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Uncovering mechanisms of immune exclusion that drive resistance

Eleftheria Maranou

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To my beloved family members who bravely fought cancer-
Your memory is the quiet force behind every page of this work

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ELEFTHERIA MARANOU: The Achilles' heel of immune checkpoint blockade: Uncovering mechanisms of immune exclusion that drive resistance
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ABSTRACT

Melanoma can affect various tissues, including the skin and the eyes. immune checkpoint inhibitors have revolutionized the treatment of many cancers, including metastatic melanoma, but uveal melanoma remains largely unresponsive, mainly due to low mutational burden. Changes in the tumor microenvironment composition can also impact immune checkpoint inhibitors' efficacy. Moreover, genetic and epigenetic alterations in cancer cells and cells of the tumor stroma can impair T-cell priming and infiltration, further compromising the effectiveness of immune checkpoint inhibitors. Therefore, many cancer patients fail to respond or eventually develop resistance to this treatment option. This doctoral thesis investigates mechanisms of immune exclusion and resistance to immune checkpoint inhibitors in uveal and cutaneous melanoma.

The first part of the thesis explores how loss of adipophilin underlies immunosuppression in uveal melanoma. Alongside *BAP1* deficiency, loss of adipophilin leads to metabolic reprogramming, promoting immunosuppression. The second part of the thesis investigates CD74, chaperone of MHC-II, in melanoma progression and its potential in regulating dendritic cell functions. This study demonstrates that CD74 deficiency enhances antitumor immunity by modulating dendritic cell migration and improving cancer cell-specific antigen cross-presentation to T cells.

By elucidating the role of adipophilin in uveal melanoma and uncovering the effects of CD74 depletion in enhancing dendritic cell functions in cutaneous melanoma, this thesis reveals novel mechanisms of immune regulation. Altogether, these findings suggest potential therapeutic targets that could enhance the efficacy of immune checkpoint inhibitors in refractory tumors.

KEYWORDS: Uveal melanoma, adipophilin, metabolism, immune regulation, cutaneous melanoma, CD74, dendritic cells, antigen cross-presentation, migration

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TIIVISTELMÄ

Melanooma voi kehittyä useisiin kudoksiin, kuten ihoon ja silmän suonikalvoon. Isännän immuunijärjestelmää elvyttävät immuunivasteen vapauttajat (immune checkpoint inhibitors) ovat mullistaneet monien syöpien hoidon. Ne tehoavat hyvin levinneessäkin ihomelanoomassa, mutta eivät silmän suonikalvon melanoomassa. Immuunivasteen vapauttajien tehoon vaikuttavat syöpäsolujen mutaatioiden lukumäärä ja kasvaimessa olevien normaalisolujen koostumuksen muutokset. Lisäksi kasvaimen syöpäsolujen ja normaalisolujen geneettiset ja epigeneettiset muutokset voivat heikentää T-solujen kulkua kasvaimeen ja T-solujen aktivaatiota kasvaimessa. Tämä väitöskirja koostuu kahdesta osata, joissa tutkitaan kasvaimen immuunisolujen puuttumista ja muita immuunivasteen vapauttajien hoitoresistenssin taustalla olevia mekanismeja silmän suonikalvon ja ihon melanoomassa.

Ensimmäisessä osatyössä havaitsin, että adipofiliinin väheneminen silmän suonikalvon melanoomassa johtaa kasvaimen mikroympäristön metaboliseen uudelleenohjelmoitumiseen, mikä heikentää immuunijärjestelmän toimintaa. Adipofiliini paljastui myös uudeksi taudin ennustetta kuvaavaksi merkkiaineeksi. Väitöskirjan toisessa osassa selvitin kudostyyppiantigeeni MHC-II:n kaitsijaproteiini CD74:n merkitystä ihomelanooman etenemisessä. Tutkimus osoitti, että CD74:n puuttuminen tehostaa kasvaimen vastaista immuunivastetta säätelemällä dendriittisolujen liikkumista ja parantamalla syöpäantigeenien esittelyä T-soluille.

Selvittämällä adipofiliinin merkitystä silmän suonikalvon melanoomassa ja paljastamalla CD74:n vaikutukset dendriittisolujen toiminnassa ihomelanoomassa tämä väitöskirja tuo esiin uusia immuunisäätelyn mekanismeja. Samalla löydökset viittaavat mahdollisiin uusiin terapeuttisiin kohdemolekyyleihin, joita voitaisiin hyödyntää immuunivasteen vapauttajien tehon parantamisessa.

AVAINSANAT: Silmän suonikalvon melanooma, adipofiliini, metabolia, immuunijärjestelmän säätely, ihomelanooma, CD74, dendriittisolut, antigeenien esittely, solujen liikkuminen

Table of Contents

Abbreviations

Ag(s)	Antigen(s)
AIRE	autoimmune regulator
ALP	All-lymphoid progenitor
AP-1	Activator Protein 1
APCs	Antigen-presenting cells
BAP1	Breast-Cancer-Associated-1 (BRCA1)-Associated Protein 1
Batf3	Basic leucine zipper transcription factor
BLP	B cell-biased lymphoid progenitor
BM	Bone marrow
BMDCs	Bone marrow-derived dendritic cells
CAR	chimeric antigen receptor
CD	Cluster of differentiation
cDC	conventional dendritic cell
cDC1	conventional dendritic cell type 1
cDC2	conventional dendritic cell type 2
CDK4	cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDPs	Common DC progenitors
cGAMP	cyclic 2′3′ GMP-AMP
cGAS	Cyclic guanosine monophosphate (GMP)-AMP synthase
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CTSS	Cathepsin S
CTV	CellTrace Violet
CXCL-	C-X-C motif chemokine ligand-
CXCR-	C-X-C chemokine receptor-
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DDT	D-dopachrome tautomerase
DEA	Differential expression analysis

DEGs	Differentially expressed genes
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FFPE	Formalin-fixed paraffin-embedded
FL-DCs	FLT3L-derived bone marrow DCs
Flk2	Fetal liver kinase-2
FLT3	Fms-like tyrosine kinase 3
FLT3L	Fms-like tyrosine kinase 3 ligand
GALT	Gut-Associated Lymphoid Tissue
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMPs	Granulocyte macrophage progenitors
GO	Gene ontology
GrB	Granzyme B
Grb2	Growth factor receptor-bound 2
GSDMD	Gasdermin D
HIF-1a	Hypoxia-inducible factor 1-alpha
HSCs	Hematopoietic stem cells
ICD	Intracellular domain
ICIs	Immune checkpoint inhibitors
ICOS	Inducible T cell co-stimulator
IFN-#	Interferon-#
IL-#	Interleukin-#
IRFs	Interferon regulatory factors
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
LAMPs	Lifestyle-associated molecular patterns
LAG3	Lymphocyte-activation gene 3
LDs	Lipid droplets
LNs	Lymph nodes
LPS	Lipopolysaccharide
MALT	Mucosa-Associated Lymphoid Tissue
MAPK	mitogen-activated protein kinase
MDPs	Macrophage dendritic cell progenitors
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
moDC	Monocyte-derived dendritic cell
MPPs	Multipotent progenitors
MΦs	Macrophages
nBAP1	nuclear BAP1

NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear Factor of activated T cells
NLRP3	NOD-like receptor family pyrin domain containing 3
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
pDC	plasmacytoid dendritic cell
PI3K	Phosphoinositide 3-kinase
PI3K	Phosphoinositide-3-kinase
polyI:C	Polyinosinic:polycytidylic acid
PRRs	Pattern recognition receptors
RLRs	Retinoic acid-inducible gene-I (RIG-I)-like receptors
ROI	Region of interest
RUNX	Runt related transcription factor
SH2	Scr homology 2
SMOCs	Supramolecular organizing centers
SPPL2a	Signal peptide peptidase-like
STING	Stimulator of interferon genes
TAMs	Tumor-associated macrophages
TAP1/2	Transporter associated with antigen processing 1/2
TCR(s)	T cell receptor(s)
tdLNs	Tumor-draining lymph nodes
Tex	Exhausted T cells
TGF-β	transforming growth factor-beta
Th	Helper CD4+ T
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIMP-1	Tissue inhibitor of metalloproteinases-1
TLRs	Toll-like receptors
TLS	Tertiary lymphoid structures
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-α
Tregs	Regulatory T cells
UM	Uveal melanoma
UV	Ultraviolet

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Maisoon Matareed[#], Eleftheria Maranou[#], Saara A Koskela, Arfa Mehmood, Helen Kalirai, Sarah E Coupland, Carlos R Figueiredo. Novel prognostication biomarker adipophilin reveals a metabolic shift in uveal melanoma and new therapeutic opportunities. *The Journal of Pathology*, 2023; 2: 203-2011. https://doi.org/10.1002/path.6076
[#]Equal contribution

II Eleftheria Maranou, Gayoung Park, Pauline Weinzettl, Jonna Alanko, Otto Pulkkinen, Sarah E. Coupland, Marko Salmi, Carlos Rogerio De Figueiredo. CD74 regulates antitumor immunity in melanoma by reprogramming dendritic cell immunogenicity and migration, 2025, *Manuscript.*

1 Introduction

The immune system is an intricate system that keeps all organisms healthy and alive. From bacteria, plants, and insects to vertebrates, including humans, the immune system employs a variety of sophisticated defense networks with the goal of providing long-lasting protection against various threats or forms of damage. Its elegance lies in its complexity, evolutionary refinement, and the delicate balance it maintains.

An oxymoron of the immune system could be that it is a highly armed and dormant defense force at the same time. During "peacetime", the immune system is ready to fight a potential threat, a state of readiness that is both defensive and dormant, as it does not launch an attack until an invader is detected. This threat is classically posed by invading microbes such as bacteria, viruses, fungi, and parasites, which are recognized as non-self and trigger immune activation. However, it can sometimes mistakenly attack the host's own cells, a condition known as autoimmune disease, creating an oxymoron of self-protection.

Among the invaders that the host's immune system can encounter in one's life is cancer. Even though the immune system is supposed to fight cancer, it has become evident that it can have completely opposite role and shape the disease through a three-phase evolutionary process, called immunoediting (Dunn et al., 2004a, 2004b). During the first phase, *elimination*, tumor antigens (Ags) are presented by professional antigen-presenting cells (APCs), mainly dendritic cells (DCs), to activate T cells in the tumor-draining lymph nodes (tdLNs), which then infiltrate the tumor microenvironment (TME) and eradicate cancer cells. However, some cancer cells survive, entering the *equilibrium* phase, during which tumors accumulate mutations or upregulate the "brakes" of immune cells, known as immune checkpoint molecules, such as programmed death-1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1), which help them escape detection and destruction by the immune system (Dunn et al., 2004b). During *escape,* immunosuppression is induced that weakens the host's immune system and makes the body vulnerable (Dunn et al., 2004a; Mellman et al., 2023).

Immunotherapy is a type of cancer treatment that uses the body's own immune system to fight diseases like cancer (Waldman et al., 2020). Immune checkpoint

inhibitors (ICIs) constitute a type of immunotherapy. They are monoclonal antibodies that block the "brakes" of immunity, such as anti-PD-L1/anti-PD-1 and anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4) and aim at reinvigorating the patients' effector cells, T cells (Waldman et al., 2020). Despite the success of ICIs, many cancer types remain refractory or develop resistance to the treatments (Huang & Zappasodi, 2022). ICIs exert immune pressure on cancer cells, driving *elimination* of the cancer cells that display immunogenic Ags (Huber & Bassani-Sternberg, 2026). Yet, cancer cells can alter their antigenic repertoire or avoid them in order to persist (*equilibrium*), escape, and continue to grow (Huber & Bassani-Sternberg, 2026). Beyond tumor-intrinsic mechanisms of immunotherapy resistance, the immune system itself facilitates the development of tumors and resistance to ICIs by suppressing antigen cross-presentation, co-stimulatory molecules of the B7 family, and proinflammatory cytokine release, the three fundamental elements required for efficient priming of naïve T cell, forming the foundation of cell-mediated antitumor immunity (Dong et al., 1999; Freeman et al., 2000; Huber & Bassani-Sternberg, 2026; Tseng et al., 2001). In addition, epigenetic modifications leading to suppression of chemokines responsible for T cell recruitment to the TME and proinflammatory cytokine signaling further hinder the infiltration and functions of T cells in the TME (Nagarsheth et al., 2016; Peng et al., 2015). Therefore, mechanisms that take place during cancer immunoediting impact the cancer-immunity cycle and are closely linked to treatment resistance.

Lastly, the TME composition, which could be a result of cancer immunoediting, can influence responses to ICIs and shape the immunotypes of tumors, characterized as immune inflamed ("hot"), immune excluded, and immune desert (Chen & Mellman, 2017; Hegde & Chen, 2020). "Cold" tumors, either immune excluded or desert, have limited or absent T-cell infiltration. The number of T cells infiltrating the TME and their spatial distribution pose another major challenge to successful ICIs responses (Giuliani et al., 2024). Even though cutaneous melanoma is considered a "hot" tumor and in patients with advanced melanoma, significantly longer overall survival has been achieved with a combination of ICIs, many patients exhibit resistance to the treatments, due to lack of T cells in their TME (Figure 1). On the other hand, uveal melanoma (UM), an aggressive intraocular cancer, is refractory to ICIs (Figure 1).

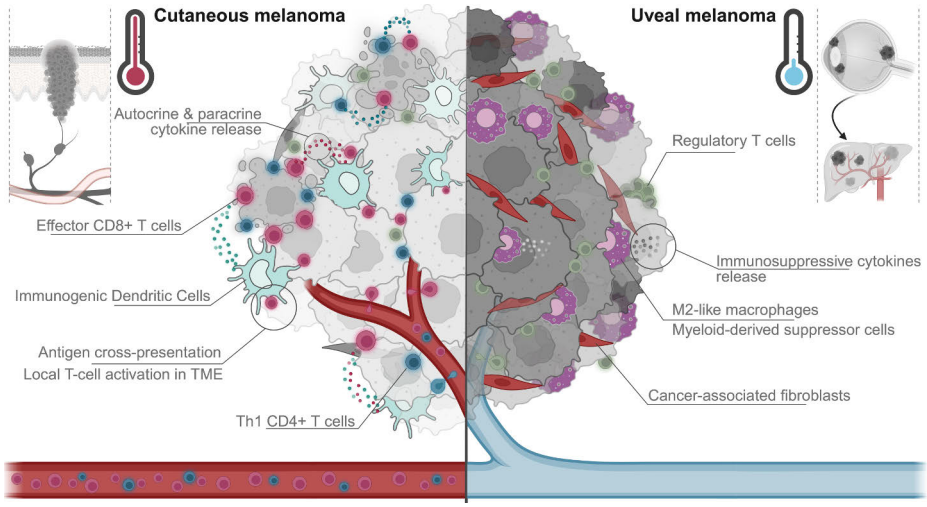


Figure 1. "Hot" versus "cold" tumor phenotypes. Schematic comparison of immune composition and mechanisms characteristic of immunologically "hot" tumors (left) and "cold" tumors (right). Although cutaneous melanoma is described as an inflamed tumor, it may develop immunosuppressive features that promote resistance to ICIs. In contrast, uveal melanoma represents a "cold" tumor paradigm and is largely refractory to ICIs. (Illustration created with BioRender).

2 Review of the Literature

2.1 A brief introduction to the immune system

The immune system guards the human body, recognizing, encountering, and neutralizing potential threats such as pathogens, cancer, and is also responsible for clearing apoptotic cells (Delves & Roitt, 2000a, 2000b). It is composed of specialized organs, cells, and molecules that work together to mediate its physiological functions and maintain homeostasis (Delves & Roitt, 2000a, 2000b). The immune system is divided into two branches: the innate and adaptive immunity. Even though each branch includes distinct cell types and molecular mechanisms, they function cooperatively to orchestrate effective immune responses. Before causing an infection, a pathogen needs to breach several surface barriers, such as epithelial layers, mucus, and antimicrobial enzymes, that either directly eradicate microbes or prevent them from attaching. The keratinized surface of skin and mucus-lined body cavities provide environments that are inhospitable to most pathogens; thus, microbes must first overcome the epithelial barrier to initiate infection. Once a pathogen breaks through this initial barrier, it encounters the innate immune system, the body's first line of defense (Abbas, 2015). The adaptive immune system is subsequently activated to mount a targeted, Ag-specific response and to establish immunological memory.

All immune cells and immunological responses are generated and initiated in the organs of the immune system that are classified into primary and secondary lymphoid organs (Drayton et al., 2006; Mercier et al., 2011; Miller, 2020). The primary lymphoid organs, the bone marrow (BM) and thymus, are responsible for the development and maturation of all immune cell precursors (Mercier et al., 2011; Miller, 2020). In contrast, the secondary lymphoid organs are the sites where immune responses are initiated against diverse foreign Ags. These include the lymph nodes, the spleen, the tonsils, the Peyer's patches, the mucosa-associated lymphoid tissue (MALT), which is distributed across various organs (Drayton et al., 2006; Mörbe et al., 2021; Ruddle & Akirav, 2009).

An effective defense against a wide range of threats depends on the development of distinct, highly specialized immune cell lineages. All immune cells originate from hematopoietic stem cells (HSCs) through a tightly regulated process known as hematopoiesis (Baum et al., 1992). Multipotent progenitors (MPPs) derived from

HSCs give rise to two major progenitor lines: the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLPs) (Figure 2) (Akashi et al., 2000; Kondo et al., 1997).

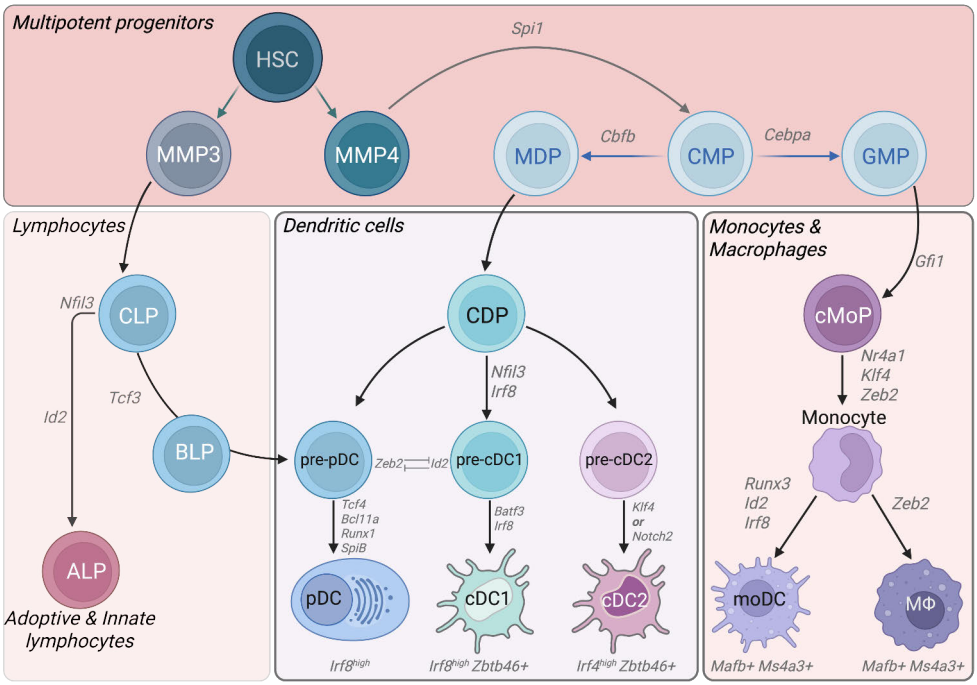


Figure 2. Hierarchical model of hematopoiesis and myeloid differentiation. Developmental pathways of mouse DCs, monocytes, MΦs originating from shared hematopoietic progenitors are shown. Hematopoietic stem cells (HSCs) generate multipotent progenitors (MPPs), which differentiate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CLPs segregate into Ly6D- ALPs and Ly6D+ B cell-biased lymphoid progenitor (BLPs). High expression of *Irf8* drives pre-pDCs and pre-cDC1 subset differentiation, whereas *Zbtb46* marks commitment to both cDC lineages. cDC2s are associated with *Irf4* expression. Monocytes and MΦs are identified through Mafb lineage tracing, while Ms4a3 distinguishes monocyte-derived MΦs from embryonically derived MΦs (adapted from (Anderson et al., 2021) using BioRender).

The myeloid lineage starts from the CMPs, which give rise to the granulocyte macrophage progenitors (GMPs) from which the macrophage-dendritic cell progenitors (MDPs) originate. The MDPs are then transcriptionally controlled to differentiate into common dendritic cell (DCs) progenitors (CDPs). The CDPs are committed to become either pre-conventional dendritic cells (cDCs), giving rise to circulating conventional dendritic cells, or pre-plasmacytoid DCs (pDCs). Pre-cDCs are restricted cDC progenitors that constantly exit the BM for the periphery to differentiate locally into cDCs (Merad et al., 2013). They do not give rise to pDCs or macrophages (MΦs) in vivo (Diao et al., 2006; Naik et al., 2006). On the other

hand, the lymphoid lineage gives rise to adaptive immune cells: B cells, T cells, and natural killer cells (Kondo et al., 1997). The CLP population can be separated into two subsets based on Ly6D expression. The all-lymphoid progenitor (ALP) has B cell, T cell, and innate lymphoid cell potential. The B cell-biased lymphoid progenitor (BLP) gives rise to B cells and pDCs (Inlay et al., 2009; Rodrigues et al., 2018). B and T cells undergo maturation in the BM and thymus, respectively. Once fully developed, mature B and T cells circulate throughout the lymphoid tissues, ready to participate in immune surveillance (Orkin & Zon, 2008).

2.1.1 The innate immune system

The innate immune system is the body's first line of defense against common pathogens. It lacks immunologic memory and provides the body's physical and biochemical barriers. In addition, it includes distinct subsets of innate immune cells, and the complement system. Physical and biochemical barriers of the body comprise soluble components, such as lytic enzymes and host defense peptides, that work as natural antimicrobial factors and prevent potential invasion from common pathogens (Salton, 1957).

The innate immune responses utilize phagocytes like neutrophils, monocytes, MΦs, DCs, as well as cells that release inflammatory mediators, such as basophils, mast cells, and eosinophils, and natural killer cells (Guermonprez et al., 2002; Kalesnikoff & Galli, 2008; Lin & Loré, 2017; Malaviya & Abraham, 2001; Novak et al., 2010; Steinman & Cohn, 1973). To identify potential threats, cells of the innate immune system rely on pattern recognition. Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), are germline-encoded and recognize conserved extracellular and intracellular microbial signatures called pathogen-associated molecular patterns (PAMPs) (Figure 3) (Kawai & Akira, 2009; Medzhitov & Janeway, 1997). Examples of such PAMPs include conserved components like bacterial lipopolysaccharide (LPS), peptidoglycan, flagellin, and viral nucleic acids (Medzhitov & Janeway, 1997). Besides PAMPs, PRRs can also detect endogenous and exogenous danger signals, such as the increase in heat-shock proteins because of necrotic cell death. These endogenous danger signals are known as damage-associated molecular patterns (DAMPs) and can activate cells of the innate immune system such as MΦs and DCs (Figure 3) (Matzinger, 1998). MΦs, DCs, and neutrophils, along with non-immune cells like epithelial and endothelial cells, contribute to the innate immune response by sensing PAMPs and DAMPs. Innate responses can also be elicited in the absence of infection through sterile inflammation, which is triggered by tissue injury, metabolic stress, DAMPs or lifestyle-associated molecular patterns (LAMPs), including oxidized low-density lipoprotein and cholesterol crystals (Zindel & Kubes, 2020).

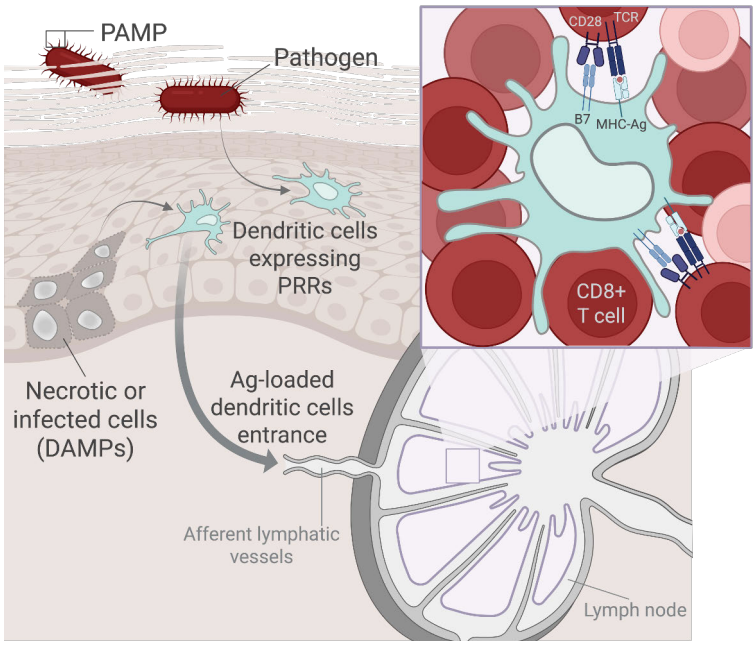


Figure 3. DCs functions. PRRs on DCs detect PAMPs and endogenous danger signals released during an infection. Upon activation, these professional APCs process Ags and present peptide-MHC complexes to CD28-expressing naïve T cells, initiating adaptive immune responses. Activation also induces upregulation of B7 costimulatory molecules, enhancing T cell priming (adapted from (Delves & Roitt, 2000a) using BioRender).

Through pattern recognition, innate immunity triggers inflammatory responses that control infection and promote tissue repair (Carpenter & O'Neill, 2024). Inflammation is driven by cytokines, chemokines, plasma enzyme mediators, and lipid inflammatory mediators released by immune cells after detecting pathogens or tissue damage (Carpenter & O'Neill, 2024). Upon infection, short-lived innate immunity cells establish and maintain long-term functional changes (Schlüter et al., 2025). This process, known as trained immunity, is characterized by an enhanced ability to eliminate pathogens upon subsequent exposure (Netea et al., 2020; Schlüter et al., 2025). Trained immunity operates independently of adaptive immunity cellular components. Innate immune cells secrete inflammatory cytokines and chemokines, such as tumor necrosis factor (TNF), IL-1beta, and IL-6, that participate in antimicrobial defense and contribute to the development of trained immunity (Crișan et al., 2016; Netea et al., 2020).

Moreover, the complement system plays a vital role in innate immunity. Its activation through the classical, lectin, and alternative pathways triggers proteolytic cascades that result in pathogen destruction by promoting opsonization and by forming the membrane attack complex, which directly lyses pathogens, while also

stimulating innate immune cells through the release of small anaphylatoxin fragments (Dunkelberger & Song, 2010; Elsner et al., 1994; Hadders et al., 2007; Morgan et al., 1993; Ricklin et al., 2010). The following chapters will focus predominantly on MΦs, DCs, and their functions, since these are central cellular components investigated in this thesis.

2.1.1.1 Macrophages

Illya Ilyich Metchnikoff is recognized as the "father of innate immunity" for his pioneering discovery of phagocytosis and the cells responsible for it, MΦs (Gordon, 2008, 2016). His findings did not only reveal a key defense mechanism of the innate immune system but also laid the foundations for the concept of cell-mediated immunity. The term *macrophage* reflects the cell's primary function, which is to engulf foreign organisms, and derives from the Greek words *makrós* (μακρός), which means large, and *phagein* (φαγεῖν), which means to eat, literally meaning "big eater" (Teti et al., 2016). MΦs have either monocyte-derived origin or are yolk-sac derived and classified as tissue-resident macrophages. They function to clear pathogens by orchestrating inflammatory responses but also play a role in repairing tissues depending on the signals they receive from the environment they reside in (Cavaillon, 2011; Varol et al., 2015).

MDPs in the BM differentiate into monocytes that enter the peripheral circulation (Ginhoux & Jung, 2014; Hettinger et al., 2013). In mice, two major subsets of monocytes have been identified: classical Ly6C^{high} (CD14^{high}CD16^{neg} in humans) and non-classical patrolling Ly6C^{low} (CD14^{low}CD16^{high} in humans) (Ginhoux & Jung, 2014; Varol et al., 2015). Ly6C^{high} monocytes migrate to inflamed tissues, where they differentiate into MΦs and monocyte-derived DCs (moDCs) (Shi & Pamer, 2011). Under homeostasis or during tissue remodeling-associated inflammation, classical monocytes can also generate short-lived, tissue-resident macrophages that lack self-renewal capacity (Varol et al., 2015).

Activation of MΦs is regulated by different environmental cues, which can influence gene expression profiles leading to a gradient of MΦ phenotypes. Transcription factors STAT1 and IRF5 are implicated in the transcription of genes leading to the M1 pro-inflammatory MΦ phenotype, while STAT6 and IRF4 promote differentiation toward the M2 anti-inflammatory phenotype (Lawrence & Natoli, 2011). This phenotypic plasticity enables MΦs to function in both pro-inflammatory and anti-inflammatory tissue repair processes. However, the distinction between M1 and M2 MΦs is very simplistic, since there is a spectrum of different phenotypes not following this black-and-white categorization of these cells.

Some tissues have distinct MΦ populations that derive from yolk sac precursors and in adult organs are renewed autonomously, independent of BM progenitors

(Varol et al., 2015). For example, brain microglia, Kupffer cells, lung alveolar MΦs and Langerhans cells are referred to as tissue-resident MΦs (Mass et al., 2016). Intestine, dermis, heart, and pancreas tissue-resident MΦs require Ly6C^{high} monocytes originated by HSCs for their maintenance (Ginhoux & Guilliams, 2016).

2.1.1.2 Dendritic cells

DCs and their functions in immunity were first identified by Ralph Steinman and Zanvil Cohn in the late 1970s (Steinman & Cohn, 1973, 1974). These cells exert the superb ability to elicit adaptive immune responses against any foreign Ag, while also contributing to the induction of immune tolerance to self-Ags. Nowadays, DCs are the forefront of cellular therapeutics, alongside approaches such as chimeric Ag receptor (CAR) T-cell therapy, which offers rapid and potent tumor reduction but, in many cases, faces challenges in tumor infiltration (Escobar et al., 2025). Their modulation is increasingly being explored for the development of vaccines targeting infectious diseases, tumors, and autoimmune disorders in both academia and industry. For his important work, Ralph Steinman was awarded the 2011 Nobel Prize in Physiology or Medicine for discovering dendritic cells and elucidating their role in adaptive immunity (Banchereau et al., 2011; Mellman & Nussenzweig, 2011; Nussenzweig & Mellman, 2011; Shortman, 2012). The second manuscript-phase publication included in the present doctoral dissertation will focus on various dendritic cell subsets and their functions in cancer.

DCs are broadly classified into conventional DCs (cDCs) and pDCs. cDCs are further divided into two main subsets: conventional dendritic cell type 1 (cDC1) and conventional dendritic cell type 2 (cDC2), each with specialized functions and distinct Ag presentation capabilities. pDCs constitute a small subset of DCs that primarily circulate in the blood and accumulate in lymphoid tissues, including lymph nodes (LNs). cDCs are also a small subset of hematopoietic cells that reside in most lymphoid and nonlymphoid tissues. The latter have an enhanced ability to detect tissue damage, capture cell-associated Ags, process them, and present them to T cells.

The differentiation and expansion of DC lineages are largely regulated by external signals, particularly hematopoietic cytokines. These cytokines control both DC lineage commitment and differentiation in the BM, as well as their maintenance in homeostasis. One key regulator is the Fms-like tyrosine kinase 3 ligand (FLT3L), which drives DC commitment in hematopoiesis (Merad & Manz, 2009; Onai, Obata-Onai, Schmid, & Manz, 2007). FLT3L is ubiquitously produced by multiple tissue stromal cells, endothelial cells, and activated T cells (Schmid et al., 2010). Its receptor, Fms-like tyrosine kinase 3 (FLT3), also known as CD135 or Flk2, is expressed on short-term repopulating HSCs, CLPs, and a subset of CMPs (Karsunky et al., 2003). FLT3 expression is also preserved on DC precursors including MDPs,

CDPs, and pre-cDCs (Fogg et al., 2006; Liu et al., 2009; Naik et al., 2007; Onai, Obata-Onai, Schmid, Ohteki, et al., 2007; Waskow et al., 2008), but it is lost when progenitors commit to non-DC lineages (Karsunky et al., 2003). Most bloodstream and tissue leukocytes are characterized by loss of FLT3 expression, with pre-cDCs, pDCs, and tissue cDCs being exceptions (Karsunky et al., 2003). FLT3 is also expressed on BM-derived DCs (BMDCs) cultured with FLT3L addition, whereas is absent in moDCs (Karsunky et al., 2003).

