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MAPPING THE IMMUNE LANDSCAPE IN SOLID TUMORS – IMPLICATIONS FOR IMMUNOTHERAPY

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Mapping of the immune landscape in solid tumors – implications for immunotherapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To all patients who have contributed to the research presented in this thesis.
Without you, none of it would have been possible.

“The more I learn, the more I realize how much I don’t know.”

– Albert Einstein

POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunsystemet är vår kropps försvar och skydd mot olika slags hot. Hot från utsidan, såsom virus, bakterier och svampar, och hot inifrån, såsom celler som har frångått sina avsedda funktioner och löper amok. När en sådan fara hotar balansen inom oss har vårt immunsystem en stor poliskår som står redo att vidta omedelbara åtgärder. Detta kallas det medfödda immunförsvaret och kompletteras av det långsammare men mycket effektiva adaptiva immunförsvaret. Inom det adaptiva immunsystemet finns de viktiga T-cellerna, som kan beskrivas som SWAT-teamet. Mycket specialiserade och med enastående färdigheter att bekämpa fienden. De är inte först på plats, men när de väl kommer, hjälper de till att lösa situationen effektivt och bär med sig minnen och lärdomar från tidigare erfarenheter med fienden.

Trots de inbyggda medfödda och adaptiva försvarsmekanismerna finns det tumörer som kan bildas och utvecklas till dödlig cancer. De flyr från den välutvecklade poliskåren och dess breda och varierande skyddsmekanismer. Det är viktigt att poängtera att tumörer inte är smarta, utan detta händer på grund av det enorma urvalstrycket som sker i en utvecklande tumör. Den förlorade kontrollen av hårt reglerade mekanismer (som till exempel styr celldelning, celldöd och reparation av DNA) och förvärvandet av fördelaktiga egenskaper (så som till exempel immun-bromsande faktorer) leder till överlevnadsfördelar för tumörceller. Faktum är att överlevande tumörceller ofta utnyttjar olika immunkomponenter och kapar immunmekanismer som kan gynna deras egna utveckling. Vissa immunceller kan till och med ombildas och börja gynna tumören. Tumörmiljön är ett stort kaotiskt maskineri som består av många delar, som alla påverkar varandra på ett komplext sätt. I allt detta har vi fortfarande SWAT-teamet, T-cellerna, som försöker bekämpa tumörcellerna. Trötta, hämmade och oförmögna att besegra tumörhotet trots sina bästa ansträngningar.

Är allt förlorat och ingenting kan göras? Nej, självklart inte. Lanseringen av cancer-immunterapi, som skett framförallt under det senaste decenniet, har lett till en revolution inom behandlingen av långt framskriden cancer. Immunterapi utnyttjar immunsystemets kraft och potential genom att riktas mot de sätt som tumörceller flyr undan immunsystemet. En kategori av immunterapi som har varit mycket framgångsrik är så kallad checkpoint-blockering. Checkpoint-blockering fungerar genom att blockera de kraftiga bromsar som hämmar T-cellerna i tumörmiljön. Detta leder till att T-cellernas förmåga att utrota tumörceller frigörs.

Det finns dock stort utrymme för förbättringar och förutsättningarna ser olika ut i olika cancertyper. Det finns fortfarande ett stort behov av att förstå grunderna i tumörimmunologi och få ökad kunskap om tumör-infiltrerande immunceller i cancer. *Vilka immunceller finns i tumörer? Vilka immunceller är fördelaktiga att ha? Är de funktionella? Hur kan vi utnyttja deras förmåga och öka deras tumörbekämpning genom immunterapi?* De artiklar som presenteras i denna avhandling syftar till att besvara dessa viktiga frågor.

Avhandlingen består av fyra artiklar där immunceller från tumörer från patienter med prostatacancer (**Artikel I**) eller äggstockscancer (**Artikel II-IV**) har undersökts. Artiklarna fokuserar på en grupp immunceller som kallas lymfocyter, och däribland mer specifikt på T-celler i tumörer från dessa patienter. I **Artikel I** och **II** fokuserade vi på att kartlägga dessa lymfocyter, vilka olika typer som fanns i tumörerna, vilka typer av receptorer de uttrycker och vilka lösliga signalämnen de omges av. I **Artikel III** utvärderade vi funktionaliteten hos T-celler och hur checkpoint-blockering kan användas för att återställa tumörbekämpande funktioner. I **Artikel IV** undersökte vi en unik grupp av T-celler som kallas gamma delta (γδ) T-celler och deras roll i äggstockscancer.

Sammanfattningsvis hittade vi stora mängder lymfocyter, och i synnerhet T-celler, i tumörer från båda undersökta cancertyperna. Dessa T-celler uttryckte ofta olika bromsande receptorer, vilket kan dämpa deras förmåga till tumörbekämpning. Tillgängligheten av dessa dämpande bromssignaler var dock vanligare i tumörer hos patienter med äggstockscancer jämfört med tumörer från patienter med prostatacancer. Detta innebär att framtida immunterapi riktad mot dessa bromsar troligtvis inte är lika effektiv för behandling av prostatacancer. Vi undersökte användningen av checkpoint-blockerande läkemedel på T-celler från patienter med äggstockscancer. Vi fann att T-cellerna ökade sin produktion av viktiga tumörbekämpande signalmolekyler men att stora utmaningar kvarstår då T-cellerna även bromsas av andra mekanismer. Detta skapar stora utmaningar för framtida behandlingar. Slutligen fann vi att γδ T-celler var viktiga tumörbekämpare i äggstockscancer och var fördelaktiga på många sätt, bland annat genom koppling till ökad överlevnad hos patienterna. Ett möjligt sätt att använda dessa γδ T-celler i framtida immunterapi är att öka deras tumörbekämpning men mer forskning behövs.

Resultaten från de ingående artiklarna ger tillgång till fördjupad kunskap om immunceller i tumörer som kan bidra till utvecklingen av framtida immunterapier. Artiklarna bidrar till den snabbt växande och spännande tumörimmunologin och dess kliniska användning i form av immunterapi, som har en enorm potential i dagens och framtidens behandling av cancer.

POPULAR SCIENCE SUMMARY OF THE THESIS

The immune system is our body's defense and protection from various threats. Threats from the outside, such as virus, bacteria and fungi, and threats from within, including cells which have gone outside of their intended program and gone rogue. When such a danger threatens the balance within, our immune system has a large police force of effectors ready to take immediate action. This is known as the innate immune system and it is complemented by the slower, but very efficient, adaptive immune system. T cells are one of the crucial effectors of the adaptive immune system and can be described as the special weapons and tactics (SWAT) team. Highly specialized, with a vast experience and outstanding skills to combat the enemy. They are not the first to arrive, but once they do, they help to resolve the situation efficiently and remember their previous encounters.

Despite the built-in innate and adaptive defense mechanisms, there are tumors which are able to form and develop into lethal cancers. They escape the police force and its broad and diverse effectors designed to protect us. Tumors are not smart, this happens due to the immense selection pressure and high-speed evolution which occurs in any developing tumor. The lost control over tightly regulated mechanisms (such as cell division, cell death and DNA repair for example) and gain of favorable features (such as expression of suppressive factors) will result in survival benefits for tumor cells. In fact, surviving tumor cells often take advantage of immune components and hijack immune mechanisms which can favor their own progress. Some immune cells even reprogram into becoming tumor-promoting. The tumor microenvironment becomes a large chaotic machinery consisting of many gears and involved components, all affecting each other in a complex way. In all of this, we still have the SWAT team, the T cells, trying to combat the tumor cells. They are weary, inhibited and unable to defeat the tumor threat despite their best efforts.

