer therapy based on unique and ubiquitous genetic aberrations in malignant cells. However, quiescent immature stem cells may survive during therapy by imatinib or other TKIs, and life-long therapy is often needed with high costs and significant treatment-related morbidity. Furthermore, TKI-resistant clones may emerge as a consequence of additional or acquired mutations.

Lymphomas are typically restricted to lymph nodes and account for almost 7% of cancer-related deaths. The main categories of lymphoma are Hodgkin’s lymphoma (a B-cell lymphoma) and the more common non-Hodgkin lymphomas (NHL, dominated by B cell lymphomas but also comprising T and NK cell lymphomas). The treatment in lymphoma is dictated primarily by the rate of proliferation of the malignant cells and by the localization of tumors. Lymphoma treatment strategies thus may range from watchful waiting to chemotherapy, radiation therapy and, for B cell lymphomas, antibodies against CD20 along with the more recently introduced inhibitors of B cell proliferation and survival. CAR-T cells engineered to express the CD19 B cell antigen was recently approved for use in Hodgkin’s lymphoma. Notably, several treatment strategies employed in B cell lymphoma, including antibodies against CD19, B cell-specific inhibitors and CAR-T cells against CD19, apply also to the treatment of B-ALL and B-CLL.
AIMS

The overall aims of the studies in this thesis were to clarify the role of NOX2-derived ROS in murine cancer models and to assess effects of genetic NOX2 inhibitors for on the expansion of malignant cells in vivo. The specific aims are listed below.

**Paper I** aimed to define effects of HDC, an inhibitor of NOX2, on the development of DCs from myeloid precursors and the impact of these mechanisms for EL-4 murine lymphoma growth in vivo.

**Paper II** aimed to evaluate the impact of genetic and pharmacologic NOX2 inhibition in NK cell-mediated control of a lung metastasis in the B16F10 in vivo model of murine melanoma model.

**Paper III** aimed at assessing the role of NOX2 for leukemic expansion in a murine model of BCR-ABL1+ leukemia.

**Paper IV** aimed to determine the impact of NOX2-derived ROS in KRAS-driven myeloid leukemia in vivo.
MATERIALS & METHODS

Animal models

All animal experiments in this study were approved by the Research Animal Ethics Committee in Gothenburg. Mice were maintained under pathogen-free conditions according to guidelines issued by the University of Gothenburg. The C57BL/6 mice (wild-type, WT) were obtained from Charles River Laboratories. B6.129S6-Cybb<sup>−/−</sup> (Nox2<sup>−/−</sup> or Nox2-KO) mice that lack the myeloid gp91<sub>pox</sub> subunit NOX2 and, thus, a functional ROS-forming NOX2 were obtained from The Jackson Laboratory. Also the B6.129S4-Kras<sup>−/−</sup>/J mice carrying a Lox-Stop-Lox (LSL) termination sequence followed by the Kras G12D point mutation and the B6.Cg-Tg(Mx1-cre)1Cgn/J mice were obtained from the Jackson Laboratory. B6.129S7-Ifng<sup>−/−</sup>/J (Ifng<sup>−/−</sup> or Ifng-KO) mice that do not produce IFN-γ (Dalton et al., 1993) were kindly provided by Prof. Nils Lycke, MIVAC, University of Gothenburg.

In paper I, mice were injected subcutaneously with 2x10<sup>5</sup> or 3x10<sup>5</sup> EL-4 lymphoma cells and treated with 1500 µg/mouse HDC intraperitoneally (i.p.) three times a week, starting the day before tumor inoculation. Tumor size was measured every second day and mice were euthanized at the end of the second week for harvesting of spleens and tumors. Paper II involves an additional transgenic mouse model. Six to twelve weeks old naïve C57BL/6, Nox2-KO and Ifng-KO mice were treated i.p. with PBS (control), HDC (Sigma, 1500 µg/mouse), IL-15 (0.04 µg/mouse), alone or combined, on the day before, the day after and 3 days after i.v. injection of B16F10 cells (5-15x10<sup>4</sup> cells/mouse). Mice were euthanized by cervical dislocation followed by harvesting of lungs and spleens after 30 min, 24 hours or 3 weeks after tumor inoculation depending on the experimental purpose. The number of metastases in lungs was assessed by counting macroscopically visible pulmonary metastatic foci under a light microscope. In paper III, the BCR-ABL1<sup>+</sup> p210 construct cloned into the MSCV-GFP<sup>+</sup> vector was kindly provided by Nikolas von Bubnoff (University of Freiburg, Germany). This vector was used to transduce bone marrow cells of WT and Nox2<sup>−/−</sup> mice to generate leukemic cells that did or did not produce ROS, respectively. Together with rescue bone marrow, these BCR-ABL1<sup>+</sup> cells were transplanted i.v. to C57BL6/J mice that were lethally irradiated. Blood
samples drawn every second week from the transplanted mice were analyzed by flow cytometry for several markers. In paper IV, B6.129S4-Kras\textsuperscript{tm4Tyj}/J mice, carrying a Lox-Stop-Lox (LSL) termination sequence followed by the Kras G12D point mutation, were bred to a strain expressing Cre recombinase under the control of the Mx1 promoter (Figure 5). When injected i.p. with pIpC, endogenous IFN production is induced and Cre recombinase in hematopoietic cells is activated to delete the transcriptional termination sequence allowing for expression of the oncogenic Kras in hematopoietic cells. As a result these mice developed pronounced myeloproliferation.

Figure 5. Kras\textsubscript{LSL-G12D} mice carry a Lox-Stop-Lox (LSL) termination sequence followed by the Kras G12D point mutation. When bred to a strain expressing Cre recombinase under the control of the Mx1 promoter, pIpC will activate the Cre recombinase in hematopoietic cells to delete the transcriptional termination sequence, allowing for expression of oncogenic Kras in hematopoietic cells.