Mice that are FLT3 or FLT3L deficient exhibit reduced numbers of MDPs, CDPs, and tissue cDCs and pDCs (Kingston et al., 2009; McKenna et al., 2000; Waskow et al., 2008), highlighting the importance of this cytokine and its receptor in DC development. Inhibition of FLT3 results in elevated serum levels of FLT3L, indicating a tightly regulated feedback loop: when cDC numbers are low, FLT3L production increases and in turn induces the generation of cDCs (Birnberg et al., 2008; Tussiwand et al., 2005). Systemic administration or overexpression of FLT3L in mice leads to a dramatic expansion of cDCs and pDCs in the circulation and across lymphoid and non-lymphoid tissues (Manfra et al., 2003; Maraskovsky et al., 1996). Beyond its role in development, FLT3L also regulates peripheral DC proliferation to maintain homeostatic DC numbers (Waskow et al., 2008).

DCs are short-lived; however, they are central mediators of the long-term immune responses, bridging innate and adaptive immunity. The role of cDCs as sentinels requires them to continuously monitor and respond to environmental cues. Under steady-state conditions, pDCs express low levels of major histocompatibility complex (MHC) class II (MHC-II) and costimulatory molecules. Upon detecting foreign Ags via their TLRs, pDCs rapidly produce large amounts of type I interferons (IFN) and acquire Ag-presenting capabilities (Reizis, 2010; Reizis et al., 2011). cDCs, on the other hand, have the unique capacity to induce immune responses against any foreign protein Ag that invades host tissues, while they also maintain tolerance to self-Ags. They function as the frontline defenders of the immune system, having strategic location in non-lymphoid tissues, where they constantly sample their environment for Ags (Figure 3). Equipped with highly efficient Ag processing and presentation machinery, they process captured Ags and subsequently migrate, laden with both self and foreign Ags, from non-lymphoid tissue to lymph nodes (LNs) (Banchereau & Steinman, 1998; Förster et al., 2012; Joffre et al., 2012; Segura & Villadangos, 2009; Villadangos & Schnorrer, 2007).

Two subsets of cDC1s, lymphoid tissue-resident CD8+ cDC1 and non-lymphoid tissue migratory CD103+ cDC1s, originate from the same progenitor and exhibit overlapping phenotypes and transcriptional profiles. CD103+ cDC1 are predominantly found in non-lymphoid tissues at the interface with the environment, from where they efficiently migrate to present Ags to T cells in LNs (Helft et al., 2010). In the spleen, CD8+ cDC1 reside in the marginal zone, an ideal location for

filtering blood-borne Ags. In contrast, a minor CD8+ cDC1 population, which comprise the LN-resident DCs, localize in the subcapsular sinus, that constitute the entry site of afferent lymphatic vessels (Gerner et al., 2012; Gerner et al., 2015; Huang et al., 2022; Merad et al., 2013; Ugur et al., 2023). This strategic positioning supports lymph sampling and the capture of Ags from peripheral tissues (Idoyaga et al., 2009; Merad et al., 2013; Qiu et al., 2009). From there, CD8+ cDC1 migrate into the T cell-rich cortical and paracortical zones of LNs, where the majority of DCs are located, to present tissue-derived Ags to T cells (Braun et al., 2011). Notably, CD8+ and CD103+ cDC1 are the only cDCs that express TLR3, sensing double-stranded viral RNA (Davey et al., 2010; Edwards et al., 2003). From this point of the text onwards, CD8+ CD103- cDC1 will be referred to as lymphoid-tissue resident or resident cDC1s, while CD103+ CD8- cDC1s will be identified as migratory cDC1s.

2.1.2 The adaptive immune system

After the innate immune system mounts a rapid defense against potential threats, the adaptive immunity acts as the second line of protection, characterized by its high specificity. Adaptive immunity also results in the formation of immunological memory, which secures a faster and more robust response upon re-exposure to the same antigenic epitope and in contrast to trained immunity it is dependent on lymphocytes and necessitates Ag specificity (Netea et al., 2020). The adaptive immunity divided into two branches: humoral and cell-mediated immunity.

Humoral immunity is mediated by antibodies secreted by B cells after they undergo terminal differentiation into plasma cells (Bottazzi et al., 2010; Edelman, 1973). Antibodies consist of four polypeptide chains, two heavy chains (50 kDa each) and two light chains (approximately 25 kDa each), connected by disulfide bonds to form a symmetrical Y-shaped molecule (Abbas, 2015; Pieper et al., 2013). The N-terminal region of both heavy and light chains exhibit significant amino acid sequence variability and are therefore referred to as the variable region, while the remaining, more conserved amino acid sequences constitute the constant region (Abbas, 2015; Pieper et al., 2013). Within the variable region of heavy and light chains, there are three hypervariable regions of complementarity-determining regions that form the antigen-binding site. Antibodies exhibit several biological functions (Abbas, 2015; Pieper et al., 2013). Antibodies function as opsonins, tagging foreign particles and enabling their destruction by other immune cells through opsonophagocytosis (Abboud et al., 2010; Guilliams et al., 2014). Moreover, antibodies can activate the classical complement pathway (Hajishengallis et al., 2017). In addition, antibodies can neutralize toxins by binding to them in body fluids and preventing their interaction with host cells (Bournazos et al., 2014; Lu et al., 2018).

Lastly, antibodies have become powerful tools in research, diagnostics, and therapy owing to their high specificity and ability to target any protein, providing countless possibilities. The therapeutic applications of antibodies will be discussed in later chapters of this thesis (Hodi et al., 2010). Here, the focus will be on cell-mediated immunity, which involves T cells that directly attack and destroy infected cells.

2.1.2.1 Cell-mediated immunity

T cells originate in the BM and they migrate to the thymus for their maturation expressing both CD8 and CD4 on their surface. These double positive T cells undergo positive and negative selection in the thymus, giving rise to CD4+ and CD8+ T cells that recognize self MHC molecules while remain tolerant to self-Ags. T cells are characterized by their T-cell receptors (TCRs), which enable them to recognize specific peptide Ags (Abbas, 2015). Positive selection ensures the survival of T cells with functional TCRs capable of interacting with MHC molecules, while negative selection eliminates T cells that strongly react to self-Ags (Abbas, 2015). Surviving T cells then released into the blood and migrate to secondary lymphoid organs, where they encounter Ags through Ag presentation by professional APCs and are differentiated into various subsets with specific functions in immune response. After activation, T cells proliferate, differentiate, and migrate to the site of inflammation (Masopust & Schenkel, 2013). Helper CD4+ T (Th) cells secrete cytokines to activate and direct other immune cells in coordinating immune responses. They further differentiate into subsets such as Th1, Th2, and Th17 cells, each with distinct roles in immune regulation and response (Abbas, 2015). Regulatory T cells (Tregs) maintain immune tolerance and prevent autoimmune diseases by suppressing immune responses (Sakaguchi et al., 2008). Cytotoxic T cells (CD8+ T cells) recognize Ags presented by MHC-I molecules and directly kill infected or cancerous cells by secreting perforin and granzymes or inducing apoptosis via the Fas/FasL pathway (Dhein et al., 1995; Waring & Müllbacher, 1999). Memory T cells including tissue-resident memory T cells form after initial Ag exposure and provide a rapid and robust response upon subsequent encounters with the same Ag (Joshi et al., 2007; Kaech et al., 2003; Yenyuwadee et al., 2022).

Among the various biological functions of T cells is the ability to secrete cytokines. For instance, Th1 cells produce IFN-γ, which boosts the functions of DCs and MΦs. Tregs produce inhibitory cytokines such as IL-10 and transforming growth factor-beta (TGF-β), thereby suppressing excessive immune responses (Jiang et al., 2022). In addition to granzyme- and perforin-mediated target cell killing, CD8+ T cells can also secret cytokines such as TNF-α to exert their cytotoxic potential (Abbas, 2015). Therefore, the fate of T cells is determined by the interaction of the

TCR, the Ag presented in MHC and the surrounding cytokine milieu (Sharpe & Freeman, 2002).

2.1.3 Antigen presentation: the bridge between innate and adaptive immunity

The innate and adaptive immunity are two separate and at the same time intertwined entities that need to communicate to mount a successful response. The connecting link between innate and adaptive immunity is primarily Ag presentation of exogenous Ags via MHC-II and endogenous Ags via MHC-I, which is expressed by all nucleated cells, as well as Ag cross-presentation of exogenous Ags via MHC-I (Guermonprez et al., 2003; Rescigno et al., 1998).

Mature DCs, monocytes, and MΦs express a variety of PRRs, scavenger receptors, and TLRs to recognize and engulf pathogens, such as bacteria. Once these immune cells are activated through PAMPs, they process the engulfed foreign information in phagolysosomes producing smaller peptides of 13 to 25 amino acids long that can be presented on MHC-II molecules expressed on their surface. These Ag peptide-MHC-II complexes are then presented to CD4+ T cells, providing the first fundamental signal required for T cells activation through a process known as Ag presentation (Abbas, 2015).

In the endogenous Ag scenario, the endogenous proteins derived from viruses or altered proteins, in case of cancer, are broken down by the proteasome in the cytosol, generating peptide fragments of 8 to 10 amino acids long. These are subsequently transferred into the endoplasmic reticulum (ER), where they are loaded onto newly assembled MHC-I molecules that are then transported through the Golgi apparatus to the cell surface. The MHC-I Ag presentation pathway allows CD8+ T cells to identify infected or transformed cells by detecting foreign or altered self Ags displayed on MHC-I. Nonetheless, naïve Ag-specific CD8+ T cells cannot directly eliminate the infected or cancer cells. To acquire effector cytotoxic functions, CD8+ T cells must first be activated by professional APCs. When professional APCs are not infected themselves, they must capture external Ags from pathogens or tumor cells and present them via MHC-I, a process known as cross-presentation. Even though different types of APCs can cross-present Ags in vitro, most studies show that DCs are the main cross-presenting APCs in vivo (Bougnères et al., 2009; Jo et al., 2025; Jung et al., 2002; Theisen et al., 2018; Villadangos & Schnorrer, 2007).

More than a decade ago, it was demonstrated that both resident and migratory cDC1 are highly capable of processing external Ags and presenting them on MHC-I molecules (den Haan et al., 2000). Effective cross-presentation relies on two key features: (*1*) a cytosolic pathway with limited degradative potential (Delamarre et al., 2005; Lennon-Duménil et al., 2002) and (*2*) a mechanism termed vacuolar

pathway that permits Ags to be transferred from the phagosome into the cytosol, enabling their loading onto MHC-I (Figure 4) (Kovacsovics-Bankowski & Rock, 1995). The cytosolic pathway can be inhibited by proteasome inhibitors, indicating that internalized proteins enter the cytosol for proteasomal degradation (Kovacsovics-Bankowski & Rock, 1995). The resulting peptides can then move into the classical MHC-I pathway, in which transporter associated with antigen processing 1 (TAP1) and TAP2 deliver peptides into the ER for loading onto the new MHC-I molecules. However, there has not been any direct evidence for ER-based peptide loading yet. Instead, the presence of TAP and MHC-I-loading machinery on phagosome and endosomes suggests that loading may occur within endocytic compartments (Burgdorf et al., 2008; Guermonprez et al., 2003; Houde et al., 2003). In contrast, the vacuolar pathway resists proteasome inhibition and does not require TAP. Nonetheless, it can be blocked by lysosomal protease inhibitor, especially cathepsin S (CTSS) inhibitors (Bertholet et al., 2006; Shen et al., 2004). This indicates that both Ag processing and MHC-I loading take place in endocytic compartments. Recent work also shows that the proteasome-dependent cytosolic pathway can proceed without TAP, potentially via an unidentified alternative peptide transporter (Merzougui et al., 2011). Determining how much each pathway contributes to cross-presentation is challenging, because MHC-I molecules are retained in the ER and remain unstable or scarce in endosomes in both TAP-deficient cells and cells exposed to proteasome inhibitors (Chefalo et al., 2003; Day et al., 1995; Van Kaer et al., 1992). Current evidence favors the cytosolic, proteasome-dependent route.

cDC1 subsets are the “conductors” of the immune orchestra. Resident cDC1 possess both abilities and have been shown to be more efficient at cross-presentation than CD8- cDC1s in steady state (Shortman & Heath, 2010). In the spleen, these cells are enriched in Rac2, a GTPase that facilitates the maintenance of an alkaline phagosomal environment, reducing protease function and supporting the cross-presentation of external Ags (Savina et al., 2009). Resident cDC1 also express more genes related to MHC-I presentation than cDC2 (Dudziak et al., 2007). They also constitute the main source of IL-12 and IL-15, two cytokines involved in the differentiation of cytotoxic CD8+ T cells (Farrand et al., 2009; Hochrein et al., 2001; Maldonado-López et al., 1999; Mashayekhi et al., 2011; Mattei et al., 2001; Pulendran, 2004). Resident and migratory cDC1 are the only hematopoietic cells that express the chemokine receptor XCR1; its ligand (XCL1) is rapidly produced by CD8+ T cells upon Ag presentation by resident cDC1 and promotes the differentiation of cytotoxic CD8+ T cells (Dorner et al., 2009). Among migratory DCs, migratory cDC1s are the most efficient at cross-presenting skin-derived Ags in draining lymph nodes (Bedoui et al., 2009; Henri et al., 2010). In contrast, mouse pDCs are generally weak APCs (Villadangos & Young, 2008). Although they can

cross-present after TLR activation ex vivo, multiple studies have not confirmed a significant role for pDCs in cross-presentation in vivo (GeurtsvanKessel et al., 2008; Lee et al., 2009; Mouriès et al., 2008; Sapoznikov et al., 2007).

During infection, Ag-specific CD8+ T cells can be primed either through cross-presentation or through direct presentation of pathogen Ags when DCs become infected. The balance between these pathways depends largely on the type and tropism of pathogen (Hickman et al., 2008; John et al., 2009; Xu et al., 2010). DCs express numerous endocytic and phagocytic receptors, enabling uninfected DCs to acquire pathogen-derived Ags via a process called "cross-dressing". During this process, DCs obtain intact peptide-MHC-I complexes from infected cells that do not require further processing (Wakim & Bevan, 2011). Cross-dressing occurs through trogocytosis where peptide-MHC-I complexes are transferred through exosomes (Théry et al., 2009).

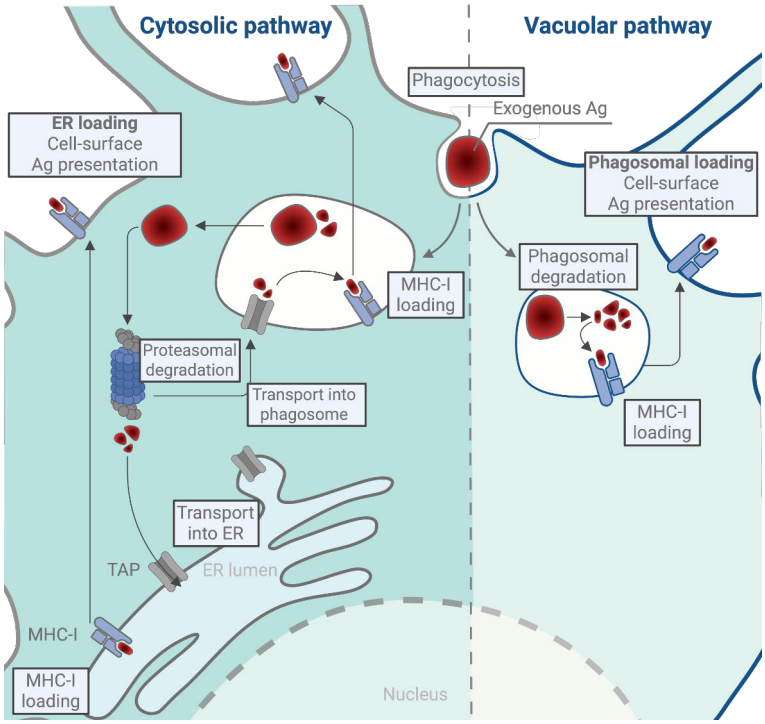


Figure 4. Antigen cross-presentation pathways. Following phagocytosis, exogenous Ags enter the cytosol for proteasomal degradation (cytosolic pathway, left). Resulting peptides are loaded onto MHC-I molecules either in the endoplasmic reticulum (ER loading) or within phagosome (cytosolic pathway with phagosomal loading). Recruitment of ER components, including TAP, to phagosomes is mediated by the SNARE protein SEC22B through interactions with syntaxin 4. Alternatively, Ags may be processed and loaded onto MHC-I molecules entirely within the phagosome (vacuolar pathway, right) (adapted from (Joffre et al., 2012) using BioRender).

In viral infection, resident cDC1s dominate cross-presentation to CD8+ T cells, while migratory DCs primarily transfer Ags from the site of inflammation to lymph nodes (Allan et al., 2003; Allan et al., 2006), implying that migratory DCs might hand off Ags to resident cDC1s for cross-presentation (Bedoui et al., 2009). For blood-borne pathogens, cross-presentation is performed exclusively by resident cDC1s (Cockburn et al., 2011; Lundie et al., 2008). The essential role of DCs in initiating antiviral cytotoxic T cell responses is highlighted by *Batf3* deficient mice, which lack both major cross-presenting DC subsets and fail to mount effective CD8+ T cell responses (Edelson et al., 2010; Hildner et al., 2008). In addition to MHC-TCR engagement, activated innate immune cells then upregulate costimulatory molecules, providing the second signal required for T cell activation. Furthermore, these cells secrete a variety of cytokines and chemokines to promote T cell activation, proliferation, and migration (Abbas, 2015). Activated DCs, as professional APCs, induce diverse T cell effector responses (Abbas, 2015).

In cancer, cross-presentation of tumor cell-associated Ags is less well understood. Even though DCs are present inside the tumor vicinity, it is not clear to which subsets these tumor-infiltrating DCs correspond. Cross-presenting DC subsets, resident and migratory cDC1s, have major role in antitumor immune responses (Hildner et al., 2008).

Ag presentation comprises the first fundamental signal for engagement with adaptive immunity; however, this is not enough for the generation of a T cell-mediated immune response. The interaction between innate and adaptive immunity also requires cytokine signaling and co-stimulation (Baxter & Hodgkin, 2002; Chen, 2004; Kapsenberg, 2003). Complex cytokine signaling pathways coordinate the immune response to pathogens while they maintain homeostasis. On one hand, innate immune cells recognize potential threats through PRRs, initiating the production of interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IFNs (Beutler, 2004; Koziczak-Holbro et al., 2007). These cytokines act as alert signals that trigger inflammation and activate adaptive immune cells. For instance, IL-1 and TNF-α facilitate the maturation and activation of DCs, which are pivotal for antigen presentation to T cells (Arroyo Hornero et al., 2025; Liu et al., 2021). Additionally, IFNs play a critical role in enhancing antigen presentation and promoting T cell differentiation to effector and memory cells. Therefore, the interaction between innate and adaptive immunity is mediated by these three crucial signals: (*1*) Ag presentation through MHC molecules, (*2*) co-stimulatory molecules, and (*3*) complex cytokine signaling.

2.1.4 The “fight-or-flight” decision of the immune system

In addition to TCR and cytokine signaling, T cells require costimulatory signals to be activated. These signals derive from various ligand-receptor interactions between T cells and professional APCs. Many receptors primarily provide co-stimulatory signals when engaged with their cognate ligands, while others deliver inhibitory signals. The CD28 and B7 families are among the best-described families of immunoglobulin superfamily. Members of these families interact with each other to provide co-stimulatory, “fight”, or co-inhibitory, “flight”, signals to T cells and determine the fate of immune responses. B7 family members are expressed mainly on the surface of APCs and CD28 family members are mainly expressed on T cells (Figure 5) (Chen & Flies, 2013).

Besides CD28 and B7 families, 4-1BB is a key co-stimulatory molecule, member of the TNF receptor superfamily, that promotes T cell activation, survival, and proliferation. Its ligand, 4-1BBL, expressed on APCs and other immune cells, binds to 4-1BB to sustain immune responses, particularly within tumors (Melero et al., 2023). In cancers, elevated 4-1BBL expression enhances antitumor immunity by stimulating T cell expansion and effector functions (Melero et al., 2023).

2.1.4.1 The CTLA-4/CD28 competition paradigm

B7-1 (CD80) and B7-2 (CD86) bind to the co-stimulatory receptor CD28 on T cells, which is characterized by an extracellular variable immunoglobulin-like domain. Other CD28 family members include inducible T cell co-stimulator (ICOS), CTLA-4, and PD-1 (Chen & Flies, 2013). The discovery of CD28 as a prototype co-stimulatory molecule supported the two-signal model of T cell activation (Bretscher & Cohn, 1970; June et al., 1987; Mueller et al., 1989). Although CD28 itself lacks immunoreceptor tyrosine-based activation motifs (ITAM), its cytoplasmic tail YMNM motif interacts with Scr homology 2 (SH2) domain to the regulatory subunit p85 of phosphoinositide 3-kinase (PI3K), initiating Akt activation and recruiting growth factor receptor-bound protein 2 (Grb2) (Boomer & Green, 2010; Prasad et al., 1994; Schneider et al., 1995). The CD28-PI3K-Akt pathway enhances T cell proliferation and survival through activation of the downstream targets, such as kappa-light-chain-enhancer of activated B cells (NF-κB), nuclear factor of activated T cells (NFAT), BCL-XL, mammalian target of rapamycin (mTOR), glucose transporter type 1 (GLUT1), and others (Boomer & Green, 2010). TCR/CD28 stimulation also induces Foxp3 by enabling NF-κB, AP-1, and NFAT binding to Foxp3 regulatory regions (Kane & Weiss, 2003; Soligo et al., 2011; Thaker et al., 2015; Vaeth et al., 2012).

CD80 and CD86 also bind to the co-inhibitory molecule CTLA-4 (CD125) (Chen & Flies, 2013; Freeman et al., 1993; Hathcock et al., 1993; Lenschow et al.,

1996; Linsley et al., 1991). Whereas CD28 amplifies TCR signaling after Ag recognition (Green et al., 1994; Shahinian et al., 1993), CTLA-4 acts as a brake on T cell activation (Lenschow et al., 1996; Schwartz, 1992). CTLA-4 has substantially higher affinity for both ligands than CD28, allowing it to suppress activation both by competing ligand binding and by delivering direct inhibitory signals (Egen & Allison, 2002; Linsley et al., 1994; Parry et al., 2005; Qureshi et al., 2011; Schneider et al., 2006; Schneider et al., 2002; Tivol et al., 1995). The precise signaling pathways used by CTLA-4 remain ambiguous. Studies have reported SHP2 association with CTLA-4 and modulation of TCR signaling in vitro, even though CTLA-4 does not contain a canonical immunoreceptor tyrosine-based inhibitory motif (ITIM) (Marengère et al., 1996; Teft et al., 2006). CTLA-4 also mediates inhibition independently of intracellular signaling by sequestering CD80 and CD86 from CD28 and by actively removing these ligands from the cell surface of APCs through trogocytosis (Qureshi et al., 2011; Tekguc et al., 2021). This mechanism is utilized by Tregs to inhibit T cell stimulatory activity of APCs (Tekguc et al., 2021).

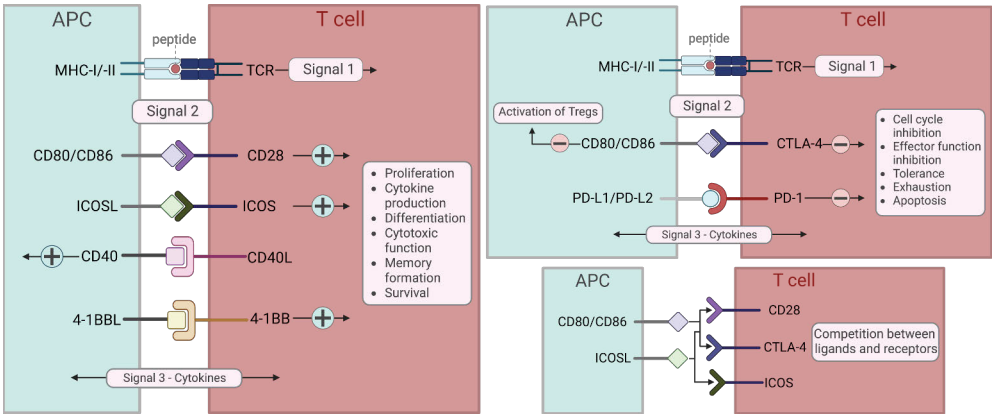


Figure 5. Co-stimulatory and co-inhibitory signaling in T cell regulation. Co-stimulatory molecules transmit activating signals to T cells through interactions with ligands expressed on APCs, with pathways, such as CD40/CD40L, operating bidirectionally (left). Co-inhibitory receptors deliver suppressive signals; CTLA-4 inhibits T cell activation by binding CD80/CD86 and may induce indoleamine 2,3-dioxygenase (IDO) and promote immune regulation (right, top). Many of these checkpoint molecule pathways unite with shared ligands and receptors, including competitive interactions highlighting the complexity of immune checkpoint regulation (right, bottom) (adapted from (Chen & Flies, 2013) using BioRender).

Besides the competing interactions of CD28 and CTLA-4 for CD80/CD86, a third ligand, B7-H2 or ICOSL, has been identified to bind CD28 and CTLA-4 in humans (Yao et al., 2011). ICOSL is broadly inducible in peripheral tissues, and it is known as binding partner for ICOS (Wang et al., 2000; Yoshinaga et al., 1999).

ICOS does not only support Th2 responses, as originally thought, but also promotes the expansion and function of Th1, Th17 and T follicular helper cells, as well as Tregs in a context-dependent fashion (Choi et al., 2011; Kroenke et al., 2012; Simpson et al., 2010). Although not required for Treg generation, ICOS promotes their proliferation and maintenance (Simpson et al., 2010). ICOS deficiency reduces memory T cells and impairs their reactivation (Simpson et al., 2010). In mice, ICOS-ICOSL interactions between activated T cells and APCs enhance T cell differentiation, survival, and inflammatory responses contributing to autoimmunity and anti-tumor immunity (Maazi et al., 2015; Mahajan et al., 2007; Peng et al., 2022; Raineri et al., 2024; Yoshinaga et al., 1999).

2.1.4.2 PD-1/PD-L1 interaction

Like CTLA-4, PD-1 inhibits T cell responses upon binding to PD-L1 or PD-L2 (Butte et al., 2007; Pardoll, 2012). PD-L1 expressed on cancer cells can also receive anti-apoptotic signals from PD-1 expressed on T cells (Azuma et al., 2008; Keir et al., 2008). Unlike CTLA-4, PD-1 contains both an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail (Ishida et al., 1992). Although both motifs appear to be phosphorylated following interaction with PD-L1, recent evidence indicate that the ITSM recruits SHP2, whereas the role of ITIM remains unclear (Ishida et al., 1992; Yokosuka et al., 2012). It was also found that PD-1 may also inhibit RAS and subsequently its downstream targets extracellular signal-regulated kinase 1 (ERK1) and extracellular signal-regulated kinase 2 (ERK2) through Src homology region 2 domain-containing phosphatase-2 (SHP-2)-independent mechanism to inhibit cell cycling (Patsoukis et al., 2012). Interestingly, PD-L1 binding to PD-1 on Tregs has been shown to inhibit Treg suppressive functions, whereas PD-1 ligation on conventional T cells has been shown to promote their differentiation into induced Tregs (Amarnath et al., 2010; Amarnath et al., 2011; Franceschini et al., 2009; Francisco et al., 2009). During the effector T cell phase many co-inhibitory receptors such as LAG3 and TIM3 are expressed to limit both CD4+ and CD8+ T cell responses (Baitsch et al., 2012).

PD-L1-PD-1 interactions are central in CD8+ T cell exhaustion, as blockade of PD-L1-PD-1 restores CD8+ effector functions following chronic infection or in the TME, whereas blockade of other co-inhibitory pathways alone in these models is less effective in rescuing T cells (Fourcade et al., 2012). However, combined blockade of PD-1 with other co-inhibitors, such as TIM3, CTLA-4 and LAG3, shows synergistic effect in reversing exhaustion (Goldberg & Drake, 2011; Sakuishi et al., 2011).

These immune checkpoint pathways are essential for central and peripheral tolerance (Okazaki & Honjo, 2006). Evidence suggests that a fine line exists between

T cell anergy and T cell exhaustion, with exhaustion representing a programmed state of anergy that develops to limit extensive pathology during chronic Ag exposure in viral infection and cancer (McAfee & Blattman, 2012). Gene-expression analyses reveal both overlap and distinction between anergic and exhausted T cells (Van Der Byl et al., 2024).

In addition to co-inhibitory receptors like CTLA-4 and PD-1, lymphocyte-activation gene 3 (LAG3), expressed on exhausted T cells, B cells, NK cells, and DCs, as well as T cell immunoreceptor with Ig and ITIM domains (TIGIT), also known as VSIG9, and expressed on T cells and NK cells, suppress T cell activation, proliferation and effector functions (Chen & Flies, 2013). Both LAG3 and TIGIT blockade are considered as major strategies in cancer immunotherapy (Chen & Flies, 2013). Therapeutic blockade of co-inhibitory molecules with antagonistic Abs is a major approach in cancer immunotherapy and will be discussed in later chapters of this dissertation.

2.2 Cancer

Cancer was first documented 4600 years ago by the Egyptian physician Imhotep. Lacking knowledge of cellular biology, the Greeks, particularly Hippocrates, believed the illness stemmed from an imbalance of black bile. In 440 BC, Herodotus described the first known excision of a breast tumor, performed on the queen of Persia, Atossa. Since then, tremendous progress has been made in diagnosing and treating the disease, from surgical tumor removal using diverse techniques to the application of X-rays for therapy, targeted therapy, and cytostatic agents. Moreover, scientific research has greatly advanced our understanding of the disease's complexity. Yet, cancer remains a formidable challenge in medicine and biomedical science, continuing to cause significant mortality worldwide. By the 20th century, it had become the second leading cause of death, following cardiovascular diseases, with 10,000,000 people dying of cancer each year. The following chapters of this dissertation will present the current scientific understanding of cancer, with emphasis on cutaneous and uveal melanoma. Attention will be given to the role of the immune system in cancer and the therapeutic strategies that harness immune mechanisms for treatment.

2.2.1 Hallmarks of cancer

The concept of the hallmarks of cancer describes the fundamental biological processes through which healthy cells acquire genetic alterations and transform into cancer cells. Hanahan and Weinberg first proposed six core capabilities that are shared by most human tumors. These traits describe how cancer cells can proliferate

relentlessly, resist apoptosis, and spread beyond the site they originate from (Hanahan & Weinberg, 2000). The original hallmarks of cancer are:

1. Sustain proliferative signaling by producing growth factors and expressing the corresponding receptors allowing the induction of an autocrine proliferation loop.
2. Evade growth suppressors by proliferating continuously. Another characteristic of cancer cells is the loss-of-function mutations in genes that code for proteins responsible for downregulating the cells' proliferation, resulting in irregular cell growth and division.
3. Activate invasion and metastasis. While a malignancy is developing, cancer cells adhere to other cells and extracellular matrix (ECM). Once the cancer cells penetrate the ECM, they escape and invade locally. Sequentially, cancer cells can be transferred through the neighboring blood and lymphatic vessels to distant tissues and create metastases. The end of the invasion-metastasis cascade is when macroscopic tumors colonize and adapt to the tissue they metastasize.
4. Enable replicative immortality by upregulating telomerase, an enzyme that elongates telomeres and prolongs division.
5. Induce vasculature. The vascular system is of major importance since it supplies all organs with nutrients and oxygen throughout life. Nutrients and oxygen are also required in tumors and for this reason tumors trigger the sprouting of new vessels known as angiogenic switch to receive nutrients and expand.
6. Resist cell death by evading programmed cell death due to loss-of-function mutations in the genes coding for proteins that regulate apoptosis. For instance, p53 serves as a damage sensor and in return to DNA damage can initiate programmed cell death.

