Novel immunotherapies for metastatic uveal melanoma

-From bench to clinical biomarkers

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Cover illustration:
Uveal melanoma metastasis patient biopsy depicting a highly multiplexed immune tumor microenvironment, by Vasu R Sah.

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If you do not believe you can do it then you have no chance at all.
Arsene Wenger

ABSTRACT

Immunotherapy has revolutionized cancer treatment, with durable long-term effects in a proportion of patients. However, in around half of these patients, there are little to no effects. Patients with metastatic uveal melanoma (mUM), a rare form of melanoma originating from the eye has lack of effective treatments. The aim of this thesis is to discover novel immunotherapies and biomarkers for treatment of metastatic uveal melanoma (mUM).
In **paper 1**, we show HDAC inhibitor increases and BET inhibitor decreases levels of HLA (human leukocyte antigen) and PDL1 (programmed death ligand -1) on uveal melanoma cell lines. The combination of Entinostat and PD-1 (programmed death -1) inhibition resulted in enhanced effects of PD-1 inhibition both with in-vitro and in-vivo studies, whereas BET inhitor JQ1 did not. Using PDL1 knockout tumor cells, combined with Entinostat, helped in gaining mechanistic understanding. This translational work from paper I, provided the foundation of a phase II clinical trial PEMDAC (NCT02697630), in metastatic uveal melanoma. In **paper 2**, we perform clinical biomarker discovery for a two year follow up of patients treated in PEMDAC trial. We observe all patients w.r.t progression free survival and overall survival, assessing the efficacy and survival long term. This led us to a comprehensive analysis of patient samples from the pre-treatment stage, followed longitudinally to the end of study. We discovered tumor and chemokine signatures as novel biomarkers predicting clinical responses. Moreover, the discovered chemokine axis, essential for T-cells migration, induce tertiary lymphoid structures (TLS)-like entities at the metastasis sites, correlating to clinical benefit. In **paper 3,** we develop patient xenografts (PDX) models of mUM, and further investigated these tumors using an ex-vivo screening platform. The PDX tumors were used to grow 3-D spheroids in-vitro, co-cultured together with their autologous and allogenic tumor infiltrating lymphocytes (TILs). Using a NOG-IL2 transgenic mice, matched tumor and TILs were assessed with subcutaneous and liver-met mUM PDX models. The ex-vivo screen and patient biopsies were evaluated further, with T cell receptor (TCR) and single cell sequencing, in identifying T- reactive clones with anti-tumor immunity. Furthermore, using a highly multiplex technique, patient biopsies were interrogated for tumor immune spatial inter-play, leading to identification of similar tumor-reactive T cell subsets, building a cross-functional discovery platform for mUM.

Keywords: Metastatic uveal melanoma, Patient-derived xenografts, immunotherapy, biomarker discovery, tumor infiltrating lymphocytes, PD-1 inhibition, histone deacetylase inhibition, Multiplex imaging, Single cell sequencing.

SAMMANFATTNING PÅ SVENSKA

Cancer är en folksjukdom med 19 miljoner förväntade nya sjukdomsfall och 10 miljoner associerade dödsfall år 2020 världen över. Den primära sjukdomen är oftast inte orsaken till de cancer-relaterade dödsfall, utan en process som kallas metastasering. Vid denna flerstegsprocess sprider sig den primära tumören till andra områden i kroppen, där dottertumörer formar metastatiska lesioner och kan påverka livsviktiga organ. Metastaserande ögonmelanom är en ovanlig form av melanom som oftast sprider sig till levern och är i regel resistent mot standardbehandling. Denna cancerform är därför en stor utmaning för onkologer och andra behandlande läkare medför ett stort lidande för patienter och anhöriga. Flera behandlingsalternativ som cellgifter, isolerad leverperfusion, riktad behandling och immunmodulerande behandling är under utvärdering för denna ovanliga cancerform.

Immunterapi är ett samlingsnamn för behandlingar med syfte att aktivera patientens egna immunförsvar till att döda tumörceller. För behandling av ögonmelanom så har immunterapier som T cell checkpointblockad (antikroppar) och adoptiv cellterapi (T celler) visat sig ge varaktiga behandlingseffekter i flertalet patienter. Däremot så utvecklar många patienter med ögonmelanom behandlingsresistens med mycket dålig prognos som följd.

Som bidrag till utvecklingen av immunterapier så har vi genomfört en translationell pre-klinisk studie beskriven i arbete I, där en kombinationsbehandling med en HDAC-inhibitor (entinostat) och Pembrolizumab (anti-PD1) har utvärderats hos patienter med ögonmelanom. Entinostat ökade antigen-presentation hos immunceller att identifiera tumörceller, och anti-PD1 inaktiverade bromsen på T-celler, vilket tillsammans genererade en kraftig inflammatorisk respons. Dessa fynd låg till grund för initiering av en fas II multicenter klinisk studie, kallad PEMDAC. Till skillnad från andra kliniska studier på ögonmelanom så resulterade kombinationsbehandling i PEMDAC-studien i högre totalöverlevnad (median overall survival), medan medianen för progressionsfri överlevnad var likvärdig. Vid 2 års uppföljning av PEMDAC-studien upptäcktes nya biomarkörer som associerade med behandlingseffekt och som presenteras i arbete II. Patientprov i form av plasma, immunceller, och tumörbiopsier erhölls och tumör- och immunbaserade biomarkörer analyserades. I dagsläget finns endast ett fåtal studier på biomarkörer i ögonmelanom och dessa rapporter rör ofta nivåer av LDH (laktat dehydrogenas) och cirkulerande DNA. Våra studier ger en mer heltäckande analys, vilket sträcker sig bortom traditionella strategier. Vi presenterar en positiv korrelation mellan chemokiner kopplade till T och dendritcells chemotaxi, tillsammans med tertiära lymfoida strukturer med överlevnad hos patienter.

Kombinationsbehandlingar är en möjlig strategi vid behandling med immunterapier. På senare år har dessutom adoptiv cellterapi hamnat mycket i fokus. I arbete III undersöker vi just adoptiv

T-cells terapi och tumörspecifika T-cellspopulationer i mikromiljön hos levermetastaser. Vi har etablerat en ny djurmodell för levermetastaser där vi kan studera mänskliga tumörer med matchande T-celler i möss (PDX musmodell). Dessutom har cellodlingar med tumörceller och T-celler i 3D-sfärkulturer använts för att identifiera specifika T-reaktiva populationer med hjälp av TCR och singel-cellsekvensering.

Genom multiplex-färgning och bildbehandling (imaging) kan vi bekräfta att T-reaktiva populationer återfinns hos patientbiopsier från levermetastaser. Behandlingsalternativen för patienter med ögonmelanom blir fler, men ännu finns flera patienter där behandlingseffekten är mycket låg. Denna avhandling har adresserat några av dessa svårigheter och presenterar vetenskapliga upptäckter och innovativa alternativ för behandling av Inte CAR-T på människa ögonmelanom.

LIST OF PAPERS

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

I. **Sah VR**, Karlsson J, Jespersen H, Lindberg MF, Nilsson LM, Ny L, Nilsson JA.

Epigenetic therapy to enhance therapeutic effects of PD-1 inhibition in therapy-resistant melanoma.

Melanoma research. vol. 32,4 (2022): 241-248.

II. **Sah VR**, Jespersen H, Karlsson J, Nilsson L, Bergkvist M, Johansson I, Carneiro A, Helgadottir H, Levin M, Ullenhag G, Ståhlberg A, Bagge RO, Nilsson JA, Ny L.

Novel biomarkers identified in patients with metastatic uveal melanoma treated with combined epigenetic therapy and checkpoint immunotherapy.

Submitted

III. **Sah VR***, Karlsson J*, Bucher V, Iqbal M, Saxena A, Johansson M, Bagge RO, Ny L, Nilsson L, Nilsson JA.

Phenotypes and spatial localization of T cells in uveal melanoma metastases.

Manuscript

Related papers not included in their thesis:

I. Karlsson, J., Nilsson, L.M., Mitra, S., Alsen, S.,Shelke, G.S, **Sah, V.R**, Forsberg E., Stierner, U., All-Eriksson,C., Einarsdottir, B., Jespersen,H., Ny, L., Lindnér,P., Larsson,E., Bagge, R.O, Nilsson, J.

Molecular profiling of driver events in metastatic uveal melanoma.

Nature Communications 11 (1), 1894 (2020).

II. Ny, L., Jespersen, H., Karlsson, J., Alsén, S., Filges, S., All-Eriksson, C., Andersson, B., Carneiro, A., Helgadottir, H., Levin, M., Ljuslinder, I., Bagge, R.O, **Sah, V. R.,** Stierner, U., Ståhlberg, A., Ullenhag, G., Nilsson, L. M., & Nilsson, J. A. (2021).

The PEMDAC phase 2 study of pembrolizumab and entinostat in patients with metastatic uveal melanoma.

Nature communications, 12 (1), 5155 (2021)

III. Revenko A, Carnevalli LS, Sinclair C, Johnson B, Peter A, Taylor M, Hettrick L, Chapman M, Klein S, Solanki A, Gattis D, Watt A, Hughes AM, Magiera L, Kar G, Ireland L, Mele DA, **Sah V**, Singh M, Walton J, Mairesse M, King M, Edbrooke M, Lyne P, Barry ST, Fawell S, Goldberg FW, MacLeod AR.

Direct targeting of FOXP3 in Tregs with AZD8701, a novel antisense oligonucleotide to relieve immunosuppression in cancer.

Journal for Immunotherapy of Cancer. 10,4, e003892 (2022)

Contents

ABBREVIATIONS

ACT	Adoptive Cell Transfer
BAP1	BRCA1 Associated Protein-1
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B β-2-Microglobulin
CAR	Chimeric Antigen Receptor
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
DC	Dendritic Cell
GNA-11	Guanine Nucleotide-Binding Protein Subunit Alpha-11
GNAQ	Guanine Nucleotide-Binding Protein G(q) Subunit Alpha
HLA	Human Leukocyte Antigen
IFN	Interferon
IL-2	Interleukin 2
In-vitro	Latin for ”in glass”, referring to research conducted in a test tube/plate
In-vivo	In-referring to research of a living organism
MHC	Major Histocompatibility Complex
NK	Natural Killer cell
NOG	NOD/SCID IL2 receptor knockout
PDX	Patient Derived Xenograft
TAA	Tumor Associated Antigen
TCR	T Cell Receptor
TIL	Tumor Infiltrating Lymphocyte
UM	Uveal Melanoma
PD-1	Programmed Cell Death Protein 1
LDH	Lactate dehydrogenase
PFS	Progression free survival
OS	Overall Survival
irAE	Immune related adverse events
TME	Tumor microenvironment
TAM	Tumor associated macrophages
MDSC	Myeloid derived suppressor cells
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor

1 INTRODUCTION

1.1 Cancer

1.1.1 Hallmarks of Cancer

Cancer is a fatal condition and the leading cause of mortality, with nearly 10 million deaths in 2020 [1]. Cancer cells are wired differently, i.e., they multiply and continue to reproduce. In contrast, normal cells are controlled for survival and expansion. The uncontrolled growth of cells is due to the genetic reprogramming that gives rise to cancerous cells, which develop mutations from the loss of tumor suppressors or gain of pro-oncogenes[2]. Cancer formation is typically depicted as a multistage process with sequential mutations, with exposures to various factors leading to these aberrations. However, it is most commonly epithelial cells located in the skin and lining of internal organs that lead to the cancerous state[3].

Cancer's origin was traced to the "Father of Medicine" Hippocrates (460-370 BC), who coined the word "circus," the Greek term for crab[4]. The Latin word for crab was translated later by a Greek-Roman physician Celsus (25 BC - 50 AD), who documented the word "cancer" and differentiated between several forms of Cancer[4, 5]. There are approximately 200 types of cancers, divided by the disease's location, cell type, and stage (https://www.nhs.uk/conditions/cancer/). In addition, there are sub-types among each cancer form, like bone cancer, i.e., osteosarcoma, Ewing sarcoma, and chondrosarcoma; lung cancer, i.e., small and non-small cell; and head & neck cancer, i.e., throat, mouth, laryngeal. However, these only represent a snapshot of all diagnosed cancers [6].

Cancer cell maintains several capabilities that develop and have been classified as their hallmarks. The Hallmarks of Cancer was first reported in 2000 and included six factors, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis[7]. Later in 2011, two provisional "emerging hallmarks" were added, including reprogramming of energy metabolism and evading immune destruction[8]. Finally, in 2022, the third update of cancer hallmarks was published, which confirmed the previous two provisional parameters and included four emerging parameters that would require further validation[9].

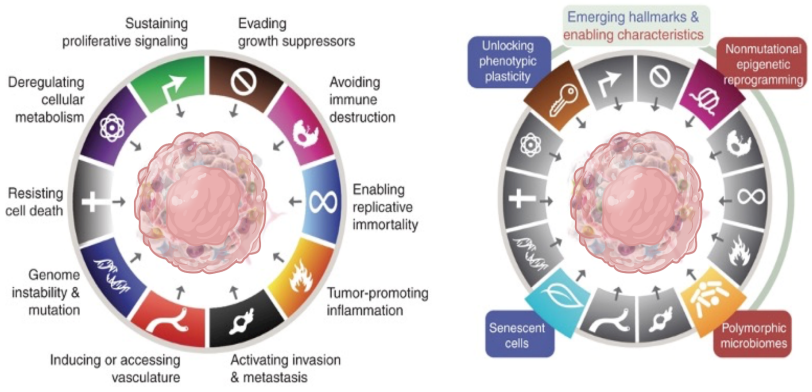


Figure 1. Adapted from Hallmarks of Cancer 2022 [9]

Private and government funding for cancer research has increased considerably, with several developed countries focusing on this area, to strengthen. There are many clinical trials in oncology, with recent advancements in identifying biomarkers, extending patient lives, and discovering novel therapies. Even though our knowledge and management of Cancer have improved over recent years, the total number of people affected is growing[10]. We must further advance our therapeutic strategies to battle cancer[11].