To study the effects of genetic NOX2 inhibition in this model, B6.129S6-Cybb\textsuperscript{tm1Din} (Nox2\textsuperscript{-/-}) mice that lack functional NOX2 were bred to B6.129S4-Kras\textsuperscript{tm4Tyj}/J mice to generate Nox2\textsuperscript{-/-} Kras\textsubscript{G12D} mice and also bred to Mx1-Cre mice to generate Nox2\textsuperscript{-/-} Mx1-Cre mice. These mice were backcrossed at least 3 times to achieve an offspring with closer genetic identity. Finally, Nox2\textsuperscript{-/-} Kras\textsubscript{G12D} mice and Nox2\textsuperscript{-/-} Mx1-Cre mice were mated to generate
Nox2<sup>−/−</sup> Kras<sub>2LSL/Mx1-Cre</sub> (Nox2<sup>−/−</sup> M-Kras<sup>G12D</sup>) mice (Figure 6). Lack of functional NOX2 in these mice was confirmed by genotyping for Nox2 gene with PCR and by measuring the lack of ROS production using chemiluminescence.

Figure 6. Breeding schema for the generating Nox2<sup>−/−</sup> Kras<sup>G12D</sup>Cre<sup>+</sup> mice.

Three to four week old M-Kras<sup>G12D</sup> or Nox2<sup>−/−</sup> M-Kras<sup>G12D</sup> pups were injected i.p. with 3 doses of pIpC every second day (250 µg pIpC per mouse per injection). At the end of the pIpC injections, M-Kras<sup>G12D</sup> or Nox2<sup>−/−</sup> M-Kras<sup>G12D</sup> mice were divided in a control and a treatment group receiving N<sup>2</sup>-methylhistamine dihydrochloride (NMH, 250 µg/mouse; Sigma) i.p. every second day. Mice were weighed and blood was collected every second week to follow the course of disease. Blood counts were analyzed on a Sysmex KX-21 Hematology Analyzer (Sysmex, Kobe, Japan). Mice were killed humanely when moribund followed by harvesting of spleen, bone marrow and thymus.
**Flow cytometry**

Flow cytometry is utilized to measure cell size, granularity, various surface structures and intracellular proteins or cell components such as DNA at the single cell level. Suspensions of cells are usually incubated with fluorescence-labeled antibodies directed to the structures of interest. After washing away unbound antibody, the flow cytometer takes up a suspension of single cells, then sheath fluid is used to hydrodynamically focus the cell suspension through a small nozzle and pass cells through a laser beam one by one. Fluorescent light that is generated as each cell passes through the laser is quantified and presented by a computer as a dot plot or a histogram. There are different fluorescent dyes that enable multiple detection of antigens in a single panel. Flow cytometry and flow cytometry-based techniques have been used extensively in all four papers of this study both to sort cells (three laser BD FACS Aria) and to assess inter- and intracellular cell markers of tumor and immune cells, ROS and cell viability (four laser BD LSR Fortessa).

**PCR**

The polymerase chain reaction (PCR) allows for the amplification of DNA segments in several orders of magnitude. PCR is based on thermal cycling. First, the template DNA is heated. High temperature causes the DNA strands to separate. Subsequently, forward and reverse primers, which are short single-stranded oligonucleotides complementary to the target DNA, anneal to the specific areas on the single-stranded DNA molecule at specific temperatures and DNA polymerase starts to synthesize new DNA. The method was used in paper II to detect presence of WT NK cells in the blood of recipient Ifng−/− mice and to confirm a successful adoptive transfer. In paper IV PCR was employed to genotype the new progeny of mice from the crosses between different transgenic mice. In the latter studies, genomic DNA was extracted from mouse ear biopsies using the mouse direct PCR kit from Biotool (Houston, USA). The following primers were used to amplify target sequences:
<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-Nox2&lt;sup&gt;−/−&lt;/sup&gt; and Nox2&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>AAGAGAAACTCCTCTGCTGTGAA</td>
</tr>
<tr>
<td>R-Nox2&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>CGCAGCTGGAACCCCTGAGAAAGG</td>
</tr>
<tr>
<td>R-Nox2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>GTTCTAATTCCATCAGAAGCTTATCG</td>
</tr>
<tr>
<td>F-Kras&lt;sup&gt;G12D&lt;/sup&gt;</td>
<td>CTTTTACAAGCGACGCAGACTGTAGA</td>
</tr>
<tr>
<td>R-Kras&lt;sup&gt;G12D&lt;/sup&gt;</td>
<td>AGCTAGCCACCATGGCTTGAGTAAGTCTGCA</td>
</tr>
<tr>
<td>F-Mx1-Cre</td>
<td>GTTTCAATTCTCTCTTGGAAAGG</td>
</tr>
<tr>
<td>R-Mx1-Cre</td>
<td>CTAGAGCCTGTGTTTGACGTTTCC</td>
</tr>
<tr>
<td>F-Kras&lt;sup&gt;LSL&lt;/sup&gt;</td>
<td>TCCGAATTCAGTGACTACAGATGTACAGAG</td>
</tr>
<tr>
<td>R-Kras&lt;sup&gt;LSL&lt;/sup&gt;</td>
<td>GGGTAGGGTTGGGATAGCTG</td>
</tr>
<tr>
<td>F-Ifng</td>
<td>AGAAGTAAGTGGAGGGCCAGAAG</td>
</tr>
<tr>
<td>R- Ifng</td>
<td>AGGGAATCGGGAAGGAGGAGAAT</td>
</tr>
<tr>
<td>F-Ifng&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>TCAGCGCAGGGGCGCCCCGTTCTT</td>
</tr>
<tr>
<td>R-Ifng&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>ATCGACAAGACCGCTTCATCCGA</td>
</tr>
</tbody>
</table>

**Table 2.** Primer sequences used in this thesis.

**qRT-PCR**

Real-time quantitative reverse transcription PCR (qRT-PCR) is a method to quantify gene expression by synthesizing cDNA from mRNA. The method allows for the detection and quantification of products generated during each PCR cycle. In addition to the conventional PCR components, there is also an oligonucleotide probe that hybridizes to the target sequence in a reaction tube. The probe contains a reporter fluorophore and its cleavage due to the 5'
nuclease activity of the Taq polymerase enables detection of amplification at the end of each cycle. In this study, GrandScript cDNA SuperMix (TATAA Biocenter) was used to synthesize cDNA and qRT-PCR was performed with a CFX384 real-time cycler (BioRad) in papers I and III to measure the expression of several genes. Each 6 µL reaction contained 1X TATAA SYBR GrandMaster Mix (TATAA Biocenter) or 1X iQ Supermix (BioRad), 400 nM of each primer (Sigma-Aldrich) or 1X TaqMan gene expression assay (Life Technologies) and 2 µL diluted cDNA. Data were analyzed with the GenEx software (MultiD) and normalization was performed against three reference genes.