These six traits alone did not fully address the complexity of cancer development. Therefore, Hanahan and Weinberg introduced two enabling characteristics underlying the hallmarks of cancer (Hanahan & Weinberg, 2011):

7. Genome instability and
8. Tumor-promoting inflammation

Moreover, in 2011 the hallmarks of cancer were revisited, and two emerging hallmarks further refined our understanding of tumor development (Hanahan & Weinberg, 2011):

9. Reprogramming of cellular metabolism and

10. Active evasion of immune destruction

Most recently, in 2022, Hanahan expanded the model by adding two new hallmarks and two new enabling characteristics (Hanahan, 2022).

Hallmarks:

11. Unlocking phenotypic plasticity/blocking differentiation
12. Cellular senescence

Enabling characteristics:

13. Non-mutational epigenetic reprogramming
14. Polymorphic microbiomes

2.2.2 Uveal and cutaneous melanoma

Uveal melanoma (UM) is one of the most aggressive intraocular malignancies, arising from aberrant extracutaneous melanocytes within the uveal tract (Carvajal et al., 2023; A. Han, Z. T. Schug, et al., 2021). Most cases affect the choroid, while fewer involve the iris or ciliary body (Figure 6**)** (Augsburger et al., 2002; Augsburger et al., 2011; Augsburger et al., 1989; Damato & Damato, 2012). UM primarily affects fair-skinned adults of older age, except for iris melanomas, which often occur in younger individuals (Damato & Damato, 2012; Jager et al., 2020; Shain et al., 2019; Shields et al., 2001). Finland shows one of the world's highest UM incidences with about 60-70 new cases annually (~8.8/million), reflecting a north-to-south gradient strongly linked to latitude, with greater frequency in less pigmented populations and adults aged 40-80 (Wu et al., 2023).

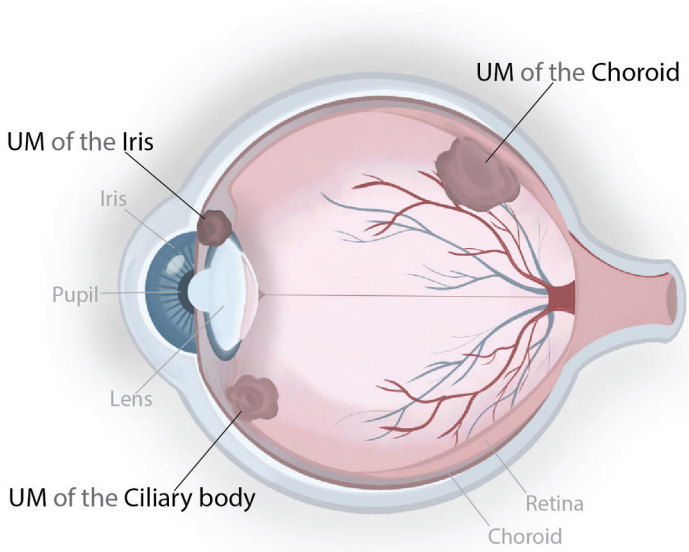


Figure 6. Development of UM. UM arises from melanocytes in the pigmented uveal tissues of the eye; the uvea is made up of the iris, ciliary body and choroid. (own illustration created using Adobe Fresco).

Environmental stressors and genetic predisposition contribute to the development of cutaneous melanoma. This cancer type accounts for only 4% of all skin cancers. Incidence varies internationally. In Europe, cutaneous melanoma is the seventh most incident cancer in men, while in women is the fifth in rank with the highest incidence countries being Nordic countries (Brochez et al., 2025). By late 2023, Finland recorded 12,324 women and 11,454 men living with cutaneous melanoma, making it the 4th and 3rd most prevalent cancer type in these groups, respectively (Figure 7). Incidence peaked in the mid-2010s, with stable mortality in women since the 70s and slower mortality growth in men. The current 5-year overall survival is ~95% but heavily dependent on the stage of the disease.

2.2.2.1 Risk factors

Risk factors for UM include uveal naevi (Eskelin & Kivelä, 2002; Kivelä & Eskelin, 2006; Singh et al., 2005), light-color irides (Houtzagers et al., 2020; Saornil, 2004), congenital ocular melanocytosis (Singh et al., 1998), melanocytoma (Reidy et al., 1985), and neurofibromatosis(Jager et al., 2020; Singh et al., 1998). Some iris melanomas show ultraviolet (UV) radiation-induced mutations, although the role of sunlight remains uncertain (Dhomen et al., 2021; Hayward et al., 2017; P. A. Johansson et al., 2020). Germline mutations in Breast-Cancer-Associated-1 (BRCA1)-Associated Protein 1 (*BAP1*) gene located on the short arm chromosome

3 (3p) and BRCA2 on the long arm of chromosome 13 (13q) predispose carriers to UM and other cancer types (Cheung et al., 2013; Johansson et al., 2019; Walpole et al., 2018).

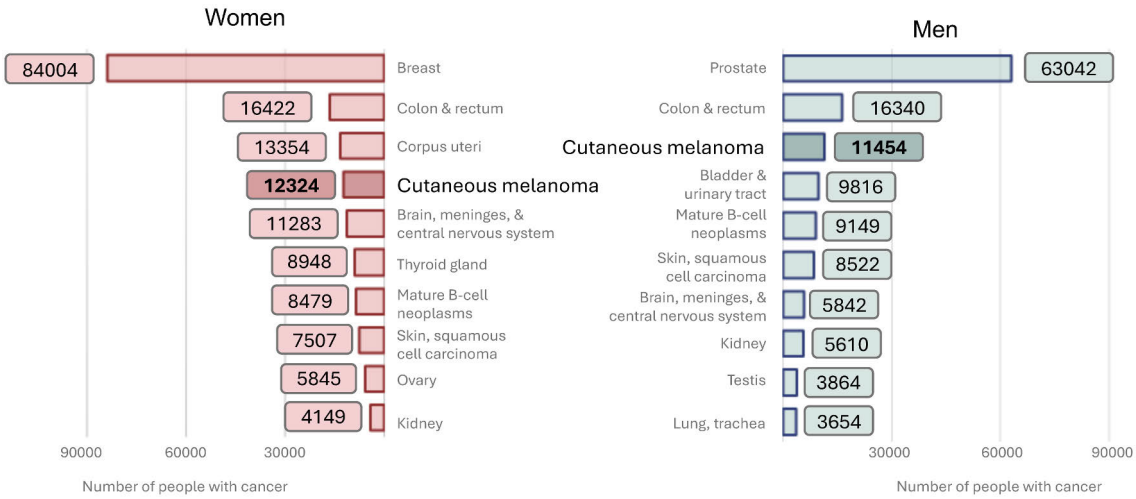


Figure 7. Number of people living with cutaneous melanoma in Finland at the end of 2023. (adapted from syoparekisteri.fi using Microsoft Excel & PowerPoint).

For cutaneous melanoma, major risk factors include family history, multiple or atypical nevi, previous melanoma, immunosuppression, UV radiation sensitivity, and UV exposure. In addition, germline mutations in cyclin-dependent kinase inhibitor 2A (*CDKN2A*) are rare but established cause of hereditary melanoma, occurring predominantly in a subset of high-risk melanoma families, while germline mutations in cyclin-dependent kinase 4 (CDK4) have also been linked to melanoma predisposition (Aitken et al., 1999; Miller & Mihm, 2006). UV radiation-induced somatic mutations affect DNA repair, impair immune functions in the skin, augment growth factors production, and induce oxidative stress, leading to keratinocyte and melanocyte damage (Gilchrest et al., 1999; Thompson et al., 2005). The tanning response, melanin transfer from melanocytes to keratinocytes, serves as a photoprotection mechanism, where UV energy is absorbed and dissipated (Gilchrest et al., 1999). UV exposure triggers α-melanocyte–stimulating hormone (α-MSH) binding to melanocortin receptor 1 (MC1R), stimulating melanin synthesis. Nonetheless, MC1R germline polymorphisms, common in fair individuals, reduces receptor activity and increases melanoma risk (Frändberg et al., 1998; Kennedy et al., 2001; Naysmith et al., 2004; Valverde et al., 1995). Clinically, variations in the pigmentation of the skin and the tanning response to UV are associated with variations in susceptibility to melanoma (MacKie, 2002; Marks, 2000). Tanning

response is dose dependent. Intense and intermittent sun exposure, especially to individuals with frequent sunburns, poses higher melanoma risk compared to low-grade sun exposure, which may induce adaptive protection against DNA damage (Gilchrest et al., 1999).

2.2.2.2 Uveal and cutaneous melanoma hallmarks

Recent advances have partially clarified the molecular landscape of UM. Unlike cutaneous melanoma, posterior UM rarely harbors *BRAF* mutations and exhibits a low mutational burden (Robertson et al., 2018; Royer-Bertrand et al., 2016). However, about 85% of UM carry mutations in guanine nucleotide-binding protein G(q) subunit alpha q (*GNAQ*) or guanine nucleotide-binding protein subunit alpha-11 (*GNA11*) (Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010), which aid the diagnosis and exclusion of cutaneous melanoma in metastatic setting, but have limited prognostic value (Bauer et al., 2009; Koopmans et al., 2013). Less common initiating mutations occur in cysteinyl leukotriene receptor 2 (*CYSLTR2*) and phospholipase C beta 4 (*PLCB4*) (Hou et al., 2020; Johansson et al., 2016; Moore et al., 2016). Cytogenic alterations such as monosomy 3, loss of BAP1, and mutations in the splicing factors Serine and Arginine Rich Splicing Factor 2 (*SRSF2*)/spliceosome factor 3b1 (*SF3B1*) and the translation initiation factor Eukaryotic Translation Initiation Factor 1A (*EIF1AX*), influence the metastatic risk (Furney et al., 2013; Krantz et al., 2017; Robertson et al., 2018; Royer-Bertrand et al., 2016).

Monosomy 3 remains primary UM's most significant prognostic marker, because it correlates with risk of death cause by metastasis (Horsman et al., 1990; Prescher et al., 1990; Sisley et al., 1992). Loss of chromosome 3 reduces the 5-year survival of stage I UM from nearly 100% to about 50% (Kivelä & Kujala, 2013; Kujala et al., 2013). Additionally, loss of chromosome 1 and gain of chromosome 8 predict poor survival outcomes (Caines et al., 2015; Dogrusöz et al., 2017; Dogrusöz et al., 2020; Patel et al., 2001; Robertson et al., 2018; Royer-Bertrand et al., 2016; Sisley et al., 2000).

In cutaneous melanoma, the Clark model describes the stepwise transformation from melanocytes to malignant cells via nevi formation, dysplasia, hyperplasia, invasion, and metastasis (Clark et al., 1984). Multiple molecular mechanisms contribute to melanoma pathogenesis(Alonso et al., 2004; Curtin et al., 2005). The initial step of melanocytic transformation is the development of benign nevi composed of neval melanocytes (Figure 8) (Clark et al., 1984). Aberrant activation of MAPK/ERK pathway, driven by *NRAS* (15%) and *BRAF* (50%) mutations, promotes proliferation (Brunet et al., 1999; Davies et al., 2002; Lin et al., 1998; Omholt et al., 2003; Welsh et al., 2001). BRAF mutation, especially replacement of

valine at position 600 in the BRAF proteins by glutamic acid (V600E), is the most prevalent in melanoma from intermittently sun-exposed skin leading to uncontrolled cell growth (Maldonado et al., 2003; Wan et al., 2004).

Despite similar *BRAF* mutation frequencies in nevi and melanomas (Pollock et al., 2003), additional genetic modifications are required for malignancy. In zebrafish, mutant *BRAF* induces melanocyte hyperplasia, while concurrent *p53* loss leads to melanoma (Patton et al., 2005). In humans, mutant BRAF triggers cellular senescence by upregulating the cell-cycle inhibitor of kinase 4A (INK4A) (Michaloglou et al., 2005). This inhibitor suppresses the excessive proliferation driven by BRAF activation. However, cell-cycle arrest mediated by INK4A can be bypassed through mutations in INK4A itself or in other regulatory cell-cycle genes (Michaloglou et al., 2005).

2.2.2.3 Metastasis and prognosis

Metastasis occurs when tumor cells detach from the primary site, infiltrate the surrounding stroma, and enter blood or lymphatic vessels to colonize distant organs.

UM exhibits a strong propensity for hepatic dissemination, which may occur at any time, from the initial diagnosis to decades later (Coupland et al., 1996; Kujala et al., 2003; Marshall et al., 2013). Approximately 50% of patients develop liver-only metastasis, and 90% of those also present metastases elsewhere (bowel, bone, lung, lymph nodes) (Lorigan et al., 1991; Rodrigues et al., 2019). The likelihood of metastasis depends on the tumor's site in the eye, morphology, and genetic alterations described above (Damato, 2012; Damato et al., 2011; Jager et al., 2020). Most UM metastases arise from choroidal and/or ciliochoroidal UM, while iridal UM spreads rarely (Figure 6) (Stadigh et al., 2017).

UM staging follows the American Joint Committee on Cancer (AJCC)/ Tumor-Node-Metastasis (TNM) system (Amin et al., 2017; Kujala et al., 2013). The TNM categories are determined by the anatomical extent of disease, including the primary tumor (T), regional lymph node metastasis (N), and distant metastases (M). In ophthalmic malignancies, the T categories are defined by size of the primary tumor and any invasion into periocular structures (Kivelä & Kujala, 2013). Specifically, classification is based on the largest basal diameter and tumor thickness, alongside ciliary body involvement and extraocular extension. Tumors are assigned to size categories ranging from small (T1) to very large (T4) (Keung & Gershenwald, 2018; Mellen et al., 2013). For example, ciliary body and choroid UM with a diameter ≤12 mm and thickness ≤3 mm, or a diameter ≤9 mm and thickness between 3.1-6 mm, are classified as category T1 (Keung & Gershenwald, 2018; Mellen et al., 2013). Each T category may be further subdivided using lowercase letter suffixes (Kivelä & Kujala, 2013). Tumor thickness strongly correlates with metastatic risk and is

incorporated into the AJCC/TNM staging system (Keung & Gershenwald, 2018; Mellen et al., 2013). In a cohort of 8033 patients, a 10-year metastasis rate was 5% for a 1-mm-thick UM, for a 2-mm-thick UM was 10%, and that for a 6-mm-thick UM was 30%(Shields et al., 2009). When 7621 UM cases were grouped into small (0-3 mm thick, 29.8%), medium (3.1-8 mm thick, 49%), or large (>8 mm thick, 20.9%) tumors, the 10-year rates of detecting metastases were 11.5%, 25.5%, and 49.2%, respectively (Shields et al., 2009). The median interval between diagnosis and metastasis detection is 2.6-3.5 years (Diener-West et al., 2004). Our knowledge of the underlying biology and genomics of metastatic UM has increased over the past 5 years but is not as advanced as that of primary UM, due to metastatic UM sample scarcity (Field et al., 2018; Figueiredo et al., 2020; Grossniklaus et al., 2016; J. Johansson et al., 2020; Krishna et al., 2020; Liao et al., 2018; McCarthy et al., 2016; Royer-Bertrand et al., 2016; Shain et al., 2019).

Moreover, the Liverpool Uveal Melanoma Prognosticator Online (LUMPO) is an online tool that predicts mortality based on tumor size, ciliary body involvement, mitotic count, monosomy 3, and extraocular extension and it has been validated across Europe and the US (Damato et al., 2011; DeParis et al., 2016). Prognosis after metastasis remains poor, with median survival of 2-12 months and a 1-year survival for 10-15% (Jager et al., 2020). Liver metastasis is the principal cause of death and while recent trials suggest modest survival improvements with new therapies, outcomes remain disappointing (Carvajal et al., 2017; Nathan et al., 2021; Sacco et al., 2018; Sussman et al., 2020; Yang et al., 2018).

Metastasis in cutaneous melanoma is likewise linked to altered cell adhesion. Normally, adhesion regulated cell migration and tissue integrity (Johnson, 1999). In cutaneous melanoma, the loss of E-cadherin and expression of N-cadherin marks the transition from radial to vertical growth (Danen et al., 1996; Hsu et al., 1996; Scott et al., 1997). This shift enables melanoma cells to interact with N-cadherin-expressing dermal fibroblasts and vascular endothelial cells, thereby promoting invasion and metastatic progression (Hsu et al., 2000). Aberrant N-cadherin also enhances survival via β-catenin signaling (Li et al., 2001; Qi et al., 2005). Common metastatic sites include lymph nodes, other skin sites, lungs, liver, bones, and brain. BRAF mutations occur at similar frequencies in nevi, primary, and metastatic lesions (Pollock et al., 2003).

Understanding the distinct metastatic behavior and molecular mechanisms of UM and cutaneous melanoma is essential for guiding therapeutic strategies, which are discussed next.

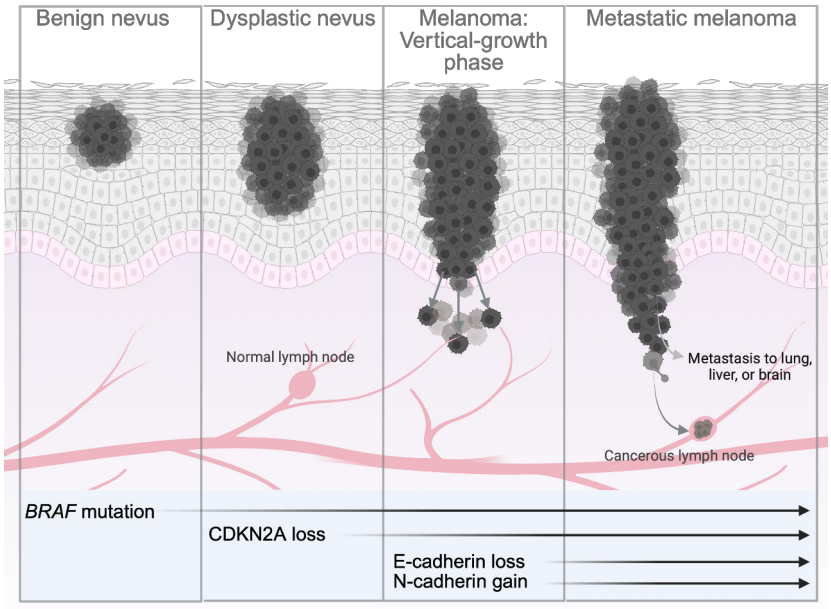


Figure 8. Biological and molecular alterations during melanoma progression. Melanoma development begins with *BRAF* mutation and MAPK pathway activation in benign nevi. Dysplastic nevi display cytologic atypia linked to alterations in CDKN2A pathways. Disease progression is marked by reduced differentiation and diminished expression of melanocytic markers. Vertical melanoma growth and metastatic stages are associated with changes in cell adhesion, including loss of E-cadherin and increased N-cadherin expression. (adapted from (Miller & Mihm, 2006) using BioRender).

2.2.2.4 Treatment options

Primary goals in therapy of UM are tumor eradication, preservation of vision, and prevention of metastasis. Enucleation is still indicated for primary melanoma. Therapeutic planning considers the tumor size, ocular function, patient age, health status, and presence of metastasis (Kaliki & Shields, 2017). Treatment options for metastatic UM remain limited (Kaliki et al., 2015; Singh et al., 2018). Conventional systemic chemotherapy has shown minimal efficacy, reflecting the inherent resistance of UM to cytotoxic agents. However, combination of Treosulfan and Gemcitabine demonstrated median survival of 14 months (Pföhler et al., 2003) but is associated with significant hematologic, neurologic, and pulmonary toxicity, which limits its use (Săftescu et al., 2020). Liver-directed therapies, such as intra-arterial Fotemustine and isolated hepatic perfusion, have yielded encouraging results (Artzner et al., 2019; Gonsalves et al., 2015; Leyvraz et al., 2014; Siegel et al., 2007). Although immunotherapy alone offers limited benefits, its combination with these treatments has improved overall survival to approximately 19 months (Najjar et al., 2020; Pelster et al., 2021).

A major advance in the treatment of metastatic UM was the development of tebentafusp (Kimmtrak), a first-in-class T-cell receptor-bispecific fusion protein, specific for glycoprotein 100 (gp100) and CD3, that redirects T cells to kill gp100-positive melanoma cells via MHC-I (HLA-A02:01) presentation. It is currently the established first-line systemic standard of care for HLA-A02:01-positive patients with unresectable or metastatic UM (Montazeri et al., 2023), who comprise approximately 45-50% of the patient population (Nathan et al., 2021). In phase III IMCgp100-202 trial (NCT03070392), tebentafusp significantly prolonged overall survival compared with control therapy, which was pembrolizumab, ipilimumab, or dacarbazine. Tebentafusp received FDA approval in 2022 and has demonstrated durable survival benefit in long-term follow-up studies (Carvajal, Butler, et al., 2022; Carvajal, Nathan, et al., 2022; Eteghadi et al., 2024; Hassel et al., 2023; Nathan et al., 2021).

In cutaneous melanoma, surgical excision remains the cornerstone for Stage I disease, followed by regular follow-up due to risks of metastasis (Rogers et al., 1986). A subset of patients with Stage I melanoma has microscopic metastases in nonpalpable lymph nodes (Day et al., 1981; Koh et al., 1986) and elective regional-node dissection improves prognosis. Following surgery, adjuvant therapy prolongs disease-free survival. Adjuvant strategies include chemotherapy, targeted agents such as Vemurafenib, Dabrafenib, and Trametinib, radiation therapy, and immunotherapy.

To better understand why immunotherapy does not achieve complete remission in these melanoma types, the following chapters will explore the role of the immune system in cancer, the evolution of immunotherapy, and its current limitations.

2.3 The immune system in cancer and blockade of immune checkpoints

2.3.1 The Cancer-Immunity cycle

The Cancer-Immunity cycle described the seven fundamental steps required for an effective immune response against tumors (Figure 9) (Chen & Mellman, 2013). The process starts when tumor-associated neoantigens, released from necrotic cancer cells, are captured and processed by DCs (1 in Figure 9)(Ferguson et al., 2011). To trigger immunity rather than tolerance, this Ag release must take place alongside immunostimulatory signals, including proinflammatory cytokines (e.g., IL-1, IFN-α), CD40/CD40L interaction, endogenous danger signals that activate the cyclic guanosine monophosphate (GMP)-AMP synthase (cGAS) and stimulator of interferon genes (STING) pathway, or microbial products acting as TLR ligands derived from the gut microbiota (Lippitz, 2013; Mellman et al., 2011: *Nature*).

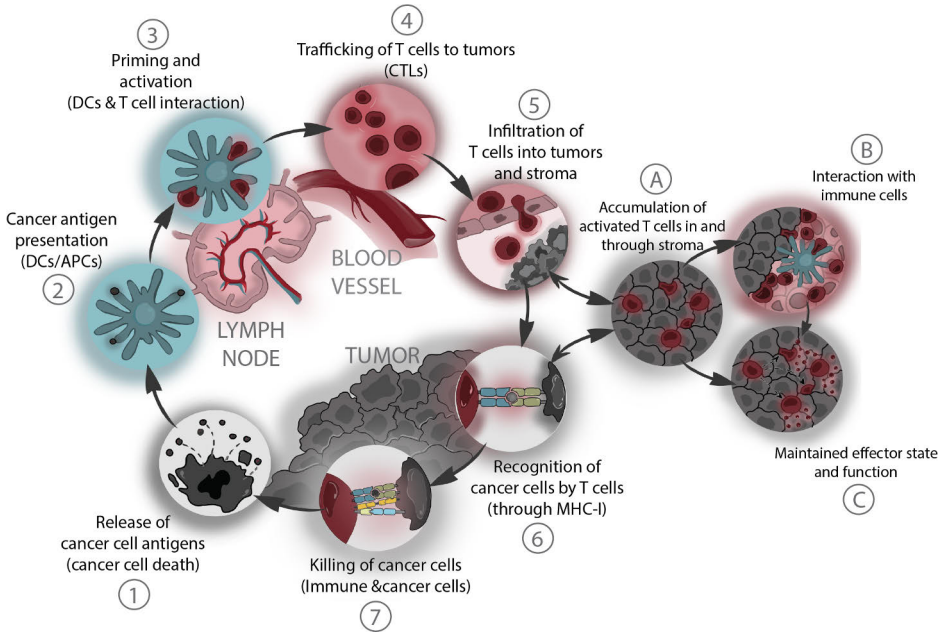


Figure 9. The cancer-immunity cycle and the TME subcycle. Advances in cancer immunology emphasize the importance of T cell trafficking, persistence, and function within the TME (A-C). Within tumors, immune responses proceed through a localized cancer-immunity subcycle that mirrors systemic antitumor immunity. Effective immunity enables T cell activation, expansion, and tumor cell killing (steps 1-7), whereas immunosuppressive cells, stromal barriers, metabolic reprogramming, and T cell dysfunction can interrupt this cycle and limit antitumor responses (adapted from (Mellman et al., 2023) using Adobe Fresco).

DCs then present these Ags on MHC-I and MHC-II molecules to naïve T cells (2 in Figure 9), promoting their priming and activation (3 in Figure 9). Besides Ag presentation, this process depends on co-stimulatory molecules interaction with their cognate receptors, such as CD28-CD80/CD86 interaction, and cytokines such as IL-2, essential for T cell proliferation, and IL-12, produced by cDC1s and boosting cytotoxic activity and immune memory (Franciszkiewicz et al., 2012; Garris et al., 2018; Wculek et al., 2020). The ratio of effector and regulatory T cells determined the nature of the response. Activated effector T cells migrate toward the tumor (4 in Figure 9) in response to chemokines such as CCL5, and IFN- γ-inducible C-X-C motif chemokine ligand 9 (CXCL9) and CXCL10, infiltrate the tumor stroma (5 in Figure 9), and recognize cancer cells via TCR-MHC-I-Ag interactions (6 in Figure 9) (Dangaj et al., 2019; Franciszkiewicz et al., 2012). Cytotoxic T cells then kill their targets by releasing perforin and granzyme B (GrB) (7 in Figure 9) (Agnello et al., 2003; Bhat et al., 2017; Lighvani et al., 2001), leading to the release of new Ags that sustain the cycle.

The Cancer-Immunity cycle often functions inadequately. Tumor Ags may go undetected, DCs and T cells may interpret them as "self", leading to tolerance, or effector T cells may fail to reach or penetrate the TME. Most critically, immunosuppressive factors within the TME can inhibit T cell functions (Motz & Coukos, 2013).

2.3.2 Tumor immunotypes

Tumor exhibit distinct immune phenotypes, also known as "immunotypes", classified as inflamed ("hot tumors"), excluded or desert ("cold tumors") (Binnewies et al., 2018; Chen & Mellman, 2017; Erdag et al., 2012; Herbst et al., 2014). Inflamed tumors contain abundant immune infiltrates; excluded tumors confine T cells to the stroma; and desert tumors completely lack immune cells (Zheng et al., 2024). Although these categories simplify a dynamic process that evolves with tumor progression and therapy, they remain a valuable framework for understanding immune responsiveness (Shen et al., 2024; Wu et al., 2024). Different cancers show different dominant immunotypes. Melanoma is typically inflamed, and uveal melanoma is immune excluded (Figueiredo et al., 2020; Kalaora et al., 2022).

2.3.2.1 The role of the TME

The TME, including immune and stromal compartments, plays a decisive role in shaping the tumor immunotypes (Caligiuri & Tuveson, 2023; Davidson et al., 2021; van Vlerken-Ysla et al., 2023). Monocytes, granulocytes, NK cells, and cancer-associated fibroblasts (CAFs) collaborate to form a fibrotic, collagen-rich stroma that physically impedes T cell infiltration and suppresses the functions of these cells (Mariathasan et al., 2018). Moreover, the TME can paradoxically enhance antitumor immunity by generating peri-tumoral lymphoid aggregates or tertiary lymphoid structures (TLSs), which are associated with improved T cell responses (Fridman et al., 2023). The presence and composition of TLSs are emerging as key predictors of better clinical outcomes and immunotherapy success.

2.3.2.2 T cell dysfunction and checkpoint regulation

Chronic Ag exposure promotes the accumulation of exhausted T (Tex) cells within tumors (Hashimoto et al., 2018). Viable but dysfunctional Tex populations are characterized by high expression of PD-1, LAG3, TIM3, and TIGIT. These receptors are also markers of T cell activation. Although blocking these coinhibitory receptors was initially believed to fully restore function (Wherry & Kurachi, 2015), Tex cells often possess irreversible epigenetic modification (Delacher et al., 2021; Philip et

al., 2017; Sen et al., 2016), suggesting that blockade of these molecules may instead prevent rather than reverse exhaustion.

Interestingly, experimental evidence from murine tumor models suggests that PD-L1 expressed by Ag-presenting DCs plays a prominent role in restraining antitumor T cell responses, particularly during T cell priming and early expansion, potentially complementing or exceeding the contribution of tumor cell-derived PD-L1 in this context (Oh et al., 2020). These findings support the concept that PD-1/PD-L1 blockade may act at early stages of T cell differentiation, maintaining effector functions and not reactivating terminally exhausted cells. Consistent with this, DCs are essential not only for priming and activating T cell responses in the draining lymph nodes (dLNs) but also for sustaining and regulating T cell activity within the TME (Sharma et al., 2023).

2.3.2.3 The Cancer-Immunity subcycle

Due to all these advances, the Cancer-Immunity cycle has been revised to include a TME-dependent subcycle, where DCs and T cells interact directly within the tumor parenchyma or TLSs (A, B, C in Figure 9). Upon infiltrating the tumor, T cells encounter local DCs that further drive T-cell differentiation into effector, memory, or Tex subsets. These interactions sustain local cytotoxic activity and shape ongoing immune dynamics. This refined model highlights the dual role of the TME in both supporting and suppressing antitumor immunity.

2.3.3 Cancer immunoediting

The Cancer-Immunity cycle outlines the steps required for effective antitumor responses. Besides its host-protective role, the immune system also shapes tumor evolution through cancer immunoediting, which consists of the elimination, equilibrium, and escape phases (Dunn et al., 2002; Dunn et al., 2004a, 2004b).

The elimination phase is summarized by the seven steps of the Cancer-Immunity cycle. Briefly, the immune system detects and destroys transformed cells through cancer immunosurveillance, a concept proposed by Paul Ehrilch (Dunn et al., 2004a). Yet, the ongoing deletion of cancer cells that display T cell targets may enable some tumor cells evade destruction and enter the equilibrium phase, remaining dormant while acquiring mutations that promote resistance. Eventually, resistant clones emerge and progress entering to the escape phase. This phase is characterized by immune evasion that occurs through multiple mechanisms, including loss of Ag recognition, resistance to immune-mediated killing, the establishment of an immunosuppressive TME induced by chronic inflammation or altered metabolism.

2.3.3.1 Immunosuppressive cells

Tumors suppress immune responses by secreting immunomodulatory cytokines such as TGF-β, IL-10 and the pro-angiogenic growth factor, vascular endothelial growth factor (VEGF), that inhibit immune activation (Barbera-Guillem et al., 2002; Batlle & Massagué, 2019; Briukhovetska et al., 2021; Goel & Mercurio, 2013). TGF-β suppresses T and NK cell proliferation and activation and promotes Tregs differentiation, reinforcing immune tolerance (Ma et al., 2024; Thomas & Massagué, 2005; Tran, 2012; Viel et al., 2016; Wang et al., 2023). IL-10 dampens MΦs and DCs cytokine production, hence blocking T cell activation while sustaining an anti-inflammatory state (Oft, 2014).

Tumors actively recruit cells with immunosuppressive phenotypes such as Tregs, myeloid-derived suppressor cells, and tumor-associated macrophages (TAMs) that participate in immune evasion (Iglesias-Escudero et al., 2023; Li et al., 2024; Qian & Pollard, 2010). TAMs are attracted by CCL2 and CSF-1 produced by the tumor (Xu et al., 2025). These cells have distinct characteristics from conventional MΦs and represent the most abundant immune cell type in the TME (Lopez-Yrigoyen et al., 2021). For instance, in primary UM Tregs and TAMs represent most immune cells within the TME (Figueiredo et al., 2020). Their phenotype is a spectrum of functional states due to their remarkable plasticity, which enables them to orchestrate inflammatory responses, then shift to tissue repair and homeostasis (Mantovani et al., 2011). M1-like and M2-like TAMs represent the two ends of a functional spectrum. The M1-like TAMs are induced by IFN-γ while M2-like are IL-4-induced (Geeraerts et al., 2021). M1-like TAMs produce TNF and IL-12 and promote inflammation, trigger antitumor immunity, and normalize tumor vasculature (Boutilier & Elsawa, 2021). In contrast, M2-like TAMs contribute to the immunosuppressive milieu with TGF-β and IL-10 production dampening immune activation, promoting angiogenesis, matrix remodeling and metastasis (Basak et al., 2023; Cassetta & Pollard, 2018).

