IMMUNOLOGICAL EFFECTS OF ISOLATED REGIONAL PERFUSION IN MALIGNANT MELANOMA

Junko Johansson

Department of Surgery
Institute of Clinical Sciences
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: *Busted!* by Junko Johansson

The cover shows s simplified illustration of an anti-tumoural immune response wherein a dendritic cell directs cytotoxic T cells towards a melphalan-exposed tumour.

junko.johansson@gu.se

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Abstract

Malignant melanoma patients with metastatic disease confined to the limbs or liver may be treated with hyperthermic isolated regional perfusion with a chemotherapeutic agent, most commonly melphalan. This procedure enables much higher tissue concentrations of the chemotherapeutic agent compared with systemic administration. Isolated limb perfusion (ILP) is approved for treatment of cutaneous metastatic melanoma, while the efficacy of isolated hepatic perfusion (IHP) is under evaluation for the treatment of liver metastases from uveal melanoma. Following ILP and IHP tumours often gradually decrease in size during a period of several months, which might be explained by a treatment-induced immunological anti-tumour response. This thesis aimed at investigating the potential role of the immune system for treatment response to ILP and IHP utilising *in vivo* analyses of patient material and mice models and *in vitro* cell cultures. As reported in **Paper I** and **Paper II**, patients who harboured a high fraction of activated and antigen-specific T cells in blood prior to ILP were more likely to achieve a complete disappearance of tumours following ILP. Furthermore, the *in vitro* and *in vivo* assays showed that melphalan exposure enhanced the activation of T cells and increased the numbers of intermediate and non-classical monocytes. This may be due to the melphalan-induced upregulation of immune-related stress markers on melanoma cells, which in turn stimulated immune cells. In **Paper III** it was reported that high levels of interferon-stimulated gene products in patient blood, including CXCL10, CCL2 and PD-L2, were predictive of a favourable treatment response to ILP, and that the receptors of these ligands increased on immune cells following treatment. **Paper IV** describes different T cell immune profiles in blood between uveal melanoma patients and healthy controls, and showed that melanoma patients harboured a lower frequency of $CD8^+$ T cells and more regulatory T cells. Uveal melanoma patients achieved a longer progression-free survival following IHP if they harboured a high fraction of activated T cells in blood. In conclusion, the findings presented in this thesis point towards a role of the immune system for treatment responses following both ILP and IHP, suggesting that it may be beneficial to combine isolated regional perfusion with immunotherapy.

Keywords: Melanoma, isolated regional perfusion, ILP, IHP, melphalan, immunogenic cell death, T cells, monocytes, ISG

Sammanfattning på svenska

Malignt melanom är en av de vanligaste formerna av cancer i Sverige. Över 90% av alla fall av melanom i västvärlden är i form av hudcancer, men melanom kan även uppstå på andra ställen i kroppen, exempelvis i ögonen. De flesta som drabbas av hudmelanom botas lätt genom kirurgiskt avlägsnande av tumören, men i de fall då metastaser, dvs. dottertumörer, uppstår blir behandlingen mycket svårare och prognosen värre. För ögonmelanom är metastasering ett stort problem då majoriteten av alla som drabbas av ögonmelanom utvecklar dottertumörer, vilket inte är fallet för hudmelanom. En behandlingsform för melanompatienter med metastaser isolerade till ett område av kroppen, framförallt till extremiter vid hudmelanom eller i lever för ögonmelanom, är hyperterm isolerad regional perfusion med cellgiftet melfalan. Under en perfusionsbehandling kopplas den drabbade kroppsdelens blodkärl till en hjärt-lungmaskin för att skapa ett separat, isolerat cirkulationssystem som är skilt från kroppens systemiska blodcirkulation. Till blodet i den isolerade kroppsdelen tillsätts höga doser av cellgift för att cirkulera runt under en timme, innan cellgiftet sköljs bort och kroppsdelen återigen kopplas till den systemiska cirkulationen. Detta upplägg gör det möjligt att enbart behandla den del av kroppen som är drabbad av tumörer, vilket minskar risken för generella biverkningar. Vissa av de tumörer som försvinner efter behandlingen minskar gradvis i storlek under flera månader innan de slutligen försvinner helt och hållet. En hypotes är att det beror på att perfusionsbehandlingen med melfalan aktiverar kroppens immunförsvar till att attackera de tumörer som överlever själva cellgiftet. Syftet med denna doktorsavhandling var att undersöka om immunförsvaret har en roll för behandlingsresponsen vid perfusion. Med hjälp av analyser av blod från perfusionspatienter, cellkulturer och försöksdjur har vi kunnat visa att melanompatienter med en stor andel aktiverade T-celler, immunceller viktiga vid försvar mot cancer och virus, innan perfusion svarar bättre på behandlingen. Perfusionsbehandlingen leder även till en högre aktivitetsgrad av T-cellerna och en högre produktion av de signalsubstanser som används för att locka T-celler och andra immunceller till tumörer. Detta kan bero på att melfalan påverkar de överlevande melanomcellerna så att de börjar visa upp och producera proteiner som aktiverar och lockar till sig immunceller. Fynden som presenteras i denna avhandling tyder på att det kan vara fördelaktigt att kombinera isolerad regional perfusion med immunstimulerande behandling för att få en ökad behandlingseffekt.

List of papers

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

I. **Johansson, J.**, Kiffin, R., Andersson, A., Lindnér, P., Naredi, P., Olofsson Bagge, R. and Martner, A. Isolated Limb Perfusion With Melphalan Triggers Immune Activation in Melanoma Patients. *Frontiers in Oncology*, 2018, 8(570)

II. Martner, A., **Johansson, J.**, Ben-Shabat, I. and Olofsson Bagge, R. Melphalan, Antimelanoma Immunity, and Inflammation—Letter. *Cancer Research*, 2015, 75(24)

III. **Johansson, J.**, Kiffin, R., Aydin, E., Nilsson, M.S., Hellstrand, K., Lindnér, P., Naredi, P., Olofsson Bagge, R. and Martner, A. Isolated limb perfusion with melphalan activates interferon-stimulated genes to induce tumor regression in patients with metastatic melanoma. *Submitted*

IV. **Johansson, J**., Kiffin, R., Siarov, J., Mölne, J., Naredi, P., Olofsson Bagge, R., Martner, A. and Lindnér, P. Presence of activated T cells in peripheral blood correlates to longer progression-free survival in patients undergoing isolated hepatic perfusion for uveal melanoma liver metastasis. *Manuscript*

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Additional publication not part of this thesis:

i. Aydin, E., **Johansson, J.,** Nazir, F. H., Hellstrand, K. and Martner, A. Role of NOX2-Derived Reactive Oxygen Species in NK Cell–Mediated Control of Murine Melanoma Metastasis.
Cancer Immunology Research, 2017, 5(9)

Content

Abbreviations

ACT	Adoptive cell transfer
APC	Antigen-presenting cell
CR	Complete response
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
HSP	Heat-shock protein
ICD	Immunogenic cell death
IFN	Interferon
IHP	Isolated hepatic perfusion
IL	Interleukin
ILP	Isolated limb perfusion
ISG	Interferon-stimulated gene
MHC	Major histocompatibility complexes
NK cell	Natural killer cell
OS	Overall survival
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD	Progressive disease
PD-1	Programmed cell death protein 1
PD-L1/2	Programmed death-ligand 1/2
PFS	Progression-free survival
PMN	Polymorphonuclear leukocyte
PR	Partial response

PRR	Pattern recognition receptor
SD	Stable disease
TCR	T cell receptor
TNF	Tumour necrosis factor
T_{reg}	Regulatory T cell
UV	Ultraviolet

1. Background

To find a solution to a problem, you must first define the problem. To find a treatment to cancer, you must first know what cancer is.

1.1. Cancer

Cancer is an umbrella term for a variety of diseases that all originate from cells with uncontrolled growth and which spread and invade other parts of the body. Often the cells will conglomerate in a mass known as a tumour. There are both benign and malignant tumours, wherein benign tumours do not invade nearby tissues or metastasise, e.g. spread, to other parts of the body, in contrast to malignant tumours. Malignant tumours are thus cancers. Depending on cell type and which tissue or organ the cells reside in, different types of cancer arise.

Cell growth is heavily regulated. In order to maintain a functional multicellular organism each cell needs to function in its proper way, for example only dividing when needed and when ordered to. Cancer cells lack or have circumvented these regulatory mechanisms, causing them to have abnormal growth. This is due to an accumulation of mutations which causes genetic instability of the cells [1]. It is often considered that a tumour originates from a single abnormal cell, which produces daughter cells that in each generation accumulate more and more mutations due to natural selection.

Genetic instability is of paramount importance in the transformation of a healthy cell to a cancer cell. It lays the foundation for eight underlying principles, the “hallmarks of cancer”, in cancerogenesis: self-sufficiency in growth signals (cancer cells develop their own signalling molecules that tell them to grow), insensitivity to anti-growth signals (cancer cells ignore signals that tell them to stop growing), evasion of apoptosis (evasion of self-destruction), sustained angiogenesis (maintaining a constant blood supply), limitless replicative potential (limitless amount of cell divisions), tissue invasion and metastasis (spreading to other tissues and organs), deregulating cellular energetics (altered energy metabolism), and avoiding immune destruction (avoiding destruction by the immune system) [2, 3]. The latter is of interest since another enabling characteristic for the hallmarks, in addition to genetic instability, is inflammation induced and promoted by the tumour itself [3]. Thus, cancer cells might promote one type of immunologic response and at the same time manage to avoid destruction by immune cells.

1.1.1. Malignant melanoma

Melanoma is cancer of the melanocytes, which are cells producing the pigment melanin. Melanocytes are originally derived from a structure called the neural crest; a transient group of cells found during the early stages of the development of an embryo [4]. The neural crest is a group of cells that are part of the early structure of the pre-cursor to the central nervous system (brain and spinal cord), the neural tube, but are pinched off during the formation of the tube and differentiate into cells that do not remain part of the central nervous system. Melanocytes are found throughout different parts of the body, such as in the skin, the eye and in mucosal membranes in the nasal cavity [5], the genital and urinary tract [6], and in the rectum [7].

Melanin protects the epidermis (the upper most layer of the skin) from harmful ultraviolet (UV) light through absorbance and scattering of the radiation [8]. Except for producing melanin, melanocytes have other functions as well, such as by interacting with and regulating the immune system [9, 10]. This may in part explain why melanocytes reside in parts of the body that do not need protection against sun-derived UV damage.

Depending on where in the body the melanocytes reside, different forms of melanoma arise. Despite originating from the same cell type, they differ in their genetic and phenotypic composition and how the disease develops in the patient. This thesis if focused on melanoma in the skin (cutaneous melanoma, **Papers I-III**), and in the eye (uveal melanoma, **Paper IV**).

1.1.1.1. Cutaneous melanoma

When discussing skin cancer, care should be taken to define what type of skin cancer that is the focus of discussion. Although all forms of skin cancer have some things in common, such as arising in the skin and being induced by UV radiation, there are big differences in incidence and mortality rates between them. In general there are three different types of cancers that are included in the term skin cancer; basal cell carcinoma, squamous cell carcinoma and cutaneous melanoma [11]. The incidence rates of basal cell carcinoma and squamous cell carcinoma are higher than for melanoma, though cutaneous melanoma has a higher mortality rate [12].

1.1.1.1.1 Epidemiology and causes

Cutaneous melanoma is most common in countries with a predominantly Caucasian population, such as in Europe, Northern America and in Oceania [13]. In 2017 it was the fifth most common type of cancer among women and the sixth most common among men in Sweden, and unfortunately the incidence rate is steadily increasing [14]. Most melanoma patients in Sweden are diagnosed when they are around 60 years old, and the most common site

for the primary tumour is the extremities for women while it is the trunk for men [15-18].

Exposure to UV radiation is a known risk factor for the probability to develop cutaneous melanoma. In WHO's list of different agents and their carcinogenic hazards to humans, UV radiation is found in group 1, where chemicals and other agents who have been shown to be carcinogenic belong [19]. For a reference, tobacco smoke and plutonium are also in group 1. The hazardous property of UV radiation is that it damages DNA.