Is all lost and nothing can be done? No, of course not. The past decades have firmly proven this with the introduction of cancer immunotherapy, leading to a revolution in the treatment of advanced cancers. Immunotherapy harnesses the power of the immune system by targeting interactions between tumor cells and the immune system. One successful category of immunotherapy is checkpoint blockade, which works by removing the massive brakes which T cells receive from the tumor environment, unleashing their abilities to eradicate tumor cells.

However, there is much room for improvement. There is still a large need to understand the basics of tumor immunology and gain knowledge about tumor-infiltrating immune cells in human cancer. *Which cells are present in tumors? Which subsets are the good guys? Are they functional? How can we harness their ability and boost their tumor-fighting functions for immunotherapy?* The work presented in this thesis aims to address these important questions.

The work consists of four papers in which immune infiltrates from prostate cancer (**Paper I**) and ovarian cancer (**Paper II-IV**) have been sampled from patients undergoing surgery. The work focuses on lymphocytes and more specifically on T cells in these tumor environments. In **Paper I** and **II**, we focused on mapping the presence of lymphocytes, what types of

receptors they express and what soluble factors they are surrounded with. In **Paper III**, we assessed the functionality of T cells and how checkpoint blockade can be used to restore anti-tumor functions. In **Paper IV**, we dived into a unique subset of immune cells called gamma delta (γδ) T cells and explored their role in ovarian cancer.

In summary, we found an abundance of infiltrating lymphocytes, and in particular T cells in the tumors of both cancer types. These T cells frequently expressed different braking receptors, which can dampen their active tumor-fighting responses. However, the availability of these dampening brake signals was more pronounced in the tumors of ovarian cancer patients compared to tumors of prostate cancer patients. This implies that future immunotherapy targeting these brakes might not be as effective in prostate cancer. We explored the use of checkpoint-targeting drugs on T cells from ovarian cancer and found them to increase their production of important tumor-fighting signaling molecules. However, the T cells were still likely inhibited by other mechanisms, which presents additional challenges for future immunotherapy. Lastly, we found γδ T cells to be potent tumor-fighting mediators in ovarian cancer and being beneficial in numerous ways. Also, their functionality was associated with patient survival. The results warrant further exploration of the possibility to boost γδ T cell function in future immunotherapy.

Overall, the findings provide new knowledge about immune cells in human tumors and have implications for future immunotherapy. The results of the presented work contribute to the rapidly expanding and exciting field of tumor immunology and its clinical translation, cancer immunotherapy, which holds an enormous potential in treatment of cancer today and in the future.

ABSTRACT

Our cells are programmed with various safety mechanisms to avoid transformation into tumor cells. In case these fail, we have a guarding immune system ready to recognize and eliminate these cells. Despite these safety measurements, cancer is one of the leading causes of death worldwide. The tumor cells find ways to escape the immune system. Paradoxically, components of the immune system can contribute to the progression of tumors by the use of various immunosuppressive pathways. However, the immune system can also be harnessed, and the anti-tumor functions restored to regain control of the tumor development. This has been highlighted in the past decade, with the introduction of novel immunotherapeutic approaches, such as checkpoint blockade, to target the naturally occurring brakes called co-inhibitory receptors.

The work presented in this thesis consists of four papers which contribute with knowledge on infiltrating immune cells in prostate cancer (**Paper I**) and ovarian cancer (**Paper II-IV**). In the work of **Paper I-IV**, we have looked into tumor-infiltrating lymphocytes and mapped the presence, composition, expression pattern and functionality of various T cell subsets in these two solid tumor types. The work was performed by retrieving material from cancer patients undergoing surgery, isolating immune cells and performing phenotypic descriptions by flow cytometry. We also have assessed the soluble environment in which the immune infiltrates reside and assessed T cell functionality by looking into cytokine secretion, cytotoxicity and/or proliferation by various readouts.

In **Paper I**, we performed phenotyping of immune infiltrates in peripheral blood and prostates with malignant, benign or healthy histology. In **Paper II**, we assessed the immunophenotype in peripheral blood, ascites and metastasized tumor tissue of advanced ovarian cancer patients. The results in **Paper I** and **II** showed lymphocyte infiltration to be common in both tumor types, in particular of $CD8^+$ effector memory T cells. PD-1, which enables inhibition of effector functions by binding to its ligands, was the most abundantly expressed co-inhibitory receptor in both tumor types. However, in **Paper I**, PD-1 expression was also common in healthy prostates indicating a role in the homeostasis of the prostate environment. In **Paper II**, we correlated our findings to patient outcome and identified eight immune-related risk factors (both cellular and soluble) in ascites and/or tumor associated with overall patient survival.

In **Paper III**, we investigated the functionality of infiltrating T cells isolated from ovarian cancer patients. We wanted to explore if functionality, in terms of cytokine responsiveness, could be enhanced using immunotherapeutic PD-1-targeting conventional monoclonal antibodies (mAbs) nivolumab/pembrolizumab and novel scaffold proteins called DARPin® proteins. The results showed improved secretion of several important effector cytokines using the PD-1 targeting reagents. A bivalent PD-1 targeting DARPin® protein showed comparable results to the clinically approved mAbs which warrants further investigation. However, despite boosted cytokine responsiveness, our results indicated that tumor-derived T cells are still highly dysfunctional, presenting challenges in restoring anti-tumor responses.

In **Paper IV**, we investigated the features of the unconventional subset γδ T cells in ovarian cancer. Our aim was to investigate their features and contribution in this cancer type. We profiled their T cell receptor (TCR) characteristics, their phenotype and functional response to various stimuli. We found the ascites-derived and tumor-derived γδ T cell repertoires to be distinct from one another. We suggested the ascites γδ T cells to be driven by adaptive TCR-driven pathways due to the observed clonal focusing in this compartment, while tumor γδ T cells displayed a high diversity and likely respond through innate pathways. In summary, we found the γδ T cells to be beneficial for the patients by anti-tumor functions including cytotoxicity and production of important effector cytokines. Importantly, we identified their functionality to be associated to outcome, where higher functionality was linked to increased patient survival. We observed a negative impact of CD39 on γδ functionality, which warrants further investigation to understand how γδ T cell functionality can be boosted.

Future optimization of immunotherapeutic approaches requires basic understanding of immune infiltrates in tumors. By learning more about these tumor-infiltrating immune cells, what they express and how their functionality can be affected, new strategies can be outlined based on this knowledge. I hope that by reading this thesis, you will obtain insight into this exciting research field and how the presented work has contributed.

LIST OF SCIENTIFIC PAPERS

I. **Rådestad E**, Egevad L, Jorns C, Mattsson J, Sundberg B, Nava S, Ericzon BG, Henningsohn L, Levitsky V, Uhlin M. Characterization of infiltrating lymphocytes in human benign and malignant prostate tissue. *Oncotarget*. 2017;8(36):60257–60269.

II. **Rådestad E**, Klynning C, Stikvoort A, Mogensen O, Nava S, Magalhaes I, Uhlin M. Immune profiling and identification of prognostic immune-related risk factors in human ovarian cancer. *OncoImmunology*. 2018;8(2): e1535730:1–17.