2.3.3.2 Metabolic reprogramming

Metabolic rewiring also supports immune escape in cancer (Arner & Rathmell, 2023; Nicolini & Ferrari, 2024). Tumor cells preferentially use aerobic glycolysis (the Warburg effect), competing with immune cells for glucose and glutamine, which impairs immune cell differentiation and function (Enríquez & Mittelbrunn, 2024; Herbel et al., 2016; Liberti & Locasale, 2016; Xia et al., 2021). Lipid metabolism further influences immune suppression and TAM polarization (Cao et al., 2022; Netea-Maier et al., 2018; Su et al., 2020). TAMs enhance fatty acid uptake, biosynthesis, and storage, often mediated by the scavenger receptor CD63 that facilitates fatty acids transport and lipid droplets (LDs) accumulation (Ménégaut et

al., 2017; Wu et al., 2019). They utilize fatty acid oxidation which results in phosphorylation and STAT6 signaling, promoting M2-like immunosuppressive phenotype (Su et al., 2020; Wu et al., 2019). On the other hand, inhibiting fatty acid oxidation or altering lipid availability can reprogram TAMs toward M1-like phenotype and hinder tumor progression (Cui et al., 2021).

2.3.4 Cancer immunotherapy

Cancer immunotherapy dates to the 1890s, when William B. Coley observed tumor regression in patients after bacterial infection. This led him to inject a mixture of toxins from killed *Streptococcus* and *Serratia marcescens* to stimulate the immune system to fight tumors, later known as Coley's toxins, making one of the earliest and most controversial efforts to harness the immune system against cancer (Coley, 1910).

Since then, remarkable progress has transformed immunotherapy into a cornerstone of modern oncology, offering effective treatment options for many cancer types (Brahmer et al., 2012; Hodi et al., 2010; Larkin et al., 2015). The goal of cancer immunotherapy is to activate or restore a self-sustaining cancer-immunity cycle, enabling immune recognition and elimination of tumor cells while avoiding harmful autoimmunity.

Major therapeutic modalities include ICIs, which are monoclonal antibodies that target regulatory pathways such as CTLA-4, PD-1 and PD-L1. Additional approaches encompass adoptive T cell therapy (Dudley et al., 2002; Morgan et al., 2006), chimeric antigen receptor (CAR) T-cell therapy(Conde et al., 2021; Ma et al., 2023; Singh & Maus, 2023), cancer vaccines, oncolytic viruses, bispecific Abs that usually bring immune and tumor cells in contact, cytokine-based immunomodulators that enhance systemic immune activation, and monoclonal antibodies including antibodies that target growth factors or growth factor receptors, like epidermal growth factor receptor or human epidermal growth factor receptor 2 (Martinelli et al., 2009; Swain et al., 2023; Yoon & Oh, 2024), or monoclonal antibodies that induce cellular toxicity (VanDerMeid et al., 2018; Waldman et al., 2020). These strategies augment endogenous antitumor defense mechanisms.

More recently, bispecific T-cell engagers, which are engineered antibodies that simultaneously bind a tumor-associated Ag on cancer cells and the CD3 receptor on T cells, have emerged as a distinct class of biologics to harness the immune system against cancer. Among these, immune-mobilizing monoclonal T-cell receptors against cancer (ImmTAC) are soluble, high-affinity T-cell receptor-CD3 bispecific fusion proteins designed to recognize intracellular tumor Ags presented on MHC-I(Carvajal, Butler, et al., 2022; Middleton et al., 2020).

Building on this paradigm, additional ImmTAC molecules are currently being evaluated in clinical trials. Preferentially Expressed Antigen in Melanoma (PRAME), a cancer-testis Ag initially identified in metastatic cutaneous melanoma (Ikeda et al., 1997), is expressed in several malignancies including uveal melanoma. PRAME-specific T cell clones have been shown to effectively kill uveal melanoma cells, supporting PRAME as a promising target for immune-based therapies (Gelmi et al., 2023; Ribeiro et al., 2024).

Beyond ImmTAC molecules, bispecific T-cell engagers have demonstrated substantial clinical activity in other immune-excluded cancers. Tarlatamab, a delta-like ligand 3 (DLL3)-targeted bispecific T-cell engager, has shown significant efficacy in small-cell lung cancer following progression on platinum-based chemotherapy, leading to FDA approval (Mountzios et al., 2025). Its success highlights the broader potential of CD3-enganging bispecific immunotherapies in overcoming resistance mechanisms associated with "cold" tumors.

Next, the present dissertation will focus on ICIs that act early in the T cell differentiation pathways to preserve effector functions and prevent T cell exhaustion.

2.3.4.1 Immune checkpoint inhibitors

T cell activation involves APCs that present Ags via MHC molecules to the TCR of naïve T cells and CD28-B7 co-stimulation, which is balanced by inhibitory checkpoints that prevent autoimmunity.

First, blocking CTLA-4 with Abs in animal models restored T cell activity and promoted tumor regression (Krummel & Allison, 1995; Leach et al., 1996). Clinical trials followed and led to the FDA approval of ipilimumab for advanced melanoma (Hodi et al., 2010; Robert et al., 2011). Follow-up showed durable responses, with approximately 21% of patients achieving survival beyond 10 years (Schadendorf et al., 2015). Subsequent efforts targeted the PD-1/PD-L1 axis, which abrogates activated tumor-reactive T cells. Clinical trials of anti-PD-1 Abs pembrolizumab and nivolumab, and the anti-PD-L1 atezolimumab, demonstrated lasting antitumor responses across multiple cancers (Okazaki et al., 2013; Zou et al., 2016).

Because CTLA-4 and PD-1 act through distinct pathways, combining their blockade has resulted in enhanced efficacy. Combination treatments have achieved response rates up to 60% in melanoma, although with higher immune-related toxicity (Das et al., 2015; Gubin et al., 2014; Wolchok et al., 2025).

4-1BB agonists are also being developed as cancer immunotherapies to potentiate T cell responses and synergize with other ICIs. Anti-4-1BB Abs enhance T cell proliferation, cytotoxic T cell-mediated tumor rejection, and depletion of Tregs within the TME, underscoring 4-1BB as a promising target in immuno-oncology (Buchan et al., 2018). Lastly, LAG3 inhibitors restore T cell ability to

attack cancer cells. LAG3 inhibitors in combination with nivolumab (anti-PD1) has been approved by the FDA and are used for treatment of advanced melanoma. These inhibitors enhance antitumor immunity and synergize with PD-1/PD-L1 inhibitors to overcome resistance (Aggarwal et al., 2023).

2.3.4.2 Resistance mechanisms to immune checkpoint inhibitors

The rapid rise of cancer immunotherapy over the past decade has transformed oncology by engaging the immune system rather than directly targeting tumor cells. Unlike the traditional therapies that exert direct selective pressure leading to rapid resistance, immunotherapies act indirectly through immune modulation, allowing for durable clinical benefits in patients. Nevertheless, primary, adaptive, and acquired resistance remain major challenges (Hugo et al., 2016; Topalian et al., 2016; Tumeh et al., 2014).

Patients who have primary resistance to ICIs do not respond to initial therapy. Studies indicate that both tumor-cell-intrinsic, such as low mutational burden, deletion in beta-2-microglobulin, silenced MHC-I, as well as tumor-cell-extrinsic factors, such as absence of T cells contribute to primary resistance. Inhibitory immune checkpoint molecules, and immunosuppressive cells contribute to the resistance mechanisms (Gubin et al., 2014; Hamid et al., 2011; Lastwika et al., 2016; Parsa et al., 2007; Spranger et al., 2015).

One major mechanism involves CD8+ T cell exhaustion, which occurs under chronic Ag exposure. This process follows a progressive loss of effector functions due to continuous tumor Ag stimulation (Chow et al., 2022; Kaech et al., 2002). The degree and timing of exhaustion are influenced by factors such as tumor cell resistance to cytotoxicity, hypoxia, and TCR signaling quality and quantity (Y. N. Liu et al., 2020; Scharping et al., 2021; Singh et al., 2020; Thommen & Schumacher, 2018). Interestingly, early evidence of exhaustion in pretreatment samples correlate with favorable responses to ICIs, as it indicates an ongoing antitumor response that can be therapeutically targeted (Attrill et al., 2022; Chalabi et al., 2020; Daud et al., 2016; Kumagai et al., 2020; Terranova-Barberio et al., 2020; Thommen et al., 2018). In contrast, persistent exhaustion after treatment signals poor prognosis (Caushi et al., 2021; Sade-Feldman et al., 2018).

Reducing antigenic load can help limit exhaustion. Combining ICIs with chemotherapy and radiotherapy can enhance efficacy by reducing tumor burden and Ag persistence in patients with advanced- stage lung cancers (Gandhi et al., 2018; Horn et al., 2018; Paz-Ares et al., 2018; Socinski et al., 2018). High baseline tumor burden correlates with poor ICIs responses in patients with metastatic melanoma or head and neck cancer receiving ICIs (Huang et al., 2017), and cytotoxic treatments

have been shown to decrease exhausted T cell populations in preclinical tumor models (Falvo et al., 2021; Hanoteau et al., 2017).

Another critical factor in ICIs response is the presence of pre-existing CD8+ T cells in the TME, which is associated with clinical benefit (Spranger et al., 2014; Tumeh et al., 2014). Tumors lacking such infiltration often exhibit defective type I IFN signaling and poor activation of the cGAS-STING pathway, which promotes DC activation, Ag presentation, and CXCR3-CXCL9/CXCL10 T cell recruitment (Fuertes et al., 2011; Harlin et al., 2009; Mikucki et al., 2015; Woo et al., 2014). *Batf3*-DCs are indispensable for this process, as tumor rejection is impaired in their absence (Broz et al., 2014a; Edelson et al., 2010; Engelhardt et al., 2012; Fuertes et al., 2011; Roberts et al., 2016).

Emerging evidence highlights the importance of PD-L1+ DCs within the tdLNs for ICI efficacy. Blockade of T cell egress using FTY720 has a minimal role in blocking overall tumor progression, suggesting that the primary antitumor action is not solely dependent on a continuous flux of new circulating T cells into the tumor. The frequency of PD-1-PD-L1 interactions in tdLNs, rather than in the tumor itself, correlates with better ICIs responses in metastatic melanoma patients (Dammeijer et al., 2020). In a mouse melanoma model, localized anti-PD-L1 delivery to tdLNs enhances anti-tumor immunity compared to systemic administration (Francis et al., 2020). Together, these findings suggest that a PD-L1+ immune cell population capable of mediating ICIs responses appears to be present within lymphoid tissues, although the precise mechanisms and they relevance to human tumor evolution remain vague.

Overall, resistance to ICIs arises from a complex interplay of T cell exhaustion, deficient Ag presentation, impaired innate signaling, and restricted T cell recruitment. Understanding where the Cancer-Immunity cycle falls behind will be key for the generation of combination therapies. Approaches aimed at restoring innate immune activation, which will be covered in the next section of this thesis manuscript, may have the potential to sensitize treatment-resistant tumors to immunotherapy.

In addition to innate immune agonists, certain cytotoxic chemotherapeutic agents, particularly platinum-based drugs and selected alkylating agents, are now recognized to exert immunogenic effects (Bracci et al., 2014). Chemotherapy-induced cancer cell death can promote the release of DAMPs, enhance tumor Ag exposure, and induce immunostimulatory DNA damage, collectively supporting DC activation and antitumor T cell responses (Barros et al., 2022; Chen et al., 2025). These immunogenic properties may contribute to the clinical efficacy observed with chemotherapy-immunotherapy combinations, as demonstrated in lung cancer and other malignancies.

2.3.5 Targeting innate immunity to overcome resistance

Therapeutic modulation of innate immunity aims either to (*1*) activate canonical signaling pathways and cytokines or (*2*) alter the abundance and function of innate immune cells, particularly DCs. Effective T cell-mediated antitumor immunity requires DCs to perform five coordinated activities:

1. MHC-mediated Ag presentation,
2. Co-stimulatory molecule expression,
3. Cytokine secretion to activate effector T cells,
4. Migration of DCs to tdLNs, and
5. Production of memory-inducing cytokines such as IL-1β and type I IFNs (Ben-Sasson, Wang, et al., 2013; Curtsinger et al., 2005; Kolumam et al., 2005; Zhivaki et al., 2020).

Distinct molecular pathways can agonize these five DC activities. However, no individual pathway activation is sufficient to elicit all five functions, necessitating combinatorial immunotherapy strategies.

2.3.5.1 Enhancing DC cytokine production and function

PRRs drive multiple DC activation steps. TLR signaling enhances Ag capture and MHC loading while it also upregulates CD40, CD80, CD86, IL-12, and type I IFNs, promoting Th1 and CD8+ T cell effector responses (Blander & Medzhitov, 2006; Schnare et al., 2001; Trinchieri, 2003; West et al., 2004). Yet, PRR agonism alone fails to fully activate DC migration or memory cytokine production (Ben-Sasson, Hogg, et al., 2013; Jain et al., 2018). During viral infection, DC motility remains intact in mice lacking MyD88 and mitochondrial antiviral signaling protein that regulate TLR and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) signaling, respectively (Bhoj et al., 2008; Rehwinkel & Gack, 2020). Similarly, TLR ligands modestly induce migration but require additional stimuli for maximum effect (Zhivaki et al., 2020).

TLR, RLRs and the intracellular enzyme and PRR, cGAS, pathways are strong inducers of type I IFNs (Rehwinkel & Gack, 2020; Sun et al., 2013), and efficiently trigger pro-IL-1β production and IL-1β release. Cytosolic supramolecular organizing centers (SMOCs), assembled in the TLR, RLR, and cGAS pathways, activate transcription factors like NF-κB, AP-1, and IFN regulatory factors (IRFs) (Kagan et al., 2014). The NOD-like receptor family pyrin domain containing 3 (NLRP3) assembles a protein complex known as the NLRP3 inflammasome, which is a SMOC, upon sensing various endogenous DAMPs. The assembly of this complex

leads to the activation of caspase-1 (Martinon et al., 2002). Activated caspase-1 cleaves pro-IL-1β into its bioactive state and also cleaves gasdermin D (GSDMD) to generate plasma membrane pores, thereby allowing IL-1β secretion and pyroptosis, while gasdermin E (GSDME) can similarly induce pore formation when activated by caspase-3 (Chan & Schroder, 2020; Ding et al., 2016; Evavold et al., 2018; Heilig et al., 2018; Liu et al., 2016). Because TLRs, RLRs, and cGAS are poor inducers of the NLRP3 inflammasome assembly, adjuvants that promote IL-1β release are valuable for generating memory CD8+ T cells (Cao & Kagan, 2023; Zhivaki et al., 2020). NLRP3 agonists include chemical 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC), naturally released by damaged cells (Di Gioia & Zanoni, 2021). TLR stimulation of murine DCs in combination with PGPC leads to production of IL-1β and enhances their migratory capabilities (Zanoni et al., 2016; Zanoni et al., 2017; Zhivaki et al., 2020).

Given the critical role of type I IFNs in shaping intratumoral protective immunity (Busselaar et al., 2024), increasing attention has turned to non-TLR pathways that elicit responses (Baccala et al., 2007). Among these, cGAS-STING pathway has emerged as a central mechanism linking cytosolic DNA sensing to antitumor immunity (Mosallanejad & Kagan, 2022; Motwani et al., 2019). DNA is frequently leaking from tumor cells due to their genomic instability, oxidative stress, or metabolic dysfunction (Alipour-Khezri et al., 2025; Klarquist et al., 2014). Upon activation, cGAS catalyzes the production of the cyclic dinucleotide known as 2′3′ GMP-AMP (cGAMP), a STING ligand that triggers robust type I IFN responses, stimulating cytolytic and inflammatory T and NK cell responses to cancer (Duong et al., 2022). Activation of cGAS-STING in DCs enhances their maturation, Ag processing and presentation, migration and T cell priming while sustaining T cell effector function (Diamond et al., 2011; Li et al., 2020; Papewalis et al., 2008; Zhivaki et al., 2023). In tumor cells, cGAS-STING signaling can exert direct antitumor effects inducing apoptosis (Vanpouille-Box et al., 2018). The therapeutic potential of this pathway is supported by numerous preclinical models demonstrating that STING activation boosts antitumor immunity (Curran et al., 2016; Falahat et al., 2019; Lv et al., 2020; Ohkuri et al., 2014; Thomsen et al., 2020). Consequently, cGAS-STING agonists are being actively developed as promising immunomodulators potentiating type I IFN antitumor responses (Lv et al., 2020).

Conversely, immunosuppressive factors such as TGF-β, IL-10, IDO, PGE2, and VEGF impair DC activation and promote tumor progression (Niu et al., 2023). Blocking these restores DC function. For example, anti-VEGF Abs restore DC function and synergize with peptide-pulsed DCs to improve survival in mice (Gabrilovich et al., 1998; Gabrilovich et al., 1996; Osada et al., 2008), and IDO silencing using siRNA enhances DC cytokine output and Ag presentation (Flatekval & Sioud, 2009).

2.3.5.2 Targeting CCR7 to modulate DC migration

CCR7+ DCs show enhanced Ag presentation, co-stimulation, and cytokine production promoting effector T cell activity (Heras-Murillo et al., 2024). After activation, DCs migrate to tdLNs, where they engage T cells to initiate immunity. This migration is primarily directed by the CCR7-CCL19/CCL21 axis (Randolph et al., 2005; Robbiani et al., 2000). CCR7, a G protein-coupled chemokine receptor upregulated by PRR signaling, directs DCs to lymph nodes via lymphatic vessels in response to CCL19 and/or CCL21 gradients produced by lymphatic endothelial cells (Britschgi et al., 2010; Randolph et al., 2005; Roberts et al., 2016; Russo et al., 2016; Sokol & Luster, 2015; Weber et al., 2013). PRR-induced expression of these ligands establishes chemokine gradients that guide DCs toward T cell-rich regions within lymph nodes (Schmid et al., 2011; Weber et al., 2013). In murine tumors, however, CCR7+ DCs can have an alternative fate, remaining within the TME and accumulating near blood vessels (Lee et al., 2024; Zitti et al., 2026). Increased intratumoral abundance of CCR7+ DC has been associated with better response to immunotherapy (Lee et al., 2024; Magen et al., 2023). This CCR7+ DCs retention into perivascular niches is supported by fibroblast-derived CCL19 (Zitti et al., 2026). Upon activation, CCR7 oligomerization triggers Src-mediated phosphorylation and downstream signaling through PI3K/Akt, MAPK- NF-κB, and HIF-1α pathways (Hauser et al., 2016; Köhler et al., 2012; Riol-Blanco et al., 2005), which coordinate cytoskeletal remodeling, metabolic adaptation, and epigenetic changes to support migration (Guak et al., 2018; Kohout et al., 2004; Liu et al., 2019; Moran et al., 2014).

Preclinical studies show that CCR7 agonism, through intratumoral CCL19 or CCL21 delivery, enhances DC and T cell infiltration and promotes antitumor immunity (Hillinger et al., 2006; Turnquist et al., 2007). However, systemic chemokine delivery can cause toxicity, prompting development of targeted approaches such as nanoparticles, gene therapy, and CAR T cell engineering. For instance, CCL21-loaded vault nanoparticles, which are cytoplasmic ubiquitous ribonucleoprotein particles and serve as naturally occurring nanocapsules in eukaryotic cells, improved antitumor responses in vitro and in vivo (Kar et al., 2011); CCL19-expressing B16-BL6 melanoma cells showed reduced tumor growth (Okada et al., 2004); and adenoviral CCL19/CCL21 delivery suppressed melanoma and colon carcinoma progression (Okada et al., 2006; Shao et al., 2015). Together, these studies highlight CCR7 and its ligands as dual regulators of tumor immunity and promising targets for immunotherapeutic intervention.

2.3.5.3 Increasing DC abundance with FLT3L administration

cDC1 and cDC2 are essential for initiating T cell responses, but their low intratumoral abundance limits Ag delivery to tdLNs (Bhandarkar et al., 2025; Broz et al., 2014b; Demaria et al., 2019; Lavin et al., 2017; Salmon et al., 2016). FLT3L promotes DC differentiation and expansion and has shown efficacy in preclinical and clinical cancer models (Chakravarty et al., 2006; Esche et al., 1998; Riediger et al., 2013; Somers et al., 2003; Watowich & Liu, 2010). However, DC expansion alone does not ensure robust antitumor immunity. FLT3L would need to be combined with other DC activators to generate effective antitumor T cells.

Adoptive cell transfer with FLT3L-expressing T cells boosted DC proliferation, type I IFN-driven inflammation, and enhanced solid tumor control in mice (Lai et al., 2020). In melanoma models, systemic FLT3L followed by TLR3 agonists resulted in expansion and activation of DC progenitors in the TME, sensitizing tumors to PD-1/PD-L1 blockade and conferred re-challenge protection (Salmon et al., 2016).

2.3.5.4 Reprogramming TME components into adaptive immunity activators

DC subsets vary in their capacity to capture, migrate, and present tumor-specific or -associated Ags to T cells in tdLNs. Moreover, tumor-residing *Batf3* DC-T cell crosstalk is essential for effector T cell differentiation and trafficking (Prokhnevska et al., 2023; Spranger et al., 2017). Overexpression of the transcription factors PU.1, IRF8, and BATF3 in components of the TME other than immune cells can potentially convert “cold” tumors into immunologically active. Ectopic expression of these factors reprograms mouse and human fibroblasts into induced DCs, capable of Ag uptake, cross-presentation to CD8+ T cells, IL-12 production, CD80/CD86 coexpression, and migration (Rosa et al., 2018). Similarly, tumor cells can be directly reprogrammed to adopt cDC1-like phenotype, enhancing Ag presentation and overcoming tumor immune evasion. In melanoma mouse models, replication-deficient viral vectors delivering PU.1, IRF8, and BATF3 reprogrammed cancer cells into Ag-presenting, cDC1-like cells, triggering robust T cell activation, tumor regression, and immunological memory, protecting against tumor re-challenge and metastasis (Ascic et al., 2024). However, limitations of this approach may be the potential for incomplete or unstable reprogramming and whether therapeutic efficacy depends on the number of cells and the fidelity of the reprogramming.

2.4 Adipophilin (PLIN2) and functions

Adipophilin, also known as perilipin 2 (PLIN2), is encoded by the *PLIN2* gene located on the short arm of human chromosome 9. This protein coats lipid droplets (LDs) together with phospholipids, facilitating the storage of lipids (Conte et al., 2016).

Functionally, adipophilin acts as a structural and regulatory gatekeeper of LDs, maintaining their stability and modulating lipid metabolism by preventing LD dissociation and inhibiting lipolysis (Bickel et al., 2009; Hickenbottom et al., 2004; Straub et al., 2010; Sztalryd & Brasaemle, 2017).

Elevated PLIN2 levels are associated with metabolic disorders such as type 2 diabetes, insulin resistance, cardiovascular disease, and other age-related conditions (Conte et al., 2016). Adipophilin also serves as a prognostic biomarker in several cancers, including colorectal, lung, breast, ovarian, clear cell renal cell carcinoma, Burkitt lymphoma and cutaneous melanoma (Ambrosio et al., 2012; Fujimoto et al., 2020; Iwahashi et al., 2021; Matsubara et al., 2011; Prieto et al., 2014; Shin et al., 2018; Tolkach et al., 2017; Yoshikawa et al., 2020). Adipophilin has also been detected at elevated levels in urine and plasma in clear cell renal cell carcinoma and colorectal cancer patient samples, respectively (Matsubara et al., 2011; Morrissey & Kharasch, 2013). Morrissey and Kharasch indicated that urinary PLIN2 is increased in patients with clear cell and papillary renal cell carcinoma and shows high diagnostic sensitivity when normalized to creatinine, supporting its potential as a non-invasive diagnostic biomarker. In colorectal cancer, Matsubara et al. show that plasma adipophilin levels are elevated even in early-stage disease (stages I or II), with moderate diagnostic accuracy, indicating possible utility for early detection. However, current evidence suggests that adipophilin as a fluid-based biomarker is cancer-type specific, and its sensitivity and specificity have not been validated across different malignancies.

In primary UM, evidence of reprogrammed lipid metabolism has been observed through differences in accumulation of LDs within the TME, and PLIN2 was used as an LD marker (Fiorentzis et al., 2017). However, whether adipophilin serves as a prognostic biomarker in UM and if its expression correlates with BAP1 and a metabolic shift that could offer new therapeutic opportunities was unclear and one of the subjects of this dissertation.

2.5 Cluster of Differentiation 74

CD74, also known as the invariant chain (Ii), is a single-spanning transmembrane protein. Its N-terminus faces the cytosol. In mice, two isoforms of the protein exist due to alternative splicing, which comprise 215 and 279 amino acids and are referred to as p31 and p41, respectively (Shachar et al., 1995). In humans, these correspond

to p33 and p41. Furthermore, in humans, the use of an alternative start codon gives rise to two additional isoforms, p35 and p43, exhibiting slightly extended N-termini with 16 additional amino acids (O'Sullivan et al., 1987; Strubin, Berte, et al., 1986; Strubin, Long, et al., 1986). Several post-translational modifications of the CD74 protein have been identified. N- and O-glycosylation has been reported for murine CD74 (Koch, 1988; Sung & Jones, 1981). The turnover of CD74 is completed by the intramembrane protease signal peptide peptidase-like (SPPL2a), which cleaves the final membrane-bound N-terminal thereby releasing an intracellular domain (ICD) into the cytoplasm, which has the capability to translocate to the nucleus and may influence transcriptional regulation (Beisner et al., 2013; Bergmann et al., 2013).

2.5.1 Functions of CD74 in Ag presentation

CD74 is a pivotal regulator of Ag presentation controlling the assembly, trafficking, and peptide loading of MHC-II molecules. Initially, it was shown that CD74 enhances Ag presentation in MHC-II-expressing fibroblasts (Stockinger et al., 1989). CD74 is primarily expressed in professional APCs, such as DCs, MΦs, and B cells, but it can also be upregulated in epithelial and endothelial cells under inflammatory conditions, particularly upon IFN-γ stimulation (Collins et al., 1984; Momburg et al., 1986; Volc-Platzer et al., 1984).

Acting as a chaperone and trafficking adaptor, CD74 ensures that MHC-II complexes are properly folded in the ER and directed to endosomal compartments for peptide loading. It simultaneously blocks the peptide-binding groove via its CLIP region (residues 81-104), preventing premature peptide association (Bijlmakers et al., 1994; Castellino et al., 2001; Koch et al., 2007). In the ER, CD74 assembles as a trimer that binds three MHC-II αβ dimers, forming a nonameric complex that facilitates MHC-II folding and ER export (Anderson & Miller, 1992; Bikoff et al., 1993; Elliott et al., 1994; Roche et al., 1991). Two dileucine-based sorting motifs in its N-terminal cytoplasmic domain recruit adaptor protein complexes, thereby directing MHC-II-CD74 complexes to endosomal compartments for Ag processing (Bakke & Dobberstein, 1990; Bonifacino & Traub, 2003; Bremnes et al., 1994; Odorizzi et al., 1994; Pieters et al., 1993). Once in endosomes, CD74 undergoes stepwise degradation to permit peptide loading (Pieters et al., 1991).

In DCs and B cells, CTSS mediates the cleavage of CLIP fragment (Driessen et al., 1999) while cathepsin L and cathepsin F perform this function in thymic epithelial cells and MΦs (Nakagawa et al., 1998; Shi et al., 2000). If this cleavage is inhibited, peptide loading and MHC-II surface expression are compromised, leading to complex retention in Ag-processing vesicles (Driessen et al., 1999). Additionally, CD74 modulates endosomal maturation and Ag degradation, thereby optimizing the

timing and precision of Ag presentation in professional APCs (Landsverk et al., 2009).

Beyond its canonical role in the MHC-II pathway, CD74 was shown to contribute to cross-presentation via MHC-I. Basha et al. demonstrated that CD74 promotes the trafficking of newly synthesized MHC-I molecules from the ER to endocytic compartments in DCs and is required for cross-presentation of cell-associated Ags (Basha et al., 2012).

2.5.2 CD74 is more than the MHC-II chaperone

2.5.2.1 CD74 as a receptor for MIF and DDT

Beyond its classical function as an MHC-II chaperone, CD74 also acts as a cell-surface receptor for macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine secreted by monocytes, MΦs, DCs, and granulocytes (Calandra & Roger, 2003). A homolog, d-dopachrome tautomerase (DDT), also referred to as MIF-2, shares overlapping functions (Merk et al., 2012). MIF stimulates the production of TNF, prostaglandin E2, and modulates TLR4 expression, amplifying inflammation (Calandra & Roger, 2003; Roger et al., 2001).

MIF binding to CD74 is necessary but insufficient for triggering downstream signaling cascades, requiring co-receptors such as CD44 or C-X-C chemokine receptor 4 (CXCR4) (Schwartz et al., 2009; Shi et al., 2006). MIF/CD74/coreceptors complexes activate extracellular signal-regulated kinase (ERK)-1/2 MAPK, phosphoinositide-3-kinase (PI3K)/Akt pathways (Lue et al., 2006; Lue et al., 2007), inhibit p53 in MΦs to prolong survival (Mitchell et al., 2002), and trigger Syk and NF-κB, enhancing inflammation, cell proliferation, and survival (Binsky et al., 2007; Gore et al., 2008; Lantner et al., 2007; Starlets et al., 2006).

In cutaneous melanoma, MIF produced by tumor cells supports growth and immunosuppression (Repp et al., 2000; Shimizu et al., 1999; Waigel et al., 2016; Yaddanapudi et al., 2013; Yaddanapudi et al., 2016; Yan et al., 2006). Notably, release of CD74 in soluble form, regulated either by disintegrin and metalloproteinase-mediated cell-surface cleavage or cysteine-protease-mediated lysosomal cleavage, suppresses melanoma growth and induces apoptosis under IFN-γ stimulation by blocking the MIF/CD74/Akt pathway, indicating a regulatory role of soluble CD74-MIF interaction in tumor progression (Fukuda et al., 2022).

2.5.2.2 CD74 as a receptor for TIMP-1

CD74 also functions as a signaling receptor for tissue inhibitor of metalloproteinases-1 (TIMP-1) (Schoeps et al., 2021). TIMP-1 interacts with CD63

and CD74, activating the PI3K/Akt and ZAP70 pathways through its N-terminal (Schoeps et al., 2021). Like MIF, TIMP-1 signaling through CD74 likely requires CD44 co-engagement, linking these pathways to lymphocyte activation (Lambert et al., 2009; Shi et al., 2006). During inflammation, soluble CD74 is shed from the membrane and potentially sequesters TIMP-1 and inhibits CD74-mediated intracellular signaling, analogous to soluble CD74-MIF regulation (Assis et al., 2014; Fukuda et al., 2022).

2.5.2.3 CD74 intracellular domain signaling

Following intramembrane proteolysis by SPPL2a, CD74-ICD, comprised by 29-46 amino acids depending on the isoform and species, can be released into the cytosol (Mentrup et al., 2015). Due to its small size and rapid proteasome degradation, its detection was challenging; however, nuclear translocation of CD74-ICD has been demonstrated using overexpression (Becker-Herman et al., 2005; Matza, Kerem, et al., 2002). It is proposed that upon CD74-ICD translocation into the nucleus, the short peptide interacts with the transcription factors Runt-related transcription factor (RUNX) and NF-κB, regulating genes involved in apoptosis, immune response, and migration (Gil-Yarom et al., 2017). Reported transcriptional downstream targets of this signaling cascade include Bcl-XL, the Tap63 protein, Bcl-2, and IL-8 (Binsky et al., 2007; Lantner et al., 2007; Starlets et al., 2006). Loss of CD74 in B cells contributes to impaired B-cell survival (Matza, Kerem, et al., 2002; Matza, Lantner, et al., 2002; Matza et al., 2001). Identifying these targets helps elucidate key pathways governing B-cell homeostasis and immune regulation.

2.5.2.4 CD74 regulates DC migration

The ability of DCs to function as APCs depends heavily on their migratory capacity, which enables them to patrol tissues and capture Ags (Heuzé et al., 2013). Upon maturation, DCs reduce migration and endocytosis as they home to secondary lymphoid organs to present Ags to T cells.

