Targeting myeloid cells in the tumor microenvironment

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“If I step onto my misfortune, I stand higher”

Friedrich Hölderlin

ABSTRACT

Myeloid cells are central regulators of the tumor microenvironment (TME), where they frequently accumulate and drive immunosuppression and disease progression. A key mechanism underlying their immunosuppressive function is the production of reactive oxygen species (ROS), predominantly mediated by the NOX2 enzyme complex, along with the secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β). Through these pathways, myeloid cells impair the activity of cytotoxic lymphocytes, including T cells and natural killer (NK) cells. Consequently, they facilitate tumor progression by promoting metastasis in solid malignancies and supporting the survival and expansion of leukemic cells. However, myeloid cells are functionally plastic and can also exert anti-tumor effects. This thesis investigated strategies to target and reprogram suppressive myeloid cells to restore anti-tumor immunity and limit disease progression across solid tumors and hematological malignancies. In **Paper I**, histamine dihydrochloride (HDC) was shown to induce the production of the anti-angiogenic factor thrombospondin-1 (TSP-1) via cAMP/PKA signaling in myeloid cells, contributing to reduced melanoma metastasis. In **Paper II**, NOX2-dependent ROS-producing myeloid cells were identified as key regulators of NK-cell-mediated control of lung and liver metastasis in pancreatic ductal adenocarcinoma (PDAC), supporting NOX2 inhibition as a potential therapeutic strategy. In **Paper III**, HDC/IL-2 treatment was associated with reduced IL-1β levels in acute myeloid leukemia (AML) patients in first complete remission, and lower IL-1β levels were associated with improved leukemia-free survival, consistent with a role for inflammatory myeloid cytokines in relapse risk. In **Paper IV**, Bruton's tyrosine kinase inhibition blocked NOX2 activation in myeloid cells and enhanced NK-cell-mediated killing of malignant cells under immunosuppressive conditions.

Overall, these studies highlight the context-dependent and plastic roles of myeloid cells across cancer types and demonstrate that targeting their suppressive programs represents a conceivable therapeutic strategy.

Keywords: Immunotherapy, reactive oxygen species, NOX2, histamine, TSP-1, NK cells, AML, PDAC, melanoma, BTK

SAMMANFATTNING

Myeloida celler anrikas ofta i tumörer och kan utveckla suppressiva egenskaper som begränsar natural killer (NK)-cellers och T-cellers förmåga att attackera och eliminera tumörceller. Cancerpatienter med en hög andel myeloida suppressorceller i blodet eller i tumörvävnad har ofta sämre prognos. De mekanismer som myeloida celler använder för att undertrycka immunitet mot cancerceller är bara delvis kartlagda, men myeloida celler producerar bland annat inflammatoriska signalämnen såsom IL-1β och reaktiva syreradikaler (*reactive oxygen species*, ROS) via enzymet NOX2, vilka bidrar till att inaktivera tumördödande lymfocyter. Att motverka myeloida cellers immunhämmande funktioner utgör en möjlig strategi för att förbättra NK-cellers och T-cellers förmåga att eliminera cancerceller.

Denna avhandling omfattar studier av hur myeloida celler reglerar immunitet mot cancer och hur dessa processer kan påverkas av läkemedel. I **arbete I** och **III** studerades mekanismer av betydelse för histamindihydroklorids (HDC) antineoplastiska effekter. I en experimentell melanommodell (**arbete I**) inducerade HDC produktion av trombospondin-1 (TSP-1) i myeloida celler, vilket kan bidra till att minska metastasering. I en klinisk studie av patienter med akut myeloisk leukemi (AML) (**arbete III**) kopplades behandling med HDC/IL-2 till minskad IL-1β-medierad inflammation, vilket korrelerade med förbättrat kliniskt utfall. I **arbete II** visades att genetisk inaktivering av NOX2 var associerat med förbättrad NK-cellsfunktion och minskad metastasering i en djurmodell för pankreascancer. På liknande sätt visade **arbete IV** att hämning av Brutons tyrosinkinas resulterade i minskad NOX2-beroende ROS-produktion i myeloida celler och förbättrad NK-cellsmedierad eliminering av tumörceller.

Sammanfattningsvis visar denna avhandling att det går att stärka kroppens antitumorala immunförsvar genom att rikta in sig på myeloida cellers immunhämmande funktioner, särskilt deras produktion av ROS och inflammatoriska signaler. Resultaten pekar mot nya behandlingsstrategier, till exempel selektiva NOX2-hämmare och kombinationsterapier, som skulle kunna minska metastasering och förbättra överlevnad hos cancerpatienter.

สรุปผลงานวิทยานิพนธ์

เซลล์ไมอีลอยด์ (myeloid cells) มักพบในปริมาณสูงในเนื้องอก และสามารถพัฒนาให้มีคุณสมบัติในการกดภูมิคุ้มกัน ซึ่งจำกัดความสามารถของเซลล์นักฆ่าตามธรรมชาติ (natural killer; NK cells) และเซลล์ T ในการโจมตีและกำจัดเซลล์มะเร็ง ผู้ป่วยมะเร็งที่มีสัดส่วนของเซลล์ไมอีลอยด์ชนิดกดภูมิคุ้มกันสูง ทั้งในเลือดและในเนื้อเยื่อเนื้องอก มักมีพยากรณ์โรคที่แย่กว่า อย่างไรก็ตาม กลไกที่เซลล์ไมอีลอยด์ใช้ในการยับยั้งภูมิคุ้มกันยังเป็นที่เข้าใจเพียงบางส่วน

เป็นที่ทราบว่าเซลล์ไมอีลอยด์สามารถผลิตสารในระบบภูมิคุ้มกันที่ส่งเสริมการอักเสบ เช่น IL-1β และสารอนุมูลอิสระ (reactive oxygen species; ROS) ผ่านเอนไซม์ NOX2 ซึ่งมีบทบาทในการยับยั้งการทำงานของเซลล์เม็ดเลือดขาวที่ทำหน้าที่กำจัดเซลล์มะเร็ง ดังนั้น การยับยั้งคุณสมบัติการกดภูมิคุ้มกันของเซลล์ไมอีลอยด์จึงเป็นแนวทางหนึ่งที่อาจช่วยเพิ่มประสิทธิภาพของเซลล์ NK และเซลล์ T ในการกำจัดเซลล์มะเร็ง

วิทยานิพนธ์ฉบับนี้ครอบคลุมการศึกษาว่าเซลล์ไมอีลอยด์ควบคุมภูมิคุ้มกันต่อมะเร็งอย่างไร และประเมินว่ากระบวนการเหล่านี้สามารถถูกปรับเปลี่ยนด้วยยาได้หรือไม่ ในงานวิจัยที่ ๑ และ ๓ ได้ศึกษากลไกที่เกี่ยวข้องกับฤทธิ์ต้านมะเร็งของฮิสตามีนไดไฮโดรคลอไรด์ (histamine dihydrochloride; HDC) โดยในแบบจำลองมะเร็งผิวหนังเมลาโนมา (งานที่ ๑) พบว่า HDC กระตุ้นการสร้างโปรตีนทรอมโบสปอนดิน-1 (thrombospondin-1; TSP-1) ในเซลล์ไมอีลอยด์ ซึ่งอาจช่วยลดการแพร่กระจายของมะเร็ง นอกจากนั้น (งานที่ ๓) ในการศึกษาทางคลินิกในผู้ป่วยมะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน (acute myeloid leukemia; AML) พบว่าการรักษาด้วย HDC ร่วมกับ IL-2 มีความสัมพันธ์กับการลดระดับโปรตีนอักเสบที่เกิดจาก IL-1β ซึ่งสัมพันธ์กับผลลัพธ์ทางคลินิกที่ดีขึ้น ในงานวิจัยที่ ๒ แสดงให้เห็นว่าการยับยั้ง NOX2 ทางพันธุกรรมมีความสัมพันธ์กับการทำงานของเซลล์ NK ที่ดีขึ้น และการลดการแพร่กระจายในแบบจำลองของหนูทดลองด้วยมะเร็งตับอ่อน ในทำนองเดียวกัน งานวิจัยที่ ๔ แสดงให้เห็นว่าการยับยั้ง Bruton's tyrosine kinase (BTK) ส่งผลให้การสร้าง ROS ผ่านเอนไซม์ NOX2 ในเซลล์ไมอีลอยด์ลดลง และช่วยเพิ่มประสิทธิภาพของเซลล์ NK ในการกำจัดเซลล์มะเร็ง

โดยสรุป วิทยานิพนธ์นี้แสดงให้เห็นว่าสามารถเสริมสร้างภูมิคุ้มกันต้านมะเร็งของร่างกายได้โดยการมุ่งเป้าไปที่หน้าที่กดภูมิคุ้มกันของเซลล์ไมอีลอยด์ โดยเฉพาะการผลิต ROS และสารสื่อการอักเสบ ผลลัพธ์ที่ได้ชี้ให้เห็นถึงแนวทางการรักษาใหม่ ๆ เช่น การใช้ตัวยับยั้ง NOX2 แบบจำเพาะ และการรักษาแบบผสมผสาน ซึ่งอาจช่วยลดการแพร่กระจายของมะเร็งและเพิ่มอัตราการรอดชีวิตของผู้ป่วยมะเร็งได้

LIST OF PAPERS

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

I. **Issdisai N**, Kaya M, Hellstrand K, Grauers Wiktorin H*, Martner A*. Myeloid cell-derived thrombospondin-1 mediates anti-metastatic effects of histamine in B16 melanoma.

Accepted for publication in ImmunoTargets & Therapy 2026

*Authors contributed equally

II. **Issdisai N***, Kaya M*, Kiffin R, Grauers Wiktorin H, Hellstrand K, Martner A. Myeloid NOX2-derived reactive oxygen species restrain NK cell-mediated control of lung and liver metastasis in pancreatic cancer.

In manuscript

*Authors contributed equally

III. Grauers Wiktorin H, Aydin A, Christenson K, **Issdisai N**, Thorén FB, Hellstrand K, Martner. Impact of IL-1β and the IL-1R antagonist on relapse risk and survival in AML patients undergoing immunotherapy for remission maintenance.

OncoImmunology. 2021, Vol. 10, No. 1, e1944538

IV. Törnell A, Waldenström J, **Issdisai N**, Kiffin R, Akhiani AA, Thorén FB, Hellstrand K, Martner A. Bruton's tyrosine kinase activates the NOX2/ROS axis to drive myeloid immunosuppression in cancer

In manuscript

Additional publications not included in this thesis:

1. Wiktorin HG, Einarsdottir S, Törnell A, Arabpour M, **Issdisai N**, Waldenstrom J, Ringlander J, Lindh M, Lagging M, Hellstrand K, Martner A. COVID-19 vaccine-induced adverse events predict immunogenicity among recipients of allogeneic haematopoietic stem cell transplantation. *Haematologica. 2022;107(10):2492–5.*

2. Kaya M, Johnsson O, **Issdisai N**, Paul S, Wiel C, Dongre A, Hellstrand K, Martner A**.** NRF2 activation by myeloid cell-derived oxidative stress induces SNAI-driven features of EMT in breast cancer.

   Accepted for publication in PNAS 2026

CONTENTS

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ARG1	Arginase-1
BTK	Bruton's tyrosine kinase
CAF	Cancer-associated fibroblast
CGD	Chronic granulomatous disease
DC	Dendritic cell
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
FPR	Formyl peptide receptors
HIF	Hypoxia-inducible factor
HSC	Hematopoietic stem cells
ICB	Immune checkpoint blockade
IFN-γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
KIR	Killer-cell immunoglobulin-like receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid derived-suppressor cell

MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NET	Neutrophil extracellular trap
NK	Natural killer
NKT	Natural killer T cells
NO	Nitric oxide
NOX2	NADPH oxidase 2
PBMC	Peripheral blood mononuclear cell
PD-1	Anti-programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
ROS	Reactive oxygen species
SKCM	Skin cutaneous melanoma
SOD	Superoxide dismutase
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophil
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
Treg	Regulatory T cell
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor

TLS	Tertiary lymphoid structure
TME	Tumor microenvironment
TSP-1	Thrombospondin-1
HDC	Histamine dihydrochloride
VEGFR	Vascular endothelial growth factor receptor

PREFACE

Cancer remains a major cause of death and is characterized by uncontrolled cell growth and immune evasion. Although advances in immunotherapy have improved outcomes for some patients, many malignancies, including pancreatic ductal adenocarcinoma (PDAC) and acute myeloid leukemia (AML), still show limited and heterogeneous responses. A major challenge is the immunosuppressive tumor microenvironment, which impairs effective anti-tumor immunity and promotes disease progression.

Myeloid cells play a central role in this immunosuppression. In cancer, they acquire tumor-promoting functions through the production of immunomodulatory factors and reactive oxygen species (ROS), thereby limiting the activity of cytotoxic lymphocytes such as natural killer (NK) cells and T cells. NADPH oxidase 2 (NOX2) is a key mediator of ROS generation in myeloid cells and contributes to immune suppression and tumor progression.

In both tumor and bone marrow microenvironments, NOX2-derived ROS establish an immunosuppressive niche that impairs immune-mediated clearance of malignant cells. In parallel, inflammatory signaling pathways further regulate myeloid cell function and influence disease outcome. However, the interplay between redox regulation, inflammatory signaling and immune suppression in cancer remains incompletely understood.

This thesis aimed to provide insight into how inflammatory and redox-regulated pathways contribute to immune dysfunction and to define potential strategies to restore anti-tumor immunity.

INTRODUCTION

1 IMMUNE SYSTEM

The immune system comprises a complex network of diverse cell types that either circulate throughout the body or reside within specific tissues. These cells have specialized roles and communicate with one another to protect the host from infections and pathogens such as viruses, bacteria, and parasites, as well as from tumor cells. The first line of defense is the innate immune system, which provides rapid protection and can subsequently initiate and shape the adaptive immune response. An overview of immune cell progenitors arising from the bone marrow is shown in Figure 1.

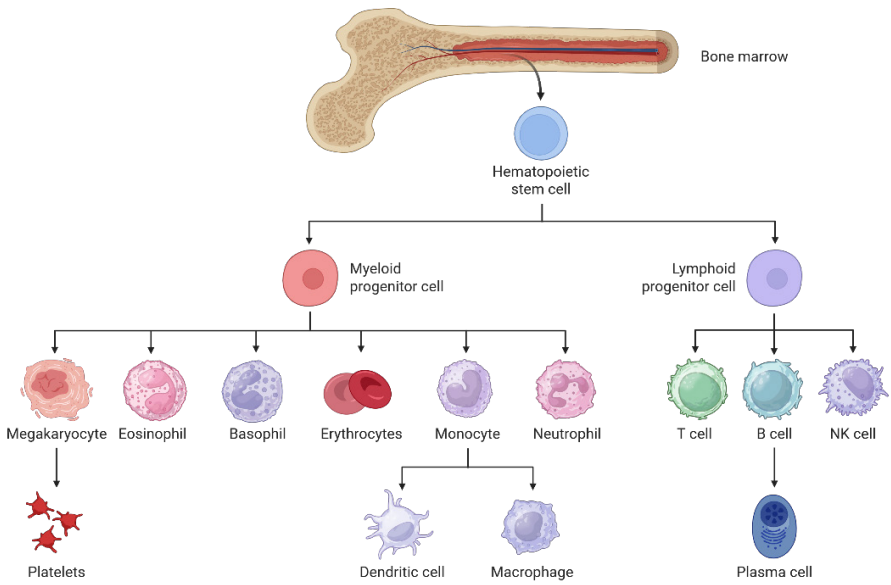

Figure 1: **Hematopoiesis and differentiation of immune cells from bone marrow stem cells.**

1.1 Innate immune system

The innate immune system provides the most immediate defense through barrier mechanisms, including physical barriers (skin and epithelial tight junctions), chemical barriers (saliva, tears and mucus containing antimicrobial factors) and microbial barriers (commensal microorganisms that limit pathogen colonization) [1]. Innate immunity also recognizes pathogens through dedicated receptors and integrates these signals via intracellular pathways to mount appropriate local and systemic responses [2].

1.1.1 Granulocytes

Granulocytes arise from hematopoietic stem cells (HSCs) in the bone marrow and differentiate into individual lineages before entering the bloodstream and migrating into tissues. They are characterized by cytoplasmic granules that contain proteases and other degradative enzymes. Granulocytes are typically divided into neutrophils, eosinophils and basophils [1].

Neutrophils are the most abundant granulocyte subset and are often among the first immune cells recruited to affected tissues during infection or injury [3]. They extravasate from the bloodstream in response to adhesion molecules and chemotactic signals, and eliminate pathogens through phagocytosis and degranulation, releasing enzymes such as hydrolases, peroxidases and lysozyme. Additionally, neutrophils can contribute to limiting excessive inflammation by producing anti-inflammatory cytokines [4, 5]. Nuclear factor kappa B (NF-κB) is a key transcriptional regulator of neutrophil activation, controlling genes involved in survival, cytokine/chemokine production and antimicrobial functions, thereby shaping the magnitude and duration of inflammation. In addition, neutrophils generate reactive oxygen species (ROS) through the NADPH oxidase 2 (NOX2) complex, which produces superoxide

and downstream oxidants as part of the oxidative burst. NOX2-derived ROS contribute to intracellular killing of phagocytosed microbes and can also modulate local inflammatory processes and immune responses in a context-dependent manner [3, 6]. Formation of neutrophil extracellular traps (NETs) via NETosis, releasing decondensed chromatin coated with granule-derived proteins such as neutrophil elastase and myeloperoxidase to trap and neutralize microbes, is an additional effector function of neutrophils. NET production is frequently associated with NOX2-derived ROS, which supports signaling events that drive chromatin decondensation and subsequent NET release. [7].

Eosinophils are large granulocytes whose primary function is to protect against parasites, particularly worms and helminths, through extracellular release of cytotoxic granules. They also contribute to allergic inflammation and tissue remodeling [8].

Basophils are rare in circulation and are known for their role in allergic reactions. Upon activation via IgE-dependent mechanisms, they rapidly degranulate and release histamine which increases vascular permeability, promotes vasodilation and contributes to symptoms such as swelling and itching. They can also produce cytokines such as IL-4 supporting Th2 responses and amplifying allergic inflammation. Additionally, they contribute to anti-helminth responses [1, 9].

1.1.2 Monocytes and macrophages

Monocytes and macrophages are components of the mononuclear phagocyte system (MPS) which originate from HSCs in the bone marrow. During hematopoiesis, these progenitors give rise to promonocytes, which subsequently differentiate into monocytes that are released into the bloodstream. Circulating monocytes can migrate into tissues, particularly

under inflammatory conditions, where they further differentiate into macrophages. Tissue-resident macrophages are distributed throughout the body and include specialized populations such as alveolar macrophages in the lungs, microglia in the brain and Kupffer cells in the liver [1].

In humans, circulating monocytes are commonly classified into three major subsets based on the expression of CD14 and CD16. Classical monocytes ($CD14^{++}CD16^{-}$) represent the predominant population and are primarily involved in phagocytosis. Intermediate monocytes ($CD14^{++}CD16^{+}$) exhibit proinflammatory properties and participate in antigen presentation and cytokine production. Non-classical monocytes ($CD14^{+}CD16^{++}$) are mainly involved in vascular surveillance and patrolling functions [10, 11].

In mice, monocytes are generally classified based on expressions of Ly6C, CCR2 and CX3CR1. Classical or inflammatory monocytes are typically characterized as $Ly6^{high}CCR2^{high}CX3CR1^{low}$ cells and are rapidly recruited to inflammatory sites. In contrast, non-classical or patrolling monocytes are defined as $Ly6C^{low}CCR2^{low}CX3CR1^{high}$ cells and are involved in immune surveillance and tissue homeostasis [11-13].

Macrophages play essential roles in maintaining tissue homeostasis and response to pathogens and tissue damage. Depending on environmental cues, they can adopt different functional states, most often referred to as classically activated M1 macrophages and alternative activated M2 macrophages. M1 macrophages are typically induced by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) or other microbial products and are associated with pro-inflammatory responses and pathogen killing through the production of ROS. In contrast, M2 macrophages are stimulated by factors such as IL-4, IL-10, IL-13 and transforming growth factor-beta (TGF-β) are involved in anti-inflammatory responses, tissue repair, wound healing and immune regulation.

Importantly, macrophages are highly plastic cells and can shift between these phenotypes depending on signals in their microenvironment [10, 14].

Mature monocytes and macrophages display a variety of receptors on their cell surfaces, such as Fc receptors, Toll-like receptors (TLRs) and formyl peptide receptors (FPRs), which enable recognition of pathogens, damaged cells and immune complexes. Ligand engagement to these receptors triggers phagocytosis, production of inflammatory cytokines and generation of ROS, thereby contributing to immediate innate immunity while also facilitating the activation and regulation of adaptive immune responses (Figure 2).