1.1.2 Metastasis

Cancer remains a significant health problem worldwide, with an estimated 19 million new cases and 10 million associated deaths in 2020[12]. However, it is not the primary disease that accounts for most cancer-related deaths but a process known as metastasis. It is a multi-step procedure where the primary Tumor circulates to secondary sites, impacting vital organs[13]. MetMap, a metastasis map of 21 tumor types, demonstrates organ-specific imprints of metastasis, unraveling the clinical and genomic patterns[14].

Cancer stem cells (CSC) and the epithelial-mesenchymal (EMT) remain significant areas of investigation in cancer research and treatment[15]. This is because cancer stem cells retain self-renewing properties and play essential roles in tumor initiation, relapse, and metastasis[16]. In addition, intrinsic factors, such as epigenetic, genetic, and extrinsic factors, such as the tumor microenvironment, contribute to renewing cancer stem cells' properties. Therefore, these stem cells plasticity, dormancy, renewal, and therapeutic potential are essential parameters to consider for developing their targeting options[17].

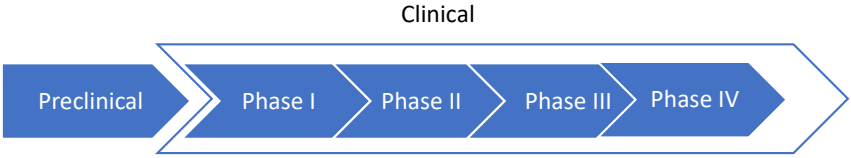
Primary disease, mechanisms of immunosuppression, extracellular matrix (ECM) remodeling, and metastatic conditioning niches have been shown to influence metastasis through the delivery of soluble elements, i.e., exosomes[18]. Surgery may be curative and prevent metastasis, especially in early dissemination[19]. Metastatic colonization has been connected to the characteristics of the disseminated cancer cells (seed) and the specific met site (soil) [19] [20]. In addition, the evolution of Cancer is very dynamic, with clones and even sub-clones proliferating, downsizing, and traveling to different organs, leading to various changes in their molecular signatures[21]. Furthermore, clonal benefits may change according to disease time as distinct requirements arise in different tumor areas. The circulating tumor cells can potentially seed metastases that strive for a long time. Likewise, the circulating tumor cell studies demonstrate smaller clusters of metastatic seeding possessing multiple clones than single cells [22].

Despite decades of cancer drug development research, survival rates with metastatic disease are dismal, with five-year survival rates ranging from 5 to 30% among all solid cancers[23]. The cancer cells travel from the primary site via lymph or blood vessels to distant organs, i.e., lungs, liver, bones, and brain, forming metastatic lesions at multiple locations within the body[24] [25]. For example, the growth of primary melanomas is unknown; however, observable metastases of patients with malignant melanomas double every approx. Fifty days, however, with immense variation[19].

Metastatic Cancer is usually resistant to therapy and challenging to cure, a significant concern for oncologists, patients, and their caregivers[26]. The extent of metastasis can be measured using staging, and the most commonly used method is the TNM system[27]. It enables the oncologists to know how severe the disease is and explore clinical trials as a viable option. In the TNM system, T would be described as the size and extent of the primary Tumor, N as the number of cancerous lymph nodes, and M as a metastasized tumor. The overall cancer stage grouping is in four stages, i.e., 0,1,2,3 and. It corresponds to the severity of Cancer, with stage IV being the most lethal, spreading to distant parts of the body[28].

1.1.3 Clinical trials

Clinical trials are research investigations that involve human subjects[29]. These studies prevent, diagnose or treat Cancer, leading to regulatory approvals, eventually coming into the market and impacting our society. New trials could also improve survival and quality of life for patients with life-threatening diseases[30]. In addition, in the modern era of oncology, the data from new cancer trials would help decipher the associated complex biology. US Food and drug administration (FDA) documents an increase of 60% from 2015 to 2020 in the number of oncology clinical trials[31]. In addition, there have been increased trials for the more prevalent cancers and even more so for rare cancer types[32].



Above is a flowchart schematic depicting the preclinical to all clinical trial phases.

The flowchart depicts the preclinical phase, which includes laboratory and animal studies. The clinical trial cycle starts at Phase I, where preliminary safety & toxicity, including dosing, is determined with a sample size in low tens[33]. With Phase II, the sample size consists of high tens to hundreds, providing intermediate efficacy and acquiring additional safety and toxicity information[34]. It is followed by Phase III, where the sample size is more expansive, i.e., hundreds to thousands, confirms efficacy, and compares the investigational to the standard therapy[35]. It also includes monitoring side effects leading to Phase IV, with additional information on long-term survival, risk, and safety. FDA approves a new treatment after a successful phase III, and a phase IV study will be analyzed to understand the post-approval surveillance[36]. Eleven clinical trials would shape Medicine in 2022; ranging from Antisense Oligonucleotides (ASOs) for Huntington's disease, antibody Nirsevimab for Respiratory syncytial virus (RSV) to antibody pembrolizumab (PD1) for triple-negative breast cancer[37].

1.2 Immunity in cancer

1.2.1 Innate immunity

The first immunological, not antigen-dependent, are innate responses that assist in combating cancer cells. It's a non-specific, rapid response that has no immunological memory[38]. It comprises cells that notify the adaptive system after recognizing deadly antigens, i.e., antigen-presenting cells (APCs). APCs are cells that recognize the pathogen, engulf and digest it, and then create multiple antigen sections. This, in turn, activates adaptive immunity as the antigenic fragments are transported to the surface of APC [39].

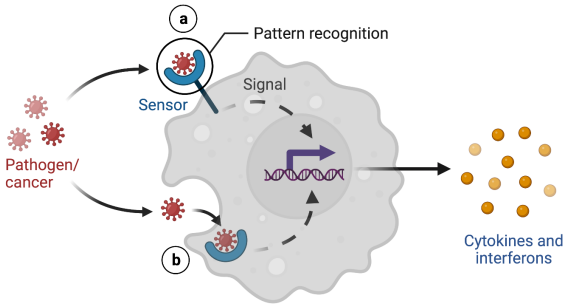


Figure 2. Depiction of innate immunity. Created using BioRender.

1.2.2 Adaptive Immunity

An immunological memory defines the adaptive system with an antigen-specific immune response. When innate immunity is not sufficient to stop the infection, adaptive immunity is triggered [39] [40]. More so, without assistance from innate immunity, the adaptive could not be effective in clearing cancer[40]. There are primarily two forms of adaptive immunity, the humoral and cellular mediated response. Humoral includes activated B cell response and antibodies; cellular mediated has a reaction controlled by T cells. Tumor antigens trigger T cells by MHC (major histocompatibility complex) machinery, leading to a cascade of events that further leads to the killing of tumor cells[41] [42].

There are diverse cells, like immature dendritic cells, which travel to regional- lymph nodes via lymphatics with the phagocytized antigen[43]. These cells next activate antigen-specific, naïve T cells, which come from blood to lymph nodes by specialized vessel endothelial venule (HEV)[44].

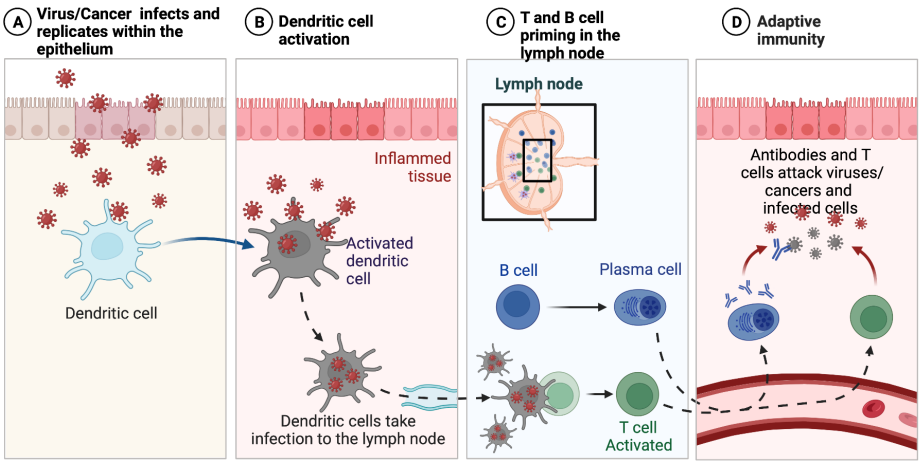


Figure 3. Depiction of adaptive immunity. Created using BioRender.

1.2.3 Tumor Antigen and recognition

In mice and humans, polymorphic molecules, i.e., MHC, also known as H2 and HLA (Human Leukocyte Antigen), present peptide antigens to T lymphocytes, which possess CD4 or CD8 positivity. HLA complexes are of two kinds, I and II, which activate CD8+ and CD4+, respectively. Class I, which consists of 8-12 amino acids, is found in all cells[45]. In contrast, Class II, which consists of up to 15 amino acids, is only found on specific cells, like macrophages, dendritic and antigen-presenting cells. It's the T cell receptor (TCR), which helps T cells to bind to surface antigens on MHC. The selectiveness of T cells is based on antigenic peptide detected, found on the groove of the MHC complex[46].

In the case of Class I molecules, proteosomes resulted in antigenic peptides, which are transported to ER (endoplasmic reticulum), further shortened by aminopeptidases, and displayed on Class I complex, presented to CD8+ cytotoxic T cells[47]. However, it's not just one crucial signal; CD80 and CD86 on the tumor cells or APCs bind CD28 on T cells, forming a second signal. IL-2, IL-7, and IL-15 exercise various effects, i.e., T-cell survival and activation, including memory-cell development and upkeep[46]. The activation, in turn, leads to degranulation (LAMP1) and the release of granzymes through perforins and interferon (IFN-g). Granzyme B could initiate caspase 3 and 7 cascades for target cell apoptosis. Granzyme B enters the target cell perforin-dependent, forming pores in the target cell's plasma membrane[48].

However, on the other hand, Class II complexes are internalized by clathrin-moderated endocytosis, i.e., engulfing the foreign body in a vesicle[49]. Following endocytosis, it contains endosomes to late endosomal–lysosomal molecules possessing antigenic proteins and peptides. Class II complexes are loaded with the peptide from the ER phagolysosome and transported to the cell surface, permitting CD4 T helper cells to identify them[50].
Tumor-specific immune reactions are stimulated by antigens, viral antigens, cancer-germline genes, or antigens resulting from a mutation. There are various cancer antigens determined by T cells, which correspond to peptides, i.e., mutated amino acid alterations originating from ubiquitous mutated proteins in tumor cells[51].

1.2.4 Immunoediting of cancer

Paul Ehrlich envisioned the notion of immunoediting in 1909 by conceptualizing that body's immune system could subdue the elevated frequency of various cancers. These findings were incorporated into the 'cancer immunosurveillance' hypothesis proposed by Macfarlane Burnet in 1957 [52]. Moving forward with decades of research within this domain, these concepts of immunoediting and immune invasion are constantly evolving. Cancer cells successfully develop and are considered to have evaded this immunosurveillance [53]. Furthermore, the field has led to more complexities as new research and mechanisms are being discovered. Cancer immunoediting transpires in three sequential phases: elimination, equilibrium, and escape. Innate and adaptive immunity work together to eliminate cancer cells before they become clinically apparent, also known as elimination[54]. In some instances, cancer cells may not be eliminated but enter an equilibrium phase where the immune cells arrest the tumor growth. Eventually, the tumor dormancy would be violated with the advancement of the cells into the escape stage. The edited tumor with less immunogenicity, which results in immune escape, proliferates and sets an immunosuppressive TME, also becoming clinically prominent[55].

Moreover, multiple human tumors can suppress the immune system to survive and grow. These tumor cells evade detection by immune cells by reducing the expression of antigen-presenting proteins (MHC) at their surface. The cytotoxic T lymphocytes cannot locate these molecules and are thus imperceptible to tumor killing [54] [56]. Notably, escape from immune control is accepted to be one of the hallmarks of cancer[55] [57] [58].

1.3 Uveal Melanoma

1.3.1 Epidemiology

Uveal melanoma annual incidence accounts for approximately eight new cases/ million in Sweden and only 2% of all melanoma cases[59]. Moreover, it corresponds to about 80 UM cases per year in Sweden and increases from south-to-north European prevalence [60].

There is only a small understanding of the etiology and associated environmental risk factors. However, evidence from epidemiological studies opposes the role of sun exposure as manifested by cutaneous melanoma. Furthermore, we and others have shown using whole genome sequencing of primary and metastatic uveal melanoma, concluding a lack of UV-light rendered mutational signature in these tumors [61].

Vision loss, cataracts, glaucoma, and intraocular hemorrhage are some complications that arise when the Tumor is at its primary site. Moreover, some established risk factors include, Fair skin color, northern European descent, light-colored eyes, ocular melanocytoma, and germline mutations in PALB2, MLH1, and MBD4 [62].

1.3.2 Eye and metastasis

Uveal melanoma (UM) is a rare condition of melanoma but the most common primary malignancy of the eye. In Europe, the incidence of UM varies from 4 to 7 per million patients annually[63] [64]. UM comprises about 85% of all ocular melanoma developed from uveal tract melanocytes (Figure 1). About 90% of UM are confined to the choroid, about 6% to the ciliary, and 4% to the iris. Around 50% of patients develop metastasis, mainly in the liver but rarely in the lungs, bones, and other organs [63] [65].

Meta-analyses demonstrate a 10–13 months median overall survival, with a closer to zero cure rate. However, survival exceeding five years is probable, assessed at 2%, either with first-line therapy or adequate supportive care [66].