Studies have shown that the risk of developing melanoma is highest during intermittent sun exposure compared to chronic sun exposure [20]. Severe sunburns and high sun exposure during childhood are also risk factors. The UV radiation which is derived from the sun fall into three categories: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). UVC is absorbed by the ozone layer and does not affect humans, while UVB penetrates only the uppermost layer of the skin (epidermis) and UVA reached down to the middle layer (dermis). UVB causes direct DNA damage by absorption into the DNA molecule where it induces molecular rearrangements while UVA causes indirect DNA damage by the formation of reactive oxygen species [21].

As for many other types of cancer, mutations in certain genes have been correlated to a higher risk of developing melanoma. Examples of such genes are the tumour suppressor genes *CDKN2A* and *BAP1*, and the oncogene *CDK4* [22, 23]. Another interesting gene where a mutated status has been linked to melanoma is *MC1R*. *MC1R* encodes for the melanocortin-1-receptor which regulates both hair and skin pigmentation in humans. People with red hair and fair skin often have mutated variants of the *MC1R* gene [24], and these features together with a high number of common and atypical naevi (i.e. moles) have been linked to a higher risk of developing melanoma [25].

1.1.1.1.2 Subtypes and staging

Cutaneous melanoma is clinically classified into four different subtypes depending on how and where the tumour grows [25]. The most common type is superficial spreading melanoma, accounting for approximately 60% of all cases in Sweden [15, 17, 18], which has a horizontal growth pattern in contrast to nodular melanoma which almost exclusively has a vertical growth. Nodular melanoma accounts for 20% of the Swedish cutaneous melanoma cases [15, 17, 18]. Acral lentiginous melanoma can be found on e.g. the fingers, the palm of the hand and the sole of the foot (acral meaning affecting or belonging to peripheral body parts), and is uncommon among Caucasian patients but is percental more common among patients of African, Asian and Hispanic descent. Lentigo maligna melanoma is correlated with long-term

UV exposure and increasing age and can grow down into the hair follicles of the skin.

To classify the spread of the disease, melanoma is categorized into different stages according to the TNM staging system [26]. T defines the depth of the primary tumour and if the primary tumour has ulceration, N explains the spread to nearby lymph nodes and M contains information about distant metastasis and is defined according to anatomical site. Based on the TNM-status the patient is then staged into a numerical staging system from stage 0 to stage IV, where the higher the number the more the metastatic spread. Stage 0 is superficial and non-invasive, stage I and II is melanoma without sign of metastatic spread, stage III indicates metastasis to lymph nodes and stage IV is when the disease has distant metastasis and thus has spread to other organs. Two other measurements that are sometimes utilised for melanoma is the Breslow thickness (describing the vertical thickness of the primary tumour) and the Clark level (how far down into the skin the primary tumour has invaded) [26].

1.1.1.1.3 Prognosis and treatment

The prognosis for cutaneous melanoma depends a lot on the characteristics of the tumour at the time of diagnosis and the state of metastasis. The melanoma-specific and overall survival decreases rapidly with increasing depth of the primary tumour and a higher degree of metastatic spread [15-18]. Among Swedish melanoma patients diagnosed with stage I and stage II melanoma, 90% are alive after five years without dying of their disease. For stage III patients, the corresponding percentage is 36% and for stage IV it is 25% [17]. Worth noticing is that over 90% of all patients are diagnosed with stage I or stage II [16, 17].

A short primer on survival:

- Overall survival (OS): Time from diagnosis until death of any cause.
- Melanoma-specific survival: Time from diagnosis until death due to melanoma.
- Progression-free survival (PFS): Time from start of treatment until progression and/or recurrence of melanoma.

The standard treatment option for patients with stage I or II melanoma without metastatic spread to lymph nodes is surgical excision of the tumour. To prevent recurrence of the tumour a certain area around it is also often removed; the margin of the excision is based on the thickness of the tumour. In case of suspicion of metastatic spread to lymph nodes, which is usually the first place of metastasis, a sentinel lymph node biopsy may be performed. The sentinel lymph node is the first draining lymph node from the primary tumour and is hypothetically the first lymph node metastases gather in. The sentinel lymph node can be found by injecting a radioactive tracer or a dye

near the primary tumour and then surgically remove the nodes that have taken up the radioactive tracer or the dye. By removing and examining the lymph node it is possible to evaluate the metastatic spread of the disease [27-29].

For patients with metastatic disease, surgery is usually not curative but can be utilised to prolong and improve the life of the patient. Depending on how many metastases and where they are located, patients may or may not benefit from surgical excision of their metastatic tumours [30]. Other treatment strategies that may be employed are targeted therapies, locoregional therapies and immunotherapies.

In addition to harbouring mutated versions of *CDKN2A*, *BAP1* and *CDK4*, 40-60% of all melanoma patients do also have a changed version of *BRAF* [31-33]. *BRAF* encodes for the protein B-raf which is a part of the MAPK/ERK pathway; a signal-transduction pathway wherein signalling molecules bind to a receptor on the cell surface and induce an intracellular signalling cascade, ultimately regulating vital cell functions such as cell growth and proliferation. The mutated *BRAF* common in melanoma encodes for a version of the protein that is highly activated [31]. Another component of the MAPK/ERK pathways is N-ras, encoded by the *NRAS* gene. It is also frequently mutated in melanoma, with 15-20% of all melanomas harbouring a mutated variant [32, 33]. Targeted therapies have been developed wherein inhibitors target the most common mutated variant of the *BRAF* gene product or other proteins in the same signalling pathway. Despite a relatively high response rate, many patients will experience progression of their disease since it is common to develop resistance to the inhibitors [34].

For in-transit melanomas, i.e. when the melanoma has started to metastasise and spread from the primary tumour via the lymph vessels but have not yet reached the nearest lymph nodes, locoregional therapies may be an option. Locoregional therapies only treat the part of the body affected by metastatic disease, compared to systemic therapies which affect the whole body. Two locoregional treatment options are isolated regional infusion and isolated regional perfusion with chemotherapeutic agents, the latter being the subject of this thesis and will be described in detail in chapter 1.2.

Immunotherapy has during recent years been an important part in the treatment of metastatic melanoma. Immunotherapy aims to boost the body's own immune system to attack the cancer cells, which might be achieved by e.g. making cancer cells more attractive to immune cells or activating immune cells. Immunotherapy in the treatment of cancer will be discussed further in chapter 1.3.

1.1.1.2. Uveal melanoma

Uveal melanoma arises in the uvea of the eye. The uvea consists of three parts; the iris which controls the size of the pupil and thus the amount of light entering the eye, the ciliary body which holds the lens and produces the fluid found between the cornea and the lens, and the choroid which is a vascular layer of the eye containing connective tissues and blood vessels.

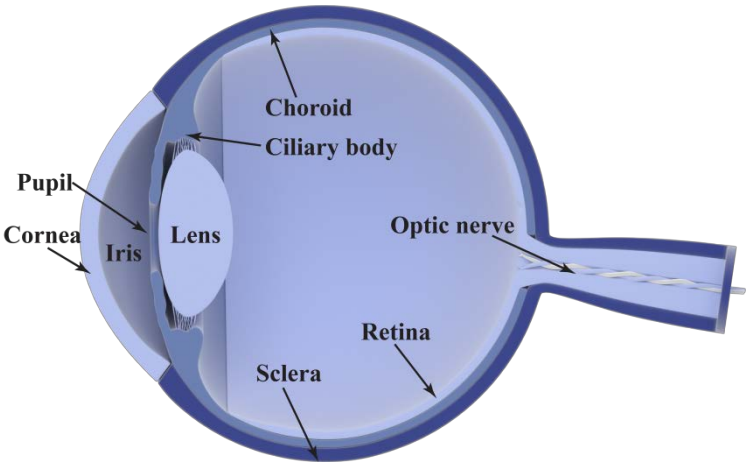

Figure 1. Schematic diagram of the human eye. The uvea consists of the iris, the ciliary body and the choroid.

1.1.1.2.1 Epidemiology and causes

Despite being a rather rare form of cancer, uveal melanoma is the most common intraocular tumour in adults and is the second most common form of melanoma after cutaneous melanoma. It constitutes approximately 5% of all melanoma cases [35, 36]. As with cutaneous melanoma, uveal melanoma is most common in Caucasian populations [37, 38], and in Sweden there are approximately 70 diagnosed uveal melanoma cases each year [12].

In contrast to cutaneous melanoma where exposure to UV radiation is a known risk factor for the development of the disease, the relationship between UV radiation and uveal melanoma is not as clear. Intermittent exposure to sun-derived UV light has not been shown to increase the risk of developing uveal melanoma [39], even though hypothetically melanocytes in the eye should be affected as well. It is known that people with fair skin and light eye colour, such as blue-eyed Caucasians, have a higher risk of developing uveal melanoma [40].

Mutations in the *BAP1* gene have shown to increase the risk of the development of uveal melanoma [41-44]. Two other genes that have shown

to be implicated in the development of cutaneous melanoma, *CDKN2A* and *CDK4*, do not seem to increase the risk of uveal melanoma [45-48], indicating differences in the genetic landscape of the two forms of melanoma.

1.1.1.2.2 Subtypes and staging

The TNM staging system is also applicable for uveal melanoma and can be utilised to further categorise the spread of the disease in a numerical system with stages from I to IV [49, 50]. If the affected eye is removed or a biopsy is taken, the additional stage G might be added. G denotes the pathological appearance of the tumour, i.e. what the tumour cells and the tissue look like when observed with a microscope. Uveal melanoma tumours consist of cells that can be classified as spindle cells or epithelioid cells [51]. An extremely brief explanation of the difference between the two cell types is that spindle cells look more slender and stream-lined, while epithelioid cells are bulkier.

1.1.1.2.3 Prognosis and treatment

The problem with uveal melanoma is that the risk of developing metastatic disease is rather high. 25% of all uveal melanoma patients will have been diagnoses with metastatic disease after five years, and among them the majority will have developed metastases in the liver [52, 53]. Only 20% of all uveal melanoma patients will be alive one year after being diagnosed with metastatic disease, and after two years only 4-8% of all patients are alive [52, 53].

It is not fully known why uveal melanoma has such a high metastasis rate to the liver. Uveal melanoma cells that form metastases travel through the circulatory system of the blood, in contrast to cutaneous melanoma cells that more often traffic the lymphatic system, which is due to the lack of lymphatics in the eye. But this does not explain why uveal melanoma cells end up in the liver, since there are other stops between the eyes and the liver if you travel via the blood road, such as the lungs. Thus, it is thought that the liver produces substances that attract and stimulate the growth of uveal melanoma cells [54]. For example, the liver produces high amounts of C-X-C motif chemokine 12 (CXCL12) which binds to the receptor C-X-C motif chemokine receptor 4 (CXCR4) on e.g. melanoma cells and recruits them to the liver.

Genetic studies of primary uveal melanomas have shown that the loss of one copy of chromosome 3 is strongly correlated to a poor prognosis and higher risk of developing metastatic disease [55-57]. Inactivating mutations in the *BAP1* gene usually occur together with loss of chromosome 3 [58]. It has recently been revealed that primary uveal melanomas can be further divided

into four genetically distinct subgroups with different prognoses; two groups with loss of one copy of chromosome 3 and two groups without loss [59].

The major treatment options for primary uveal melanoma are either removal of the eye (enucleation) or radiotherapy. Enucleation used to be the standard treatment, but it has been shown that there is no difference in survival between patients undergoing enucleation or more conservative treatments such as radiotherapy [60, 61], resulting in enucleation now being utilised only for big tumours where more conservative treatments will not be able to save the patient's vision [62]. One commonly used type of radiotherapy is brachytherapy in which a sealed radiation source is placed next to the tumour. Other types of conservative therapies where the affected eye is not removed include photodynamic therapy, wherein light is used to induce the production of radicals and reactive oxygen species, and thermotherapy, wherein heat is used to treat the tumour [63].

Due to the high rate of liver metastasis formation, management of metastatic disease is of outmost importance. Unfortunately, no specific treatment option has of yet been proven successful in improving survival, despite many different types of therapies being developed and tested [64]. As for cutaneous melanoma, locoregional therapies, targeted therapies and immunotherapies have been tested in various studies. Worth noting is that *BRAF* mutations are not common in uveal melanoma, subsequently rendering B-raf inhibitors useless. Instead, in uveal melanoma mutations in the genes *GNAQ* and *GNA11* are much more common, with over 90% of all primary tumours harbouring mutations in *GNAQ* or *GNA11* [65]. Both genes encodes for proteins that are part of the intracellular IP_3 pathway which regulates the release of calcium inside the cell.