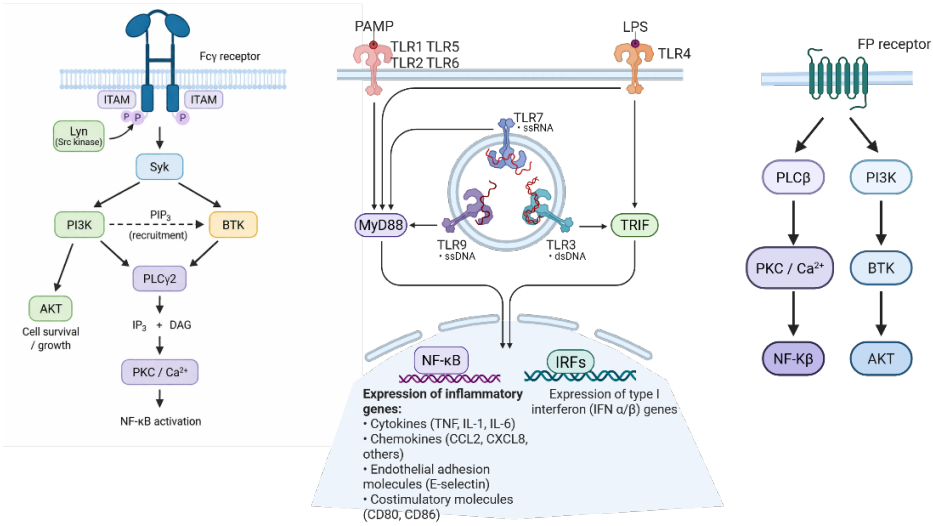

Figure 2: **Signaling pathways of FcγR, TLR and FPR**

Fcγ receptors are IgG-binding receptors that enable myeloid cell recognition and response to antibody-opsonized targets. In humans, the major activating FcγRs include FcγRI (CD64), FcγRIIA (CD32A), FcγRIIc (CD32C) and FcγRIIIA (CD16A), which signal via immunoreceptor tyrosine based-activating motifs (ITAMs). In contrast, FcγRIIB (CD32B) is the major

inhibitory FcγR and has immunoreceptor tyrosine based-inhibitory motifs (ITIM) in its cytoplasmic tail. Upon receptor engagement, ITAM-dependent signaling activates downstream pathways involving kinases such as phosphatidylinositol 3-kinases (PI3K), phospholipase C gamma 1 (PLCγ) and Bruton's tyrosine kinase (BTK) promoting effector functions including phagocytosis, oxidative burst, cytokine production, antigen presentation and antibody-dependent cellular cytotoxicity (ADCC) [15, 16].

Toll-like receptors detect pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs). They can be divided into two main groups: cell surface TLRs and endosomal TLRs. Cell surface TLRs, including TLR1, TLR2, TLR4 and TLR5, detect microbial lipids and proteins, whereas endosomal TLRs, including TLR3, TLR7, TLR8, TLR9 and TLR13, mainly recognize microbial nucleic acids. One of the best-known TLRs is TLR4, which detects LPS, a component of the outer membrane of Gram-negative bacteria. During ligand engagement, TLRs recruit adaptor proteins such as myeloid differentiation primary response 88 (MyD88) or TIR-domain-containing adaptor inducing interferon-β (TRIF), which activate downstream signaling pathways including NF-κB or interferon regulatory factors (IRFs), leading to the production of pro-inflammatory cytokines, type I interferons and co-stimulatory molecules [17].

Formyl peptide receptors are G protein-coupled receptors that recognize formylated peptides containing *N*-formyl methionine which are derived from bacteria or released by injured cells. In humans, three FPR family members have been identified: FPR1, FPR2 and FPR3. FPR1 and FPR2 are expressed by both monocytes and neutrophils, whereas FPR3 is expressed by monocytes but not neutrophils. Upon ligand binding to FPR1, G-protein activation induces dissociation of the Gα and Gβγ subunits, which trigger downstream signaling

pathways including PI3K, mitogen-activated protein kinase (MAPK) and PLC. These pathways activate mediators such as BTK, Akt, PKC and intracellular calcium signaling, leading to chemotaxis, inflammation, degranulation, transcriptional responses and ROS production. Thus, FPR signaling plays a significant role in immune surveillance, host defense and regulation of inflammation [18, 19].

1.1.3 Dendritic cells

Dendritic cells (DCs) originate from HSCs in the bone marrow and develop from myeloid and some subsets from lymphoid progenitors. As professional antigen presenting cells (APCs), they serve as a crucial link between innate and adaptive immunity. They are commonly classified into conventional (classical) DCs, plasmacytoid and monocyte-derived DCs. The latter arises mainly under inflammatory conditions. After development in the bone marrow, DCs enter the peripheral blood and populate tissues, where they continuously survey the environment [20-22].

The primary function of DCs is to capture, process and present antigens to naïve T cells on major histocompatibility complex (MHC) molecules (including presentation on MHC class I and II) to initiate adaptive immune responses. Upon activation, DCs upregulate co-stimulatory molecules and migrate to draining lymph nodes, where they prime naïve T cells. In addition to antigen presentation, DCs secrete cytokines and chemokines that recruit and shape other immune cells, thereby coordinating effective immune responses to pathogens and infection. DCs also involve immune tolerance by presenting self-antigens under steady-state conditions, induing anergic or regulatory T cells, which helps maintain immune homeostasis and prevent harmful autoimmunity [1, 5].

1.1.4 Natural killer cells

Natural killer (NK) cells are a subset of large granular lymphocytes that develop from HSCs in the bone marrow. After differentiation, they circulate primarily in the peripheral blood but can also migrate to and reside in various tissues including the liver, spleen, lungs and lymph nodes. NK cells are important components of innate immunity and play key roles in the early defense against virus-infected and malignant cells. Human NK cells are characterized by expression of CD56 (NCAM) and are commonly divided into two major subsets: $CD56^{bright}$ and $CD56^{dim}$ NK cells. $CD56^{bright}$ NK cells are primarily known for their ability to produce large amounts of cytokines, such as IFN-γ and tumor necrosis factor-alpha (TNF-α), as well as chemokines upon activation. They also proliferate efficiently in response to the cytokines IL-2 or IL-15 but exhibit limited cytotoxic capacity against tumor cells [23]. In contrast, most NK cells in peripheral blood are $CD56^{dim}$ NK cells which produce lower levels of cytokines but exhibit greater cytotoxic activity and are considered more effective in tumor cell eliminations. However, while $CD56^{bright}$ NK cells are thought to give rise to $CD56^{dim}$ NK cells, these two subsets are believed to have distinct functions and complementary roles in immunity [24]. In mice, NK cells do not express CD56 but are commonly identified by the expression of NKp46, a highly conserved member of the natural cytotoxicity receptor (NCR) family of NK cell-activating receptors that can be used across mammalian species [25].

In contrast to T cells, NK cells can eliminate target cells without prior sensitization or antigen presentation by APCs. Their activity is highly controlled by the balance between stimulation via activating and inhibitory receptors that recognize membrane-bound ligands on target cells. Inhibitory receptors, such as killer-cell immunoglobulin-like receptors (KIRs) and

NKG2A, recognize MHC-I molecules and suppress NK-cell activation. In contrast, activating receptors including NKp46, NKp30, NKp44, NKG2C, NKG2D, DNAM-1 and activating KIRs promote NK-cell activation [26, 27]. Infected or malignant cells often downregulate or lose MHC class I expression to evade $CD8^+$ T cell surveillance. Reduction in MHC class I decreases inhibitory signaling to NK cells and shifts the balance toward NK-cell activation, a mechanism known as "missing-self" recognition [28]. Once activated, NK cells release IFN-γ and TNF-α thereby promoting target-cell killing and initiating inflammatory response. In addition, NK cells can kill target cells by releasing of cytotoxic granules containing perforin and granzymes, as well as through death receptor mediated pathways such as Fas ligand (FasL)-Fas and TNF-related apoptosis-inducing ligand (TRAIL)-TRAIL receptor interactions and through ADCC, in which CD16 (FcγRIII) recognizes the Fc portion of antibodies bound to the surface of target cells to initiate a cytotoxic response [28, 29] (Figure 3).

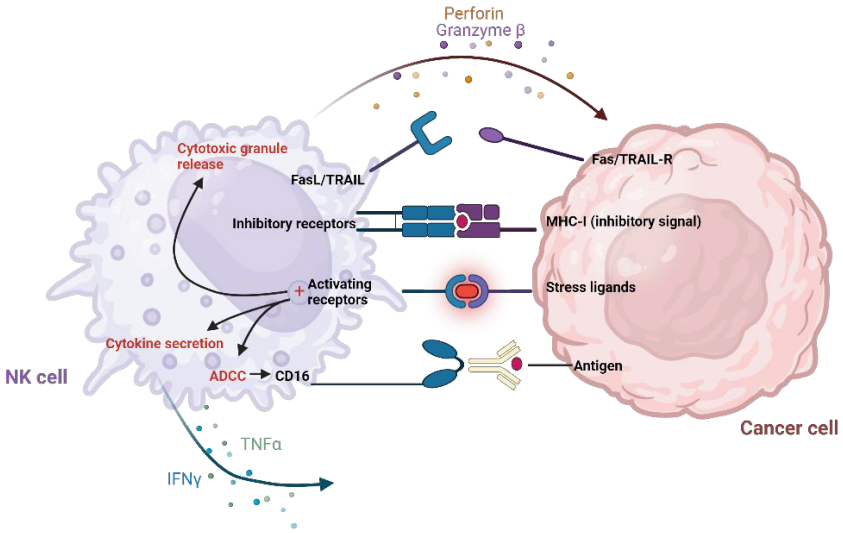

Figure 3: **Mechanisms of NK cell-mediated cytotoxicity against cancer cells**

1.2 Adaptive immune system

The adaptive immune system comprises cellular immunity mediated by T and B cells. These cells are essential for mounting targeted defenses and creating immune memory that aid in immediate and long-term protection against pathogens.

1.2.1 T cells

T cells are distributed throughout lymphoid organs, peripheral tissues and bloodstream. They originate from HSCs in the bone marrow and mature in the thymus through a series of tightly regulated developmental stages. During this process, developing T cells are subjected to both positive and negative selection, to ensure T cell receptor (TCR) recognition of MHC molecules while eliminating self-reactive T cells. These selection mechanisms are critical for the establishment of a functional and self-tolerant T cell repertoire. Following maturation, T cells differentiate into distinct subsets with specialized immunological functions, most notably $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells.

$CD4^+$ T cells, also known as helper T cells, play a central role in regulating and coordinating immune responses. These cells recognize antigenic peptides presented by APC cells in the context of MHC class II molecules. Upon activation, $CD4^+$ T cells secrete cytokines that regulate the activity of other immune cells and guide the development of appropriate immune responses. Depending on the cytokine environment and nature of antigenic stimulation, $CD4^+$ T cells can differentiate into several functional subsets upon activation.

Th1 cells produce IFN-γ, which enhances macrophage activation and supports cell-mediated immunity. Th2 cells secrete cytokines such as IL-4, IL-5 and IL-

13, promoting humoral immunity, including B cell activation and class switching to IgE and driving responses against helminths and allergic inflammation. T follicular helper (Tfh) cells produce IL-21 and play a key role in supporting B cell activation, germinal center formation and antibody production. Th17 cells produce IL-17, thereby contributing to recruitment of neutrophils and amplification of inflammatory responses at sites of infection. Collectively, these subsets are essential for orchestrating immune defense against a wide range of pathogens.

In addition to effector T cell subsets, regulatory T cells (Treg) are crucial for maintaining immune homeostasis and self-tolerance. Treg cells suppress excessive or inappropriate immune activation and thereby help prevent autoimmune pathology. Their immunosuppressive effects are mediated, in part, through the secretion of inhibitory cytokines such as IL-10 and TGF-β. Consequently, Treg cells are indispensable for limiting immune-mediated tissue damage and preserving immunological balance [1, 30].

$CD8^+$ T cells, also known as cytotoxic T lymphocytes, are primarily responsible for the direct elimination of infected and malignant cells. Naïve $CD8^+$ T cells are primed by APCs through recognition of antigenic peptides presented on MHC class I molecules, together with appropriate costimulatory signals. Since MHC class I is expressed on nearly all nucleated cells, $CD8^+$ T cells can broadly survey tissues for abnormal cells. Activated, $CD8^+$ T cells induce target-cell death through several mechanisms, including the release of perforin and granzymes, as well as activation of apoptotic pathways through FasL-Fas interactions. In addition, $CD8^+$ T cells produce cytokines such as IFN-γ, which further enhance their cytotoxic activity and contribute to the overall immune response. Through these mechanisms, $CD8^+$ T cells serve as

key contributors to host defense against intracellular pathogens and malignancies [1, 5, 31].

1.2.2 B cells

B-cell development occurs in a step wise process initiated in the bone marrow from HSCs. In the bone marrow, B cells differentiate and mature giving rise to a diverse repertoire of B cells with B-cell receptors capable of recognizing a wide range of antigens. During this process, B cells undergo negative selection to remove self-reactive clones. Once fully mature, they enter the circulation and migrate to peripheral lymphoid organs, including the spleen and lymph nodes, where they remain naïve until encountering their specific antigen. Activation of B cells is triggered by antigen binding to the B-cell receptor and is further supported by signals from helper T cells, in particular Tfh cells. After activation, B cells can differentiate into plasma cells, which produce antibodies that help neutralize and eliminate pathogens. In addition, B cells secrete cytokines that regulate immune responses. They also contribute to long-term immunity by forming memory B cells, which enable a faster and stronger response upon future exposure to the same antigen [5, 31].

2 CANCER

Cancer refers to a diverse group of diseases, including both solid tumors and hematologic malignancies, characterized by uncontrolled cell proliferation and the ability to disrupt normal cellular and tissue homeostasis, with many also having the capacity to invade surrounding tissues and spread to distant sites. In solid tissues and organs, this abnormal growth can form a mass known as a tumor, whereas in the blood and bone marrow, malignant cells expand and can outcompete normal hematopoietic cells [32]. Importantly, cancer does not arise in a single step. It develops through a multistage evolutionary process that

often begins with an oncogenic mutation in one somatic cell. As the mutant clone expands, it accumulates genetic and epigenetic alterations, ultimately giving rise to an increasingly heterogenous and invasive disease state. Mutations that confer a selective growth advantage and promote malignant progression are referred to as driver mutations. Defining these driver mutations and their functional consequences remains a central objective in cancer genome research [33].

However, oncogenic mutations alone are likely insufficient to initiate tumor formation, suggesting that additional factors contribute to early tumorigenesis. Among these, aging and environmental exposures are important contributors to cancer risk, yet how these influences drive early tumorigenesis and how they interact with specific oncogenic mutations remain incompletely understood. As cells integrate epigenetics remodeling with intrinsic and external factors, they may acquire aberrant properties that enable malignant transformation and uncontrollable growth [34]. This process involves three mechanisms (1) a permissive cell of origin, typically a long-lived stem or progenitor cell with strong self-renewal capacity that can accumulate genetic mutations and epigenetic alterations over time [35, 36], (2) release from the original differentiation program and (3) the ability to control and navigate intermediate cell stages. Ultimately, transformed cells can become increasingly autonomous, gaining control over cell fate and survival programs, including proliferation, cell state regulation, senescence, resistance to apoptosis, responses to DNA damage and metabolomic changes [33]. Together, these factors allow transformed cells to escape normal differentiation and acquire autonomous programs that support malignant growth and survival.

3 METASTATIC CANCER

After malignant transformation, solid tumors can progress from a benign or localized phase to an invasive state [37], where they acquire the ability to disseminate from the primary site and establish macroscopic secondary lesions in distant organs in a process known as metastasis. The term derives from the Greek *meta* (change) and *stasis* (standing or state), reflecting both the process of spread and its result [38]. Clinically, metastasis is a major determinant of patient prognosis, and most cancer-related deaths result from metastatic disease rather than growth of the primary tumor [39, 40].

Metastasis can occur before primary tumors are clinically detectable. Within a heterogeneous tumor, certain subpopulations are evolutionary selected for that can survive interactions with surrounding tumor cells and stromal components and ultimately adapt to new environments [38]. Even before tumor cells arrive at distant organs, the primary tumor can "prime" future metastatic sites by the release of soluble molecules (e.g., via paracrine factors) that remodel distant tissues [41]. This remodeled environment, referred to as the premetastatic niche, is a complex environment shaped by interactions between hematopoietic cells and mesenchymal stromal/stem cells and extracellular components of the local tissue [42-44]. Prior to the arrival of tumor cells, recruited cells can modify the secondary microenvironment by restructuring the extracellular matrix and promoting conditions that support metastatic outgrowth, including immunosuppression, increased vascular permeability and angiogenesis [45].

Successful metastasis is commonly described as a five-step process: (1) invasion, (2) intravasation, (3) circulation, (4) extravasation and (5) colonization [46] (Figure 4).

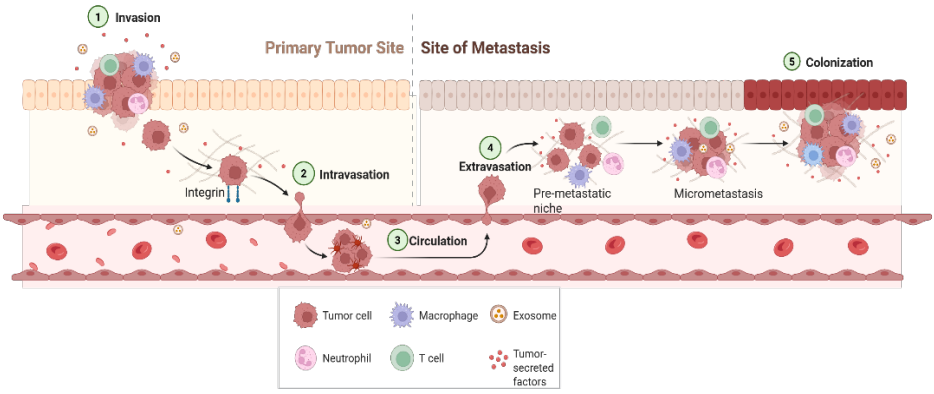

Figure 4: **Metastatic cascade**

During invasion, tumor cells undergo morphological and phenotypic changes and actively remodel their surrounding microenvironment to facilitate tissue penetration [38]. This step is driven by coordinated changes in cell adhesion, extracellular matrix (ECM) reorganization and cell motility. In epithelial tumors, invasion is often enabled by epithelial–mesenchymal transition (EMT), during which cells lose epithelial features such as tight junctions and desmosomes and acquire a more mesenchymal, migratory phenotype [47, 48]. These shifts are regulated by numerous structural and signaling proteins that modulate cell-cell and cell-ECM interactions and thereby determine the strength and dynamics of cellular adhesion [49, 50].

During intravasation, invasive tumor cells enter the blood or lymphatic circulation. This step is facilitated by continued ECM and basement membrane remodeling, as well as dynamic interactions with endothelial cells and stromal cells [51]. Tumor-associated macrophages (TAMs) and other inflammatory cells can promote intravasation by releasing cytokines and proteases and by increasing local vascular permeability thereby loosening endothelial junctions [51, 52]. Once the endothelial barrier is compromised, tumor cells migrate

through the vessel wall and gain access to the circulation as circulating tumor cells (CTCs), either as single cells or as clusters, with the latter often exhibiting enhanced survival and metastatic potential [39, 53].

During circulation, tumor cells that enter bloodstream or lymphatics must survive multiple processes associated with cellular stress, including shear forces and immune surveillance [39, 53, 54]. CTCs often associate with platelets, neutrophils and other stromal components, which can shield them from immune detection and enhance survival, particularly when traveling as multicellular clusters with increased metastatic potential [39, 53].

During extravasation, CTCs arrive at distant sites, adhere to the vascular endothelium and exit the vasculature into the surrounding tissue. Endothelial cells form the first barrier to vessel exit and vascular beds differ across organs, including continuous, fenestrated and discontinuous (sinusoidal) vessels. Because endothelial cells exhibit tissue-specific junctional properties and surface molecules, tumor cells can preferentially adhere to particular organ sites, contributing to metastatic organotropism [38]. Extravasation is mediated by receptor-ligand interactions that promote firm adhesion (e.g., selectins and integrins), followed by transmigration through endothelial junctions or directly through endothelial cells [39, 55].

The last step in the metastatic process, colonization, requires disseminated tumor cells to adapt to the new environment and establish conditions that support sustained growth [38, 39, 48]. Many cells fail to expand and instead undergo apoptosis or enter dormancy, sometimes persisting as micro metastases for prolonged periods [38, 56]. Successful colonization is promoted by a supportive premetastatic niche, which can be enriched in vascular endothelial growth factor receptor ($VEGFR^+$) bone marrow-derived progenitors, myeloid derived-suppressor cells (MDSCs) and neutrophils.

These cells help create a permissive, pro-angiogenic and immunosuppressive environment that supports tumor cell survival and outgrowth into clinically detectable metastases [38, 57-60].

4 MELANOMA

Cutaneous melanoma is the most aggressive type of skin cancer and is responsible for a disproportionate share of skin cancer-related deaths. Melanoma develops from the malignant transformation of melanocytes, the pigment-producing cells that originate from the neural crease that synthesize melanin from tyrosine in response to stimuli such as ultraviolet (UV) which is a major environmental risk factor for melanoma [61, 62]. Early detection of melanoma or localized lesions can often be cured by complete surgical excision, with near-100% survival [63, 64]. However, melanoma can progress rapidly to a vertical growth phase, invade the dermis and metastasize to distant organs, most commonly the lymph nodes, lungs, liver and bone, which is associated with a marked decline in prognosis, with median 5-year survival dropping to ~30% [64, 65].

Melanoma typically develops through stepwise genetic changes, often beginning with activating mutations in oncogenes such as BRAF or NRAS, followed by loss of tumor suppressor function (e.g., CDKN2A, PTEN, TP53) that enables progression to an invasive disease stage. These alterations frequently activate MAPK signaling to drive proliferation; BRAF mutations predominantly stimulate the MAPK signaling, whereas NRAS mutations can engage both MAPK and PI3K survival pathways [66]. Targeted therapies such as BRAF and MEK (MAPK pathway) inhibitors are widely used to treat patients with advanced melanoma, alongside immune checkpoint blockade (ICB) therapies such as anti-programmed cell death protein 1 (PD-1)- targeting antibodies. However, despite these treatment options, and even with

combination regimens, many patients with metastatic melanoma develop therapy resistance. Resistance can be driven by tumor cell-intrinsic mechanisms as well as host- and microenvironmental factors, including cell-extrinsic signals from the tumor microenvironment (TME), which can blunt therapeutic efficacy. Therefore, strategies that target or reprogram the TME may help overcome resistance and improve outcomes for patients with advanced metastatic melanoma [67, 68].

