# Novel immunotherapies for metastatic melanoma

## *– from mouse models towards clinical trials*

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Life is what happens while you pipet.
– Lisa Nilsson

# Abstract

Immunotherapies including checkpoint blockade and adoptive T cell transfer (ACT) show great promise for the treatment of melanoma, with long-term effects in some patients. However, around half of the patients with metastatic malignant melanoma will not be cured with available therapies today, and these patients require other treatment strategies. For metastatic uveal melanoma (a rare melanoma of the eye), available immunotherapies are less effective, and there is currently no approved therapy for these patients.

To be able to study immunotherapies in mice, we in Paper I developed an immune-humanized mouse model called patient-derived xenograft (PDX) version 2 (PDXv2). In this model, tumor cells and tumor infiltrating lymphocytes (TILs) from the same patient were grafted in IL-2 transgenic NOD/SCID IL2 receptor gamma knockout (NOG) mice, and we found that responses in the mouse model correlated to responses in the corresponding patients in a clinical trial of ACT.

So far, no chimeric antigen receptor T cell (CAR-T) therapy is approved for use in solid tumors. In Paper II we tested the potential for CAR-T therapy in melanoma. First, we used TCGA to determine the expression in melanoma biopsies of targets for commercially available CAR-T cells. We found that HER2 is expressed in both cutaneous and uveal melanoma biopsies. HER2 CAR-T cells were then used to treat skin melanoma and uveal melanoma patient-derived xenografts in the PDXv2 mouse model resulting in curative responses, even in models resistant to TIL therapy. However, CAR-T cells were only effective in IL-2 transgenic mice and not in regular NOG mice.

In order to facilitate translation of the findings from Paper II into a treatment strategy for patients with melanoma, we developed CAR-expressing autologous TILs (called CAR-TILs). In Paper III, we demonstrate that this strategy could overcome resistance to treatment with autologous TILs in melanoma. Current CAR-T therapies use blood-derived T cells as a substrate for CAR-T cell production. We hypothesized that by using TILs instead, we might facilitate homing to the tumor and potentially also utilize the fact that some TILs can recognize melanoma antigens, enabling a dual targeting of CAR-TILs. We also developed an automated

production protocol for TILs and CAR-TILs utilizing a bioreactor, enabling safe and less variable production of the drug product.

## Keywords

Metastatic melanoma, uveal melanoma, patient-derived xenograft, immune-humanized mouse model, immunotherapy, adoptive T cell therapy, tumor-infiltrating lymphocytes, chimeric antigen receptor T (CAR-T) cells

# Sammanfattning på svenska

Immunterapi är en grupp behandlingsmetoder som syftar till att aktivera patientens egna immunförsvar för att döda cancerceller. I hudmelanom har denna typ av terapier, framför allt antikroppsbaserade (så kallade checkpoint blockad) och cellbaserade metoder (adoptiv T-cellstransfer; ACT) visat sig kunna ge långvariga effekter hos många patienter. Dessvärre svarar inte alla patienter med hudmelanom på denna typ av behandling, varför nya behandlingsmetoder behöver utvecklas. Uvealt melanom uppkommer i ögat och vid spridd sjukdom är den mycket svårbehandlad. För denna patientgrupp är överlevnaden låg och dagens behandlingsmetoder fungerar ej.

I syfte att kunna studera immunterapi i möss utvecklade vi i delarbete I en ny typ av immun-humaniserad musmodell, som vi kallar patient-deriverade xenograft version 2 (PDXv2). I denna musmodell används immunkomprimerade möss som överuttrycker humant IL-2, vilket leder till att både humana cancerceller och humana immunceller (så kallade tumör-infiltrerande lymfocyter; TIL) från samma patient med kutant melanom kan transplanteras till mössen. Denna metod är en modell av ACT, och vi har visat att då tumör och TIL kommer från en patient som svarar på ACT i kliniken, så dödas även tumören i mössen. Då cellerna däremot kommer från en patient som inte svarade på ACT i kliniken så svarar heller inte mössen på behandlingen. Detta visar att PDXv2 kan användas för att studera immunterapi i melanom, och ger oss ett verktyg för forskning som syftar till att förbättra och förnya immunterapi mot melanom.

CAR-T är en typ av immunterapi som fungerar väldigt bra i patienter med blodcancer, men som än så länge inte är en godkänd behandling för patienter med solid cancer. I delarbete II studerade vi CAR-T behandling i PDXv2 och fann att CAR-T celler som känner igen HER2 effektivt dödade svårbehandlade melanom i mössen, inklusive både hudmelanom som inte svarade på ACT och uveala melanom.

I delarbete III utvecklade vi en ny behandlingsmetod som kombinerar ACT med TIL och CAR-T (så kallad CAR-TIL) och visade att denna metod kan övervinna resistens mot ACT med TIL i melanom. Detta tillvägagångssätt utnyttjar fördelar med både ACT och CAR-T, genom två olika sätt att döda cancerceller. Vi utvecklade också en automatiserad

metod för att producera CAR-TIL i en bioreaktor, för att göra tillverkningen säkrare och mer standardiserad.

# List of papers

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

I. Henrik Jespersen*, Mattias F Lindberg*, Marco Donia, Elin MV Söderberg, Rikke Andersen, Ulrich Keller, Lars Ny, Inge Marie Svane, Lisa M Nilsson and Jonas A Nilsson.
*Equal contribution.

Clinical responses to adoptive T-cell transfer can be modeled in an autologous immune-humanized mouse model.

*Nature communications, 2017,* 8 (1), 707[#]

II. Elin MV Forsberg*, Mattias F Lindberg*, Henrik Jespersen, Samuel Alsén, Roger Olofsson Bagge, Marco Donia, Inge Marie Svane, Ola Nilsson, Lars Ny, Lisa M Nilsson and Jonas A. Nilsson.
*Equal contribution.

HER2 CAR-T cells eradicate uveal melanoma and T cell therapy-resistant human melanoma in interleukin-2 (IL-2) transgenic NOD/SCID IL-2 receptor knockout mice.

*Cancer research, 2019,* 79 (5), 899-904

III. Elin MV Forsberg, Samuel Alsén, Larissa Rizzo, Henrik Jespersen, Roger Olofsson Bagge, Marco Donia, Inge Marie Svane, Anders Lindahl, Lars Ny, Lisa M Nilsson and Jonas A Nilsson.

Chimeric antigen receptor expressing tumor infiltrating lymphocytes can eradicate immunotherapy resistant melanoma.

*Manuscript*

# Content

# Abbreviations

| | |
|---|---|
| ACT | Adoptive T Cell Transfer |
| BAP1 | BRCA1 Associated Protein-1 |
| BRAF | V-Raf Murine Sarcoma Viral Oncogene Homolog B |
| B2M | β-2-Microglobulin |
| CAR | Chimeric Antigen Receptor |
| CM | Cutaneous Melanoma |
| CTLA4 | Cytotoxic T Lymphocyte Antigen-4 |
| DC | Dendritic Cell |
| ERK | Extracellular Signal-Regulated Kinase |
| GEMM | Genetically Engineered Mouse Model |
| GNA11 | Guanine Nucleotide-Binding Protein Subunit Alpha-11 |
| GNAQ | Guanine Nucleotide-Binding Protein G(q) Subunit Alpha |
| GPCR | G Protein Coupled Receptor |
| HLA | Human Leukocyte Antigen |
| IFN | Interferon |
| IL2 | Interleukin 2 |
| *In vitro* | Latin for ”in glass”, referring to research conducted in a test tube |
| *In vivo* | Latin for ”within the living”, referring to research of a living organism |
| MAPK | Mitogen-Activated Protein Kinase |
| MEK | Mitogen-Activated Protein Kinase Kinase |
| MHC | Major Histocompatibility Complex |
| MM | Malignant Melanoma |
| NK | Natural Killer cell |
| NOG | NOD/SCID IL2 receptor knockout |
| N-RAS | Rat Sarcoma N |
| PD1 | Programmed Cell Death Protein 1 |
| PDX | Patient Derived Xenograft |
| TAA | Tumor Associated Antigen |
| TCR | T Cell Receptor |
| TIL | Tumor Infiltrating Lymphocyte |
| UM | Uveal Melanoma |

# 1. Introduction

## 1.1 Cancer

Cancer is a group of diseases arising due to uncontrolled growth of genetically alterered, malignant cells. The development of cancer is a complex and step-wise line of events altering the regulation of normal cells in the body. Normal cells are tightly controlled when it comes to growth and survival. Cancer cells, however, have mutations in their genetic material (DNA) to achieve uncontrolled growth and deregulation of cell death. In order for cancer to develop, several capabilities are required of the cancer cell. These capabilities are commonly referred to as The Hallmarks of Cancer, and include: sustaining proliferative signaling, evading growth supressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis [1]. More recently, two additional hallmarks have been suggested: reprogramming of energy metabolism and evading immune destruction [2].

Cancer can develop in any tissue but with different likelihood. Most often, cancer develops in epithelial cells (called carcinoma). Epithelial cells are located in the skin and in tissues lining internal organs and body cavities. Cancer can alo develop in connective and supportive tissues (for example bone, muscle, fat and blood vessels; called sarcoma) or in blood cells of the immune system (called lymphoma and leukemia).

The development of cancer is mainly caused by genetic misfortune owing to the propensity of cell division and DNA replication to spontaneously go wrong. Since these events happen rarely, cancer is more common in older individuals. However, there are several things that increase the probability of cancer development, including genetic pre-dispositions and some environmental factors such as smoking and exposure to UV-radiation or chemicals. Even though our knowledge and management of cancer has improved over recent years, the total number of people affected is increasing [3], and we need to advance our therapeutic strategies further to battle cancer.

### 1.1.1. Cancer metastasis

Cancers have the ability to dessiminate from the primary site (the tissue from where the cancer originated) to distant sites of the body, a process called metastatic spread. This is in contrast to benign tumors that do not possess or have not yet developed this property. Metastasis is the leading cause of cancer mortality [4]. Cancer is often classified according to the spread of disease, from phase I/II (localized to the primary site) to phase III (lymphatic spread) and finally to stage IV (distant metastases) [5]. In general, the earlier the stage at which a cancer is discovered, the better the prognosis.