Detection of ROS

We used a cytometry-based method that involves staining of cells with a cell-permeant fluorogenic reagent, 2′,7′-dichlorofluorescin diacetate (DCFDA, Invitrogen). DCFDA is first deacetylated to a non-fluorescent form by esterases, but in the presence of ROS it is oxidated to fluorescent 2′,7′-dichlorofluorescein (DCF) that can be quantified by FACS. We also detected extracellular and intracellular ROS by the chemiluminescence method developed by Dahlgren et al. (146). This method is based on quantifying the emitted light as a result of adding a chemiluminescent reagent to the reaction mixture containing cells and horseradish peroxidase. In this second method, selection of the chemiluminescent agent (luminol or isoluminol) allows for the distinction between intracellular and extracellular ROS. Single cell suspensions of cells were diluted to 10⁷ cells/ml in Krebs-Ringer glucose buffer supplemented with isoluminol (10 mg/ml; Sigma-Aldrich) and horseradish peroxidase (HRP, 4 U/ml, Boehringer Mannheim, Germany) and added to 96-well plates that were incubated at 37°C. Phorbol myristate acetate (PMA, 5x10⁻⁸ M, Sigma-Aldrich, Missouri, USA) or the formyl peptide receptor agonist WKYMVm (Tocris Bioscience, Bristol, UK) were added to trigger ROS production. Light emission was recorded continuously using a FLUOstar Omega plate reader (BMG, Ortenberg, Germany). In some experiments HDC or NMH were added 5 min prior to the addition of WKYMVm. The methods were used in all four papers.
Co-culture experiments and ELISA

The co-culture experiment in paper II aimed at detecting the IFN-γ production by lung cells cultured with melanoma cells. WT mice were treated with HDC or PBS (control). On the next day, B16F10 cells were injected i.v. to all mice and lungs were harvested after 30 min. Single cell suspensions of lung cells from treated or untreated mice were co-cultured with B16F10 melanoma cells overnight at effector to target ratios of 1:1 to 50:1. Supernatants were collected after 24 hours and the IFN-γ content was estimated using the enzyme linked immunosorbent assay (ELISA).

Cell culture

All cell lines used in this study were cultured under sterile conditions at 37 °C and 5% CO₂ and checked and found negative for mycoplasma contamination. B16F1 and B16F10 cells were obtained from the Cell Culture Laboratory at the department of Virology, University of Gothenburg. PLB-985 cells were kindly provided by Dr. Mary Dinauer (Washington University, USA). Complete media were prepared with Iscoves’ medium supplemented with 10% FCS (human AB serum for PLB-985 cells), 2 mmol/L L-glutamine, 1mmol/L sodium pyruvate, 100 U/mL penicillin and 100 mg/mL streptomycin. PBMCs were prepared from buffy coats using Ficoll-Paque density centrifugation. Single cell suspensions of splenocytes were prepared by mashing spleens through a 70 µm strainer followed by treatment with RBC lysis buffer (Sigma-Aldrich). Lung tissues were dissociated into single cells with the lung tissue dissociation kit (Miltenyi, Lund, Sweden).

Isolation of NK cells, adoptive transfer and NK cell depletion

In paper II, we used adoptive NK cell transfer to confirm that the anti-metastatic properties of HDC relied on IFN-γ production by NK cells. NK cells were isolated from the spleens of WT mice and transferred to Ifng⁻/⁻ mice. Splenocytes were initially enriched for NK cells by passage through nylon wool columns (Polysciences, Warrington, USA). NK cells were then negatively selected using the NK cell isolation kit II (Miltenyi Biotech) to a purity of >70%. NK cells were depleted using i.p. injections of 250 µg anti-
NK1.1 antibody (BioXCell, West Lebanon, USA, Clone PK136) 4 and 2 days before B16 cell inoculation.

**Depletion and isolation of Gr1⁺ myeloid cells**

In paper I, bone marrow Gr1⁺ cells were isolated using Gr1⁻ biotinylated antibodies followed by streptavidin-conjugated MACS beads (Miltenyi, Sweden) to a purity of >95%. For depletion of Gr1⁺ cells (paper II), mice were injected i.p with 400 µg anti-Gr1 antibody (BioXCell, West Lebanon, USA, Clone RB6-8C5) 2 days before B16 cell inoculation.

**Statistical methods**

**Student’s t-test**

The Student's t-test is a commonly used parametric statistical method that compares the means of two data sets. The test requires that the values in two populations follow a normal distribution with equal variance in both groups. Two-tailed, paired and unpaired t-tests have been used in all four papers, mostly for comparisons between treatment groups.

**Logrank test**

The logrank test is a nonparametric test employed in the analysis of a survival difference between two samples. The logrank test was used in paper III to compare the difference in survival between mice transplanted with WT or Nox2⁻⁻ BCR-ABL⁺ bone marrow and in paper IV for analysis of the impact of treatment with NMH on the survival of mice with KRAS-driven leukemia.