5 PANCREATIC CANCER

Pancreatic cancer is one of the most lethal malignancies, largely due to late diagnostic, early metastatic dissemination and limited responsiveness to systemic therapy. The dominant histological subtype is pancreatic ductal adenocarcinoma (PDAC), which arises from the exocrine pancreas and accounts for most pancreatic cancer cases [69, 70]. PDAC develops through stepwise progression from noninvasive precursor lesions to invasive carcinoma [71]. The most common precursors are pancreatic intraepithelial neoplasia (PanIN), progressing from low-grade lesions (PanIN-1) through high-grade dysplasia (PanIN-3, carcinoma *in situ*) and ultimately to invasive PDAC [72]. Additional developmental routes involve cystic precursor lesions including intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) [73, 74].

At the molecular level, PDAC development is driven by genetic alterations that accumulate. Activating mutations in KRAS occur early and are present in most tumors (often reported in ~90% of cases) with G12D (40%), G12V (33%), and G12R (15%) being the most common variants [75] which are associated with aggressive clinical features [76]. As the disease progresses, the loss of major tumor suppressors such as CDKN2A, TP53 and SMAD4 drives PDAC

progression (Figure 5). Together, these changes promote uncontrolled proliferation, genomic instability, invasion and metastatic competence [69].

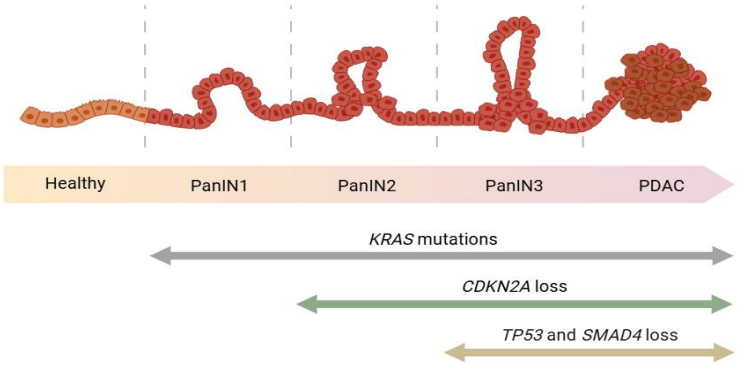

Figure 5: **PDAC progression and genetic alterations**

Beyond tumor-intrinsic alterations, PDAC is distinguished by a pronounced desmoplastic and fibroinflammatory microenvironment. Activation of pancreatic stellate cells and expansion of cancer-associated fibroblasts drive extensive extracellular matrix deposition, increased tissue stiffness, hypoxia and impaired drug delivery. Only 20-30% of patients are eligible for curative surgical resection and despite improvements in adjuvant chemotherapy, long-term clinical responses remain uncommon [69, 77]. Clinically, adjuvant chemotherapy has been associated with improved 5-year survival, particularly with FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan and oxaliplatin), which has stronger supporting evidence than gemcitabine plus albumin-bound paclitaxel (nab-paclitaxel) [78, 79]. However, despite the success of ICBs in other solid tumors (e.g., melanoma and lung cancer), PDAC is largely unresponsive to ICBs, in part due to its dense desmoplastic stroma and profoundly immunosuppressive milieu [80, 81]. This stroma-rich environment fosters immune suppression through the accumulation of suppressive myeloid

populations, including MDSCs, which can generate ROS and other inhibitory mediators that blunt cytotoxic lymphocyte function [82, 83]. Consequently, T cells and NK cells which are key effectors of antitumor immunity, are frequently dysfunctional or excluded from the tumor, reducing immune-mediated tumor clearance and thereby facilitating PDAC progression, therapeutic resistance and relapse [84-86].

6 HEMATOLOGIC MALIGNANCIES

Hematologic malignancies comprise a diverse group of cancers that arise from hematopoietic and lymphoid cells and primarily involve the bone marrow, peripheral blood and lymphatic tissues. These can occur across the life span, from children to older adulthood. Unlike solid tumors, hematologic cancers are typically diffused rather than forming a discrete mass and develop within immune-rich compartments. Disease initiation and progression are strongly shaped by interactions between malignant cells and the surrounding hematopoietic microenvironment, including stromal cells, cytokines and immune effector populations. Hematologic malignancies are classified into myeloid and lymphoid neoplasms, a distinction that reflects both the cell of origin and key cellular biological features [87, 88].

6.1 Acute myeloid leukemia

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by the rapid expansion and accumulation of immature myeloid blasts in the bone marrow and peripheral blood. The disease is highly heterogeneous and arises from a wide range of molecular and cytogenetic abnormalities, which has limited development of targeted therapies [89, 90]. Consequently, AML treatment still largely relies on intensive induction and consolidation chemotherapy, with hematopoietic stem cell transplantation

(HSCT) offered to eligible younger and medically fit patients. Although many patients achieve complete remission (CR) after initial therapy, relapse remains frequent and is associated with poor long-term outcomes. Relapse rates are especially high among older patients, with approximately 60–70% of individuals over 60 years experiencing recurrence [91-93], while relapse occurs in roughly 40–50% of younger patients [92]. These data underscore the need for improved post-remission strategies aimed at preventing relapses and extending overall survival in AML [94, 95].

Relapses are thought to arise from the persistence of therapy-resistant leukemic cells, including leukemic stem-like populations, that evade detection by conventional morphologic assessment and can later re-emerge and drive disease recurrence. In this context, measurable residual disease (MRD) assessed by flow cytometry and/or molecular methods has emerged as a key tool to define relapse risk, predict survival and inform post-remission treatment decisions [96, 97].

With high relapse burden, particularly in elderly patients who receive less intensive therapy and are more seldom candidates for allogeneic HSCT, novel maintenance and lower-intensity treatment strategies are needed [92]. Hypomethylating agents (HMA), such as azacitidine have been shown to prolong relapse-free and overall survival, although only a small portion of patients achieve durable remission [98]. In addition, several targeted and lower-intensity therapies have been introduced, including venetoclax in combination with HMA or low-dose cytarabine (AraC), as well as glasdegib in combination with low-dose AraC, both which are FDA-approved for newly diagnosed elderly or unfit AML patients. Furthermore, mutation-specific therapies, such as FLT3 inhibitors (e.g., midostaurin, gilteritinib, quizartinib) and IDH1/2 inhibitors (e.g., ivosidenib, enasidenib), have expanded the

therapeutic landscape for selected patient subsets [99-102]. More recently, menin inhibitors have emerged as a novel targeted approach for NPM1-mutated AML, particularly in the relapsed or refractory setting [103, 104]. However, despite these advances, only a fraction of patients responds and the durability of responses to these therapies remains to be fully evaluated [92].

Beyond leukemia-intrinsic genetics, AML progression and relapse are also influenced by the bone marrow microenvironment. This environment is enriched by inflammatory mediators that can promote leukemic cell survival, drug resistance and immune dysfunction. Among these pathways are IL-1 signaling that has been implicated a key regulator of myeloid expansion and microenvironmental crosstalk. This provides a rationality to investigate how IL-1β and its endogenous IL-1RA relate to relapse risk and clinical outcomes in the post-remission setting [105, 106].

6.1.1 IL-1 signaling in AML: inflammation

Inflammation is a central feature of many hematologic malignancies that can shape disease initiation, progression and treatment response by rewiring cytokine signaling, modulating immune surveillance and altering the bone marrow microenvironment. In AML, inflammatory signals produced by leukemic blasts together with stromal and immune cells can generate a supportive niche that enhances leukemic cell survival, facilitates adaptation to stress and disrupts normal hematopoiesis. Thus, inflammation is not merely a byproduct of malignant expansion but can actively contribute to leukemic persistence and relapse [105].

6.1.2 IL-1β in AML

Among pro-inflammatory mediators, IL-1β has emerged as a central cytokine with relevance to AML biology. IL-1β is produced as an inactive precursor (pro-IL-1β) and requires inflammasome-dependent activation of caspase-1 to generate mature, secreted IL-1β [106]. Importantly, ROS can act as second messengers in inflammasome activation, although their effects on IL-1β production are context-dependent and influenced by their cellular source and subcellular localization [107-109] (Figure 6). Once released, IL-1β can amplify inflammatory circuits by inducing additional cytokines and chemokines and by modulating stromal and myeloid cell function, which may further reinforce a leukemia-supportive microenvironment [106]. Because NOX2 is a major enzymatic source of ROS in myeloid cells, NOX2-dependent redox signaling may intersect with IL-1β-driven inflammation, providing a mechanistic bridge between ROS biology, innate immune activation and AML persistence.

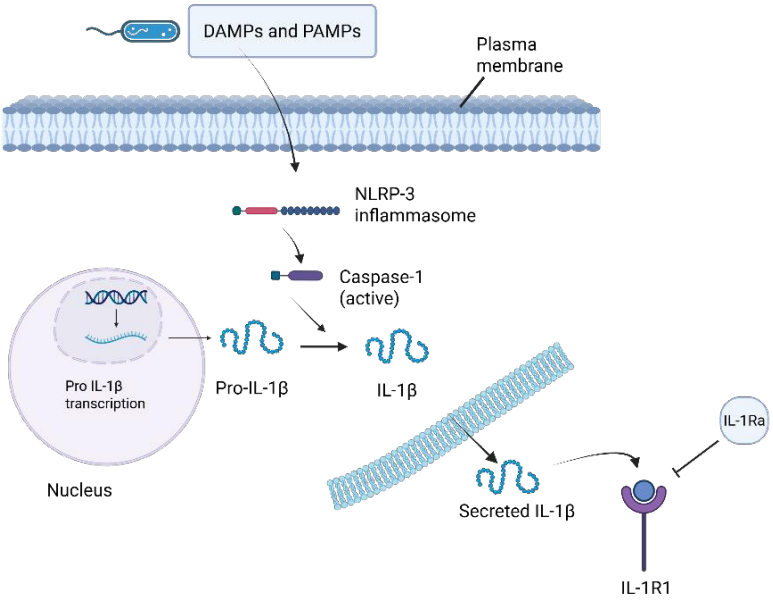

Figure 6: **IL-1β signaling pathway**

In the context of AML, IL-1β signaling has been associated with enhanced leukemic cell growth and survival and with microenvironmental changes that favor malignant over normal hematopoiesis [105, 106]. Elevated levels IL-1β in the bone marrow or peripheral blood have been reported in subsets of patients, although the specific subsets and their association with overall survival have not been fully defined. Experimental studies suggest that IL-1β can promote leukemic clonogenicity while adversely affecting normal hematopoietic stem and progenitor cell function [110]. Together, these observations support a model in which inflammatory IL-1β-associated pathways contribute to AML maintenance and therapy resistance.

6.1.3 IL-1Rα in AML

IL-1 signaling is physiologically restrained by the IL-1 receptor antagonist (IL-1Ra), which competitively binds IL-1R1 without triggering downstream signaling and thereby limits IL-1β-driven inflammation. This counter-regulatory axis may be particularly relevant in AML, where IL-1β–dependent programs have been linked to leukemic cell fitness and microenvironmental support [106]. In agreement, treatment with IL-1Ra purportedly reduce spontaneous clonogenicity of AML cells and suppress clonogenic growth after antagonist exposure [111]. Clinically, elevated IL-1Ra levels in patients undergoing relapse-preventive immunotherapy have been associated with favorable outcomes; notably, patients with low IL-1β combined with high IL-1Ra were reported to be remarkably protected against leukemic relapse, as shown in **Paper III** [112]. Together, these findings suggest that the balance between IL-1β and IL-1Ra may influence AML persistence and recurrence and point toward further investigation of IL-1-targeted approaches to reduce relapses and improve survival in AML.

6.2 B cell malignancies

B-cell malignancies are a heterogenous group of cancers, including B-cell lymphomas, B-cell leukemias and plasma cell dyscrasias that arise from abnormalities in B-cell differentiation. These abnormalities lead to malignant transformation within the peripheral blood, bone marrow and lymphoid tissues [113, 114]. Among B-cell leukemias, chronic lymphatic leukemia (CLL) is the most common form in adults in Western countries, with an incidence of about 4.7 cases per 100,000 individuals per year [115]. Immunophenotypically, CLL cells typically express the B-cell marker CD19 together with CD5, CD23, CD43 and CD200, while showing characteristically reduced expression of

surface CD20, surface immunoglobulin (Ig) and CD79b compared with normal B cells [116]. Despite the relatively low CD20 expression in CLL, anti-CD20 monoclonal antibodies such as rituximab have been widely used, often in combination regimens, to enhance immune-mediated elimination of malignant B cells by triggering ADCC, complement dependent cytotoxicity and to a lesser extend direct induction of apoptosis [117].

Treatment options for B-cell malignancies include chemotherapy, radiotherapy, targeted therapies, immunotherapy and HSCT [118]. In recent years, targeted inhibition of B-cell receptor signaling, critical for B-cell proliferation and survival, has become a major therapeutic advance, most notably with BTK inhibitors. The first-in-class BTK inhibitor ibrutinib was followed by second-generation agents such as acalabrutinib, orelabrutinib, tirabrutinib and zanubrutinib. Developed with the intention to reduce toxicity and off-target effects. More recently, third generation (non-covalent) inhibitors including pirtobrutinib and nemtabrutinib have also been introduced [119]. However, despite these advances, many patients relapse, and curative treatments remain limited for several disease subtypes [118].

7 TUMOR MICROENVIRONMENT

TME is a complex ecosystem that, in addition to malignant cells, comprises a wide range of non-cancerous cellular and non-cellular components located within and around the tumor. These components include immune cells, cancer-associated fibroblasts (CAFs), endothelial cells, pericytes and ECM, along with tissue-specific stromal elements depending on the organ of origin. Cells within the TME secrete a broad range of signaling molecules, including cytokines, chemokines and growth factors that shape tumor development, progression and responses to therapy [120, 121]. Importantly, TME components can exhibit both tumor-promoting and tumor-suppressive

functions, and their net effect depends on context and dynamic interactions. Moreover, the cellular composition and functional characteristics of the TME can vary substantially across tumors and patients. Therefore, therapeutic strategies that target key mechanisms in the TME, particularly those regulating immune activity, can improve the efficacy of other treatments, such as immunotherapy, by enhancing immune cell infiltration, reducing immunosuppression and/or enhancing drug delivery [121].

7.1 Tumor-infiltrating immune cells

Tumor-infiltrating immune cells are immune populations that migrate into the tumor and become part of the TME. Their abundance and functional state often correlate with patient prognosis and can help predict treatment responses.

7.1.1 Myeloid cells

Myeloid cells are a major cellular component of the TME and play a central role in shaping tumor immunity, angiogenesis and tissue remodeling. This compartment includes MDSCs, TAMs, neutrophils (often termed tumor-associated neutrophils, TANs) and DCs.

7.1.1.1 Myeloid-derived suppressor cells

MDSCs comprise a heterogenous group of activated immature myeloid cells often found at elevated levels in cancer patients. In this activated state these cells can be classified into polymorphonuclear or granulocytic (PMN-MDSCs) and monocytic (M-MDSCs) subsets. In humans, M-MDSCs are characterized by the expression of CD14 and low levels of the antigen-presenting receptor HLA-DR [122, 123], whereas PMN-MDSCs are typically defined as $CD11b^{+}CD15^{+}CD14^{-}$ (or $CD66b^{+}$) cells with low HLA-DR expression. In mice, MDSCs are broadly defined as $CD11b^{+}GR1^{+}$ cells and can be further

subdivided into $Ly6G^{+}Ly6C^{low}$ for PMN-MDSCs and $Ly6G^{-}Ly6C^{high}$ for M-MDSCs [124] (Figure 7).

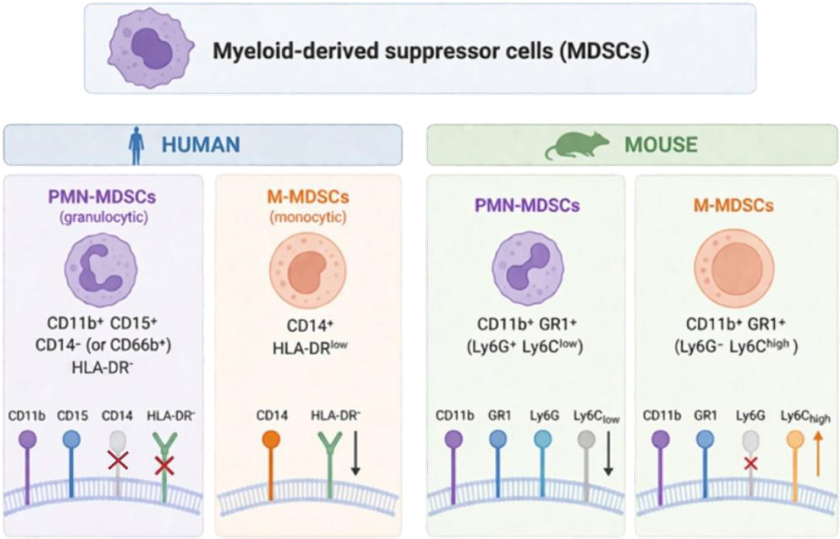

Figure 7: **Phenotypic characterization of MDSCs in human and mouse**

These cells often expand and accumulate in the peripheral blood and TME largely due to tumor-driven systemic inflammation. Tumor and stromal-cell-derived cytokines, such as GM-CSF, G-CSF, IL-6, IL-1β and VEGF promote myelopoiesis in the bone marrow, inhibit normal myeloid differentiation and mobilize immature myeloid cells into the circulation [125-128].

In parallel, cytokines such as GM-CSF and IL-6 activate signaling pathways including STAT3, which promote MDSC survival and expansion and increased levels of circulating MDSCs [129]. STAT3 signaling has also been linked to increased angiogenesis [130] and inhibition of STAT3 can reduce MDSC expansion [131].

Functionally, MDSCs acquire a potent immunosuppressive phenotype, characterized by the production of inhibitory mediators such as ROS, nitric oxide (NO) and arginase-1 (ARG1), which suppress T cell and NK cell functions [132-134]. Clinically, elevated levels of MDSCs have been associated with advanced disease stage, poor prognosis and reduced response to immunotherapy, highlighting their critical role in tumor progression and immune evasion [135-138] (Figure 8).

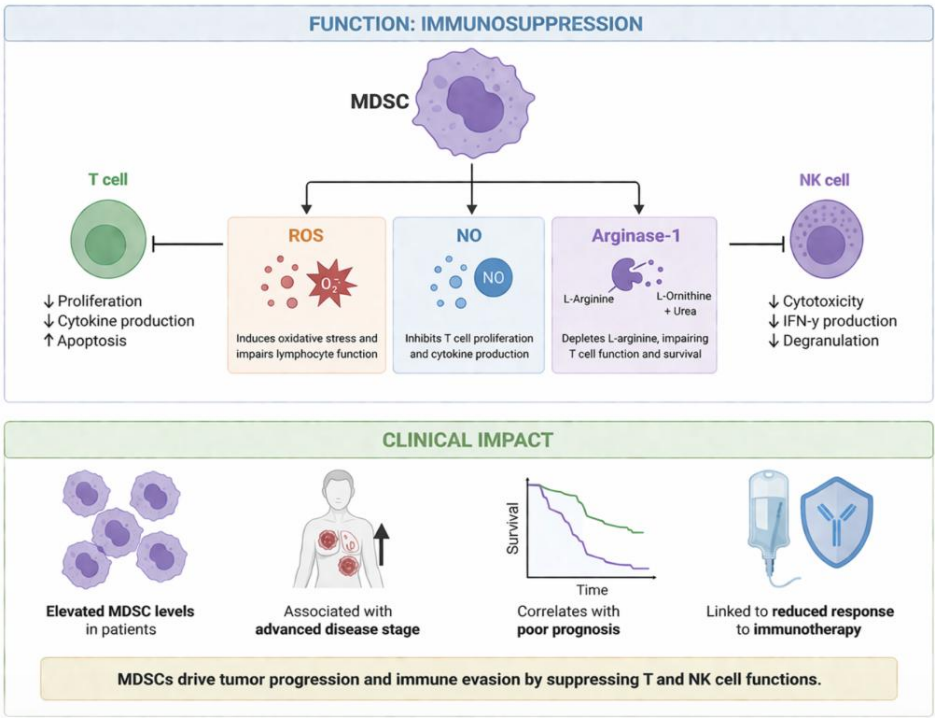

Figure 8: **Functions of MDSCs and their clinical implications**

Main suppressive mechanisms of MDSCs

ARG1 and inducible nitric oxide synthase (iNOS) are key suppressive enzymes employed by MDSCs to inhibit anti-tumor immunity. ARG1 depletes

extracellular L-arginine, an amino acid required for effective T cell activation, thereby reducing T cell proliferation and downregulation of CD3ζ chain. iNOS produces NO which impairs cytotoxic T cell function and can promote apoptosis [129, 139]. These suppressive programs are often associated with STAT1 signaling, often induced by inflammatory cytokines such as IFN-γ and IL-1β, which support iNOS expression and reinforces MDSC-mediated suppression of cytotoxic lymphocytes within the TME [128, 129].