1.2. Isolated regional perfusion

Hyperthermic isolated regional perfusion with chemotherapeutic agents is a locoregional chemotherapy wherein the treatment only affects the part of the body where tumours are located. It is used for cancer patients with tumours confined to a body part or an organ, such as a limb or the liver. The main idea behind isolated regional perfusion is that it enables the administration of much higher concentrations of a chemotherapeutic drug compared to regular systemic chemotherapy; if the drug is only locally administered, systemic side effects and toxicity can be avoided.

At the Sahlgrenska University Hospital in Gothenburg, the only centre in Sweden where isolated regional perfusion is performed, isolated limb perfusion (ILP) is utilised for cutaneous in-transit melanoma with metastatic disease confined to the limbs, while the usage of isolated hepatic perfusion (IHP) in the treatment of uveal melanoma liver metastases is currently being investigated in a randomised clinical trial. Melphalan is the most commonly used chemotherapeutic drug for ILP and IHP, and the procedure is conducted under mild hyperthermia (40^{o}C). Patients with bulky disease or who has undergone repeated perfusions might also receive tumour necrosis factor α (TNF-α).

1.2.1. The technique

Isolated regional perfusion is an old method, first described and used already in the 1950's [66]. Since then the method has been refined and adapted, and today it is used in the treatment of malignant melanoma and other type of solid cancers, such as soft-tissue sarcomas.

A detailed description of the technical aspects of ILP and IHP is beyond the scope of this thesis but can be found elsewhere [67]. The main idea is to redirect the major blood flow to the limb or the liver by clamping the artery and veins, insert cannulas and connect the cannulas to a heart-lung machine, thus creating an isolated circulatory system. The blood that is perfused through the limb or the liver is oxygenated and supplied with high doses of the chemotherapeutic agent melphalan, and the treated tissue is held at a temperature of 40^{o}C. Radioactive isotopes are added to the perfusate to enable monitoring of potential leakage to the systemic circulation. The total perfusion time is 60 minutes, followed by rinsing of the limb or the liver with a salt-based solution to get rid of the melphalan residues.

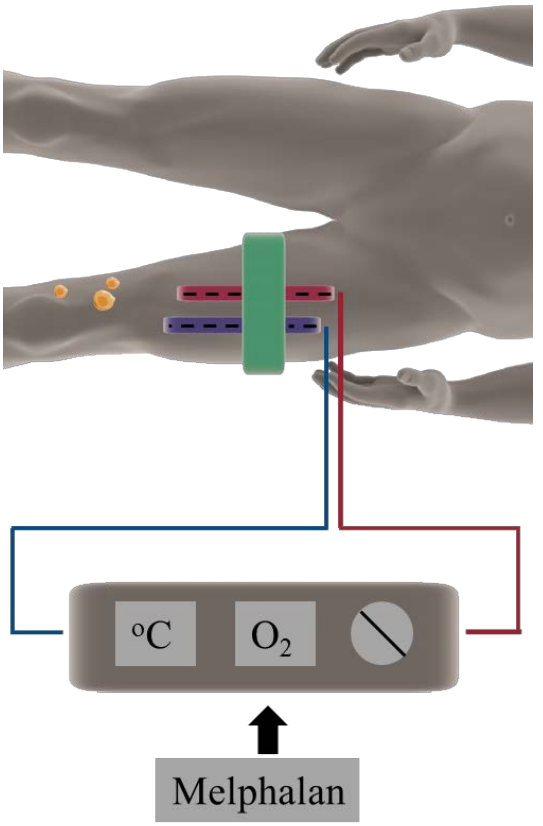

Figure 2. Schematic representation of the set-up for ILP.

1.2.2. Treatment response

Clinical response to the treatment regarding tumour burden is evaluated after three months according to WHO's criteria as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD) [68]. CR is defined as disappearance of all tumours, PR is a decrease of more than 50% of total tumour burden, PD is an increase of more than 25% in existing tumours or the appearance of new tumours, and SD is where none of the criteria for CR, PR or PD are met. ILP with melphalan has a CR rate of 50-70%, with over 85% of all patients achieving an overall response (CR or PR) [69-71]. IHP with melphalan has a CR rate of 20%, with an overall response rate of 70% [72]. Five years after treatment around 30-40% of ILP patients will still be alive [69, 70], while the effectiveness of IHP in improving overall survival is currently being investigated in a clinical study. The SCANDIUM trial (ClinicalTrials.gov identifier number: NCT01785316) is a

clinical study evaluating if IHP improves overall survival compared to best alternative care for uveal melanoma patients with liver metastases [73].

1.2.3. Melphalan

Chemotherapy is the usage of chemical compounds which inhibit cell growth and induce cell death in the treatment of cancer. Chemotherapeutic agents were first used in cancer treatment around the time of World War II. During World War I mustard gas was effectively used as a chemical warfare agent, and during World War II there was continued interest in researching mustard gas. There was an academic interest in the cytotoxic properties of the gas, and the question arose whether or not it could also be used to kill cancer cells. The first time a derivative of mustard gas was intravenously used to treat cancer was in 1942, and the rest, as they say, is history [74-76].

Melphalan, also known as phenylalanine mustard, is a nitrogen mustard derived from mustard gas. It is an alkylating agent and adds an alkyl group to the guanine base of DNA, causing linkage between DNA strands and subsequently inhibition of DNA synthesis and cell death. Is it used for systemic treatment in multiple myeloma and ovarian cancer [77], but systemic administration of melphalan is not feasible for melanoma since the effective dose is higher than what the body tolerates [78]. Thus, melphalan is only administered with ILP or IHP in the treatment of malignant melanoma.

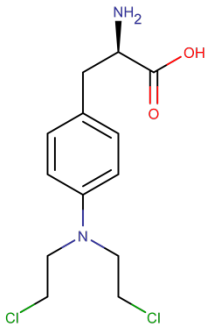

Figure 3. Structural formula for melphalan.

1.2.4. Hyperthermia

ILP and IHP with melphalan is often performed under mild hyperthermia (40^{o}C). There are synergistic effects in play between hyperthermia and melphalan, resulting in enhanced death of the cancer cells [79-81]. There are many underlying factors behind the synergy, such as a higher uptake of melphalan in tumour tissue during hyperthermia [82] and an increase in the amount of DNA crosslinks produced by melphalan [79].

Furthermore, hyperthermia has in itself anti-tumoural properties. In addition to the direct killing of cancer cells by heat, hyperthermia inhibits angiogenesis, thus preventing tumours from forming new blood vessels which are critical for their survival [83, 84]. Hyperthermia has also been indicated to induce anti-tumoural immune responses, as reviewed in [85, 86]. This is due to many different factors, for example the increased expression of heat-induced stress ligands, such as heat-shock proteins (HSPs), which bind

to and activate immune cells such as dendritic cells (DCs) and natural killer (NK) cells.

1.2.5. Tumour necrosis factor α

Tumour necrosis factor α (TNF-α) is a cytokine, a cell signalling protein, and a major driver of the inflammatory response. It has a dual role in the context of cancer; it is anti-tumoural since it directly can kill cancer cells by triggering apoptosis (programmed cell death) in cancer cells, and it is pro-tumoural due to activation of the transcription factor NF-κB and by sustaining an inflammatory tumour environment (remember, inflammation is one of the enabling characteristics for the hallmarks of cancer) [87-91].

TNF-α is sometimes utilised in ILP and IHP due to its ability to enhance the uptake of melphalan into tumour cells by affecting the tumour vasculature and increasing the blood vessel permeability [92, 93]. It is most often utilised for patients requiring multiple perfusions or for patients with big and bulky tumours where uptake of melphalan might be limited due to the tumour size.

1.3. The immune system

The immune system is the body's own defence against disease. It protects us from foreign pathogens, such as bacteria and viruses, but does also provide protection against diseases arising from within the body itself, most notably against cancer. The immune system is very complex and comprises several levels of protection, from the small proteins of the complement system via immune cells to the skin and mucosa, which are our first and biggest barriers against invading pathogens.

There is both innate and adaptive immunity. Briefly described, innate immunity is the part of the immune system which first reacts to an invading pathogen. It provides a swift response with broad specificity. In contrast, adaptive immunity is highly specific and contains immune cells which upon the first encounter with a pathogen develop a "memory" of the pathogen, prompting the cells to react much faster and stronger subsequent times the pathogen is encountered [94].

1.3.1. Immune cells

The immune cells (white blood cells or leukocytes) are cells dedicated to protect the body and are found circulating in blood or stationed in tissues. All blood cells (immune cells, red blood cells and platelets) are produced in the bone marrow and are derived from the same hematopoietic stem cell. The stem cell forms immune cells of two different lineages; lymphoid cells and myeloid cells [94]. The cells have different functions and mature cells can be found in different parts of the body.

Another common classification of immune cells purified from peripheral blood is into peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs). A very common way to separate immune cells from whole blood is by density gradient separation in which blood is placed on top of a hydrophilic polymer solution in a tube and then centrifuged. Due to differences in density between different cells, some cells will sediment through the polymer while others will not, creating a layered solution in the tube after centrifugation. The bottom layer contains red blood cells and PMNs, the layer above is the polymer, on top of the polymer is a thin layer with PBMCs and the uppermost layer contains plasma [95].

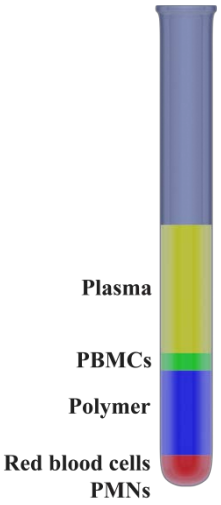

Figure 4. Diagram of density gradient separation of whole blood.

Table 1. Cheat sheet for different classifications of the most common immune cells found in blood.

	Lymphoid	Myeloid	Adaptive	Innate	PBMC	PMN
B cell	X		X		X	
Basophil		X		X		X
Dendritic cell (DC)		X		X	X	
Eosinophil		X		X		X
Monocyte		X		X	X	
Neutrophil		X		X		X
Natural killer (NK) cell	X			X	X	
T cell	X		X		X	

There are many different types of immune cells and many of them cooperate during an immunological response. Below follows descriptions of the three major immune cell populations of relevance for this thesis; dendritic cells, monocytes and T cell.

1.3.1.1. Dendritic cells

Dendritic cells (DCs) are of myeloid lineage and are found in blood in an immature form and in tissues and lymph nodes as more mature cells. They are relatively rare in blood and constitute <1% of all PBMCs [96-98]. DCs are professional antigen-presenting cells (APCs) and are thought to be required for activation of naïve T cells (see section 1.3.2 for more information about antigen presentation). They also secrete many cytokines which help to further stimulate an immune response.

DCs are often classified into three different subtypes; plasmacytoid DCs, conventional $CD1c^+$ DCs and conventional $CD141^+$ DCs [99, 100]. The plasmacytoid DCs are excellent producers of cytokines, especially of type I and III interferons. Of note, type I interferons are of importance for the findings in **Paper III**. The conventional DCs are potent stimulators of T cells. $CD1c^+$ DCs are more abundant than $CD141^+$ DCs, and while both are good at stimulating $CD4^+$ helper T cells, the $CD141^+$ DCs are much better at cross-presenting antigens to cytotoxic $CD8^+$ T cells. During inflammatory conditions or when cultured *in vitro*, DCs might also be derived from monocytes.

1.3.1.2. Monocytes and macrophages

Monocytes do also belong to myeloid-lineage cells, and they share some similarities with DCs. They constitute approximately 10% of all immune cells in blood [101]. Monocytes are APCs and can during certain conditions be converted into DCs. They are also potent cytokine producers. Furthermore, when migrating into tissues they are transformed into macrophages; large immune cells which are stationed in tissues, patrolling the area looking for invading pathogens. Macrophages and monocytes are excellent phagocytes, meaning that they are cells capable of engulfing and subsequently destroying cells and structures detrimental to a healthy body, such as pathogens, cell debris and dead cells (macrophage actually means "big eater" in Greek).