III. **Foord E**, Klynning C, Schoutrop E, Förster JM, Krieg J, Mörtberg A, Müller MR, Herzog C, Schiegg D, Villemagne D, Fiedler U, Snell D, Kebble B, Mattsson J, Levitsky V**, Uhlin M**. Profound Functional Suppression of Tumor-Infiltrating T-Cells in Ovarian Cancer Patients Can Be Reversed Using PD-1-Blocking Antibodies or DARPin® Proteins. *Journal of Immunology Research*. 2020;Article ID: 7375947:1–12.

IV. **Foord E***, Arruda LCM*, Gaballa A, Klynning C, Uhlin M. Characterization of ascites- and tumor-infiltrating γδ T cells reveals distinct repertoires and a beneficial role in ovarian cancer. *Science Translational Medicine*. 2021;13, eabb0192:1–14.

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SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

V. Gaballa A, Arruda LCM, **Rådestad E**, Uhlin M. CD8+ γδ T Cells Are More Frequent in CMV Seropositive Bone Marrow Grafts and Display Phenotype of an Adaptive Immune Response. *Stem Cells International*. 2019, Article ID 6348060:1–13.

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VIII. Stikvoort A, Chen Y, **Rådestad E**, Törlén J, Lakshmikanth T, Björklund A, Mikes J, Achour A, Gertow J, Sundberg B, Remberger M, Sundin M, Mattsson J, Brodin P, Uhlin M. Combining flow and mass cytometry in the search for biomarkers in chronic graft-versus-host disease. *Frontiers in Immunology*. 2017;8(June):1–14.

IX. Norström MM*, **Rådestad E***, Sundberg B, Mattsson J, Henningsohn L, Levitsky V, Uhlin M. Progression of benign prostatic hyperplasia is associated with pro-inflammatory mediators and chronic activation of prostate-infiltrating lymphocytes. *Oncotarget*. 2016;7(17):23581–93.

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XI. **Rådestad E**, Wikell H, Engström M, Watz E, Sundberg B, Thunberg S, Uzunel M, Mattsson J, Uhlin M. Alpha/beta T cell depleted grafts as an immunological booster to treat graft failure after hematopoietic stem cell transplantation with HLA-matched related and unrelated donors. *Journal of Immunology Research*. 2014;Article ID: 578741:1–14.

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CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
BCR	B cell receptor
BPH	Benign prostatic hyperplasia
BTN	Butyrophilin
CA-125	Cancer associated antigen 125
CARs	Chimeric antigen receptors
CCL	Chemokine (C-C motif) ligands
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
CDR3	Complementarity-determining region 3
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C motif chemokine receptor
DARPin®	Designed ankyrin repeat protein
DC	Dendritic cell
DNAM-1	DNAX accessory molecule 1
EMA	European Medicines Agency
FACS	Fluorescence-activated cell sorting
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HMBPP	(E)-1-Hydroxy-2-methyl-2-butenyl 4-pyrophosphate lithium salt
ICS	Intracellular cytokine staining
IFN-γ	Interferon γ
IL	Interleukin
IP-10	IFN-γ induced protein 10
IPP	Isopentenyl pyrophosphate
LAG-3	Lymphocyte-activation gene 3
MAb	Monoclonal antibody

MACS	Magnetic-activated cell sorting
MCP-1	Monocyte chemoattractant protein 1
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MICA/B	MHC class I chain-related protein A/B
MIP-1β	Macrophage inflammatory protein 1-β
MMR	Mismatch repair
MSI	Microsatellite instability
NGS	Next-generation sequencing
NK	Natural killer
NKG2D	Natural killer group 2 member D
PAg	Phosphorylated antigens
PARP	Poly ADP ribose polymerase
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1
PD-L1/2	Programmed death-ligand 1/2
PMA/I	Phorbol 12-myristate 13-acetate and ionomycin
PSA	Prostate-specific antigen
SHP-2	Src homology region 2 domain-containing phosphatase-2
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TGF-β	Transforming growth factor β
TIL	Tumor-infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TRG	TCRγ-chain
TURP	Transurethral resection of the prostate
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

"The failure in cancer is due not to any weakness of the organism but to a change in the character of the cells, rendering them in one way or another insusceptible to the normal control." – Sir Frank MacFarlane Burnet 1957[1], pioneer developing the theory of immunosurveillance.

1.1 THE IMMUNE SYSTEM AND CANCER

We have an amazing immune system. It is constantly busy, doing things we are not even aware of. It is fully capable of eliminating not only bacteria and viruses (instances where we might be more aware of the full activity of it) but also when things go wrong in our own body. When cells undergo transformation and start doing things they should not be doing, we have a guarding immune system ready to take action. This is an important part of the immune system's job which is sometimes forgotten, so I want to start off by saying "*Thank you, immune system.*"

However. Cancer is one of the leading causes of premature death (ages 30-69) in the majority of countries worldwide[2], so the system is evidently not bulletproof. Clearly far from it. Transformed cells are able to stay under the radar and escape detection and elimination by the immune system. So, let's establish that the immune system is not perfect. But neither is cancer. Since the quest "to cure cancer" was launched in the 1970's, the scientific community and healthcare sector have gained remarkable knowledge about cancer, immunology and the interplay within the tumor microenvironment giving rise to the field of tumor immunology. Also, immunotherapy has evolved as a completely new category of treatment and helps to prolong the life of cancer patients considerably. Immunotherapy holds an enormous potential by unleashing (or manipulating depending on type) the power of the immune system and utilizing what we already have within. However, there is much room for improvement.

1.1.1 The hallmarks of cancer

So, what goes wrong in cancer? Many things to say the least. All is impossible to cover in the scope of this thesis and the aim is not to do so. Instead, the goal is to emphasize the role and potential of the immune system in all of this.

Let's start with emphasizing that tumor development is a complex gradual multistep process in which a cell loses control of tightly regulated mechanisms. It requires the failure of intrinsic tumor-suppressing mechanisms, with which cells themselves should be able to identify internal damage and, if unable to repair, induce apoptosis and die. In a model called "Hallmarks of cancer" presented by Douglas Hanahan and Robert Weinberg in early 2000, six capabilities were highlighted as enabling the development of lethal tumors[3]. These hallmarks involve resistance to cell death and increased proliferation capacity, among others. In 2011, the model was updated with two additional hallmarks reflecting the advancements in research. In the updated model, "avoiding immune destruction" and "deregulating cellular energetics" received recognition for their involvement in tumor development[4]. Genomic

instability and tumor-promoting inflammation were also added to the model as enabling characteristics, described to underlie the hallmarks and being drivers in tumor development[4] (***Figure 1***).