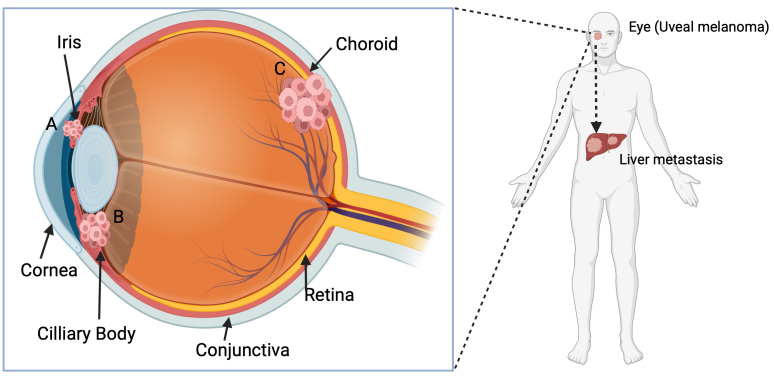


Figure 4. Locations of uveal melanoma. Created using BioRender.

1.3.3 Genetics and Molecular profile

Uveal and Cutaneous melanoma is derived from melanocytes, but they are drastically different in their biology and genetics. Monosomy3 (loss of one copy of chromosome 3) and disomy3 (presence of both copies of chromosome 3), with mutations in the BAP1, EIF1AX, and SF3B1and SRSF2 genes, are common in different UM cases and result in varying patient outcomes with metastatic risk [61] [65] [67]. Uveal melanoma (UM) has different genetics and immune microenvironment than cutaneous melanoma. Patients with UM metastases are not predicted to respond to the same therapies as patients with cutaneous melanoma since UM does not have BRAF mutations [68]. Molecular profiling of metastatic uveal melanoma has been limited, but it is imperative for identifying vital genetic drivers within tumors.
UM is divided into two distinct molecular subtypes, class I with a good prognosis with low to the medium metastatic risk and class II with poor prognosis and much higher risk of metastasis [64] [69]. BAP1 (BRCA1-associated protein), a tumor suppressor gene, is mutated in most poor prognosis UM cases and is associated with class II UM tumors[70]. Loss of this gene located on chromosome 3 corresponds to higher metastatic risk. BAP1 has various functions, involving DNA damage responses, transcription activation, remodeling of chromatin, and cell cycle regulation [64] [71]. Unlike BAP1, the mutations of guanine nucleotide-binding proteins Q polypeptide (GNAQ) and Guanine nucleotide-binding protein subunit alpha-11 (GNA11) are not associated with the molecular classes of UM tumors [72] [73].

With UM, genetics has primarily been investigated in the primary tumors of the eye, i.e., analysis within the TCGA consortium [72]. Frequent mutations in GNAQ or GNA11 are typical, whereas mutations in CYSLTR2 and PLCB4 are witnessed occasionally, downstream and upstream of GNAQ/11 [74] [75]. These mutations which drive tumor growth are all mostly mutually exclusive. Additional recurring mutations have been uncovered in EIF1AX, SF3B1, and a small proportion revealed CDKN2A and SF3B1 mutations[76] [77] [78].

1.3.4 Tumor microenvironment

A tumor is not just an amalgamation or group of cancer cells but a heterogeneous array of infiltrating and host cells, defined as the tumor microenvironment (TME). Tumor cells elicit significant cellular and physical adaptions within their environment to sustain tumor growth and progression[79]. The TME is paramount in cancer initiation, evolution, metastasis, and relapse[80].

Liver metastasis is a usual clinical course and the primary reason for high mortality[81]. Disseminating UM cells that penetrate the liver are subjected to the reaction of a unique immune response, with the liver being a site to destroy gastrointestinal-derived pathogens and sustain benign food antigens[82]. The TME of uveal melanoma liver metastases is highly complex and requires more comprehensive studies. Compared to other cancers, not much is known about the TME of mUM, mainly challenging because specimens are usually hard to attain and often small, i.e., needle biopsies. [83, 84]

The tumor microenvironment differs between different tumor types, but hallmark characteristics include immune cells, stromal cells, blood vessels, and extracellular matrix [84, 85]. A specific kind of immune cell, Tumor-associated macrophages (TAMs), facilitate tumor development and may be necessary for angiogenesis, invasion, and metastasis [86]. A high TAM coverage commonly correlates with poor prognosis [87] and is divided into M1 and M2 macrophages. M1 macrophages are activated by IFN-g and depict high levels of proinflammatory cytokines, major histocompatibility, and iNOS (inducible nitric oxide synthase)[88]. M2 macrophages have a low histocompatibility class II and interleukin (IL)-12 expression but have heightened anti-inflammatory cytokine IL-10 and arginase[89]. Cytokines produced by cells in the tumor microenvironment determine whether TAMs become M1 or M2[90]. Furthermore, potent predictors of metastasis in UM, with class II classification and PRAME expression, have been reported to strongly associate with an inhibitory TME [91]. It has been documented that primary suppressive cells within mUM are CD163+ tumor-associated macrophages (TAMs), i.e., pro-tumourigenic M2 phenotype [92]. Predominant Tumour infiltrating lymphocytes (TILs) in mUM were CD8+ T-cells (CTLs) with low expression of Granzyme B, not present within the tumor core [92, 93]. The portion of active CTLs among CD8+ GranzymeB+ cells within intra-tumor nests is also a predictive indicator, as patients have better outcomes. Specific findings supported by various studies suggest CD8+ T-cell exhaustion and dysfunction in UM [61 92 94] . Liver Metastasis portrayed UM cells adjacent to CTLs and TAM, with T cells in proximity to TAMs, indicating a deformed immune response. Additionally, the LAG3 checkpoint gene is correlated to a subset of Class II tumors [91] and has been hypothesized to have significant activity with mUM TME [94].

Recently discovered entities, Tertiary lymphoid structures (TLS), were found in solid cancers. These structures are defined majorly by rich B cell zones, T cells, and endothelial venules (HEVs) [95 96, 97]. Studies have documented showing TLS in Melanoma [98], lung [99], colorectal [100], pancreatic[101], and endometrial cancers[102] corresponding to favorable correlative survival. Various cytokines can advance or inhibit tumor growth, such as CCL17 and CCL22, which recruit T Regulatory (TReg) cells into tumors[103]. Tregs have an inhibitory role within the TME, significantly impacting the cytotoxic T cells, and in many studies, correspond to poor clinical outcomes[104]. Similarly, CCL2 has a role in attracting macrophages and enabling their survival and M2 polarization[105]. However, other cell types, like Natural killer (NK) and B cells, are scarce in uveal melanomas[106]. In addition, non-immune cells, such as stromal and endothelial cells, also play critical roles in cancer TME, including, but not limited to, immunosuppressivecancer-associated fibroblasts (CAFs) [107].

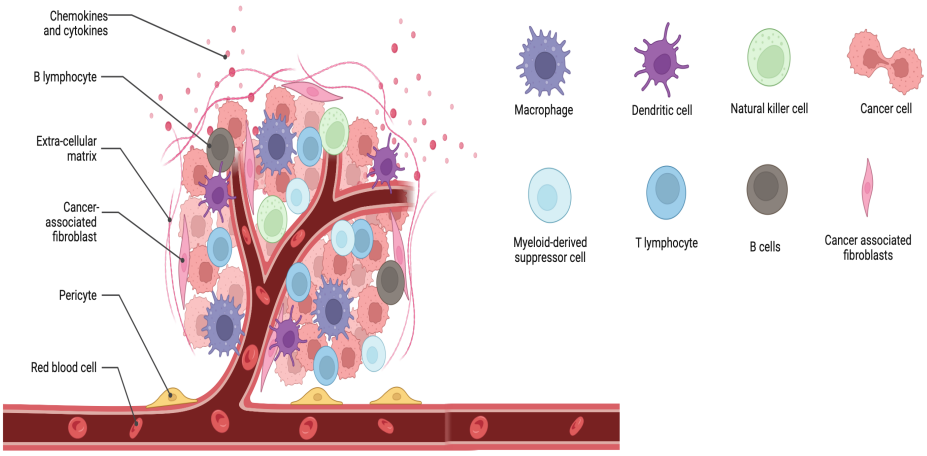


Figure 5. Tumor microenvironment landscape. Created using BioRender.

The advent of new state-of-the-art techniques has given rise to studies dissecting the TME of various cancers. Advances like Single-cell RNA sequencing (RNA-seq) [108], single-cell pathology[109], and spatial transcriptomics[110] have transformed the ability to investigate the TME in an advanced manner. This has led to the discovery of new cell subsets, biomarkers, and cancer pathways, significantly impacting many Cancer of unmet needs[111 112 113].

1.3.5 Clinical stratification

The most appropriate for patients who have a high risk of relapse would be frequent screening, as would be recommended by most clinicians. Patients with primary UM tumors with a class II gene expression profile, monosomy 3, or >8 mm apical size are in the most heightened risk group[114]. These individuals may aid from a management regimen that includes CT or MRI-based hepatic imaging and liver function tests obtained at periodic monthly intervals for the first initial years[115]. Although surveillance of patients could vary, some investigations suggest that lactate dehydrogenase (LDH) and γ-glutamyl transferase are the most acute liver process ordeals for uveal melanoma and are advanced in patients with progressive hepatic mets[116]. Given the lack of evidence for the benefit of any direct surveillance approach, especially metastases for late recurrence, suggestions for these patients and their providers remain uncertain[117]. In Sweden patients diagnosed with a primary tumor, uveal melanoma, are included in a metastatic screening program over at least five years. In this imaging of the liver (MRI/ultrasound) are carried out approximately every 6 months.

1.3.6 Treatment options

The primary goal of treatment is to destroy the Tumor, prevent recurrence and metastasis, and preserve vision. Unfortunately, while the treatment of primary uveal melanoma has continually improved over time and additional irradiation procedures have successfully replaced Enucleation in designated cases, the therapeutic possibilities for metastatic disease are nevertheless disappointing [118]. Various techniques like Proton beam radiotherapy, Photon radiotherapy, Transpupillary thermotherapy (TTT), and Photodynamic therapy (PDT) have been employed, but either were unsuccessful or still lack long-term efficacy data. A satisfactory regional control is supplied by Enucleation or brachytherapy, but 50% of patients develop metastasis, majorly to the liver [66].

Molecular-targeted therapy is an appropriate strategy for uveal melanoma due to the unique genetic profile, with mutations in the GNAQ and GNA11 genes, stirring cell proliferation[119]. Protein kinase C (PKC), YAP (Yes-associated protein), and MAPK (Mitogen-activated protein kinase) are activated downstream by mutated GNAQ-GNA11. MEK (mitogen-activated extracellular signal-regulated kinase) inhibitors like Trametinib and Selumetinib have encouraging data in preclinical models but haven't shown clinical benefit [120] [121]. For example, the study (NCT01143402) of MEK inhibitor selumetinib compared to chemotherapy did not improve overall survival in the trial [69]. Recently a study shows co-targeting Gaq (G-protein alpha-q) and MEK, resulted in enhanced efficacy in UM [122]. Current research efforts to develop new therapies for UM patients are underway, but there is still an unmet need for this disease.

Isolated hepatic perfusion (IHP) and percutaneous hepatic perfusion [123] are presently examined in phase 3 trials. Provisional data suggest significantly durable partial responses that exceed benchmark data and the control groups in those trials. But overall survival data are not yet fully matured and will be reported in the future.

With the recent advances, and the emergence of checkpoint blockades, the treatment landscape for cutaneous melanoma has fiercely changed, but the success in uveal melanoma (UM) has been minimal[124]. Cancer immunotherapies unleash the immune system by cancer antigen targeting vaccines, blocking immune checkpoints, adoptive T cell transfer, and CAR-T. The success of anti-CTLA4 ipilimumab (Yervoy; Bristol-Myers Squibb) and pembrolizumab (Keytruda; Merck)), two checkpoint antibodies, have revolutionized the treatment landscape in oncology[125]. Pembrolizumab has been approved for treating diverse tumors, and also Yevroy. The combination of PD1 and CTLA has also been approved recently for certain tumors[126] [127]. However, combination treatment of PD-1/CTLA-4 [126] inhibitors or PD-1/HDAC inhibitors [128] have demonstrated longer overall survival.

Tebentafusp (IMCgp100) is a unique therapy designed to treat metastatic UM based on a particular ImmTAC TCR-based bispecific scaffold [129]. Tebentafusp has a binding region designed to target intracellularly emanated melanoma-associated gp100280–288 peptide in complex with human leukocyte antigen (HLA)-A*02:01 found on the surface of cancer cells [130]. Gp100 is represented more frequently, uniformly, and at higher levels on UM cells than

CM cells [131]. In addition, HLA-A2*02:01 positivity is an inclusion bar for patients in tebentafusp clinical trials and is present in roughly 50% of Caucasian patients. In human studies, tebentafusp recruits a broad spectrum of T cells into the Tumor via its anti-CD3 single-chain variable fragment region[132]. This, in turn, is conceived to simulate an immune synapse and triggers T-cell-mediated cancer death. In 2022, Tebentafusp, a T-cell engager suitable for patients with HLA-A2 genotype, was approved by FDA[133]. It has demonstrated more prolonged overall survival than historical benchmark data for metastatic UM [129] [134].

The number of mutations in UM is deficient, unlike cutaneous melanoma, which has a high mutational load and has already been FDA approved for T cell checkpoint blockades. Low mutational burden results in lower expression of neoantigens which could explain the lack of efficacy. UM's response to monotherapy with these checkpoint antibodies is reported to be in the <5% range [69] T-cell therapies like Adoptive cell therapy (ACT) or CAR-T cells have not been extensively studied clinically or pre-clinically in UM [135] but hold promise for this disease with unmet need. As of 2021, there were 16 clinical trials involving checkpoint inhibitors, one with a vaccine and 7 involving T cell therapies, planned or recruited [136].

1.4 Cancer Epigenetics

1.4.1 Epigenome with Cancer

Epigenetics is a dynamic and heritable genome transformation. The term epigenome emanated from the Greek word epi, which denotes "above" the genome. It requires interchanges with diverse enzymes and molecular components. Unusual epigenetic alterations can lead to an inappropriate beginning of genetic expressions and facilitate tumorigenesis[137]. However, these changes are bound to DNA and do not alter the sequence of DNA building blocks. DNA methylation, histone modification (acetylation, methylation, phosphorylation), bromodomain, and noncoding RNAs are the primary epigenetic tools that regulate gene activity in several cancers[138]. Cancer is a genetic disease; chromatin and epigenetic anomalies play crucial roles in tumor initiation, maintenance, and progression. Disruption of the epigenome is essential in Cancer, and various epigenetic drugs are in clinical trials[139].