CD74 has been recognized as regulator of DC motility (Faure-André et al., 2008). Using granulocyte-macrophage colony stimulating factor (GM-CSF) induced BMDCs, resembling moDCs, and after stimulation with LPS, Faure- André et al. demonstrated that CD74-deficient DCs migrate continuously at higher speed, lacking alteration between high and low migration velocities (Faure-André et al., 2008). They conferred this ability on the cytosolic N-terminal domain of CD74, as CTSS-deficient DCs, which accumulate the CD74 N-terminal fragment display slower migration than wild-type cells. Mechanistically, CD74 interacts with myosin

II, a motor protein essential for DC migration (Faure-André et al., 2008; Vascotto et al., 2007).

Importantly, migration speed and Ag uptake through micropinocytosis are inversely regulated (Chabaud et al., 2015). During fast migration, DCs show reduced Ag uptake, whereas slower phase enhances micropinocytosis. Front-end enrichment of myosin-II drives migration that supports macropinosome formation (Chabaud et al., 2015). In CD74-deficient DCs, myosin II fails to localize in the front end, resulting in constant migration speed, increased vesicle numbers, and slower intracellular transport (Chabaud et al., 2015). These disruptions likely influence the diversity of Ags presented by the MHC-II pathway in CD74-deficient DCs (Chabaud et al., 2015).

2.5.3 Consequences of CD74 deficiency

The generation of invariant chain knockout mice confirmed the pivotal role of this protein in MHC-II pathway (Bikoff et al., 1993; Elliott et al., 1994; Shachar & Flavell, 1996). The B6.129S-*Cd74*^{tmlLiz}/J mice were generated by homologous recombination, leading to replacement of the wild-type locus resulting in a 3.8-kb deletion removing the first intron and 11 nucleotides of exon 2, thereby abolishing expression of *Cd74*. Upon loss of the invariant chain, the assembly and surface expression of MHC-II molecules were significantly impaired, resulting in defective Ag presentation (Bikoff et al., 1993; Viville et al., 1993). MHC-II was retained to a significant degree in the ER and Golgi compartment (Elliott et al., 1994).

Since MHC-II surface expression by cortical and medullary thymic epithelial cells is critical for thymic selection of CD4+ T cells, *Cd74-/-* mice exhibit a significant reduction of thymic and peripheral CD4+ T cells (Bikoff et al., 1993; Viville et al., 1993). T cells are positively selected when weak interaction occurs with appropriate MHC molecules expressed on cortical thymic epithelial cells (Klein et al., 2014), which express both MHC-I and MHC-II, have differences in proteasome subunits, for processing of Ags for presentation on MHC-I, and endosomal proteolytical enzymes compared to other cells. The different Ag-derived peptides produced in cortical thymic epithelial cells tend to bind to MHC molecules weakly as compared to peptides produced in other cell types (Klein et al., 2014). Positive selection is associated with lineage commitment of CD4 versus CD8 single positive T cells. In contrast, T cells are negatively selected when strongly interacting with MHC-self Ag complexes expressed by medullary thymic epithelial cells (von Boehmer et al., 1989). This process is supported by autoimmune regulator (AIRE), a transcription factor driving the expression of tissue-specific Ags (Kyewski & Derbinski, 2004; von Boehmer et al., 1989).

Detailed phenotypic analyses further revealed alterations in the architecture of thymus in adult *Cd74-/-* mice, including severe medullary atrophy and loss of normal medullary and cortical organization (Wang et al., 2021). Although the total number of thymic epithelial cells remained comparable to wild-type mice, the proportion and absolute number of medullary thymic epithelial cells, particularly MHC-II^{high} and CD80+ subsets, were significantly reduced while the proportion of cortical thymic epithelial cells was increased (Wang et al., 2021). CD40+ and AIRE+ medullary epithelial cell populations were preserved. Consistent with this, *Cd74-/-* mice do not display predisposition to autoimmunity, indicating that central tolerance regulated by AIRE is not compromised (Wang et al., 2021).

In addition, these mice display a distinct defect of B cell maturation and function (Shachar & Flavell, 1996). Ag-induced humoral immune responses including those to T cell-independent Ags which are supposed to act independently of presentation by MHC-II were impaired in these mice (Shachar & Flavell, 1996). This functional deficit correlates with a significant reduction of phenotypically mature B cells in the periphery (Maehr et al., 2004; Matza, Lantner, et al., 2002; Schneppenheim et al., 2013; Shachar & Flavell, 1996).

Since the full ablation of all MHC-II genes does not affect B cell differentiation and maturation in mice, it was proposed that the B cell phenotype induced by the loss of CD74 may be unrelated to the role of this protein in MHC-II assembly and trafficking (Madsen et al., 1999; Shachar & Flavell, 1996).

2.5.4 CD74 in cancer

CD74 is detected in several hematological malignancies including multiple myeloma, and in various solid tumors such as breast, gastrointestinal, lung, kidney, bladder, prostate, pancreas, and central nervous system cancers (Burton et al., 2004; Choi et al., 2013; Cuthbert et al., 2009; Greenwood et al., 2012; Koide et al., 2006; McClelland et al., 2009; Meyer-Siegler et al., 2006; Porter et al., 2003; Tamori et al., 2005; Young et al., 2001). Moreover, the presence of CD74 in tumor cells (Collins et al., 1984; Momburg et al., 1986; Volc-Platzer et al., 1984) suggests potential roles beyond Ag presentation, possibly influencing intracellular signaling, endocytic trafficking, and cell migration.

In melanoma, blockade of MIF-CD74 axis using the C36L1 peptide, which is a short sequence from the complementary-determining region 1 (CDR1) of the light chain of the anti-vaccinia clone 36 immunoglobulin G (IgG) that directly binds to CD74, restored antitumor and immunogenic activities of MΦs and DCs, thereby inhibiting metastasis in the lungs. Treatment with C36L1 reprogrammed M2-like TAMs into pro-inflammatory M1-like TAMs, increased the population of immunogenic DCs and activated cytotoxic T cells, and reduced Tregs and myeloid-

derived suppressor cells. C36L1 binds to CD74 and alters its structural dynamics, blocking the MIF-CD74 interaction and signaling (Figueiredo et al., 2018).

Another recent study demonstrated that CD74 promotes the expansion of tumor-infiltrating tolerogenic dendritic cells and regulatory B cells and therefore supporting tumor progression (Pellegrino et al., 2024). Using constitutive *Cd74-/-* mice, and conditional Cre-CD23 x $Cd74^{fl/fl}$ and Cre-CD11c x $Cd74^{fl/fl}$, and a CD74 inhibitor, they elucidated the mechanisms underlying CD74-mediated suppression of antitumor immunity. Tumor-derived MIF activates CD74 on DCs, leading to the binding of CD74 ICD to the SP1 promoter that subsequently binds to the IL-1β promoter, suppressing its transcription. Reduced IL-1β levels diminish antitumor immunity by promoting tolerogenic DCs expansion. This study identified CD74+ CD11c+ DCs as a key regulator of triple-negative breast cancer progression and suggest CD74 as a potential therapeutic target in this cancer type (Pellegrino et al., 2024).

Although CD74 is mainly expressed by APCs, it is also detected at mRNA and cell-surface protein levels in tumor-infiltrating Tregs (Bonnin et al., 2024). Membrane CD74 overexpression was specific to tumor Tregs and absent in Tregs from healthy donors, suggesting a candidate tumor-specific function. In vitro, CD74 knockout Tregs maintained their survival, phenotype, and suppressive capacity. Similarly, in vivo, CD74 knockout Tregs effectively prevented graft-versus-host disease in xenogeneic models, indicating that CD74 is not essential for Treg-mediated suppression. In contrast, in tumor models, CD74 knockout Tregs failed to accumulate in tumors and displayed reduced ability to suppress antitumor responses. These tumor Tregs exhibited decreased expression of activation markers CD25, 4-1BB, CTLA-4, lower Foxp3 levels, and increased PD-1 expression, IFN-γ production, indicating a loss of their suppressive function. Syngeneic mouse model confirmed that tumor Tregs express higher levels of CD74 than peripheral Tregs or conventional CD4+ T cells. Adoptive transfer of CD74 knockout Tregs had impaired tumor infiltration compared to wild-type Tregs. CD74 emerges as a regulator of tumor Tregs activation, stability, and accumulation (Bonnin et al., 2024).

3 Aims

Despite the immense advances in tumor immunology research and the success of ICIs, immune exclusion is the "Achilles' heel" of immunotherapy, a significant impediment that limits durable clinical responses. Understanding the molecular and cellular mechanisms that drive exclusion is crucial for improving therapeutic efficacy. To uncover new mechanisms implicated in immune exclusion, this thesis investigates the prognostic and metabolic role of adipophilin in UM and the immunoregulatory functions of CD74 in melanoma.

The main objectives of this thesis were to:

1. Determine whether loss of adipophilin expression indicates a lipid metabolic shift contributing to the immune suppression in BAP1-negative UM
2. Examine the role of CD74 in melanoma progression and define how its loss reshapes the immune landscape in the TME and tdLNs
3. Investigate how CD74 deficiency influences immune cell dynamics under steady-state and inflammation

4 Materials and Methods

4.1 GDC-TCGA data analysis and uveal melanoma secretome analysis (I)

Publicly available mRNA data from 80 primary UM patients were obtained from the GDC-TCGA-UM cohort (Robertson et al., 2017) via UCSC Xena browser (http://xena.ucsc.edu/). mRNA expression, generated using the Illumina HiSeq 2000 platform, was normalized with HTSeq/FPKM-UQ. Data on *PLIN2* mRNA expression, patient age, *BAP1*, and chromosome 3 status were extracted for further analysis.

Additionally, UM secretome data from a Liverpool Ocular Oncology Research group (LOORG) cohort (Eye and Vision Sciences, University of Liverpool, Liverpool, England) (Angi et al., 2016)were analyzed. Protein with significant fold-change expression were assigned risk scores based on their association with metastatic potential: high risk (3), low risk (2), and no risk (1). A clinical score for each protein was calculated by comparing mean expression values between risk groups, yielding four categories: no risk (-2), low risk (-1), mid risk (+1), and high risk (+2) (**I:** Supplementary Table S1). Protein identifiers were converted to gene codes and analyzed in the GCD-TCGA-UM dataset using the Xena browser to generate gene expression clusters supervised by *PLIN2.* Kaplan-Meier survival analysis was performed using the same cohort, and results, including log-rank p-values and prognostic classification, were compiled into a matrix (I: Supplementary Table S3). Genes with consistent clinical scores across the LOORG and TCGA datasets were subsequently used for network analysis.

4.2 Ethics statement and licenses (I & II)

Access to human UM samples was provided by the Ocular Oncology Biobank (REC Ref21/NW/0139) (Eye and Vision Sciences, University of Liverpool, Liverpool, England) with project specific ethical approval from the Health Research Authority

(REC Ref 15/SC/0611). Peripheral blood samples from healthy donors were obtained from the Finnish red cross blood service (license: 16/2022).

For the second study, all animal experiments were conducted in a pathogen-free facility at the Central Animal Laboratory (CAL), University of Turku, following the Finnish Act (62/2006), the 3Rs principle, and approved by the institutional ethical committee (license: ESAVI/39072/2021).

4.3 Cell culture (I & II)

4.4 Maintenance of immortalized cell lines (I & II)

The immortalized cell lines used in study I and II were cultured using the culture media in (Table 1). All cell lines were maintained at 37ºC and 5% CO_2.

Table 1. Immortalized cell lines and required growth medium for their maintenance and expansion.

CELL LINE	CELL GROWTH MEDIUM	STUDY
MEL285	RPMI-GlutaMAX (Gibco), supplemented with 10% newborn calf serum (NBCS)(Gibco), and 1% Penicillin/Streptomycin (pen/strep) (Gibco)	I
MP46	RPMI-GlutaMAX, supplemented with 20% NBCS, and 1% pen/strep	I
B16-OVA **B16F10**	DMEM (Gibco), supplemented with 10% NBCS, 1% pen/strep, and 4mM of ultraglutamine (Lonza)	II
YUMM1.G1	DMEM/F12 (ATCC), supplemented with 10% NBCS, 1% pen/strep, 1% NEAA (Gibco)	II

4.4.1 Human monocyte-derived MΦs (I)

Peripheral blood was purchased from the Finnish Red Cross Blood Service. One part of blood was diluted with one part of RPMI-1640 without serum. The diluted blood was then layered on top of Histopaque-1077 (Sigma Aldrich) and centrifuged at discontinuous gradient centrifugation at 774 x g for 30 min at room temperature without brake. The buffy coat was collected and the cells were washed twice with sterile PBS. The cell pellet was resuspended and the cell yield and viability were determined using an TC20 automated cell counter (Bio-Rad). For the isolation of monocytes, magnetic activated cell sorting was employed using the human Pan Monocyte isolation kit (Miltenyi Biotec), which allows negative selection of monocytes, according to manufacturer's instructions. The yield and viability of the enriched monocytes (CD14+CD16+) were then determined. 10^6 monocytes were

cultured per well of 6-well plates in complete RPMI supplemented with 10% heat-inactivated NBCS, 1% of pen/strep, and 50 ng/mL recombinant human M-CSF (Miltenyi Biotec). Fresh differentiation medium was added after 3 days and cells were cultured for total of 6 days.

4.4.2 Bone marrow-derived dendritic cells preparation with FLT3L (II)

To expose the bone marrow, femora and tibiae from mice were flushed with RPMI. Red blood cells were removed with ACK Lysing Buffer (Lonza). The bone marrow cells were then cultured at a density of $2x10^6$/mL in complete RPMI supplemented with 10% heat-inactivated NBCS, 1% pen/strep, 1X Non-Essential Amino Acids Solution (Gibco), 1mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), and 0.06 mM 2-Mercaptoethanol (Gibco) in the presence of 150 ng/mL recombinant mFLT3L (SinoBiologicals) at 37ºC and 5% CO_2. The FL-DCs were cultured in these conditions for 8 days. For the maturation of FL-DCs, loosely adherent cells from the cultured were harvested and incubated with either with 100 ng/mL LPS (Sigma) for 24 hours or 50 ug/mL polyinosinic:polycytidylic acid (pI:C) (InVivoGen) for 24 hours, both followed by a 1-hour of resting in 10% RPMI. The matured FL-DCs and resting controls (no LPS or pI:C) were then used for the agarose migration assay or the evaluation of CCR7 cell surface levels.

4.5 In vivo experiments (II)

4.5.1 Experimental animals (II)

All mice were maintained and bred in a pathogen-free facility according to the guidelines of the Central Animal Laboratory (CAL), University of Turku. C57BL/6J and B6.129S-*Cd74tm1*$^{Liz/J}$ mice were purchased from the Jackson Laboratory (strain #002729). Briefly, mice homozygous for the *Cd74*^{tm1Liz} are viable and fertile. Constitutive *Cd74* knockout mice (*Cd74-/-*) have defects in MHC-II assembly, transport, surface expression, and Ag presentation. Mature splenic DCs from *Cd74*-deficient mice also display reduced MHC-II surface expression. *Cd74-/-* mice do not display predisposition to autoimmunity, indicating that central tolerance regulated by AIRE is not compromised (Wang et al., 2021).

C57BL/6-Tg(TcraTcrb)1100Mb/J (OT-I) mice were purchased from Charles River Laboratories (Charles River Laboratories). All experiments were conducted with 6–10-week-old mice that were age and sex matched. Genotyping primers for B6.129S-Cd74tm1$^{Liz/J}$ were the following: a) CAC TCA AGG CAA CCT TCC TG (wild type reverse), b) ATT CGC CAA TGA CAA GAC G (mutant reverse), c) CGC

CTG TGG GAA AAA CTA GA (common). The primers were ordered from Integrated DNA Technologies (Integrated DNA Technologies).

4.5.2 Subcutaneous, metastatic melanoma models and treatments

B16-OVA cells, 10^5, were suspended in 100μL sterile PBS and subcutaneously injected on the right flank of 6-10-week-old male mice. Tumor-bearing mice were euthanized either on day 14 (early time point) or day 20 (endpoint). For specific assays assessing Ki-67 and CCR7 expression in vivo, female mice were inoculated with $2x10^5$ B16-OVA cells and sacrificed at days 7 and 14.

For the metastatic B16-OVA model, 10^6 B16-OVA cells were resuspended in 50 μL of PBS and injected intravenously in the left or right lateral caudal vein of 6-10-week-old male mice. Tumor-bearing mice were euthanized after 14 days and the lungs of the mice were collected for further analysis.

For anti-PD-1 treatment experiments, mice received subcutaneous injections of 10^6 YUMM1.G1 or $5x10^5$ B16F10 cells in their right flank. Once the tumors reached a volume of 50-100 mm3, animals were randomized and administered 200 μg of anti-PD-1 Ab (clone J43, BioXCell) or isotype control Ab (polyclonal Armenian hamster IgG, BioXCell) via intraperitoneal injection, given in three or four doses every other day. Mice were euthanized when tumor volumes exceeded 1500 mm^3.

4.5.3 In vivo tumor-associated Ag cross-presentation assay

On day 0, B16-OVA melanoma cells were incubated with 1 μg/mL (1.8 μM) of doxorubicin hydrochloride (Sigma Aldrich) to induce apoptosis. On the same day, spleens and lymph nodes were harvested from OT-I mice. Following tissue dissociation, the naïve CD8+ T cells were enriched using Magnetic Cell Separation and the CD8a+ T cells isolation kit for mouse (Miltenyi Biotec). The enriched naïve OT-I CD8+ T cells were then labeled with 1 μM of CellTrace™ Violet Cell (CTV) (ThermoFisher) according to the manufacturer's protocol. Subsequently, 2.5 x 10^6 CTV-labeled OT-I CD8+ T cells were intravenously injected into *Cd74-/-* and *Cd74+/+* recipient mice. On day 1, the doxorubicin-treated (apoptotic) B16-OVA melanoma cells were harvested and 1 x 10^6 cells were subcutaneously injected on the right flank of each recipient mouse. After 5 days, blood, draining lymph nodes (dLN, right inguinal LN), and spleens were collected. Single-cell suspensions were prepared from secondary lymphoid organs. Blood samples were stained using the protocol described previously, and OT-I CD8+ T cell proliferation was assessed by CTV dilution within the CD8+ SIINFEKL Dextramer+ population. Single-cell suspensions of the dLNs and spleens were stained using LIVE/DEAD Yellow

viability dye, anti-CD45, anti-CD8, anti-TCRb5.1 5.2, and anti-CD44, as detailed in Table 2. Proliferation was quantified via flow cytometry based on CTV dilution within the $CD44^{high}$ CTV+ population of OT-I CD8+ T cells.

4.6 Cell-based assays (I & II)

4.6.1 Human MΦs & UM cells co-culture (I)

UM cell lines Mel285 or MP46 were co-cultured with human monocyte-derived MΦs at a 1:4 ratio ($8x10^4$ cancer cells to $3.2x10^5$ MΦs) in RPMI supplemented with 10% NBCS. Cultures were treated with IC50 of carvedilol (58.1 μM). After 24 h, RNA was isolated for downstream qPCR analysis.

4.6.2 In vitro Ag presentation (II)

FL-DCs were generated as described above. On day 8-9 of differentiation, loosely adherent FL-DCs were harvested and pulsed for 24h with 1 or 2 mg/mL chicken ovalbumin (OVA) (MP biomedicals), or for 1h at 37ºC with varying doses of SIINFEKL (OVA 257-264) (Invivogen), the H-2Kb-restricted peptide epitope of ovalbumin, prior to co-culture with OT-I CD8+ T cells. Naïve OT-I $CD8^+$ T cells were enriched and labeled with CellTrace™ Violet (CTV) as previously described. Following labeling, 2 x 10^5 OT-I CD8+ T cells were co-cultured with 4 x 10^4 OVA-pulsed FL-DCs at 1:5 and/or 1:10 ration (DCs: T cells) ratios in 96-well U bottom plates. After 3 days, co-cultured cells were stained with anti-CD8, Zombie NIR™ Fixable Viability Kit, and anti-CD44 (Table 2) at 4ºC for 20 min in the dark. OT-I CD8+ T cell proliferation was evaluated via flow cytometry based on CTV dilution within $CD44^{high}$ population. Data were acquired using a 3-laser NovoCyte flow cytometer (Agilent) and analyzed using FlowJo software.

4.6.3 Under agarose migration assay (II)

The under-agarose migration assay was performed as previously described (Alanko et al., 2023). Briefly, 1% UltraPure Agarose (Invitrogen) was mixed in phenol-free RPMI medium with 1x Hanks' balanced salt solution (pH 7.3; Sigma Aldrich) and 10 % FBS. The agarose containing medium was poured onto custom-made chambers with a diameter of 17 mm and allowed to polymerize for 35 min. Two holes of 2 mm diameter were then punched into the agarose and either 10^6 FL-DCs or 17.5 ng of CCL19 in phenol-free RPMI were added into each hole. FL-DCs were stained with NucBlue (Invitrogen) for 15 min at 37ºC and washed once with RPMI before injecting into the agarose hole. Cell migration was recorded with an inverted Nikon

Eclipse Ti2-E wide-field fluorescent microscope at 37ºC with 5 % CO_2 for 6 to 12 hours with 1-min intervals and 4x objective. Fiji was used for image processing and analysis, and all graphs and statistical analyses were done with GraphPad Prism. Cell migration was quantified using TrackMate plugin for automated cell tracking with carefully adjusted parameters and filtering to exclude unmoving dead cells and bad tracks, as in Alanko et al. 2023 (Alanko et al., 2023). The same parameters were applied consistently in different stimulation conditions among the samples to obtain comparable results. The NucBlue channel (405 mm) was used for cell tracking.

4.7 Samples (I & II)

4.7.1 UM patients' samples (I)

Enucleated primary UM specimens (n=43) were selected based on tissue availability in the Liverpool Ocular Oncology Biobank, with informed consent from patients treated between 2016 and 2018 at the Liverpool Ocular Oncology Center (REC Ref 15/SC/0611). Formalin-fixed paraffin-embedded (FFPE) sections were analyzed using conventional histological and immunohistochemical methods (Krishna et al., 2017). Adipophilin and nuclear BAP1 (nBAP1) expression were evaluated by immunohistochemistry using specific anti-human Abs (Santa Cruz, Lifespan Biosciences). Detection was performed with 3-Amino-9-ethyl carbazole peroxidase substrate (AEC) or 3,3'-diaminobenzidine (DAB) chromogens, and slides were scanned using an Aperio CS2 slide scanner. Clinical and genetic data were pseudonymized. The cohort included 24 male and 19 female patients with a median age of 71 years. Tumors had a mean largest basal diameter of 15 mm and a mean ultrasound height of 8.3 mm; 38% showed ciliary body involvement and 26% demonstrated extraocular extension. Epitheloid cell morphology was present in 42% of cases. Chromosomal alterations were determined using multiplex ligation-dependent probe amplification and microsatellite analysis, revealing monosomy 3 in 64% of tumors, disomy 3 in 22%, and partial chromosome 3 loss in 12% (**I:** Table 1).

4.7.2 Machine learning digital analysis of full scanned images using NIS-Elements (I)

LDs quantification was performed on digitally scanned slides using NIS-Elements Advanced software (Nikon), following previously established protocols(Figueiredo et al., 2018; Goodwin et al., 2017; Nosavanh et al., 2015). Tumor regions of interest (ROI) were manually defined, and areas with tissue distortion or artefacts were excluded based on predefined criteria, including size, shape, and signal interference.

Automated image analysis generated binary masks using color-based thresholds derived from hue, intensity, and saturation of RGB values. In samples containing endogenous melanin, background pigment was subtracted using unstained serial sections to correct DAB signal interference. The total tumor area and adipophilin-positive regions were measured in square pixels and expressed as relative frequency, with the entire tumor area normalized to 100%.

4.7.3 Mouse tissue single-cell suspension preparation (II)

Mice were euthanized and organs were immediately harvested. Dissected tumors and lungs were cut into small pieces and digested in RPMI supplemented with Liberase TL (Roche) and DNase I (Sigma Aldrich) at 37ºC for 30 min while shaking. RPMI supplemented with 2% NBCS was added in the digested tissues. These were then passed through a sterile cell strainer with 70 um nylon mesh (FisherBrand). The samples were washed and the pellets were resuspended with PBS and were purified on a Histopaque-1077 (Sigma Aldrich) discontinuous gradient centrifugation at 774 x g for 20 min at room temperature without brake. The collected buffy coats were then washed and the cell pellets were quantified using dual-chamber cell counting slides (Bio-Rad) and a TC20 automated cell counter (Bio-Rad).

Lymph nodes were cleaned from the fat tissue and were mechanically dissociated. After the dissociation, they were enzymatically digested using 1 mL RPMI supplemented with Liberase TL and DNase I at 37ºC for 20 min. RPMI supplemented with 2% NBCS was used to stop the digestion and the tissue was passed through a sterile cell strainer with 70 μm nylon mesh. The cell numbers were then determined using the same method described for the tumor tissue.

4.8 Tissue immunofluorescence (I)

Immunofluorescence was performed on FFPE UM tissues (n=12) following antigen retrieval using the Dako PT link system. Sections were incubated with anti-adipophilin (1:250) and anti-CD163 (1:400) Abs, followed by Alexa Fluor 555 (red) and Alexa Fluor 488 (green) secondary Abs (Leica). Nuclei were counterstained with 10 μg/ml Hoechst 33342.

4.9 Transcriptomic network analysis of metabolic adaptation (I)

Genes involved in normoxia, hypoxia, lipid metabolism, and lipolysis were analyzed in relation to BAP1 expression to assess their prognostic value using Kaplan-Meier survival analysis in the Xena browser. Based on differential expression patterns,

genes were classified as being associated with either favorable or poor clinical outcomes. Network visualizations of prognostication-relevant gene sets were generated using the circlize package in R(Gu et al., 2014). Genes linked to hypoxia and lipid-related pathways were curated from published studies and the NanoString PanCan Immune oncology multigene panel, as previously reported(Figueiredo et al., 2020). In the network plots, link width reflects gene expression variance (σ^2) across samples, calculated from expression scores derived from GDC-TCGA-UM cohort while link color corresponds to the log-rank p-value from survival analysis.

4.10 L1000FWD drug prediction studies (I)

Normalized RNA-seq data of PLIN2-supervised multigene signatures from the TCGA cohort were analyzed using the L1000FWD platform(Wang et al., 2018). This tool compares query signatures with transcriptional profiles from >20.000 compounds in the LINCS project. Drug clusters were visualized as scaffold networks, where red clusters represent compounds mimicking the metabolic shift in UM and blue clusters denote drugs predicted to reverse it.

4.11 Viability assay (I)

From the predicted compounds from the L1000FWD platform selected ones were used from further investigation. Those were: batimastat, bosutinib, carvedilol, palbociclib, fenbendazole, forskolin, selumetinib, tamibarotene, and vemurafenib. Stock solutions were prepared in DMSO (10-50mM). Mel285 and MP46 cells (10^4/well of 96-well plate) were treated with increasing drug concentrations (0-500 μM) for 24 h. Cell viability was assessed using the CellTiter96 Aqueous One Solution (MTS) assay (Promega) and measured at 490 nm (Victor, PerkinElmer). IC50 values were calculated by non-linear regression using GraphPad Prism v8.

4.12 Transcriptome analysis (I & II)

4.12.1 Reverse transcription PCR & quantitative real-time PCR (I)

Total mRNA from UM cells- MΦ co-cultures was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA concentration, purity, and integrity were determined using a Nanodrop UV-Vis spectrophotometer (ThermoFisher Scientific). For the reverse transcription PCR (RT-PCR), 1000 ng of isolated RNA in V_{max}=10 μL were mixed with 100 ng of Oligo dT (Invitrogen) and RNAse free water. The RNA-Oligo dT mix was incubated for 10 min at 70°C using

a thermocycler. After incubation the RNA-Oligo dT mix was chilled on ice and a master mix was prepared containing: dNTPs (Thermo Scientific), 5X First Strand Buffer (Invitrogen), 0.1 M DTT (Invitrogen), Superscript II enzyme (Invitrogen), Riboblock (Thermo Scientific), and RNAse free water. The master mix was added into the RNA-Oligo dT mix and was incubated for 50 min at 42°C followed by 15 min at 70°C in a thermocycler.

The cDNA from the RT-PCR reaction was diluted 1:5 with pure water. A master mix was prepared for the qPCR containing: iTaq Universal SYBR Green Supermix (BioRad), forward and reverse primer (Integrated DNA Technologies), the template, and RNAse-free water. The strip tubes were centrifuged and loaded into CFX96 Touch Real-Time PCR Detection system (Bio-Rad). The programmed followed included pre-incubation step for 10 min at 95°C, amplification for 37 cycles for 10 sec at 95°C followed by 20 sec at 60°C and 30 sec at 72°C and a melting curve for 5 sec at 65°C, 1 min at 95°C and 97°C continuous. Relative expression levels were normalized to *GAPDH* expression.

4.12.2 Bulk RNA sequencing (I)

RNA from Mel285 and MP46 cells (RIN≥9) was processed using the Illumina Standard mRNA library kit and sequenced on an Illumina NovaSeq platform (2x100 bp, 41-47M reads/sample). Read quality was checked using a FastQC (v0.11.9), and transcript quantification was performed with Kallisto (v0.48.0) against the GRCh38 reference genome(Bray et al., 2016). Fold-changes between lines were calculated, and expression heatmaps were generated using the R pheatmap package: $GeneTPM_{Mel285}$-$GeneTPM_{MP46}$/$GeneTPM_{MP46}$.

4.12.3 Single-cell RNA (scRNA) sequencing (II)

Single-cell suspensions were prepared as described above from *Cd74*+/+ and *Cd74*-/- naïve and tumor-draining lymph nodes, as well as from naïve spleens and spleens of tumor-bearing mice. Dead cells from the cell suspensions were removed using the Dead Cell Removal kit (Miltenyi Biotec) following the manufacturer's instructions. Sample integrity and RNA quality were assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies), and the concentration was measured with Qubit ®/Quant-IT® Fluorometric Quantitation (Life Technologies). All samples passed the quality assessment.

Single-cell RNA-seq libraries were prepared using the Chromium Next GEM Single Cell 3', v3.1 reagent kit (10XGenomics) according to the manufacturer's instructions. 10,000 cells per sample were loaded on the Chromium controller

(10XGenomics) to achieve optimal single-cell encapsulation. Dual indexing was performed used the Dual Index Kit TT Set A (10XGenomics).

Library fragment size and concentration were evaluated with Agilent Bioanalyzer 2100 and Qubit®/Quant-IT® Fluorometric Quantitation, respectively. The average library fragment size was 471 bp.

Sequencing was performed at Finnish Functional Genomics Centre (FFGC, Turku Bioscience Centre) on an Illumina NovaSeq 6000 instrument using a S4 v1.5 flow cell. Libraries were sequenced with paired-end sequencing (2x50 bp) and a ≥ 90% Q30 base quality threshold. Base calling and demultiplexing were performed with CellRanger v7.2.0 (10XGenomics) using cellranger mkfastq. Raw data were delivered in FASTQ format.

4.12.4 scRNA sequencing analysis (II)

4.12.4.1 Data processing and quality control (II)

Raw sequencing data was processed using the 10x Genomics Cell Ranger pipeline. Initial dataset comprised 8 samples: *Cd74-/-* and *Cd74+/+* tumor-draining lymph node (tdLN), naive lymph node, spleen from tumor-bearing mice, and naive spleen. Quality control metrics included: minimum threshold of 100 genes per cell, mitochondrial gene content < 20%, ribosomal gene content < 50%, hemoglobin gene content < 2%. Cells were filtered based on gene count distribution with lower cutoff at 1st percentile and upper cutoff at 98th percentile. Doublet detection was performed using Scrublet. Expected doublet rates were calculated using a linear model based on cell recovery numbers. Doublet scores were computed for each sample independently, and cells predicted as doublets were removed from downstream analysis. Detected doublet rates varied slightly between samples: lymph nodes: 1.1-3.4%, and spleen: 1.5-2.3%. Data integration was performed using Scanorama. Highly variable genes (HVGs) were identified using Seurat v3 methodology preserving genes that were highly variable at least in one sample. Integration steps included: normalization to 10,000 counts per cell, log-transformation, PCA computation (30 components), neighbor graph construction using Scanorama-transformed data, UMAP and t-SNE visualization.