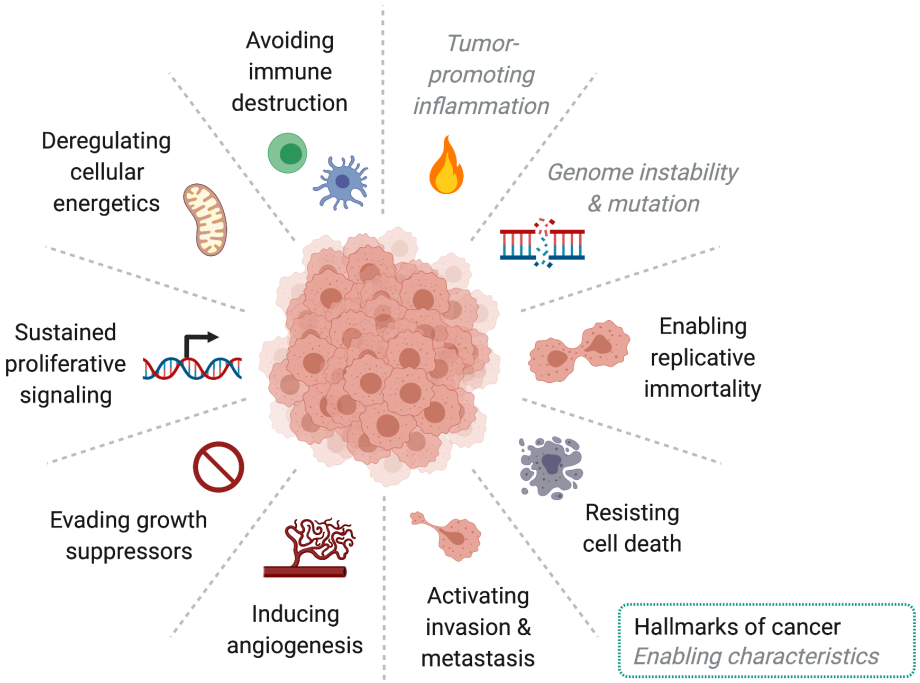

Figure 1. *The eight hallmarks of cancer cover the foundation for how tumors are able to develop. The model also contains two enabling characteristics. Figure inspired by Hanahan and Weinberg[4].*

Interestingly, each aspect of the hallmark model presents an opportunity to interfere with the tumor development. So, by learning more about how tumors avoid and escape the immune system, we can develop approaches to block, reverse and maybe even prevent the escape. This is where immunotherapy comes in.

1.2 CRASH COURSE IN IMMUNOLOGY

Before digging deeper into how tumors can avoid immune destruction (section **1.3 Tumor Immunology**) and the ways to interfere with this (section **1.4 Cancer Immunotherapy**), let's take a step back and cover a short overview of some basic immunological concepts.

As already mentioned, the immune system is able to distinguish external threats such as bacteria and viruses, but also internal threats such as transformed, potentially dangerous cells. To tackle such threats, the intricate immune system has two arms: the innate and the adaptive. These arms complement each other in numerous ways as they differ in speed, specificity, memory development, and more. Both include many cellular and non-cellular components[5] ***(Figure 2***). The innate arm is needed to launch the adaptive arm. Cytokines and chemokines are important signaling molecules in the crosstalk and help to direct and recruit different components of the immune system.

The cellular immune system is derived from hematopoietic stem cells in the bone marrow. These cells develop into a lymphoid or myeloid lineage. A common lymphoid progenitor gives rise to four groups of lymphocytes: T cells, B cells, natural killer (NK) cells and the

innate lymphoid cells. All other cells of the immune system are derived from a common myeloid progenitor. A common term for the nucleated cells derived from the lymphoid or myeloid lineage is leukocyte, which essentially means "white blood cell".

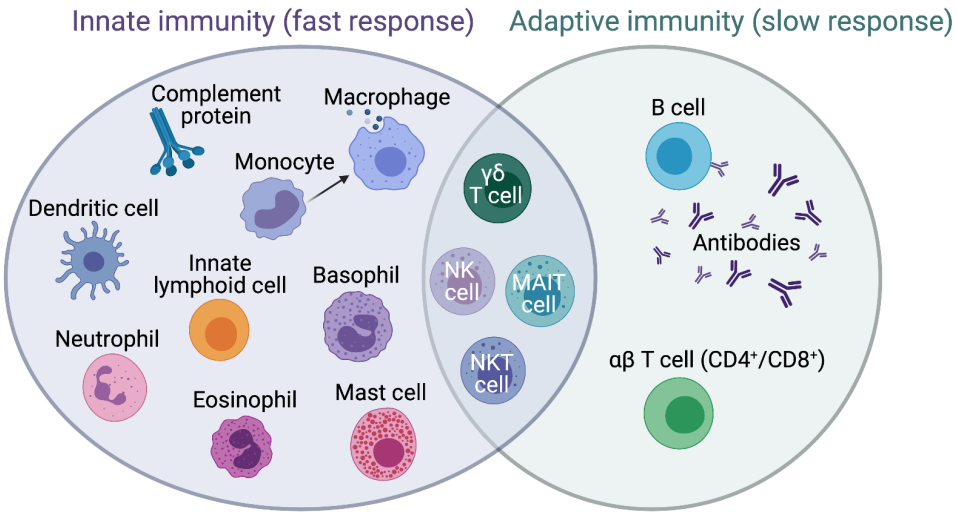

Figure 2. *An overview of the soluble and cellular components of the innate and adaptive arms of the immune system. Figure modified from Dranoff*[5].

B cells and T cells are the effectors of the adaptive arm (***Figure 2***), while NK cells are usually described as being part of the innate arm. The reason for the phrasing "usually" is because in immunology things are not always easily categorized and it might be more appropriate to say that these cells are on the border of the innate and adaptive arms. This also goes for a subset of T cells called gamma delta (γδ) T cells which have both innate and adaptive features, more on this subset later on. A main feature of adaptive responders is the expression of a B cell receptor (BCR) or T cell receptor (TCR), which is unique to each B or T cell. This expression is essential for the highly antigen-specific responses linked to adaptive immunity. Another feature of adaptive immunity is the development of memory. Upon recognition of cognate antigens, the cell clonally expands into many copies. Upon clearance of the threat, only a minor percentage continues as memory cells while the others die.

Unlike TCRs, which requires both processing of the peptide antigen and its presentation by a major histocompatibility complex (MHC or human leukocyte antigen (HLA) in humans), BCRs binds to its cognate antigens without processing or presentation. Also, the antigens can be various types of molecules, there is no limitation to peptides as for T cells. In addition to the surface expression of BCRs, B cells can secrete soluble forms of the BCRs which are then referred to as immunoglobulins or antibodies. These antibodies act as floating receptors, ready to bind to their target and initiate a variety of events. B cells, NK cells and all components of the innate arm are incredibly important for our immune system to fully function. However, with that said, we leave these cells and will only dig further into T cells.

1.2.1 T cell development

After commitment to the T lymphoid lineage in the bone marrow, the T cell progenitors will go to the thymus where they continue their development and maturation[6]. Here, the elegant

and complex somatic variable diversity joining (VDJ) recombination takes place, giving rise to an enormous TCR diversity. It can be described as a cut and paste process with different gene segments, insertion of additional nucleotides and a set of other diversity-boosting events, resulting in a unique TCR to each T cell. This TCR can only recognize its specific antigen, giving rise to the specificity of the expressing cell. Once the TCR is in place, the maturation continues.