**Analysis of variance (ANOVA)**

ANOVA enables the statistical comparison of more than two groups. There are two different types of ANOVA, one-way ANOVA and two-way ANOVA referring to the number of independent variables in the test. ANOVA was used in paper I to compare CD86 and CD11b expression on DCs and monocytes and in paper II to compare the number of metastatic foci and IFN-γ levels between experimental groups.
Post-hoc tests

Multiple parallel analyses require correction of p-values to avoid spurious significance. For this purpose, we used Bonferroni correction following one-way ANOVA in papers I and IV.
RESULTS & DISCUSSION

Paper I

In cancer, the differentiation of myeloid cells is frequently impaired along with dysfunction of myeloid cell regulation (40, 147). Dendritic cells (DCs), the immune cell population in focus in paper I, are key players in the initiation and tuning of adaptive tumor-specific immune responses. The consequences of cancer-induced deficiencies in myeloid cell differentiation comprise inefficient formation of mature, functional DCs along with accumulation of immature, and often immunosuppressive, myeloid cells (e.g. MDSCs) (148, 149). Experimental animals that are genetically deprived of histamine formation (histidine decarboxylase knockout mice) are characterized by accumulation of immature myeloid cells along with a markedly increased susceptibility to chemically induced cancer (150, 151). In light of these earlier studies, the aim of paper I was to define effects of exogenously added histamine dihydrochloride (HDC) on myeloid cell differentiation, in particular DC differentiation, in addition to assessing effects of the administration of HDC on the in vivo growth of a murine lymphoma (EL-4) where tumor expansion has been reported to entail accumulation of immature myeloid cells (45).

In initial in vitro experiments we investigated whether HDC affected the differentiation of human monocytes into functional DCs. We exposed monocytes to IL-4 and GM-CSF, which facilitate the differentiation of monocytes into DCs, in the presence or absence of HDC. Our first observation was a stretched morphology of the DCs that were generated in the presence of HDC, which is reportedly a characteristic of mature and functional DCs. The altered DC morphology was accompanied by an increase in differentiation markers, including the antigen presentation receptor HLA-DR and the co-stimulatory molecules CD86 and CD40 at the transcriptional and protein levels (Figure 7A-B).
Figure 7. (A-B) Expression of the maturation markers (A) HLA-DR and (B) CD86 on monocytes (MO) or monocyte-derived DCs (DC) cultured in the absence (control, CON) or presence of HDC. Panels (C-D) show the in vivo growth of subcutaneously implanted EL-4 lymphoma tumors in (C) WT and (D) Nox2<sup>-/-</sup> C57BL/6 mice treated with HDC (red bars) or NaCl (CON; white bars).

We next asked if the maturation of DCs observed after treatment with HDC translated into improved T cell activation capacity. DCs that were generated in the presence or absence of HDC were co-cultured with allogeneic T cells.
The HDC-generated DCs were more efficient than control DCs in inducing T cell activation, as indicated by enhanced T cell proliferation and by enhanced formation of IFN-γ and IL-4. The addition of neutralizing antibodies against CD86, a costimulatory molecule that was induced on DCs by HDC, markedly decreased T cell proliferation. Based on the finding that Th1 as well as Th2 type T cells were enhanced by HDC-treated DCs, we propose that HDC stimulated DCs towards induction of Th0 responses.

In a next series of experiments, we aimed at defining the mechanism underlying the pro-differentiating properties of HDC. Our group has previously reported that HDC inhibits the formation of NOX2-derived ROS from myeloid cells via its agonist activity at H2Rs (113, 132, 152). We confirmed lower ROS levels in DCs generated in the presence of HDC by use of DCFDA staining. However, the pro-differentiating properties of HDC appeared not to depend on the removal of extracellular ROS as DCs generated from monocytes in the presence of catalase, a ROS scavenger, did not mimic the enhanced DC maturation noted after exposure to HDC.

To shed further light on the involvement of NOX2-derived ROS for the observed pro-differentiating action of HDC, we utilized WT and NOX2−/− variants of the human myelomonoblastic cell line PLB-985. In WT PLB-985 cells, treatment with HDC induced a more mature phenotype as reflected by upregulation of CD11b and CD14 expression. In contrast, HDC had no effect on the expression of these maturation markers on NOX2−/− PLB-985 cells. To clarify if H2R signaling was involved also in the pro-differentiating actions of HDC, we used an H2R-specific antagonist, ranitidine, which antagonized the ability of HDC to reduce NOX2-derived ROS production as well as the HDC-induced differentiation of myeloid cells. AH20239AA, an inert structural analogue of ranitidine, was devoid of these effects, thus further supporting that these effects of HDC were mediated by H2R.

To investigate if HDC induced DC activation and maturation also in an in vivo setting, we used the EL-4 lymphoma mouse model where DCs previously have been ascribed a significant role in controlling tumor growth (45). EL-4 cells were thus implanted into C56BL/6 mice, and the systemic administration of HDC treatment significantly reduced tumor growth (Figure 7C). In addition, HDC treatment entailed accumulation of intratumoral CD11c+MHCII+ DCs, and the occurrence of these cells in tumors was inversely correlated to the size of EL-4 tumor growth in vivo.
The same experimental setup was employed in $\text{Nox}^{2-}$ mice. In these mice, which are genetically depleted of NOX2, HDC treatment did not affect tumor growth (Figure 7D) or intratumoral DC infiltration. Taken together, these results suggest that (i) HDC, acting via H$_2$R, improves DC maturation by targeting NOX2 and (ii) controls EL-4 tumor growth in vivo in a NOX2-dependent manner.

**Paper II**

In this study, we aimed at defining the mechanisms underlying the anti-metastatic efficacy of HDC in B16F10 murine melanoma with particular reference to the role of NOX2. We utilized $\text{Nox}^{2-}$ mice that do not produce ROS and observed a markedly reduced engraftment of melanoma cells in lungs in these NOX2-deficient mice. Thus, 150,000 intravenously injected B16F10 cells produced equal numbers of metastatic foci in the lungs of $\text{Nox}^{2-}$ mice as did 100,000 melanoma cells in WT mice. From these findings, we propose that NOX2-derived ROS, produced by myeloid cells, may facilitate metastasis formation in this model.