1.4.2 Epigenetic drugs

There are three classes of epigenetic proteins—readers, writers, and erasers which are potential druggable targets. In addition, there are multiple epigenetic drugs in clinical trials for cancer treatment, such as inhibitors of DNA methyltransferases, histone deacetylases, histone methyltransferases, lysine-specific demethylases, and BET (bromodomain and extra-terminal domain) family proteins[140]. DNA methyltransferase (DNMTs) are essential enzymes that mediate DNA methylation, especially DNMT1, 3A, and 3B. DNMT inhibitors can revive the expression and function of tumor suppressor genes by blocking DNA methylation activation, hindering the development of tumor cells and causing their apoptosis[141].

The acetylation of multiple lysine residues in the histones core is moderated by histone acetyltransferases (HATs). It is further identified by proteins carrying bromodomains (BRDs) which improve the rate of transcription of associated genes. BET proteins (Histone reader proteins) identify histone acetylation through one of their bromodomains, BD1 or BD2. BET inhibitors (BETi) are therapeutic agents that restore regulated gene expression by suppressing bromodomain–acetyl-lysine protein-protein interaction[141].

Histone acetyltransferase enzyme (HAT) unwraps the DNA from histones by transferring an acetyl group to the N terminal, and HDACs (Histone Deacetylases) is an enzyme that reverses HAT's action. HDACs are often overexpressed in Cancer, where they may silence tumor suppressor genes[142]. It has been shown that HDAC inhibitors can have therapeutic potential for differentiation and enhanced dormancy of metastatic disease in UM. HDAC (Histone deacetylases) inhibitors are tested as single agent therapies in various cancers. HDAC inhibitors are approved by FDA for T-cell lymphomas and are also currently investigated for multiple cancers[143]. HDAC are enzymes that detach acetyl groups from core histones (H2A, H2B, H3, and H4), strengthening the chromatin structure and minimizing access to transcription factors. Inhibiting the HDACs leads to hyperacetylated histones accumulating into the chromatin, resulting in transcriptional activation of many genes[142]. Class I HDAC comprises of HDACs 1,2,3 and 8; class II comprises HDACs 4, 5, 6, 7, 9, and 10, and class IV comprises HDAC 11. Pan HDACi, such as Vorinostat, inhibit HDAC Class I, II, and IV, whereas Entinostat is more specific and inhibits HDAC Class I [144]. Within class I, the transcription of HDACs 1 and 2 are coordinated, but HDAC2s are cell-specific in lung cancer. With the siRNA screen, BAP1 knockdown resulted in a decrease in HDAC2 and an increase in HDAC1 levels, implying the correlation between HDAC2 and BAP1[145]. Epigenetic changes like silencing tumor suppressor genes, e.g., BAP1 with histone modifications, are a field of active cancer research [146].

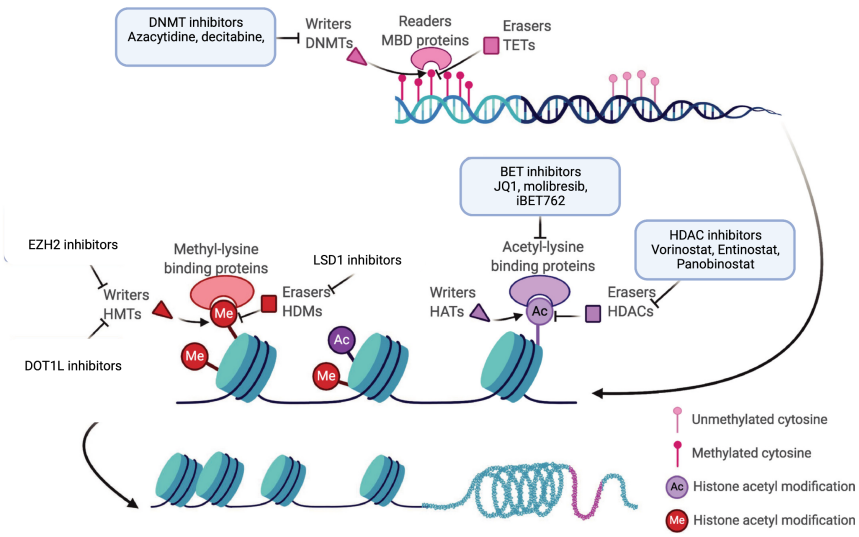


Figure 6. Epigenetic remodeling. Adapted from [147]

1.4.3 Epigenetic and Immunotherapy

As some of these HDACi have not been promising in various clinical trials as a single agent in multiple cancers, their role in combination with immunotherapy is being explored[148]. HDAC inhibitors boost anti-tumor immune responses and, with the advent of checkpoint blockers, are promising for combination therapy. Tumors evade immune responses by down-regulation of MHC molecules, activating suppression of anti-tumor immune responses at the cancer site by creating an immune suppressive tumor microenvironment (TME)[149].

As discussed before, several HDACs have the potential to regulate diverse genes within the tumor microenvironment. Pan HDACs inhibitors have shown efficacy with an immunotherapy combination. HDACi has been shown to enhance PDL1 gene expression on tumor cells, thereby suppressing T cell activity [150] [151] and enhancing antigen presentation by introducing HLA molecules on cancer cell lines[150] [152]. In addition, DNMT and HDAC6 Combination Inhibitor therapy improves the cytotoxic immune response in ovarian Cancer [153]. Furthermore, DNA Methylation Upregulates PD-1 and Decreases PD-L1/L2 and Lysine-Specific Histone Demethylase 1A (LSD1) negatively influences anti-cancer immune reaction, i.e., a negative correlation with CD8+ infiltration [154]. EZH2 inhibitor reduces PD-1 Expression[155], and BET inhibitors decrease PD-L1 expression (PMID: 31653272). There are strong indications that immunotherapy should be combined with epigenetic therapies to overcome these tumor-induced immune escape mechanisms[156].

1.5 Immune targeting therapies in Cancer

1.5.1 Vaccines

Therapeutic cancer vaccines exhibit efficacy and the possibility to aid patients resistant to other standard-of-care immunotherapies, but they have yet to discover their maximum potential. Therapeutic cancer vaccines aim to facilitate adaptive immunity against distinct tumor antigens to contain tumor growth, cause regression and eradicate minimal residual disease[157]. Cancer vaccines generally apply the administration of specified tumor antigens integrated with adjuvants that activate dendritic cells. The fundamentals required for positive therapeutic vaccination against tumors comprise antigen delivery to DCs, leading to intense and sustained CD4+ T helper cell and cytotoxic T lymphocyte (CTL) responses by penetrating the TME and attaining durability and maintenance of treatment[158].
Cancer vaccines are divided into two distinct domains, outlining predefined vaccines (personalized) and anonymous vaccines (shared), utilizing two different antigens, tumor-specific antigens (TSA) and tumor-associated antigens (TAA), respectively. In the case of predefined vaccines, the success of the neoantigen platform relies upon the tumor mutational burden (TMB), which is the number of mutations in the Tumor[159].

There are various vaccine classes, and different technologies are implemented for manufacturing a cancer vaccine [160]. The first is cell-based vaccines, i.e., developing an

engineered tumor cell vaccine model is the GVAX vaccine, wherein the tumor cell is transfected with the GM-CSF gene, regulating immune cells such as DCs and NK cells [161] [162]. Moreover, clinical trials of GVAX in combination with anti- PD-1 are ongoing (NCT03190265, NCT02451982). Secondly is the use of viruses to develop vaccines. The most common adenovirus-based vaccines that deliver tumor antigens (TAAs) or bear immune-instigating genes to induce solid antitumor immunity are being studied in preclinical and clinical trials [163]. The third is a class of peptide-based vaccines, i.e., incorporating multiple targets or epitopes to activate T cells that identify various targets and minimize tumor-immune escape. The fourth and fifth classes are DNA and mRNA-based vaccines, respectively. DNA vaccines are primarily described as personalized vaccines that encode neoantigens. For example, VGX-3100, a DNA vaccine against Human Papillomavirus Associated Cancers (HPV), is currently being assessed [164] for safety and efficacy in two Phase III clinical trials (NCT03185013, NCT03721978). However, mRNA-based vaccines have certain advantages over DNA-based, like the ease of design and accessibility of scaling. mRNA vaccines for Sars-Cov2, by Moderna and BioNTech/Pfizer, have significantly impacted humanity in fighting this deadly virus [165] [166]. However, cancer mRNA vaccines require more studies and can potentially change the landscape of cancer treatments [167]. For example, mRNA-4157, a personalized cancer vaccine encoded with neoantigens within lipid nanoparticles, produced by Moderna, alone or combined with Pembrolizumab, has shown promising antitumor efficacy in Phase I trials in patients with solid tumors (NCT03313778). Another example is BNT122 (BioNTech), a neoantigen cancer vaccine based on the mRNA encoded for patient-specific neoantigens, currently in Phase 1a/1b trial, assessed as monotherapy or in combination with immune checkpoints in solid tumors (NCT03289962) [168].

1.5.2 Immune checkpoint blockade

Checkpoint blockade is an innovative therapy that uses drugs known as immune checkpoint inhibitors to address several cancer types. It releases the brakes on one's own body's natural immune system to improve its ability to fight off infections and diseases. Specifically, the drugs used in checkpoint blockade treatment can aid the immune system in identifying and attacking cancerous cells. Checkpoint antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein-1 (PD-1), and programmed death-ligand-1 (PD-L1) are now approved treatments in multiple cancer types[169]. They work by stimulating antitumor immunity and reversing tumor-specific T-cell dysfunction. The 2018 Noble Prize in Medicine for discovering T cell checkpoints [170] [171], PD-1, and CTLA-4, combined with FDA approval of new checkpoints, has resulted in an escalation of immuno-oncology clinical studies with these molecules [171] [172] and there combinations [173] [174] [175]. In addition, a new checkpoint, LAG-3, has achieved approval [176] for advanced melanoma and reinforces the science d checkpoints. Furthermore, promising T checkpoints like TIGIT, PVRIG, TIM3, and myeloid targets like CSF1R and CD47/Sirpa have shown therapeutic benefits and are being investigated in the clinic [177]. However, among the promising T checkpoints, only TIGIT is in the phase III clinical trial, being studied together with PDL1[178].

There are many downstream mechanisms induced during initial antigen-mediated activation, i.e., peptide–MHC arrangement of the T cell receptor (TCR) and positive costimulatory signals such as exchanges between CD28 on T cells and CD80 (B7.1) and/or CD86 (B7.2) on antigen-presenting (APCs) or tumor cells[179]. Negative regulators are induced early during the activation process to counteract the activation. Cytotoxic T lymphocyte antigen 4 (CTLA4; CD152) directly contests with CD28 for the ligands CD80/CD86 [180]. Programmed cell death protein 1 (PD1; CD279) is also expressed during T cell activation and opposes positive signals through the TCR and CD28 by encountering its ligands programmed cell death one ligand 1 (PDL1; CD274 and B7-H1) and/or PDL2 (CD273 and B7-DC). PD1 can impede T cell functions by drafting phosphatases, including SHP2, to the immunoreceptor tyrosine-based switch motif (ITSM) in the PD1 tail [181]. These phosphatases can contradict the positive signaling being activated by the T cell receptor (TCR) on interaction with peptide-MHCI complex, impeding ZAP70 and the phosphoinositide 3-kinase (PI3K)–AKT and RAS signaling [182]. Collectively, the outcome is a reduced activation level of transcription factors, i.e., activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT), and atomic factor-kB (NF-kB), crucial for causing T cell activation, expansion, and effector functions[183]. Mice genetically lacking gene Pdcd1 (which encodes PD1) produce accelerated autoimmunity [184] [185].

1.5.3 Adoptive T-cell therapy

Adoptive T-cell therapy, also known as cellular immunotherapy, is a kind of treatment that utilizes the cells of one's immune system to eradicate Cancer. These processes are applied by isolating T cells and further expanding them for either using them similarly or modifying them, i.e., genetically engineering (via gene therapy) to enhance their cancer-fighting capabilities[186].

Cell therapies are continually maturing and improving, providing new possibilities to cancer patients. These therapies are presently being assessed, independently or in combination with additional treatments, in a mixture of cancer types in clinical trials[187].

Largely, ACT (adoptive cell therapy) can be carried out using three T-cell strategies. The first approach is when the TILs are expanded from a patient's Tumor before being infused into the same (autologous) or a different (allogenic) patient [188]. The second approach uses T-cell receptors (TCRs) engineered to identify specific tumor antigens; however, this approach is restricted to major histocompatibility complex (MHC)-expressed antigens. Finally, chimeric antigen receptors (CARs) comprise an extracellular antigen recognition domain, a transmembrane, and a cytoplasmic signaling part, to identify a class of tumor surface antigens unassisted by MHCs [189]. Adopting cell therapy (ACT) in solid tumors has various challenges. i.e., Tumor heterogeneity, immune escape, T-cell trafficking, and an immunosuppressive environment collectively illustrate barriers in solid tumors ACT outcome.

However, autologous CAR T cell products are challenging to manufacture efficiently, with the process taking weeks, which patients may be incapable of affording. As a result, allogeneic CAR T cells are manufactured in advance and distributed in an "off-the-shelf" manner[190]. This

prospect poses a distinct set of positive and negative consequences. While it would take a few days to get allogeneic CAR T cells ready instead of weeks, their foreign disposition puts patients at a threat. This is usually because they could contract graft-versus-host disease or one's immunity to eradicate the transfused cells[191].

CAR-T has shown the most promising results and resulted in FDA approvals in blood cancers [192] [193] i.e., the Kymriah for acute lymphoblastic leukemia and Yescarta for large B cell lymphoma. In addition, it holds immense promise in next-generation therapies for Cancer [194].