Monocytes are found in blood while macrophages are present in tissues. Monocytes are usually divided into three different subtypes based on the expression of the cell surface receptors CD14 and CD16 [101-103]. Classical monocytes ($CD14^{++}CD16^{-}$) are the most common type of monocyte, constituting around 80-90% of all monocytic cells, and often migrate into tissues to become macrophages. They can also convert into the intermediate ($CD14^{++}CD16^{+}$) and nonclassical ($CD14^{+}CD16^{++}$) monocytes which accumulate during infections and inflammatory conditions and produce high levels of the pro-inflammatory cytokines TNF-α and interleukin 1 β (IL-1β). The different monocyte populations are discussed in **Paper I**.

The majority of tissue-resident macrophages are not descendants of monocytes, but are instead developed before birth from their own pre-cursor cells and are present throughout the whole adult life. During inflammatory conditions the pool of tissue-resident macrophages are maintained through the addition of monocyte-derived macrophages. Macrophages have different functions and different gene signatures depending on which tissue or organ they reside in, suggesting that they are highly adapted to their surrounding environment [104]. They are also given different names depending on tissue type, such as Kupffer cells in the liver, microglia in the brain and osteoclasts in bone.

1.3.1.3. T cells

T cells are lymphocytes essential for adaptive immunity. They activate and regulate various type of lymphocytes (including themselves), and are important in the eradication of cancer cells and virus-infected cells. The "T" in T cell stands for thymus. Like all other immune cells T cells are first developed in the bone marrow, but they mature and are transformed into functional T cells in the thymus. The fraction of T cells in peripheral blood during healthy conditions varies a lot between different individuals, but is

typically around 60-80% of all PBMCs. T cells are of importance for **Papers I-IV** in this thesis.

There are two major T cell subpopulations; helper T cells and cytotoxic T cell. They are distinguished and denoted by the receptors CD4 for helper T cells and CD8 for cytotoxic T cells.

> What's up with all the CDs?
>
> In immunology CD is an abbreviation for "cluster of differentiation" and not for a disc-shaped storage device. It is basically a naming system for cell surface molecules used for identifying different types of cells. A CD structure is usually a receptor or a ligand found on the cell surface, and often their function and relevance to the cell is known. For example, all T cells express CD3, which is a co-receptor to the main T cell receptor which T cells need to recognise antigens. By combining different CD markers it is possible to identify different immune cells, e.g. a cell expressing both CD3 and CD4 but not CD8 (written as $CD3^+CD4^+CD8^-$) is a helper T cell.

1.3.1.3.1 $CD4^+$ helper T cells

$CD4^+$ T cells are regulators of immunity; activating or inhibiting responses from e.g. $CD8^+$ T cells and the antibody-producing B cells by receptor interactions and secretion of cytokines. The $CD4^+$ T cells are further classified into different groups based on which cytokines they produce and which transcription factors that are responsible for the development of that particular $CD4^+$ T cell subtype. T_{h1} cells produce interferon γ (IFN-γ) and interleukin 2 (IL-2) which activate $CD8^+$ T cells and macrophages. They are important for the development of immune responses against intracellular pathogens. In contrast, T_{h2} cells mediate responses against extracellular pathogens by the production of e.g. interleukins 4 and 5 (IL-4, IL-5) which, among many things, activate B cells, basophils and eosinophils. T_{h17} cells primarily produce interleukin 17 (IL-17) which recruits and activates neutrophils to help in the eradication of extracellular bacteria and fungi. T regulatory cells (T_{regs}) are another member of the $CD4^+$ T cell family. Compared to the other $CD4^+$ T cells, T_{regs} have a different function; instead of activating an immune response, T_{regs} are responsible for suppressing it. T_{regs} are responsible for curbing the activity of other immune cells, particular other T cells, that otherwise would lead to damage; such as overreactive T cells or T cells that mount a response against healthy tissue, causing autoimmune disorders. This is done through many mechanisms, one is the secretion of immunosuppressive cytokines such as transforming growth factor β (TGF-β) and interleukin 10 (IL-10) [105, 106].

$CD8^+$ cytotoxic T cells

While $CD4^+$ T cells regulate and facilitate the induction of an immune response, $CD8^+$ T cells directly kills abnormal cells. After being activated

and told which cell to target (via antigen-presentation by APCs), the $CD8^+$ T cell kill the target cell through induction of apoptosis via the release of cytotoxic molecules or direct cell-to-cell contact. They contain and release perforin, which creates pores in the cell membrane of the target cell, and granzyme B, which enters the target cell through the pores and induces apoptosis. Alternatively, the structure Fas ligand (FasL) on the surface of activated $CD8^+$ T cells can bind to the receptor Fas on the target cell. Upon binding the FasL/Fas complex triggers the activation of an intracellular signalling cascade, ultimately leading to apoptosis of the target cell [107].

1.3.2. Immune responses against pathogens

What really happens during an infection or when the immune system mounts a response against cancer cells? Do the immune cells just circulate throughout the body and attack everything that seems suspicious? The truth is that the immune system is a highly complex and dynamic system, where different cells have different roles. The threats the immune cells need to recognise and eliminate are of widely different natures; from foreign bacteria and parasites, to virus that infiltrate the body's own cells and to cancer cells which are altered host cells, meaning that different kinds of immune responses need to be initiated. One thing that is common for all immune responses is that they always include collaborations between different kinds of immune cells.

1.3.2.1. Extracellular pathogens

During an infection by foreign pathogens, e.g. bacteria, the bacteria are first recognised by innate immune cells patrolling the area which happened to be infected. Among the first responders are the tissue-resident macrophages that try to eliminate the pathogens by e.g. phagocytosis. Macrophages, and other myeloid cells, express pattern recognition receptors (PRR) on the cell surface which bind to certain patterns, or structures, found only on microbes, so called pathogen-associated molecular patterns (PAMPs). The stimulation of PRRs triggers the activation of transcription factors in the myeloid cells, which among other things leads to production and secretion of cytokines that regulate other parts of the immune system. One important group of cytokines are chemokines, which are proteins stimulating other cells to migrate to the area of infection. By following increasingly higher concentrations towards the source of the chemokine, immune cells stationed far from the site of infection can find their way to the pathogen. Innate myeloid cells, such as neutrophils, are abundant in blood but not in tissues. However, during an infection neutrophils rapidly respond and migrate to the area of infection and can rapidly start eliminating the pathogen [94]. In contrast to the myeloid cells, the activation and recruitment of T cells is much more complex.

Naïve T cells are mainly present in secondary lymphoid organs such as lymph nodes or the spleen (the primary lymphoid organs are the bone marrow and the thymus where immune cells are created and mature), waiting to be activated. An APC, for example a DC, needs to take up a part of the pathogen (the antigen), travel through the lymph vessels to the lymph node and present the antigen for the T cell. By presenting the antigen on cell surface structures called major histocompatibility complexes (MHC), the antigen becomes recognisable by the T cells. T cells have different T cell receptors (TCRs) that recognise different antigen-MHC complexes, and if a T cell with the correct TCR recognises the antigen in the context of MHC, the T cell becomes activated and starts to proliferate. This is a process called antigen-presentation. There are two kinds of MHC; class I and class II. All APCs express MHC class II and use it to present antigens to $CD4^+$ T cells, while $CD8^+$ T cells can only recognise antigens presented on MHC class I. After being activated towards its particular antigen for the first time, T cells differentiate into effector and memory T cells. The memory cells are long-lived and will react rapidly the second time they encounter the same antigen, thus enabling them to mount a faster response [94].

1.3.2.2. Viruses and cancer

In contrast to the restricted expression of MHC class II, almost all cells in the body have MHC class I. This is because all cells need to be able to send a message to the immune system in case they become intracellularly infected, for example by viruses. By constantly sampling their own insides and presenting peptides on the MHC class I molecules, infected cells show circulating memory $CD8^+$ T cells what is inside them. If the presented peptide is a "normal" peptide, the T cells will not interfere. But if the presented peptide is of foreign origin, e.g. virus-derived, and the $CD8^+$ T cell has been pre-activated against this peptide by an APC, the $CD8^+$ T cell will start to eradicate the infected target cell [94].

While normally only intracellular peptides are presented on MHC class I, APCs can present both extracellular and intracellular antigens on MHC class I. In order for the naïve T cells, which have no memory functions, to recognise an intracellular antigen from a target cell for the first time, an APC must present the peptide (which for the APC is extracellular) on MHC class I to a naïve $CD8^+$ T cell. DCs are the APCs that most effectively can do this kind of antigen-presentation, which are known as cross-presentation [94]. Cross-presentation is of importance for the eradication of tumours since cancer cells do not have any "foreign" antigens that may trigger an immune response.

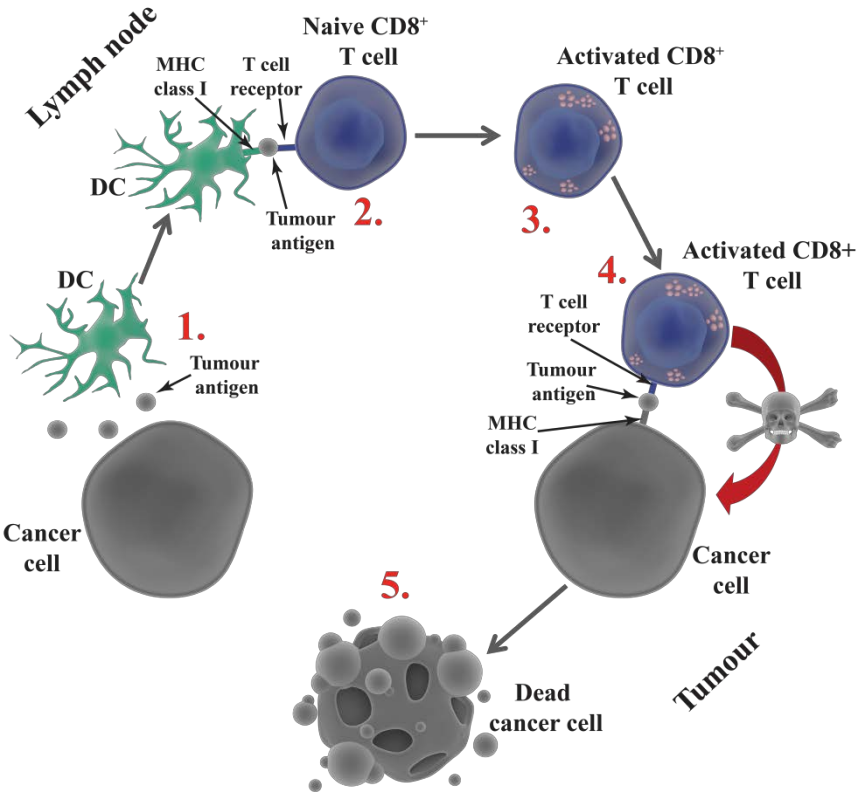

Figure 5. Very simplified depiction of initiation and implementation of an anti-tumour response. **1)** An APC, here a DC, takes up a tumour antigen (e.g. a DAMP) from a cancer cell and travels to the lymph node where **2)** it cross-presents the antigen to a naïve tumour-specific $CD8^+$ T cell. **3)** The T cell becomes activated and travels to the tumour where it searches for the same antigen presented on MHC I by the tumour. **4)** When the T cell finds the same antigen it will **5)** kill the cancer cell.

One form of antigen that DCs can take up from the tumour microenvironment and cross-present to naïve $CD8^+$ T cells to start an immune response is a damage-associated molecular pattern (DAMP). In contrast to the PAMPs which are extracellular structures found on microbes, DAMPs are intracellular molecules that are released in the extracellular environment or expressed on the cell surface when the cell is stressed or injured. DAMPs are generally structures which during normal conditions only can be found inside the cell, such as RNA/DNA, the energy-carrying molecule adenosine triphosphate (ATP) and the calcium-binding protein calreticulin [108-110]. If a cell dies from non-immune related causes, it may release, for example, its DNA into the environment.

During a particular type of cell death called immunogenic cell death (ICD), dying cells expose and release high amounts of DAMPs, which stimulate the recruitment and activation of DCs and facilitate the cross-presentation to naïve $CD8^+$ T cells, thus initiating an anti-tumour response [108, 109]. Certain chemotherapeutic agents are known to induce ICD, and this will be further discussed in the next section.

1.3.3. Cancer immunology

During recent years it has been increasingly more known that the immune system is important in the fight against cancer. Different immunotherapies boosting and regulating the immune response have successfully been applied to the treatment of different cancers. The academic journal *Science* awarded cancer immunotherapy the prestigious "Breakthrough of the Year" award for 2013 [111], and the Nobel Prize in Physiology or Medicine 2018 was awarded jointly to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation" [112].