### 1.1.2. The immune system in cancer

The immune system seems to play a vital role in surveilling the body for alterations in cellular behavour, including malignant transformation of normal cells into cancer cells. Evidence for immunosurveillance include the fact that immunocompromised individuals (including people receiving organ transplants and HIV patients) have a slightly higher incidence of cancer compared to healthy controls [6-11]. Second, immune cells are found at the tumor-site and can recognize tumor-assiciated antigens (TAA) [12]. In many tumor-types, infiltration of immune cells (lymphocytes) in the tumor is correlated to good prognosis [13-16]. There is also evidence showing that mice lacking different components of the immune-system more frequently develop tumors compared to immune-competent mice [17-21].

Even though the immune system can recognize and eliminate cancer cells, there are ways for the cancer cell to evade immunity, which results in the formation of clinically evident tumors. The complex interactions between the immune system and developing tumors (called immunoediting) can be summed up by three processes: Elimination, Equilibrium and Escape [22]. The Elimination phase corresponds to immunosurveillence, including recognition and elimination of tumor cells by the immune system. During Equilibrium, tumor cells that managed to survive Eliminiation experience immune selection, favoring the development of clones resistant to immune attack. The last phase of immunoediting is called Escape, when tumor cells manage to escape control of the immune system, leading to tumor formation. Cancer cells have different strategies to escape the immune system. Some of these evasion mechanisms can be overcome by immunotherapy, a group of treatment strategies aiming to activate the patients own immune system to fight off cancer.

### *1.1.2.1 Organization of the immune system*

The immune system is comprised of many different cell types with the general purpose to defend our body against foreign and intrinsic threats such as pathogens and cancer cells. Broadly, the immune system can be divided into innate immunity and adaptive immunity. Innate immunity provides quick and unspecific defences. It is comprised by cellular and biochemical defences, present at all times in healthy individuals. Upon detection of a threat, the innate immune system can subsequently stimulate adaptive immunity [23]. The adaptive immune system consists of lymphocytes and their products, and can also use and enhance effector mechanisms of the innate immune system. Adaptive immunity is specific to the current threat, and has the ability to induce immunogenic memory.

### *1.1.2.2 Innate immunity*

Innate immunity consists of primitive defences including:

- Physical and chemical barriers (epithelial cells and antimicrobial substances produced by the same)
- Innate immune cells: granulocytes, phagocytes and NK cells
- The complement system (blood proteins)
- Cytokines (regulate and coordinate the activity of immune cells)

Innate immunity is highly conserved in animals and plants, and all multicellular organisms have innate immunity [24]. Innate immunity is an instant first-line of defence protecting the body against infectious agents, while the induction of adaptive immunity takes longer to develop and would give infectious agents too much time to replicate. Epithelial surfaces act as physical barriers protecting against pathogens, including the skin and mucosal tissues. If pathogens manage to break this barrier, innate immune cells in blood and tissues wait to defend the body against the intruder. Blood also contains proteins called the complement system, with the purpose to bind to conserved structures on pathogens. Complement proteins can induce pore-formation in bacterial membrane inducing cell lysis, as well as bind to pathogens and attract immune cells (phagocytes) [25].

Phagocytes are innate immune cells capable of ingesting (phagocytosing) bacteria, including neutrophils, mast cells, macrophages and dendritic cells. Phagocytes identify their target cells by recognition of pathogen-associated molecular patterns (PAMPs) or by coating by complement proteins or

antibodies. PAMPs are evolutionary conserved structures on pathogens including for example bacterial lipids and double-stranded RNA found in some viruses [26].

Some cells of the innate immune system contain intracellular granules containig proinflammatory molecules and effector molecules capable of killing pathogens [27]. These cells are called granulocytes and include neutrophils, mast cells, eosinophils and basophils.

NK cells can recognize virus-infected cells and kill them by secreting molecules and proteins capable of lysing the target cell. NK cell recognition is mediated by receptors recognizing MHC I (KIR), stress receptors MICA/B (NKG2D) and bound antibodies on the target cell [28]. NK cells have also been shown to play a role in the detection and control of cancer cells. Cancer cells can downregulate MHC I or the antigen presentation pathway, as one way to evade immune recognition. NK cells recognize a lack of MHC I on their cell surface, for example pathogens or cancer cells downregulating MHC I. This is called the "missing self" hypothesis [29].

### *1.1.2.3 Adaptive immunity*

Adaptive immunity consists of lymphocytes, and can be further divided into humoral immunity (B-lymphocytes and their antibody products), and cell-mediated immunity (T-lymphocytes). Adaptive immunity is specific to variable elements called antigens. The adaptive immune response is initiated when phagocytes engulf pathogens or abnormal cells, alternatively take up soluble antigens, and present these antigens on MHC II, representing a link between innate and adaptive immunity [30]. Cells with this ability are called antigen presenting cells (APCs) and include dendritic cells (DCs), B cells, macrophages and monocytes. Conventional DCs are the most efficient APC in terms of antigen uptake, presentation and activation of T cells [31]. APCs are specialized to present antigens to CD4+ T cells on MHC II, but can also present antigens on MHC I to CD8+ T cells (so called cross-presentation) [32].

T cells mature in the thymus, and can recognize antigens via their T cell receptor. T cells are generally categorized into T helper cells (CD4+) and cytotoxic T cells (CD8+). T helper cells produce cytokines and thereby "help" other cells of the immune system to perform their function, including cytotoxic T cells [33]. Another group of CD4+ T cells are regulatory T cells, able to suppress an immune-response and playing an important role in maintaining self-tolerance, preventing autoimmune disease [34]. Cytotoxic T cells induce lysis of altered cells upon activation, such as virus-infected cells or cancer cells [35].

The interactions between T cells and cancer cells will be more thoroughly discussed in the upcoming sections.

B cells develop in the bone marrow, and can be activated by recognition of its cognate antigen by the B cell receptor [36]. In response to activation, B cells mature into plasma cells capable of producing antibodies that can neutralize pathogens, inhibiting them from infecting human cells. Antibody binding to pathogens can also activate other componenents of the immune system by facilitating phagocytosis and cytotoxicity. Humoral immunity is efficient in eliminating free virus and bacteria, but cannot efficiently target intracellular threats, as in the case for virus-infected cells or cancer cells.

Both B and T lymphocytes are able to proliferate in response to antigen recognition, thus producing large clonal armys recognizing a specific threat. Importantly, activated lymphocytes can also develop into long-lived memory cells [37, 38]. This property results in immunological memory, enabling a quicker and more efficient immune response upon re-encounter of the same threat.

### *1.1.2.4 Immune recognition of tumor cells by T cells*

In general, cancer cell recognition by the immune system is based on antigen presentation on receptor complexes called MHC I. All cells in the body have MHC class I, consisting of one alpha chain (encoded by the HLA A/B/C genes in humans) and one β-2-microglobulin chain (encoded by the *B2M* gene). MHC I present cellular antigens on the cell surface which can be recognized by cytotoxic (CD8+) T cells via their T cell receptor (TCR) (*Figure 1 (b)*) [39]. Antigens are protein fragments (peptides) produced in the proteasome by breakdown of a fraction of all synthesized proteins in the cell. The antigens are subsequently transported to the endoplasmatic reticulum (ER) and loaded onto MHC I complexes. The loaded MHC I complexes are then transported to the cell surface via vesicles, where they display fragments of the proteins synthesized within the cell to the immune system [40]. Genetically altered cells can be detected by the immune system via presentation of mutated antigens on MHC I to cytotoxic T cells.

In order for a cytotoxic T cell to become activated, three different signals are required [41]. The first signal is mediated by MHC I and bound antigen recognized by the TCR. Additionally, co-stimulatory receptors CD80 and CD86 on the target cell bind CD28 on the T cell, mediating a second signal. Finally, Interleukin 2 (IL-2), a cytokine produced by activated helper (CD4+) T cells is required for subsequent clonal expansion of the activated T cell.

Once the primed cytotoxic T cell encounter an altered cell presenting it´s cognate antigen, it is activated to degranulate and release cytotoxic substances targeting the target cell, including perforins, granzymes and gamma-interferon (IFN-γ). Perforins create pores in the plasma membrane of target cells, allowing for passive diffusion of granzymes, which in turn cleave and activate pro-apoptotic caspases. Cytotoxic lymphocytes can also kill target cells by another mechanism, mediated by the Fas death receptor on tumor cells [42]. Fas ligand (FasL) on cytotoxic T cells can bind the Fas receptor, inducing apoptosis of the target cell.

Downregulation of the antigen presentation pathway by cancer cells disable T cell recognition, representing an important immune evasion strategy. In addition, inhibitory receptors on cancer cells can bind to and inactivate cytotoxic T cells. These and other mechanisms to evade immunity are presented in the next section.

### *1.1.2.5 Immune evasion*

In order for cancer to develop, cancer cells need to evade immune destruction, representing one of the hallmarks of cancer [2]. Many different mechanisms of immune evasion have been described, including hiding strategies, immune regulation and resistance to immune cell killing.

One obvious way for the cancer cell to evade detection by the immune system is to downregulate the antigen presentation machinery (hiding). Strategies to do so include downregulation of tumor-associated antigens, MHC I genes (*HLA-A/B/C* and *B2M*) or other proteins involved in the transportation of MHC I complexes to the cell surface (for example TAP1/2) [43, 44]. However, complete lack of MHC I expression can cause NK-cell mediated killing, and therefore tumor cells might selectively downregulate some but not all MHC I molecules to decrease expression of specific antigens [45].

Another strategy for tumor cells to evade immunity is to inhibit T cell function. This can be done in several ways. Checkpoints on T cells (including Cytotoxic T Lymphocyte Antigen-4; CTLA4 and Programmed Cell Death Protein 1; PD1) negatively regulate their activity. CTLA4 bind CD80/CD86 (also known as B7-1/B7-2), the same ligands that bind CD28 mediating the second signal required for T cell activation [46]. PD1 is another checkpoint on T cells that bind PDL1 on tumor cells, aslo resulting in inhibition of T cell function. Upregulation of PDL1 can be adopted by cancer cells as a way to evade T cell mediated killing [47].