We next assessed the efficiency of systemic treatment with HDC in reducing melanoma metastasis in WT and $\text{Nox}^{2-}$ animals. Monotherapy with HDC significantly decreased the number of pulmonary metastases in WT mice but not in $\text{Nox}^{2-}$ mice. In contrast, the NK cell-activating cytokine IL-15 showed as potent anti-metastatic properties in $\text{Nox}^{2-}$ mice as in WT mice (Figure 8A). These findings imply that the action of HDC on NOX2 function is critical for its anti-neoplastic efficacy in the B16F10 metastasis model. The results thus cohere with those presented in paper I showing that HDC reduced the subcutaneous growth of EL-4 lymphoma tumors in WT, but not in $\text{Nox}^{2-}$ mice.
Figure 8. (A) Formation of B16F10 metastases in lungs of Nox2\(^{-/-}\) or WT mice after treatment with HDC and/or IL-15. One-hundred thousand B16 cells were injected intravenously to WT mice and 150,000 cells to Nox2\(^{-/-}\) mice (n=15 for WT mice, n=8 for control and single treatments of Nox2\(^{-/-}\) mice, n=3 for treatment with HDC + IL-15 in Nox2\(^{-/-}\) mice). Results were analyzed by Repeated Measures ANOVA followed by Bonferroni’s post-hoc test. (B) Effects of treatment with HDC on the formation of B16F10 lung metastasis in Ifng\(^{-/-}\) mice (left, n=9 for each group, t-test); and in Ifng\(^{-/-}\) mice that received WT or Ifng\(^{-/-}\) NK cells (right, n=6 for each group, t-test).

In the B16F10 metastasis model, immune-mediated clearance of malignant cells in the early phase after tumor inoculation is likely critical to control the formation of macroscopic metastases (153, 154). To define effects of HDC on myeloid cell populations and ROS formation in lungs following injection of B16F10 cells, lung tissue was prepared before and 30 min, 4 h or 24 h after the injection of melanoma cells, followed by analyses of myeloid cell content and the capacity of myeloid cells to generate extracellular ROS \textit{ex vivo}. Thirty minutes after tumor cell injection, there was a striking influx of CD11b\(^+\) and Gr1\(^+\) immature myeloid cells into the lung tissue. While the administration of HDC on the day before tumor cell injection did not affect the magnitude of the early influx of Gr1\(^-\)CD11b\(^+\) cells into lungs, we observed that Gr1\(^-\)CD11b\(^+\) recovered from HDC-treated animals displayed reduced ROS-forming capacity.
These results indicate that HDC treatment may reduce the availability of ROS in lungs during the early phase of metastasis. To further define the effects of Gr1+CD11b+ cells in melanoma metastasis, we depleted these cells \textit{in vivo} using an anti-Gr1 antibody before treatment of mice with HDC and inoculation of B16F10 cells. The administration of anti-Gr1 enhanced metastasis, thus supporting that presence of Gr1+CD11b+ cells may promote melanoma metastasis in this model. In agreement with the view that HDC exerted anti-metastatic efficacy by regulating the myeloid cell NOX2, HDC did not affect metastasis in anti-Gr1-treated animals.

We next aimed at elucidating downstream mechanisms of relevance to the anti-metastatic action of HDC. Earlier studies imply that treatment with HDC improves clearance of B16 melanoma cells in mice, and that HDC is devoid of efficacy in this model when NK cells are depleted (155). We thus depleted NK cells \textit{in vivo} using an anti-NK1.1 antibody and observed (i) that the extent of lung metastasis after inoculation of B16F10 cells was enhanced in the absence of NK cells, and (ii) that HDC did not reduce metastasis after NK cell depletion. In addition, we noted that \textit{in vivo} treatment with HDC significantly increased the number of intrapulmonary NK cells of WT, but not of Nox2−/−, mice as measured 3 weeks after tumor cell inoculation, with a similar trend noted already at 24 h. These results imply that treatment with HDC is associated with enhanced survival, influx or proliferation of NK cells at the site of the tumor.

Unexpectedly, the lungs and spleens of Nox2−/− mice contained significantly fewer NK cells than corresponding tissues from WT animals. While the reason for this finding is unknown, it may be speculated that the genetic absence of NOX2 affects the dynamics and distribution of immune populations. Notably, Nox2−/− mice showed markedly increased melanoma metastasis after NK cell depletion and also responded to the NK cell-stimulatory cytokine IL-15, thus supporting that NK cells contribute in controlling melanoma metastasis also in Nox2−/− mice.

Earlier studies have identified IFN-γ as a key mediator of NK cell-dependent elimination of tumor cells (156). Additionally, \textit{in vitro} studies imply that HDC increases the formation of IFN-γ in human NK cells by targeting the production of NOX2-derived ROS (157). To assess the IFN-γ production of murine pulmonary NK cells in the melanoma metastasis setting, we co-
incubated B16F10 cells with lung cells recovered from HDC-treated and control WT mice. It was observed that treatment with HDC in vivo was associated with increased formation of IFN-γ from lung cells ex vivo. We therefore assessed the potential antitumor efficacy of HDC treatment in Ifng−/− mice. In Ifng−/− mice, the degree of melanoma metastasis was augmented, thus supporting the notion that IFN-γ is crucial in controlling metastasis. To achieve a comparable number of lung metastases, 50,000 and 100,000 B16F10 cells were injected to Ifng−/− mice and WT mice, respectively. With this design, we observed that treatment with HDC did not affect melanoma metastasis formation in Ifng−/− mice. Additionally, when NK cells isolated from WT animals were adoptively transferred to Ifng−/− mice the anti-metastatic efficacy of HDC was restored, thus supporting that the reduction of metastasis achieved by HDC relied on the availability of IFN-γ (Figure 8B).