1.3.3.1. Immunoediting

Immunoediting is a balance between tumour progression and immune-derived tumour suppression. It is a dynamic process showing how immune cells can both suppress and promote tumour growth, and it also shows how cancer cells are both negatively and positively affected by the inflammatory environment they reside in. Immunoediting consists of three phases, called the three E's of cancer immunoediting: elimination, equilibrium and escape.

Elimination, also known as cancer immunosurveillance, is when the immune system actively fights cancer cells, as previously described. During the elimination phase the immune system might succeed in eradicating all cancer cells, in which the immunoediting process finishes and does not progress to equilibrium. If some malignant cells survive, the process enters the equilibrium phase. During equilibrium the immune cells are trying to eradicate the remaining cancer cells, succeeding in containing them but not fully destroying them. This phase might persist for many years; illustrating that the time lag from the appearance of malignant cells to the formation of tumours may be very long. Due to the high selection pressure exerted by the immune system on the cancer cells, some cancer clones arise which are immunologically resistant. These clones escape the immunological attack, proliferate and form tumours which cannot be contained by the immune system. One commonly employed immune escape mechanism is the downregulation of MHC class I on cancer cells, thus hindering $CD8^+$ T cells from recognising the cancer cells. Moreover, tumours may actively suppress the immune system by secreting immunosuppressive cytokines, express ligands to inhibitory receptors found on immune cells, and recruit

immunosuppressive immune cells, such as T_{regs} and myeloid-derived suppressor cells (MDSCs) [113, 114].

1.3.3.2. Immunogenic cell death

For a long time cell death was thought to include only apoptosis, a programmed and regulated non-inflammatory death, and necrosis, a non-programmed death caused by trauma and injury and which elicits an inflammatory response. It is now known that there are many different types of regulated cell death in addition to apoptosis; all triggered by different events, driven by different intracellular pathways [115].

Immunogenic cell death (ICD) is a regulated type of cell death which induces an immunological response. It is caused by certain chemotherapeutic drugs, but also by other treatments for cancers, such as radiotherapy and photodynamic therapy. During ICD the dying cancer cells release or express DAMPs which recruit and activate APCs [108, 109, 116].

Studies have shown that exposure to melphalan causes the expression of some of the markers for ICD in different cancers, indicating that melphalan induces ICD, or at least some sort of regulated and immunogenic or inflammatory type of cell death. Treatment with melphalan in models of B cell lymphoma and colorectal cancer caused the cells to release high mobility group box 1 (HMGB1), an intracellular protein important for DNA transcription and a prototypical marker for ICD, and the upregulation of surface-expressed calreticulin [117]. In melanoma models exposure to melphalan did not cause any significant upregulation of surface-expressed calreticulin or release of extracellular ATP, though it did induce the expression of the immunogenic-related stress marker heat shock protein 90 (Hsp90) and caused release of pro-inflammatory cytokines. Moreover, when immunocompetent mice were injected with dying melphalan-exposed melanoma cells and subsequently challenged with live non-exposed melanoma cells in a vaccination trial, the vaccinated mice had slower tumour growth. The protective effect of the vaccination was dependent on the presence of $CD8^+$ T cells in the mice [118].

1.3.3.3. Immunotherapy

Cancer immunotherapy includes a wide variety of different treatments which enhance or regulate an already existing anti-tumour immune response or induce an immune response if one if lacking. Immunotherapies can include treatment with proteins, such as cytokines and antibodies, and also the administration of modified and activated immune cells.

Two examples of cytokines which are approved for the treatment of various cancers are IL-2 and interferons. Both are approved for the treatment of

advanced metastatic melanoma. To achieve durable responses during monotherapy high concentrations of cytokines are often needed, which cause severe systemic toxicities. Therefore, cytokines are more commonly being utilised in combination therapies rather than as monotherapies [119].

Monoclonal antibodies are extensively used in both treatment and research of cancer. There are unconjugated antibodies and conjugated antibodies, the latter are antibodies modified to carry other molecules, such as radioactive isotopes or dyes. Antibodies may act in a number of ways. They can bind directly to antigens on the cancer cells and cause death or inhibit cell growth through e.g. by binding to receptors or ligands and inhibit important intracellular signalling, label the cancer cells so that immune cells can find them, or deliver drugs such as radioactive isotopes or chemotherapeutic agents directly to the cancer cells. They can also bind to immune cells and regulate their activities, such as the highly successful immune checkpoint inhibitors which will be discussed in the next section. It is also possible to alter the tumour microenvironment with antibodies, for example by inhibiting angiogenesis [120].

Some antibody-related terminology:

- Antibody: Protein produced by B cells which recognises and binds to a unique molecule on a pathogen.
- Antigen: The structure on pathogens recognised by antibodies and the T cell receptor.
- Epitope: The specific part of an antigen that an antibody binds to; one antigen may have many epitopes.
- Monoclonal antibodies: Antibodies produced by one specific clone of B cells, recognise only one epitope on an antigen.
- Polyclonal antibodies: Antibodies produced by many B cell clones, recognise the same antigen but different epitopes.

Modified and activated immune cells have also been of interest in the treatment of cancer. In these kinds of treatments, the immune cells of interest are recovered from the patient, modified and expanded to more efficiently fight cancer cells, and transferred back into the patient. DC-based cancer vaccines are treatment options where DCs, or monocytes which can be converted into DCs, are extracted from the patient and cultured *ex vivo*. The immature DCs are loaded with tumour-specific antigens, such as killed tumour cells or tumour cell lysates, peptides or nucleic acids. They are then matured in culture and transferred back into the patient, aiming to induce an anti-tumoural T cell response. The only DC vaccine which is approved for clinical use today is a vaccine targeting prostate cancer [121].

It is also possible to directly target T cells instead of first activating DCs. In adoptive cell transfer (ACT) T cells are recovered from the patient, get heavily expanded *ex vivo*, and are then transferred back into the patient who

has undergone lymphodepletion to remove existing lymphocytes, thus removing immunosuppressive T_{regs} and making space for the expanded T cells. The T cells can be derived from resected tumours or from peripheral blood. Before expansion the T cells are first screened in order to identify clones reactive against tumour antigens, or if none can be found, the T cells can be genetically engineered to be tumour-specific [122]. For cutaneous melanoma patients it is possible to find and expand tumour-specific T cells directly from tumour biopsies. Cutaneous melanoma is known to be a highly immunogenic tumour, possibly due to melanoma being one of the forms of cancer harbouring the highest numbers of mutations [123]. A high mutational burden is correlated with a high amount of neoantigens; newly formed antigens negatively selected against in the thymus. Thus, a tumour with many neoantigens has in theory many new antigens for T cells to target.

1.3.3.3.1 Immune checkpoint inhibitors

The success of immune checkpoint inhibitors in the treatment of cancers, particular cutaneous metastatic melanoma, is one of the main reasons why the field of cancer immunotherapy has grown significantly during recent years.

Immune checkpoint inhibitors can very briefly be described as antibodies releasing the brakes on T cells. Activated T cells need to be suppressed and inhibited to not cause unwanted damage. This might be achieved through the suppressive T_{regs}, but also through inhibitory receptors present on the surface of T cells, so called inhibitory immune checkpoints. Upon binding to their cognate ligands, which are often expressed on myeloid cells, the receptors inhibit T cell activity through different pathways. The two most known and targeted immune checkpoint receptors are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), though many more exist and are currently being researched.

Antibodies targeting CTLA-4 were the first immune checkpoint inhibitors to be developed for therapeutic purposes. CTLA-4 binds to the ligands CD80 and CD86 on APCs, which are also ligands to the T cell receptor CD28. In contrast to CTLA-4 which is an inhibitory receptor, CD28 mediates activating signals, and the binding of CD28 on a naïve T cell to CD80 or CD86 on an APC is necessary for the T cell to be fully activated. CTLA-4 and CD28 competes with each other for binding to the ligands, thus regulating the activation status of the T cell. CTLA-4 is expressed on T_{regs}; the competitive binding to CD80/CD86 being one of the mechanisms by which T_{regs} can suppress the function of other T cells. CTLA-4 primarily regulates T cells in lymph nodes or other secondary lymphoid organs, thus playing a part mainly during the early stages of the immune response [124, 125].

The ligands to PD-1, programmed death-ligands 1 and 2 (PD-L1, PD-L2), are not only expressed on myeloid cells, but can also be upregulated on cells not part of the immune system. They can be induced by inflammatory cytokines such as IFN-γ, which are produced by various immune cells upon activation, thus rendering the PD-1 pathway more important during an already ongoing immune response, in contrast to the early stage CTLA-4 pathway. Upon binding to its ligands, the PD-1 receptor transmits inhibitory signals, leading to inhibited activity and exhaustion wherein the T cell has limited functionality in presence of its antigen [124, 125]. The PD-1/PD-L1 axis has also been shown to promote the conversion of naïve $CD4^+$ T cells and T_{h1} cells into T_{regs}, thus contributing to an additional suppressive function of PD-1 [126, 127]. Many tumour cells are known to express PD-L1 and PD-L2 on their surfaces, thus being able to themselves inhibit T cell functionality through the PD-1 pathway and escape anti-tumoural immune responses.

Checkpoint inhibitors were first approved for the treatment of melanoma in 2011, but have since then been approved for the treatment of e.g. non-small cell lung cancer, renal cell carcinoma and Hodgkin lymphoma [124]. In a large study wherein patients with stage III and IV cutaneous melanoma were treated with an anti-PD-1 inhibitor, an anti-CTLA-4 inhibitor or a combination of both, it was seen that patients treated with an anti-PD-1 agent, either alone or in combination with anti-CTLA-4, had a longer overall survival compared with patients treated with only anti-CTLA-4 inhibitors. Moreover, after four years approximately 50% of all patients treated with anti-PD-1 alone or in combination with anti-CTLA-4 were alive [128]. This can be compared with the five year survival rate in Sweden for stage III and stage IV melanoma patients which before the advent of immune checkpoint inhibitors were 36% respectively 25% [17].

Despite the success of immune checkpoint inhibitors in the treatment of cutaneous melanoma, they have not worked as well in uveal melanoma. In clinical trials wherein patients with stage IV uveal melanoma were treated with anti-PD-1/PD-L1 antibodies alone or together with anti-CTLA-4 antibodies, only 4-5% of all patients had a response to anti-PD-1/PD-L1 monotherapy, while 17% responded to the combination therapy [129, 130]. For patients with stage III or IV cutaneous melanoma, 45% respond to anti-PD-1 monotherapy and up to 58% respond to combination therapy [128]. This discrepancy between cutaneous and uveal melanoma regarding efficacy of immune checkpoint inhibitors implies that immune control mechanisms may be fundamentally different in these two diseases.