4.12.4.2 Clustering and iterative refinement (II)

Clustering was performed using the Leiden algorithm (sc.tl.leiden) at multiple resolutions to capture both broad and fine cell populations. Initial clustering was performed at resolution = 1.0 (leiden_initial). Clusters containing DCs, monocytes, and macrophages were further refined by subsetting and re-clustering at lower

resolutions (e.g., 0.3 for cDC1 and cDC2 sub-clusters in spleen samples). The final cluster assignment was set to the most refined clustering. Uniform Manifold Approximation and Projection (UMAP) was used to visualize cell populations (sc.pl.umap).

4.12.4.3 Cell type annotation (II)

Initial cell type predictions were performed using CellTypist (v1.3.0) with the Immune_All_Low.pkl model. Mouse-to-human gene symbol conversion was conducted using the Jackson Laboratory's HomoloGene mapping file, ensuring compatibility with CellTypist's human reference models. Clusters were annotated based on differential expression analysis (DEA) and canonical marker genes curated from PanglaoDB, Tabula Muris, ACT (Annotation of Cell Types, formerly CellMarker). DEA was performed using the Mann-Whitney U test (i.e. Wilcoxon rank-sum test, sc.tl.rank_genes_groups with method = 'wilcoxon', corr_method='benjamini-hochberg'). Genes with adjusted p-values p_adjusted < 0.03 and log2 fold-change > 1.0 were considered significant. Custom annotation dictionaries were created for both low- and high-resolution cell type assignments, mapping cluster IDs to biological cell types (e.g., "Naive B cells," "Lymphoid-resident cDC1,"). These annotations were stored in the cell_type_low_res and cell_type_high_res columns of the AnnData object. Gene module scores for key immune populations (B cells, T cells, DC subsets, monocytes, macrophages, etc.) were calculated using sc.tl.score_genes. Marker sets were derived from published literature and public databases (Anderson et al., 2018; Brown et al., 2019; Jackson et al., 2011; Schlitzer et al., 2015). UMAPs displaying module scores were generated to validate cell type assignments and subpopulation identities.

4.12.4.4 Differential gene expression of *Cd74-/-* and *Cd74+/+* in various cell types (II)

Differential gene expression between conditions was assessed using the Mann–Whitney U test (two-sided) for each gene within each cell type or aggregated group, requiring at least two cells per group for inclusion. p-values were corrected for multiple testing using the Bonferroni method. For combined or subsetted cell populations (e.g., "Combined DCs"), data from all relevant subtypes were aggregated prior to statistical testing.

4.12.4.5 Pseudobulk differential gene expression analysis with interactions (II)

For each spleen sample and cell type group from DC subtypes, raw count matrices were aggregated by summing counts across all cells belonging to each sample-cell type combination. To increase robustness and account for biological variability, each sample was randomly subdivided into four pseudobulk replicates. For each replicate, cells were randomly assigned and counts summed, yielding four pseudobulk samples per condition (naïve/pathogenic, referring to tumor-bearing) per group (*Cd74-/-* and *Cd74+/+*). The resulting pseudobulk count matrices were used as input for downstream DEA. DEA was performed using the PyDESeq2 package, which implements the DESeq2 methodology for bulk RNA-seq adapted to pseudobulk single-cell data. The design formula included genotype, pathogenicity, and their interaction: ~genotype + pathogenicity + genotype:pathogenicity. The interaction analysis was carried out using R version of DESeq2 because the python implementation of interaction designs was not available at the time of the analysis. For each comparison, Wald tests were performed to estimate log2 fold changes and significance, Multiple testing correction was applied using the Benjamini-Hochberg false discovery rate (FDR) method, and a pseudocount (eps = 1e-300) was added for numerical stability in log-transformation of p-values. Genes were considered significantly differentially expressed if they met both log2 fold change and adjusted p-value thresholds $|\log2FC| > 0.5$ and $padj < 0.05$. Volcano plots were constructed to visualize the relationship between log2 fold change and significance (log-transformed adjusted p-values), with top genes labeled directly on the plots. Clustermaps (heatmaps) were generated using log1p-normalized pseudobulk expression for the top differentially expressed genes, comparing *Cd74+/+* and *Cd74-/-* across conditions.

4.12.4.6 Gene Ontology (II)

Differentially expressed genes from DC pseudobulk interaction DEA were used for gene ontology analysis (GOA) with python package GOATOOLS. GO terms were filtered for immune relevance by including all GO terms higher in the tree for lists of GO terms determined as core immune processes, DC-specific processes, tumor microenvironment relevant, cellular processes relevant to DCs, and additional immune processes, and further by a long list of immune-related keywords. Finally, only GO terms with at least 5% of the pathway genes represented by our list of DEGs, and with $p\_corrected<0.01$ (FDR: Benjamini-Hochberg), were included. The GO terms were then clustered into clusters of Jaccard similarity greater or equal to 0.4, and the clusters were given descriptive names.

4.13 Flow cytometry (II)

Table 2. Antibodies and reagents used in flow cytometry experiments (**II:** Table S1).

TARGET	REACTIVITY	FLUOROPHORE	CLONE	SUPPLIER
CD8A	MOUSE	FITC	53-6.7	Biolegend
CD62L	MOUSE	PE	MEL-14	Biolegend
CD44	MOUSE/HUMAN	PE/CY7	IM7	Biolegend
CD25	MOUSE	APC	PC61	Biolegend
CD3	MOUSE	APC/CY7	17A2	Biolegend
CD45	MOUSE	violetFluor™ 500	30-F11	CyTEK
CD4	MOUSE	cFluor® V610	RM4-5	CyTEK
FOXP3	MOUSE/HUMAN	PerCP/eFluor™ 710	FJK-16s	eBioscience
KI-67	MOUSE/HUMAN	Brilliant Violet™ 786	SolA15	Invitrogen
PD-1	MOUSE	PE/eFluor™ 610	J43	eBioscience
CD69	MOUSE	Brilliant Ultra Violet™ 737	H1.2F3	eBioscience
GRANZYME B	MOUSE/HUMAN	APC	QA16A02	Biolegend
IFN-γ	MOUSE	PE	XMG1.2	Biolegend
LY6C	MOUSE	PerCP/Cyanine 5.5	HK1.4	Biolegend
CD11C	MOUSE	PE/Cyanine 7	N418	Biolegend
CD3	MOUSE	PE/Cyanine 5	17A2	Biolegend
CD11B	MOUSE/HUMAN	APC	M1/70	Biolegend
B220	MOUSE/HUMAN	redFluor™ 710	RA3-6B2	CyTEK
MHC-II	MOUSE	APC/Cyanine 7	M5/114.15.2	Biolegend
LY6G	MOUSE	violetFluor™ 450	1A8	CyTEK
CD80	MOUSE	Brilliant Violet™ 480	16-10A1	eBioscience
MHC-I	MOUSE	Super Bright 702	AF6-88.5.5.3	eBioscience
CD103	MOUSE	Brilliant Ultra Violet 737	2E7	eBioscience
CD24	MOUSE	Super Bright 645	M1/69	eBioscience
CD44	MOUSE/HUMAN	Brilliant Violet 605™	IM7	Biolegend
CD11C	MOUSE	Alexa Fluor® 700	N418	Biolegend
B220	MOUSE/HUMAN	Pacific blue	RA3-6B2	Biolegend
CD24	MOUSE	APC	M1/69	Biolegend
CCR7	MOUSE	APC	4B12	eBioscience
CCR7	MOUSE	PE	4B12	Biolegend
TCR Vβ5.1, 5.2	MOUSE	PE/Cyanine 7	MR9-4	Biolegend
TruStain FcX™ (CD16/32)	MOUSE	N/A	N/A	Biolegend
MHC-I Dextramer SIINFEKL (H-2Kb)	MOUSE	APC	N/A	Immudex
MHC-I Dextramer SSYSYSSL (H-2Kb) Negative control	MOUSE	APC	N/A	Immudex

TARGET	REACTIVITY	FLUOROPHORE	CLONE	SUPPLIER
LIVE/DEAD™ Fixable Yellow Dead Cell Stain	N/A	405/570 NM	N/A	Invitrogen
ViaDye™ Red Fixable Viability Dye	N/A	615/740 NM	N/A	CyTEK
Zombie NIR™ Fixable Viability Kit	N/A	640/743 NM	N/A	Biolegend

4.13.1 Cell surface staining

Cells were washed with 1X PBS and centrifuged at 300 x g for 5 min. Cell pellets were first incubated with either yellow or red fixable viability dye depending on the experiment. After a washing step, cells were incubated with anti-mouse CD16/32 for 10 min in 4ºC and then incubated with an antibody cocktail, as listed in the antibodies and reagents used for flow cytometry table (**II:** Table S1), diluted in flow cytometry staining buffer (2% BSA, 0.5 mM EDTA, in PBS). For the staining of the blood samples, the procedure was the following. First, blood samples were collected in EDTA tubes (BD). Then the samples were incubated with SIINFEKL MHC Dextramer (Immudex) or negative control peptide (SSYSYSSL) MHC Dextramer (Immudex) in staining buffer (PBS, 1 mM EDTA, 2% NBCS, pH 7.4) at RT in the dark, for 10 min. Without washing the samples were blocked with anti-mouse CD16/32 in RT and protected from light for 10 min followed by incubation with relevant antibodies in 4ºC for 20 min (Table 2). After the staining, red blood cells were lysed with 1X BD FACS™ Lysing Solution (BD) following manufacturer's instructions. When the samples' turbidity changed, 1 mL of staining buffer was added to wash followed by a centrifugation. After centrifugation, the supernatants were discarded, and the wash step was repeated. In the final step, 150 μL of staining buffer were added per sample.

4.13.2 Intracellular staining

For the intracellular staining of T cells in the TME, we first incubated 10^6 cells from tumor single-cell suspension from each sample with 1X brefeldin A (BioLegend) and 25 ng/mL PMA (Sigma Aldrich) diluted in 10% RPMI at 37ºC for 4 hours. Then, the samples were stained for surface markers following the above-mentioned protocol. After the last washing step, the cells were fixed and permeabilized by resuspending the cell pellet in BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD) following the manufacturer's instructions. Briefly, cells were incubated with Fixation/Permeabilization solution at RT in the dark, for 30 min. After this incubation, cells were washed with 1X BD Perm/Wash Buffer and centrifuged. The cell pellets were incubated with the relevant antibodies in 1X Perm/Wash Buffer at RT

in the dark, for 30 min. Two washing steps followed using 1X Perm/Wash Buffer and centrifugation. Finally, the cell pellets were resuspended in flow cytometry staining buffer.

Regarding the intracellular staining of transcription factors, Foxp3 and Ki-67, in T-cell populations, the cells were primarily stained for surface markers. After the final wash, the cell pellets were completely dissociated via pulse vortex in the residual volume before the use of Foxp3/Transcription Factor Staining Buffer Set (Invitrogen). First, Foxp3 Fixation/Permeabilization working solution was added into each sample followed by pulse vortex. The samples were incubated at RT for 30 min protected from light. Then, samples were washed with 1X Permeabilization Buffer and centrifuged to discard the supernatant. The cell pellets were resuspended in the residual volume and the relevant antibodies were added for incubation at RT in the dark, for 30 min. This procedure was followed by two washing steps using 1X Permeabilization Buffer. Finally, samples were resuspended in ion-free PBS containing 1 mM EDTA.

4.13.3 Intratumoral cytokine quantification

Intratumoral cytokine quantification was performed via flow cytometry and the LEGENDplex™ Mouse Anti-Virus Response Panel (Table 2) according to manufacturer's instructions.

4.13.4 Acquisition & analysis

Flow cytometry data acquisition was performed using a 3-laser Northern Lights spectral flow cytometer (CyTEK Biosciences) for samples derived from the in vivo Ag presentation assay (Section 4.5.3 of the thesis manuscript) or tumor challenge experiments that were stained as described in Sections 4.12.1, 4.12.2, and 4.12.3 of the thesis manuscript. Data acquisition for in vitro Ag presentation assay (Section 4.6.2 of the thesis manuscript) was performed using a 3-laser NovoCyte flow cytometer (Agilent). All flow cytometry data were analyzed using FlowJo software (version 10.10.0).

4.14 Statistical analysis (I & II)

GraphPad Prism (GraphPad Software, version 8 for I & version 10 for II) was used to perform all statistical analyses.

4.15 Artificial intelligence (Thesis manuscript)

An artificial intelligence (AI)-based language model (ChatGPT) was used to assist with language editing in limited sections of the literature review. The AI tool was not employed to generate text. All text was reviewed and edited by the author, who takes full responsibility for the contents of the thesis manuscript.

5 Results

5.1 Metabolic and immune consequences of PLIN2 loss are linked to prognosis and therapeutic vulnerabilities in UM

Reduced expression of adipophilin in UM correlates with *BAP1* loss, monosomy 3, and poor overall survival, supporting a link between lipid metabolism and tumor progression and immune suppression. Transcriptomic and proteomic analyses revealed that PLIN2 downregulation is associated with hypoxia and a shift from lipid storage to lipolysis, consistent with a metabolically reprogrammed, immunosuppressive TME in high-risk UM. Integrative network analysis identified *PLIN2*-based multigene signatures that predict prognosis and revealed potential therapeutic compounds, including adrenergic, MEK, and RAF inhibitors, predicted to reverse this metabolic profile. Among these, carvedilol restored PLIN2 expression while it reduced hypoxia-associated gene expression in UM cells-MΦ co-cultures.

5.1.1 Adipophilin expression correlates with survival and clinical outcomes in pUM

Previous work had reported adipophilin expression in a cohort of primary uveal melanoma (pUM) samples (Fiorentzis cohort, n=28). When adipophilin staining scores (intensity x proportion) were stratified by chromosome 3 status (disomy 3 versus monosomy 3), we observed a reduction in adipophilin expression in monosomy 3 tumors; however, this difference was not statistically significant within this cohort (**I:** Figure S1A). To determine whether adipophilin expression is significantly reduced in monosomy 3 tumors in a larger dataset, we analyzed transcriptomic data from the GDC-TCGA-UM cohort focusing on PLIN2 expression. We assessed the association between *PLIN2* expression and patient survival, chromosome 3 copy number, *BAP1* expression levels, and patient age. Reduced *PLIN2* expression was significantly associated with worse overall survival in UM patients (**I:** Figure 1A), as well as with monosomy 3 and *BAP1* loss (defined as *BAP1* expression below the cohort median) (**I:** Figure 1B). In addition, *PLIN2* expression showed an inverse correlation with patient age, with lower expression

observed in older individuals. These findings are consistent with those previously reported in the Fiorentzis cohort (Fiorentzis et al., 2017) and demonstrate a significant association between *PLIN2* downregulation, decreased survival, *BAP1* loss, monosomy 3, and age.

We next examined adipophilin protein expression in a second, independent UM cohort (LOORG cohort, n=43) (**I:** Table 1). This cohort had been previously characterized for nBAP1 expression by immunohistochemistry and chromosome 3 copy number alterations. FFPE tumor sections were stained for adipophilin, digitally scanned, and quantitatively analyzed based on size and circularity, as previously described(Figueiredo et al., 2018; Ireland et al., 2018). Representative immunohistochemical images using AEC red chromogen, along with particle detection thresholds across the entire tumor area, are shown for nBAP1-negative (blue) and nBAP1-positive (red) tumors (**I:** Figure 1D). Consistent with the findings from the Fioretzis and GDC-TCGA-UM cohorts, adipophilin expression was significantly lower in tumors with monosomy 3 and nBAP1-negative status (**I:** Figure 1E).

We also identified a negative correlation between adipophilin expression and patient age in this cohort (**I:** Figure S1B). Whether this association reflects tumor-specific metabolic changes or a broader ageing-related phenotype requires further investigation in larger RNA-seq datasets of normal ocular tissues spanning comparable age ranges, which are currently unavailable. Nonetheless, preliminary analysis of the NIH Eye Integration cohort RNA-seq data (n=8) (Swamy & McGaughey, 2019)demonstrated significantly higher *PLIN2* expression in fetal retinal tissue compared to adult retina (**I:** Figure S1C), suggesting that age-related decreases in *PLIN2* expression may contribute to increased cancer susceptibility with advancing age (Chatsirisupachai et al., 2021).

The consistent association of this clinical phenotype with adipophilin at both the RNA and protein levels across two independent cohorts indicates that adipophilin abundance in UM may be directly regulated by *PLIN2* expression. No associations were observed between adipophilin expression and copy number alterations of chromosomes 6p, 6q, 8p. or 8q (**I:** Figure S2A-D), nor with tumor patient's tumor ciliary body involvement (CBI) defined as tumor extension or presence within the ciliary body located behind the iris, cell type, extraocular extension, patient sex, necrosis profile, tumor height, largest basal diameter, or mitotic count (**I:** Figure S2E-L).

5.1.2 BAP1 and PLIN2 deficiency associates with elevated HIF1A expression and MΦ abundance in pUM

Previous works indicate that loss of *BAP1* affects cellular metabolism and immune-related functions (Carbone et al., 2020; A. Han, T. J. Purwin, & A. E. Aplin, 2021). More recently, *BAP1* loss was identified as a major driver of TAM-dependent immunosuppression in UM (Figueiredo et al., 2020), suggesting a connection between loss of *BAP1*, adipophilin expression, and thus potentially lipid metabolism, as well as TAMs infiltration. First, CD163, a MΦ marker, was examined for its association with survival outcomes in the GDC-TCGA-UM cohort. Elevated CD163 expression was associated with reduced overall survival in UM patients (**I:** Figure 2A). CD163+ M2-like TAMs rely heavily on lipid uptake as a terminal metabolic pathway (S. Li et al., 2022; Ma et al., 2022), particularly under hypoxic conditions, which are largely regulated by hypoxia-inducible factors such as hypoxia-inducible factor 1-alpha (HIF-1a) (Munir et al., 2019).

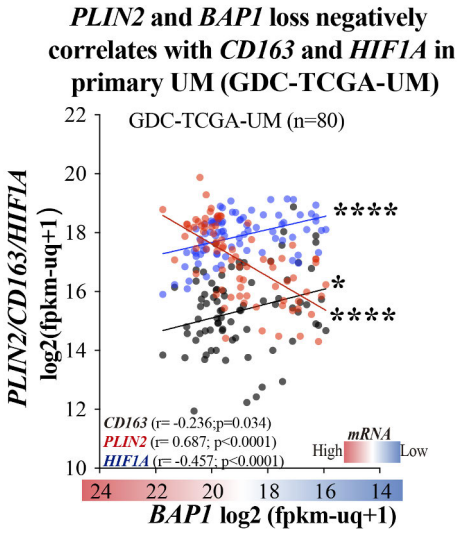


Figure 10. Correction for Figure 2B (I). The red-blue color axis was corrected to represent *BAP1* expression levels, with red indicating high BAP1 expression and a transition to blue indicating decreasing *BAP1* expression.

Consistent with this, lower *BAP1* expression in the GDC-TCGA-UM cohort was associated with increased expression of CD163 and HIF-1a, alongside reduced *PLIN2* expression (**I:** Figure 2B, Figure 10). Furthermore, LDs containing adipophilin appeared diffusely distributed within the cytoplasm of nBAP1-negative UM samples, suggesting LD breakdown potentially caused due to increased infiltration of CD163+ TAMs (I: Figure 2C, D). Together, these results indicate that

BAP1 loss is linked to metabolic reprogramming from normoxic lipid storage toward lipid consumption under hypoxic conditions in high-risk UM, characterized by a reduction in adipophilin-positive LDs (**I:** Figure 2E).

5.1.3 Enhanced protein secretion uncovers novel metabolic signatures in pUM

To investigate whether metastatic risk in UM is associated with reduced lipid storage and enhanced lipolysis in high-risk tumors, we re-analyzed the secretome profiles of a subset of pUM samples from the LOORG cohort (n=14) that had been cultured ex vivo (Angi et al., 2016). The analyzed secretome comprised proteins with clinical relevance to hypoxia and lipid metabolism (**I:** Table S1 and S2). We found that increased overall secretory activity, measured as the mean protein fold-change, correlated with higher metastatic risk scores, suggesting elevated metabolic activity in high-risk UM (**I:** Figure 3).

Each secreted protein was assigned a clinical risk score ranging from -2 (no risk, blue) to +2 (high risk, red), visualized in a heatmap sorted by total protein expression levels (**I:** Figure 3B). When ordered by expression, a clear shift from low- to high-risk profiles emerged. The corresponding gene symbols were then analyzed using the Xena browser to assess transcript levels in the GDC-TCGA-UM cohort, stratified by *BAP1* expression. This analysis revealed two main clusters, genes upregulated with preserved *BAP1* expression and associated with favorable outcomes as well as genes upregulated following *BAP1* loss, which are associated with poor prognosis. Good-outcome predictors were linked to patient survival (white) while poor-outcome predictors were associated with death (black) (**I:** Figure 3).

To validate the prognostic relevance of these secretome-derived proteins and identify those involved in metabolic regulation, we assessed their impact on survival in the GDC-TCGA-UM cohort. Proteins and genes consistently associated with poor outcomes in both cohorts were selected for downstream analyses (**I:** Table S3). A meta-analysis was conducted to categorize these genes according to metabolic pathways, including normoxia, hypoxia, lipid storage, and lipolysis. Each category was linked to gene expression variance (σ^2) within the GDC-TCGA-UM cohort, as a measure of transcriptional plasticity (**I:** Table S2) and to Kaplan-Meier survival p-values (**I:** Table S3). These parameters were integrated into a network-category chord plot, ordered by survival impact and variance, with chord thickness reflecting gene expression σ^2. Genes with statistically significant survival associations were color-coded (blue, orange, red).

Notably, genes predicting favorable outcomes clustered within normoxia and lipid storage pathways (**I:** Figure 3D). On the other hand, genes associated with poor survival grouped with hypoxia and lipolysis pathways (**I:** Figure 3D). These

metabolic signatures were further examined in UM cell lines with retained BAP1 expression (Mel285) or BAP1 loss (MP46). While several genes mirrored expression patterns observed in bulk RNA-seq data from the GDC-TCGA-UM cohort, discrepancies were also noted. For example, *SNTB2* was upregulated in BAP1-loss tumors in patient data (**I:** Figure 3C, D) but downregulated in MP46 cells compared with Mel285 cells (**I:** Figure 3E). These differences likely reflect contributions from non-tumor components of the TME. Supporting this, *SNTB2* and *FN1* expression increased in co-cultures of MP46 cells with macrophages derived from human peripheral blood monocytes (**I:** Figure S3A), suggesting that *BAP1* loss in tumor cells may induce metabolic signatures in stromal cells such as TAMs.

Quantitative analysis showed that 65.5% of the normoxia/lipid storage signature originated from tumor cells, while 34.4% was attributable to the TME in *BAP1*-positive tumors (**I:** Figure 3E). Conversely, in *BAP1*-negative tumors, tumor cells accounted for 33.3% of the hypoxia/lipolysis signature, with the TME contributing 66.7% (**I:** Figure 3E). Representative Kaplan–Meier curves for selected genes included *SPP1* and *LAMA4* (good prognosis) and *HTRA1* and *SNTB2* (poor prognosis) (**I:** Figure 3F). Collectively, these results indicate that genes inversely correlated with *BAP1* and *PLIN2* expression are associated with a metabolic shift from normoxia/lipid storage to hypoxia/lipolysis in UM.

5.1.4 Metabolic reprogramming linked to PLIN2 suggests new therapeutic opportunities in UM

The identification of adipophilin-based multigene metabolic signatures provides a foundation for developing targeted metabolic therapies for *BAP1*-deficient UM (A. Han, T. J. Purwin, N. Bechtel, et al., 2021). Repurposing existing drugs capable of reversing tumor metabolic evolution may offer a therapeutic benefit. Metabolic signatures with significant survival impact and high expression variability in the GDC-TCGA-UM cohort (**I:** Figure 4A) were submitted to the L1000FWD platform to identify small molecules predicted to shift the hypoxia/lipolysis transcriptional profile toward a normoxia/lipid storage state.

The resulting drug signature was visualized in a scaffold distribution plot, where red clusters represented compounds capable of mimicking the adverse metabolic signature, while blue clusters (c1-c4) contained drugs with the greatest potential to mimic *BAP1*-positive metabolic profiles or reverse the metabolic shift in *BAP1*-negative UM (**I:** Figure 4B). Notably, compounds within these clusters shared common mechanisms of action, including adrenergic receptor agonists (c1), PI3K, CDK, and tubulin inhibitors (c2), MEK and RAF inhibitors (c3), and retinoid receptor agonists (c4). Most identified drugs were either approved or in Phase II/III clinical development (**I:** Figure 4C). The top five compounds from each cluster,

along with their mechanisms of action and clinical status, are summarized in **I:** Table 2.

Given the vulnerability of cancer cells to metabolic perturbations (Ackerman & Simon, 2014), we tested the cytotoxic effects of nine candidate drugs (**I:** Table 3) in UM cell lines in vitro: batimastat, bosutinib, carvedilol, palbociclib, fenbendazole, forskolin, selumetinib, tamibarotene, and vemurafenib. Dose-response curves (**I:** Figure S3B) with IC_{50} values and curve-fitting parameters (R^2) are reported in Table 3. Five of the nine compounds (55.5%) significantly reduced Mel285 cell viability, including bosutinib (IC_{50}=8.8 μM), palbociclib (IC_{50}=37.8 μM), carvedilol (IC_{50}=58.1 μM), vemurafenib (IC_{50}=200.3 μM), and forskolin (IC_{50}=266.9 μM) (**I:** Table 3; Figure S3B). Among these, carvedilol demonstrated moderate cytostatic effects (**I:** Figure 4D) and has previously been shown to counteract HIF-1-mediated hypoxia in cardiac models (Shyu et al., 2005).

We therefore investigated whether sub-cytotoxic doses of carvedilol could reverse poor-prognosis metabolic signatures or enhance favorable ones in co-cultures of *BAP1*-negative MP46 cells with human macrophages derived from peripheral blood (**I:** Figure 4E). Carvedilol treatment restored *PLIN2* expression, increased *LAMA4* levels (normoxia/lipid storage signature), and reduced *FN1* and *SNTB2* expression (hypoxia/lipolysis signature) (**I:** Figure 4F; Figure S3C). Together, these findings indicate that selected repurposed drugs can reduce UM cell viability, restore *PLIN2* expression, and potentially reverse metabolic signatures associated with poor clinical outcomes in *BAP1*-deficient tumors.

5.2 CD74 restrains antitumor immunity by limiting DC functions and CD8+ T cell responses in melanoma

5.2.1 CD74 deficiency reshapes the immune organization of naïve lymph nodes

CD74-MIF interaction has been implicated in tumor-driven immune suppression in melanoma and breast cancer (Figueiredo et al., 2018). While this study identify CD74 as a potential regulator of myeloid cell function, it remains critical, particularly for therapeutic targeting, to determine whether CD74 also controls DC functions under homeostatic conditions and independently of MIF. To address this question and define the impact of CD74 deficiency on myeloid cells and lymphoid tissue organization at steady state, we utilized a constitutive *Cd74-/-* mouse model (Bikoff et al., 1993).

We first investigated whether constitutive depletion of CD74 affects DC and T cell populations in naïve lymph nodes, which represent a central site for DC–T cell

interactions relevant to tumor immunity. Total cellularity of naïve lymph nodes, including both immune and stromal compartments, was reduced in *Cd74-/-* mice compared to *Cd74+/+* controls (**II:** fig. S1A, fig. S2A). Among DC subsets, the frequency of migratory cDC1s was increased in naïve inguinal lymph nodes of *Cd74-/-* mice, whereas their absolute numbers, as well as those of total and lymphoid tissue-resident cDC1s, were comparable between genotypes (**II:** fig. S1A, fig. S2B). In contrast, while the frequencies of cDC2s and pDCs remained unchanged, the absolute number of cDC2s was reduced in *Cd74-/-* mice (**II:** fig. S2B).

Phenotypic analysis revealed increased CD40 expression on lymphoid tissue–resident cDC1s and reduced CD80 expression on migratory cDC1s in *Cd74-/-* mice, whereas PD-L1 expression was unchanged across DC subsets (**II:** fig. S2C). Notably, migratory cDC1s displayed elevated cell-surface MHC-I expression, suggesting potentially enhanced antigen presentation capacity (**II:** fig. S2D). A similar trend toward increased MHC-I expression was observed in lymphoid tissue–resident cDC1s and cDC2s (**II:** fig. S2D). Collectively, these data indicate that total DC numbers in naïve lymph nodes are preserved in *Cd74-/-* mice, with the observed differences reflecting altered subset distribution and increased MHC-I expression per cDC1 rather than expansion of MHC-I+ cDC1s.

Consistent with the increased frequency of migratory cDC1s expressing higher levels of MHC-I, *Cd74-/-* mice exhibited remodeling of their T cell compartment. The proportion of CD8+ T cells was increased in *Cd74-/-* mice, while their absolute numbers remained unchanged (**II:** fig. S2E, F). Conversely, CD4+ T cells were reduced both in frequency and absolute number, in line with impaired MHC-II–dependent homeostatic maintenance (Bikoff et al., 1993; Wang et al., 2021) (**II:** fig. S2E, F). Under steady-state conditions, markers of activation and exhaustion, as well as the distribution of naïve, effector, and central memory subsets, were comparable in both $CD4^+$ and $CD8^+$ T cells between *Cd74-/-* mice and *Cd74+/+* controls (fig. S2G, H).

Altogether, these findings demonstrate that constitutive loss of CD74 remodels the immune architecture of naïve lymph nodes, characterized by reduced total cellularity, increased CD8+ T cell frequency, and diminished CD4+ T cells. Despite preserved overall DC numbers, cDC subsets in *Cd74-/-* mice display elevated basal MHC-I expression, potentially supporting enhanced immune readiness associated with an increased proportion of immunogenic cDC1 subsets.

5.2.2 Host CD74 deficiency limits melanoma growth and permits effector CD8+ T cell infiltration

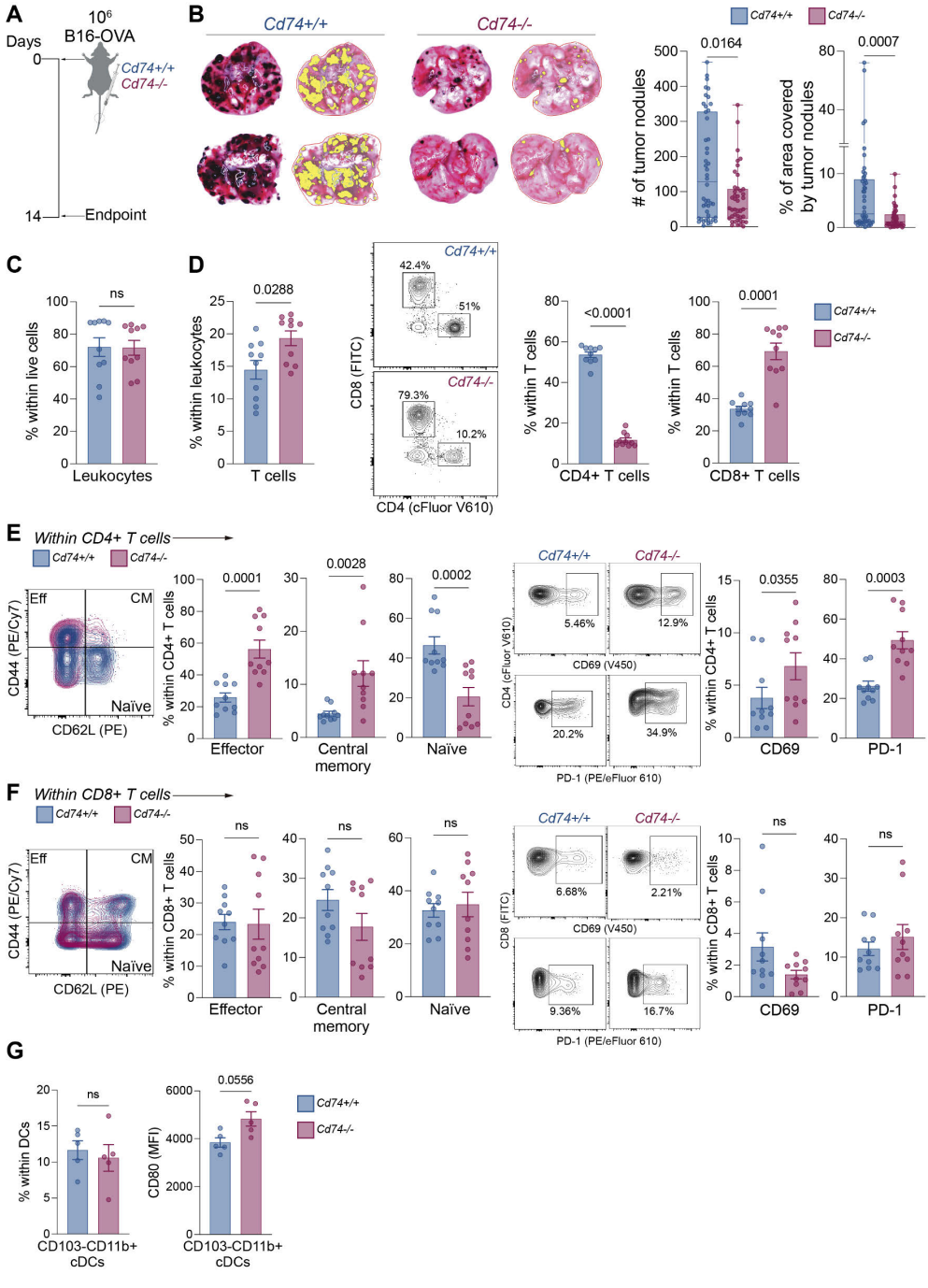
Based on these observations, we next evaluated whether the immune alterations detected in naïve lymph nodes of *Cd74-/-* mice translate into enhanced antitumor

immunity in vivo. *Cd74-/-* mice and *Cd74+/+* controls were subcutaneously challenged with ovalbumin-expressing B16 melanoma cells (B16-OVA) (**II:** Fig. 1A), and we observed that *Cd74-/-* mice exhibited reduced tumor growth compared with control animals (**II:** Fig. 1B).