It should be pointed out that the experimental metastasis model employed in these studies, where a single or related clone of tumor cells are injected into the blood stream of mice, does not encompass critical steps of metastasis formation such as intra- and extravasation. To address these mechanisms, and thus to more closely mimic the process of metastasis in humans, we are currently in the process of studying the impact of genetic or pharmacological targeting of NOX2 in a murine model of spontaneous melanoma metastasis.

**Paper III**

CML is a myeloproliferative disorder resulting from the generation of a constitutively active tyrosine kinase, encoded by the fusion gene BCR-ABL1, whose activity enhances the proliferation and survival of mutated myeloid cells with ensuing accumulation of mature granulocytes in bone marrow and blood. In several hematopoietic cancers caused by genetic abnormalities associated with increased tyrosine kinase activity, including BCR-ABL1, JAK2 and FLT3-ITD, the transformed cells reportedly display elevated intracellular ROS levels (158-160). The activity of tyrosine kinases is normally counter-balanced by protein tyrosine phosphatases (PTPs) that remove phosphate groups from phosphorylated tyrosine residues on proteins. As the catalytic domain of PTPs contains redox-sensitive cysteine residues,
ROS are assumed to inhibit the action of PTPs and may thus reinforce tyrosine kinase activity within the malignant cells (161, 162).

With this background, the results presented in paper III were generated to determine the potential impact of NOX2-derived ROS for the course of CML. Increased ROS levels in CML cells may be the result of increased ROS generation via NOX enzymes, or by enhanced ROS production during mitochondrial respiration (159, 163, 164). Although BCR-ABL-dependent signaling may activate also other NOX isoforms (163), NOX2 is, as discussed above, the most abundantly expressed isoform in mature myeloid cells (102).

We thus transduced the BCR-ABL1 gene into bone marrow cells from WT and Nox2−/− mice. As a result, BCR-ABL1+ myeloid cells were generated that were either capable or incapable of producing NOX2-derived ROS. The transduced WT and Nox2−/− cells expressed equal amounts of BCR-ABL1, but the clonogenic capacity of Nox2−/− BCR-ABL1+ cells was markedly reduced compared to WT BCR-ABL1+ cells (Figure 9A). In experiments designed to assess the capacity of BCR-ABL1+ expressing WT and Nox2−/− cells to cause leukemia in vivo, equal amounts of leukemic cells were transplanted to lethally irradiated WT mice together with WT rescue bone marrow. While both WT and Nox2−/− BCR-ABL1+ cells engrafted, there was a significant difference in the onset of leukemia. In mice that received transformed Nox2−/− cells, the increase of BCR-ABL1+ cells in blood thus occurred at significantly later time points compared with mice receiving leukemic cells capable of producing NOX2-derived ROS.
Figure 9. (A) Colony-forming capacity of Nox2−/− or WT BCR-ABL1+ leukemic cells. (B) Survival of mice transplanted with Nox2−/− or WT BCR-ABL1+ leukemic cells (n=6, logrank test).

The delayed engraftment translated into a significantly prolonged survival of animals receiving Nox2−/− BCR-ABL1+ cells (Figure 9B). The mice were sacrificed when moribund and splenocytes, livers and bone marrow were isolated for further analysis. All tissues of the diseased mice were infiltrated with BCR-ABL1+ cells out of which the majority were CD11b+ Gr1+ mature myeloid cells along with a smaller fraction of CD11bGr1− blast-like cells. The malignant cells in the bone marrow of mice receiving Nox2−/− BCR-ABL1+ cells exhibited a more mature phenotype, which is in agreement with previous studies suggesting that NOX2 is involved in myeloid differentiation (165, 166).

**Paper IV**

*Kras* is one of three homologues of the *Ras* family of oncogenes. Oncogenic *Kras* mutations are frequently detected in monocytic forms of acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CMML) and other myeloproliferative diseases (MPD). The principal aim of paper IV was to evaluate the efficacy of a NOX2 inhibitor, Nα-methyl histamine (NMH),
which is a histamine derivative with high selectivity at H\textsubscript{2}R (166), in a murine model where oncogenic \textit{Kras} expression was induced in hematopoietic cells of LSL-\textit{Kras}\textsuperscript{G12D} and Mx1-\textit{Cre} double transgenic mice (M-\textit{Kras}\textsuperscript{G12D} mice) with ensuing development of MPD.

To determine the role of NOX2 in \textit{Kras}-mutated hematological disease, we mated \textit{LSL-Kras}\textsuperscript{G12D} and Mx1-\textit{Cre} mice to generate M-\textit{Kras}\textsuperscript{G12D} pups. Three to six week old M-\textit{Kras}\textsuperscript{G12D} pups were injected with pIpC to initiate leukemia. Two weeks after pIpC injections, the mice showed progressive leukocytosis along with a significant reduction of RBC counts and hemoglobin. At the end of the experiment spleens of M-\textit{Kras}\textsuperscript{G12D} mice were markedly enlarged and infiltrated with CD11b\textsuperscript{+}Gr1\textsuperscript{+} myeloid cells.

To assess the potential ROS-inhibitory efficacy of \textit{NMH} in \textit{Kras}-mutated cells, CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells were isolated from the spleens of M-\textit{Kras}\textsuperscript{G12D} mice and stimulated with a NOX2-inducing dipeptide in the presence of absence of \textit{NMH}. These cells produced significant amount of ROS, which was dose-dependently reduced by \textit{NMH}. The addition of ranitidine, an H\textsubscript{2}R receptor antagonist, blocked the \textit{NMH}-induced inhibition of ROS production in these cells.