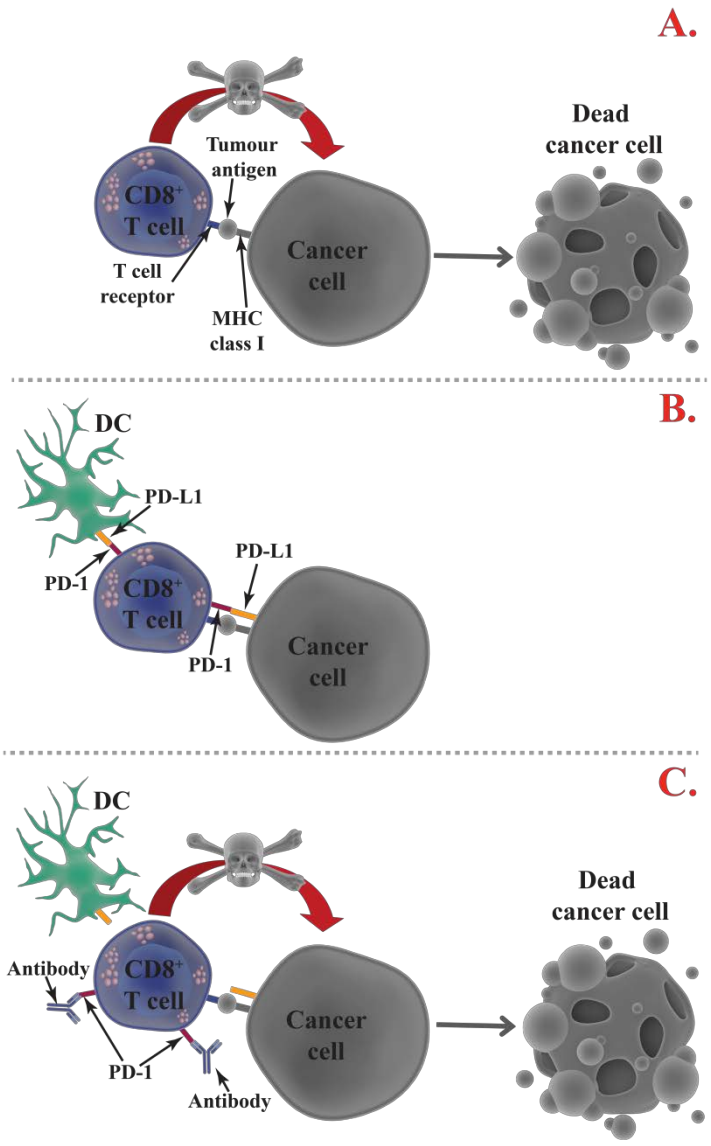

Figure 6. A simplified depiction of the mechanisms behind anti-PD-1 treatment. **A)** shows a $CD8^+$ T cell attacking a cancer cell without any inhibitory mechanisms involved. In **B)** the T cell is inhibited through binding of its PD-1 receptors to PD-L1 on myeloid cells, here a DC, and on the cancer cell. In **C)** the PD-1/PD-L1 interaction is blocked through antibodies targeting PD-1, thus enabling the T cell to kill the cancer cell.

1.4. Melanoma, isolated regional perfusion, and immunology

It is known that melanoma, at least cutaneous melanoma, is a very immunogenic tumour, as evidenced, for example, by the success of the immune checkpoint inhibitors. Couple that with the knowledge that some chemotherapeutic agents induce an anti-tumoural immune response via ICD and it is not hard to imagine that chemotherapy in the treatment of melanoma may function through two ways; via direct killing of the tumour cells and via induction of an anti-tumoural immune response.

The strength of utilising isolated regional perfusion to administer chemotherapeutic agents is that the administration is local and not systemic. In addition to achieving high tissue concentrations of the chemotherapeutic drug, only the isolated body part should be affected by the drug, in theory preventing the development of systemic side effects, such as bone marrow suppression which leads to a decreased production of e.g. immune cells. While systemic chemotherapy might render the surviving cancer cells immunogenic, it might also wipe out the immune cells, which theoretically should be less of a problem during locoregional chemotherapy.

The potential impact of the immune system on the clinical outcome to isolated regional perfusion with melphalan is supported by the frequently slow regression of melanoma tumours. It may take several months until a melanoma patient achieves a complete response (CR) after ILP [131], suggesting that the last remaining tumours are not directly killed by the cytotoxic effects of melphalan, but rather of an activated immune response. In a pilot study investigating if ILP with melphalan in the treatment of cutaneous metastatic melanoma induced an anti-tumoural immune response, it was shown that patients who achieved a CR after ILP had higher amounts of cytotoxic $CD8^+$ T cells before treatment and that these patients also had more activated T cells [132]. These findings indicate that there might be an immunological component at play. If this is the case, it suggests that it might be beneficial to combine isolated regional perfusion with immunotherapy in the treatment of metastatic melanoma.

2. Aims

The main objective for this thesis was to investigate the role of the immune system for the treatment response after isolated regional perfusion with melphalan in patients with metastatic melanoma. Specific aims for each paper are as follows:

- **Paper I** and **Paper II** aimed to define whether or not a cellular immune response is induced and is of importance for the anti-tumoural effects of isolated limb perfusion (ILP) with melphalan.

- **Paper III** aimed at defining serum factors of relevance for the treatment response to ILP, in particular chemokines encoded by interferon-stimulated genes (ISGs).

- The aim of **Paper IV** was to define how the composition of immune cells in blood and tumour of patients with uveal melanoma liver metastasis affected the treatment response to isolated hepatic perfusion (IHP) with melphalan.

3. Methodology

The data presented in this thesis were generated from experiments and analyses of blood and tumour samples from melanoma patients treated with ILP and IHP, from *in vivo* murine vaccination models and from *in vitro* cell culture models of perfusion.

3.1. Isolated regional perfusion

Papers I-III contain data generated from analyses of blood samples and tumour biopsies from patients with cutaneous melanoma in-transit metastases undergoing treatment with melphalan-based ILP. PBMCs and serum were obtained before and after ILP, and a limited number of metastatic tumour biopsies were obtained during the surgical procedure. The majority of the patients underwent perfusion of the leg, with a few undergoing perfusion of the arm. All perfusions were performed at hyperthermia (40^{o}C).

Paper IV contains data from patients with uveal melanoma liver metastases treated with melphalan-based IHP. Patients were enrolled from the IHP-treatment arm of the SCANDIUM trial, and PBMCs were collected before IHP from each patient. A limited number of metastatic tumour biopsies were obtained during the surgical procedure.

Patients were treated at the Sahlgrenska University Hospital, with clinical responses evaluated at each patient's referring hospital after three months. The studies were approved by the Regional Ethical Review Board in Gothenburg, and patients gave written informed consent before enrolment.

3.2. *In vivo* murine model

In **Paper I** a murine melanoma model was utilised to investigate if injection of melphalan-exposed dying melanoma cells into mice could function as a protective vaccine against tumour growth. Immunocompetent C57BL/6 mice were injected with B16-F1-OVA cells which had previously been pre-exposed to sub-lethal concentrations of melphalan. The B16F1-OVA cell line is a murine melanoma cell line originally derived from the C57BL/6 mouse strain, engineered to express the chicken egg protein ovalbumin (OVA). Ovalbumin in itself does not cause any harm to the mice, but it does induce a T cell-dependent immune response [133]. As the mice have not previously been exposed to ovalbumin, all ovalbumin-specific immune responses detected in this model must originate from the reactions towards the B16F1-OVA cells. Therefore, measurements of OVA-specific T cells may be utilised as a means to quantify tumour-specific T cell responses.

Approximately one week after injection of the cell-based vaccine the mice were subcutaneously injected with live B16-F1-OVA cells to generate tumour growth. The tumour growth was monitored, and at the end of the experiment all tumours were excised and analysed for immune cell infiltration by flow cytometry.

The murine experiments were approved by the Animal Ethics Research Committee in Gothenburg.

3.3. *In vitro* perfusion model

In order to in more detail investigate the effect of a short-term exposure of melanoma cells to melphalan and the subsequent effects on immune cells, an *in vitro* model of isolated regional perfusion was created wherein human melanoma cell lines were exposed to sub-lethal concentrations of melphalan during one hour at hyperthermia. After the exposure the cells were thoroughly washed to remove the chemotherapeutic drug. PBMCs obtained from healthy donors were added to the melanoma cell culture for two days, after which supernatants were analysed for soluble factors and effects on myeloid cells were analysed by flow cytometry. For experiments regarding effects of melphalan-exposed melanoma cells on T cells, the non-adherent PBMCs were transferred from the melanoma cell co-culture and cultured in a monoculture in the presence of IL-2 for an additional four days or two weeks. This model was utilised in **Paper I** and **III**.

3.4. Methods

Different experimental methods were utilised to generate data in this study. The main methods were flow cytometry which was utilised for characterisation of immune cell phenotypes, immunoassays (Luminex-based multiplex assays and ELISAs) for detection of soluble factors in serum and cell culture supernatants), RT-qPCR for quantification of gene expression and immunohistochemistry for detection of immune cells in tumour biopsies.

More detailed information about the experimental procedures can be found in **Papers I-IV**.

4. Results and discussion

Several different immune cell populations were investigated in the studies that constitute this thesis. Apparent throughout all papers was that a short-term exposure of melanoma cells to melphalan, as during isolated regional perfusion, appeared to have an impact on nearby APCs, such as monocytes and dendritic cells, and on T cells. Moreover, it was found that melanoma patients harbouring immune cells of certain phenotypes showed a more favourable outcome after treatment with isolated regional perfusion.

4.1. Melphalan induces an immunogenic-type of cell death in melanoma cells

When chemotherapeutic treatments induce ICD in cancer cells, they upregulate or release DAMPs which facilitate the initiation of an anti-tumour immune response. In **Paper I** we investigated if melphalan could induce ICD in melanoma cells. To this end, human melanoma cell lines were exposed to sub-lethal concentrations of melphalan, causing around 15-30% cell death, for one hour at 40^{o}C to mimic the conditions for hyperthermic isolated regional perfusion. Approximately 24 hours after the end of the exposure the melanoma cells were analysed by flow cytometry in order to investigate whether they expressed DAMPs typically associated with ICD on their cell surfaces. Upon treatment with melphalan, melanoma cells upregulated the expression of MHC class I, heat-shock protein 70 (Hsp70) and PD-L1. Hsp70 is an ICD-associated DAMP that promotes an anti-tumoural response through e.g. facilitating cross-presentation of tumour antigens by APCs and stimulating DC maturation [134]. MHC class I and PD-L1 are not prototypical DAMPs but are important in the regulation of anti-tumoural immune responses; expression of MHC class I is necessary for $CD8^+$ T cells to recognise cancer cells, and cancer cells can upregulate the expression of PD-L1 to inhibit the functionality of $CD8^+$ T cells.

In contrast, melphalan did *not* cause an induction of surface-expressed calreticulin on melanoma cells, which is one of the major markers for ICD [116]. This is in line with reports from other studies [118]. When the same melanoma cells were exposed to sub-lethal concentrations of daunorubicin, a chemotherapeutic agent belonging to the class of anthracyclines which are known to be potent inducers of ICD [116], the cells showed a high expression of calreticulin, as expected during ICD.

To further investigate the immunogenicity of melphalan-exposed melanoma cells, these cells were injected into immunocompetent mice to form a cancer

vaccine. When the mice were re-challenged with non-exposed live melanoma cells injected subcutaneously, the mice who had received the cell-based vaccine experienced reduced tumour growth (**Figure 7**). As a control, one group of mice were instead injected with melanoma cells exposed to the alkylating agent mitomycin C, which has shown to be a very weak inducer of ICD [135, 136]. In comparison to the mitomycin C-based vaccine, the melphalan-based vaccine was much more potent, indicating that the protective effects of the vaccines is not simply due to the presence of dying melanoma cells, and that the way in which the cells are dying matters. Moreover, tumours from mice vaccinated with the melphalan-based cell vaccine contained a higher fraction of total and of tumour-specific $CD8^+$ T cells, suggesting that the protective effects of the vaccine are of an immunological origin.

Even though dying melphalan-exposed melanoma cells do not express all the prototypical markers for ICD, they express some of them, and they provide protection against tumour growth in a murine vaccination model, which is considered to be a gold-standard method to monitor ICD [116]. Taken together it is apparent that melphalan causes an immunogenic or inflammatory type of cell death in melanoma cells.