Cancer cells also have additional strategies to regulate the immune system. They can for example attract regulatory immune cells to the tumor site, including regulatory T cells (Tregs) or myeloid-derived suppressor cells that in turn inhibit the activation of cytotoxic T cells. Treg inhibition can be achieved in several ways, including production of inhibitory cytokines or cytotoxic granzyme B, inhibition of DC function, checkpoint expression or by binding all available IL2 with high affinity receptors, depriving other T cells of necessary IL2 stimulation [48]. Tregs normally inhibit CD8+ T cells in tissue, and a lack of Tregs lead to development of autoimmune disease [34]. Cancer cells can also produce immune-inhibitory molecules such as IL-10 and TGF-β [49], and induce lymphocyte apoptosis by secreting soluble FasL [50].

Finally, cancer cells can become insensitive to T cell induced apoptosis, for example by inhibiting the caspase cascade or acquire resistance to FasL-mediated killing [51].

## 1.2 Immunotherapeutic strategies

Cancer immunotherapy is a group of therapeutic strategies aiming at activating the patient's own immune system to achieve tumor eradication. This can be done in several ways, and the main approaches are described below.

### 1.2.1. Immune system modulators

Immune system modulators are a group of treatment strategies that act by a general immune stimulation, in order to boost an already existing immune response against the tumor.

Interleukin 2 (IL2) is the T cell growth factor, required for activation and clonal expansion of T cells. High dose bolus IL2 have been approved for use in patients with renal cell carcinoma [52] as well as melanoma [53]. In a small fraction of patients, this treatment leads to tumor shrinkage and long-term survival. Toxicities included cardiopulmonary toxicities but was most often reversible [54].

Interferon α (IFN-α) is another molecule that can stimulate immune responses in some patients with renal cell carcinoma [55], but is also associated with low response rates and toxicities, limiting the usefulness of this treatment. In melanoma, IFN-α treatment did not improve the patient outcome [56]

Bacillus Calmette-Guérin (BCG) is a weakened form of Mycobacterium bovis virus, which can be used to successfully induce immune responses in patients with bladder cancer [57].

### 1.2.2. Cancer vaccines

Cancer vaccines can be either prophylactic or therapeutic. The first act in the same way as other vaccines, that is to produce an immune response against a weakened virus or viral antigen, and hence only work in virus-induced cancers. One example is human papilloma virus (HPV) vaccine which is successfully used to prevent the development of cervical cancer [58].

In the case for non-viral-induced cancer, other types of vaccination approaches are required, and these treatments are used in patients with already existing lesions. Great efforts have been made to develop therapeutic vaccines against cancer, largely with modest success. Early studies using free peptides suffered from lack of immunization, resulting in no or low therapeutic benefit [59].

In order to improve immunization in cancer vaccines, dendritic cell-based vaccines have been developed [60]. So far, the most promising results for this approach has been achieved in prostate cancer with sipuleucel-T, a vaccine comprised by autologous peripheral blood mononuclear cells cultured in vitro with a fusion protein composed of PAP (prostatic acid phosphatase; a prostate tumor associated antigen) and GM-SCF (Granulocyte-macrophage colony-stimulating factor, a cytokine). Sipuleucel-T caused a survival benefit of about 4 months in patients with metastatic, castration-resistant prostate cancer [61].

### 1.2.3. Oncolytic viruses

Oncolytic viruses work by infecting and causing lysis of tumor cells, while largely sparing normal cells. Many different strains of oncolytic viruses exists that are currently under pre-clinical and clinical investigation, often based on adenoviruses or herpes simplex viruses genetically altered to optimize safety and tumor cell tropism [62].

So far, this type of treatment has been approved for use in patients with head and neck cancer (called H101, based on adenovirus) [63] and melanoma (called T-VEC, based on herpes simplex virus) [64]. These viruses are administered via intra-tumoral injections, and can cause tumor shrinkage especially in the injected lesions but also in non-injected lesions [65]. Treatment with oncolytic viruses

are generally well tolerated, but the major challenge with this treatment strategy is to induce a systemic immune response required for effects on patient survival [66].

## 1.2.4. Antibody based immunotherapy

Antibody-based immunotherapies can be divided into two types; tumor targeting antibodies and checkpoint inhibitors.

### *1.2.4.1 Tumor targeting antibodies*

Monoclonal antibodies targeting cancer-associated proteins have been approved for treatment of several malignancies. They work by opsonizing (coating) cancer cells, leading to activation of an immune response. Several mechanisms for immune activation by monoclonal antibodies have been revealed, including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and the induction of adaptive immune responses [67]. ADCC typically involves NK cells, which recognize the constant (Fc) portion of an antibody via their Fc receptor (CD16) leading to activation and release of cytotoxic substances including perforin and granzymes. CDC depends on complement proteins binding to the antibodies and initiating a cascade leading to cellular lysis. Monoclonal antibodies can also activate the adaptive immune system, since peptides derived from lysed tumor cells (for example through ADCC or CDC) can be loaded onto MHC II and MHC I (via so called cross-presentation) on DCs, leading to T cell activation.

There are several tumor targeting antibodies approved for use in cancer patients, which increase survival in some patient groups. These include HER2 antibodies used for HER2 positive breast cancer [68, 69], CD20 antibodies used in B cell malignancies [70-72] and EGFR antibodies used in colorectal cancer [73, 74].

### *1.2.4.2 Checkpoint blockade*

Checkpoint inhibitors are antibodies that bind to and inhibit inhibitory molecules (so called checkpoints) on T cells, and hence enable the T cell to become activated and attack cancer cells. Several checkpoints have been discovered. Inhibitors against CTLA4 [75, 76] and PD1 [77, 78] can lead to durable cancer

regression and increased survival, and have been approved for use in patients with melanoma. PD1 inhibitors are also efficacious in other cancer types, including non-small cell lung cancer (NSCLC) and renal cell carcinoma [79, 80].

A number of other checkpoints have been described and novel inhibitors are being evaluated preclinically; including (but not limited to) LAG-3 [81], TIM-3 [82], TIGIT [83] and Siglec-15 [84].

### 1.2.5. Cell based immunotherapy

Cell based immunotherapy utilizes tumor reactive T cells, which can be expanded and activated *in vitro* before infusion at large numbers to the patient.

These tumour-specific T cells can be derived from the tumour environment, called tumour-infiltrating lymphocytes (TILs), or from peripheral blood. They can also be modified to express high affinity antitumour T cell receptors (TCR) or genetically engineered chimeric antigen receptors (CAR) with the aim to improve their anti-cancer activity.

#### *1.2.5.1 Adoptive T cell transfer*

Adoptive T cell transfer (ACT) utilizes the anti-cancer properties of T cells found in the tumor, so called tumor-infiltrating lymphocytes (TILs). By extracting TILs from human tumors, expanding them in vitro to large number in the presence of the T cell growth factor IL-2 and finally infusion of the cellular product back into the same patient, tumor-specific T cells can be reactivated and mount an attack on the tumor. ACT was the first cell-based therapy successfully used in the clinic, and this approach show great efficacy in about 50% of patients with cutaneous melanoma [85, 86].

Approaches have also been used to determine the tumor antigen specificity of transferred T cells. In melanoma, Melanoma antigen by T cells 1 (MART1) and GP100 specific T cells have been used for adoptive transfer [87, 88]. In patients with melanoma and synovial cell sarcoma, Cancer/testis antigen 1 (also known as NY-ESO-1) specific T cells have been used [89]. So far, these therapies are generally not more efficacious compared to ACT with a pool of TILs (with unknown TCR specificity) but instead correlated to more serious adverse events.

### *1.2.5.2 CAR-T*

Another approach to cause T cell mediated killing in cancer patients is to equip blood-derived T cells with a chimeric antigen receptor (CAR). A CAR-receptor is a genetically modified receptor consisting of a binding moiety and T cell activating components including the intracellular part of CD3 and co-stimulatory molecules [90]. The binding domain is a single-chain variable region from a monoclonal antibody with the potential to bind to a specific surface protein of the target cell (*Figure 1 (a)*). The signaling domain in the first generation of CAR receptors including the CD3z intracellular signaling domain [91]. Later, the functional properties of CARs have been further optimized by including co-stimulatory domains, most often either CD28 or 4-1BB (in second generation CARs) or a combination of both (in third generation CARs). A fourth generation of CARs have also been generated by adding IL-12 to the base of the second-generation constructs, known as T cell redirected for universal cytokine-mediated killing (TRUCKs) [92].

The most encouraging results for this treatment strategy are the successes achieved with the use of CD19 CAR-T cells in adult and pediatric patients with B cell malignancies [93, 94]. A majority of patients with B cell lymphoma and leukemia respond to CD19 CAR-T treatment, often resulting in durable remissions, leading to approval of these treatments. However, adverse events in CAR-T treatments are frequent, and include cytokine release syndrome (CRS) and neurological toxicities. These adverse events can be severe and lead to hospitalization. Most often they are managed with immunosuppressive treatment, but can sometimes be fatal.

A few CAR-T cell therapies have also been used in patients with other malignancies, including solid cancers. CEA CAR-T therapy has been tested in patients with gastrointestinal cancers [95, 96], MUC16 CAR-T therapy in ovarian cancer [97], MUC1 CAR-T therapy for seminal vesicle cancer [98] and GD2 CAR-T therapy in patients with neuroblastoma [99]. HER2 CAR-T therapy has been evaluated in patients with HER2 positive sarcoma [100], glioblastoma [101] and biliary tract cancer and pancreatic cancer [102]. Approaches to treat solid tumors with CAR-T therapy have so far been much less impressive compared to CD19 CAR-T treatment for B cell malignancies. Important challenges need to be overcome in order to be able to use CAR-T therapy successfully in patients with solid tumors. These include finding appropriate CAR targets, achieving CAR-T cell tumor penetration and sustaining persistence and cytotoxicity in the hostile tumor microenvironment [103].