Another major suppressive mechanism involves formation of ROS via the NOX2 enzyme, which is a key mediator of G-MDSCs driven immunosuppression. STAT3 signaling has been shown to regulate NOX2-dependent ROS generation, contributing to elevated ROS levels in cancer [127, 140]. In the bone marrow environment, NOX2 activity in immature myeloid cells contributes to the establishment of an immunosuppressive niche by impairing NK cell function and limiting immune-mediated clearance of leukemic cells. Notably, inhibition of NOX2 has been shown to restore NK cell activity and enhance the elimination of leukemic cells in AML, highlighting its therapeutic potential [141-143].

In multiple tumor models, tumor-induced MDSCs display markedly increased ROS production which has been linked to MDSC-induced dysfunction of adjacent lymphocytes [133, 134, 137, 144]. Consistently, administration of NOX2 inhibitors was reported to reverse MDSC-mediated suppression of T cell responses indicating the importance of ROS for MDSC-induced immunosuppression [145, 146]. Because many ROS are short-lived and highly reactive, their suppressive effects are primarily local and are most pronounced in microenvironments where MDSCs accumulate, such as primary tumors and metastatic niches [147, 148]. Mechanistically, MDSC-derived ROS and peroxynitrite (formed when superoxide reacts with nitric oxide) can modify the

TCR and CD8 molecules, thereby impairing TCR signaling with resulting reduced antigen-specific activation of $CD8^+$ T cells [149]. In addition, myeloid cell-derived ROS has been shown to suppress NK-cell effector functions [150].

7.1.1.2 Tumor-associated macrophages

TAMs represent one of the most abundant immune cell populations within TME. In tumors, TAMs primarily arise from bone marrow-derived monocytes. Recent studies have shown that circulating monocytes can be recruited to the tumor site by chemokines such as CCL2 and CCL5 and by the cytokine CSF-1, which promotes accumulation of inflammatory monocytes and can drive the differentiation of $CCR2^+$ monocytes into TAMs [151, 152]. TAMs are often described along a functional spectrum from M1-like to M2-like macrophages. M1-like macrophages are considered anti-tumorigenic and can be activated by bacterial products and/or IFN-γ. They can kill tumor cells through multiple mechanisms, including (i) direct cytotoxicity mediated by the release of ROS and NO [153] (ii) secretion of pro-inflammatory cytokines such as TNF and (iii) antibody-dependent effector functions via Fc receptor engagement, which can promote tumor cell phagocytosis and the release of cytotoxic mediators [154]. In contrast, M2-like macrophages are typically pro-tumorigenic and promote tumor cell proliferation and invasion by producing immunoregulatory and pro-tumor cytokines [155]. M2-like macrophages can also facilitate metastasis by degrading endothelial and extracellular matrix barriers through the secretion of proteases, including cathepsins and matrix metalloproteinases (MMPs), thereby promoting tumor cell migration [156, 157]. In addition, they can promote EMT [155] and angiogenesis by producing pro-angiogenic factors and enzymes, including MMP-2, MMP-7, MMP-9, MMP-12 and cyclooxygenase-2 [157, 158]. Clinically, high infiltration of TAMs has been

associated with resistance to chemotherapy and immunotherapy, as well as poor prognosis [159, 160].

7.1.1.3 Tumor-associated neutrophils

TANs are tumor-infiltrating neutrophils. Similar to macrophages, TANs are described along a functional spectrum ranging from N1-like (anti-tumor) to N2-like (pro-tumor) phenotypes. N1-like TANs can exert anti-tumor activity by releasing ROS, NO and TNF through neutrophil degranulation [161]. They can also release catalytically active neutrophil elastase (ELANE) which has been reported to contribute to tumor cell killing in several cancer models *in vitro* and *in vivo* [162]. Whereas N1-like TANs can have anti-tumor functions, N2-like TANs can promote tumor growth and progression by producing factors such as TGF-β, neutrophil elastase, IL-17a, CCL-2 and IL-8 [163], supporting angiogenesis and suppressing cytotoxic lymphocyte responses. Neutrophils phenotypes can vary depending on the tumor type and disease stage. During early tumor development, they are often inflammatory, whereas at advanced stage they may acquire a more immunosuppressive profile [164]. In many cancers, an increased infiltration of pro-tumor TANs is associated with poor prognosis and resistance to therapy [161, 165].

7.1.1.4 Dendritic cells

DCs are professional APCs that play a central role in cancer immunosurveillance and immunoediting by initiating anti-tumor immune responses. Tumor-infiltrating DCs can capture tumor-derived antigens, undergo activation and maturation and migrate to the draining lymph nodes, where they prime naïve T cells to mount anti-tumor immunity [166-168]. In addition, some tumors contain tertiary lymphoid structures (TLS), which can support local antigen presentation and T cell priming within the TME. The

presence of TLS is often associated with improved clinical outcomes [169, 170]. However, soluble factors (e.g TGF-β and IL-10) in the TME secreted by tumor cells may suppress the maturation and differentiation of DCs and may also inhibit recruitment of DCs to the tumor [171]. As a result, tumor-infiltrating DCs often display an immature phenotype characterized by reduced expression of co-stimulatory molecules and diminished production of type-1 T cell–activating cytokines, including IL-12 [168, 172]. Collectively, tumors may impair DC functions through multiple mechanism with resulting impaired DC functions and survival ultimately supporting tumor immune evasion [167].

7.1.2 T cells

Despite their anti-tumor potential, the function of T cells is frequently impaired within tumors. Chronic antigen stimulation together with inhibitory signals in the TME can drive T cell exhaustion. This dysfunctional state is characterized by reduced proliferation, diminished cytokine production and impaired cytotoxic activity. Exhausted T cells typically express high levels of inhibitory receptors on the surface, such as PD-1, cytotoxic T lymphocyte antigen-4 (CTLA-4), T cell immunoglobulin and mucin domain containing-3 (TIM-3), lymphocyte activation gene 3 (LAG-3), B and T lymphocyte attenuator (BTLA) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) [173, 174]. Additionally, immunosuppressive cells in the TME including Tregs, MDSCs, M2-like TAMs, N2-like TANs in the TME can further suppress T cell responses through inhibitory cytokines such as IL-10 and TGF-β as well as through metabolic restriction and redox stress [173]. Sears *et al.* defined exhausted T cells by three key criteria; (i) reduced effector function, (ii) sustained expression of inhibitory receptors and (iii) a transcriptional profile distinct from that of effector or memory T cells [175].

7.1.3 NK cells

The two major human NK cell subsets are commonly defined as $CD56^{hi}$ $CD16^{\pm}$ and $CD56^{lo}$ $CD16^{hi}$. $CD56^{hi}$ $CD16^{\pm}$ NK cells secrete inflammatory cytokines, while $CD56^{lo}$ $CD16^{hi}$ NK cells are highly cytotoxic and thereby contributing to limitation of metastatic dissemination [176]. NK cells kill target cells primarily though perforin and granzyme-mediated cytotoxicity and by inducing death receptor-mediated apoptosis [177].

Tumors can evade NK surveillance through several mechanisms. For example, remodeling of the ECM, including increased collagen deposition on cancer cells, can promote inhibitory signaling in NK cells and interactions with platelets can “cloak” circulating tumor cells and reduce NK cell recognition [178]. In TME, both NK subsets have shown reduced cytokine production and impaired cytotoxic activity. In addition, tumors and stromal cells secrete immunosuppressive mediators that inhibit NK cell function and may promote the development of regulatory-like NK subtypes (often referred to as NKregs), further dampening anti-tumor immunity in the TME [179].

In parallel, natural killer T (NKT) cells represent a distinct lymphocyte population exhibiting both NK and T cell features. NKT cells are often subdivided into type I (invariant) NKT cells, which are frequently associated with anti-tumor immunity and type II NKT cells, which can promote immunosuppression depending on context [179]. The balance between these NKT cell subsets can shift during tumor progression, and type I NKT cells have been shown to limit metastasis in a breast cancer metastasis model [180], whereas NKT type II cells have been shown to support MDSC-mediated immunosuppression in B-cell lymphoma models [181, 182].

7.2 Hot and cold tumor microenvironment

Tumor microenvironments are often described as ‘hot’ and ‘cold’ based on cytotoxic T cell infiltration within the tumor. Hot tumors are characterized by abundant T cell infiltration and proinflammatory cytokines, and patients with hot tumors are considered more likely to respond to ICB therapies such as anti-PD-1/PD-L1 and/or anti-CTL4 targeting antibodies. In contrast, cold tumors show limited anti-tumor immunity, often due to poor antigen presentation and barriers that restrict T cell entry to the tumor core (immune-excluded) or result in few tumor-infiltrating T cells (immune-desert) [183, 184]. Many immunotherapeutic strategies aim to transform cold tumors into hot tumors by improving antigen presentation, promoting T cell recruitment and infiltration and reducing local immunosuppression [185].

Melanoma and PDAC represent contrasting TME states; while pancreatic tumors often display a cold signature, melanomas are regarded as prototypic hot tumors (Figure 9).

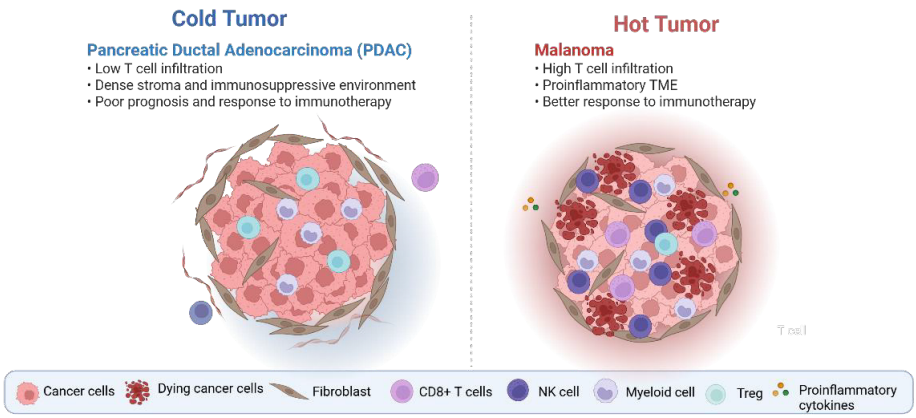

Figure 9: **Hot and cold tumor microenvironments**

7.2.1 Tumor microenvironment in melanoma

Melanoma is one of the most immunogenic cancers and has shown strong responsiveness to immunotherapy, partly due to its high mutational burden, which facilitates antigen presentation to T cells. Accordingly, melanoma is often considered an immunologically hot tumor because many lesions contain high levels of tumor-infiltrating lymphocytes, particularly cytotoxic $CD8^+$ T cells. Nevertheless, the melanoma TME can become strongly immunosuppressive, which contributes to therapeutic resistance and limits the efficacy of ICB in a subset of patients [186]. CAFs, including immunomodulatory CAFs (iCAFs), can secrete factors such as IL-1β, IL-6, IL-8, platelet-derived growth factors (PDGFs), TGF-β and VEGF, promoting tumor invasion and shaping immune cells toward suppressive phenotypes. MDSCs further support tumor progression through multiple mechanisms, including angiogenesis and establishment of premetastatic niches [187]. Clinically, increased MDSC levels in melanoma patients have been associated with poorer responses to ICB therapy [188], suggesting that targeting MDSCs may improve treatment outcomes. In addition, TAMs, often polarized towards M2-like phenotypes, are abundant in the melanoma microenvironment and can contribute to immune suppression and tumor progression [189, 190].

Melanoma most commonly spreads first to lymph nodes and nearby skin followed by spreads to distant organs, including lungs, liver, brain and bone [191]. In the lung, metastatic melanoma lesions develop within an organ-specific microenvironment shaped by resident immune and stromal cells. Lung metastases may remain immune-infiltrated, but suppressive myeloid populations and inhibitory cytokine signaling can limit effective T cell function and reduce responsiveness to immunotherapy [188, 192].

7.2.2 Tumor microenvironment in pancreatic ductal adenocarcinoma

PDAC is widely considered a classic cold tumor, marked by poor infiltration of cytotoxic T cell infiltration and a strongly immunosuppressive, immune-excluded microenvironment [80, 193]. A hallmark of PDAC is its pronounced desmoplastic stroma, in which abundant CAFs deposit excessive ECM components such as collagen and hyaluronan, driving fibrosis, elevated interstitial pressure and poor vascular perfusion [194]. This dense, fibrotic architecture may not only promote tumor growth and invasion but also creates a physical barrier that restricts drug penetration and immune-cell trafficking, thereby reducing the efficacy of immunotherapy and other systemic treatments [195, 196]. TGF-β contributes to desmoplasia [197] and is considered to play a central role in pancreatic carcinogenesis by promoting fibroblast activation and reinforcing stromal and ECM accumulation [198].

Consistent with this immune-refractory milieu, the PDAC TME is enriched in immunosuppressive cell populations, including MDSCs, N2-polarized neutrophils, regulatory T cells, M2-like macrophages and Th2 cells. In parallel, several effector immune compartments that could restrain tumor growth such as $CD8^+$ T cells, conventional dendritic cells, NK cells, M1-like macrophages, Th1 cells and N1-polarized neutrophils are often functionally impaired or excluded, contributing to ineffective anti-tumor immunity [80].

Importantly, the degree and nature of immunosuppression can differ across metastatic sites. The liver and lung are among the most common metastatic organs in PDAC, yet patients with liver metastases generally have poorer outcomes than those with lung metastases [199, 200], likely reflecting differences in local tissue architecture and immune composition. Consistent with this, metastatic PDAC mouse models suggest that the hepatic metastatic

niche is more immunosuppressive, whereas lung metastases tend to show greater immune infiltration and more active immune signaling [199].

In one such model, liver metastases contained fewer NK cells, dendritic cells and T cell subsets than lung metastases and also showed higher expression of inhibitory immune markers such as PD-L1 and LAG-3. Although MDSCs and Tregs were present at similarly high levels in both sites, the functional impact of immunosuppression appeared to differ. These findings suggest that in liver metastases, immunosuppressive myeloid cells may more effectively limit NK and T cell infiltration and function, whereas the lung microenvironment remains more permissive to immune cell recruitment and activation. This difference may be driven by the local chemokine release, as the hepatic TME was also enriched for pro-tumorigenic chemokines, including CCL5, CCL22 and CXCL12 [80, 199]. In addition, the liver is known to be rich in macrophages and other ROS-producing myeloid cells, which may further contribute to an immunosuppressive environment. In contrast, lung metastases displayed higher levels of immune-stimulatory chemokines such as CXCL9 and CXCL10, supporting recruitment of effector T cells and a more inflamed microenvironment [80, 199].

7.3 Angiogenesis and vascularization

Angiogenesis is considered a hallmark of cancer and plays a crucial role in tumor growth and metastasis. In the context of tumorigenesis, angiogenesis refers to the development of new blood vessels from pre-existing vasculature enabling tumors to obtain sufficient oxygen and nutrients for continued growth and to facilitate dissemination of distant organs. Hypoxia is a major driver of this process, as reduced oxygen availability stimulates the expression of several hypoxia-inducible factors (HIFs), which regulate oxygen homeostasis and promote neovascular growth. Angiogenesis is a complex and highly

coordinated process that depends on interactions among the ECM, cytokines, growth factors and multiple cell types. It involves vascular invasion into previously avascular regions of the tissue and requires tightly regulated communication between endothelial cells, which form the vessel lining, pericytes, which support the endothelium and stromal cells such as fibroblasts. In parallel, localized degradation of the ECM and vascular basement membrane is necessary to permit cell migration and vessel sprouting [201, 202]. Under hypoxic conditions, HIF-1α is upregulated and promotes the expression of major pro-angiogenic mediators, including VEGFA and VEGFR2 [203] released by tumor cells. This signaling axis stimulates endothelial-cell proliferation and promotes the formation of new vascular sprouts, which often remain persistent and poorly resolved due to continuous stimulation by tumor-derived factors and tumor hypoxia [204]. Tumor-associated blood vessels are typically disorganized and more permeable than normal vessels [205, 206]. These abnormalities contribute to elevated interstitial fluid pressure, which in turn may impair drug delivery to tumor cells [207]. This process is reinforced by the angiogenic switch, driven by an imbalance between increased levels of pro-angiogenic factors, such as VEGF and reduced levels of anti-angiogenic factor, such as thrombospondin-1 (TSP-1), thereby sustaining neovascularization and supporting tumor progression [208].

Anti-angiogenic therapy was among the earliest TME-targeted treatment strategies, initially developed to target VEGF and PDGF. Bevacizumab, a monoclonal antibody targeting VEGFA, was the first anti-angiogenic agent approved by FDA in 2004, initially in combination with chemotherapy for metastatic colorectal cancer. Subsequent approvals expanded its use to additional malignancies, including non-squamous NSCLC, glioblastoma and metastatic renal cell carcinoma [202]. Although anti-angiogenic approaches

showed substantial promise in preclinical studies, their clinical impact has been limited. This discrepancy may be explained by the fact that many murine tumor models fail to capture the vascular heterogeneity, vessel maturation and microenvironmental complexity characteristic of human cancers [202, 209].

7.4 Role of thrombospondin-1 in the tumor microenvironment

TSP-1 is a large matricellular glycoprotein secreted by multiple cell types, including platelets, endothelial cells, fibroblasts, myeloid cells and certain cancer cells. Within the TME, TSP-1 plays a complex and highly context-dependent role and is considered a pleiotropic regulator of tumorigenesis, with both tumor-suppressive and tumor-promoting functions. TSP-1 can suppress angiogenesis by inhibiting vascular formation, and its expression is reduced near sprouting neovasculature [210-212].

Structurally, TSP-1 contains several functional domains, including the N-terminal domain, a procollagen-like domain, type 1 repeats (TSRs), type 2 repeats, type 3 repeats and the C-terminal globular domain [213] (Figure 10). These domains interact with different receptors expressed on a variety of cell types, thereby explaining the diverse functions of TSP-1 in the TME.

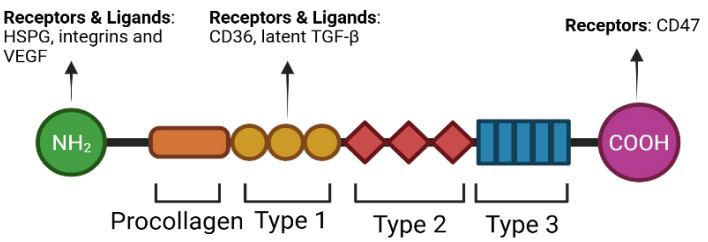

Figure 10: **Domain structure of TSP-1 and its main receptor interactions**

TSP-1 exerts inhibitory effects on tumor angiogenesis in both VEGF-dependent and VEGF-independent manners [212]. As an example, TSP-1 contains a heparan sulfate proteoglycan (HSPG)-binding region that can engage VEGF directly and promote its internalization and clearance through low-density lipoprotein receptor-related protein 1 (LRP-1) [214]. Besides, the TSR1 domain is important for the anti-angiogenic and anti-metastatic functions of TSP-1. Interactions between the TSR1 region and CD36 on endothelial cells can induce apoptosis through Fyn/Src-dependent signaling, resulting in reduced micro vessel density. In addition to inducing endothelial apoptosis, TSP-1 can inhibit endothelial-cell proliferation and migration, further contributing to its anti-angiogenic activity. In some contexts, TSP-1 may also promote apoptosis in tumor cells that express CD36, such as ovarian cancer cells [215]. The anti-angiogenic effect of TSP-1 may further contribute in suppressing tumor growth [216]. Furthermore, TSP-1 has been implicated in the regulation of myeloid cells. In a melanoma xenograft model, tumor-derived TSP-1 was shown to increase macrophage recruitment and promote M1-like macrophage polarization [217]. In addition, tumors with low metastatic potential were reported to promote TSP-1 expression in bone marrow-derived $Gr1^+$ myeloid cells, which in turn contributed to suppression of metastatic spread in preclinical models [218].

Expression of TSP-1 is frequently reduced during tumor progression, particularly in malignant cells, through multiple mechanisms. This induces epigenetic silencing, such as promoter methylation, as well as alterations in tumor suppressors and oncogenic pathways. Loss of p53 function has been associated with decreased *THBS1* transcription, whereas activation of oncogenic pathways, including KRAS signaling, can indirectly suppress TSP-1 expression through downstream effectors [210, 211, 219]. In addition, reduced serum TSP-1 in patients with advanced lung cancer has been

associated with poor prognosis [220]. Reduced TSP-1 expressions may contribute to tumor progression by removing an important endogenous inhibitor of angiogenesis. In addition, TSP-1 may limit metastatic dissemination by modulating stroma and myeloid cells in the premetastatic niche [212].

However, pro-tumor roles of TSP-1 in the TME have also been increasingly described, particularly through its ability to modulate cytokine production and immune signaling [211]. TSP-1 regulates TAM activity through interactions with CD36 and CD47 on macrophages. Binding to CD36 can increase macrophage IL-10 expression [221], whereas interaction with CD47 promotes IL-1β production [222] and suppresses IL-12 release, thereby reducing the activation of T cells, NK cells and APCs. These changes may favor a more M2-like TAM phenotype [223]. Nevertheless, the effects of TSP-1 on TAM polarization appear to be context-dependent, with the potential to influence both M2-like immunosuppressive functions and M1-like antitumor responses [212]. Beyond macrophage polarization, TSP-1/CD47 signaling also contributes to tumor immune evasion. As CD47 functions as a ‘do not eat me’ signal, this pathway can inhibit macrophage phagocytosis of tumor cells. TSP-1 binding to CD47 can additionally suppress the effector activity (e.g., granzyme production) of NK cells and cytotoxic T cells, while inhibitory CD47 signaling in macrophages and dendritic cells limits antigen presentation to T cells. In addition, TSP-1 is known to activate latent TGF-β, thereby generating its biologically active form [224]. In the TME, TGF-β has dual roles which suppresses tumor development in early carcinogenesis but promotes metastasis at later stages, in part through induction of EMT [225]. Furthermore, several integrins expressed on malignant and stromal cells can bind TSP-1 and these interactions have been implicated in the regulation of

cell adhesion, migration and vascular responses associated with tumor progression [211].