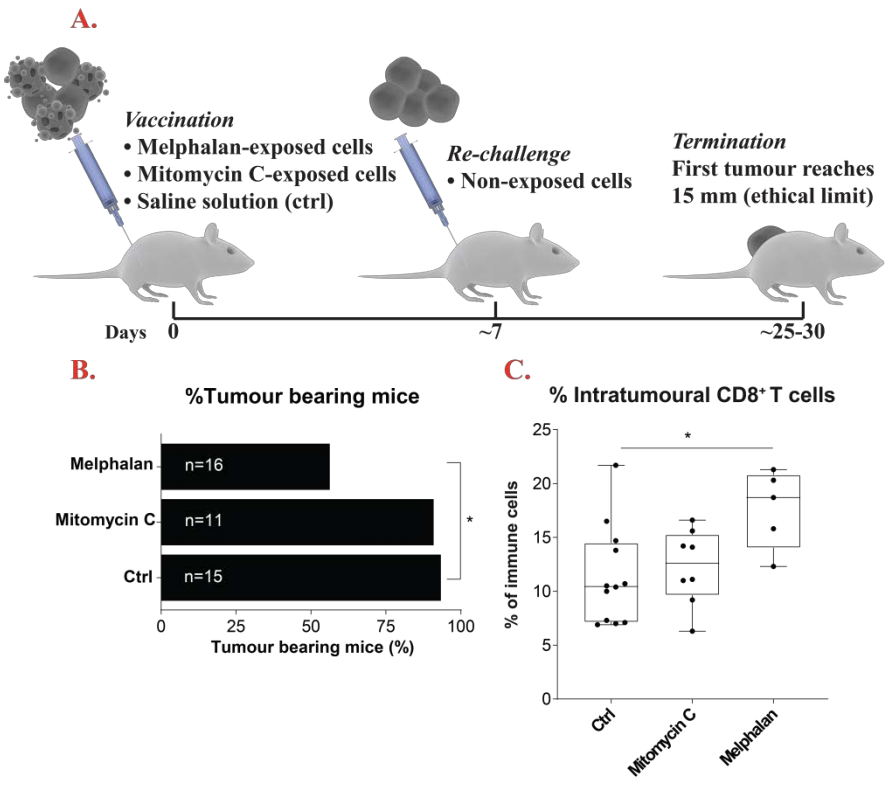

Figure 7. A) Set-up for a murine vaccination model wherein immunocompetent mice were injected with melphalan- or mitomycin C-exposed melanoma cell or a saline control before being re-challenged with live non-exposed melanoma cells. At the end of the experiment when the first tumour reached the ethical limit (15 mm), all mice were euthanized and the tumours were excised. **B)** Mice which had been vaccinated with the melphalan-based cell vaccine were protected against, or experienced less, tumour growth. **C)** Those mice did also harbour a higher fraction of $CD8^+$ T cells in their tumours.

4.2. Melphalan-exposed melanoma cells triggers induction of $CD16^+$ monocytes and interferon-stimulated gene products

In order to successfully mount an anti-tumoural T cell response, it is of uttermost importance to have activated and functional APCs. In **Paper II** we analysed various myeloid cell populations in peripheral blood from cutaneous melanoma patients treated with ILP and *in vitro* cell culture models of perfusion to investigate how a short-term exposure of melanoma cells to melphalan affects surrounding myeloid cells. Even though no correlation could be found between treatment response to ILP and percentage of different myeloid cell populations in peripheral blood of melanoma patients, it was seen that the fraction of $CD16^+$ monocytes, but not of classical $CD14^{++}CD16^-$ monocytes, increased after ILP. This was also apparent in the *in vitro* model where the presence of melphalan-exposed melanoma cells drove the expansion of $CD16^+$ nonclassical monocytes. In the *in vitro* model there was also an increase in the percentage of DCs, though this could not be seen in blood of melanoma patients.

As previously discussed, one important function of DCs and monocytes in addition to provide antigen-presentation to T cells is the production of cytokines. In **Paper III** we investigated the profile of cytokines and other soluble factors in serum from ILP patients and correlated them to clinical outcome. We saw that patients who achieved complete response (CR) after ILP had higher serum levels of the chemokines C-X-C motif chemokine 10 (CXCL10) and C-C motif chemokine ligand 2 (CCL2), and of soluble PD-L2, compared to patients who did not achieve CR (**Figure 8**). The levels of these proteins did also tend to increase after treatment. Patients who had high levels of all three proteins generally had a better clinical outcome than patients with low levels. It was seen in an *in vitro* model of hyperthermic isolated perfusion that the presence of melphalan-exposed melanoma cells caused a very high production of CXCL10 and CCL2 in a co-culture setting with PBMCs, where the presence of non-exposed melanoma cells did not trigger the same chemokine production. We did also investigate the serum level of other cytokines, such as C-C motif chemokine ligand 4 and 5 (CCL4, CCL5), but these chemokines did not show the same patterns as CCL2 and CXCL10.

One common feature of CXCL10, CCL2 and PD-L2 is that they are all encoded by interferon-stimulated genes (ISGs). ISGs are genes induced by interferons through the intracellular JAK-STAT pathway [137] and encode for proteins important in the defence against virus, bacteria and other

pathogens. They also encode for proteins important in immune modulation and chemotaxis. CCL2 is mainly produced by monocytes and macrophages, and its expression is induced by type I and type II interferons [138, 139]. CXCL10 is also produced by monocytes, in addition to other cell types, but it is mainly induced by type II interferons [140]. The expression of PD-L2 has been shown to be stimulated by both type I and type II interferons, in contrast to PD-L1 which mainly is induced by type II [141]. The interferons themselves are primarily produced by e.g. DCs (the type I interferons) and by activated T and NK cells (the type II interferons) [142]. Of note, $CD16^+$ monocytes can produce both type I interferons and ISG products upon stimulation [143-145].

Human interferons:

- Type I: IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω
- Type II: IFN-γ
- Type III: IFN-λ

Type I and II interferons have been shown to be important regulators of anti-tumoural immune responses. One explanation is that many ISGs encode for chemokines which recruit immune cells, such as T cells, to the tumour. In melanoma patients, a high level of chemokines encoded from ISGs, such as CXCL10, correlate with a higher infiltration of T cells [146]. It has also been shown that chemotherapeutic treatment with anthracyclines of various solid cancers causes the cancer cells to produce type I interferons which upregulates the expression of ISGs within the cancer cells themselves [147]. However, in our experiments we did not observe any induction of CXCL10 or CCL2 production from melanoma cells following exposure to melphalan, which is of note not an anthracycline.

To find a link between chemokines and tumour-infiltrating T cells, we correlated expression levels of CCL2 produced by cell cultures from tumour biopsies obtained during the ILP procedure with the amount of tumour-infiltrating T cells from the same biopsies. We found that high CCL2 levels correlated with a decreased fraction of infiltrating $CD4^+$ T cells and an increased fraction of $CD8^+$ T cells among T cells within the tumours.

Thus, a short-term exposure of melanoma cells to melphalan, as during ILP, drives the expansion of a subpopulation of monocytes. In addition, it also causes an induction of ISG products, such as chemokines, which are predictive for treatment response. This might be due to recruitment by the chemokines of cytotoxic immune cells into the tumour.

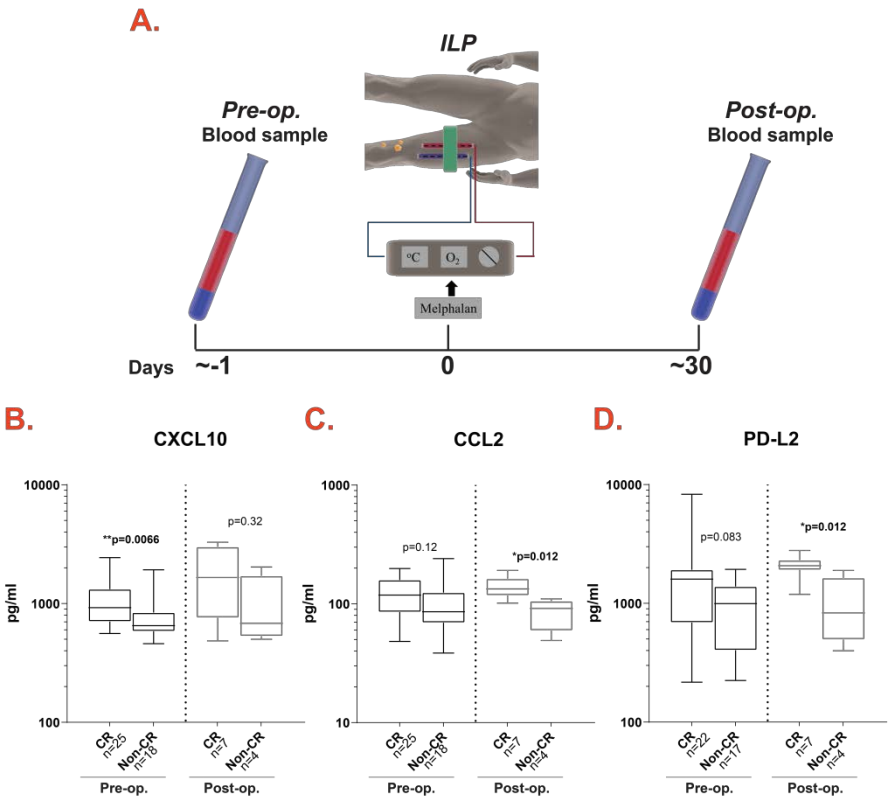

Figure 8. **A)** Serum samples were obtained from cutaneous melanoma patients approximately the day before and one month after ILP. The expression of ISG products, such as **B)** CXCL10, **C)** CCL2 and **D)** PD-L2 were measured in all serum samples. Treatment response was evaluated three months after ILP, and patients who achieved CR had higher serum levels of **B)** CXCL10 before ILP and higher levels of **C)** CCL2 and **D)** PD-L2 one month after ILP.

4.3. Favourable outcome after ILP is correlated with activated and antigen-specific T cells

After investigating the impact of isolated regional perfusion with melphalan on melanoma cells and on myeloid cells, focus shifted to T cells and their anti-tumoural properties. It was seen in **Paper I** that ILP did not cause an expansion of either $CD4^+$ T cells or $CD8^+$ T cells in peripheral blood of cutaneous melanoma patients, as measured one month after perfusion, even though an *in vitro* co-culture model of PBMCs with melphalan-exposed melanoma cells drove the expansion on $CD8^+$ T cells. In **Paper II** it was also observed that the total amount of $CD8^+$ T cells in blood did not affect treatment response, even though there seemed to be a trend towards beneficial outcome with higher numbers of $CD8^+$ T cells. Further analyses of the T cells revealed that although ILP did not affect the numbers of T cells in blood, it did activate them.

HLA-DR is an MHC class II molecule which is upregulated on T cells during activation. T cells always express MHC class I, but expression of MHC class II is usually reserved for APCs. It is thought that T cells might upregulate HLA-DR upon activation and that they utilise it for IL-2 dependent proliferation [148, 149]. In **Paper II** we saw that patients who achieved CR had higher HLA-DR expression on T cells prior to ILP. Furthermore, in **Paper I** we showed that the expression of HLA-DR on both $CD4^+$ and $CD8^+$ T cells in peripheral blood of melanoma patients increased after ILP.

Another sign indicating that ILP causes an activation of T cells is that it increased the expression of the chemokine receptors C-C chemokine receptor type 4 and 5 (CCR4, CCR5) on $CD4^+$ T cells in peripheral blood, as observed in **Paper III**. It did also increase the expression of CCR5 and C-X-C motif chemokine receptor 3 (CXCR3) on NK cells, another lymphocyte with anti-tumoural properties. These receptors are found on e.g. activated T cells and NK cells and are utilised during the recruitment of these cells to sites of inflammation or cancer. Both CCR4 and CCR5 bind to the ISG chemokines CCL4 and CCL5, while CCR4 also binds to CCL2. CXCR3 is the receptor for CXCL10. Even though we could find no correlation between the chemokine receptors and treatment response, the ligands to these receptors had an impact on treatment outcome, as seen in section 4.2.

Using the aforementioned *in vitro* co-culture model with melphalan-exposed melanoma cell lines and PBMCs it was apparent that lymphocytes cultured together with melphalan-exposed melanoma cells showed a higher expression

of chemokine receptors and PD-1 compared to lymphocytes cultured with non-exposed melanoma cells. In this setting there was also an impact on $CD8^+$ T cells, with upregulation of CXCR3 and CCR4, which was not seen in the patient material.

Furthermore, the $CD8^+$ T cells from the *in vitro* model did also show signs of high functionality. In **Paper I** we saw that $CD8^+$ T cells from a co-culture with melphalan-exposed melanoma cells had very high expression levels of IFN-γ, granzyme B and perforin – all mediators of T cell cytotoxicity (**Figure 9**). When these T cells were further cultured in a short-term culture with new (non-exposed) melanoma cells, they were capable of killing the melanoma cells.

To further investigate the impact of melanoma-specific T cells on treatment response after ILP, the percentage of antigen-specific $CD8^+$ T cells in peripheral blood from melanoma patients was investigated. ILP did not seem to cause an induction of melanoma-specific $CD8^+$ T cells, and the presence of these cells did not differ between patients who achieved CR or not. Interestingly patients who achieved CR did harbour a higher fraction of $CD8^+$ T cells specific for common viral antigens in blood before ILP, suggesting that activated and functional T cells are important for treatment response. The fact that we could not detect any differences between patients achieving CR or not regarding melanoma-specific $CD8^+$ T cells might be due to e.g. translocation of melanoma-specific $CD8^+$ T cells from peripheral blood into tumours or a to a low frequency of tumour-specific T cells in blood.