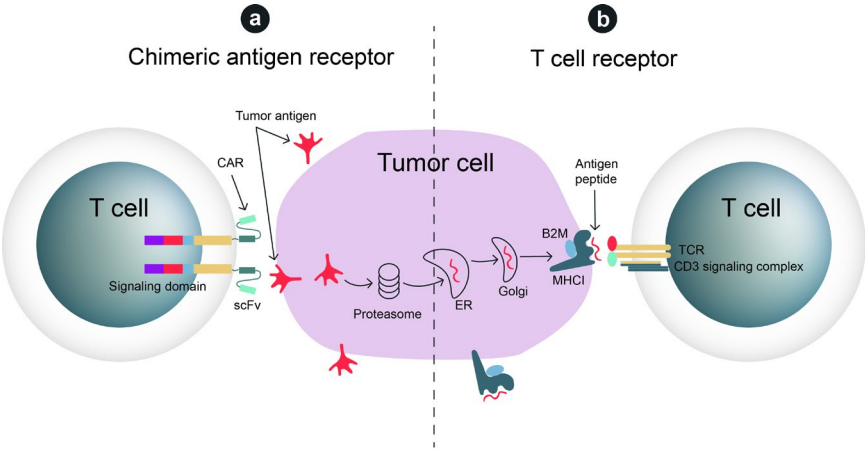


***Figure 1.*** *Tumor cell recognition by chimeric antigen receptor (CAR) T cells (a) and T cell receptor (TCR) mediated T cells recognition (b). The CAR is comprised by a single chain antibody domain (scFv) recognizing native proteins on the tumor cell surface, linked by a hinge and transmembrane domains to several intracellular signaling domains. The TCR instead recognizes antigen peptides, processed by the proteasome, loaded onto MHC I protein complexes, and transported to the tumor cell surface. Adapted and modified from [104].*

# 1.3 Cutaneous (skin) melanoma

Melanoma (also called malignant melanoma) is a cancer arising from melanocytes. Melanocytes are melanin-producing cells found mainly in the skin, but also in other tissues such as the uvea of the eye, in mucosal tissues and in the leptomeninges. Melanoma arising in the skin is called cutaneous melanoma. Cutaneous melanoma is the deadliest form of skin cancer.

## 1.3.1 Normal melanocytes

Melanocytes are derived from the neural crest, and migrate to the skin and other sites of the body during embryonic development [105]. Melanocytes in the skin have the function to produce melanin in order to protect underlying (subcutaneous) cells from UV-damage from sun-exposure. UV-radiation cause melanocytes to produce melanin [106], a process called melanogenesis.

### 1.3.2 Epidemiology

Melanoma affects mainly the Caucasian population, and the incidence is increasing worldwide [3]. Also in Sweden, the incidence has increased over the last decades, and affects about 4000 people yearly [107].

### 1.3.3 Etiology

The most important risk factors for cutaneous melanoma include fair skin and concomitant sun exposure. UV radiation from the sun can cause DNA-damage leading to mutations, increasing the risk of cancer development. Melanomas frequently show UV signature mutations with frequent C→T transitions at dipyrimidine sites [108]. Frequent genetic alterations important for development of melanoma are summarized in *Figure 2*.

Cutaneous melanoma commonly harbor mutations in V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF, 66% of patients) [109] or Rat Sarcoma N (N-RAS, 20% of patients) [108], causing mitogen-activating protein kinase (MAPK) pathway activation. Activation of the MAPK pathway leads to increased proliferation and survival in melanoma cells [110, 111].

BRAF is a serine/threonine protein kinase activating the MAPK pathway by phosphorylation of another kinase (mitogen-activated protein kinase kinase; MEK), which in turn phosphorylates and activates extracellular signal-regulated kinase (ERK). ERK can phosphorylate many different proteins including transcription factors such as c-myc [112]. BRAF mutations most commonly occur in V600E, causing constitutive activation of the kinase as well as insensitivity to negative feedback mechanisms [109].

N-RAS is a GTPase upstream of BRAF. Most commonly, mutations in melanoma cells occur at Q61 and disrupt the GTPase activity of N-RAS, locking it in its active conformation [113].

KIT mutations are found in some subtypes of melanomas, including mucosal, acral and melanoma on chronic sun-damaged skin [114].

Cutaneous melanomas frequently also have mutations in tumor suppressor genes including CDKN2A, P53 and PTEN [115]. PTEN loss in turn lead to upregulation of the PI3K-AKT pathway, also involved in increased proliferation and migration of melanoma cells [116].

### 1.3.4 Clinical classifications and prognosis

In cutaneous melanoma, the stage at which the cancer is detected has great importance for the prognosis of the patient [117]. About 90% of melanomas are detected as primary tumors, and these patients have a good 10-year survival rate of 75-85% [118].

In contrast, metastatic melanoma (stage IV) historically had a dismal prognosis, but the outcome for these patients has significantly improved over recent years with the development of targeted therapies and immunotherapies [117].

### 1.3.5 Treatment options

#### *1.3.5.1 Surgery*

Primary cutaneous melanomas can be surgically removed with good outcome [118]. This is a curative treatment when the primary tumor has not yet spread to other sites (metastasized). However, metastatic disease requires additional treatment strategies. Options include chemotherapy, targeted therapy and immunotherapy.

#### *1.3.5.2 Chemotherapy*

Dacarbazin is approved for treatment of metastatic cutaneous melanoma, and was for many years the only approved therapy. Response rates of 15-20% have been reported but no survival benefit [119]. Dacarbazin is often used in combination with carboplatin. Other drugs used historically or at relapse following other treatments include taxanes.

Isolated limb perfusion is a localized chemotherapy treatment possible when melanoma metastases are confined to a limb. Response rates in clinical trials are very good, around 90%, however a survival benefit has not been established [120].

### *1.3.5.3 Targeted therapy*

BRAF inhibitors have been approved for use in patients with BRAF mutated melanoma, including Dabrafenib [121] and Vemurafenib [122]. High response rates of about 50% are achieved with BRAF targeted treatment.

For N-RAS mutated melanoma, no inhibitor targeting N-RAS directly has been developed. However, MEK, a protein kinase downstream of both N-RAS and BRAF has been targeted with the MEK1/2 inhibitor Trametinib [123]. Response rates to Trametinib are 28%.

Especially good clinical outcome for patients with BRAF mutated melanoma was achieved by combining BRAF and MEK inhibitors [124], leading to an impressive response rate of 67% for patients with BRAF mutated melanoma.

Pre-clinical research also indicate a possibility to target ERK, downstream of MEK, in combination with BRAF and MEK to further lower the fitness threshold for melanoma cells in PDX mice [125]. The ERK inhibitor Ulixertinib has been tested in a phase I clinical trial and was shown to induce partial responses in some patients [126].

In conclusion, targeted therapies have very good response rates in cutaneous melanoma and increase survival in patients, but there is a problem of development of resistance in most treated patients [127].

### *1.3.5.4 Immunotherapy*

Cutaneous melanoma is the disease where immunotherapy has been researched and tested for the longest time. In this disease, lymphocyte infiltration is correlated to good prognosis [128, 129], and a high mutational burden also suggests high immunogenicity [130]. Several immunotherapies tested thus far has had limited effects on survival, including IL-2, vaccines, interferons and histamine [59]. T-Vec (Talimogene Laherparepvec) is an oncolytic virus that increased survival in patients with melanoma with about 4 months [65]. So far, the most promising approaches have been antibody based (checkpoint inhibitors) [131-133] or cell based (adoptive T cell transfer; ACT) [86].

Immune checkpoint activation is one way for tumor cells to evade immunity and checkpoint blockade overcome negative regulation of T cell function [134-137]. Immunotherapy using checkpoint inhibitors targeting CTLA4 and PD1 have been approved for use in patients with cutaneous melanoma. The CTLA4 inhibitor Ipilimumab achieved an objective response rate of 60%, and could increase the survival of patients [75]. Importantly, this was the first therapy to increase survival in patients with metastatic melanoma. PD1 inhibitors have also been developed and approved for treatment of metastatic melanoma, including

Pembrolizumab [77] and Nivolumab [78]. PD1 inhibitors show better response rates compared to Ipilimumab, and less side effects [131]. A combination of CTLA4 (Ipilimumab) and PD1 blockade (Nivolumab) has even higher effect, but also more severe side effects compared to monotherapies [132, 133]. Importantly, immunotherapy with checkpoint inhibitors can induce long-term responses in patients with metastatic melanoma.

Adoptive T cell transfer (ACT) is another immunotherapeutic strategy tested in patients with cutaneous metastatic melanoma in clinical trials, showing responses in about 50% of patients [85, 86, 138]. In 20% of all patients, responses are even durable [85].

In conclusion, long-term follow-up indicates that immunotherapies can cause durable responses in patients with metastatic melanoma. However, not all patients will be cured with available immunotherapies today, pointing to the need of developing additional strategies.

## 1.4 Uveal melanoma

Melanoma arising in the uveal tract of the eye is called uveal melanoma. The uvea is comprised of the iris, the ciliary body and the choroid. Uveal melanoma is a rare form of melanoma (less than 5% of all melanoma cases) but still the most common malignancy of the eye [139]. It most commonly develops in the choroid (90% of cases) and less frequently in the iris (4%) or ciliary body (6%) [140].

### 1.4.1 Normal melanocytes

Melanocytes in the iris determine the color of the eye, but the function of eye melanocytes is not fully understood. Aside from the iris, melanocytes are also found in the choroid (a vascularized layer situated between the retina and the sclera; supporting the eye with oxygen and nutrients) and the ciliary body (contain ciliary muscles determining the size of the pupil). Melanocytes in the uvea, alike other melanocytes in the body, are derived from neural crest and migrate to their final destination during embryonic development [141].

### 1.4.2 Epidemiology

Uveal melanoma affects mainly the Caucasian population. Unlike skin melanoma, the incidence is stable and has not increased over recent years [142]. Uveal melanoma shows a south-to-north increase in prevalence in Europe [143]. The incidence in Sweden is 70-80 cases per year [144].

### 1.4.3 Etiology

The cause of uveal melanoma is not fully understood. Unlike cutaneous melanoma, UV radiation could not be shown to be a risk factor for the development of uveal melanoma [145]. Welding has been revealed to be a risk factor for developing uveal melanoma [145].