In experiments designed to determine the effects of \textit{NMH} on the progression of disease in M-\textit{Kras}\textsuperscript{G12D} mice, animals received \textit{NMH} thrice weekly during 5 weeks, starting one week after the first pIpC injection. Treatment with \textit{NMH} entailed a milder course of disease as determined by the degree of mobility, fur condition and posture and also delayed the development of MPD and prolonged survival of M-\textit{Kras}\textsuperscript{G12D} mice (Figure 10A-B).
Figure 10. (A) Survival of control (CON) and NMH-treated WT M-Kras\textsuperscript{G12D} mice (n=22-25, logrank test). (B) Counts of white blood cells in blood before and after the onset of treatment for WT M-Kras\textsuperscript{G12D} mice (n=22, t-test). (C) Survival of control and NMH-treated Nox2\textsuperscript{-/-} M-Kras\textsuperscript{G12D} mice (n=7-8, logrank test). (D) Counts of white blood cells in blood before and after the onset of treatment with NMH for Nox2\textsuperscript{-/-} M-Kras\textsuperscript{G12D} mice (N=7-8, t-test).

To shed further light on the role of NOX2 in Kras-related leukemia, we generated triple transgenic mice that expressed Mx1-Cre inducible LSL-Kras\textsuperscript{G12D}, thus lacking the gp91\textsuperscript{phox} subunit that is crucial to NOX2 function (167), by mating Nox2\textsuperscript{-/-} mice with LSL-Kras\textsuperscript{G12D} and Mx1-Cre mice.
followed by several backcrosses (see Figure 6). The efficiency of the depletion of Nox2 was confirmed by genotyping and the absence of ROS production was confirmed by chemiluminescence upon dipeptide stimulation. In these triple transgenic mice, there was a markedly reduced myeloproliferation vs. M-Kras$^{G12D}$ WT mice that did not translate into improved survival. In vivo treatment with NMH did not alter the development of leukemia or survival in the triple transgenic mice (Figure 10C-D).

By virtue of NOX2 inhibition HDC, acting via H$_2$R, has been reported to protect anti-neoplastic lymphocytes such as NK cells and T cells from ROS-induced inactivation and death (112, 168). To determine the potential contribution by cellular immunity for the observed anti-leukemic properties of NMH in KRAS-related leukemia we utilized flow cytometry for the analysis of peripheral blood samples during the course of disease. In these experiments, bone marrow and splenocytes were analysed for T cell, NK cell, B cell, Gr$^+$ myeloid cell, monocyte and dendritic cell populations. These populations of immune cells did not differ between control and treatment groups.

FLT3/ITD mutations in AML, expression of BCR-ABL in CML and RAS mutations in MPD are associated with elevated ROS formation that may generate DNA injury with ensuing genomic instability (169-171). Among the different types of DNA damage, DNA strand breaks (DSB) have been proposed as a significant contributor in carcinogenesis (172). We therefore determined in vivo effects of NMH treatment on oxidative stress on DNA by use of an antibody against 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is a product of DNA oxidation. We observed M-Kras$^{G12D}$ mice showed increased oxidation of DNA that was significantly inhibited by in vivo treatment with NMH. We also determined whether the reduced DNA oxidation translated into protection against DSBs and observed that treatment with NMH entailed reduced DSBs in the peripheral blood. These effects were not replicated by systemic NMH treatment in Nox2$^{-/-}$ M-Kras$^{G12D}$ mice.
CONCLUDING REMARKS

This thesis aimed at contributing to the understanding of the role of NOX2 in cancer, in particular hematopoietic cancer (lymphoma, myeloid cell malignancies) and malignant melanoma. For this purpose, we used experimental animals that were genetically deprived of NOX2 along with NOX2-deficient tumor cells and inhibitors of NOX2-derived ROS formation. Broadly, the results support that the targeting of NOX2 function is associated with an aggravated course of cancer, but the underlying mechanisms may differ between different malignancies. In murine lymphoma, our results imply that presence of NOX2 may limit the maturation of DCs translating into a less efficient T cell-mediated elimination of tumor cells. In melanoma, presence of NOX2 in the tumor microenvironment was found to reduce the effectiveness of NK cell-mediated clearance of melanoma cells to augment metastasis. In the BCR-ABL1 model of CML and in the M-Kras<sup>G12D</sup> model of myeloid leukemia, the malignant cells themselves expressed NOX2; in this setting elevated intracellular ROS levels triggered DNA damage and mutagenesis, thus likely contributing to the progression of leukemia.

Each of these experimental models has significant limitations. In brief, the results presented in paper I were derived from heterotopic implantation of lymphoma cells and may not adequately reflect lymphoma development. In paper II, we used a method to induce metastasis that does not take aspects of intra- or extravasation of tumor cells into account. In paper III, the results reflect the development of acute BCR-ABL leukemia rather than the chronic phase of CML. Also, the survival of mice reported in paper IV, in particular of mice carrying simultaneous mutations of Kras and Nox2 may have been confounded by deaths from other causes than leukemia.

In all employed tumor models, NOX2 inhibition reduced tumor progression. The results thus point towards the possibility of utilizing pharmacological inhibitors of NOX2 (HDC, NMH) in cancer treatment. Notably, however, the role of ROS in cancer is likely complex, as shown by studies using antioxidants that improve the survival and metastatic capacity of the malignant cells (129-131). These findings may, in part, reflect enhanced resistance to ROS-mediated toxicity in cancer cells that are loaded with antioxidants. If so, the results are coherent with the results presented in paper
IV, where inhibition of NOX2 yielded reduced oxidation of DNA and reduced DNA strand breaks, thus illustrating that NOX2 inhibition may reduce potentially lethal events in cancer cells. Hence, the success of targeting NOX2-derived ROS for therapeutic purposes may be dependent on *inter alia* the sensitivity of the particular cancer cells to redox modulation, as well as on the sensitivity of cancer cells to elimination by immune effector mechanisms. With these reservations, NOX2 may constitute an attractive target for improved cancer therapy.
ACKNOWLEDGEMENTS

There have been many people that have walked alongside me during my PhD journey and time in Sweden. I would like to thank each of them for making this journey remembered beautifully.

"Don't pick a job. Pick a Boss. Your first boss is the biggest factor in your career success. A boss who doesn't trust you, won't give you opportunities to grow." ~ W. Raduchel.