To make things a little more relatable, T cells can be described as the special weapons and tactics (SWAT) team in the body's line of defense against different threats. Highly specialized, with a vast experience and outstanding skills to combat the enemy. They are not the first to arrive, but once they do, they help to resolve the situation efficiently and don't forget about their previous encounters. Being part of this team requires graduation from a tough training school. The cells undergo two intense selection processes in which their binding capacity is tested. The main goal of the two selection processes, called positive and negative selection, is to generate functional self-tolerant T cells which bind in a "lagom" fashion. "Lagom" is a Swedish expression which translates to "just enough" or "not too much, not too little". If they bind too weak, they will fail the positive selection and if they bind too strong, they will fail the negative selection due to being a potential threat by being auto-reactive[7]. The T cell SWAT education is a complex chain of events and all credit goes to the thymus and the components in there which aid to accomplish the proper education and selection[7]. The T cell SWAT graduates are released into the blood stream as naïve/unexperienced T cells to circulate until they are called in for duty (meeting their cognate antigen).

1.2.2 αβ and γδ T cells

Let's make things a bit more complicated and introduce more layers to the T cells. What has been described so far is relevant for the so-called alpha beta (αβ) T cells. In fact, T cells can be classified into two groups based on the type of TCR they carry: the first one is known as the αβ T cells as they carry a heterodimeric TCR consisting of an α chain and a β chain (***Figure 3***). These are the conventional members of the SWAT team (with some exceptions which are regarded as unconventional T cells including mucosal associated invariant T cells and NKT cells, but these will not be discussed further). The second type of T cells, which is considered a more unconventional subset is called γδ T cells, due to the TCR consisting of γ and δ chains (***Figure 3***). This is a specialized unit of the SWAT team and makes up 0.5-10% of the total T cell pool in human peripheral blood[8]. These γδ T cells are more frequent in epithelial tissues (such as for example the colon[9,10], liver[11], skin[12] and breast[13]). Their development is different than the αβ T cells, and much remains elusive due to differences between γδ T cells in rodents compared to humans[14,15].

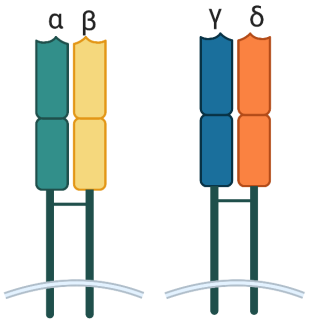

Figure 3. *There are two types of T cell receptors; the αβ TCR which defines αβ T cells and the γδ TCR defining γδ T cells.*

These two groups of T cells are distinct in numerous ways[16]. Conventional αβ T cells are strictly adaptive responders in the sense that they react and bind to processed peptides for which they have TCR specificity. Furthermore, this binding is, as briefly mentioned earlier, dependent on presentation by a MHC/HLA complex and requires co-stimulation[17]. γδ T cells on the other hand can be described as being a hybrid between an innate-acting NK cell and an adaptive-acting αβ T cell[16,18]. Similar to NK cells, γδ T cells frequently express various traditionally NK cell-associated receptors including natural killer group 2 member D (NKG2D), DNAX accessory molecule 1 (DNAM-1), NKp30 and NKp44 which can trigger cytotoxicity upon binding with corresponding ligands[19]. Like NK cells, they also frequently express the Fcγ receptor III (FcγRIII, also known as CD16), which enables binding of IgG antibodies, giving rise to antibody-dependent cell-mediated cytotoxicity (ADCC)[19].

However, the γδ T cells also carry rearranged TCRs, characteristic of T cells and adaptive immunity. Interestingly, the γδ TCRs have similarities with immunoglobulins, both in terms of structure and recognition of antigens[20]. γδ T cells are unique in the sense that they can become activated in both TCR-independent/-dependent ways along with not being restricted by antigen processing/presentation by MHC/HLA molecules, like their αβ equivalents are. The list of identified activating ligands for γδ T cells includes many kinds of molecules (not only peptides, which is similar to immunoglobulins), and includes both self-expressed ligands as well as non-self ligands (exemplified later in section **1.2.2.2**). The list of ligands is continuously increasing along with our understanding of their recognition[20].

γδ T cells have been recognized to play a key role in lymphoid stress surveillance, making them central for tissue homeostasis and anti-microbial/tumor immunity[21]. By sensing cell dysregulation in various ways (by TCR or NKG2D for example), γδ T cells provide a first line of defense, complementing the conventional first defense line of innate myeloid cells engaging adaptive cells[21]. Although the γδ T cell subset is old, estimations say 400-500 million years[22], our knowledge about it is young (about 35 years). The discovery of the TCRγ chain in mid 1980's initiated a chain of events leading to the discovery of γδ T cells[23,24]. Although our knowledge about this subset is still limited, increased understanding on their innate and adaptive biology is emerging[14,25]. Also, we know that γδ T cells are not a homogeneous group of cells, quite the opposite, and have pleiotropic functions[26,27]. Complicating the studies of these cells is the fact that the γδ subset appears to have evolved with some differences between species, which makes it difficult to translate findings from animal models to humans[16,20,27,28]. "Enigmatic", "mysterious" and "paradoxical" are common adjectives found in the literature about γδ T cells, which shed light on our incomplete understanding of this subset.

To summarize, the γδ T cells are puzzling and contribute in various important ways on their own or to other parts of the immune system[27] (***Figure 4***). More on their functions will be presented in section **1.3.7**.

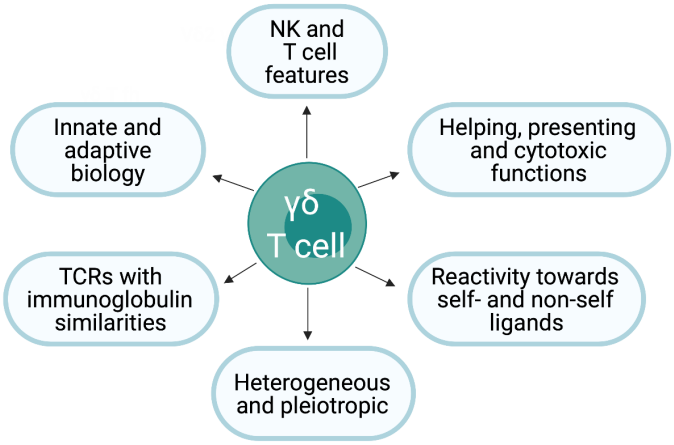

Figure 4. *Some of the puzzling characteristics of γδ T cells.*

1.2.2.1 Subsets of αβ T cells

αβ T cells are classified into subsets depending on the expression of either the cluster of differentiation (CD) 4 or CD8 co-receptor, which is obtained during the development in the thymus (more specifically during the positive selection process)[7]. These co-receptors aid the binding to MHC*; CD4 to MHC class II receptors expressed by professional antigen-presenting cells (APCs, such as dendritic cells, (DCs) and B cells) while CD8 aids the binding to MHC class I receptors (expressed by all nucleated cells) (***Figure 5***). MHC class II molecules present processed peptide antigens which have been sampled from outside the presenting cell. MHC class I molecules traditionally present processed peptide antigens sampled from within the cell[29]. Additionally, by a process known as cross-presentation, extracellular proteins can be processed and presented by MHC class I molecules (primarily by DCs) and therefore render response to $CD8^+$ T cells[29].