The genetics of uveal melanoma are different from cutaneous melanoma (*Figure 2*), and *BRAF* and *NRAS* mutations are non-existent [146]. Instead, most uveal melanomas have mutations in Guanine Nucleotide-Binding Protein G(q) Subunit Alpha (*GNAQ*) [147] or Guanine Nucleotide-Binding Protein Subunit Alpha-11 (*GNA11*) [148]. Mutations in *GNAQ* or *GNA11* occur in 83% of patients and are mutually exclusive. Less frequently, driver mutations are found in *CYSLTR2* [149] or *PLCB4* [150].

GNAQ and GNA11 are Gα GTPases which form complexes with Gβ and Gγ subunits and bind G-protein coupled receptors (GPCRs). In this state, the Gα is bound to a GDP and inactive. Upon activation, Gα exchanges the GDP to a GTP and dissociates from the complex. Mutations in uveal melanoma occur at R183 or Q209 and leads to a decreased GTPase activity, preventing them from deactivation and rendering them constitutively active [147]. Activated Gα in turn activates phospholipase C-β (PLC-β) leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG) [151]. DAG stimulate the mitogen activated protein kinase (MAPK) pathway by protein kinase C (PKC) whereas IP3 mediates Ca2+ signaling and is dephosphorylated into inositol monophosphate (IP1) [152]. MAPK activation drive proliferation and survival [146]. Activated GNAQ and GNA11 also stimulates the ADPribosylation factor 6 (ARF6)–TRIO–RHO/RAC pathway implicated in cytoskeletal organization, and yes-associated protein 65 (YAP), also driving cancer initaiation and progression [153, 154]. Another common mutation in uveal melanoma is the BRCA1 Associated Protein-1 (BAP1) tumor suppressor, which is inactivated in the majority of uveal melanomas [155]. This gene encodes a deubiquitinating enzyme that interacts with many proteins. Its inactivation occurs late and is associated with metastasis.

Based on genetics, uveal melanoma can be divided in two groups with diverse prognosis (class 1 and 2). Mutations in EIF1AX and SF3B1 are connected to low or intermediate risk of metastasis and to a better prognosis (class 1). Monosomy 3 and loss of the tumor supressor BAP1 are linked to high risk of metastasis and poor prognosis (class 2) [156]. Recently, we and others have also shown that additional changes affecting copy-numbers of certain gene segments (including the one encoding CDKN2A) can be seen in metastases as compared to the primary tumors [157, 158].

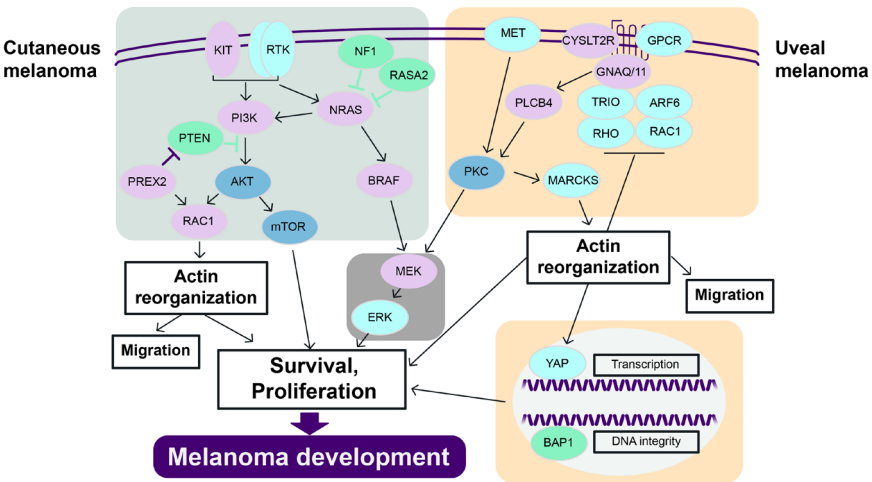


***Figure 2.*** *Signalling pathway alterations differ in cutaneous (CM, left in light gray box) and uveal melanoma (UM, right in yellow boxes). Frequent activating (gain-of-function) mutations are shown in pink and loss-of-function mutations are shown in green. In CM, mutations in NRAS and BRAF frequently occur and lead to MAP-kinase pathway activation (dark gray box), increased proliferation and survival and subsequent melanoma development. In UM, mutations most frequently occur in GNAQ and GNA11 activating the MAP-kinase pathway (dark gray box). Activated GNAQ and GNA11 also stimulate the ARF6–TRIO–RHO/RAC pathway implicated in cytoskeletal organization, as well as YAP, also driving cancer initaiation and progression. BAP1 is frequently lost in uveal melanoma which is strongly correlated to metastatic spread. Adapted and modified from [159].*

## 1.4.4 Clinical classifications and prognosis

Uveal melanoma most often affects the choroid, and less frequently the ciliary body or iris [140]. Iris melanomas show a better prognosis compared to those

arising in the ciliary body or choroid [160]. It is not clear, however, if this is due to easier detection and hence earlier treatment of iris melanomas compared to posterior uveal melanomas.

The vast majority of uveal melanomas are detected before any signs of metastatic disease [161]. Nevertheless, 50% of patients will later present with metastases implicating a poor prognosis with a median survival of less than 6 months [162, 163]. Uveal melanoma most frequently metastasizes to the liver (89% of patients), but also to other sites including lung (29%), bone (17%), skin (12%) and lymph node (11%) [161].

## 1.4.5 Treatment options

Primary uveal melanoma is treated with either brachytherapy or enucleation with very good local control [164]. Unfortunately, effective treatment of the primary tumor does not inhibit subsequent cancer progression, and around half of the patients diagnosed with uveal melanoma will later develop metastases [165]. Patients with metastatic uveal melanoma completely lack approved therapies today, because of poor effects of tested treatments so far [166, 167]. As a consequence, the prognosis for patients with uveal melanoma has not improved over the last decades [142]. There is therefore an unmet medical need for developing novel treatment strategies for these patients.

### *1.4.5.1 Brachytherapy and enucleation*

Primary uveal melanoma was historically treated with enucleation. Of note, there have been concerns that enucleation may not just remove the primary tumor, but also enhance metastatic spread the years following enucleation [168]. Later there has been a shift toward eye-preserving treatments including local radiation to the eye, called brachytherapy. Brachytherapy with Rhutenium-106 or Iodine-125 is most commonly used, and was shown to be equally efficient as enucleation for control of the primary tumor and is therefore the preferred treatment used today [161].

Even though good local control is achieved in 96% of patients after treatment of the primary tumor [164], about 50% of patients will later present with metastatic disease. For patients with metastatic uveal melanoma, no approved therapies exist today. Most therapies tested have shown disappointingly limited efficacy, indicating that metastatic uveal melanoma is notoriously therapy-

resistant. The coming sections will describe treatments that has been used in these patients, and their potential.

### *1.4.5.2 Chemotherapy*

Systemic chemotherapy has been used in patients with metastases of uveal melanoma, but with no proof of a survival benefit [166, 167].

Because of the high frequency of liver metastases in patients with metastatic uveal melanoma, treatment can be targeted specifically to the liver. Intra-hepatic perfusion (IHP) with melphalan can be performed in patients with isolated hepatic metastases and increase survival (median survival 22 months) [169]. This and other loco-regional treatments, including liver resection [170] showed both feasibility and high response rates, but recurrence occurs in most patients indicating that they are not curative.

### *1.4.5.3 Targeted therapy*

There are no approved targeted therapies for patients with uveal melanoma. The frequent mutations in GNAQ and GNA11 make these interesting targets for inhibitors, but unfortunately no such molecules has been developed [171]. However, activation of the MAPK pathway in uveal melanoma has implications for inhibitors targeting Mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK). The MEK inhibitors Selumetinib [172] and Trametinib [173] have been tested in patients with uveal melanoma, but unfortunately, no overall survival benefit was achieved with this treatment.

### *1.4.5.4 Immunotherapy*

Interestingly, lymphocyte infiltration has been correlated with poor prognosis in patients with uveal melanoma [174, 175] as opposed to the opposite finding in most other cancer types [176]. TILs were mainly CD8+ T cells, and less CD4+ T cells, Tregs and B-cells [177]. Tumor infiltrating macrophages has also been correlated to poor prognosis in patients with uveal melanoma [178], and the main type of macrophages was found to be of the anti-inflammatory M2 phenotype [179]. Furthermore, high expression of MHC I and II in uveal melanoma was also associated with poor survival [180, 181], which might indicate a role for NK cells in controlling metastatic spread. An inflammatory

phenotype in uveal melanoma, characterized by high expression of MHC I and II and infiltration of macrophages was associated with monosomy of chromosome 3 [182], and hence highly correlated to metastatic progression [183].

Immunotherapy with checkpoint inhibitors targeting CTLA4 and PD1 show great efficacy in patients with cutaneous melanoma [75, 78, 184], while these treatments are less promising in uveal melanoma patients [185, 186]. It has been suggested that uveal melanoma is a non-immunogenic cancer due to its origin in the eye which is an immune-privileged site [187]. Indeed, uveal melanomas have a low mutational burden [150, 188], especially compared to cutaneous melanoma which is one of the most highy mutated cancers [130]. A low mutational burden indicates a lack of immunogeneity [189]. Nevertheless, studies have shown that there is an immunogenic subset in uveal melanoma [190].

Another immunotherapy that was efficatious in 50% of patients with cutaneous melanoma is adoptive T cell transfer (ACT) with tumor infiltrating lymphocytes (TILs) [85, 86, 138]. ACT has also been tested in uveal melanoma in a clinical trial [191]. Response rates of 35% were reported, demonstrating potential for T cell-based immunotherapy in uveal melanoma. However, only 1 patient out of 21 reached complete and long-term response (>20 months), indicating a need for improving response frequency and durability.

# 2. Aims

The overall aim of this thesis was to use mouse models to study novel therapeutic strategies to treat metastatic melanoma. Specifically, the aims of the papers incuded in this thesis were:

Paper I

To create novel mouse models to study immunotherapy in melanoma

Paper II

To use these models to find novel treatment strategies for melanoma

Paper III

To conduct the preclinical experiments needed to enable a clinical trial

# 3. Methods

## 3.1 Preclinical research

### 3.1.1. *In vitro* models

Cancer cell lines have been used extensively in pre-clinical cancer research [192]. They are cheap to use compared to other preclinical models, and provide a fast and easy-to-use system, especially for screening of novel drugs or combinational therapies. Cell lines can also be grown in immune-compromised mice as cell-line derived xenografts [193]. The problem with cell lines is that they usually do not represent the complex reality in tumors very well, because of the different selection pressure on cells *in vitro* compared to *in vivo* [194, 195]. Hence, findings made using cancer cell lines should also be validated in more accurate *in vivo* models.