TSP-1 based therapeutics have been developed to exploit the antitumor activity of TSP-1 without requiring delivery of the full-length matricellular protein. Most of these agents are peptide mimetics designed to reproduce the anti-angiogenic properties of the type I repeat domain of TSP-1. Among the best-characterized examples are ABT-510 and CVX-045, both of which reached early clinical testing in melanoma, advanced solid tumors and other cancer types [226-230]. However, in melanoma, ABT-510 demonstrated only limited single-agent efficacy [228]. More recently, approaches aimed at blocking TSP-1/CD47 interaction, such as TAX2, have shown encouraging preclinical results in melanoma models by inhibiting metastatic dissemination, although these strategies have not yet translated into established clinical use [231].

8. HISTAMINE

Histamine is a biogenic amine with diverse functions in immunity, inflammation, neurotransmission and gastric acid secretion. It is synthesized from the amino acid histidine by histidine decarboxylase and is produced predominantly by mast cells, basophils and certain myeloid cells. Under inflammatory conditions, other cell types can also produce histamine. Histamine mediates its effects through four G protein-coupled receptors, H1R, H2R, H3R and H4R, which are differentially expressed across tissues and organs and account for its pleiotropic roles in health and disease [232].

8.1 Histidine decarboxylase

Histidine decarboxylase is the key enzyme for histamine synthesis and catalyzes the conversion of L-histidine to histamine. Since histamine

production depends on histidine decarboxylase activity, regulation of this enzyme is an important determinant of histamine availability across different tissues. Histidine decarboxylase-mediated histamine synthesis is therefore essential for both physiological histamine signaling and histamine-dependent responses during inflammation and immune reactions [232, 233].

8.2 Histamine physiology

Under physiological conditions, histamine plays an important role in immune regulation, as well as in nervous and gastrointestinal systems. Histamine is synthesized and stored in granules of mast cells and basophils, from which it can be rapidly released upon activation. In addition, other immune cells, including monocytes, macrophages and dendritic cells, can produce histamine, although they generally lack substantial storage capacity and instead release it following *de novo* synthesis. Its biological effects are mediated through four receptors with distinct tissue distribution and functions. H1R is widely expressed and is involved in allergic reactions, increased vascular permeability and smooth muscle contraction. It signals through $G\alpha_{q/11}$ proteins, activating the PLC/IP_3/DAG/PKC pathway and increase intracellular Ca^{2+} levels. H2R couples to $G\alpha_s$ proteins and stimulates cAMP production. It is expressed mainly in the stomach, smooth muscle and cardiac tissue, where it regulates gastric acid secretion, vasodilation and immune responses. In contrast, both H3R and H4R signal through $G\alpha_i$ proteins and suppress cAMP production, whereas H3R is primarily expressed in the nervous system, where it functions as a presynaptic receptor. H4R is mainly expressed in hematopoietic cells and bone marrow and is involved in immune cell migration and inflammatory responses [232-234].

8.3 Histamine in cancer

Histamine has been reported to exert both pro-tumor and anti-tumor effects in cancer, depending on the tumor type, disease stage and the pattern of histamine receptor expression on tumor cells and stromal or immune cells. Elevated histamine levels have been observed in several cancer types [235]. Consistent with this, increased histidine decarboxylase activity has been detected in certain malignancies [236, 237], and patients with newly diagnosed solid tumors exhibit higher blood histamine levels that decline after tumor removal [238]. Together, these findings suggest that histamine production is increased during tumor development [235].

In the TME, histamine can shape myeloid-cell function in a context-dependent manner [232, 234]. One study showed that tumor-derived or allergy-associated histamine can signal through H1R on TAMs, promoting an M2-like immunosuppressive phenotype characterized by increased VISTA expression, reduced $CD8^+$ T cell activity, enhanced tumor growth and weaker responses to ICB [239]. Other studies suggest that interference with H2R signaling can reduce tumor-promoting myeloid-cell activity. In mouse models, blockade of H2R signaling with cimetidine increased apoptosis of MDSCs and reduced their expression of arginase 1 and iNOS [240], whereas ranitidine altered myeloid-cell populations and was associated with reduced breast tumor growth and metastasis [241].

In contrast, histamine may also mediate anti-tumor effects by regulating the differentiation and suppressive activity of myeloid cells. Mice lacking histidine decarboxylase have been shown to exhibit increased susceptibility to colon and skin carcinogenesis, which is linked to impaired myeloid maturation and accumulation of $CD11b^+Ly6G^+$ immature myeloid cells, supporting a role for histamine in regulating myeloid differentiation during early stages of cancer

development [242]. Moreover, experimental studies using exogenously added histamine dihydrochloride (HDC) as a treatment, have shown that HDC inhibits NOX2-derived ROS in myeloid cells, reduces the suppressive activity of MDSCs, and promotes dendritic cell differentiation to strengthen anti-tumor immune responses [146, 243]. In several mouse tumor models, HDC also improved the efficacy of PD-1/PD-L1 checkpoint blockade and further reduced tumor growth [146].

9 REACTIVE OXYGEN SPECIES AND NOX ENZYMES

ROS can be produced by different cell types but are generated in particularly high amounts by innate immune cells such as monocytes, neutrophils and eosinophils and are now recognized as important regulators of adaptive immune cells [244]. In immune cells, ROS are produced both by NOX enzymes and via mitochondria. Mitochondrial ROS arises mainly from respiratory chain where electrons are transferred through the electron transport chain to oxygen as the final acceptor [245]. Incomplete reduction of oxygen or electron leakage during this process leads to superoxide formation [246-248]. In phagocytes, NOX2-derived ROS play a key role in host defense by killing microbes during phagocytosis [244]. However, excessive or dysregulated ROS production within cells can result in oxidative stress, damaging macromolecules, such as proteins, lipids and nucleic acids ultimately impairing tissue function, neighboring cells, and promote chromosome instability and mutations [245, 246]. Under physiological conditions, ROS levels are tightly controlled by intracellular antioxidant systems within the cell, including superoxide dismutase (SODs), catalase, thioredoxin and glutathione peroxidase-1, which limit oxidative damage and help maintain redox

homeostasis in cells and tissues. Autophagy can further mitigate ROS-induced DNA damage by removing damaged organelles and proteins, thereby reducing oxidative stress [245, 249-251].

9.1 NOX enzymes

NOX2 belongs to the NOX/DUOX family of enzymes (NOX1-5 and DUOX1-2), which differ in tissue distribution, subunit composition and subcellular localization. NOX1 is expressed mainly in the colon, NOX2 is highly expressed in myeloid cells, NOX3 is found predominantly in the inner ear and fetal tissues, NOX4 is abundant in the kidney and NOX5 is expressed in testis and lymphoid tissues [246]. Dual oxidases (DUOX1 and DUOX2) are expressed in the thyroid and mucosal surfaces. The primary function of NOX/DUOX enzymes is to generate ROS, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), thereby contributing to cellular redox signaling and host defense [252].

NOX2 was the first member of this enzyme family to be identified. Myeloid cells such as macrophages, neutrophils and monocytes express high levels of NOX2 [252]. The NOX2 complex consists of membranes bound (catalytic subunit) and cytosolic subunits. The membrane bound catalytic core is formed by gp91^{phox} (also known as CYBB or NOX2) together with p22^{phox} (CYBA). The cytosolic regulatory components include p47^{phox} (NCF1), p67^{phox} (NCF2) and p40^{phox} (NCF4) which translocate to the membrane upon activation to assemble the functional NOX2 complex and generate O_2^- [246, 253, 254] (Figure 11). In phagocytes, activation of NOX2 triggers a rapid “oxidative burst,” resulting in production of high levels of ROS within phagosomes. These reactive species contribute to killing of engulfed pathogens and represent a key component of the antimicrobial immune defense. In addition, NOX2 can generate extracellular ROS, which further contributes to killing

of pathogens but may also affect neighboring cells by causing damage or modulate cellular responses.

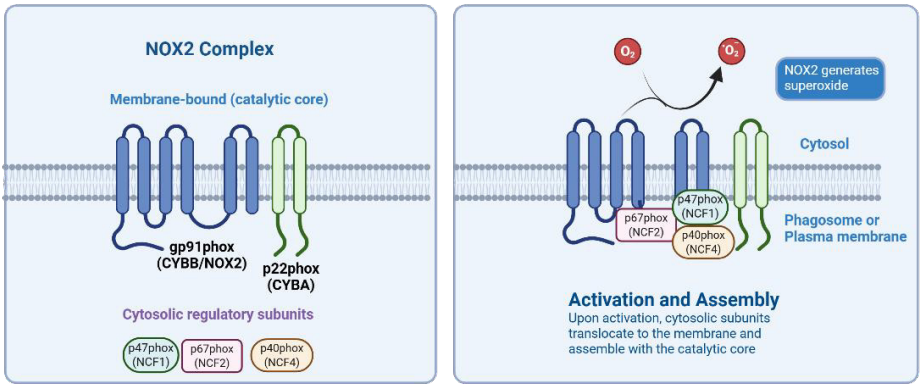

Figure 11: **Structure and activation of the NOX2 complex**

Defective NOX2 activity leads to chronic granulomatous disease (CGD), which is characterized by impaired respiratory burst, leading to reduced ROS production. While this compromises microbial killing, it paradoxically also promotes dysregulated inflammation due to impaired resolution of immune responses, altered autophagy and enhanced inflammasome activation [245, 255].

9.2 NOX and ROS in cancer

NOX enzymes have been implicated both in promoting tumorigenesis and, under certain conditions, in triggering apoptosis of malignant cells [246]. During tumor development, dysregulated NOX activity in malignant cells can lead to increased ROS production that acts as a signaling mediator to promote proliferation, survival and metabolic reprogramming in cancer cells. In addition, ROS produced by neighboring cells within the tumor microenvironment can influence tumor cell behavior. In particular,

NOX2/myeloid cell-derived ROS contribute to a dysregulated redox environment that can impair the function of cytotoxic lymphocytes, including T and NK cells [150, 246, 256-258].

In the TME, growth factor signaling through integrins, PDGF and EGF can further enhance ROS production by NOX enzymes, thereby activating PI3K-AKT and RAS-MEK-ERK pathways that drive cell proliferation and survival [259-261]. ROS can also modulate PKC-dependent signaling and has been shown to enhance cancer cell invasion by regulating the mitogen-activated protein kinase activity through oxidation of protein tyrosine phosphatases (PTPs) and PKC. Although excessive ROS can induce tumor apoptosis, many cancer cells adapt by upregulating antioxidant systems to counteract ROS toxicity. Mutations that enhance antioxidative responses can therefore confer resistance to oxidative stress and support tumor progression [246].

Nrf2 is a key transcription factor that regulates the cellular antioxidant response. Under basal conditions, Nrf2 is sequestered by Keap1 and targeted for proteasomal degradation [262]. Oxidative modification of key cysteine residues in Keap1 promotes the release and stabilization of Nrf2, allowing its translocation to the nucleus, where it binds antioxidant response elements to drive expression of detoxifying and antioxidant enzymes (Figure 12). Mutations in Keap1 or Nrf2, frequently observed in cancer, including lung cancer, result in constitutive Nrf2 activation and enhanced antioxidant capacity [246, 263, 264]. The action of Nrf2 is believed to protect from tumor initiation [265].

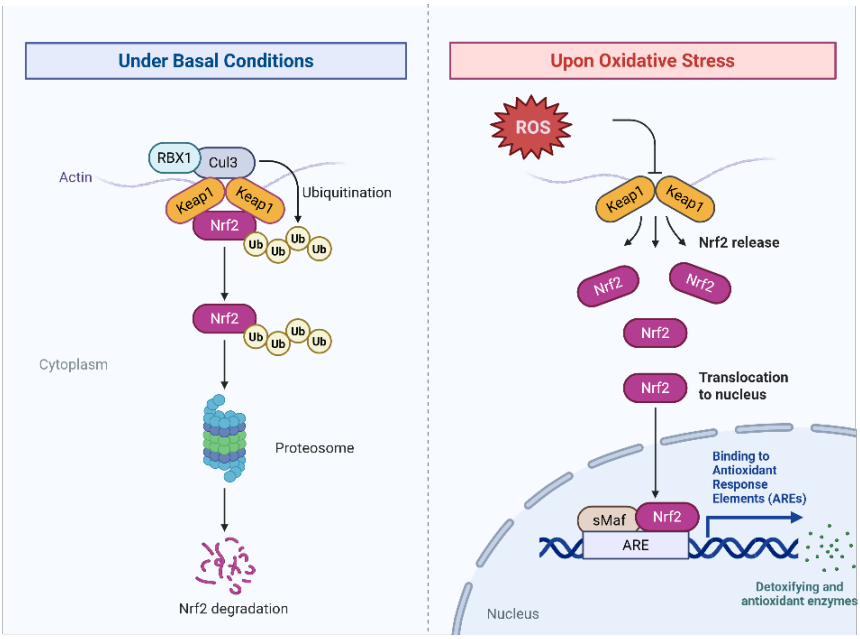

Figure 12: **Nrf2 regulation under basal and oxidative stress conditions**

BACH1 is another redox-sensitive transcription factor functionally interacting with Nrf2 signaling. Although often described as a transcriptional repressor of antioxidant genes, the relationship between Nrf2 and BACH1 is dynamic and context-dependent, rather than strictly antagonistic. Under acute oxidative or heme-rich conditions, BACH1 is exported from the nucleus and degraded, permitting transient activation of Nrf2-driven antioxidant programs. In contrast, during chronic oxidative stress, such as in tumors with sustained Nrf2 activation, increased expression of heme oxygenase-1 (HMOX1), which encodes the heme-degrading enzyme HO-1, reduces intracellular heme levels. This can stabilize BACH1 and permit its re-accumulation. In this setting, Nrf2 and BACH1 can be co-expressed and regulate distinct, but partly overlapping transcriptional programs. Functionally, Nrf2-driven gene expression promotes antioxidative defense, metabolic adaptation and cell survival, but its persistent

activation in tumor cells is associated with tumor progression, therapy resistance and, in some contexts, metastatic behavior [265-268]. In parallel, BACH1 has been linked to metastatic behavior through the induction of gene expression programs that promote migration, invasion and metabolic rewiring [246, 263, 269]. Thus, redox signaling in cancer reflects a dynamic balance between cytoprotective Nrf2-dependent programs and BACH1-associated pro-metastatic transcriptional states. High activity of antioxidant pathways may further protect cancer cells from apoptosis and contribute to resistance to chemotherapy and other therapies [268].

9.2.1 Roles of ROS in pancreatic cancer

Some *in vitro* studies have investigated ROS-inducing strategies as potential therapeutic approaches in pancreatic cancer, highlighting important roles of ROS in pancreatic biology. These strategies aim to elevate intracellular ROS levels beyond a tolerable threshold, thereby inducing oxidative stress-mediated apoptosis in cancer cells [267, 270, 271]. ROS can exert dual effects in pancreatic cancer: moderate, sustained ROS can support tumor cell survival and proliferation, and promote resistance to apoptosis by promoting adaptive antioxidant responses, whereas excessive ROS can overwhelm these defenses system, cause macromolecular damage and trigger cell death [272]. Consistent with this, pancreatic cancer cells with lower basal ROS levels have been reported to be more resistant to chemotherapy [273].

Elevated ROS levels in pancreatic cancer may arise through multiple mechanisms. Mutations in the tumor suppressor gene p53 can impair antioxidant defenses, allowing ROS to accumulate [274]. In addition, oncogenic KRAS mutations, present in over 90% in pancreatic cancer, drive metabolic reprogramming and increased ROS production, contributing to genomic instability and tumor progression [275]. Inflammatory responses

further amplify ROS levels in the TME. Pancreatic tumors are characterized by substantial infiltration of myeloid cells, which produce high levels of ROS via NOX2 [276, 277]. Moreover, inflammatory responses can be further enhanced by surgical interventions, which have been shown to promote the expansion of $NOX2^{+}$ MDSCs that produce high levels of ROS and may facilitate metastatic dissemination by limiting immunosurveillance [276, 278].

Hypoxia is another hallmark of pancreatic cancer that interacts closely with ROS signaling. Due to poor vascularization and dense stromal architecture, pancreatic tumors are highly hypoxic. Importantly, hypoxia does not imply low ROS levels; instead, ROS production can persist under low oxygen conditions through mitochondrial activity and NOX enzymes, as well as through infiltrating inflammatory cells. Under hypoxic conditions, ROS contribute to the stabilization of HIF-1α partly via PI3K/Akt/p70S6K signaling, thereby promoting pro-tumorigenic pathways [279]. In addition, ROS contributes to desmoplasia by activating PSCs [280, 281]. In this context, ROS-driven stabilization of HIF-1α, together with upregulation of Gli1, can stimulate PSCs to secrete factors such as IL-6, SDF-1 and VEGF-A, which support tumor invasion [282]. ROS has also been reported to suppress autophagic cell death through AKT/mTOR signaling in pancreatic cancer although increased intracellular ROS may enhance sensitivity to mTOR inhibitors currently being tested in clinical trials [280, 281] .

9.2.2 Roles of ROS in AML

In AML, elevated ROS levels contribute to leukemic cell signaling by promoting activation of kinase pathways and reversibly inactivating redox-sensitive phosphatases that normally restrain these cascades. This redox wiring is considered particularly relevant in genetically defined AML subsets such as FLT3-ITD mutated AML, where constitutive oncogenic signaling can be

further reinforced by ROS-mediated dampening of phosphatase activity, thereby supporting leukemic cell proliferation and therapeutic tolerance [283-285]. While multiple sources of ROS contribute to these effects, NOX enzymes, including NOX2, may participate in shaping this redox environment.

Beyond cell-intrinsic effects, NOX2-derived ROS also shape the bone marrow microenvironment where they can contribute to immune dysfunction and impaired cytotoxic lymphocyte activity, thereby promoting leukemic persistence and relapses. The clinical relevance of this axis is illustrated by HDC, which inhibits NOX2-dependent ROS production and, when combined with low-dose IL-2 to enhance cytotoxic lymphocyte activity, reduces relapse risk in AML. This position NOX2 as a therapeutically actionable link between redox signaling, immune regulation and AML recurrence [286-289].

In addition, ROS act as key second messengers in inflammasome activation, promoting the maturation of IL-1β and shaping downstream inflammatory signaling, thereby linking redox balance to inflammatory cytokine programs that may be relevant in AML (see previous section on IL-1β) [107, 108].

9.2.3 NOX2 mouse model in cancer studies

To investigate the role of NOX2-derived ROS in cancer, several NOX2 knockout (KO) mouse models have been utilized, including mice with defect catalytic subunit Cybb ($gp91^{phox}$/NOX2) or regulatory components such as Ncf1 ($p47^{phox}$). In these mouse models, disruption of Ncf1 has been reported to suppress the growth of Lewis lung carcinoma and melanoma, whereas no effect was observed in prostate carcinoma or methylcholanthrene-induced sarcoma [290, 291]. Similarly, mice deficient in Cybb have been reported to exhibit reduced lung metastasis [255, 256, 292]. Supporting the role for NOX2 in hematogenous spread, systemic treatment with the NOX2-targeting agent

HDC reduces lung melanoma metastases in wild-type (WT) mice but not in NOX2-deficient animals. The anti-metastatic effect of HDC was abolished upon NK-cell depletion or in IFN-γ–deficient mice, consistent with the idea that NOX2-derived ROS can suppress NK-cell–mediated tumor clearance [256]. In coherence, hematogenous metastasis was also markedly reduced in mice lacking major NOX2 subunits (Cyba, Cybb, Ncf1, Ncf2, or Ncf4) and tumors in NOX2-deficient mice showed increased infiltration of antitumor lymphocytes [246, 255]. Furthermore, in two murine cancer models, no differences in primary tumor growth were observed between WT and NOX2-deficient mice. However, reduced number of metastatic nodules were observed in NOX2-deficient mice. This reduction was linked to NOX2-derived ROS-dependent expression of thymosin β4, which can promote tumor cell invasion [292].

10 IMMUNOTHERAPIES

Immunotherapy has become a crucial strategy in the treatment of cancer by enhancing and/or restoring immunosurveillance. In addition to ICBs, this broad therapeutic category also includes cytokine-based therapies and agents designed to modulate the TME to overcome immune suppression and improve antitumor immunity. More recently, combination approaches have gained increasing attention, as the integration of immunotherapy with other treatment modalities can produce synergistic effects and has been shown to improve survival in patients with metastatic disease. For example, ICBs can be combined with other treatment regimens, such as chemotherapy, radiotherapy, cytokines, targeted agents or TME-modulating therapies, to enhance immune cell activation, promote tumor infiltration and restore immune-mediated tumor control [293-296].

10.1 Cytokines

Cytokines are polypeptides or glycoproteins (typically <30 kDa) that act on immune cells and enable communication between the innate and adaptive immunity through autocrine and paracrine signaling, but also via endocrine mechanisms under certain conditions. A single cytokine can exert pleiotropic effects depending on the target cell type and the receptor(s) it engages. Cytokine-based cancer therapies have been developed to enhance immune activation and killing of tumor cells. Cytokine therapy such as IFN-γ, GM-CSF, IL-2, IL-12, IL-15 and IL-21 have shown efficacy in preclinical studies. IFN-α was the first cytokine used clinically in human cancer therapy, for the treatment of hairy cell leukemia in 1986 [297-299].