1.5.4 CRISPR-Cas9

The explicitness of CRISPR-Cas9 technology in genome editing highlights its prospect to treat various conditions like Cancer and cardiovascular and neuronal disorders [195] [196] [197]. Furthermore, in the last two decades, scientists have contributed to the knowledge of CRISPR and the blossoming of CRISPR technologies, including milestone articles for programmable DNA editing in human mammalian cells [198] [199] [200].

CRISPR-Cas9 system can both activate and repress a target gene. Cas9 is an RNA-guided endonuclease that identifies and cleaves target DNAs, with a template strand pairing to the guide RNA and a trans-activating crRNA (tracrRNA). TracrRNA enables crRNA binding and processing, forming a single molecule known as the single guide RNA used in genome editing (sgRNA) [201]. crRNA, which holds a nucleotide sequence complementary to the target DNA, and tracrRNA, which serves as a Cas nuclease binding platform.

Genome editing with CRISPR-Cas9 can be employed with Tumor and immune cells for downstream, in-vitro, and in-vivo analysis[202]. Tumor cell lines genetic alterations using CRISPR, by either knock-in or knock-out of specific genes, provide a deeper mechanistic understanding of biology. CRISPR in T cells can be employed in various ways by producing CAR-T cells via knocking out dysfunctional genes and CAR-T Cell Functionality Augmentation via CRISPR/Cas. Moreover, immune T cell checkpoints have been genetically modified via CRISPR/Cas9 and subsequently utilized in adoptive T cell therapy [203]. Recent work has shown a highly efficient way to disrupt PD-1 using the CRISPR-Cas9 system for tumor-infiltrating lymphocyte-based adoptive T-cell therapy [204]. Additionally, endogenous T cell receptor (TCR) chains, TCRa and TCRb, were knocked-out in T cells to lower TCR mispairing and to improve the expression of a cancer-specific TCR transgene, i.e., NY-ESO-1 [205]. CRISPR-Cas9 immune-related adverse effects pose certain limitations. The delivery system, Cas9 protein, and guide RNA can all stimulate the host's immune system.

1.5.5 Tumor-associated macrophages

Macrophages depict one of the significant tumor-infiltrating immune cell types and are commonly classified into two functionally opposing subtypes, classical activated M1-like and alternatively activated M2-like macrophages. They can stem from different sources and play

distinct pro (M1) -or anti (M2)-tumoral roles, with each class having a distinctive transcriptional landscape established on the type, stage, and immune composition of the TME[206]. M1 and M2 macrophages have a heightened degree of plasticity and, therefore, can be transformed into each other upon TME adaptations or therapeutic interventions [207]. M2 Macrophages facilitate cancer cell proliferation and invasion, described as tumor-associated macrophages (TAMs). The association between TAMs and malignant tumors is maturing, and TAMs have evolved as an extremely promising target for designing new cancer therapies.

Meta-analyses revealed that increased infiltration of macrophages in solid tumors is associated with poor clinical outcomes. Moreover, the expression of macrophage growth factors and chemoattractants, i.e., CSF1 [208] and CCL2 [209], within the TME, is usually associated with poor prognosis, and inhibitors of these molecules are promising [210]. TAMs moderate suppression in the TME upon the expression of T-cell immune checkpoint ligands, which directly impede T-cell processes; moreover, they secrete chemokines such as CCL2, CCL3, CCL4, CCL5, and CCL20, which lead to the recruitment of T regulatory cells [211] [212].

B7 family checkpoints ligands, i.e., PDL-1 (B7-H1) and PD-L2 (B7-DC) were discovered to be expressed on TAMs, as well as other checkpoints ligands such as galectin-9 and V-domain Ig-containing suppressor of T cell activation (VISTA) [213] [214]. The PD-L1+ TAMs, triggered by tumor-derived IL-10, moderate CD8+T cell dysfunction based on the PD-1/PD-L1 axis[215]. Moreover, Tregs can enhance the immunosuppressive properties of TAMs by promoting the differentiation of monocytes into an immunosuppressive phenotype. Additionally, TAMs favor chemokine/cytokine-mediated recruitment of Tregs to the TME, using CCL20 to grow CCR6+Tregs in Colorectal Cancer [216] [217].

Hypoxia affected the upregulation of stromal cell-derived factor 1 alpha (SDF-1α), i.e., CXCL12, which contributed to the infiltration of the immunosuppressive M2 macrophages. In addition, the SDF-1α receptor (CXCR4) inhibition using AMD3100 (CXCR4 antagonist) reduced local immunosuppression and nurtured anti-PD-1 therapy in sorafenib-resistance Cancer[218].

2 AIMS

This research aims to investigate novel immunotherapies and biomarkers for the treatment of metastatic uveal melanoma. The specific aims of this thesis were as follows :

- To develop a novel immunotherapy combination for uveal melanoma, a translational and pre-clinical study (Paper I)
- Discover novel biomarkers and signatures correlating to clinical benefit for a Phase II immunotherapy clinical trial of metastatic uveal melanoma (Paper II)
- Investigate tumor-reactive T cells in uveal melanoma patient's metastases (Paper III)

3 METHODS

3.1 In-vitro models and research

3.1.1 Cell lines

In vitro systems such as cancer cell lines, i.e., "2D system," are extensively used in oncology research and drug discovery. However, their usefulness is primarily linked to their capacity to provide an endless source of biological material for experimental goals[219]. Unfortunately, establishing a unique cell line is a very intricate process that is still not well comprehended with the hit rate for its establishment, which is low and erratic. When taking the biopsy, an individual cancer cell line offers a snapshot of the Tumor. "HeLa," i.e., Henrietta Lacks, a young black woman impacted by cervical cancer, emanated from HeLa cells. HeLa cell line can be viewed as another landmark in biomedical sciences, unlocking new boundaries in cancer research. Beginning from their inception, the HeLa cells formed the first instance of a "human cancer in a test tube" [220].

Cancer cell lines are instrumental in functional drug inhibition or genetic manipulation studies. Each unique cell line is utilized to understand a new attribute of the cancer disease and to experiment with the effectiveness of anti-cancer drugs[221]. For example, individual genes can be knocked-in or out for a deep understanding of gene reliance. CRISPR-Cas9 system has revolutionized biology and could be employed in cancer cell lines to elucidate specific genes, thus increasing the ease of genome editing for studying complex mechanisms [222]. (PMID: 11252900)

Oncology drugs that demonstrate encouraging cytotoxicity in the 2D in-vitro system, i.e., cell lines advance to in-vivo, i.e., animal (mice) models of human cancers. Unfortunately, the most promising drugs have limited efficacy in real-world patients, resulting in a significant hold on the drug development process. One of the primary factors underlying this poor success is the inadequacy of the preclinical 2D cultures and animal models to recapitulate the human tumor microenvironment (TME). Therefore, with varying genetics, mouse cell lines B16F10 (BRAF negative melanoma) and human primary uveal melanoma 92-1, MP-41, and MEL-202 were tested for this work. Moreover, a human metastatic uveal melanoma cell line, i.e., UM22, was derived from a patient from Sahlgrenska Hospital and was also employed in this research. Experimentation of these cell lines and others were used in papers 1 and paper 3.

3.1.2 Tumor Spheroids

Tumor spheroids (TS) are typically formed by speeding patient cancer cells, PDX material, or cell lines derived cells on ultra-low attachment adhesive or agarose-coated culture plates, resulting in 3D systems[223]. Spheroids have an external proliferating and inner quiescent area, potentially with a necrotic middle at their center. Spheroids don't capture the vasculature and cellular heterogeneity in representations of mutations and clonal growth. However, molecular

fingerprints of 3D spheres closely correspond to those of original tumors compared with 2D cell cultures[224].

While cell lines (monolayer cell cultures) have been used for decades of cancer research, they have been condemned for being unreliable predictors of therapy response with animal studies (in vivo). The culture geometry of a fixed, flat substrate restricts the number of neighboring cells, resulting in less inter-cellular communication compared with cells in vivo. In addition, 2D culture requirements drive cells to extend, expanding the surface area directly exposed to the culture medium. Despite these constraints, benefits such as easy application, low cost, increased throughput, and control over microenvironmental elements have made 2D cultures the classic technique for assessing cellular reactions.
In this research work, tumor spheroids are developed from Patient-Derived Xenografts (PDX) tumors. Patient biopsies (metastasis) in fragments or single cell suspension were implanted in a mice model. Unfortunately, the formation of palpable human tumors in mice was only reported for very few, i.e., about 15% of all samples tested. Therefore, downstream investigations of these patient-based spheroids are essential, especially with sparse mUM materials. This has been demonstrated in paper 3 with further downstream studies.

3.1.3 Co-Culture systems

To study cancer immunotherapies, 2D and 3D cancer systems, i.e., cell lines and spheroids, will be examined together with immune cells[225]. The tumor immune microenvironment is crucial in building novel cancer therapies. Immune cells release chemokines and cytokines in patients' blood, which are potential biomarkers for predicting signatures of inflammation and survival in cancer clinical trials. Moreover, immune cells can be cultured in vitro, with the cytokines essential for their growth and survival. Concerning this work, a specific immune cell, i.e., Tumor-infiltrating lymphocytes, was studied. TILs were extracted from patient metastasis samples in a medium supplemented with high doses of IL-2 [226] [227]. Using a standard small-scale Rapid Expansion Protocol (REP), these TILs are expanded to larger quantities for further experiments. The REP includes stimulating the TILs with CD3 antibodies in the presence of high dose IL-2 and pooled PBMCs (inactivated through radiation), and after 14 days, TILs are typically 1000 fold more [228]. This TIL product is used for analyses in its original form or genetically modified using the CRISPR-Cas9 system.
Moreover, downstream investigations involve additional in-vitro or in-vivo analysis, i.e., mice studies. This helps understand the immune responses to various mUM cell lines and patient spheroids. A co-culture system is required to elucidate this biology, where cancer and immune cells (i.e., TILs) are jointly incubated, further leading to the regulation of specific surface proteins and-or cancer cell death, measured using downstream experiments[229].

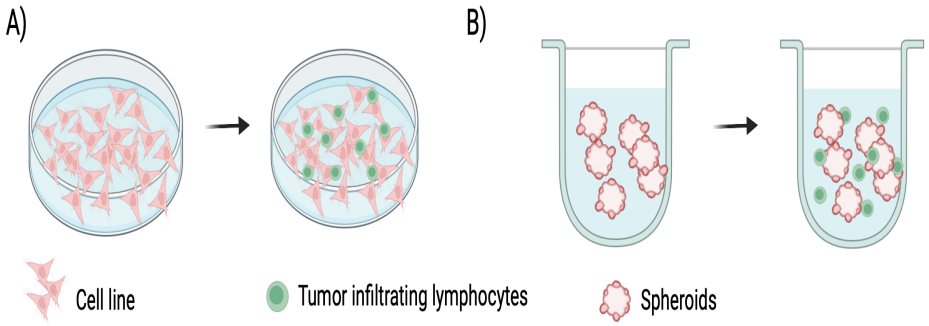


Figure 7. Depicting cancer cell line and spheroids coculture setup with autologous or allogenic TILs, with or without experimental drugs. Created using BioRender.

3.1.4 Multicolor Flow Cytometry and Sorting

Flow cytometry involves using an antibody matched with an antigen on the surface of the targeted cell. The cells are passed constantly through a laser beam, with each cell scattering some laser light, and cells labeled with the identifying antibody emit fluorescent light. Subsequently, cells are analyzed based on their fluorescence pattern and are studied individually or with several other proteins, leading to a multicolor system [230]. Moreover, FACS (fluorescent activated cell sorting) is defined as the usage of cytometry merged with sorting various cell types based on the protein of interest[231]. These techniques are employed in papers 1, 2, and 3.

3.1.5 CRISPR-Cas9 gene editing

Genetic editing is a powerful technique that involves the knockout of target genes from either cancer cells or the immune (ex. TILs) using CRISPR (clustered regularly interspaced short palindromic repeats) /CRISPR-associated protein (Cas9). Hence, this technique becomes an effective tool to dissect dependency on specific genes of interest within the cancer immunity landscape[232]. CRISPR/Cas9 is used here in this research by knocking out PDL1, i.e., the receptor of PD1, in the B16F10 cancer cell line, for paper 1. This led to in-vitro investigations and further in-vivo experiments by implanting both wild types and knockout cell lines in mice, followed by drug treatment. Moreover, CRISPR/Cas9 was employed to knockout genes in UM patient TILs and further analysis of their reliance for more effective cancer killing.

3.1.6 Inhibitors and drugs

Patients diagnosed with cancer will require some treatment with anti-cancer drugs. Most targeted cancer treatments are either small-molecule drugs or monoclonal antibodies[233]. This research involves using both treatments and studying the effects of mono or combination therapy. For both invitro and in-vivo investigations, cells and mice respectively were treated with epigenetic drugs, i.e., HDAC inhibitors (Entinostat) and BET inhibitors (JQ1), immunotherapeutic drugs, i.e., Pembrolizumab (PD-1) or in combination. Pertaining to in-

vivo mice studies, Entinostat was administered orally and anti PD-1 antibody was given intraperitoneally. This translational work was followed by PEMDAC, a phase 2 clinical trial combining Entinostat and Pembrolizumab in mUM patients. All patients in this trial (NCT02697630) were dosed orally with Entinostat and intravenously with Pembrolizumab.

3.1.7 Next generation sequencing

RNA sequencing is a critical tool; bulk RNA sequencing has been employed in cancer research, including a depiction of cancer heterogeneity and evolution, drug resistance, cancer immune microenvironment, cancer neoantigens, and biomarker discovery[234]. In addition, RNA sequencing has significantly advanced, evolving into a critical method for transcriptome profiling to recognize differentially expressed genes, demonstrating their role and molecular mechanisms. Moreover, it could assist in detecting premature mutations and also high molecular risk mutations. This NGS method was employed in Paper 1,2 and 3. T cell receptors (TCR) are present on T cells, comprised of TCRa and B chains, which help recognize MHC-antigen complexes, causing antigen-specific immune reactions to cancer. However, an individual TCR repertoire is highly diverse, thus requiring a high-throughput NGS method to determine TCR sequences, as TCR sequencing. TCR chains contain varying parts, essential for antigen recognition, and a constant region. The variable region of TCRα and δ chains is encoded by several variable (V) and joining (J) genes, while diversity (D) genes further encode TCRβ and γ chains. During VDJ recombination, one arbitrary allele of each gene component is recombined with the others[235]. Recombination of the variable area with a constant gene segment results in a functional TCR chain. In addition, a TCR sequence can be employed as a distinctive identifier of T cell clones.