There are many commersially available cell lines from metastatic cutaneous melanoma. Cell lines from metastatic uveal melanoma have been more difficult to obtain. Instead, cell lines from primary tumors (grown *in vitro* or as PDXes) have been commonly used in pre-clinical research of uveal melanoma.

### 3.1.2. *In vivo* models

Many different model organisms are used to study cancer, including (but not limited to) yeast, fruit flies, zebrafish, mice, rats and dogs.

The mouse as a model organism has many advantages. It has a relatively short reproduction time and is easily housed. There is a high similarity between mouse and human genetics (80% of mouse genes have human orthologues) [196]. Furthermore, there are many well-characterized mouse models already developed for cancer research [197]. Genetically engineered mouse models (GEMMs) generally model disease progression more accurately compared to *in vitro* models [197]. On the other hand, GEMMs cannot recapitulate the heterogenous nature in human cancer patients. To study these intra-patient characteristics, many different syngenic transplant models of spontaneous or chemically induced tumor models can be used. An alternative is patient-derived

xenograft mouse models, where human tumors are engrafted to immune-compromised mice.

### *3.1.2.1 GEMMs*

Mice can be genetically altered to spontaneously develop cancer, called genetically engineered mouse models (GEMMs). In these models, disease progression can be studied (from spontaneous tumor formation to metastatic spread) in an *in vivo* setting.

Several GEMMs have been established for cutaneous melanoma [198]. For example, activation oncogenic *Braf* V600E together with *Pten* loss generate cutaneous metastatic melanoma in mice [199]. There are two uveal melanoma GEMM model, driven by inducuble oncogenic GNAQ Q209L [200] or GNA11 Q209L [201]. In these models, mice develop neoplasms of the uvea and central nervous system as well as pigment anomalies in skin and impaired hearing and balance. Loss of BAP1 leads to acceleration, akin to the human situation [201].

GEMMs can be used for studying spontaneous cancer development in mice, and accurately model interactions between the tumor and microenvironment, including interactions with the immune system. The limitations of GEMMs include differences in human and mouse biology, for example treatments tested in research using mice might not be directly applicable for human use.

### *3.1.2.2 PDX models*

Patient derived xenograft (PDX) models are mice with human tumor transplants. In order for successful engraftment of human tumors, mice are altered to be immunocompromised (they lack a functional immune system). There are different strains of immunocompromised mice. Nude mice lack thymic tissue and were the first immunocompromised mice successfully used to transplant human tumors [202]. The lack of thymus in nude mice results in the abcense of T lymphocytes and alterations in B lymphocyte function. NOD/SCID mice harbor defects in many components of the immune system, they completely lack functional T and B lymphocytes and have reduced macrophage and NK cell function [203]. NOD/SCID/interleukin-2Rγ mutant mice (called NOG [204] or NSG [205]) are further immunocompromised and are currently the models with the highest engraftment rate of human tumors. NOG/NSG mice completely lack functional NK cells as well as T and B lymphocytes.

PDX mouse models can be readily established for cutaneous melanoma [206] and especially well in NOG mice [207, 208]. In general, metastatic cutaneous melanoma PDXes have a take-rate of about 90% and can be established in a few months time. Metastatic uveal melanoma has a lower take rate in NOG mice and generally takes longer to develop. Liver metastases have a take rate of about 10% and take 6-12 months to develop. Cutaneous metastases, on the other hand, behave more like cutaneous melanoma with good take rate (Figure 3).

PDXs can accurately model human tumors, including intra-patient heterogeneity, and correlate to therapeutic responses in patients [208-210]. PDX models also enable testing of human specific reagents directly in the mouse model. An important disadvantage of PDX models is the challenge to study interactions of tumor and the microenvironment, especially since the mice are immune-compromised and lack a functional immune system.

Recently, efforts have been made to create so called immune-humanized mice, were human immune cells are grafted in immune-compromised mice [211]. These models often depend on engraftment of human peripheral blood leukocytes (PBLs) or hematopoetic stem cells (HSCs). These models develop a subset of human immune systems, but there is a challenge of immune cell education in the mice. Also, these mice frequently develop symptoms of Graft versus Host disease – posing a challenge for using these models [212].

## 3.2 Clinical trials

Ultimately, the goal of pre-clinical cancer research is to find novel therapeutic approaches to benefit cancer patients. In order for a treatment strategy to be approved for use in patients, it has to be tested in phase I through III clinical trials. Phase I trials focus on safety, and usually starts with a low dose and treatment of a small number of patients (one at a time). Phase IV trials are conducted with approved therapies on large cohorts of patients, in order to better understand efficacy and safety of the treatment. In order to reach the next level of clinical testing, a benefit for the patients needs to be established. Most anti-cancer drugs fail to prove efficacy in clinical trials, leading to clinical approval in only about 10% [213]. The low success rate in clinical trials points to the importance of using accurate pre-clinical models in the search for novel cancer treatments.

For metastatic cutaneous melanoma, several treatment strategies have already been approved for patient use, including targeted therapies inhibiting BRAF and MEK as well as immunotherapies blocking checkpoint proteins CTLA4 and PD1. For metastatic uveal melanoma on the other hand, no potential treatment

strategy has so far reached clinical approval because of failure to prove survival benefit. A rare disease such as uveal melanoma also poses a challenge for conveying clinical trials, because of the limited number of available patients.

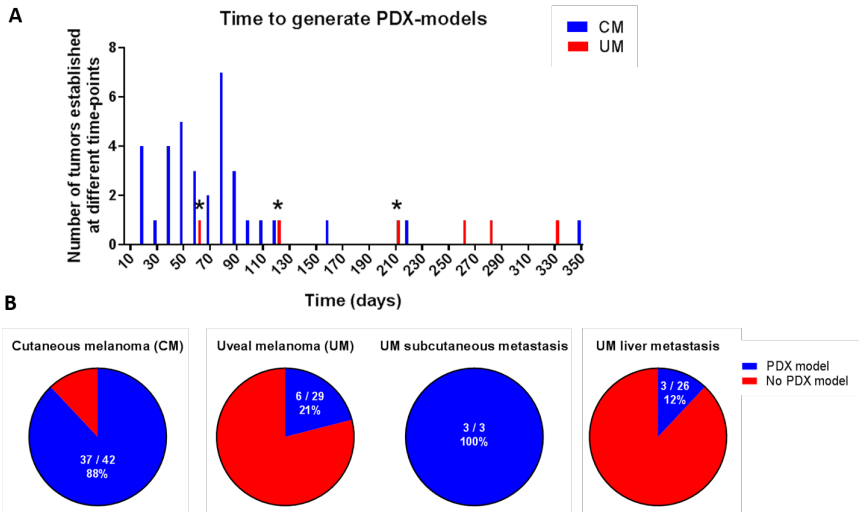


***Figure 3.*** *Generation of patient-derived xenograft (PDX) models of cutaneous melanoma (CM) and uveal melanoma (UM). A) Number of tumors from cutaneous melanoma (CM, blue) and uveal melanoma (UM, red) established at different timepoints after engraftment in NOG mice (days). Asterisks above 3 UM samples indicates subcutaneous metastases, the other 3 UM are liver metastases. B) Number of tumors successfully engrafted on mice (blue) and tumors that were not (red) between 2012 and 2017 from cutaneous melanoma (CM) and uveal melanoma (UM). Out of the 6 uveal samples that were successfully engrafted on mice, 3 were subcutaneous metastases and 3 were liver metastases.*

# 4. Results

## Paper I

Patient-derived xenograft (PDX) mouse models have been used extensively in pre-clinical cancer research, and have been shown to mimic therapeutic effects in patients [209, 210]. In melanoma specifically, PDX models can be readliy established [206], and have been shown to accurately model the disease and even develop fast enough to guide treatment decisions [208]. A requirement for human tumors to engraft in mice is that the mice are immune-compromized, in order for the graft not to be rejected by the host immune system. With recent advances in treatment of melanoma using immune-therapies including antibody-based and cell-based techniques, classical PDX models are no longer good pre-clinical tools simply because the mice lack a functional immune system, making studies of immunotherapies impossible.

The aim of this paper was to create immune-humanized mouse models of malignant melanoma, to model adoptive T cell transfer (ACT) in PDX mice. In order to meet this goal, we created a model called PDX version 2 (PDXv2), where immune cells (tumor-infiltrating lymphocytes; TILs) and tumor cells from the same patient with malignant melanoma were grafted in the same mouse.

### Etsablishing the PDXv2 model

We aquired TILs and tumor cells from malignant melanoma patients enrolled in a clinical trial studying ACT in Herlev, Denmark [138]. The tumor cells were transplanted to NOG mice while TILs simultaneously were isolated from tumor pieces and further expanded to large numbers in vitro using a rapid expansion protocol (REP). Subsequently, TILs were adoptively transferred to NOG mice carrying autologous tumors.

## T cells can home to, but not kill, tumors in wt NOG mice

In vitro studies were conducted, assessing tumor cell viability and TIL IFN-γ production when co-culturing autologous tumor cells and TILs in vitro from a patient with malignant melanoma (MM33). The tumor cells could be readily killed by T cells in vitro, accompanied with high IFN-γ production by the T cells.

We next tested ACT treatment in NOG mice, by engrafting MM33 tumor cells and infusing autologous TILs consecutively. Mice were treated with hIL-2 (45 000 IU on the day of TIL infusion and the next two days, and then biweekly for two weeks) to resemble the protocol used in patients undergoing ACT in the clinic. Although using a tumor sample which was effectively killed by autologous TILs in vitro, no effect of ACT could be achieved in NOG mice.