In conclusion, a short-term exposure to melphalan, as during ILP, causes an activation of $CD4^+$ and $CD8^+$ T cell. This might lead to an increase in the functionality of the T cells and may recruit them to the tumour microenvironment.

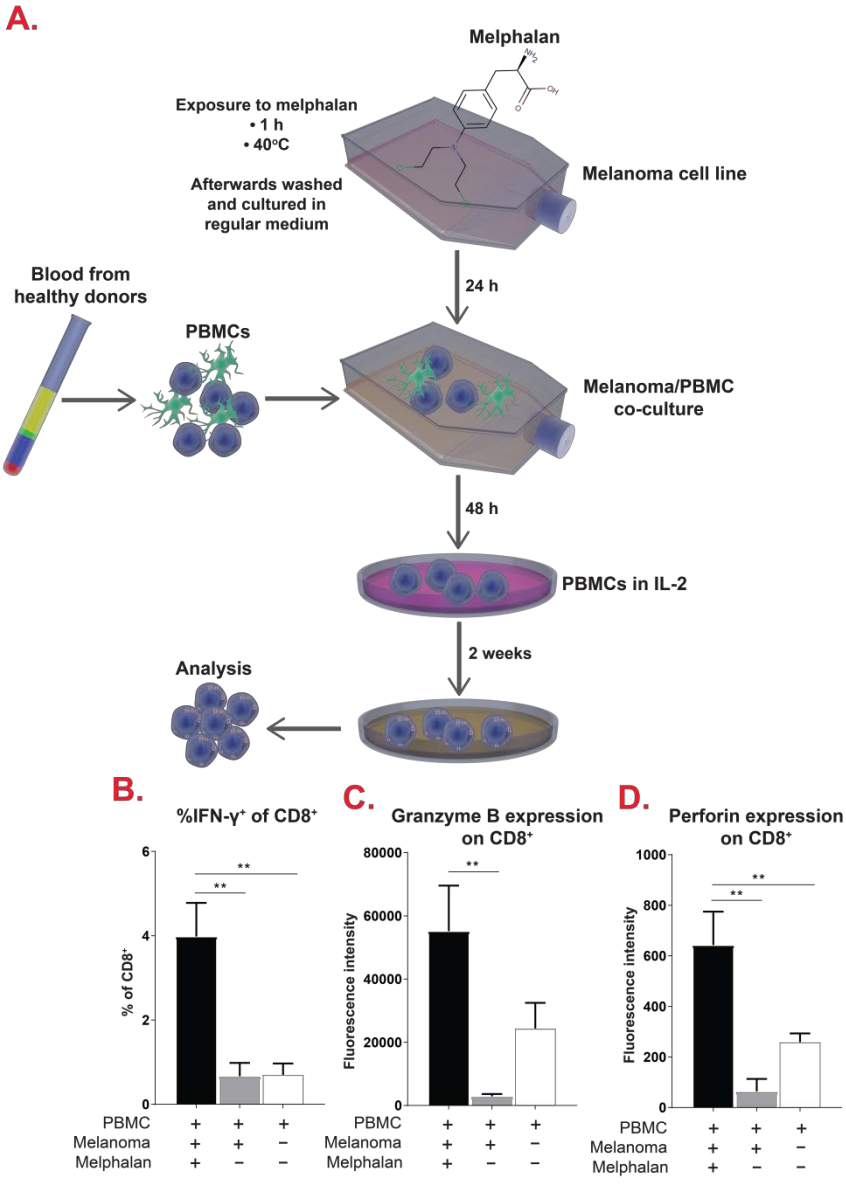

Figure 9. A) A schematic diagram of an *in vitro* model of ILP. Cells from a human melanoma cell line were exposed to a sub-lethal concentration of melphalan for one hour at 40°C. The cells were washed and cultured in regular medium for 24 hours. PBMCs from healthy donors were added to the melanoma cells, and the cells were co-cultured for 48 hours. Thereafter, the non-adherent PBMCs were transferred to a new culture and incubated in medium with IL-2 for two weeks. $CD8^+$ T cells from a co-culture with melphalan-exposed melanoma cells showed higher levels of **B)** IFN-γ, **C)** granzyme B and **D)** perforin compared to T cells cultured alone or in the presence of non-exposed melanoma cells.

4.4. Favourable outcome after IHP is correlated with activated T cells

In addition to analysing samples from cutaneous melanoma patients treated with ILP, we did also have access to blood and tissue samples from uveal melanoma patients with liver metastases who were treated with IHP (**Figure 10**). This material was the basis for the data presented in **Paper IV**.

A comparison of peripheral blood obtained from patients before IHP and peripheral blood from healthy blood donors revealed that the melanoma patients had a different T cell profile. Melanoma patients harboured a lower percentage of $CD8^+$ T cells among their PBMCs, and a higher fraction of T_{regs}. Moreover, the T cells from melanoma patients expressed higher levels of PD-1, however there was no difference in the other analysed activation marker HLA-DR.

To investigate the impact of activated T cells on clinical outcome, we divided all IHP patients into two groups based on high or low expression of PD-1 or HLA-DR on different T cell subpopulations and looked at progression-free survival (PFS), e.g. the time from treatment until progression of disease, for each group. Patients with activated T cells, e.g. high expressions of PD-1 or HLA-DR, showed a longer PFS. This was especially apparent for $CD4^+$ T cells where both a high expression of PD-1 and of HLA-DR was associated with a longer PFS. Interestingly, there was no impact of the percentages of the different T cell subpopulations on PFS; it was only their activation status that mattered.

Even though uveal melanoma patients as a group had a lower fraction of $CD8^+$ T cells in peripheral blood compared to healthy controls, further examination showed that it mostly was melanoma patients who did not respond to the IHP treatment that had low levels of $CD8^+$ T cells. The responders showed levels more similar to healthy controls. This was also reflected in tumours, where responders had a higher degree of tumour-infiltrating $CD8^+$ T cells compared to non-responders. The $CD8^+$ T cells did also infiltrate tumour tissue to a much higher degree than normal liver tissue.

Thus, it seems as if activated T cells play a role for the treatment response to IHP as well as to ILP. Due to the low number of available tumour biopsies it was not possible to correlate the amount of tumour-infiltrating $CD8^+$ T cells with PFS as we did for T cells in peripheral blood. However, it would have been a very interesting analysis to perform since it is known that a high degree of lymphocytic tumour-infiltration in uveal melanoma patients rather unexpectedly is correlated to a higher mortality; for cutaneous melanoma it is

the opposite [150-153]. Even though the percentage of $CD8^+$ T cells in peripheral blood had no impact on survival, the finding that responders to IHP seem to harbour a higher amount of tumour-infiltrating $CD8^+$ T cells in their metastases might indicate that the presence of tumour-infiltrating lymphocytes might be beneficial for uveal melanoma patients in a perfusion setting.

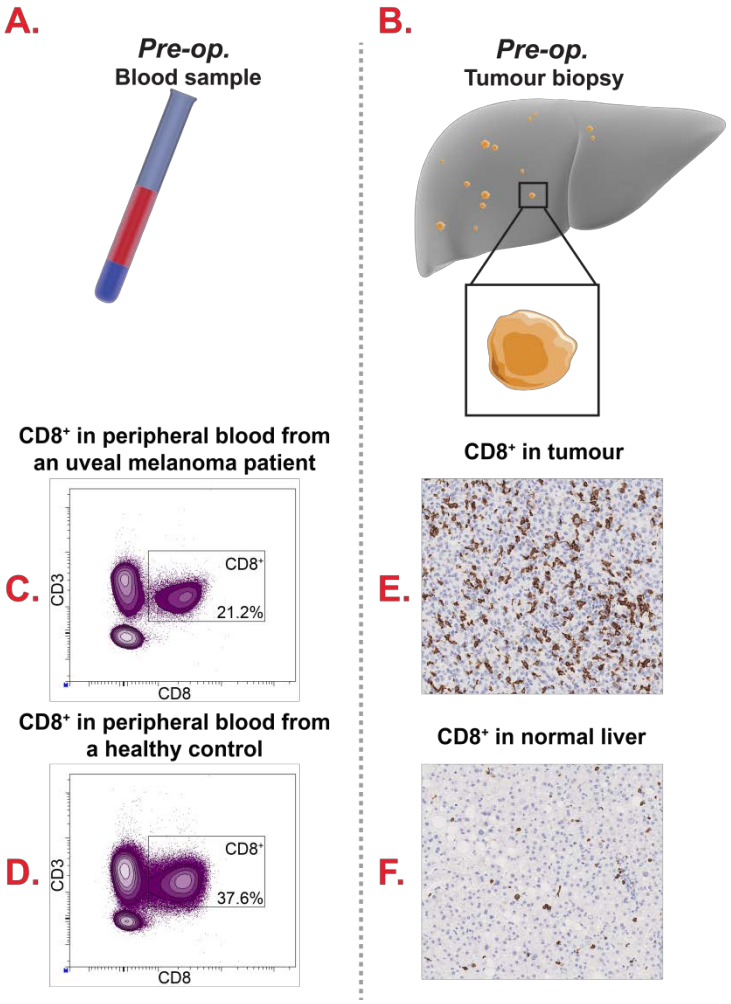

Figure 10. A) Peripheral blood samples and **B)** tumour biopsies were obtained from uveal melanoma patients before IHP. PBMCs were purified from the blood and analysed by flow cytometry, while the biopsies were analysed by immunohistochemistry. **C)** Uveal melanoma patients harboured lower percentages of $CD8^+$ T cells among immune cells **D)** than healthy controls. **E)** The tumours from the uveal melanoma patients contained a higher degree of $CD8^+$-infiltration (brown staining) than **F)** nearby normal liver tissue within the same biopsy.

5. Concluding remarks

The objective for this thesis was to investigate the role of the immune system for the treatment response to isolated regional perfusion with melphalan in patients with metastatic melanoma. Our main finding is that the treatment outcome following ILP and IHP partly depends on an immunological component, which most probably is executed through the action of cytotoxic $CD8^+$ T cells. We showed that melanoma patients who harboured activated and antigen-specific T cells in peripheral blood prior to treatment had a better response and a longer progression-free survival. It was also observed that ILP causes an activation of T cells, together with an induction of the percentage of $CD16^+$ monocytes. We did also find a melphalan-associated increased production of chemokines and of chemokine receptors on lymphocytes, which might facilitate the recruitment of the immune cells into tumours.

These effects on the immune system might be due to a melphalan-induced increased immunogenicity of melanoma cells. After exposure to melphalan, melanoma cells expressed and upregulated DAMPs and other immune-related stress markers, which might stimulate an immune response. Thus, melphalan is likely to induce an immunogenic-type of cell death in melanoma cells, which in turn stimulate and activate the immune system to launch an attack against the remaining cancer cells.

It is important to recognise the limitations of the study and its confounding factors. A major factor which must be taken into consideration is the chronic inflammatory environment of tumours. Tumours are known to cause non-specific chronic inflammations, which might conceal and mislead data on chemotherapy-induced tumour-specific immune responses. Most of the sample material we had access to came from peripheral blood, with a limited number of tumour biopsies where none was obtained after perfusion. This makes it more difficult to monitor tumour-specific immune responses since the immune profile in peripheral blood might not fully reflect the immune profile in tumours. It was also noted during the course of this thesis work that the time points for sampling might not have been optimal for all analyses. For the measurement of chemokines in serum it would likely have been better to obtain post-perfusion samples sooner after perfusion since the serum levels of chemokines induced by the perfusion might have declined after one month.

In conclusion, the findings presented in this thesis indicate that the treatment response to ILP and IHP partially is due to the induction of an immune response. This proposes a potential beneficial effect of combining isolated regional perfusion with immunotherapy, such as immune checkpoint inhibitors. There is in fact a newly launched clinical trial investigating this

combination treatment of metastatic melanoma; the NivoILP trial (ClinicalTrials.gov identifier number: NCT03685890) which combines ILP with the neutralising anti-PD-1 antibody Nivolumab. Hopefully the addition of an immune checkpoint inhibitor will increase the efficacy of ILP in the treatment of malignant melanoma.

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"It takes a village to raise a child" is a common proverb about the communal efforts of childrearing, and I strongly think that there should be a similar saying for the growth of a PhD student in academia. This thesis would never have been written without the support and the enriching environment of those around me during the last couple of years.

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