However, a much more powerful single-cell RNA (sRNA) sequencing technique has transformed the landscape of translational biomedicine research [236]. TCR V(D)J sequencing combined with RNA sequencing permits analysis of paired TCRα and TCRβ chains at the single-cell level, with high throughput. scRNA seq enables characterizing global gene expression in the same cell, identifying T cell clonal expansion in a stable and a disease condition[237]. The corresponding analysis of transcriptome changes and adaptations of the same clone, with or without treatments, is a crucial application of this technique. Moreover, the exact clones could be investigated at the in-vitro, in-vivo, and patient levels, dissecting pathways within the immune tumor microenvironment.
The evolution of a dominant malignant clone, as well as of less abundant clones, during the disease or metastasis, provides the framework of the neoplastic and reactive clones[238]. In addition, several cancer-specific or immune-related pathways could be upregulated, controlling tumor cell growth, expansion, survival, and migration by individual malignant clones[239]. TCR sequencing and sc-RNA sequencing are utilized in Paper 3.

3.1.8 Immunohistochemistry

Disease diagnosis using histopathology is the 'gold standard in most diseases, particularly cancers. However, tissue analysis by a pathologist is the only conclusive method for confirming the presence of cancer and grading or staging the cancerous lesion[240]. FFPE (Formalin fixed paraffin embedded) tumor sections are incubated with an antibody that will bind to the antigen of interest, followed by a secondary antibody, which is visualized using a fluorescent or light microscope. FFPE tissue materials from both in-vivo studies and patient biopsies are evaluated in this research and utilized in papers 2 and 3.

3.1.9 Multiplex Tissue Imaging

The present state of art technologies, such as scRNA-seq (single-cell RNA sequencing), lacks spatially resolved data in describing cellular networks and cell-cell communications within the tumor microenvironment [108] [241]. Therefore, single-cell pathology and spatial cellular network analysis [112] [242] [243] is at the forefront of current technologies in medicine and health. Multiplex techniques like Imaging mass cytometry – IMC and Co-Detection by Indexing - CODEX co-stain a single tissue section with 30 or more markers. IMC uses metal-tagged antibodies [112], and CODEX [243] uses DNA-tagged fluorochromes, resulting in high-quality data. These techniques help us communicate patterns within individual phenotypes and their possible interactions at a single-cell level. At present, histology stratification is critical for clinical decision-making. However, it doesn't reflect multiple biomarkers' spatial architecture and network. Here CODEX multiplex technique is employed on a TMA (tissue microarray) comprising various mUM biopsies for paper 3.

3.2 In-vivo models and research

3.2.1 Syngeneic mouse models

Mouse syngeneic tumor models are widely used to study the mechanisms and activity of novel cancer immunotherapies. Syngeneic models are allografts perpetuated from mouse cancer cell lines using an inbred immunocompetent mouse strain, i.e., Balb/c, C57Bl/6. The matching host and cell line strain imply that tumor rejection doesn't occur, creating an immunocompetent prototype for immunotherapy examination[244]. An element that defines syngeneic, possessing fully qualified immunity, is a valuable tool but also a disadvantage, with all the cells, i.e., Tumor, immune, and stroma of a mouse. This corresponds to difficulty analyzing and forecasting how a mouse immune response translates back to patient immunity. Therefore, syngeneic mouse models were employed in Paper 1 of this thesis.

3.2.2 PDX models

Patient-derived xenograft models are a system for establishing a human tumor in immunocompromised mice, i.e., lacking a functional immune system. Nude mice lack thymus with no T cells and alterations in B cells. NOD/SCID mice carry deficiencies in immune cells, with no functional T and B lymphocytes and reduced macrophages and natural killer cells. In addition, NOD/SCID mice with a targeted mutation in the interleukin-2 (IL-2) receptor gamma chain (Il2rg) gene, resulting in a complete absence of NK, T, and B lymphocytes. These are also called NOG/NSG mice and have the highest take rate of human cancers. However, we require advanced humanized mice models, where immunodeficient mice engrafted with both human immune and cancer cells offer the tools to investigate various cancer immunity mechanisms [245] [246]. PDXv2.0 was developed using extended humanization of the NOG mouse with tumor-infiltrating T lymphocytes and human IL2 [247]. The key to T-cell survival and impact in this model is the continued existence of interleukin-2 (IL-2). We have reported that melanoma using PDXv2.0 respond to its autologous TILs, from the same patient who responded to ACT in the clinic [228]. Moreover, surgical techniques such as hemi-splenectomy, splenectomy, and intra-splenic injections were tested to build a liver metastasis UM model successfully. Both NOG and NOG-IL2, with subcutaneous and liver metastasis UM models, were conceived and shown together with TILs therapy in paper 3.

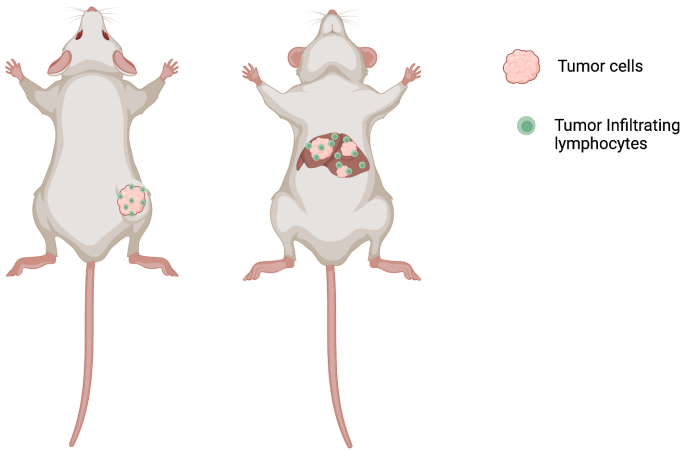


Figure 8. From left to right, depicting subcutaneous and liver metastases model of UM based PDXv2, involving both patient tumor and tumor infiltrating lymphocytes (TILs). Created using BioRender.

3.3 Patient samples

Patient samples are the epicenter of highly translational cancer research, playing a crucial role in all research efforts. Biobanking and managing patient samples from clinical trials and studies provide a vital framework for unraveling mechanisms of biological processes[248]. It gives deeper insights and leads to better-targeted therapies for patients in the future. These investigations involved extensive use of patient materials and data and involved a Phase-II clinical trial, PEMDAC (NCT02697630). A rare disease, i.e., uveal melanoma, poses a challenge in steering clinical trials due to the limited number of available patients. This work represents a highly translational creation that involved biobanking, managing, and performing various downstream experimental analyses on clinical samples. It included PBMCs (White blood cells), Blood Plasma, tumor fragments, and biopsies for IHC and sequencing. All papers involved in this research work had a considerable amount of patient samples, thus having direct relevance to human disease and health.

3.4 Ethical aspects

Ethical considerations in research are a collection of principles that drive the research design and practices and should not be an afterthought or side directive to the research study. The vital ethical considerations include validity, voluntary participation, informed consent, sampling, confidentiality, risk of harm, and research methods. Informed consent is a method designed to enlighten probable human subjects about the nature of a research assignment, including its processes, duration, benefits, and probable risks. Clinical data, such as the individual's diagnosis, drug regimen, outcomes, age, race, and gender, are also collected to enhance the usefulness of the biospecimens. The patients had informed consent about blood sampling and biopsy resection, under the guidance of trained nurses, surgeons, pathologists and oncologists. The studies involving human material, were conducted in accordance with Good Clinical Practice guidelines, and the ethical provisions of the Declaration of Helsinki. To sum up, all experiments performed for this translational research, were compliant to ethical approvals for animal studies and patient sampling.

3.5 Statistics

Implementing statistical methods is imperative for governing scientific and medical research. These methodologies are used for data analysis and outlining valid conclusions from research-based studies. High quality statistics, assists reviewers, scientists, clinicians and readers to assess the integrity and credibility of the research, in addition to the quality. In this thesis, and in all the papers, we have performed high-level statistical analysis, more so with multiple testing corrections, providing clarity and accuracy for results obtained.

4 RESULTS AND DISCUSSION

4.1 Paper I

The tumor growth has been related to epigenetic changes, and HDAC (Histone deacetylases) inhibitors are being tested as single-agent or in combination therapies in various cancers [149] [249] [137]. However, the recent developments in immunotherapy have led to various promising combination-based studies with epigenetic drugs [250] [251] [252]. This emergence has been due to the FDA approvals of T cell checkpoints in cancer, i.e., pembrolizumab (PD1) and Ipilimumab (CTLA4) [253] [254].

Panobinostat (Pan HDACi) has previously been reported to enhance PD-L1 and PD-L2 gene expression in melanoma cells [151] [255], and a combination with anti-PD1 has shown to be significantly effective [151]. Furthermore, Vorinostat (Pan HDACi) can enhance antigen presentation by induction of HLA molecules on glioma cancer cells [256], and Valproic acid (Class I HDACi) upregulates HLA signatures on the cervical cancer cells [257]. Additionally, various studies have been reported combining immunotherapy with HDACi in solid cancers [151, 258] [259] [260]. In uveal melanoma, Quisinostat (pan HDACi) has been shown to elevate HLA expression in tumor cells [261]; however, more studies are mandated.

This paper shows significant upregulation of PDL1, HLA-A, B, C (Class I) and HLA-DR, DP, and DQ (Class II) in multiple UM cell lines at both the protein and RNA levels, after treatment with Entinostat (Class I HDACi). These changes were observed in human primary (three BAP1 WT), human metastatic (one BAP1 mutant) uveal melanoma cell lines, and BRAF-negative mouse melanoma cell lines. Enhanced transcription of these genes by entinostat induced the tumor cells to enrich HLA presentation and suppress T cells via PD-1/PD-L1 signaling. However, this requires using immune cells together with tumor cells, both as invitro and in-vivo systems. Therefore, a co-culture assay was set up to analyze various cell death and T cell activation markers in a combination of the tumor, entinostat, TILs (Tumor-infiltrating lymphocytes), and pembrolizumab (anti PD1). yTILs (young TILs) extracted from a UM patient tumor were sorted for MART1+ tetramer and expanded substantially by rapid expansion protocol (REP). We observed a significant increase in tumor killing, i.e., Cleaved caspase-3 (CC3) levels, when combining Entinosat and HLA-A*02 TILS, more so with the addition of pembrolizumab. We also report that this CC3 induction in tumor cells relied on Granzyme B, implying enhanced deposition of granzyme B within the tumor cells. Three human uveal melanoma cell lines (92-1, MP-41, and UM22) were examined to verify the mechanism and its robustness. Since Entinostat improved TIL-based cancer death in the BAP1 mutant cell line UM22, it implies that the BAP1 status may pertain to a role in TME but not the tumor-intrinsic signaling. However, when we treated these cell lines with JQ1 (BET inhibitor), there were no effects or changes at the protein and RNA levels of PDL1 and HLA molecules. It did not match the transcriptional changes as compared to the entinostat regime.

To confirm our observation, we performed co-culture experiments with the MP41 cell line, and JQ1 did not show added benefit with TILS and pembrolizumab.

Moreover, we utilized a BET inhibitor, Molibresib (iBET-762), in an in-vivo setting using a syngeneic mouse immunocompetent model (C57BL6). This experiment combined immune checkpoint antibodies, i.e., CTLA-4 and PD-1, with Molibresib using a BRAF-negative B16F10-Luc mouse melanoma. The results suggested that BET inhibition does not improve immunotherapy in-vivo, resulting in poorer survival than immunotherapy.

As noted above, we gathered substantial evidence around Entinostat and its possible combination with anti-PD1; we tested our hypothesis in experimental animal models of uveal melanoma. Unfortunately, there is a scarcity of mouse uveal melanoma cell lines which could be utilized in an immunocompetent mouse, i.e., consisting of an entire immune system similar to humans. This limitation leads us to use the most relevant and closely associated UM model, i.e., BRAF-negative B16F10 melanoma cell line, which could help us build and study new cancer immunotherapies. Our lab previously developed a PDX v2 (PMID: 28955032), studying human tumors and TILS, in addition to checkpoint blockades in mice. This model loses the depiction of other immune system components but crucially assists in providing a deeper understanding of the 'human-specific' disease mechanisms.

Uveal melanoma immune tumor environment has shown to have essential roles in the disease biology and treatment modalities [92] [93, 262] [263]. Furthermore, it has been reported that the identification and role of T cell checkpoints in uveal melanoma could have clinical significance [61] [94] [264]. Here we report that Entinostat improved the anti-tumoral effects of anti-PD1 in-vivo, using the B16F10-Luc cell line in an immunocompetent mouse. This combination resulted in significant tumor reduction and improved survival compared to monotherapies. Additionally, HDAC inhibitors have previously been shown to impact the reprogramming of myeloid-derived suppressor cells (MDSCs), making solid cancers responsive to immune checkpoints [265] [266]. Entinostat led to a decrease of MDSCs (Myeloid-Derived Suppressor Cells) and improved effects of PD-1 Inhibition in mouse models of lung and renal cancer [267]. Moreover, an increase in CD8-specific genes with Nanostring analysis with Entinostat and PD1 combo has been reported in a PDAC (pancreatic ductal cancer) liver met model [265].