To investigate wheather the TILs were able to reach the tumor in NOG mice, tumors from vehicle or TIL treated mice were sacrificed, and the tumors analyzed with immunohistochemistry and flow cytometry. The IHC revealed T-cells in tumors treated with TILs as assessed by positive staining of human CD3. Furthermore, an upregulation of the PD1/PDL1 axis was observed in TILs after tumor encounter, as revealed by PDL1 upregulation in TIL treated tumors by IHC as well as an increased PD1 expression in TILs from the tumors compared to the infusion product. This fact encouraged subsequent treatment of the mice with the PD1 inhibitor pembroluzimab, but still without resulting in tumor regressions.

In order to rule out the possibility that the MM33 TILs were non-functional in vivo for any unknown reason, additional experiments were performed with 4 samples which were known from the clinical trial to be one complete responder to ACT (MM11), one partial responder (MM24) and two non-responders (MM29 and MM46). Mice were transplanted with tumor cells and subsequently treated with TILs with or without the addition of pembrolizumab. There was a slight decrease in tumor growth in MM46 treated with TILs and pembrolizumab, as well as in MM24 treated with TILs (with and without pembrolizumab). However, still no tumor regressions were achieved.

## hIL-2 transgene expression enables tumor eradication in NOG mice

We hypothesized that some factor was missing in our mouse model, in order for the T cells to be able to kill the tumor cells. A potential reason for the lack of effect of ACT in NOG mice included additional tumor evasion mechanisms aside from PD1/PDL1 engagement and insufficient viability of TILs in vivo. One important factor for T cell viability and expansion is IL-2. Potentially, the half-life of IL-2 was too short or the dosing scheme used was insufficient in mice. We found that IL-2 plasma levels in mice peaked 2 hours after injection and was almost completely vanished after 6 hours. In order to circumvent this problem, we purchased NOG mice transgenic for human IL-2 (hIL2-NOG; Taconic). The hIL2-NOG mice had various levels of IL-2 in plasma ranging from 0 to 8 ng/ml.

We found that mice with >2 ng/ml IL-2 in plasma could support survival and expansion of TILs in mice for more than 6 weeks. TILs were shown to selectively home to the tumor site in IL-2 transgenic mice. When repeating adoptive T cell transfer in IL-2 mice carrying MM33 tumors, treatment with TILs and Pembrolizumab could eradicate the tumors. Additional experiments showed that TILs alone could eliminate the tumors in MM33, and no additional benefit could be shown with Pembrolizumab treatment.

## PDXv2 accurately model ACT in patients with malignant melanoma

Additional experiments were performed with samples from patients in the clinical trial testing ACT in IL-2 transgenic mice. 3 samples were responders to ACT in the clinic (MM11, MM05 and MM24) and 3 samples were non-responders to ACT (MM29, MM46 and MM04). The samples from ACT responders in the clinic were able to eradicate autologous tumors in IL-2 transgenic NOG mice, while samples from non-responding patients did not cause tumor-shrinkage in mice.

In order to investigate whether TILs could also eradicate melanoma in other sites than the skin, MM33 engrafted mice were treated with surgery to remove the primary tumor and allow for metastatic spread in the same mouse. We found that metastatic spread to lymph nodes as well as the liver could be targeted effectively with TILs in PDXv2 in 3 out of 4 mice.

# Paper II

The next step after creating PDXv2 (Paper I), was to use this model in order to study immunotherapy in melanoma. In the mouse model, just as in the malignant melanoma patients in the clinical trial, not all tumors were responders to ACT with TILs. We set out to find a way to treat non-responders of ACT in this model. One approach to overcome several immune-evasion strategies used by tumors, including downregulation of antigen presentation, lack of suitable neoantigens and expression of inhibitory receptors is to use CAR-T cells. CAR-T cells are genetically manipulated to express a chimeric receptor (CAR) able to recognize specific surface proteins on tumor cells. This recognition is independent of antigen presentation and circumvents normal regulatory mechanisms inhibiting T cell function.

CAR-T cells have been very useful in the treatment of B-cell malignancies [93, 94], and CD19 targeting CAR-T treatments have already been approved for use in patients. CAR-T therapies have been less successful in pre-clinical models of solid tumors, and consequently no CAR-T therapy has been approved for use in solid cancers [103]. In this paper, we tested CAR-T cell therapy in PDXv2.

## Expression of HER2 in melanoma

In order to predict useful CAR-T targets, we used The Cancer Genome Atlas (TCGA) to look at expression of genes encoding published CAR-T targets. Among the available targets HER2 (ERBB2), CD20 (MS4A1), CD19, VEGF (KDR), Glypican-3 (GPC3), CD133 (PROM1) and EpCAM, HER2 was the most highly expressed both in cutaneous and uveal melanoma. We also found HER2 to be expressed in tumor samples from patients and in PDX models from mice as well as in commersially available melanoma cell lines. HER2 protein expression could also be detected in cutaneous and uveal melanoma PDX tumors from mice using immunohistochemistry.

We obtained HER2 CAR-T cells (ProMab) and tested them *in vitro* in two melanoma cell lines, one with high HER2 expression (HS695) and one with low expression (SK-MEL-1). The cell line with high HER2 expression was more sensitive to CAR-T cell degranulation and CAR-T cell killing *in vitro* compared to the HER2 low cell line, suggesting target specific killing.

## HER2 CAR-T cells kill melanoma cells in vitro and are target-specific

To test the killing capacity *in vitro* of the HER2 CAR-T cells in patient-derived cells from malignant melanoma and cell lines from uveal melanoma. We found that the CAR-T cells could kill both malignant melanoma cells and uveal melanoma cell lines, assesed by decreased viability of cancer cells and CAR-T mediated degranulation and IFN-γ production.

In order to validate the specificity of the CAR-T cells, we used CRISPR/Cas9 technology to disrupt HER2 genetically in cutaneous and uveal melanoma cells. HER2 negative cells were unresponsive to CAR-T cell killing, and did not provoke degranulation and IFN-γ production by the CAR-T cells, indicating that the CAR-T cells were target-specific.

## ACT-resistant cutaneous and uveal melanoma can be eradicated by HER2 CAR-T cells in PDXv2

We next treated NOG and hIL2 NOG mice carrying cutaneous melanoma PDXes with CAR-T cells. We found that all 5 tested samples were responsive to CAR-T cell killing *in vivo*, but only in hIL2-transgenic NOG mice and not in wt NOG mice. Importantly, treatment with CAR-T cells was effective irrespectively of whether the samples were from responders or non-responders to ACT with autologous TILs. Furthermore, CAR-T cells could eradicate one cell line-derived xenograft and one PDX of uveal melanoma in hIL2 mice.

# Paper III

Encouraged by the finding that HER2 CAR-T cells were able to eradicate both malignant melanomas that were non-responders to ACT and uveal melanomas (Paper II), we wanted to further explore the possibilities of CAR-T treatment and facilitate the translation into clinical testing.

Current CAR-T therapies use T cells from peripheral blood as basis for CAR-T cell production (including the HER2 CAR-T cells used in Paper II; ProMab). We hypothesized that it would be possible to use patient's autologous TILs as CAR-T substrate instead of blood T cells. At least theoretically, this strategy could combine the advantages of both ACT and CAR-T therapy in the same treatment. ACT with TILs has the advantage that most TILs retain their ability to home to the tumor even after *in vitro* expansion [214]. TILs are also trained T cells and might therefore be safer to use in patients compared to blood-derived T cells. Moreover, some TILs might recognize the tumor via their intrinsic T-cell receptor (TCR). On top of this, the added CAR-receptor can recognize proteins on the tumor surface independent of antigen presentation, and can elicit effective killing of less immunogenic tumors, including ACT non-responding melanomas and uveal melanomas (Paper II). In this paper, we developed a novel approach to treat melanoma, by equipping patient-derived TILs with improved means to target autologous tumors.

## TILs can be manipulated in vitro to express a CAR construct

With the aim to equip TILs with a HER2 CAR receptor, we used the same lentiviral vector as used in the commercial production of the HER2 CAR-T cells (Promab, Paper II). TILs could be successfully transduced before rapid expansion (REP) or transduced on day 1 and 2 of the REP. The CAR construct was detected in CAR transduced TILs (CAR-TILs) at the end of expansion by qPCR and flow cytometry. Lentiviral transduction enables the CAR construct to be incorporated into the genome at random, resulting in CAR-TILs retaining their normal TCR allowing dual tumor cell targeting.

## CAR-TILs can kill melanoma cells independent of MHC I presentation

As a proof-of-concept for CAR-mediated killing, CRISPR/Cas9 technology was used to disrupt the gene encoding β-2-Microglobulin, *B2M*. B2M is part of the

MHC I complex and required for assembly of MHC I on the cell surface. Indeed, *B2M* knockout MM33 tumor cells were completely insensitive to TIL mediated killing (assessed by tumor cell viability and TIL degranulation), while the parental MM33 cells are sensitive. Intriguingly, MM33 CAR-TILs were able to degranulate in response to and kill B2M negative MM33 tumor cells both *in vitro* and *in vivo*, proving that the antigen presentation dependency for TIL mediated killing was overcome by the CAR-receptor.

## CAR-TILs and TILs are equally efficient to treat ACT responders

To compare the efficacy of CAR-TILs and TILs in ACT-responsive samples, TILs and CAR-TILs were produced as described previously, and used to treat IL-2 transgenic mice with autologous tumors. We found that TILs and CAR-TILs were equally efficient to treat one cutaneous and one uveal melanoma sample that responded to autologous TILs. This indicated that CAR-TIL production does not impact negatively on TCR mediated tumor killing. Therefore, CAR-TIL treatment can be a conceivable treatment strategy for patients, irrespecitive if they would respond or not to regular ACT treatment. Another cutaneous melanoma sample (FOHO) was completely refractory both to autologous TILs and to CAR-TILs, indicating that not all immune evasion strategies used by melanoma can be overcome by CAR mediated tumor recognition. Other immune evasion strategies might be responsible for the lack of response, including the ability of tumor cells to undergo T-cell mediated apoptosis or factors in the tumor microenvironment suppressing T cell function.