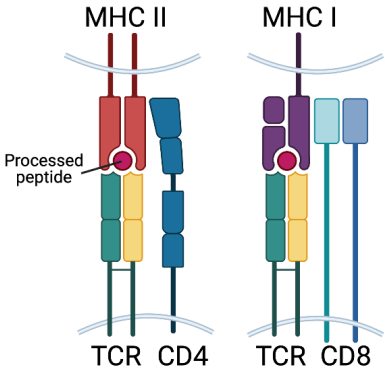

Figure 5. *Co-receptor CD4 facilitates in the binding of the TCR to MHC class II, while CD8 is needed for the MHC class I and TCR complex.*

The $CD4^+$ and $CD8^+$ T cell subsets occupy different niches. To revisit the SWAT team metaphor one last time, we can now further specify that there are $CD4^+$ helper SWAT members which recognize extracellular threats and mainly function by helping others. They serve as team leaders and provide their essential support to B cells, DCs and $CD8^+$ T cells by cytokine production and expression of co-stimulatory ligands for example[30]. We also have the $CD8^+$ killer SWAT members, the true snipers of the team. They are known for their efficient cytotoxicity towards identified intracellular threats, such as virus-infected or malignant cells expressing foreign antigens. Upon activation they utilize their main weapons, namely the release of granzymes and perforin, to eliminate the target cell. The $CD8^+$ T cells

*For simplicity will only MHC class I and II be mentioned in this part, not human HLA-equivalents (HLA-A, B and C are equivalent for MHC class I while HLA-DP, HLA-DQ and HLA-DR are equivalent to MHC class II).

are also important cytokine producers[31]. However, no T cell is an island. Both $CD4^+$ and $CD8^+$ T cell subsets are vital parts of the immune system and complement each other to fight off threats together with other parts of the immune system.

1.2.2.2 Subsets of γδ T cells

In humans, γδ T cells are divided into subsets based on their TCR δ chain expression[16]. In human peripheral blood, 50-95% of all γδ T cells express the Vδ2 chain and are referred to as the $V\delta2^+$ subset[19]. This $V\delta2^+$ subset is usually characterized by co-expression of Vγ9 in the TCR. This semi-invariant $V\gamma9^+V\delta2^+$ subset is the most well-studied human γδ subset (it is absent in rodents[20]) due to the dominant presence and ease of retrieval from blood. Also, the identified $V\gamma9^+V\delta2^+$ subset-specific reactivity towards phosphoantigens (pAgs) makes them easy to expand *ex vivo*.

PAgs are a group of non-peptide phosphorylated antigens which are intermediates in the synthesis of isoprenoids (which can be used for cholesterol synthesis)[19]. The pAgs can be produced endogenously (internally) through the mevalonate pathway or exogenously (by microbial organisms) through the non-mevalonate pathway. An increased synthesis of pAgs can be detected in transformed tumor cells as a result of altered metabolism or during microbial invasion. Either way, the resulting pAgs isopentenyl pyrophosphate (IPP) or (E)-1-hydroxy-2-methyl-2-butenyl 4-pyrophosphate lithium salt (HMBPP) can activate the $V\gamma9^+V\delta2^+$ subset, which makes this subset important in the immunosurveillance of these threats[19]. The endogenous IPP activates $V\gamma9^+V\delta2^+$ T cells at much higher concentrations (micromolar) compared to the very low concentrations (picomolar) of exogenous HMBPP required for activation[19,32]. This enables $V\gamma9^+V\delta2^+$ to distinguish between normal and abnormal self-production and immediate production by microbes.

Recent years have proven that it is not the pAgs themselves which are being recognized by the $V\gamma9^+V\delta2^+$ T cells. Instead, other molecules are involved, bridging between the $V\gamma9^+V\delta2^+$ T cells and pAgs. Extending on earlier studies recognizing a role of butyrophilin (BTN) 3A1 in the sensing of pAgs[32–34], two recent studies demonstrated the pAg sensing to be dependent on the co-binding of BTN2A1 and BTN3A1 to the Vγ9Vδ2 TCR[35,36]. However, there is still uncertainty on how presence of pAgs alters these BTN molecules to become stimulatory to the $V\gamma9^+V\delta2^+$ subset. Rigau *et al.* have suggested remodeling and/or conformational changes of BTN3A1 while Karunakaran *et al.* have suggested an additional yet unidentified $V\gamma9^+V\delta2^+$ TCR ligand[35,36].

As for the other γδ T cells, they are commonly referred to as non-Vδ2. These have been shown to have distinct functions from the $V\delta2^+$ subset, and provide important anti-microbial and anti-tumor surveillance by other means[37]. The most common subset in the non-Vδ2 group (and the second most common γδ subset in peripheral blood) is the $V\delta1^+$ subset. While the $V\delta2^+$ subset dominates in peripheral blood, the $V\delta1^+$ subset is more associated with presence in tissues, as exemplified earlier in **1.2.2**, along with the less frequently discussed and more rare $V\delta3^+$ and $V\delta5^+$ subsets[11,16,38–40]. The list of activating ligands for these subsets

is heterogeneous and largely unknown[20], but includes various lipid-presenting CD1 molecules[41,42] and endothelial protein C receptor (EPCR)[40], vitamin B metabolite-presenting MHC-related protein 1 (MR1)[39] and stress molecules including annexin A2[43] and MHC class I chain-related protein A/B (MICA/B)[20]. Some of these requires the engagement of the γδ TCR, such as annexin A2[43], while the recognition of MICA/B for example requires NKG2D. The NKG2D-associated activation of γδ T cells has been shown to be independent of the TCR[44], but have also been shown to act co-stimulatory[45,46]. The identification of ligands and other necessary/accessory molecules for activation is still very much an ongoing hunt[47].

1.2.3 Co-stimulation and co-inhibition

Co-stimulation and co-inhibition of T cells[**] are essential means for the induction and downregulation of responses that are required upon activation. These positive and negative signals orchestrate how T cells will behave and can be compared to traffic signals, giving the T cell a go-signal or a brake/stop-signal (***Figure 6***). Upon binding of the TCR with its cognate antigen presented by MHC, the T cell requires a second signal for activation, namely a co-stimulatory signal derived from interaction between the co-stimulatory receptor CD28 and its ligand CD80/CD86 (B7-1/B7-2) expressed by the APC. This is a safety check, to ensure that the activation is called for.

Due to the imperfect central tolerance (elimination of auto-reactive clones) taking place during development in the thymus (or bone marrow for B cells), there is a need for a backup system; peripheral tolerance. This is where co-inhibitory receptors come into play (among other peripheral tolerance mechanisms). These negative signals, or checkpoints as they can be called, are important to maintain self-tolerance and thus prevent autoimmunity, but are also important in the normal control of an active immune response[48]. An activated state needs to deactivate and "cool off" at some point. The engagement of a co-inhibitory receptor can lead to, for example, inhibition of cell cycle progress and effector functions[48]. These checkpoints also become important in the context of tumor challenge as tumors take advantage of this natural brake/stop-system. Suppression of immune reactivity by increased expression of co-inhibitory receptors and their ligands have been discussed as a major reason to why the immune system fails to control tumor development. A lot more on this will come.

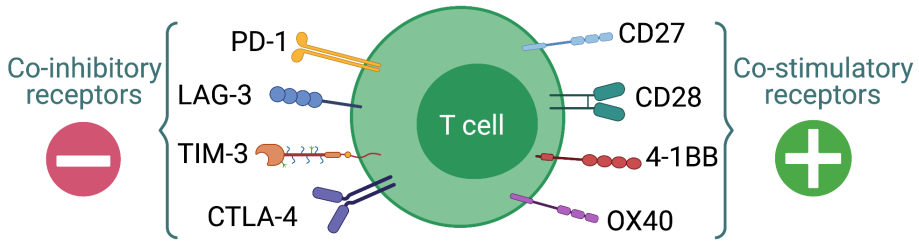

Figure 6. *Examples of co-inhibitory and co-stimulatory receptors which upon binding to corresponding ligand affect the T cell response.*

[**] When referring to T cells, it is the conventional αβ T cells which are intended unless stated otherwise.