Entinostat combination with pembrolizumab mechanistic understanding within UM TME is still poorly understood. We report a significant intra-tumoral CD8+ T cells increase but no CD4+ changes. Furthermore, a decrease in monocytic MDSCs and tumor-infiltrating myeloid cells was observed. In addition, there was a gain in the macrophage phenotype, i.e., a pro-tumor cell type, "M2-like" tumor-associated macrophages (TAMs), showing a possible targeting pathway, which may have added benefit towards the combination. However, while not reaching complete regressions in our study, a possible hypothesis could be due to an exhaustive CD8 signature which requires an additional T cell checkpoint blockade. To gain further understanding, we created a knockout of PD-L1 (CD274) in the B16-F10- cell line using CRISPR/Cas9. These cells were implanted in C57BL6 mice and, when treated with entinostat, resulted in a slower growth rate than the control arm. PD-L1 is the ligand for PD-1, and deleting

that gene equips us to block this axis and study treatment effects with Entinostat, both in invitro, and invivo systems. This additionally assists us in recapitulating the <1% tumor PDL1+ tumor environment in syngeneic models, as witnessed with patient samples.

The preclinical and translational data encouraged us to initiate a clinical trial PEMDAC (NCT02697630) with this combination in patients with metastatic UM. Collectively, using in-vitro and in-vivo models, we decipher the biology behind the combination of Entinostat and anti-PD1 in this rare cancer, providing new insights and findings.

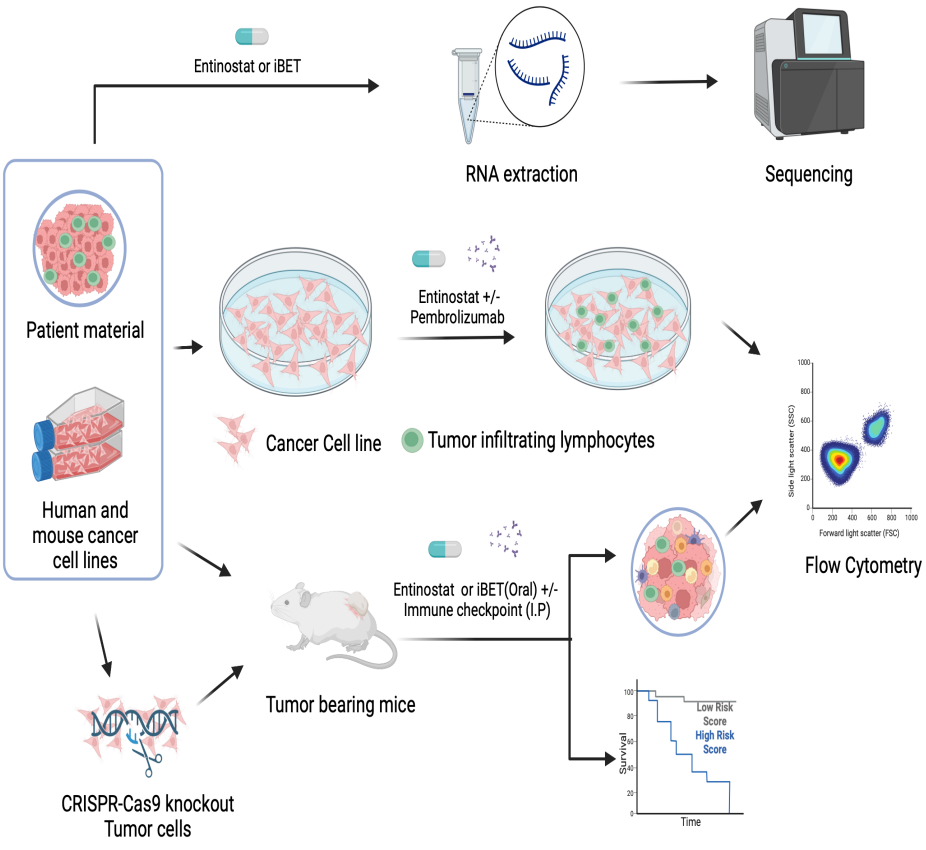


Figure 9. Workflow schematic for Paper I. Created using BioRender.

Deciphering the combination of HDAC inhibitor and anti-PD1checkpoint blockade in uveal melanoma

4.2 Paper II

PEMDAC phase II trial combining Entinostat (HDACi) and Pembrolizumab (PD-1) trial has been reported previously and tests this novel combination in a rare disease, metastatic uveal melanoma [128]. In addition, we have previously shown in Paper I the translational understanding of this combination using in-vitro and in-vivo tools [150]. In paper II, we demonstrate the patients two-year follow-up clinical data and identify novel biomarkers of responses or survival associated with the trial (NCT02697630) [128]. This research paper reports the follow-up median progression-free survival (PFS) of 2.1 months and overall survival (OS) of 13.7 months. About 25% (7 out of 28) patients were still alive at the database lock timepoint (31st January 2022). Among those, three are currently receiving chemotherapy, two receiving checkpoint blockades, and two are not receiving any therapy. In all 29 patients, adverse events were reported, overall grade 3-4 toxicity in about 60% of patients and immune-related grade 3-4 toxicity in about 35% of patients. Furthermore, there were no differences in health related quality-of-life measurements between pre-treatment and last assessed timepoint.

In recent years, T-cell checkpoint combinations have been tested for the treatment of metastatic uveal melanoma. For example, a single-arm, phase II trial of Ipilimumab plus Nivolumab reported a median OS was 12.7 months and PFS of 3.0 months [268], and another phase-II demonstrated ipilimumab and nivolumab combo with median OS of 19.1 months and a PFS of 5.5 months [269]. This latter study showed one confirmed complete response and five confirmed partial responses, with an ORR of 18% [269]. Additionally, the FDA approved Tebentafusp, a bispecific gp100 peptide-directed CD3 T cell engager, for HLA-A*02:01–positive metastatic uveal melanoma. However, the landmark approval is a designation applicable to only patients with a specific genotype, more clear effects on overall survival, not treatment-specific progression-free survival [130]. These necessary clinical trials in metastatic uveal melanoma [136] have been impactful, but still, there is a lack of extensive biomarker discoveries. A recent study in Nature Medicine, shows ctDNA as a potential biomarker for the Tebentafusp clinical trial however, does not provide any additional biomarkers [270]. Also, compared to other cancers, there is less understanding for predicting clinical outcomes and patient responses in this rare unmet disease [268 271] . Therefore, we perform a comprehensive analysis for finding novel tumor and immune-based biomarkers using plasma, PBMC, and metastases biopsies. All patient samples from this PEMDAC multicenter clinical trial were collected, documented, biobank created, and used for downstream analysis.

Thymidine kinase 1 (TK1) is a critical enzyme in DNA precursor synthesis, upregulated during the S phase of the cell cycle, and is an indicator of cell proliferation. The levels of TK1 in the plasma of patients are associated with tumor burden and are exhibited in multiple cancers [272 273 274]. In addition, this biomarker has been previously reported in studies on metastatic cutaneous melanoma [275 276]. However, there are no studies on metastatic uveal melanoma, more so on immunotherapy-based investigations. Lower TK1 activity at the pre-treatment stage

indicated survival benefit, with significant progression-free survival (PFS) and overall survival (OS). Furthermore, the levels were higher in short-term survival patients compared to long-term survival patients (n=7). In addition, we found that TK1 groups differed during individual patient disease, similar to those observed with ctDNA in the PEMDAC study [128]. Additionally, our findings showed a positive correlation between pre-treatment TK1 and ctDNA values, whose importance has been re-instated in recently in a UM trial corresponding to FDA approval [270].

It's imperative to study additional biomarkers, especially immune-related ones. This will collectively assist scientists and clinicians in better stratification of the patient population in immunotherapy-focused trials. Previously it has been reported, at the pre-treatment stage, that activated T cells correlated with clinical benefit [277] and further revealed an increase in T cells and monocytes following treatment with HDAC inhibition and pembrolizumab in Lung cancer [278]. However, no studies are focused on uveal melanoma immune signature analysis, with more investigations warranted. We conducted a highly multiplex ELISA on pre-treatment and on-treatment plasma samples, also utilizing Flow cytometry, RNA sequencing, and Immunohistochemistry based tools. We report the discovery of the chemokine CCL21 as a potential biomarker for predicting survival in metastatic uveal melanoma, showcasing its receptor CCR7 (Naive T cells) as equally vital. CCL21, in serum, was significantly elevated in patients with responders compared to progressive disease patients. CCL21 has been established as a crucial mediator of T cell chemotaxis and immune cell trafficking through high endothelial venules (HEV's) [279] [280] [281]. Recently, in a phase I Trial, autologous dendritic cells-CCL21 (Ad-CCL21-DC) in Lung Cancer orchestrated CD8 infiltration and tumor-specific immune reaction[282]. CCL21 receptor CCR7 was assessed on PBMCs, using Flow cytometry, and a CD3+CD45RA+CCR7+ population was observed to be higher for patients with more prolonged survival, also implying a positive clinical benefit. Next, we further dissected the role of these chemokines, using tumor biopsies, of patient metastases. We discovered formations, i.e., tertiary lymphoid structures (TLS) within the metastatic tumor microenvironment (TME). As previously reported in scientific literature, the composition of a TLS includes cells segregated into T- and B-cell zones within cancer biopsies [95]. They possess B-cell follicles and germinal centers surrounded by a T-cell region, share structural and functional characteristics with traditional secondary lymphoid organs. [97] [102] [283]. In addition, mature dendritic cells (DCs) are also present within the T-cell zone with high endothelial venules (HEVs), permitting the infiltration of immune cells from the blood, such as DCs, T, and B cells. However, in a recent study, they are classified into various stages, from immature early TLS (eTLS), primary follicle-like TLS (pTLS), and secondary follicle-like TLS (sTLS) [284]. TLS entities have been reported in uveal melanoma in one research article; however, in an unobtrusive manner and was not correlative with survival [93]. Our study shows the importance of TLS entities and links to survival benefits in an immunotherapy-based metastatic uveal melanoma trial. In this paper, we describe these entities as 'TLS-like' due to the presence of a wide range of states, from early to mature forms. Their detection was achieved by Immunohistochemistry (IHC) in FFPE tissues, a robust and comprehensive method for studying these entities and related histopathology. We report the identification of TLS-like entities in liver metastasis biopsies, with a varied expression of B and T cells and an expression of CD68+, PDL1+, and SOX10-

in certain available biopsies. RNA sequencing from TLS-positive cancer tissue reaffirmed our understanding of the specific gene signatures for verifying their presence and associated signaling [98] [285]. When divided into TLS-like and no-TLS patient groups, we see a highly significant increase of previously published TLS-associated genes (i.e., CD79a, CCL19, CCL21) [98] [284].

We altogether demonstrate the clinical benefit with high CCL21 and its receptor CCR7 in blood but also in TLS-like locations in liver metastasis tissue, reaffirming the function of these proteins in formation of these entities. We report the interconnected cellular mechanism and signaling of these molecules, and with this biomarker discovery, we, for the first time, show the correlation of clinical benefit with TLS, more so the CCL21 axis, both suggesting crucial roles in metastatic uveal melanoma. These findings, combined with TK1, provide the framework for future studies with these biomarkers, potentially leading to their use in clinical practice and assisting in patient stratification.

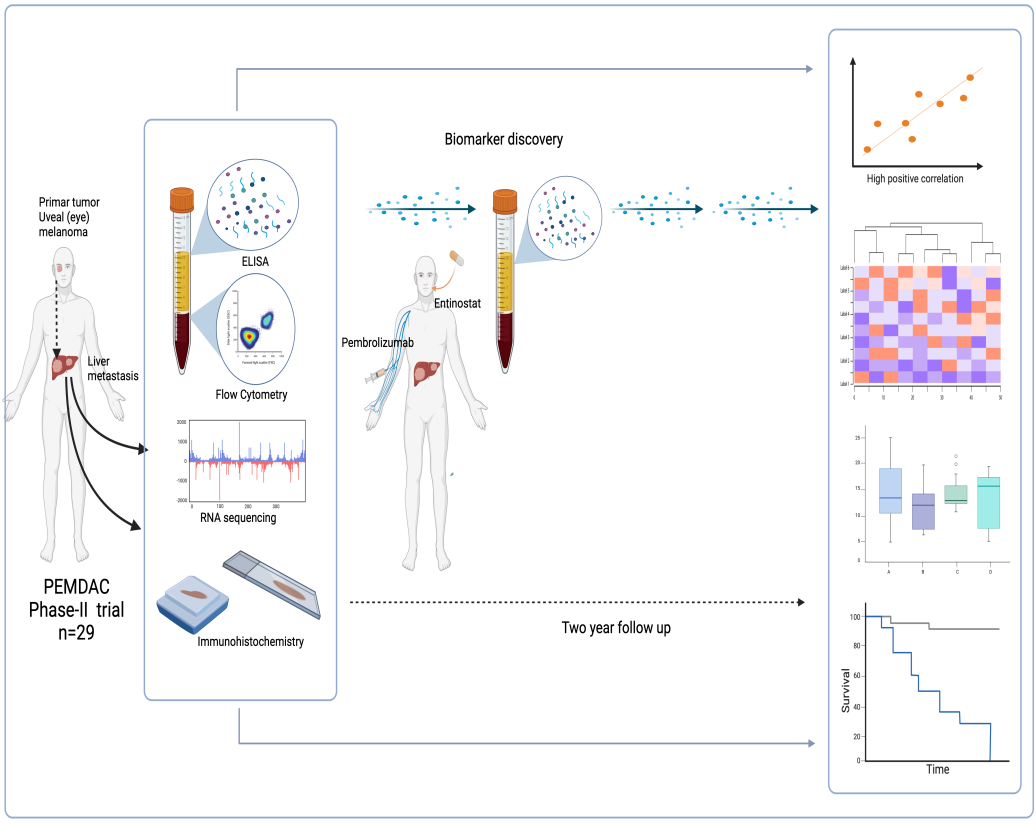


Figure 10. Schematic for Paper II. Created using BioRender.