## Automated production of CAR-TILs facilitates clinical translation

The requirements for sterility and conformity during production of advanced therapy medicinal products (ATMPs) for patient use are of utmost priority. In order to simplify and ensure safety during CAR-TIL production, we used a bioreactor (CliniMACS Prodigy; Miltenyi) for expansion and transduction of CAR-TILs. The bioreactor is a closed system ensuring minimal risk of contamination during production. Also, the automated protocol used for all processes during expansion including transduction, washing of cells, media exchange etc reduces variations during production. The process used to produce CAR-TILs must also include only materials approved for clinical use, and all processes involved in the production must be performed according to good manufacturing practice (GMP). GMP requirements include all handling of the

product to be performed in controlled environments, called cleanrooms, and by educated personel. Taken together, all of these requirements necessitate changes to the protocol and materials used for CAR-TIL production for clinical use compared to research use.

In order to facilitate translation of the CAR-TIL treatment strategy to reach acceptance for clinical testing, we developed an automated protocol for CAR-TIL production including expansion in a bioreactor. The protocol was validated by testing the functionality of cells produced with the automated protocol compared to cells produced with the standard (manual) protocol in PDXv2. First, TILs were produced from melanoma sample M160811 with both protocols, and then used to treat IL-2 transgenic mice carrying autologous tumors. TILs produced with both protocols caused tumor regressions in all mice. Next, CAR-TILs were produced from MM33 using the automated protocol and compared to MM33 CAR-TILs produced with the manual protocol by injection to IL-2 transgenic mice carrying MM33 *B2M* KO tumors. CAR-TILs produced with both protocols included a population of CAR positive cells (14% with the manual protocol and 11% with the automated protocol), and supported complete tumor rejections in mice carrying autolougus *B2M* KO tumors.

# 5. Discussion

## Paper I

PDXv2 is, to our knowledge, the first PDX mouse model to achieve complete responses of human tumor cells by autologous immune cells. Importantly, this model could accurately recapitulate responses to ACT in patients in a clinical trial, confirming usability of this model in pre-clinical research.

Other immune-humanized mouse models have been previously developed, using either hematopoetic stem cells or peripheral blood mononuclear cells (PBMCs) [211]. These models frequently develop symptoms of Graft versus Host disease (GvH) [212]. In contrast, no GvH disease was detected in the mice treated with TILs. Instead, IL-2 transgenic mice treated with TILs often presented with enlarged spleens and sometimes developed lympho-proliferative disease with enlarged lymph nodes and high blood cell count. These side-effects were generally late events, appearing after TILs had completely eradicated the tumors (in the case for ACT responding samples). It is possible that TILs are less toxic to mice compared to less educated T cells, explaining the relatively mild side-effects in PDXv2.

We found that human IL-2 is required for ACT in mice, and IL-2 transgenic mice facilitate experiments. The dependency of IL-2 in this model indicates the importance of IL-2 treatment to achieve efficacy of ACT. However, high dose IL-2 can lead to serious side effects in patients, stressing the importance of accurate IL-2 dosing. Lower but continuous IL-2 dosing was shown to support efficacy of TILs in a clinical trial [215].

Interestingly, PD1 inhibition could not replace IL-2 in NOG mice. This could be because a missing component – dendritic cells - are not present in the NOG mice [216]. Furthermore, PD1 inhibition (pembrolizumab) impose no additional effect in this model, probably because IL-2 overrides the PD1/PDL1 axis, as was previously reported [217]. Therefore, PDXv2 might not be the optimal model to study PD1 blockade. Potentially, checkpoint blockade using other antibodies could be studied in the PDXv2 mouse model.

# Paper II

HER2 is expressed in cutanous and uveal melanoma, and represents a novel CAR target for melanoma. HER2 expression was equally high as in sarcoma, another disease where HER2 targeted CAR-T therapy has been tested [100], but not as high as in HER2 overexpressing breast cancer. We show that HER2 CAR-T cells can kill ACT refractory cutaneous melanoma and uveal melanoma *in vitro* and in IL-2 transgenic NOG mice. HER2 as a target for cutaneous and uveal melanoma has not previously been studied, representing an interesting possibility for developing novel therapeutic strategies for melanoma patients that do not benefit from available therapies, and uveal melanoma patients with no current available therapies.

Human IL-2 is required for tumor eradication in mice, and lack of IL-2 is a potential reason for poorer responses to CAR-T therapies in solid tumors compared to hematologic malignancies. Possibly, models like PDXv2 can facilitate pre-clinical studies of CAR-T therapies.

In the immune-humanized mouse model used here, IL-2 transgenic NOG mice are used to engraft human tumor cells and human CAR-T cells. This system is informative for showing interactions between CAR-T cells and the tumor, but cannot recapitulate the complexity of a normal immune system. Limitations to this type of immune-humanized mouse model include difficulties to study toxicities in this model (for example on-target off-tumor effects), because normal tissues in the model are mouse origin. It is also difficult to use this model to study persistence of T cells because of the constitutive expression of IL-2 driving T cell proliferation. It might also be of interest to develop a mouse model with inducible expression of IL-2 in order to facilitate studies on aspects including long-term treatment effects after T cells are “turned off/disappear” after tumor eradication. Hence, efficacy of the treatment can be well modeled in the PDXv2 mouse model, while other characteristics such as safety and durability are more difficult to assess. These factors are in general difficult to study in cancer models, and might not be accurately measured anywhere else than in a clinical trial.

# Paper III

Challenges for immunotherapies include how to target non-immunogenic tumors, for example those with low mutational load (lack of neoantigens) or immune evasion strategies including downregulation of the antigen presentation pathway. Cutaneous melanoma should be an optimal disease for immunotherapies, because of the high mutational load, and indeed immunotherapies including checkpoint blockade and ACT show great efficacy in this patient group. Still, not all patients with cutanoues melanoma will benefit from available immunotherapies. Uveal melanoma is a less immunogenic type of melanoma compared to cutaneous melanoma, with lower mutational load [150, 188] and disappointingly poor effects were achieved with checkpoint blockade in these patients [185, 186]. ACT showed some effect in uveal melanoma, but markedly less compared to cutaneous melanoma [191]. Importantly, metastatic uveal melanoma completely lacks approved therapies today, stressing the urgent need for improving therapeutic strategies for this patient group.

Here we present a novel approach to target melanoma by equipping TILs with a CAR targeting HER2, called CAR-TILs. This approach could enhance TIL killing capacity of autologous tumors, circumventing poor efficacy of some TILs. CAR-TILs were able to target autologous tumor cells in a completely MHCI independent manner, since CRISPR/Cas9 disruption of B2M in melanoma cells (B2M KO) were resistant to TILs but sensitive to CAR-TILs. This is highly relevant since loss or downregulation of B2M in melanoma has been correlated to resistance in melanoma patients treated with different immunotherapies, including ACT [218] and PD1 inhibition [219]. The fact that B2M KO cells are sensitive to CAR-TILs shows that CAR-mediated killing is possible, even in the absence of MHC-TCR interactions. However, dual targeting in CAR-TILs inflicts an additional challenge to determine if killing is TCR or CAR mediated (or both). In one sample refractory to ACT with autologous TILs (FOHO), CAR-TILs too did not affect tumor growth, indicating that additional immune evasion strategies can be employed by tumor cells rendering them insensitive to CAR-TIL mediated killing. Since this sample express HER2, the unresponsiveness might be due to other factors besides T cell recognition. Potential reasons include tumor-intrinsic resistance to T cell killing or other factors in the tumor microenvironment supressing T cell function.

The immune-humanized PDX model used here (PDXv2) was previously shown to accurately model ACT (Paper I), indicating a usefulness in this model for testing novel treatment strategies. However, it is difficult to study off-target and on-target off-tumor effects in this model, simply because normal tissues in the model are of mouse origin. Mouse HER2 is not completely identical to

human HER2 (identity of 86%), making it impossible to accurately assess safety in this model. One phase 1/11 study used HER2 CAR-T cells in patients with HER2 positive sarcoma, and found that this treatment was safe, although with limited clinical responses [100]. However, when using the same treatment but in combination with a conditioning chemotherapy, responses could be achieved in patients with sarcoma (clinical trial ref NCT00902044, abstract nr LB-147/4 presented on AACR, 2019). HER2 CAR-T cells have also been evaluated in phase I trials of HER2 positive glioblastoma [101] as well as biliary tract cancers and pancreatic cancers [102], and both trials found that this treatment was safe and indicated some clinical activity. In an early case-study, one patient with colon cancer was treated with a dose of HER2 CAR-T cells that is much higher than used nowadays, resulting in that the patient suffered from pulmonary distress leading to death [220]. This event clearly underlines the danger in using novel CAR T cells at high doses, without doing a proper dose-escalation study, starting with low doses and only increasing the dose after safety could be proven. The HER2 CAR-T construct used in the current study has not been previously tested in patients.

Automated production of TILs and CAR-TILs using a bioreactor (CliniMACS Prodigy, Miltenyi) facilitates clinical translation. It also minimizes the risk of contaminations and operator-dependent differences between consecutive samples. Reassuringly, TILs and CAR-TILs produced in an automated fashion using the bioreactor was equally efficient in eradicating autologous tumors in mice as compared to cells produced with the standard protocol. Taken together, these findings potentiate future clinical testing of HER2 CAR-TIL treatment in patients with cutaneous and uveal melanoma that did not benefit of currently available therapies.

# 6. Conclusion and future perspective

We have developed an immune-humanized mouse model called PDXv2, where human tumor xenografts and human T cells are engrafted in the same IL2 transgenic NOG mouse. This model could accurately model ACT with autologous TILs in cutaneous melanoma, since effects in the mouse model correlated with clinical responses in the corresponding patients in a clinical trial.

PDXv2 could also be used to discover a novel and efficatious CAR-T target for melanoma (HER2). HER2 CAR-T treatment was capable of eradicating both ACT resistant cutaneous and uveal melanoma xenografts in IL-2 transgenic NOG mice, proving for the first time that complete regressions of solid tumors can be achieved by CAR-T treatment in mice.

Finally, PDXv2 was used to validate a novel immunotherapeutic approach to overcome resistance to immunotherapy in melanoma, called CAR-TIL treatment. By equipping autologous TILs with a HER2 CAR-construct, TILs could kill melanoma cells independent of antigen presentation and enabled killing of an ACT non-responding sample. Furthermore, CAR-TILs could be produced in an automated fashion facilitating the translation from pre-clinical findings into clinical testing. A clinical trial is warranted to learn the true potential for CAR-TIL treatment in patients with metastatic melanoma.

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