To determine whether this effect correlated with changes in the immune landscape at the endpoint (day 20), we profiled tumor-infiltrating leukocytes using spectral flow cytometry. This analysis revealed a trend toward increased numbers of tumor-infiltrating leukocytes in *Cd74-/-* mice (**II:** Fig. 1C, D) (Ishihara et al., 2019; Lacher et al., 2024; X. Liu et al., 2020). Further analysis demonstrated that CD74 deficiency increased the frequency of CD8+ T cells within the tumor (**II:** Fig. 1E). Quantification of immune cells per 100 mg of tumor tissue confirmed elevated infiltration of CD8+ T cells in *Cd74-/-* mice (**II:** Fig. 1F).

Although Tregs also showed a trend toward increased numbers in the TME of *Cd74-/-* mice, the ratio of CD8+ T cells to Tregs was elevated in absence of CD74 (**II:** Fig. 1G), indicating a shift toward an immune landscape associated with enhanced immunosurveillance. Both CD4+ and CD8+ T cells displayed increased frequencies of Ki-67+ cells, consistent with enhanced proliferation in the TME of *Cd74-/-* mice (**II:** Fig. 1H, I). Furthermore, increased numbers of tumor-infiltrating $CD8^+$ T cells exhibited effector (CD44+CD62L-) and central memory (CD44+CD62L+) phenotypes in *Cd74-/-* mice compared with controls (**II:** Fig. 1J, fig. S3A, B), whereas no differences were observed in naïve CD4+ or CD8+ T cells (**II:** fig. S3C). Notably, the TME of *Cd74-/-* mice contained higher numbers of CD69+ CD8+ T cells (**II:** fig. S3D, E), suggesting enhanced local activation or prolonged tissue residency (Hayashizaki et al., 2016; Mackay et al., 2015; Walsh et al., 2019).

Figure 11.► CD74 deficiency reduces experimental tumor metastasis and modulates the T cell compartment in lungs. (**A**) Experimental design for the B16-OVA subcutaneous challenge of 6-10-week-old male Cd74-/- and Cd74+/+ mice. (**B**) Representative images of lungs from tumor-bearing Cd74-/- and Cd74+/+ mice captured with Zeiss AxioZOOM.V16 and analyzed using NIS-Elements (left) and number of tumor nodules on both surfaces of the lungs of each mouse and percentage of area covered by the tumor nodules calculated using the selected ROI as 100% (right) (n=21-23 mice per group resulting in 42 and 46 data points from two sides of lungs, data from three independent experiments, Mann-Whitney U test). (**C**) Frequency of leukocytes in lungs (n=10 mice per group, data from two independent experiments, Mann-Whitney U test). (**D**) Frequency of T cells calculated within leukocytes, representative flow cytometry contour plots for analyzing the frequencies of CD4+ and CD8+ T cells in the lungs (n=10 mice per group, data from two independent experiments, Mann-Whitney U test). (**E, D**) Representative flow cytometry contour plots and frequencies of effector (Eff), central memory (CM), and naïve CD4+ (E) and CD8+ (D) T cells; as well as representative flow cytometry contour plots and frequencies of CD69+ and PD-1+ CD4+ (E) and CD8+ (D) T cells in lungs (n=10 mice per group, data from two independent experiments, Mann-Whitney U test). (**G**) Frequency of CD103-CD11b+ cDCs calculated in DCs and median fluorescence intensity (MFI) of CD80 calculated in CD103-CD11b+ cDCs in lungs (n=5 mice per group, data from one experiment, Mann-Whitney U test).


A
10^6
B16-OVA
Days
0
Cd74+/+
Cd74-/-
14
Endpoint
B
Cd74+/+
Cd74-/-
Cd74+/+
Cd74-/-
0.0164
0.0007
# of tumor nodules
% of area covered by tumor nodules
C
ns
% within live cells
Leukocytes
D
0.0288
% within leukocytes
T cells
Cd74+/+
42.4%
51%
Cd74-/-
79.3%
10.2%
CD8 (FITC)
CD4 (cFluor V610)
<0.0001
% within T cells
CD4+ T cells
0.0001
% within T cells
CD8+ T cells
Cd74+/+
Cd74-/-
E
Within CD4+ T cells
Cd74+/+
Cd74-/-
Eff
CM
Naïve
CD44 (PE/Cy7)
CD62L (PE)
0.0001
0.0028
0.0002
% within CD4+ T cells
Effector
Central memory
Naïve
Cd74+/+
Cd74-/-
5.46%
12.9%
20.2%
34.9%
CD4 (cFluor V610)
CD69 (V450)
PD-1 (PE/eFluor 610)
0.0355
0.0003
% within CD4+ T cells
CD69
PD-1
F
Within CD8+ T cells
Cd74+/+
Cd74-/-
Eff
CM
Naïve
CD44 (PE/Cy7)
CD62L (PE)
ns
ns
ns
% within CD8+ T cells
Effector
Central memory
Naïve
Cd74+/+
Cd74-/-
6.68%
2.21%
9.36%
16.7%
CD8 (FITC)
CD69 (V450)
PD-1 (PE/eFluor 610)
ns
ns
% within CD8+ T cells
CD69
PD-1
G
ns
0.0556
% within DCs
CD80 (MFI)
CD103-CD11b+ cDCs
CD103-CD11b+ cDCs
Cd74+/+
Cd74-/-

Consistent with these findings, intratumoral cytokine quantification revealed increased levels of the T cell–recruiting chemokines CCL5 and CXCL10 in *Cd74-/-* mice (**II:** Fig. 1K), supporting enhanced T cell infiltration. Altogether, these results indicate that CD74 deficiency promotes increased proliferation, retention, and memory formation of tumor-infiltrating CD8+ T cells, which could associate with the reduced melanoma growth.

In the metastatic B16-OVA model, *Cd74-/-* mice and *Cd74+/+* controls were intravenously challenged (Figure 11A**)** and the tumor nodules on the lungs of the mice were quantified using the NIS-Elements software (Figure 11B). CD74 deficiency efficiently reduced experimental tumor burden in the lungs as compared with control mice (Figure 11B), indicating enhanced systemic tumor control. Despite comparable leukocyte frequencies between genotypes, the immune composition of the lungs differed (Figure 11C). *Cd74-/-* mice exhibited increased T cell frequency, characterized by dramatic reduction of CD4+ T cells and a corresponding increase in CD8+ T cells (Figure 11D). Within the CD4+ T cell compartment, a higher proportion displayed effector and central memory phenotypes, accompanied by elevated proportion of CD69+ and PD-1+ cells (Figure 11E). In contrast, CD8+ T cells did not show significant alterations in differentiation states or activation between genotypes (Figure 11F). Analysis of cDCs revealed that CD103-CD11b+ (cDC2) cells in *Cd74-/-* mice demonstrated a trend for increased CD80 expression levels (Figure 11G), implying potentially better co-stimulation and presentation to CD4+ T cells (Gao et al., 2013; Guilliams et al., 2016; Persson et al., 2013; Shin et al., 2020; Williams et al., 2013). Together, these results demonstrate that loss of CD74 confers a more immunostimulatory lung environment, characterized by increased CD8+ T cells, activated CD4+ T cells, and potentially enhanced co-stimulatory potential in cDC2s. These coordinated changes are consistent with the reduced metastatic tumor burden in the lungs of CD74-deficient mice.

5.2.3 Increased CD8+ T cell cytotoxicity and sensitivity to PD-1 blockade are consequences of CD74 deficiency

To further assess intratumoral T cell function in the absence of CD74, we analyzed intracellular expression of IFN-γ and granzyme B (GrB), as well as surface expression of PD-1, in tumor-derived lymphocytes from *Cd74-/-* and *Cd74+/+* control mice. While the frequencies of IFN-γ+ CD4+ and CD8+ T cells were similar between genotypes, analysis of absolute cell numbers per 100 mg of tumor tissue revealed a significant increase in IFN-γ+ CD8+ T cells in *Cd74-/-* mice (**II:** Fig. 2A, Figure 12). Similarly, the number of CD8+ T cells expressing GrB was increased in the TME of *Cd74-/-* mice (**II:** Fig. 2A, Figure 12). In addition, the absolute number of PD-1+ CD8+ T cells was elevated in the TME of *Cd74-/-* mice (**II:** Fig. 2B).

These data indicate that while the proportion of cytotoxic T cells remains comparable, CD74 deficiency supports increased accumulation of activated, cytotoxic $CD8^+$ T cells, going toward exhaustion, within the TME.

Because *Cd74-/-* mice bear increased numbers of PD-1+ CD8+ T cells within the TME, we next assessed their response to PD-1 blockade. Tumor growth was evaluated in *Cd74-/-* and *Cd74+/+* mice treated with anti-PD-1 using B16F10 and YUMM1.G1 melanoma models, both of which exhibit resistance to PD-1 blockade. In the B16F10 model, treatment with anti-PD-1 delayed tumor progression in *Cd74-/-* mice compared to *Cd74+/+* controls (**II:** Fig. 2C), demonstrating improved therapeutic efficacy in the absence of CD74. A similar trend was observed in the YUMM1.G1 model, where PD-1 blockade resulted in a reduction in tumor volume in *Cd74-/-* (**II:** Fig. 2D), despite the resistance of this model to ICIs. Notably, across both melanoma models, the greatest degree of tumor control was consistently achieved in *Cd74-/-* mice receiving PD-1 blockade. Together, these findings show that CD74 deficiency potentiates the effects of PD-1 blockade to enhancing antitumor responses.

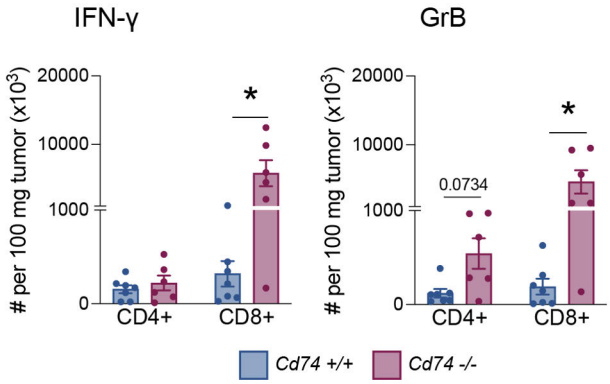


Figure 12. Correction for Figure 2A (II). The numbers of IFN-γ+ and GrB+ T cells per 100 mg of tumor tissue were calculated by applying the frequencies of IFN-γ+ and GrB+ cells within the CD4 and CD8 T cell population to the corresponding cell counts per 100 mg of tumor, as determined by extracellular staining of the same samples.

5.2.4 Selective upregulation of MHC-I on DCs is observed in *Cd74-/-* mice

The enhanced intratumoral T cell responses led us to hypothesize that CD74 deficiency alters DC composition or function to promote immune activation locally within the TME and tdLNs(Garris et al., 2018). Although DC frequencies within the TME were comparable between genotypes (**II:** Fig. 3A, B, fig. S1C), there was a trend toward increased DC numbers per 100 mg of tumor tissue in *Cd74-/-* mice (**II:**

Fig. 3A, B). Subset analysis revealed no differences in cDC subset abundance (**II:** Fig. 3C), and CD40 expression was similar across all DC subsets between genotypes (**II:** Fig. 3D). However, migratory cDC1s in the TME of *Cd74-/-* mice displayed a higher frequency of PD-L1 expression, along with a trend toward increased PD-L1 MFI (**II:** Fig. 3E). Furthermore, both the frequency and expression level of MHC-I on cDC1s, particularly the migratory subset, were elevated in *Cd74-/-* mice (**II:** Fig. 3F).

Cd74-/- B16-OVA-bearing mice also showed a trend toward increased pDCs frequency and augmented absolute pDCs number within the TME (**II:** fig. S5A). This accumulation of pDCs could be explained by the increased CCL2 concentration in the TME of *Cd74-/-* mice (Figure 13) (Drobits et al., 2012). pDCs from *Cd74-/-* mice expressed more CD80, indicating enhanced activation (**II:** fig. S5B). In line with these results, intratumoral IFN-α levels were elevated in the TME of *Cd74-/-* mice (**II:** fig. S5C), consistent with the pDCs origin of type I interferons (Arroyo Hornero et al., 2025; Wimmers et al., 2018). pDCs also exhibited increased frequency and expression of MHC-I in *Cd74-/-* compared to *Cd74+/+* control mice (**II:** fig. S5D).

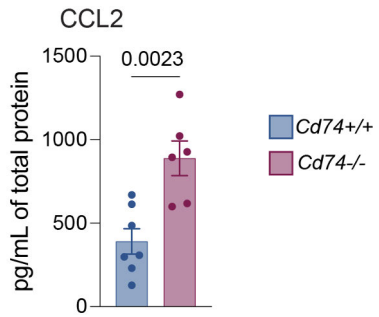


Figure 13. CCL2 concentration in the TME. Intratumoral CCL2 quantification in B16-OVA tumors in the endpoint of the tumor challenge experiments (n=6-7 mice per group, from two independent experiments, mean ± SEM, unpaired t-test).

Given that type I interferons promote cDC1 migration to tdLNs and facilitate tumor antigen transport (Busselaar et al., 2024), we hypothesized that elevated IFN-α in the TME of *Cd74-/-* mice might enhance cDC1 trafficking to tdLNs, thereby supporting systemic immune responses. At the early time point (day 14) of the tumor challenges, *Cd74-/-* mice exhibited increased frequency of total, migratory, and lymphoid tissue–resident cDC1s in tdLNs, while absolute numbers of migratory cDC1s were comparable and resident cDC1s trended lower (**II:** Fig. 4A). Absolute numbers of cDC2s were reduced despite unchanged frequencies, and total tdLNs' cellularity was similar between genotypes (**II:** fig. S6A). By the endpoint (day 20),

migratory cDC1 frequencies trended downward in the tdLNs of *Cd74-/-* mice (**II:** Fig. 4B), whereas absolute numbers of DC subsets were comparable. pDCs' frequency increased in the tdLNs of *Cd74-/-* mice (**II:** fig. S5F), and MHC-I expression on lymphoid tissue–resident cDC1s was elevated (**II:** Fig. 4C). These findings suggest that CD74 deficiency induces a transient reorganization of DC populations in tdLNs during early tumor growth rather than expansion of cDC1s, potentially reflecting altered DC trafficking that contributes to the modified tumor immune environment.

Analysis of T cells in the tdLNs revealed genotype-specific dynamics. At early time point, *Cd74-/-* mice showed a trend toward increased T cell frequency (**II:** fig. S6B), and both genotypes exhibited comparable expansion in absolute T cell numbers over time. *Cd74+/+* mice consistently displayed higher CD4+ T cell frequencies and numbers (**II:** fig. S6C), whereas CD8+ T cells prevailed in *Cd74-/-* mice (**II:** fig. S6D). Increased CD69 expression was observed on CD4+ T cells in the tdLNs of *Cd74-/-* mice during early tumor development, with more pronounced differences at the endpoint (**II:** fig. S6E). In contrast, CD69+ CD8+ T cell frequencies did not increase over time in *Cd74+/+* mice. PD-1+ CD4+ and CD8+ T cells were enriched in *Cd74-/-* mice during early tumor development, with PD-1+ CD4+ T cells remaining elevated at the endpoint (**II:** fig. S6F). These observations suggest that combined targeting of PD-1 and CD74 may offer therapeutic potential (Dammeijer et al., 2020).

5.2.5 CD74 deficiency augments CD8 T cell proliferation in vivo

Endpoint phenotyping indicated that CD74 deficiency influences the expression of MHC-I on cDC1 subsets. Elevated MHC-I levels on cDC1s, together with increased intratumoral CD8+ T cells, may imply enhanced antigen cross-presentation in the absence of CD74. To test this hypothesis, we assessed the role of CD74 in cross-presentation of tumor cell-associated Ags.

Using an in vivo proliferation assay, CellTrace Violet (CTV)–labeled OT-I T cells were adoptively transferred into *Cd74-/-* and *Cd74+/+* mice, followed by immunization with apoptotic B16-OVA (**II:** Fig. 5A). Five days later, OT-I T cells were detected at higher frequencies in the peripheral blood, spleen, and tdLNs of *Cd74-/-* mice (**II:** Fig. 5B, C, fig. S7A). OT-I cells in the peripheral blood and tdLNs also exhibited enhanced proliferation, as determined by SIINFEKL dextramer binding and CTV dilution (**II:** Fig. 5D–E, 5H–I).

To confirm that these effects were DC-intrinsic, we performed in vitro antigen presentation assays using FLT3L-derived bone marrow DCs (FL-DCs). *Cd74-/-* FL-DCs pulsed with specific concentration of ovalbumin (OVA) induced greater OT-I

proliferation than *Cd74+/+* DCs (**II:** Fig. 5I, fig. S7C). A similar enhancement was observed when DCs were pulsed with the lowest concentration of SIINFEKL peptide (0.01 pg/mL) (**II:** Fig. 5J).

To exclude genotype-specific differences in Ag uptake or proteolysis, the DQ-OVA assay was employed. *Cd74-/-* FL-DCs displayed comparable Ag processing to *Cd74+/+* FL-DCs under both resting and LPS-matured conditions (**II:** fig. S9A, B). Together, these data demonstrate that loss of CD74 enhances DC-mediated cross-presentation of tumor Ags without affecting Ag processing capacity.

5.2.6 CD74 deficiency is associated with upregulation of AP-1 members in response to melanoma

To elucidate the molecular mechanisms underlying the functional alterations observed in CD74-deficient DCs, we performed single-cell RNA sequencing (scRNA-seq) on lymph nodes and spleens from *Cd74-/-* and *Cd74+/+* mice under steady-state conditions and following B16-OVA tumor challenge.

Cell clusters were first annotated, and DC populations in spleen and lymph node samples were identified using established subset-specific gene signatures (Anderson et al., 2018; Brown et al., 2019; Jackson et al., 2011; Schlitzer et al., 2015) (**II:** Fig. 6A, fig. S10A–C). Aggregated DC clusters from spleen samples, excluding macrophages and monocytes, were then subjected to differential expression analysis (DEA) comparing naïve and tumor-bearing *Cd74-/-* mice with their *Cd74+/+* counterparts. This approach enabled identification of differentially expressed genes (DEGs) because of the interaction between genotype and disease state (**II:** Fig. 6B). Accordingly, an interaction-effects statistical model was applied to determine whether CD74 deficiency alters transcriptional responses to tumor challenge(Duda et al., 2023).

To gain insight into the biological pathways affected by loss of CD74, we performed gene ontology (GO) enrichment analysis on the DEGs derived from the interaction DEA. This analysis revealed enrichment of immune-related pathways, including negative regulation of IL-1 production and NF-κB signaling (**II:** Fig. 6B). In addition, components of the AP-1 transcription factor complex were enriched as a cellular component, driven by differential expression of *Jun*, *Junb*, *Fos*, and *Jund* (**II:** Fig. 6B). Notably, *Jun* and *Junb* were significantly upregulated across cDC2s, cDC1s, migratory cDC1s, and the total DC population in the spleen of *Cd74-/-* B16-OVA-bearing mice (**II:** Fig. 6C).

Jun and *Junb* are encoding for transcription factors that are critical for the maintenance and function of lymphoid tissue–resident cDC1s in mouse spleen (Novoszel, Drobits, et al., 2021) and have been linked to expression of *Wdfy4*, a gene essential for cross-presentation of cell-associated Ags by cDC1s and cDC2s (Jo et

al., 2025; Novoszel, Drobits, et al., 2021; Theisen et al., 2018). Consistent with this, *Wdfy4* expression was increased when analyzing all DC clusters together and within the cDC2 cluster, with a trend toward elevated expression in migratory cDC1s in the spleen of *Cd74-/-* B16-OVA-bearing mice (**II:** Fig. 6C). Furthermore, *Asb2*, *Gpr183*, and *Eps8* were identified among the DEGs and mapped to the GO term "dendritic cell migration" (GO:0036336; p=0.0125) (**II:** fig. S10D). Despite enrichment of the migration-related pathway, *Ccr7* mRNA levels in splenic DCs did not differ significantly between *Cd74-/-* and *Cd74+/+* B16-OVA-bearing mice (**II:** Fig. 6C).

In tdLNs, *Junb* was upregulated in cDC2s of *Cd74+/+* B16-OVA-bearing mice, whereas *Jun* expression was increased in the combined DC cluster of *Cd74-/-* B16-OVA-bearing mice. In contrast, *Wdfy4* and *Ccr7* mRNA expression did not differ between genotypes in tdLNs (**II:** fig. S10E). Collectively, these data suggest that CD74 deficiency is associated with transcriptional reprogramming of DCs, prominently involving members of the AP-1 transcription factor complex.

5.2.7 Migration and CCR7 expression are compromised by CD74 in DCs

The scRNA-seq analysis of spleens from naïve and B16-OVA-bearing mice identified differential regulation of genes associated with NF-κB and AP-1 signaling pathways. Both transcriptional programs have previously been implicated in controlling *Ccr7* expression (Krappmann et al., 2004). Given the early accumulation of migratory cDC1s in the tdLNs of *Cd74-/-* mice, we next investigated whether CD74 directly influences DC migration and trafficking by regulating the protein expression of key homing receptors such as CCR7 under steady-state conditions and during melanoma progression (Dieu et al., 1998; Sallusto et al., 1998).

At steady state, lymph nodes of *Cd74-/-* mice contained a significantly higher proportion of CCR7+ MHC-II^{high}CD11c^{int} migratory DCs compared to *Cd74+/+* controls (**II:** Fig. 7A, fig. S11A). Although scRNA-seq analysis did not reveal differences in *Ccr7* mRNA expression in DCs from spleens or tdLNs of *Cd74-/-* and *Cd74+/+* mice, we assessed the expression of CCR7 surface levels during different stages of tumor progression, as mRNA and protein levels may diverge. At both early and late time points of tumor development, higher frequency of CCR7+ MHC-II^{high}CD11c^{int} migratory DCs was present in the tdLNs of *Cd74-/-* mice (**II:** Fig. 7B). These findings suggest that CD74 regulates CCR7 protein expression during DC migration potentially through post-transcriptional or post-translational mechanisms.

To further investigate DC migratory capacity, we generated FL-DCs from *Cd74-/-* and *Cd74+/+* mice, which resemble steady-state cDCs and pDCs. FL-DC cultures were analyzed under resting conditions or following maturation with LPS or polyI:C. Flow cytometric analysis revealed that resting *Cd74-/-* FL-DCs contained a higher

proportion of CCR7+ cells compared with *Cd74+/+* FL-DCs (**II:** Fig. 7C, D). Following LPS stimulation, *Cd74-/-* cDC-like cells (CD11c+B220-) showed a trend toward increased CCR7 expression and higher frequencies of CCR7+ cells (**II:** Fig. 7D). Similarly, polyI:C-matured *Cd74-/-* cDC-like cells displayed a tendency toward elevated CCR7 expression compared to *Cd74+/+* cDC-like cells (**II:** fig. S11D). In contrast, CCR7 expression on pDC-like cells (CD11c+B220+) was not altered by CD74 deficiency under either resting or LPS-stimulated conditions (**II:** fig. S11B, C). These data indicate that CD74 loss selectively enhances CCR7 expression in specific DC subsets under both steady-state and maturation conditions. This increase in CCR7 expression in *Cd74-/-* cDC-like cells is consistent with our in vivo observations and supports a role for CD74 in regulating DC migratory potential.

To assess the functional consequences of elevated CCR7 expression, we performed an under-agarose migration assay toward CCL19, a key CCR7 ligand produced in lymph nodes, using resting and LPS-matured *Cd74-/-* and *Cd74+/+* FL-DCs (**II:** Fig. 7E). Quantitative analysis demonstrated increased mean migration speed (**II:** Fig. 7E, F) and a greater number of migrating cells (**II:** Fig. 7G) among *Cd74-/-* FL-DCs. Together, these results indicate that CD74 negatively regulates DC migration, potentially through modulation of CCR7 expression and function.

6 Discussion

Aging does not merely add years to life; it fundamentally alters the defense mechanisms designed to protect the host, increasing vulnerability to life-threatening diseases such as cancer (Montégut et al., 2024). Despite remarkable biomedical innovations over recent decades, the incidence of cancer continues to rise, particularly among individuals above 60 years of age (Bray et al., 2024; Siegel et al., 2025). This persistent correlation between aging and cancer emphasizes the urgent need for a shift in how we conceptualize and treat malignancies.

Immune system decline is seen as an inevitable consequence of aging, and cancer as an unavoidable part of life (Goronzy & Weyand, 2017; Goyani et al., 2024). This way of thinking was like how people once accepted disease like smallpox and polio as an inescapable part of life until scientific breakthroughs redefined what was possible. Immuno-oncology research is challenging cancer fatalism, exploring how to restore immune function and reinvigorate immune surveillance against tumors even in older age.

When the immune system is "young", DCs efficiently prime T cells, and cytotoxic CD8+ T cells remain vigilant against malignant cells (Bhandarkar et al., 2025). However, with aging, DC function declines, T cell diversity is diminished, and overall immune responsiveness wanes (Bailey et al., 2019; Pereira et al., 2011). This immunosenescence not only increases cancer susceptibility but also limits the efficacy of conventional immunotherapies in older individuals (Lian et al., 2020). Animal model studies mirror these findings, showing that aged mice exhibit reduced antitumor immunity and impaired immune memory formation (Haynes et al., 2004; Haynes et al., 1999; Nakajima et al., 2021). Immunosenescence leads to treatment resistance, particularly in cancer immunotherapy. Thus, the following question arises: Can we reprogram the immune system to maintain its lifelong surveillance against cancer? Achieving durable immune memory, something that current cancer therapies have yet to fully accomplish, could eliminate the chronic fear of recurrence that shadows many cancer survivors.

Despite the clinical success of ICIs in melanoma, therapeutic resistance remains a significant challenge. The majority of cancer research focuses on cytotoxic CD8+ T cells that normally fight cancer very well at young age. However, a complementary approach to sustain lifelong immune vigilance may involve innate

immune cells, particularly professional APCs and their functions, alongside the established role of adaptive immune memory.

6.1 PLIN2 as a prognostic biomarker in UM

Adipophilin, a lipid droplet (LD)-associated protein expressed in nearly all mammalian cells containing LDs, has gained increasing attention for its prognostic value across multiple cancer types. Its expression has been linked to disease progression and outcomes in colorectal, lung, breast, ovarian, renal cancer, and cutaneous melanoma (Fujimoto et al., 2020; Iwahashi et al., 2021; Matsubara et al., 2011; Shin et al., 2018; Tolkach et al., 2017; Yoshikawa et al., 2020). In UM, prognosis typically depends on the integration of clinical, histological, and genetic factors, with loss of BAP1 and chromosome 3 being the most consistent predictors of metastasis and survival (Damato et al., 2011; Eleuteri et al., 2018; Gallenga et al., 2022).

In this study, we validated adipophilin as a prognostic marker in UM, revealing *PLIN2*-dependent metabolic alterations associated with *BAP1* loss and monosomy 3. Notably, *PLIN2* expression was inversely correlated with patient age, suggesting a link between aging, lipid metabolism, and tumor immune regulation. Previous research has demonstrated that *Bap1* deficiency impacts metabolism in mice and loss of *BAP1* in UM underlies the immunosuppressive microenvironment (Figueiredo et al., 2020). Therefore, the association of *PLIN2* with *BAP1* expression potentially conceals the mechanisms governing *BAP1* loss that drive a metabolic reprogramming responsible for the TAMs accumulation and immune suppression observed in UM (Figueiredo et al., 2020).

6.2 PLIN2-dependent metabolic reprogramming and MΦ infiltration

Proteomic studies have shown that *Bap1* depletion alters metabolic pathways, increasing cholesterol biosynthesis while reducing proteins involved in gluconeogenesis and lipid homeostasis in the liver (Baughman et al., 2016). These changes coincide with hepatic lipid deficiency and elevated markers of inflammation and pancreatitis. Interestingly, liver-specific deletion of *Plin2* alleviates diet-induced nonalcoholic steatosis and inflammation through a phosphatidylethanolamine N-methyltransferase-mediated mechanism that compensates for lipid imbalance and ER stress (Najt et al., 2016).

One hallmark of cancer is the ability to exploit metabolic pathways to sustain growth and survival, either directly within tumor cells or by reprogramming other cellular components of the TME, such as immune cells. Fatty acids are crucial for cell proliferation, being converted by fatty acyl-CoA synthetases into

triacylglycerols for LD storage. Adipophilin stabilizes these LDs by regulating lipase access, thereby slowing lipid hydrolysis (Listenberger et al., 2007).

In UM, PLIN2 loss in BAP1-negative tumors was linked to lipid storage dysregulation and unfavorable clinical outcomes, which may contribute to the liver's susceptibility as a metastatic site. The association between *PLIN2* and *BAP1* emphasizes a shared role in regulating metabolism and immune dynamics, where *BAP1* loss induces a lipid-rich, hypoxic niche beneficial for M2-like TAM infiltration and therefore immune suppression. Consistently, reduced *PLIN2* expression correlates with hypoxia-induced lipid metabolism changes, enhancing metastatic potential and promoting an immunosuppressive TME. Under hypoxic conditions, PLIN2 degradation coexists with increased accumulation of CD163+ TAMs, which rely on lipid catabolism to maintain their M2-like TAM phenotype (Figueiredo et al., 2020; Krishna et al., 2020; Viola et al., 2019). These TAMs utilize fatty acid oxidation to sustain energy production (Rabold et al., 2017). Pharmacological inhibition of fatty acid oxidation has been shown to reprogram M2-like TAMs toward an M1-like phenotype in vivo, demonstrating the critical role of fatty acids in maintaining tumor-promoting TAMs (Hossain et al., 2015).

The frequent presence of TAMs in UM is partly driven by hypoxia, which also promotes M2-like polarization (Bai et al., 2022; Chen et al., 2023). Analysis of the GDC-TCGA-UM dataset revealed that high *CD163* and *HIF1A* expression correlate with indicators of poor prognosis and reduced *PLIN2* expression. This supports a model where PLIN2 acts as a metabolic gatekeeper: its genetic loss or proteasomal degradation (Masuda et al., 2006) accelerates lipolysis, allowing TAMs to exploit free fatty acids under hypoxic conditions. Under hypoxic and inflammatory conditions, stabilization of HIF-1a further reprograms tumor metabolism toward oxygen-independent energy pathways, including enhanced de novo lipogenesis (Baenke et al., 2013; Eales et al., 2016; Rius et al., 2008; Schug et al., 2015). Elevated fatty acid levels during hypoxia, combined with *PLIN2* downregulation, contribute to lipid accumulation and lipotoxic stress, particularly in the liver (Imai et al., 2012; Tsai et al., 2017). The inability to sequester excess fatty acids into LDs not only drives cellular damage but also impairs immune function, leading to CD8+ T cell exhaustion (Manzo et al., 2020), while Tregs adapt to increased fatty acid uptake and develop resistance to lipotoxicity (Pacella et al., 2018; Wang et al., 2020). We therefore propose that PLIN2 loss under hypoxia disrupts lipid storage, enabling TAMs to adopt a pro-tumorigenic phenotype that further contributes to immune evasion in BAP1-negative UM.