In contrast to the co-inhibitory receptors, co-stimulatory receptors are important for alleviating the immune response by promoting differentiation, development of memory responses, proliferation, cytokine production and more[48]. There are functional differences and similarities between the wide array of different receptors. Both co-stimulatory and co-inhibitory receptors are classified according to the internal signaling they initiate, dividing receptors into different super families. For example, co-stimulatory receptor CD28 is part of the immunoglobulin superfamily (together with co-inhibitory receptors PD-1, CTLA-4, LAG-3 and TIM-3) which signals using tyrosine kinases, similar to the TCR[48]. Another large superfamily is the tumor necrosis factor receptor superfamily which signals using TNF-receptor-associated factors. CD27, 4-1BB and OX40 are examples of co-stimulatory receptors which belong to this family[48]. Despite using different signaling cascades, the end result is regulation of important stimulatory factors such as nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase[48].

Since co-signaling pathways play an essential role in the direction and fine-tuning of T cell responses, it has become a great field of interest in the development of immunotherapy, which will be discussed further later on. There are many receptors involved in the co-signaling of T cells and a brief overview on a number of them will follow with emphasis on co-inhibitory receptors and specifically PD-1 due to the work presented later in the thesis.

1.2.3.1 PD-1

Programmed cell death protein-1 (PD-1) is expressed by activated T cells[49,50] and serves to dampen an ongoing response, preventing overactivation. It can also be expressed by various other immune cells including B cells and NK cells as well as several other cell types[51]. Beyond regulating acute infection, it plays an important role in self-tolerance[51].

PD-1 was identified in 1992 by Tasuku Honjo and colleagues, who thought the primary function was, as the name indicates, in cell death regulation[52]. However, a number of years later, the Honjo lab demonstrated that PD-1-deficient mice developed organ-specific autoimmune disease, indicating a primary role in immune regulation[53]. In the following years, PD-1 was recognized as a co-inhibitory receptor and programmed death-ligand 1 (PD-L1)[54] and 2 (PD-L2)[55] were defined as the ligands.

PD-L1 and PD-L2 can be expressed by a range of cell types including T cells, B cells, DCs, macrophages, endothelial cells and various other non-immune cell types[51,54]. Expression of these ligands is induced by interferon γ (IFN-γ), which is produced upon T cell activation as a negative feedback mechanism[54,55]. Activation-induced PD-1 expression is downregulated upon clearance of antigen and homeostasis is reinstated[56,57]. In the context of tumors and chronic infection/inflammation, expression can remain high and PD-1 is therefore commonly used to distinguish so-called exhausted T cells[56–58].

The molecular mechanisms by which PD-1 inhibits the T cell response are still being investigated, but the knowledge has increased in past years[59]. Upon binding with its ligand, the cytosolic domain of the PD-1 receptor becomes phosphorylated leading to a two-step

binding process resulting in the recruitment (step 1) and activation (step 2) of Src homology region 2 domain-containing phosphatase-2 (SHP-2)[59]. Lately, a dimerization model has also been suggested. In this model, SHP-2 can be activated by the phosphorylated cytosolic parts of two PD-1 molecules[59]. Regardless of the activation model, SHP-2 will dephosphorylate (thereby inactivate) important positive signaling molecules primarily delivered from the TCR[51] and CD28[60]. This will result in decreased expression of transcription factors involved in T cell activation (such as activator protein-1, nuclear factor of activated T-cells and NF-κB for example), affecting cell cycle progression, effector functions and metabolic activity negatively[51,61].

The importance of the PD-1/PD-L1 pathway as an immune escape mechanism by tumors was highlighted by Lieping Chen and colleagues in 2002[62]. This initiated the exploration of modulating this pathway. Restoring function of PD-1^+ dysfunctional T cells was described in 2006 by Rafi Ahmed and colleagues in a chronic virus mouse model using PD-1 blockade[57]. More research in chronic infection was translated into the context of tumor immune escape. Since then, PD-1 has become a successful target to reinvigorate T cell responses in cancer patients as will be outlined later.

1.2.3.2 CTLA-4

Human cytotoxic T lymphocyte antigen 4 (CTLA-4) was first described in 1987[63] and in the years that followed, the importance of CTLA-4 in self-tolerance, regulation of activation and tumor immunity was demonstrated[64,65]. Mice deficient of CTLA-4 quickly develop lethal autoimmunity, highlighting the importance of CTLA-4[66]. CTLA-4 is upregulated on T cells in response to activation and helps to control the amplitude of the activation[67]. It is also described to play an important role for regulatory T cells (Tregs), which, with their suppressive activities, serve to dampen immune responses and prevent autoimmune reactions (another example of peripheral tolerance)[67].

The inhibitory molecular mechanisms are distinctly different compared to those initiated by PD-1[68,69]. The ligands of CTLA-4, namely CD80/86, are the same as for co-stimulatory receptor CD28. Upon expression of CTLA-4, it outcompetes CD28 due to a higher affinity for the ligands. This results in a dampened activation, as CD28 will be unable to bind its ligands to the same degree. CTLA-4 has been described to act as a checkpoint in early phases of activation, in the priming phases occurring in lymphoid tissues, compared to PD-1 which is described to be important in later stages of activation, acting on primarily effector T cells in peripheral tissues[67].

1.2.3.3 LAG-3

Co-inhibitory receptor lymphocyte-activation gene 3 (LAG-3) is similarly to its checkpoint cousins upregulated upon activation on T cells and NK cells[70]. It was described first in 1990[71] and was later described to resemble co-receptor CD4, binding to MHC class II, but with higher affinity[72]. Recent years have led to the discovery of additional ligands, such as liver

and lymph node sinusoidal endothelial cell C-type lectin (LSECtin)[70], galactin-3[73] and fibrinogen-like protein 1 (FGL1)[74]. The regulatory role of LAG-3 includes inhibition of cell proliferation, reduced cytokine release and inhibited expression of the CD3/TCR complex[75]. Research on LAG-3-deficient mice has shown that LAG-3 plays an important role in regulating the survival and expansion of activated T cells[76]. LAG-3 is also discussed to play an important role for Tregs in promoting their inhibitory functions[70].

1.2.3.4 TIM-3

T cell immunoglobulin domain and mucin domain 3 (TIM-3) is another co-inhibitory receptor which was first described in 2002[77]. A couple of years later, galactin-9 was described as the main ligand[78]. Since then, three additional ligands have been described; phosphatidyl serine[79], high mobility group protein B1 (HMGB1)[80] and carcinoembryonic antigen related cell adhesion molecule-1 (Ceacam-1)[81]. TIM-3 expression is found on many cell subsets including $CD4^+$ and $CD8^+$ T cells, Tregs, NK cells, macrophages and DCs[82]. Numerous studies have shown that TIM-3 acts as a checkpoint, negatively regulating TCR signaling and other events[82]. TIM-3 is also expressed by exhausted dysfunctional T cells, and co-expression of TIM-3 and PD-1 identifies the most severely dysfunctional T cells in viral infection[83] and cancer[84]. The biology of TIM-3 is still being explored as it can function directly and indirectly in various ways, through a number of ligands and expressing cell subsets[82].