Elucidating novel biomarkers for metastatic uveal melanoma

4.3 Paper III

Personalized medicine is an evolving field, and its effect on cancer patients has vast potential [286] [287]. Adoptive T cell and CAR-T therapies are studied extensively in both blood and solid cancers [288] [289] [290]. With heterogeneous antigens in solid cancers, it has impeded T-cell therapy efficacy and thus is challenging to study compared to Hematologic malignancies. These cell therapies are not studied extensively both pre-clinically or clinically in UM. One clinical study reported in Lancet Oncology using T- adoptive cell therapy (ACT) for the first time in metastatic UM showed encouraging results [135]. Additionally, we have shown that HER2 CAR-T cells can eliminate UM in patient xenografts mice models [291]. Nevertheless, the results are not yet highly encouraging for utilizing ACT, with UM, due to the scarcity of powerful in-vitro and in-vivo tools combined with only a few patient-derived xenografts (PDX). TILs have been shown to have crucial roles in the patient liver metastases UM microenvironment [61] [93] [94] [292]. However, there is a shortage of reactive TILs with the tumor due to the low mutational burden of this melanoma type compared to cutaneous[293].

For this paper, we gathered pre-treatment uveal melanoma metastases samples from patients in clinical trials of the SCANDIUM I & II, i.e., intra-hepatic perfusion [123] and PEMDAC, i.e., a combination of the PD-1 inhibitor, pembrolizumab, and the HDAC inhibitor, entinostat [128]. With the limited biopsies available, we utilized them for multiple purposes. One part for cryopreservation of finely minced tumor used for flow cytometry and single-cell RNA sequencing (scRNA seq), one for DNA/RNA preparation followed by sequencing, one for producing TILs, another one for developing PDX mice models, and lastly, one part for histopathology and multiplexing. Recent publications such as Durante et al. and Tosi et al. analyzed approx. 10-20 UM patient samples to understand their cancer-immune interactions. Likewise, with the rarity of this disease, there is a limitation to sample accessibility, especially compared to cutaneous melanoma. Here we report 26 UM patient samples, all metastases, either to the liver or extra-hepatic, and perform further downstream interrogation.

PDX models are efficient for studying cancer biology and, lately, cancer immunology (PMID: 32070860). Previously UM PDXs have been reported, mainly using the primary patient tumor (eye), scarcely using metastasis material [294] [295]. We demonstrate developing UM PDXs using metastases material in NOG mice. They indicated a lower take rate than cutaneous melanoma, previously reported [296] and additionally, exhibit lengthier timeframes in establishing tumors in mice[291]. We have further shown the development of a NOG-IL2 (PDXv2) mice model in assessing matched human tumors and TILs [228]. Likewise, using a subcutaneous implant, we showed UM1 was eradicated with their autologous TILs; although, UM22 wasn't. However, due to the slow-growing nature of these PDX models, it became challenging to conduct PDXv2 (NOG-IL2) Tumor-TILS experiments routinely. We thus developed a strategy to grow tumor spheroids from the PDX material and screen for TIL therapy. Tumors were harvested, and 3D spheres were successfully grown in 10/11 PDX models. Autologous or allogenic TILs were

cocultured with tumor spheroids, observing cancer killing and markers for T cell activation[297]. In addition, 3D imaging and quantification by incorporating tags in cancer cells and TILs provided evidence of a lesser number of tumor spheres after TILs treatment for some, but not others. This sphere data correlated with our in-vivo PDXv2 investigations for UM1 and UM22. Within the sphere coculture, using flow cytometry, we detected only a portion of these spheres-TILS cocultures resulted in T activation signatures (degranulation and 41BB). Therefore, we hypothesized that specific samples reflected T-activated phenotypes, potentially steering a reactive TILs population. Single-cell and TCR sequencing are powerful tools to elucidate molecular signatures of tumor-reactive T cells [298] [299].
These T cell proportions (CD3+41BB+) were sorted from the coculture and utilized for T cell receptor (TcR) sequencing. To examine if the TCRs could be discovered in the biopsies, we explored the single-cell RNA sequencing from patient biopsies and TILS. We could successfully map these TRLs (Tumor reactive lymphocytes) clones from our ex-vivo investigation to patient samples. The UM22 and UM46 MART1 distinct TCRs belonged to the exhausted and late-activated T cells; however, the UM1 and UM9 TCRs were mostly confined among exhausted cells. The mapping of TCRs among exhausted and late-activated T cells among all samples, was performed at a single cell level. In our work, some known TCRs were discovered, reactive to other antigens than MART1. Moreover, several TCRs were shared between two or more patient samples.

scRNA-seq is at the forefront of current methods to decipher cancer biology at the single-cell level [300]. However, new domains like single-cell pathology and spatial proteomics are emerging and impacting how we associate findings based primarily on DNA/RNA data [109] [301]. A tissue microarray (TMA) was created using twenty UM metastasis biopsies from the FFPE material. It consisted of the same patient samples for in-vitro and in-vivo investigation and further single-cell sequencing. In addition, highly multiplex staining and imaging technology, i.e., CODEX (Co-detection by indexing) [243] [302], was employed on the TMA, with a 30- plex staining which included 29 protein biomarkers, and nuclear stain DAPI. A publication recently showed 7-plex staining on a UM material [93]; hence, we would be the first to show a high multiplex of 30 plex of UM metastasis. These technical advances depict the landscape of UM metastasis TME to identify relevant protein expression. We discover exhausted and activated TIL phenotypes in all patient biopsies with varying expression. This shows the presence of these TRLs and provides links to the single-cell RNA data discussed before. Furthermore, we discovered in UM9 that the TRL proportions were higher intra-tumoral than in the stromal compartment. However, our in-vitro-based 3-D sphere imaging found that UM9 were resistant to their autologous TILs, uncovering similar clones in biopsies. It provides a platform to interrogate TRLs and their possible resistance to UM.

In certain cancers, not uveal melanoma, it has been shown that similar T cell clones were identified among in-vitro analysis and more so, mapped to original patient samples [298] [299] . We further interrogated these TRL clonotypes within an in-vivo liver metastasis PDX model using patient human and matched autologous TILs. A liver metastasis model was developed, and after testing various modalities, a validated procedure involving surgery followed by the splenic injection method was found [303] [304]. UM22 liver metastasis model was set up, and two

different TILs were intravenously injected, i.e., autologous and MART1 specific autologous. We report utilizing IHC and FACS to show the presence of tumor met's in the liver, interestingly showing significantly activated T cells proportions (CD69+ and 41BB+) in the MART1 group as compared to the non-specific. We hypothesize that MART1-specific clones were more tumor-reactive in liver met TME and could be mapped to TCR and single-cell data from the sphere and original patient analysis. Henceforth, the single-cell sequencing and analysis are underway and would provide an even more comprehensive platform.

The intricate interplay between cells of the tumor-immune ecosystem is necessary to dissect, for developing future precision medicine treatments. Although future work is needed in uveal melanoma, we provide a cross-functional approach with single-cell genomics and pathology that lets us identify meaningful reactive cell subpopulations.

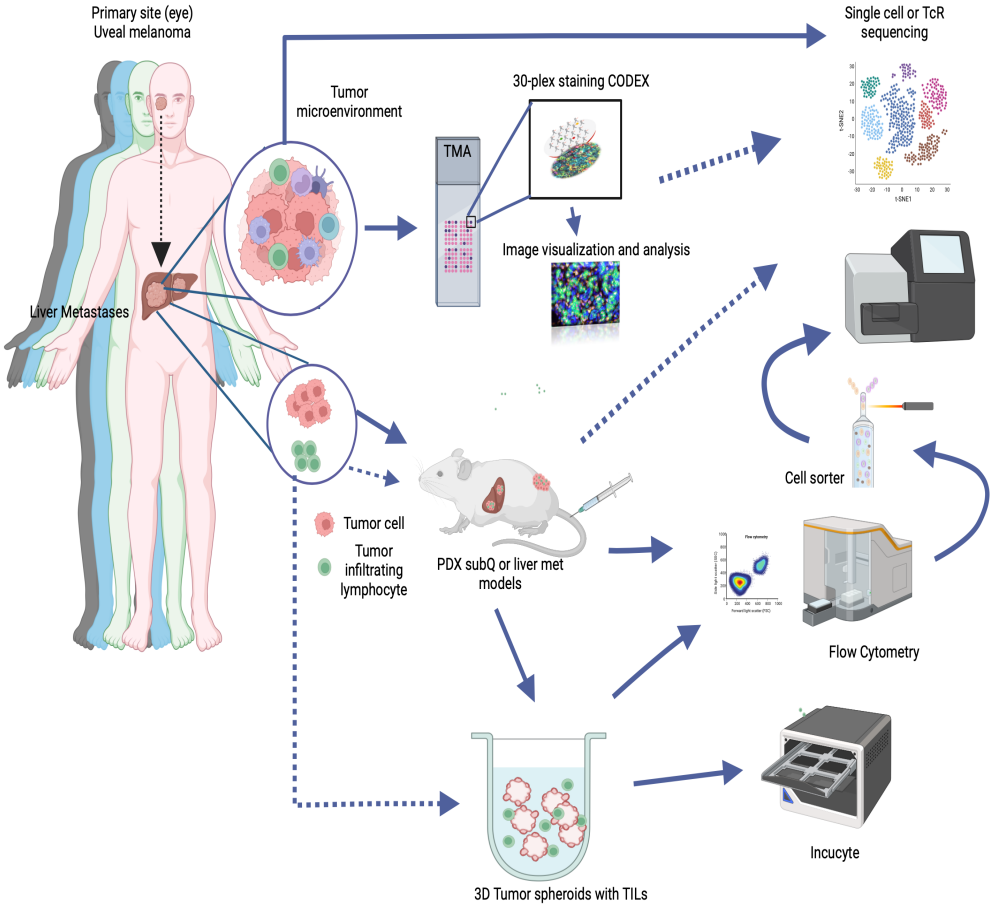


Figure 11. Workflow schematic for Paper III. Created using BioRender.

Investigating T reactive clones using a multi-faceted approach to build strategies for studying metastatic uveal melanoma

5 CONCLUSIONS AND FUTURE PERSPECTIVE

In paper I, we show a novel immunotherapy combination in uveal melanoma, a translational understanding of the PEMDAC (Pembrolizumab and HDACi) phase II clinical trial. Entinostat, a class I specific HDAC inhibitor, showed a significant upsurge in both RNA and protein levels of MHC Class I, MHC Class II, and PD-L1 of multiple human (primary and metastatic) and mouse cell lines. With in-vitro co-culture assays with entinostat, TILs and pembrolizumab (anti-PD1), cells resulted in elevated cancer killing and increased granzyme B, compared to monotherapies. However, BET inhibitor treatment didn't show any changes to HLA and PD-L1 molecules and didn't synergize in the in vitro co-culture setting. Moreover, adding BET inhibitor to immunotherapy reduced efficacy and survival in a mouse model. In the same in-vivo model, we show in a mouse model that a combination of entinostat and anti-PD1 resulted in higher efficacy and survival. This was due to increased CD8+ cytotoxic cells and decreased proportions of myeloid-derived suppressor cells. We also demonstrate CRISPR-Cas9 knockout of PD-L1 in tumor cells, resulting in slower tumor growth when treated with Entinostat than WT cells. Collectively this paper establishes Entinostat as a potent combination agent with anti-PD1, and not BET inhibitor, for the treatment of therapy-resistant melanoma or uveal melanoma.

Paper II has led to new biomarker discoveries with the clinical follow-up evaluation of the PEMDAC trial in metastatic uveal melanoma. This paper documents the follow-up data, with a median progression-free survival (PFS) of 2.1 months and overall survival (OS) of 13.7 months. Seven out of twenty-eight patients were still alive, offering a unique set of patient samples to perform further research investigation. First, we discover TK1 as a predictor of progression-free survival (PFS) and overall survival (OS), marking it as a potential serum-based biomarker with the trial. Furthermore, pre-treatment lower TK1 levels correlated to better survival and vice-versa. Likewise, TK1 was also related to patients circulating DNA (ctDNA) levels. Next, we reveal a chemokine, CCL21, is of considerable importance with links to clinical benefit with the PEMDAC trial. More so, we additionally report that its receptor CCR7 shows indications of being equally essential. This axis has a role in T and dendritic cell chemotaxis and migration. Subsequently, we document finding Tertiary lymphoid structures (TLS)-like entities within metastasis biopsies that positively correlated with overall survival. We also show gene signatures of CD79a, CCL19, CCL21, and CCR7 being significantly elevated in the TLS-like group compared to the no-TLS group. The novel biomarker discoveries in this paper, i.e., TK1 or CCL21 axis or TLS-like entities, exhibit potent data to classify them as future screening strategies in uveal melanoma.

Paper III provides a comprehensive framework of tools to understand and build new immunotherapies with metastatic UM. We report eleven PDX models with varying genetics from metastasis samples. This is remarkable, with the low accessibility of UM, rare cancer; a low take rate in mice; and an incredibly prolonged time in developing PDXs. With these slow-growing models, we report a high-throughput 3-D invitro tumor spheres system and

subsequently culture them with their autologous or allogenic TILs and experimental therapies. We identify similarly activated and reactive TILs (TRL) clonotypes within sphere in-vitro analysis and original patient biopsies. Furthermore, using CODEX, a highly multiplex imaging system, we stained UM met biopsies with a 30 plex marker panel. We confirmed the presence of the TRLs protein markers, as shown with single-cell sequencing, constructing an essential link between these approaches. Moreover, we created a disease-relevant in-vivo liver-met PDXv2 model and showed the reaction of human UM liver Mets with autologous TILS, interestingly again depicting a signature of activated T phenotype. These samples are being processed and analyzed with single-cell sequencing. Finally, we document various state-of-the-art techniques to define the immune cell arrangement, particularly the TRLs of uveal melanoma metastases. This has led us to identify novel immunotherapies and a multi-faceted platform to unravel TILs activity.

In this thesis, we study various immunotherapies to build novel treatment and biomarker strategies associated with liver metastasis, and associated clinical trials of metastatic uveal melanoma. This work would lay the foundation for future research studies in this rare cancer form with high unmet needs, potentially improving the lives of cancer patients and their caregivers.

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