6.3 Lipid metabolism: an appealing target for UM management

Metabolic reprogramming in high-risk UM presents an opportunity for therapeutic interventions in a disease with primary resistance to ICIs (Sacco et al., 2018). Integrated transcriptomic and secretome analysis led to the identification of various drug clusters potentially capable of reversing the hypoxia/lipolysis metabolic state toward normoxia/lipid storage phenotype in BAP1-deficient UM. These include adrenergic, retinoid, and glucocorticoid receptor agonists, as well as PI3K, MEK, and RAF inhibitors. Some drugs, such as the non-adenosine triphosphate competitive inhibitor of MEK1 and MEK2, selumetinib, have been tested in patients with advanced UM in a randomized clinical trial; however, it did not improve overall survival (Carvajal et al., 2014). Notably, 50 μM of carvedilol has been tested in 3D UM spheroids with monosomy 3 and completely prevented the spread of spheroid cells (Farhoumand et al., 2022).

These findings suggest that restoring metabolic control through pharmacologic modulation of lipid metabolisms may reshape the TME, enhance immune responsiveness, and improve outcomes for patients with *BAP1*-deficient UM. Targeting tumor metabolism thus emerges as a promising strategy to overcome immune exclusion and improve disease control in UM.

6.4 CD74 deficiency favors DC quality over quantity

Concerning CD74, an important unresolved question is whether systemic ablation of CD74 compromises or strengthens antitumor immunity, given that CD74 is mainly expressed by professional APCs and exerts dual functions, supporting adaptive immunity through MHC-II while also mediating immunosuppression through its interactions with MIF or DDT (Figueiredo et al., 2018; Pellegrino et al., 2024). The phenotypes observed in this study resemble those described in MHC-II-deficient mouse models, despite demonstrated mechanistic differences (Chaoul et al., 2018; Shi et al., 2024; Wohn et al., 2020). However, our data demonstrate that CD74 deficiency reprograms cDC1 subsets and pDCs toward enhanced migratory and/or cross-presenting states, thereby strengthening melanoma immunosurveillance and promoting CD8 T cell priming, tumor infiltration, and effector activity. Thus, CD74 regulates the immune microenvironment not only through its classical MHC-II-dependent pathways but also through cell-mediated immunity modulation independent from MHC-II.

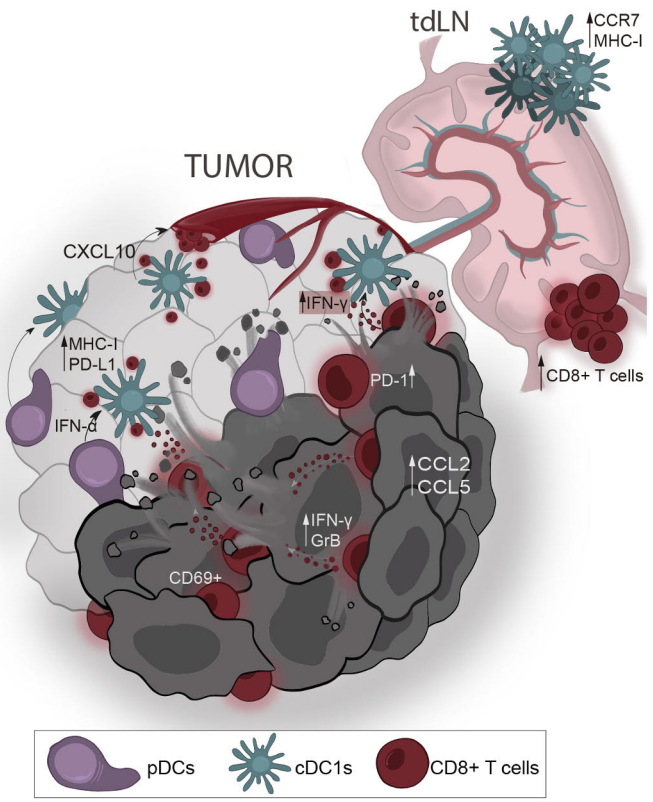


Figure 14. Schematic representation of our current working model for the key events underlying the enhanced antitumor response in CD74-deficient mice. In the TME of *Cd74-/-* mice, elevated CCL2 levels promote the recruitment of pDCs. Tumoral pDCs secrete IFN-alpha, which drives upregulation of MHC-I on APCs, including cDC1s and pDCs, thereby enhancing their Ag cross-presentation capacity. Tumoral cDC1s express PD-L1 associated with Ag uptake and processing. Improved Ag presentation within the TME leads to more efficient priming of CD8+ T cells, which display increased activation, proliferation, and cytotoxic effector functions, characterized by CD69 expression as well as IFN-gamma and GrB production. Simultaneously, tumor-cell derived CCL5 and IFN-gamma-inducible CXCL10 produced by cDC1s promote further recruitment of CD8+ T cells into the TME, amplifying local cytotoxic immunity. Migratory cDCs in tdLNs exhibit increased CCR7 and MHC-I expression, supporting enhance Ag cross-presentation and proliferation of CD8+ T cells. These T cells subsequently replenish the effector pool in the tumor. (own illustration created using Adobe Fresco).

CD74 deficiency amplified DC-mediated adaptive immune responses across multiple sequential stages of tumor immunity (Figure 14). In the TME, migratory PD-L1+ cDC1s, which are linked to Ag uptake and processing (Peng et al., 2020) were enriched in *Cd74-/-* mice. Although PD-L1 can attenuate PD-1+ T cell function, it also confers protection to DCs against CD8 T cell-mediated cytotoxicity, thereby supporting antitumor immunity and responsiveness to ICIs (Peng et al., 2020).

In addition, an accumulation of activated pDCs was observed in the TME of *Cd74-/-* mice. Beyond their canonical role in IFN-α secretion following TLR stimulation, pDCs possess Ag-presenting capabilities that can elicit immunogenic T cell responses (Figure 14) (Swiecki & Colonna, 2015). In murine models, virus-activated pDCs promote Th1 polarization, and CpG-stimulated pDCs expand naïve CD8+ T cells in response to both endogenous and exogenous Ags (Boonstra et al., 2003; Dalod et al., 2003; Salio et al., 2004; Schlecht et al., 2004). In cancer, appropriately activated pDCs can support antitumor immunity; for example, vaccination with activated pDCs loaded with tumor-associated peptides induces robust CD4+ and CD8+ T cell responses in metastatic melanoma patients, while TLR-stimulated pDCs mediate tumor killing through TRAIL, GrB, and promote NK cell activation in the B16 melanoma model (Drobits et al., 2012; Liu et al., 2008; Tel et al., 2013). In contrast, tumor-associated pDCs are frequently nonactivated and characterized by low CD80/CD86 expression (Ito et al., 2007). CD74 seems to participate in the maintenance of lower pDC activation, since its deficiency confers elevated CD80 and MHC-I expression in tumor-associated pDCs, suggesting a potential role in local T cell activation.

In combination with the elevated frequency and density of pDCs in the TME of *Cd74-/-* mice, elevated IFN-α levels, likely derived from pDCs, may further contribute to the local MHC-I upregulation observed in intratumoral migratory cDC1s (Figure 14). CD74 has previously been characterized as indispensable in noncanonical endolysosomal MHC-I cross-presentation pathway (Basha et al., 2012). Using a viral infection model, Basha et al. reported that CD74 is required for efficient antigen cross-presentation by directing MHC-I trafficking from the ER to endolysosomes. In contrast, our findings demonstrate that CD74 deficiency enhances cross-presentation of tumor cell-specific Ags and augments CD8+ T cell proliferation (Figure 14).

Already in steady-state, CD74 deficiency indicates remodeling of the immune populations in the naïve lymph nodes. More apparent in melanoma, CD74 deficiency enhances the activation DC-population responsible for type I IFN production, leads to enhanced DC surface MHC-I, and cross-presentation of tumor-specific Ags to T cells. Even though the populations of immunogenic cDC1s are not affected in quantity, CD74 seems to have an effect in the quality of these cells, further justified by cytotoxic CD8+ T cell-mediated melanoma control.

6.5 CD74 restrains chemokine-mediated recruitment of cytotoxic and memory CD8 T cells

Our data indicated that CD74 regulates the activation and migratory behavior of lymphoid-tissue resident and migratory cDC1 subsets under homeostatic conditions,

thereby shaping adaptive immunity. Within the TME, cDC1s also serve as a major source of CXCL10 in response to IFN-γ and type I IFNs, a chemokine essential for recruitment of effector CD8+ T cells (Figure 14) (Reschke & Gajewski, 2022).

In the TME Cd74-/- mice, increased levels of CCL5 and CXCL10 were observed, which are consistent with enhanced infiltration of effector CD8+ T cells. CXCL10 expression has been associated with tumor-infiltrating lymphocytes, Th1-polarized immunity, and favorable immunotherapy responses in melanoma (Harlin et al., 2009). Similarly, constitutive CCL5 production has been shown to promote lymphocyte recruitment in ovarian cancer (Dangaj et al., 2019). Notably, CCL5-mediated T cell recruitment can initiate a positive feedback loop in which Ag recognition by T cells induces IFN-γ release, leading to DC activation and CXCL10 secretion, thereby further amplifying local T cell activation (Dangaj et al., 2019). This mechanism likely explains the observed IFN-γ+ CD8 T cell accumulation in the *Cd74-/-* mice, which also show elevated CXCL10 levels and cytotoxic CD8+ T cells in their TME. Therefore, DC-derived chemokines are central in sustaining CD8+ T cell recruitment in *Cd74-/-* mice (Figure 14). Moreover, the dominance of central memory CD8 T cells in *Cd74-/-* mice support long-term T cell persistence and rapid response upon Ag re-exposure, features that are critical for immune surveillance and effective ICIs response (J. Han et al., 2021).

6.6 CD74 negatively regulates CCR7-dependent DC migration

Beyond its role in Ag presentation, our tdLN analyses uncovered an additional CD74-dependent regulatory layer involving cDC migration. The increased frequency of migratory cDC1s at the early time point in the tdLNs of *Cd74-/-* mice suggests that CD74 deficiency may improve DC trafficking from melanoma to the draining lymph node. Previous work identified CD74 as negative regulator of moDC motility using LPS-stimulated, GM-CSF BMDCs and attributed the impaired migration to retention of uncleaved CD74 within endolysosomal compartments following CTSS deletion, which disrupted myosin II function (Faure-André et al., 2008).

Here we evaluated DC migration using FL-DCs and an under-agarose migration assay toward CCL19 (Alanko et al., 2023). *Cd74-/-* FL-DCs exhibited enhanced migration toward CCL19 under both resting and LPS-matured conditions. This study further supports previous observations linking CD74 to DC migration regulation. Unlike earlier studies that focused solely on cytoskeletal effects after moDC maturation, our data suggest for the first time that increased CCR7 expression and a higher proportion of CCR7+ migratory DCs were enriched in *Cd74-/-* FL-cDCs in vitro as well as in both naïve lymph nodes and tdLNs of *Cd74-/-* mice, strengthening the link between CD74 and CCR7-dependent DC migration.

6.7 Limitations of the studies

6.7.1 Methodological considerations for the PLIN2 study

Although this study integrates transcriptomic and proteomic analysis to identify metabolic vulnerabilities in UM, several limitations must be acknowledged. The limited size of available UM cohorts restricts statistical power and generalizability across heterogeneous patient populations.

In addition, functional assays were largely performed in immortalized UM cell lines and co-culture systems with healthy-donor monocyte-derived MΦs that may not fully capture the complexity of tumor-immune interactions in the host. The lack of an established preclinical UM model further impedes mechanistic investigation.

The precise relationship between BAP1 loss, adipophilin degradation, and lipid metabolism also remains partially defined, particularly regarding the causal hierarchy of these alterations. Finally, while drug screening identified compounds capable of restoring PLIN2 expression, in vivo validation and pharmacodynamic studies are required to assess translational feasibility.

Small molecules frequently interact with other proteins (off-targets), leading to complex and sometimes adverse phenotypes. Many small molecules are "multiple ligands," meaning they act on multiple receptors or enzymes simultaneously (Drygała et al., 2024; Wang et al., 2006).

Moreover, most small-molecules exert pleiotropic and systemic effects across multiple cell types, complicating selective therapeutic targeting of tumor-intrinsic metabolic pathways. This complexity is particularly relevant for pathways involved in cellular responses to hypoxia and metabolic stress. Hypoxia-inducible factors (HIFs) signaling in MΦs and other myeloid populations is a major driver of hypoxia-associated immunosuppression within tumors (Chiu et al., 2017; Kumar & Gabrilovich, 2014), whereas HIFs activity in effector CD8+ T cells has been shown to support glycolytic metabolism, cytotoxic function, and antitumor immunity (Liikanen et al., 2021; Palazon et al., 2017). Thus, pharmacologic manipulation of shared metabolic pathways may produce divergent effects on tumor cells and immune populations.

6.7.2 Biological and technical consideration for the CD74 study

This study employed a constitutive germline *Cd74-/-* mouse model as a proof-of-concept to assess how complete loss of CD74 impacts DC-mediated antitumor immunity and responsiveness to PD-1 blockade. Because CD74 is deleted from early development onward, this model integrates both developmental and functional consequences of CD74 loss and does not allow temporal separation of these effects

in mature immune cells. Complementary in vitro experiments using FL-DCs support attribution of the observed effects to DCs and their specific subsets.

A further limitation is that DEA could not be conducted on lymph node samples from naïve and B16-OVA-bearing *Cd74+/+* and *Cd74-/-* mice due to the insufficient DC numbers. Therefore, the transcriptional changes in DC-T cell interaction within tdLNs could not be evaluated. The same is applicable for the *Cd74+/+* and *Cd74-/-* tumor samples. Due to low leukocyte yield, especially in the TME of *Cd74+/+* mice, DEA was not feasible. These could be avoided if the scRNA-seq experiments were carried out after characterizing the TME and tdLNs with flow cytometry analysis to have an estimation of the cell populations and their total number is these tissues.

In addition, differences between our cross-presentation findings and those reported by Basha et al. are likely attributed to context-specific differences between viral and tumor Ag processing. Although MHC-I assembly typically occurs in the ER with cytosolic peptides, MHC-I molecules can also traffic through the Golgi apparatus and endolysosomal compartments (Joffre et al., 2012). In infections involving endosomal viruses that depend on vacuolar processing, loss of CD74 may restrict MHC-I delivery to endolysosomes, thereby limiting CD8 T cell priming. On the other hand, tumor Ag cross-presentation predominantly proceeds via the cytosolic pathway, with peptide loading taking place in the ER (Jhunjhunwala et al., 2021). Here, CD74 deficiency may therefore favor MHC-I retention within the ER, enhancing peptide loading and cell surface expression. This is consistent with the elevated MHC-I levels detected on cDC1s and pDCs in the TME, as well as on cDC1s in tdLNs at endpoint in *Cd74-/-* mice.

In addition, Ag source and experimental design differed between the studies. Whereas previous work relied on viral Ags or irradiated OVA-pulsed DCs, we used tumor cell-specific Ags derived from apoptotic B16-OVA cells, which resemble Ags encountered withing the TME. Methodological differences in the in vitro Ag presentation assay may also contribute to divergent outcomes, including the use of GM-CSF-derived BMDCs by Basha et al., which resemble inflammatory moDCs, compared with FL-DCs used in our study, which more closely resemble steady-state cDCs and pDCs with cross-presentation capacity (Angelov et al., 2005; Mayer et al., 2014).

Finally, discrepancies between cell frequencies and absolute cell numbers across naïve lymph nodes, tdLNs, and TME samples may derive from the dramatic reductions in certain immune populations, such as CD4+ T cells in *Cd74-/-* mice, as reported by us and others (Bikoff et al., 1993; Wang et al., 2021). Dramatic reduction of these population in lymphoid tissues may increase the proportional representation of other cell types, including cDC1s, thereby explained by altered cellular trafficking rather than expansion in cell numbers.

As with all experimental studies, these limitations highlight important areas for future investigation and continued innovation.

6.8 Future perspectives

6.8.1 Research implication for PLIN2 in UM

Future studies should validate the adipophilin-based metabolic signatures in larger UM cohorts and integrate spatial transcriptomics to delineate the cellular sources of lipid metabolism dysregulation. Parallel mechanistic investigations are needed to clarify how *BAP1* loss alters lipid droplet dynamics and to determine whether restoration of adipophilin expression can reverse immunosuppressive reprogramming. In addition, the identified drug candidates, particularly adrenergic inhibitors, should be further evaluated in preclinical UM models to assess their efficacy in modulating lipid metabolism, potentially reversing immunosuppression and improving immunotherapy responsiveness.

Given the cell-specific and sometimes opposing roles of metabolic pathways in cancer cells versus immune populations, future investigations should assess how adipophilin-modulating interventions affect distinct immune subsets, including MΦs, other myeloid cells, and CD8+ T cells. Such analyses will be essential to avoid unintended suppression of antitumor immune responses when targeting processes such as metabolic reprogramming.

Progress in UM research is currently restricted by the limited interventions that can be employed to better understand this aggressive cancer type. Therefore, there is need for the establishment of a preclinical UM model that will bear the genetic alterations that dictate metastatic risk. Advanced experimental methods, such as UM tumor organoids or tumor-on-chip technology, incorporating stromal and immune components, may provide a better representation of the complex TME and offer a relevant setting to interrogate lipid metabolism, tumor-immune cross-talk, and therapeutic responses prior to in vivo studies.

Beyond UM, elucidating the shared roles of adipophilin and BAP1 in regulating lipid metabolism and subsequently immune function may uncover conserved mechanisms relevant to other immune-excluded cancer types. Recent clinical successes with CD3-enganging bispecific T-cell therapies, including tebentafusp in uveal melanoma, demonstrate that immune-excluded tumors can be responsive to immunotherapy. These advances highlight the broader relevance of identifying tumor-intrinsic and extrinsic barriers to immune engagement, such as metabolic reprogramming, and support continued investigation of combinatorial approaches that integrate metabolic modulation with immunotherapy.

6.8.2 Next steps and translational opportunities for CD74

The use of constitutive *Cd74-/-* permits the evaluation of integrated immune effects that may better reflect therapeutic targeting of CD74 and which immune cells and functions are influenced by this intervention. To better define the specific DC subsets that participate in the Ag cross-presentation and migration, there is need for further studies employing conditional knockout models or adoptive cell transfer experiments after ablation of specific DC populations. Currently, the only available conditional CD74 knockout model is the *Cd74*$^{\Delta/\Delta}$*Itgax-Cre* used by Pellegrino et al. (Pellegrino et al., 2024). The authors generated the model by crossing *Cd74*$^{fl/fl}$ littermates into *Itgax* (CD11c)-Cre mice to investigate how CD74 regulates antitumor immunity and which APCs control tumor growth. However, CD11c is expressed not only by DCs but also by other immune populations, including monocytes and macrophages. Although the authors show that monocytes and macrophages retain CD74 while total DCs lack it, this validation was only performed using PBMCs, leaving the efficiency of CD74 deletion in tumor-infiltrating and tissue-resident APCs uncertain(Herbst et al., 2024). Consequently, the *Cd74*$^{\Delta/\Delta}$*Itgax-Cre* model offers limited resolution for defining which DC subset mediates the antitumor effects.

To address this limitation, *Batf3-/-* mice could be used to examine T cell responses following adoptive transfer of *Cd74-/-* cDC1s; however compensatory *Batf* expression could restore wild-type cDC1 development (Hildner et al., 2008; Tussiwand et al., 2012). A more definitive approach would involve *Irf8*-deficient mice, which completely lack cDC1 without compensation, combined with adoptive transfer of *Cd74-/-* cDC1s (Durai et al., 2019). Adoptive transfer of ex vivo-activated and tumor Ag-pulsed DCs can elicit both effector responses and memory T cell formation; however, the functional profile of the memory T cells induced by DC vaccination remains poorly understood, particularly in the presence of pre-existing antitumor immunity. A potential limitation of this approach is that ex vivo-activated DCs may not fully resemble the migratory behavior, tissue localization, or activation status of intratumoral DCs. Moreover, the route of administration may lead to inefficient lymph node homing, potentially affecting the spatial dynamics of T cell priming and thereby influence effector responses.

Generation of a *Cd74*$^{\Delta/\Delta}$ *Zbtb46-Cre* mouse model would allow more precise interrogation of which cDC subset drives cancer cell-specific Ag cross-presentation and migration (Satpathy et al., 2012). Furthermore, selective pDC ablation in the constitutive *Cd74-/-* model would help clarify whether pDCs contribute to tumor control via Ag presentation to CD8+ T cells, due to their increased MHC-I and CD80 levels in the TME of *Cd74-/-* mice, or through IFN-α production within the TME (Cervantes-Barragan et al., 2012).

In addition, we observed an increased density of IFN-γ-producing CD8+ T cells within the TME of *Cd74-/-* mice. DCs can respond to IFN-γ released by neighboring T cells by producing IL-12, which in turn reinforces cytotoxic CD8+ T cell immunity. This represents a reciprocal cross-talk that is essential for effective anti-PD-1-mediated responses (Everts et al., 2016; Garris et al., 2018). The elevated IFN-γ detected in CD8+ T cells of Cd*74-/-* mice may therefore promote enhanced IL-12 production by DCs, in parallel with CXCL10, further supporting efficient cross-presentation of tumor-specific Ags and sustained CD8+ T cell cytotoxicity. To investigate this cross-talk in the context of CD74 deficiency, future studies should quantify IL-12 production and frequency of IL-12+ DCs, and assess how these parameters are influenced by IFN-γ neutralization in tumor-bearing mice, both at baseline and during anti-PD-1 treatment. Such analyses would clarify whether cDC1s in the TME of *Cd74-/-* mice exhibit increased IL-12 production in response to the enhanced IFN-γ. IL-12 production by DCs in this context could also involve *c-Jun*/AP-1 signaling and pDC recruitment within the TME, both of which were observed in our model. *c-Jun* has been shown to be essential for DC development and function (Novoszel, Drobits, et al., 2021), and c-Jun/AP-1 mediated TLR7/8 signaling in type I IFN-primed DCs by inducing the pDC-recruiting chemokine CCL2 and promoting IL-12 production (Drobits et al., 2012; Sanlorenzo et al., 2025). Consistent with this, expression of both IL-12 subunits, IL-12A and IL-12B, is *c-Jun* dependent, and pDCs infiltration into the TME is impaired in mice lacking *c-Jun* in DCs (Sanlorenzo et al., 2025). Similar defects in pDCs recruitment have been reported in CCL2-deficient mice, further supporting a central role for *c-Jun*-dependent CCL2 regulation in this process (Novoszel, Holcmann, et al., 2021). Therefore, the increased expression of *c-Jun* observed in DCs of the CD74-deficient mice might be directly connected with the CCL2 levels in the TME and the increased numbers of activated pDCs.

We also observed a temporal increase of pDCs to tdLNs. CXCR3-dependent recruitment of circulating pDCs to lymph nodes has been reported following inflammatory stimulation (Yoneyama et al., 2004). Whether a similar mechanism takes place in the tdLNs of *Cd74-/-* mice remains unknown and could be determined.

One of the most promising translational implications of CD74 deficiency is the accumulation of activated PD-1+ CD8 T cells within the TME, a key determinant of ICIs efficacy (H. Li et al., 2022). Importantly, PD-1 expression on CD8+ T cells does not necessarily indicate terminal exhaustion. In a study of chronic viral infection, a distinct subset of PD-1+ CD8+ T cells was identified with preserved proliferative capacity and long-term self-renewal potential (Im et al., 2016). These stem-like cytotoxic CD8+ T cells serve as a renewable source of effector cells, contributing to antitumor immunity (Siddiqui et al., 2019; Zehn et al., 2022). Evidence further suggests that tdLNs are the hub of continuous generation and maintenance of these stem-like CD8+ T cells, which then replenish tumor sites and

expand to effector populations (Connolly et al., 2021; Rahim et al., 2023; Schenkel et al., 2021). Emerging evidence indicates that sustained antigenic stimulation via TCR signaling does not necessarily enforce terminal T-cell differentiation but can instead preserve a pool of stem-like CD8+ T cells with ongoing proliferative capacity (Hor et al., 2026). In this context, PD-1 signaling is a key regulator by fine-tuning TCR signal strength, enabling selective expansion of high-affinity stem-like clones. Disruption of this regulatory axis through PD-1 blockade may lead to terminal differentiation and death of highly avid stem-like T cells, raising the possibility that ICIs may provide short-term tumor control at the potential cost of depleting high-affinity TCR precursors (Hor et al., 2026). Complementary data indicate that in the absence of CD4+ T cell help, these stem-like CD8+ T cells accumulate in lymphoid tissues without efficiently converting into cytotoxic effectors (Busselaar et al., 2026).

An alternative is that the expanded PD-1+ CD8+ T-cell population reflects an enrichment of tissue-resident memory T cells characterized by CD103 expression (Edwards et al., 2018; Szabo et al., 2019). This T cell subset display high PD-1 levels while they retain their cytotoxic effector functions (Corgnac et al., 2020). Tissue-resident memory T cells have been associated with favorable prognosis and improved responses to ICIs (Corgnac et al., 2020; Damei et al., 2025; Djenidi et al., 2015; Jaiswal et al., 2022). As CD103 expression was not evaluated in the present study, their contribution cannot be excluded. Future attempts should incorporate CD103 to precisely identify this CD8 T-cell subset.

Migratory PD-L1+ cDC1s which are linked to Ag uptake and processing were simultaneously enriched in TME of *Cd74-/-* mice. Together, these findings suggest that CD74 ablation creates an immune permissive TME for improved ICIs responsiveness by promoting a more inflamed yet regulated immune landscape, potentially preceding the onset of T cell dysfunction. Notably, the increased infiltration of PD-1+ CD8 T cells translated into improved response to PD-1 blockade in two melanoma models, highlighting the role of CD74 in restraining effector T cell immunity and identifying its loss as a sensitizing factor for ICI-mediated tumor control. Future studies combining CD74 inhibition with PD-1/PD-L1 blockade in preclinical models will be essential to define therapeutic synergy and optimize treatment timing. Given its predominant expression on professional APCs, CD74 represents an attractive immunomodulatory target with the potential for reduced off-target toxicity, which is more likely to happen when targeting cytokines. Moreover, elucidating the role of CD74 in type I IFN signaling may inform strategies to enhance immune activation in immunologically “cold” tumors.

The translational potential of targeting CD74 is further supported by the prior development of milatuzumab (hLL1), a humanized anti-CD74 monoclonal antibody originally generated from the murine LL1 monoclonal antibody (IgG1κ) following immunization of BALB/c with Raji lymphoblast-like cells derived from Burkitt

lymphoma patient (Pawlak-Byczkowska et al., 1989). LL1 binds a cell-surface epitope of CD74 with high affinity and undergoes rapid internalization, with continuous replacement of newly synthesized CD74 molecules at the plasma membrane (Hansen et al., 1996; Ong et al., 1999). This internalization property, which is linked to the physiological role of CD74 in MHC-II trafficking and Ag processing, enabled efficient lysosomal delivery and encouraged the development of CD74-targeted antibody conjugates for the delivery of toxin drugs and radioisotopes (Cresswell, 1994; Sapra et al., 2005).

Milatuzumab retained comparable Ag-binding and internalization properties and demonstrated significant therapeutic efficacy in preclinical xenograft B-cell malignancies models, including non-Hodgkin lymphoma and multiple myeloma (Stein et al., 2004). Milatuzumab induces minimal antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity, likely due its rapid internalization rather than IgG constant sequences since hLL1 has the same IgG1 heavy chain constant regions as IMMU-106, an anti-CD20 monoclonal antibody, which mediates antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity (Stein et al., 2004).

While milatuzumab has been developed primarily for B-cell malignancies, rapid CD74 internalization has been also observed across CD74-positive non-hematologic tumor cell lines, including melanoma models (Ong et al., 1999).

In solid tumors such as melanoma, anti-CD74 antibodies would be expected to function predominantly through immune modulation and not tumor cell cytotoxicity. Hence, repurposing of CD74-directed antibodies for melanoma would require careful validation of CD74 expression and function across intratumoral APCs, as well as assessment of antibody accessibility and activity within solid tumors. Given the established role of CD74 in B cell functions including survival, potential effects of B-cell population would need to be evaluated, as B cells were not examined in the present study.

Finally, our observation that CD74 deficiency increases both the proportion of CCR7-expressing migratory DCs and surface CCR7 levels on cDCs reveals a previously unrecognized connection between Ag presentation and DC migratory reprogramming. Future studies should determine whether CD74 perturbation restores the migration of early and, critically, late-stage intratumoral cDCs towards CCL19 and CCL21 gradients, a process that progressively deteriorates during tumor progression and limits effective tumor-specific T cells priming and replenishment within the TME. Because DC-dependent initiation and maintenance of the cancer-immunity cycle are key for both endogenous and therapeutically induced antitumor responses, sustained CCR7-depedent DC trafficking remains a central determinant of immune-mediated tumor control.

It will also be crucial to assess whether increased CCR7 expression in the absence of CD74 promotes the accumulation and spatial organization of tumor-

resident CCR7+ DCs within perivascular immune niches, which serve as hubs for intratumoral T cell interactions (Chen et al., 2024; Onder et al., 2025). Recent study has shown that the ability of tumor-resident CCR7+ DCs to drive effective immune responses is regulated by their CCL19-CCL21-dependent positioning in blood vessels, with venous-associated fibroblasts acting as major source for CCL19 (Lee et al., 2024; Zitti et al., 2026). These perivascular DC niches closely resemble lymph node architecture, where CCL19- and CCL21-producing fibroblastic reticular cells guide DC localization within T-cell rich paracortical zones (Braun et al., 2011). Based on these observations, future work should examine whether CD74 deficiency alters the relative sensing of CCL19 versus CCL21, thereby influencing whether CCR7+ DCs are retained within intratumoral niches or migrate to tdLNs.

7 Summary/Conclusions

This study reveals that loss of adipophilin in UM contributes to a metabolic reprogramming potentially associated with the immunosuppressive profile of BAP1-defient tumors. Reduced *PLIN2* expression was consistently associated with loss of *BAP1*, monosomy 3, hypoxia, and poor overall survival across independent patient cohorts. These alterations reveal a metabolic shift from lipid storage toward lipolysis, promoting M2-like TAM polarization and an immunosuppressive TME. Integrative analysis identified *PLIN2*-based metabolic gene signatures predictive of prognosis and revealed drug repurposing as a viable option for reverting the adverse metabolic shift. Notably, carvedilol partially restored *PLIN2* expression and reduced hypoxia-related gene expression in co-culture of UM cells and MΦs, suggesting that targeting lipid metabolism may offer a viable therapeutic option for high-risk, *BAP1*-deficient UM.

In parallel, this dissertation identified CD74 as a critical regulator of DC functions in antitumor immunity. By limiting cytotoxic CD8 T cell responses and suppressing memory-inducing cytokine production, CD74 expression in immune cells promotes melanoma progression. In contrast, systemic deletion of CD74 in mice makes the TME immune permissive by enhancing MHC-I expression and CCR7-mediated migration of DCs, thereby improving tumor-specific Ag cross-presentation and subsequent proliferation and activation of cytotoxic CD8+ T cells. Therefore, host CD74 deficiency limits melanoma progression through a localized cancer-immunity subcycle by enhancing the quality of innate immune response and reshaping the adaptive immune landscape of melanoma tumors. Finally, potential molecular mechanisms underlying the cellular events involve AP-1 family members, *c-Jun*, *Junb*, and *Fos*, besides NF-κB, which influence DC functions in the TME and secondary lymphoid organs. Altogether, these findings suggest two mechanisms potentially leading to immune exclusion and contributing to ICIs resistance, (*1*) metabolic rewiring through loss of PLIN2 in UM and (*2*) negative innate immune regulation through CD74 in melanoma that could both be further investigated for their therapeutic potential.

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