10.1.1 Interleukin-2

IL-2 is a central immunoregulatory cytokine that signals through the IL-2 receptor (IL-2R) which consists of 3 subunits; IL-2Rα (CD25), IL-2Rβ (CD-122), which is shared with IL-15, and IL-2Rγ (CD132). IL-2 is primarily produced by activated $CD4^+$ T cells and is critically involved in the regulation of both innate and adaptive immune system. It promotes T cell proliferation, survival and differentiation, while also contributing to immune self-tolerance through the maintenance of regulatory T cells and the induction of activation-induced cell death [297-300]. Besides its effects on T cells, IL-2 also modulates the activity of NK cells and B cells [297-299].

Following IFN-α, IL-2 became the second cytokine approved for cancer immunotherapy. High-dose IL-2 received FDA approval for the treatment of metastatic renal cell carcinoma in 1992 and metastatic melanoma in 1998, producing complete response rates of approximately 7% and 12%, respectively [301, 302]. Despite these clinical benefits, the therapeutic application of IL-2

is constrained by its short serum half-life, which requires frequent administration, as well as by substantial toxicities such as vascular leak syndrome, which has been a concern in patients receiving high-dose IL-2 [303, 304]. Furthermore, IL-2 may preferentially stimulate Tregs rather than cytotoxic $CD8^+$ T cells [305], thereby potentially limiting effective antitumor immunity. Low-dose IL-2 was investigated as a better tolerated alternative to high-dose regimens, with the aim of achieving immune stimulation while reducing toxicity. These approaches were largely based on the ability of low-dose IL-2 to expand $CD56^{bright}$ NK cells, which express the high affinity IL-2 receptor. Although prolonged low-dose treatment increased NK cell numbers, it was generally insufficient to fully activate their antitumor function [306-308]. To enhance its efficacy, low-dose IL-2 has been evaluated and approved in combination with the NOX2 inhibitor HDC in AML, as discussed below. In addition, IL-2 is being explored in combination with immune checkpoint blockade, particularly PD-1 inhibitors, as well as in next-generation engineered forms designed to improve selectivity, pharmacokinetics, and tolerability [297]. Furthermore, IL-2 remains an important component of adoptive cell therapies, including the transfer of tumor-infiltrating lymphocytes [309] and engineered chimeric antigen receptor (CAR) T cell therapies [310].

10.1.2 Interleukin-15

IL-15 is a central immunoregulatory cytokine that signals through the IL-15 receptor (IL-15R), which consists of the IL-15Rα chain, IL-2Rβ (CD122), and common γ-chain (CD132) subunits shared with IL-2 [297, 298]. The IL-15/IL-15Rα complex is presented in trans to the IL-2Rβγ complex on NK cells and T cells, where it signals through JAK1 and JAK3, leading to activation of STAT3 and STAT5 [311]. IL-15 is mainly produced by monocytes, macrophages and DCs and plays a crucial role in the regulation of both innate

and adaptive immunity. Its main function is to stimulate NK cells, although it also promotes T cell proliferation and IFN-γ production [311]. In contrast to IL-2, IL-15 does not significantly stimulate Tregs, as it does not bind the IL-2Rα (CD25) chain, making it an attractive candidate for cancer immunotherapy (Figure 13) [312]. In addition to its effects on NK and $CD8^+$ T cells, IL-15 also contributes to the maintenance of long-lived immune memory and enhances cytotoxic lymphocyte function [299].

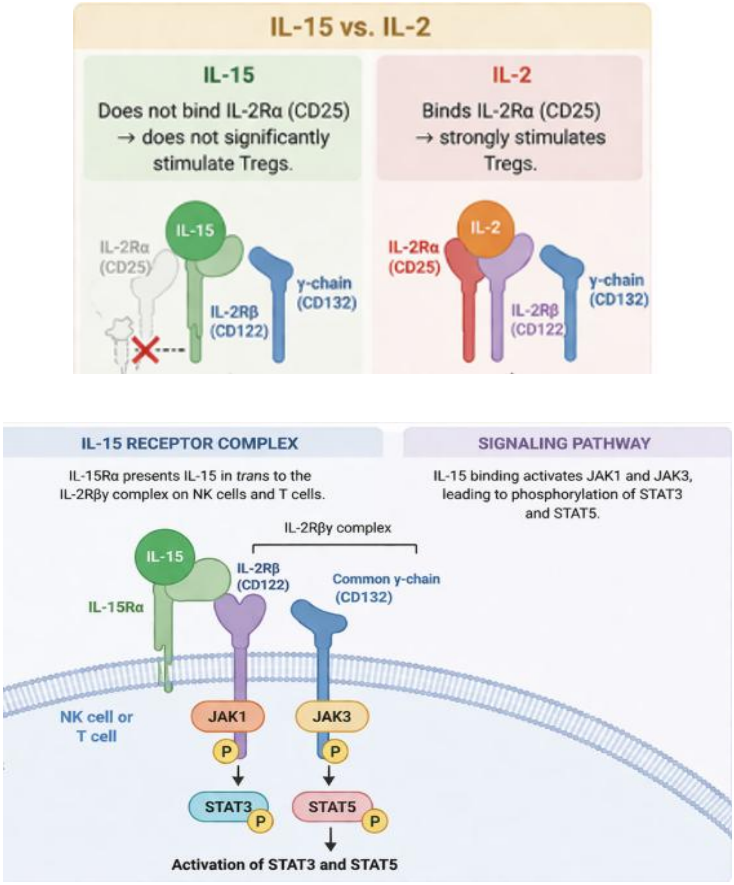

Figure 13: **IL-15 signaling pathway and structural comparison with IL-2**

Despite these promising immunostimulatory properties, the clinical use of IL-15 has been limited. In patients with melanoma and renal cell carcinoma, IL-15 therapy via intravenous bolus administration increased serum cytokine levels and promoted the proliferation of NK cells and $CD8^+$ T cells in the blood, but these effects were accompanied by notable toxicities, including thrombocytopenia, hypotension, high fever and resulted in only limited tumor regression [313, 314]. Although subcutaneous administration reduced toxicity and was better tolerated at higher doses than intravenous bolus injection, it did not lead to clear clinical responses and primarily resulted in disease stabilization [315]. In addition, recombinant IL-15 is rapidly cleared from the circulation because of its small molecular size. Therefore, current strategies focus on engineered IL-15 agonists, IL-15 superagonists and combination approaches designed to improve pharmacokinetics and bioactivity, thereby prolonging exposure and reducing peak systemic cytokine levels, which enables more controlled immune activation and improved antitumor efficacy. Many of these engineered IL-15 formats combine IL-15 with IL-15Rα, or its sushi domain, and fuse them to stabilizing elements such as Fc domains or other linker-based protein scaffolds. These constructs have been evaluated in mouse models and in phase I and II clinical trials, where they have shown promising results, particularly in hematological malignancies, bladder cancer and metastatic NSCLC. The treatment reportedly also induces expansion of circulating NK cells and $CD8^+$ T cells with manageable toxicity profiles [316-321].

10.2 NOX2 inhibitors and ROS-targeting therapy

HDC is currently the only clinically used NOX2-targeting inhibitor in cancer. HDC combined with low-dose IL-2 is approved as immunotherapy to prevent relapse in AML patients in Europe and Israel [286, 289]. The IL-2 component

of the therapy intends to enhance antitumor activity of cytotoxic T and NK cells. HDC is thought to counteract immunosuppressive NOX2-derived ROS released by myeloid cells, thereby protecting nearby NK and T cells from ROS-induced dysfunction [146, 246, 256, 289, 322]. In addition, HDC has been proposed to promote differentiation of leukemic blasts and potentially relieve the block in differentiation block characteristic of leukemic clones [323]. Other yet incompletely defined mechanisms may also contribute to its anti-leukemic effects. Clinically, a randomized phase III trial demonstrated that HDC/IL-2 significantly improves leukemia-free survival [324], with particularly pronounced benefit reported in chemo-responsive AML subgroups and in patients with normal karyotype AML in post hoc analyses [287, 288]. In a phase III trial of patients with metastatic melanoma, HDC/IL-2 treatment significantly improved overall survival compared with IL-2 alone [325].

In preclinical murine models, HDC suppresses lung metastasis of NK cell–sensitive tumor cells and mitigates surgery-induced myeloid inflammation in metastatic melanoma [256, 257, 276, 326]. In addition, in primary tumor models such as lymphoma, colorectal cancer and breast cancer, HDC delays tumor growth and, when combined with PD-1/PD-L1 ICB, further enhance antitumor immunity [146].

Additionally, NOX2 inhibitors and ROS-targeting agents have been investigated in preclinical models. DPI is one of the most widely used classic pan-NOX inhibitors. However, because it broadly inhibits flavoproteins, its effects cannot be attributed specifically to NOX2. In addition to NOXs, DPI can also inhibit other enzymes, including nitric oxide synthase (NOS), xanthine oxidase and components of the mitochondrial respiratory chain [327-329]. Similarly, apocynin, a natural compound, has been widely used as NOX inhibitor and a ROS scavenger, but its limited specificity, off-target effects and

intrinsic antioxidant activity complicate mechanistic interpretation [330, 331]. Naloxone, an FDA-approved drug used to treat opioid overdose, has also been proposed as a selective NOX2 inhibitor, although its activity against other NOX isoforms has not been thoroughly evaluated. Preclinically, naloxone has been investigated in Parkinson's disease models, where it appears to reduce inflammation and inhibit microglial NOX2 activation [332].

A commonly used strategy for developing putative NOX2-selective inhibitors is to disrupt the interaction between $p47^{phox}$ and $p22^{phox}$, thereby blocking assembly of the active NOX2 complex. 7-azaindole compound (GSK2795039) represents a more NOX2-directed approach, as it binds to the catalytic site rather than acting through nonspecific thiol reactivity [333]. This inhibitor has shown efficacy in a mouse model of traumatic brain injury, where it reduced NOX2 expression and improved neurological outcomes [334]. Nevertheless, its poor bioavailability and relatively rapid clearance may limit its therapeutic potential [330, 335]. To overcome these limitations, newer analogues have been developed, including NCATS-SM7270, which showed approximately twofold greater potency against NOX2 than GSK2795039. Nevertheless, its lower activity in primary mouse neutrophils suggests that its efficacy may be species-dependent and potentially less pronounced in mice [330, 336].

Beyond selective NOX2 inhibitors, several broader ROS-targeting approaches have been investigated to modulate oxidative stress more direct. Commonly used approaches include ROS scavengers such as N-acetylcysteine (NAC), which supports glutathione-dependent antioxidant defenses and catalase-based therapies, which decompose hydrogen peroxide into water and oxygen, and pharmacological interventions such as high-dose vitamin C or E. In contrast to NOX2 inhibitors, which primarily target ROS production by myeloid cells in

the TME, these interventions affect redox balance in malignant as well as non-malignant cells.

The consequences of such interventions are complex and context-dependent. In some experimental settings, antioxidant-based approaches have demonstrated promising antitumor effects, for example by limiting ROS-induced proliferation of cancer cells or by protecting immune cells [337, 338]. However, direct antioxidants may also protect tumor cells from oxidative injury and can potentially reduce the efficacy of chemotherapy or radiotherapy when these treatments rely, at least in part, on ROS-mediated cytotoxicity [339, 340].

Clinically, progress in ROS-scavenging therapy has been limited. Among the various redox-modulating approaches, high-dose intravenous vitamin C is one of the most extensively studied, with some encouraging results in cancer treatment, particularly when used in combination with standard therapies such as chemotherapy [341-344]. Nevertheless, the current evidence is considered insufficient to support its routine clinical use [345].

Importantly, the therapeutic value of antioxidant-based strategies in cancer remains controversial. Several studies have reported paradoxical findings, showing that antioxidant supplementation may, under certain conditions, promote rather than suppress tumor progression [345]. For example, long-term administration of NAC and vitamin E has been shown to enhance metastasis in KRAS-driven lung cancer models [269], and NAC has been reported to increase the progression of lung cancer and melanoma metastasis in preclinical studies [346]. These findings suggest that broadly reducing ROS may attenuate stress signaling pathways that restrain tumor progression or selectively favor survival of more aggressive cancer cell populations.

Taken together, ROS-scavenging therapies cannot be viewed simply as strategies to either decrease or increase ROS. Their effects depend on the source, magnitude, duration and cellular compartment of ROS, as well as on whether the intervention targets tumor cell-intrinsic redox signaling or microenvironmental ROS that shape immune function. While global ROS scavenging may influence tumor cell-intrinsic redox balance, more selective approaches such as NOX2 inhibition target microenvironmental sources of ROS that regulate immune function [345, 347].

10.3 Bruton's tyrosine kinase inhibitors: B cell targeting and myeloid modulation

BTK is best known for its role in B-cell receptor signaling, but it also serves as an important mediator of myeloid cell activation in monocytes, macrophages, neutrophils, dendritic cells and MDSCs. BTK integrates signals from innate immune receptors, and its inhibition can exert anti-inflammatory effects by dampening pathways such as NF-κB, the NLRP3 inflammasome and Akt signaling [348, 349]. In cancer, suppressive myeloid subsets, including MDSCs and NOX2-expressing TAMs, accumulate within the TME and, in several hematologic malignancies, are also expanded systemically. A key mechanism by which these myeloid cells dampen anti-tumor immunity is the release of NOX2-derived ROS, which can impair ROS-sensitive NK- and T cell effector functions [150, 256, 257, 350].

These observations have increased interest in BTK inhibitors, such as ibrutinib, not only as B cell-targeting therapies but also as immunomodulatory agents that may counteract myeloid-driven immunosuppression. Consistent with this, ibrutinib treatment has been associated with reduced MDSC levels in CLL patients [351] and preclinical/clinical studies in solid tumors and metastatic

models suggest enhanced anti-tumor immunity, particularly in combination with ICB therapies [352, 353]. Mechanistically, BTK may regulate NOX2 activity upstream by engaging PI3K/Akt-dependent pathways that promote phosphorylation of $p47^{phox}$, facilitating NOX2 complex assembly and ROS generation in activated myeloid cells [260, 354]. In line with this, ibrutinib treatment has been associated with reduced stimulus-induced ROS production in granulocytes, supporting the idea that the BTK-NOX2 axis contributes to myeloid-driven immune suppression [351, 355].

AIM

The overall aim of this thesis is the targeting of myeloid cells within the tumor microenvironment and to investigate the therapeutic potential of reprogramming these cells from a tumor-promoting to an anti-tumor phenotype, thereby enhancing anti-tumor immunity. This is investigated using histamine, NOX2 inhibitors and BTK inhibitors. The specific aims of each paper are outlined below:

Paper I: To investigate whether HDC regulates TSP-1 expression in myeloid cells, to elucidate the underlying mechanism and to assess whether this contributes to its anti-metastatic effects.

Paper II: To define how NOX2 regulates NK cell function and its impact on metastasis in murine models of PDAC.

Paper III: To assess the roles of IL-1β and IL-1RA in disease progression in AML patients receiving relapse-preventive combination immunotherapy comprising the NOX2 inhibitor HDC and low-dose IL-2.

Paper IV: To investigate how Bruton's tyrosine kinase inhibition affects NOX2 activation and to evaluate its potential as a NOX2-targeted therapeutic strategy.

MATERIALS AND METHODS

CELLULAR ASSAYS

In **Paper I**, we utilized the human myelomonocytic cell line PLB-985, derived from a patient with AML, together with a NOX2-knockout (NOX2-KO) variant of the cells, to explore effects of HDC on TSP-1 induction. Transcriptomic data from our previous study showed that *THBS1*, the gene encoding TSP-1, was the most strongly upregulated transcript in HDC-treated PLB-985 cells [323]. We therefore aimed to examine the underlying mechanism for HDC-induced TSP-1 formation by assessing kinetics, dose-response and receptor involvement using agonist and antagonist conditions, as well as stimuli linked to the PKA/cAMP signaling pathway. Supernatants from PLB-985 cultures (WT and NOX2-KO) were collected, and secreted TSP-1 protein was quantified by ELISA. In parallel, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and patients with pancreatic cancer, as well as primary monocyte and non-monocyte fractions from healthy donors, were stimulated with HDC *ex vivo* and TSP-1 was quantified by ELISA in cell culture supernatants. In addition, $Gr1^+$ cells isolated from naïve mouse bone marrow were stimulated with HDC *ex vivo*, and TSP-1 expression assessed by western blot.

In **Paper II**, NK cell cytotoxicity toward the PDAC cell line PDA30364 was assessed. Murine NK cells were isolated from naive WT mice and co-cultured with PDA30364 cells. NK cell degranulation was subsequently analyzed by flow cytometry.

In **Paper III**, PBMCs isolated from healthy donors were used to investigate the *in vitro* effects of HDC and IL-2 on IL-1β and IL-1Ra production. Cells

were cultured in the presence or absence of LPS, HDC, DPI, or IL-2 and cytokine levels in cell culture supernatants were measured by ELISA.

MOUSE MODELS

The murine B16F10 melanoma cell line was used in **Paper I** and **IV** as a model of experimental lung metastasis (Figure 14). In **Paper I**, the model was used to investigate whether HDC-induced TSP-1 contributes to suppression of metastatic outgrowth in lungs. WT and TSP-1-KO mice were intravenously injected with B16F10 cells and were treated with HDC or NaCl every other day for three doses, starting one day before tumor cell inoculation. TSP-1 protein levels in lung lysates were analyzed by western blot. Metastatic lesions in lungs were enumerated for three weeks post tumor cell inoculation. In **Paper IV**, the same model was used to assess the anti-metastatic effects of ibrutinib. WT and NOX2-KO mice intravenously infused with B16F10 cells were treated with ibrutinib or vehicle control for five consecutive days beginning one day before tumor cell inoculation. Metastatic burden was assessed after three weeks by quantifying metastatic foci in lungs. To determine whether the anti-metastatic effects of ibrutinib were NK cell dependent, NK cells were depleted in WT mice using anti-NK 1.1 targeting antibodies.

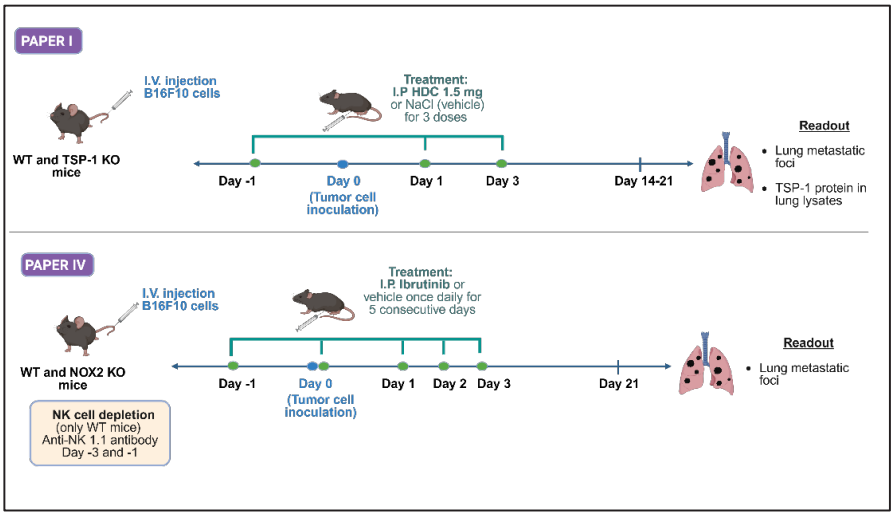

Figure 14: **B16F10 metastatic models**

In **Paper II**, a murine PDAC metastasis models were developed to explore the role of NOX2-derived ROS for metastatic tumor spread and the importance of NK cell–mediated protection against PDAC-metastasis (Figure 15). The luciferase-tagged murine PDAC cell line, PDA30364, originally established from a tumor arising in a KPC mouse model carrying $KrasG^{12D}$ $p53^{R172H}$ mutations, was used to induce experimental lung and liver metastases in WT and NOX2-KO mice. In the lung metastasis model, PDA30364-luc cells were injected intravenously. After two weeks, metastatic burden was defined by bioluminescence imaging, while the immunophenotype of spleen- and lung-infiltrating cells was determined by flow cytometry. In some experiments, the therapeutic effect of IL-15 was explored. Tumor-bearing mice received treatment with low- or high-dose IL-15 for 5 consecutive days (5 doses), starting one day before tumor injection. NK cell depletion, using anti-NK1.1 antibodies, were conducted to explore if the model was sensitive to NK cell–mediated killing.

In the liver metastasis model, PDA30364-luc cells were injected intrasplenically followed by splenectomy. After two weeks, liver metastatic burden was assessed by bioluminescence imaging and immune cell populations in liver were analyzed by flow cytometry.