My main supervisor, Anna Martner, I am very happy that you are my first boss. It is not easy to find a supervisor that trusts her students and provide freedom to develop their ideas. Many thanks for giving me to opportunity to work in your lab, for excellent supervision and your friendly attitude. Our scientific discussions always inspired and motivated me at times I felt down.

My co-supervisor, Kristoffer Hellstrand, for encouragement, sharing your outstanding knowledge and expertise. I admire how you support and inspire people around you. I have learned a lot from you and gained new perspectives. It feels so safe when you and your problem-solving skills are around. Without your positive attitude, music and jokes, the TIMM lab would clearly be a less attractive work place.

My co-supervisor, Mats Brune, for your guidance and support.

All my colleagues at the TIMM lab, you are more than colleagues for sure. I have spent more time with you than with my family and friends during the last four years. Without you, it would have been very difficult for me to adapt to a new country and a new city. Thank you for making Sahlgrenska Cancer Center 3rd floor a great place. You have always been there for me whenever I needed help. I love you all. Alexander, my desk partner, sorry for invading your part all the time, you were always very nice to me when I try to glue all my Kras blood counts around. Hanna, your warmth, spirit and support always made us a real team. You are a great friend and it is so good to have someone that will understand when I complain about mice. Elin, we started our PhD studies together and it has been a great pleasure going through this together. I really admire how you care about people and stand up for the values you believe in. Fredrik, your valuable inputs to every discussion widened my perspective. Also, your positive point of view, kindness and support contribute so much to the lab. Johan, Rebecca and Anna R., thank you for introducing me the lab and all your guidance during my first years.
Junkooooo, I love our good-morning hugs (I mean some time around 11.00 when I say morning), before-lunch hugs, after-lunch hugs, hugs at the analysis computer, hugs in the kitchen, goodbye hugs etc. I love all the hugs you provided us with, all the cat pictures, all those games with no real objective. I also love your strong personality and that you keep your unique style with confidence. You bring so much joy and relaxation to our lab. Roberta, your patience and warm smile always made my day. I remember when you started you were told that we want you to know everything. But, seriously, you are so good at everything. You are an open-hearted, fun and caring friend. I love our failures in gluten-free recipes and EBM frustrations. Besides, I love talking about my art classes with you (not because you are the only person that likes my art). Malin N., you have a beautiful energy. Your natural and unique sense of humor make our fika and lunch times great. Ali, you are one of the most helpful people I know, you always do your best to guide us and share your expertise generously. In addition, I really enjoy watching people when you and me talk about our strange traditions and politics. Brwa, I hope one day we will solve all our bureaucratic problems and never do paperwork again, on the other hand it’s so much fun to complain with you about all the visa problems. I admire your brave decisions to make your dreams come true. Belson, so nice to have a late-lunch company. You have a good heart and make our lab a nicer place. Annica, it has been a short since you joined the lab but it feels like that you have been here forever. You create a friendly atmosphere in the lab and I always feel your support. Karin, we have a common nightmare that unites us. You are a great person with lots of knowledge and you share it in the most humble way. It was a real pleasure to work with you.

Of course all the former lab members and students in the lab, Malin P., Anna S., Olle, Brianna, Frida, Charlotta, Britta, Adi, Linnea, Isabel. I had great time with you, thank you for all your contribution.

Mia, Sofia, Jessica, Haamid; life would be very hard during PhD without your contributions in taking care of all the mice. Your understanding, positivity and attention mean a lot. Thank you for the great job.

My dear friends Pinar and Ezgi, I am so upset that we met so late but I am sure we will never fall apart. Everything we lived was so surreal with you. You made my final year so much fun. I couldn’t have written this thesis without your support and the late night lahmacun therapies. Bilal, I am really grateful for all your help with the language.
Gulay ablam, I don’t have words to describe my gratitude. I am so lucky to meet you. You and Havva Gul made me feel home, provided the family with spirit and have been with me whenever I needed your help. But the best moments I enjoyed was of course painting and studying Swedish with Havva Gul. I never felt alone thanks to your company.

Alpirinim, you are a great friend, I laugh so much with you. Your support during all the writing period is priceless, or maybe values 300 TL.

Sabiha Akmanoglu, my first teacher, thank you for your encouragement and believing in me. I always felt your love and support.

The Kurtulmus and Ozturk families, I am very happy that you are welcoming me. I am looking forward to spend more time together.

Canim anneannem Fatma ve dedem Halil, iyi ki varsiniz, uzakta da olsak sizin sevginiz bana hep kuvvet verdi. Grandma, Seniye, I know you would be proud of me, I miss you so much.

Abdurrahman amcam, Serpil teyzem, Helinsu, Silosum, you are an important part of my life. Thank you all for raising me and being a second family for me.

Selen, my best, you mean so much to me. I couldn’t be happy without your care, love and support. You made me follow my dreams and made this thesis possible just like almost every good thing in my life.

Cagla’m, you are my precious, the joy in my life, the only person that can make me laugh regardless of how sad I feel. Thanks for being the best sister ever.

Sultan, mom; and Yucel, dad; you believed in me, you trusted me, supported in every sense, you loved me and I know you will forever. You are the best parents in the world. Annecigim, babacigim sizleri çok seviyorum. Siz olmadan basaramazdim.

Lastly, Bahtiyar’im, without your love and encouragement it wouldn’t be possible to write this thesis. It’s hard to believe it’s over. We dreamed so much about our life together, I am looking forward starting our new adventure. You are my home, I am coming home. I love you.
REFERENCES


55. Ishida T, Oyama T, Carbone DP, Gabrilovich DI. Defective function of Langerhans cells in tumor-bearing animals is the result of defective maturation from hemopoietic progenitors. Journal of Immunology. 1998;161(9):4842-51.


57


Limon-Pacheco J, Gonsebatt ME. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat Res. 2009;674(1-2):137-47.