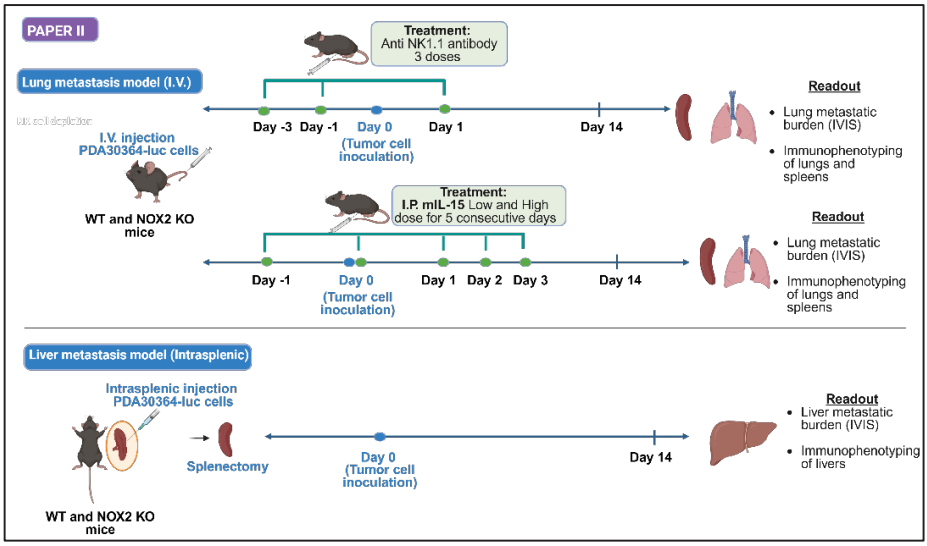

Figure 15: **PDA-30364 luciferase metastatic models**

BIOINFORMATICS

In **Paper I**, the correlation between *THBS1* and genes associated with the cAMP/PKA signaling pathway was analyzed using mRNA expression data from skin cutaneous melanoma (SKCM) in The Cancer Genome Atlas (TCGA) database, accessed through cBioPortal (cbioportal.org). The same dataset was also used for overall survival analysis by Kaplan-Meier curve generation. In addition, *THBS1* expressions in normal and tumor tissues were evaluated using the TNMplot database (tnmplot.com). To further define *THBS1* expression across immune cell populations, single-cell RNA-seq data

from melanoma patients were analyzed using the Broad Institute Single Cell Portal (singlecell.broadinstitute.org).

In **Paper II**, overall survival in pancreatic cancer was analyzed in relation to the expression of NOX2 subunits and NK cell receptors using data available in the KMplot database (kmplot.com). In addition, single-cell RNA-seq data from lung tissue of WT and *Ncf2*-deficient mice were analyzed to characterize NK cell populations and expression of NK cell–related markers. A published single-cell RNA-seq dataset from patients with PDAC was also analyzed to assess cell type-specific expression patterns, with particular focus on *CYBB* expression in myeloid cells.

SAMPLES FROM CLINICAL STUDIES

In **Paper III**, IL-1β and IL-1Ra levels were measured in serum from patients with AML in remission, enrolled in the Re:Mission trial (NCT01347996). In brief, the trial included 84 adult AML patients in first CR. Patients received a maximum of ten treatment cycles of low-dose IL-2 and HDC, each cycle consisting of a three-week treatment period with twice daily injections followed by a three- or six-week rest period. Serum samples were collected from 78 patients before initiation of immunotherapy, from 73 patients after the first treatment cycle, from 55 patients before the third cycle, and from 53 patients after the third cycle (Figure 16). IL-1β and IL-1Ra levels were analyzed in serum samples obtained before and after treatment during cycles 1 and 3 using a 27-plex cytokine panel based on Luminex technology. The study was conducted in accordance with the Declaration of Helsinki, and all patients provided written informed consent prior to enrollment and had the right to withdraw consent at any point.

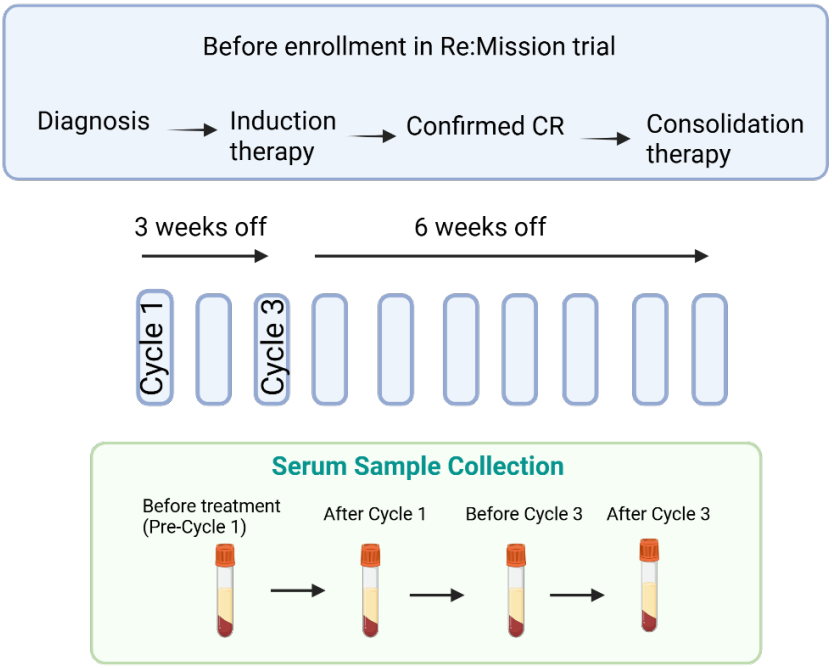

Figure 16: **Re:Mission trial design**

In **Paper IV**, peripheral blood samples were collected from two patients with X-linked agammaglobulinemia (XLA) and one patient with CLL who was asymptomatic, classified as Binet stage A, and had not received treatment. At the time of sampling, both patients with XLA were receiving IgG replacement therapy. All participants provided written informed consent. PBMCs and monocytes were isolated from XLA patients, and ROS formation was measured in response to different stimuli in the presence or absence of ibrutinib. Additionally, primary CLL samples were used as target cells for NK cell-mediated cytotoxicity assays in the presence or absence of ibrutinib, a NOX2 inhibitor and a ROS scavenger.

STATISTICS

For *in vitro* experiments, statistical comparisons between two groups were performed using paired or Welch's unpaired t-tests, as appropriate, whereas comparisons among more than two groups were analyzed by one-way ANOVA or mixed-effects model, followed by Holm–Šidák's multiple-comparisons test. *Ex vivo* data were analyzed using the Wilcoxon signed-rank test.

For *in vivo* experiments, normality was assessed using the Shapiro–Wilk test. In the murine metastasis models, two-group comparisons were analyzed using the Mann–Whitney U test or Welch's t-test, whereas comparisons involving more than two groups were analyzed by one-way ANOVA followed by Dunn's or Holm-Šidák's multiple-comparisons tests. Flow cytometry data were analyzed by one-way ANOVA with Holm–Sidak's multiple-comparisons test in the lung metastasis model and by Welch's t-test in the liver metastasis model.

For clinical data, overall survival and leukemic free survival were analyzed using the log-rank test. Associations between serum cytokine levels and overall and leukemic free survival were evaluated using Cox univariable and multivariable regression models.

ADDITIONAL TECHNIQUES

For additional methodological details, including cell culture, flow cytometry, ELISA, western blotting, qPCR, ROS measurement and cytotoxicity assays, please refer to the Materials and Methods sections of the individual papers.

RESULTS AND DISCUSSION

PAPER I

Previous microarray analysis of HDC-treated human myeloid leukemia PLB-985 cells identified *THBS1* as one of the top HDC-induced genes [323]. We aimed to assess the mechanism, receptor involvement and downstream signaling cascade behind HDC-induced TSP-1 formation. Given that HDC has previously been shown to inhibit lung metastasis in the B16F10 model [256, 326], and that TSP-1 production by $GR1^+$ myeloid cells in premetastatic niches has been linked to suppression of metastasis in preclinical models [215, 218], we also sought to determine whether the anti-metastatic effect of HDC might be mediated by induced formation of TSP-1.

We first showed that HDC robustly induced TSP-1 protein expression in the myeloid PLB-985 cell line as well as in primary human monocyte cultures (Figure 17A–B). We next examined the receptor involved and found that the effect was mediated through H2R, as TSP-1 induction was abolished by the H2R antagonist ranitidine but not by the H1R antagonist fexofenadine (Figure 17C), indicating receptor specificity. Further mechanistic studies demonstrated that HDC-induced TSP-1 production was transduced through the cAMP/PKA signaling pathway. Treatment with different cAMP inducers increased TSP-1 production, whereas pharmacological inhibition of PKA reversed this effect (Figure 17D), supporting the involvement of cAMP-dependent signaling. The proposed signaling mechanism is summarized in (Figure 17E).

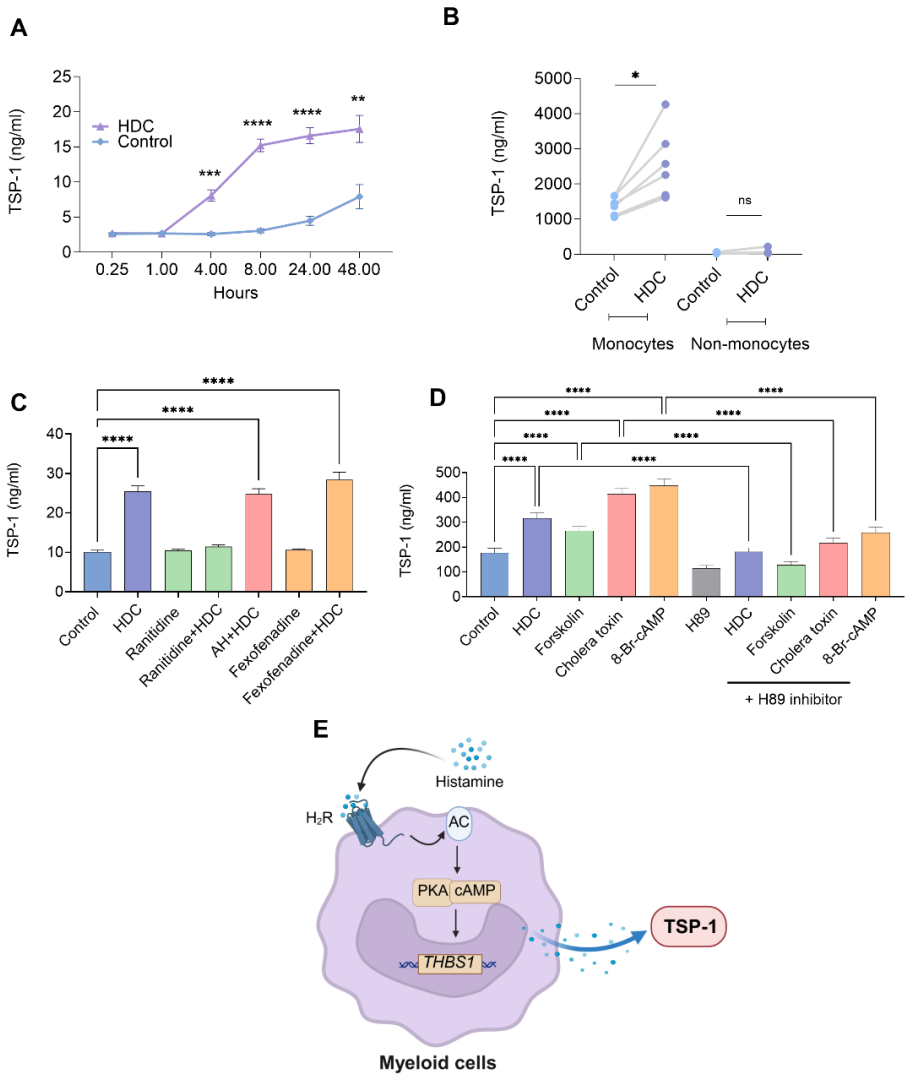

Figure 17: **HDC induces TSP-1 production in myeloid cells through histamine type 2 receptor mediated cAMP-PKA signaling. (A)** PLB-985 cells were stimulated with 100 µM HDC and TSP-1 production was determined at different time points ranging from 15 minutes to 48 hours. Levels of TSP-1 in the cell supernatants were measured by ELISA (n=6); Welch's unpaired t-test. **(B)** Freshly isolated monocytes from PBMCs (n=6) and non-monocyte fractions (n=4) were stimulated with 100 µM HDC and TSP-1 levels in supernatant were measured after 48 hours by ELISA; Wilcoxon signed rank test. **(C)** PLB-985 cells were stimulated for 48 hours with 100

μM HDC, 50 μM ranitidine, 50 μM AH (a chemically inert control for ranitidine) and 10 μM fexofenadine or a combination thereof (n=6-12). TSP-1 levels were measured in the cell supernatant by ELISA; one-way ANOVA followed by Holm-Šidák's test. **(D)** Human monocytes were treated with 100 μM HDC and the known cAMP inducers as follows: 20 μM forskolin, 10 nM cholera toxin and 500 μM 8-Bromo-cAMP, either alone or in combination with the 10 μM PKA inhibitor H89. TSP-1 levels were measured in the supernatant after 24 hours of incubation by ELISA (n=7-11). One-way ANOVA followed by Holm-Šídák's test. **(E)** Proposed mechanism of HDC-induced TSP-1 in myeloid cells.

We next aimed to explore the contribution of HDC-induced TSP-1 to the anti-metastatic action of HDC in the B16F10 metastatic lung model as outlined in Figure 18A. Consistent with our previous findings, HDC treatment resulted in reduced lung metastatic burden in WT mice [256, 326]. However, the anti-metastatic effect was reduced or absent in TSP-1-KO mice, suggesting that induction of TSP-1 might contribute to HDC-mediated suppression of lung metastasis (Figure 18B). Importantly, HDC treatment increased levels of TSP-1 in lungs of WT mice (Figure 18C). Finally, analysis of a clinical mRNA dataset from melanoma patients showed that high *THBS1* expression was associated with improved overall survival (Figure 18D), supporting a beneficial role for TSP-1 in melanoma.

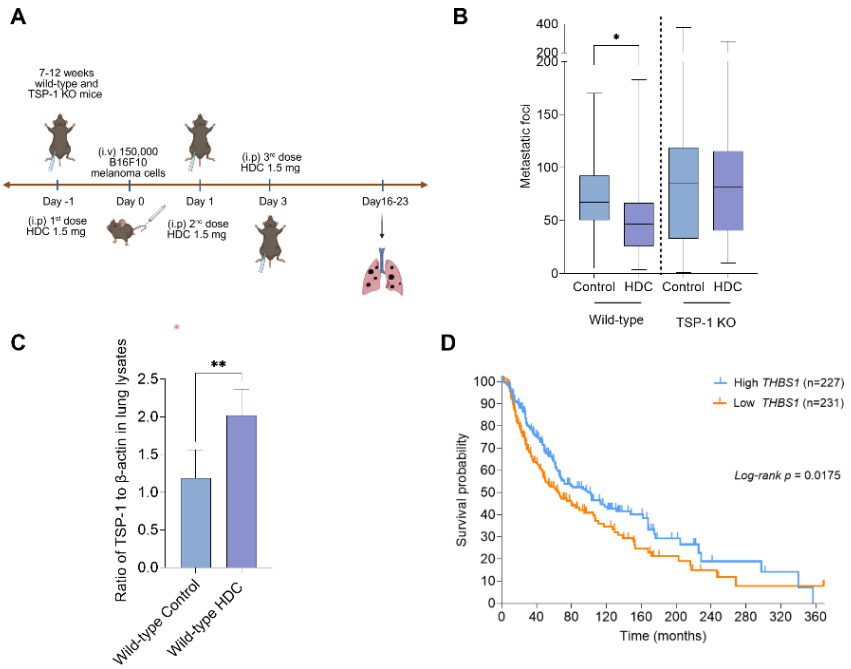

Figure 18: **HDC reduces melanoma metastasis in a TSP-1-dependent manner and high *THBS1* positively correlates with better overall survival in human melanoma.** WT and TSP-1 KO C57BL/6 mice were treated with HDC or NaCl (control) one day before and one and three days after i.v. inoculation of 1.0–1.5 × 10^5 B16F10 cells **(A)** (created with BioRender). **(B)** Number of metastatic foci in the lungs three weeks after tumor inoculation. Results are presented as box-and-whisker plots with boxes representing the interquartile range, and whiskers indicate the minimum and maximum values. **(C)** Quantification of TSP-1 protein levels normalized to β-actin in lung lysates of B16F10 tumor-bearing mice: WT control (n = 24), WT HDC (n = 25), TSP-1-KO control (n = 23) and TSP-1-KO HDC (n = 24). Statistical significance was determined using the Mann–Whitney U test. Data are presented as mean ± SEM. **(D)** Kaplan–Meier analysis of overall survival in the TCGA SKCM cohort stratified by *THBS1* mRNA expression. Expression z-scores were obtained from cBioPortal, where a z-score of 0 approximates the cohort median. Patients were divided into high (EXP > 0; n = 227) and low (EXP < 0; n = 231) *THBS1* expression groups. Survival

differences were assessed using the log-rank (Mantel–Cox) test. The number of patients at risk is shown below the curves.

The function of TSP-1 in the TME appears to be highly context-dependent and can vary with cancer type and disease stage. This likely reflects its multidomain structure and ability to interact with several receptors and extracellular binding partners, thereby influencing angiogenesis, stromal remodeling and immune responses [210, 212]. In early tumor progression, TSP-1 is generally considered protective, as it suppresses angiogenesis, supports tumor dormancy and may help restrain the outgrowth of newly seeded micrometastases within the premetastatic niche [211, 212]. Consistent with this, TSP-1 produced by myeloid cells has been shown to create a metastasis-refractory microenvironment and limit metastatic progression [215, 218].

In contrast, in advanced cancer, the TME is often dominated by strong immunosuppressive and pro-tumorigenic signals that may blunt the protective effects of TSP-1. In addition, cancer cells may exploit TSP-1 as a matricellular communication molecule to reprogram the TME through interactions with receptors such as CD47, integrins as well as via activation of latent TGF-β. However, these effects are highly context dependent and may vary across tumor types and stages as previously mentioned [210-212].

Our findings support previous studies showing that induction of TSP-1 by myeloid cells contributes to suppression of lung metastasis in melanoma. Induced formation of TSP-1 may represent an additional, non-NOX2-related, anti-neoplastic mechanism of HDC that is mediated via engagement of H2R and activation of the cAMP/PKA pathway.

PAPER II

In **Paper II**, we used murine PDAC models of lung and liver metastasis to examine NK cell function in the metastatic niche in the presence or absence of functional NOX2. Building on our previous studies [326, 356], we sought to determine how NOX2-derived ROS regulate NK cell-mediated immune surveillance and metastatic progression in PDAC.

PDAC is characterized by early dissemination of tumor cells, poor prognosis for long-term survival and a highly immunosuppressive TME that limits effective immunosurveillance and therapeutic response. NK cells are key mediators in anti-tumor immunity and may be particularly relevant in controlling growth and spread of PDAC cells as these cells commonly show reduced expression of MHC class I, which facilitates NK cell function [357]. The PDAC TME is, however, characterized by the presence of suppressive myeloid cells and NK cells are reportedly highly sensitive to inactivation by NOX2-derived ROS. Hence, myeloid cell-derived ROS may contribute to immune evasion and metastatic progression [146, 326, 358]. These observations propose that targeting myeloid NOX2-derived ROS formation may represent a potential therapeutic strategy.

We first assessed whether the murine PDAC cell line PDA30364 is susceptible to NK cell-mediated killing and observed increased NK cell degranulation ($CD107a^+$) upon co-culture, indicating sensitivity to NK cell cytotoxicity (Figure 19A). Using intravenous and intrasplenic metastasis models, we next evaluated NK cell-mediated control of metastasis under NOX2-deficient conditions (Figure 19B). These models enabled assessment of early immune surveillance on metastatic colonization in lungs and liver. Using WT and NOX2-KO mice, we found that NOX2 deficiency significantly reduced metastatic burden at both sites (Figure 19C & Figure 20A). Phenotypic

analysis further revealed enhanced NK cell activation in NOX2-KO mice, as indicated by increased expression of NK cell activation markers at both lung and liver sites (Figure 19D & Figure 20B), supporting a role for NOX2 in regulating NK cell function.

We next tested whether pharmacological activation of NK cells could further modulate lung metastasis. Treatment with IL-15 reduced lung metastasis. While high-dose IL-15 effectively controlled metastatic burden in both WT and NOX2-KO mice (data not shown), low-dose IL-15 showed efficacy only in NOX2-KO animals (Figure 19E), suggesting increased NK cell activation upon stimulation under NOX2-deficient conditions.

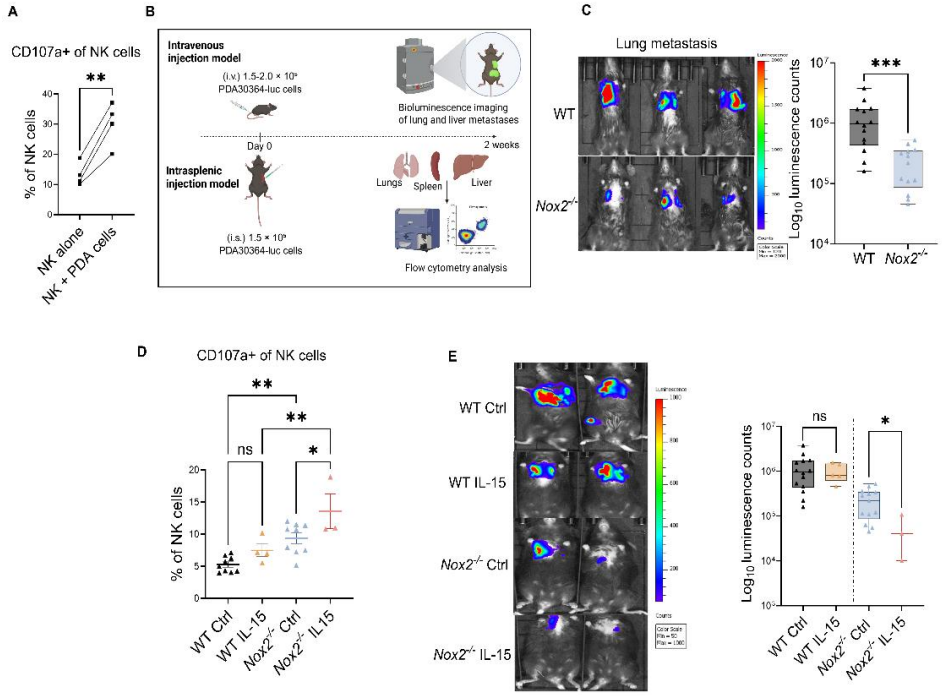

Figure 19: **NOX2 inhibition synergizes low-dose IL-15 to support NK cell-mediated control of lung PDAC metastasis. (A)** Frequency of CD107a⁺ murine NK cells after four hours of co-culture with or without PDA30364 PDAC cells (n = 4);

paired t-test. **(B)** Schematic of experimental lung and liver metastasis models. WT and *Nox2*$^{-/-}$ mice were injected with PDA30364-luc cells either intravenously (i.v.) via the tail vein, or intrasplenically, followed by splenectomy. Lungs and livers were analyzed by bioluminescence imaging 14-15 days after tumor inoculation. Tissues (lungs or liver and spleen) were harvested for phenotypic analysis by flow cytometry. **(C)** Representative IVIS images (left) and quantification of lung bioluminescence (right) in WT ctrl (n = 14), *Nox2*$^{-/-}$ ctrl (n = 13) mice; Mann-Whitney U test. **(D)** Flow cytometric analysis of splenocytes from the lung metastasis model: WT ctrl (n = 9), *Nox2*$^{-/-}$ ctrl (n = 9), WT IL-15-treated (n = 4) and *Nox2*$^{-/-}$ IL-15–treated (n = 3) mice. Frequencies of CD107a^{+} NK cells are shown; one-way ANOVA followed by Holm-Šidák's multiple-comparisons test. **(E)** Representative IVIS images (left) and quantification of lung bioluminescence (right) in WT ctrl (n = 14), *Nox2*$^{-/-}$ ctrl (n = 13), WT IL-15-treated (n = 5) and *Nox2*$^{-/-}$ IL-15-treated (n = 3) mice; Mann-Whitney U test. Data are presented as mean ± SEM.

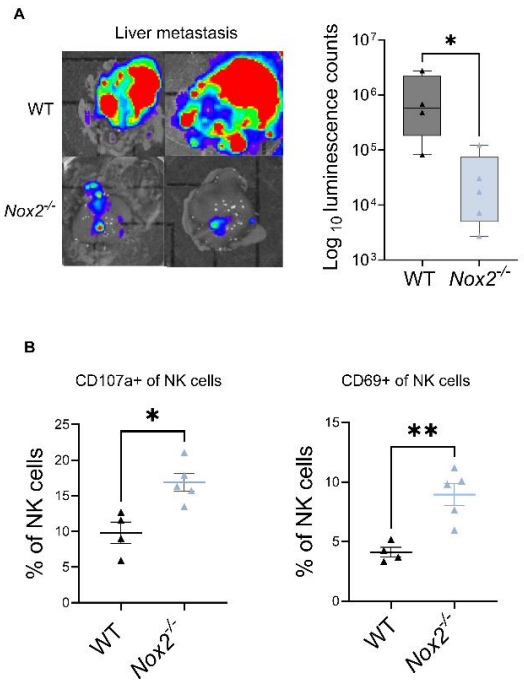

Figure 20: **NOX2 inhibition supports NK cell-mediated control of liver PDAC metastasis. (A)** Representative IVIS images of liver (left) and quantification of liver bioluminescence (right) in WT ctrl (n = 4) and *Nox2*$^{-/-}$ ctrl (n = 5) mice; Mann-Whitney U test. **(B)** Flow cytometric analysis of hepatic lymphocytes from WT ctrl (n

= 4) and *Nox2*$^{-/-}$ ctrl (n = 5) mice. Frequencies of CD107a^{+} and CD69^{+} NK cells are shown; Welch's unpaired t-test. Data are presented as mean ± SEM.

While these results point to NOX2 as a mediator of immunosuppression, with ensuing aggravation of PDAC metastasis, it is important to note that NOX2-deficient mice exhibit a CGD-like phenotype characterized by chronic inflammation and altered immune homeostasis, which may influence metastatic outcomes. Such baseline alterations in inflammation and tissue remodeling could affect tumor cell seeding, survival and outgrowth following intravenous challenge [245, 255, 356]. Thus, confirming anti-metastatic efficacy in wild type mice using pharmacological inhibition of NOX2^{+} myeloid cells would be a valuable addition to the study.

In addition, although IL-15-based therapies have shown promise in enhancing NK cell function, their clinical efficacy has been limited by a short half-life, low bioavailability and dose-limiting toxicity. Ongoing efforts to develop improved or engineered IL-15 formulations may help to overcome these limitations. In this context, combining IL-15-based approaches with NOX2 inhibition may represent a promising strategy to further enhance anti-tumor immunity [297, 299].

In summary of **Paper II**, our findings demonstrate that NOX2-derived ROS suppress NK cell-mediated antitumor immunity in PDAC. Genetic ablation of NOX2 enhances immunosurveillance mediated by NK cells reduces metastasis and may act synergistically with IL-15 to further reduce metastatic burden. The results warrant further evaluation of pharmacological inhibition of NOX2 in PDAC.

PAPER III

In **Paper III**, we investigated how HDC/IL-2 treatment modulates IL-1β and IL-1Ra levels in AML patients in remission and whether the dynamics of these cytokines are associated with clinical outcome. AML is characterized by high relapse rates despite the achievement of complete remission after initial chemotherapy, and pro-inflammatory cytokines such as IL-1β have been implicated in leukemic progression and therapy resistance, whereas IL-1Ra acts as a natural inhibitor of IL-1β signaling [106, 359].

We observed that the first treatment cycle of HDC/IL-2 led to a significant reduction in serum levels of IL-1β, which remained stable at later time points (Figure 21A). In contrast, HDC/IL-2 did not significantly affect levels of IL-1Ra (Figure 21B). Notably, the most favorable clinical outcome was observed in patients presenting with low IL-1β levels in conjunction with high IL-1Ra levels (Figure 21C-D), suggesting that the balance within the IL-1 axis may have prognostic relevance.

Given the proposed roles of IL-1β in promoting leukemic cell proliferation, survival and inflammation [110, 359], it may be speculated that HDC, in conjunction with IL-2, contributes to improved outcomes in AML by reducing IL-1β-driven inflammation. Mechanistically, this effect may be mediated, at least in part, by inhibition of NOX2-derived ROS, thereby limiting myeloid-driven inflammation while enhancing NK and T cell functions. Consistent with this hypothesis, histamine has been shown to suppress pro-inflammatory production of cytokine and NOX2-derived ROS in myeloid cells [150, 256, 358].

Importantly, this interpretation does not contradict the enhanced IL-1β responses observed in genetic NOX2 deficiency, such as in chronic

granulomatous disease (CGD). In this context, lifelong absence of NOX2-derived ROS leads to dysregulated inflammatory signaling, characterized by enhanced NFκB-dependent priming and increased activation of the NLRP3 inflammasome, driven by secondary events such as mitochondrial damage, release of mitochondrial DNA, increased K^+ efflux and pyroptosis [109, 360]. These findings support a model in which NOX2-derived ROS do not simply promote inflammation but instead contribute to the maintenance of cellular homeostasis in phagocytes, that restrains excessive inflammasome activation. Thus, whereas genetic NOX2 deficiency results in chronic inflammatory rewiring and exaggerated IL-1β production, pharmacological modulation of NOX2 activity by HDC likely exerts a context-dependent effect, dampening inflammatory signaling in activated myeloid cells without inducing the dysregulated inflammatory responses observed in CGD.

In summary, **Paper III** highlights the IL-1 axis as a potential biomarker and therapeutic target in AML. Targeting IL-1β-driven inflammation, in combination with immunotherapy, may represent a strategy to improve relapse prevention in the post-chemotherapy phase.

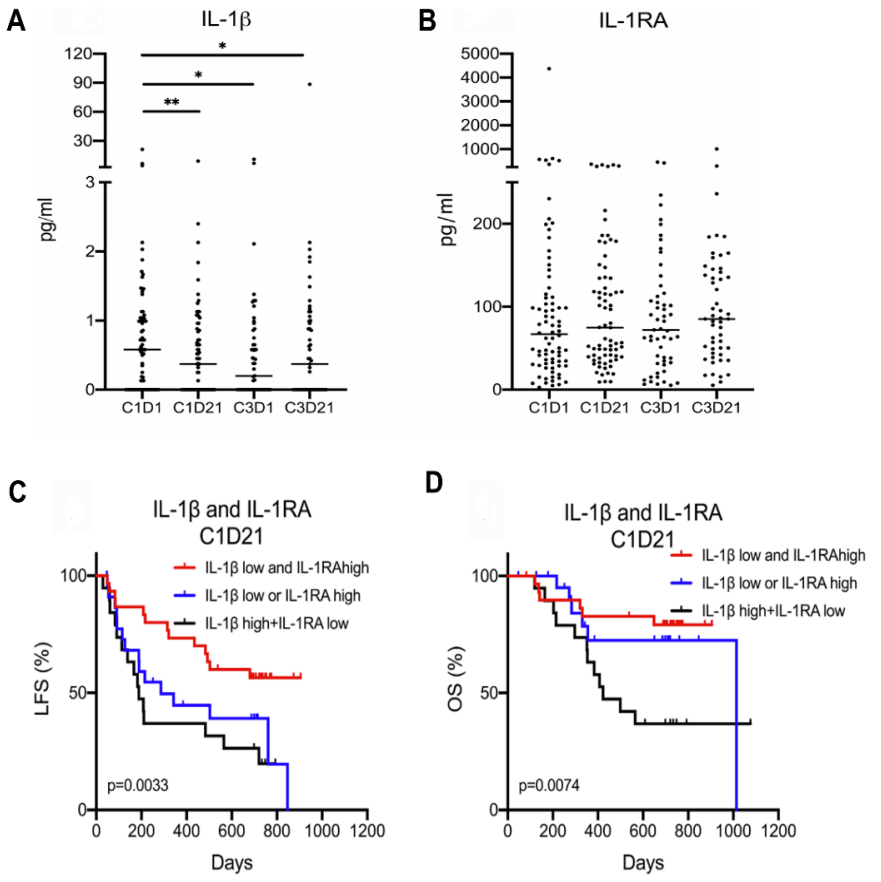

Figure 21: **HDC/IL-2 immunotherapy reduces serum IL-1β levels in AML patients. (A-B)** Serum levels of IL-1β **(A)** and IL-1Ra **(B)** measured before (day 1, D1) and after (day 21, D21) cycles 1 (C1) and 3 (C3) of HDC/IL-2 treatment (C1D1 n = 78, C1D21 n = 73, C3D1 n = 55, C3D21 n = 53); Wilcoxon matched-pairs test. **(E-F)** Patients were stratified into three groups based on IL-1β and IL-1Ra serum levels, using cut-offs 0.6 pg/ml for IL-1β or 66 pg/ml for IL-1RA after the first treatment cycle: low IL-1β and high IL-1Ra (n = 30), high IL-1β and low IL-1Ra (n = 24) and an intermediate group comprising the remaining of the patients with other combinations of IL-1 β and IL-1Ra levels (n = 19). Leukemia-free survival **(E)** and overall survival **(F)** were analyzed using the log-rank test for trend. The figures are adapted from **Paper III** [112].

PAPER IV

BTK inhibitors, including ibrutinib and related compounds, are widely used to treat B cell malignancies such as B cell lymphoma and chronic lymphocytic leukemia. In **Paper IV**, we investigated whether BTK inhibition modulates NOX2-derived ROS activity in myeloid cells, thereby conferring additional anti-neoplastic effects in solid tumors.

We first showed that stimulation of human PBMCs with immobilized IgG (rituximab) induced ROS production via the Fcγ receptor, which was significantly inhibited by ibrutinib (Figure 22A). Given the immunosuppressive properties of myeloid-derived ROS, we examined the impact of BTK inhibition on NK cell function. Ibrutinib protected NK cells from ROS-mediated suppression, reflected by a strong reduction of NK cell apoptosis in co-culture with monocytes (Figure 22B). Furthermore, ibrutinib enhanced NK cell-mediated cytotoxicity against primary human CLL cells under rituximab induced ROS formation. Similar effects were observed with a NOX2 inhibitor (GSK) and the ROS scavenger catalase, indicating that the NK-cell protective effect of ibrutinib is mediated through inhibition of NOX2-derived ROS (Figure 22C). These findings show that NOX2 is a downstream effector of BTK signaling in myeloid cells. Consistent with these findings, treatment of mice with ibrutinib reduced metastatic burden in the B16 melanoma metastasis model. The effect was dependent on both NOX2 and NK cells as it was absent in NOX2-KO and NK cell-depleted mice (Figure 22D).

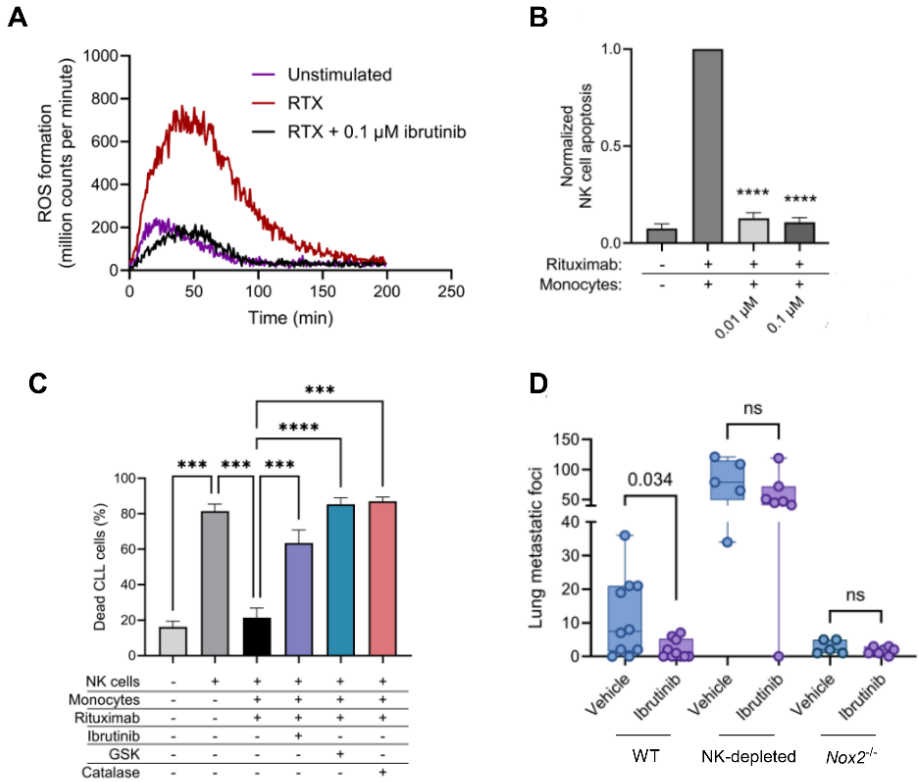

Figure 22: **The BTK inhibitor ibrutinib protects NK cells from monocyte-induced immunosuppression by inhibiting NOX2-derived ROS and enhancing NK cell-mediated tumor clearance in murine melanoma. (A)** Representative kinetics of ROS production. PBMCs were stimulated with rituximab in the presence or absence of inhibitors were compared; one-way ANOVA followed by Holm-Šidák's multiple comparisons test. **(B)** Autologous NK cells and monocytes from healthy donors (n = 6) were co-cultured with immobilized rituximab in the presence or absence of ibrutinib. NK cell apoptosis was assessed by flow cytometry and normalized to rituximab-stimulated controls without inhibitors; one-way ANOVA with Holm-Šidák's multiple comparisons test. **(C)** Autologous NK cells and monocytes were co-cultured with primary CLL cells (n = 4-5) under rituximab stimulation. NK cell cytotoxicity was measured by target cell lysis; mixed-effects model with Holm-Šidák's multiple comparisons test. **(D)** WT or *Nox2*$^{-/-}$ mice were injected intravenously with B16F10 melanoma cells and treated with ibrutinib or vehicle. In one experiment, NK cells were depleted from WT mice using anti-NK1.1 antibodies. Lung metastases were quantified three weeks after tumor inoculation; Mann-Whitney U test. Data are presented as mean ± SEM.

BTK inhibitors such as ibrutinib have been shown to modulate the generation and function of immunosuppressive myeloid cells, including MDSCs, in both B cell malignancies and solid tumors [361-363]. Ibrutinib has been reported to reduce MDSC frequencies and enhance the efficacy of ICB, such as anti-PD-L1 therapy in preclinical models [362]. Notably, first-generation BTK inhibitors like ibrutinib also exhibit off-target effects due to inhibition of other kinases with similar ATP-binding domains, including IL-2-inducible T cell kinase (ITK) and related Tec-family kinases. These unintended interactions may contribute to adverse effects, including potential impacts on NK cells, and appear to be less pronounced with second-generation BTK inhibitors [361, 364].

Collectively, our findings suggest that BTK inhibition enhances anti-tumor immunity by targeting NOX2-driven immunosuppression in myeloid cells, thereby improving NK cell-mediated tumor clearance beyond its direct effects on tumor cells. These data raise the possibility that the clinical efficacy of BTK inhibitors such as ibrutinib may not solely reflect inhibition of malignant B cell proliferation but also improved immune surveillance through relief of myeloid-derived suppression. This dual mechanism may be particularly relevant in settings where immune evasion contributes to disease persistence. Nevertheless, the off-target effects of ibrutinib should be considered, and future studies using more selective BTK inhibitors are needed to validate these mechanisms *in vivo*.

CONCLUDING REMARKS

Myeloid cells constitute a major component of the TME and often exert immunosuppressive effects that limit cytotoxic immune responses against cancer cells. Through the production of pro-inflammatory cytokines, such as IL-1β, and NOX2-derived ROS, myeloid cells can contribute to immune dysfunction and are frequently associated with poor clinical outcomes in both solid tumors and hematological malignancies. Targeting myeloid-driven immunosuppression therefore represents a potential strategy to restore NK and T cell function and enhance tumor clearance.

In **Paper I** and **Paper III**, we demonstrate that HDC exerts anti-neoplastic effects beyond its established role as an inhibitor of NOX2-derived ROS, by modulating myeloid cell function in a context-dependent manner. In melanoma (**Paper I**), HDC was found to induce TSP-1 production by myeloid cells, potentially promoting anti-angiogenic activity and suppressing metastasis. In AML (**Paper III**), treatment of patients in remission after chemotherapy with HDC/IL-2 reduced IL-1β-driven inflammation, which correlated with improved clinical outcomes. These findings suggest that HDC can reprogram myeloid cells toward a less immunosuppressive and more tumor-protective phenotype.

In **Paper II** and **Paper IV**, we further highlight the role of NOX2-derived ROS in mediating myeloid-driven immunosuppression. Inhibition of NOX2 restored NK cell function and reduced metastatic burden in PDAC models, while BTK inhibition similarly suppressed NOX2-driven ROS production in myeloid cells and also enhanced *in vivo*-clearance of NK cell-sensitive melanoma cells. These findings support the concept that the NOX2-ROS axis is a key regulator of immune suppression within the TME.

Importantly, the findings in this thesis also suggest that therapeutic modulation of myeloid cells, for example by HDC, may involve multiple interconnected mechanisms, rather than a single dominant pathway. While inhibition of NOX2-derived ROS represents a central component, HDC also influences additional signaling pathways, including cytokine production and angiogenesis regulation, highlighting the functional plasticity of myeloid cells. This complexity makes it challenging to attribute observed anti-tumor effects to a single mechanism and underscores the need for further studies to define how these pathways interact across different tumor contexts.

STRENGTHS AND LIMITATIONS

This thesis aimed to elucidate the roles and underlying mechanisms of immunosuppressive myeloid cells, with a particular focus on inflammatory signaling and NOX2-derived ROS, as well as strategies to modulate these cells to suppress tumor progression. A major goal of this work was to integrate *in vitro*, *in vivo* and clinical data approaches across multiple cancer models, aiming to provide a comprehensive evaluation of the biological and translational relevance of the findings. The use of specific genetic mouse models enabled mechanistic insights into the role of specific pathways during tumor progression and allowed evaluation of targeted therapeutic strategies. Furthermore, functional immune analyses, including NK cell activity and cytokine profiling, combined with patient-derived datasets, strengthens the clinical relevance of the findings.

However, several limitations should be acknowledged. The use of complete genetic knockout models may not fully capture the complexity and heterogeneity of human cancer, and caution is required when translating these findings to clinical settings. Baseline differences in immune function in NOX2- and TSP-1-deficient mice may influence the observed tumor outcomes. Furthermore, some pharmacological agents used in this work, such as ibrutinib, are known to exhibit off-target effects. Although second-generation BTK inhibitors may mitigate these limitations, further studies are warranted to evaluate their specificity and *in vivo* efficacy.

In addition, the TME comprises a complex network of multiple cell types beyond myeloid cells, and the current models may not fully recapitulate this complexity Future studies incorporating more physiologically relevant systems, such as organoid models or advanced co-culture systems that better recapitulate the full immune context, may provide deeper insights into cellular

interactions. Finally, further clinical validation is required to confirm the translational potential of these findings.

In summary, this thesis highlights the importance of myeloid cell-mediated immunosuppression in the TME and identifies potential strategies to target these mechanisms to improve clinical outcomes.

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Use of Generative AI

ChatGPT (OpenAI, Edu version) was used for language assistance, including grammar correction and improving clarity. All research content, analysis, interpretations and conclusions are original work of the author.

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