1.2.3.5 Some examples of co-stimulatory receptors

CD28 is expressed on naïve T cells and is crucial to initiate a primary T cell response as it binds to CD80/CD86 on activated APCs. This binding delivers positive signals into the T cell, stimulating and "confirming" activation. To balance the activation, CD28 downregulation and CTLA-4 upregulation upon activation will provide possibilities to modulate the magnitude of the response[48].

Other important co-stimulatory receptors include CD27, 4-1BB (CD137) and OX40 (CD134). In common, they provide signals for survival, expansion and memory development. However, their expression pattern differs. CD27 is, similar to CD28, expressed on naïve T cells and with increased differentiation, expression can be downregulated or lost. 4-1BB and OX40 on the other hand are not expressed constitutively on naïve T cells and are upregulated rapidly in response to activation[85]. Optimal expression of OX40 occurs after approximately 24-48 hours and is important for the production of interleukin (IL)-2 and upregulation of the IL-2 receptor[86]. 4-1BB and OX40 (to a lesser degree) have been found to be important for cell survival and improving effector functions in human melanoma-derived T cells[85].

Overall, there are many co-signaling molecules involved in modulating the T cell response. The highly complex synergistic or even antagonistic effects of different receptor interactions help to modulate the response by different kinetics, timing of expression, levels of expression and availability of ligands.

1.3 TUMOR IMMUNOLOGY

"*Tumors are imprinted by the immunologic environment in which they form*" – Shankaran *et al.* 2001[87], concluding remark in a seminal paper which sparked modern tumor immunology.

Tumor immunology describes the interplay between tumor cells and the immune system. It includes the unpleasant fact that the immune system, aimed to combat tumors, also works to shape the tumor and, ultimately, enhance tumor growth. There have been important theories proposed in the past, such as in 1863 when Rudolf Virchow was the first to hypothesize about the contribution of inflammation in tumor development; or the theory of the immune system's role in preventing tumor formation proposed by Paul Ehrlich in 1909 and last to be mentioned is the hypothesis of immunosurveillance presented by Sir Frank MacFarlane Burnet and Lewis Thomas in the late 1950's[1,88,89]. Despite these theories (and numerous others, nicely reviewed by Galon *et al.*[89]), there have been times of limited enthusiasm for the field of tumor immunology due to identified flaws in the theories or inadequate understanding. However, new essential discoveries and methods paved the way for an increased understanding about basic immunology. In the past couple of decades, groundbreaking discoveries have enabled the merging of oncology and immunology, leading to a skyrocketing interest in tumor immunology[89].

1.3.1 Cancer immunoediting

The concept of cancer immunoediting was key for the renewed interest of the field and is essential to understanding the role of the immune system in tumor development[87,88,90]. The idea was introduced by Robert Schreiber and colleagues in 2001[87] and was established in a landmark review in 2002[88]. The concept was an elaboration of the immunosurveillance hypothesis with new highlights resulting from their own results[87] as well as numerous advances made in the 1990's and early 2000 (nicely reviewed by Vesely *et al.*[91]). So, what is the concept about? Well, cancer immunoediting is a dynamic process with three distinct phases: elimination, equilibrium and escape (***Figure 7***).

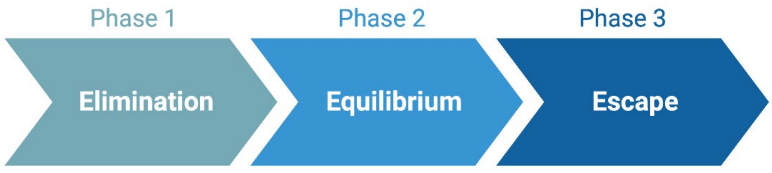

Figure 7. *The three E's of cancer immunoediting: elimination, equilibrium and escape.*

1.3.1.1 Elimination

In the elimination phase, the immune system protects its host from tumor development. Recognition of foreign transformed antigens, stress-induced ligands and danger signals, or even absence of antigen-presenting receptors, are examples which can trigger and activate immune responses which will eliminate the potential threat[90].

In this phase, both innate and adaptive arms of the immune system work to eradicate transformed cells and prevent tumor growth[90]. This is achieved by macrophages, DCs,

T cells (both γδ and αβ subsets) and NK cells as important effectors[91]. Studies of knockout mice in the 1990's shed light on the role for the anti-tumor cytokine IFN-γ in this phase (again, nicely reviewed by Vesely *et al.*[91]). In the elimination phase, the immune system is in control.

1.3.1.2 Equilibrium

In the equilibrium phase, a state of tumor cell dormancy is entered[90,92]. If, for some reason, tumor cells are not eliminated, they can enter this dormant state in which there is no net growth of the tumor. The tumor cells might not carry all the necessary characteristics needed to continue the tumor development. The adaptive arm of the immune system maintains control in this equilibrium phase which can last for a very long time[90]. In fact, the tumor cells might never pass this state and become active again.

However, this phase enables editing of the immunogenicity of the tumor[90]. Immunogenicity can be described as the ability to provoke an immune response. From the perspective of a tumor cell, having a lower immunogenetic signature will yield survival benefits because there is less risk of being recognized as a threat by immune effectors. Back in 2001, this was the seminal discovery leading to the development of the cancer immunoediting concept[87]. The identified role of the immune system in altering the tumor cells, or "*sculpting*" as the Schreiber and colleagues elegantly phrased it[87], was pioneering. This results in changes of the "quality" or characteristics of the tumor. If the tumor immunogenicity is altered or if the immune system's ability to maintain the tumor cells is affected, the tumor cells can leave the equilibrium phase and enter the next[90].

1.3.1.3 Escape

In the escape phase of cancer immunoediting, the immune selection pressure will have favored tumor cells which are able to avoid elimination and get past the equilibrium phase. Thus, genomic instability and immune selection pressure are important drivers in this transition[90]. Let's be clear, tumor cells are not smart, but it almost appears that way when a rare tumor clone has acquired a new property (or lost control of one) that will make it survive better than its sister clones. This selection pressure applies to any new mutation that becomes advantageous to the tumor cell and can involve acquisition of any of the hallmarks of cancer (***Figure 1***), including altered immunogenicity and escaping the immune system.

With establishment of a suppressive tumor microenvironment and suppression of anti-tumor immune responses (which will be exemplified in the next section), tumor cells are able to escape and expand. The outgrowth is no longer restricted and controlled by the immune system[93]. Similar to the elimination phase, both innate and adaptive components of the immune system are involved in this escape phase[90]. The paradox is a fact; the system which was designed to control tumor cell development has in fact enabled its escape.

1.3.2 Escape mechanisms

There are many examples of how tumor cells can successfully escape the immune system in both direct and indirect manners; by changes in or on the tumor cells themselves, by

changes in the tumor microenvironment or by changes on surrounding cells including immune cells, mesenchymal cells and fibroblasts[94] (***Figure 8***). The induced changes can drive additional escape mechanisms to develop. One effective escape is by simply staying under the radar to reduce the possibility of being recognized. This can be done by, for example, loss of tumor antigens, downregulation of antigen-presenting molecules MHC class I and II (and equivalent HLA-molecules for human) or changes in the antigen-presentation machinery causing dysfunctional presentation[95,96].

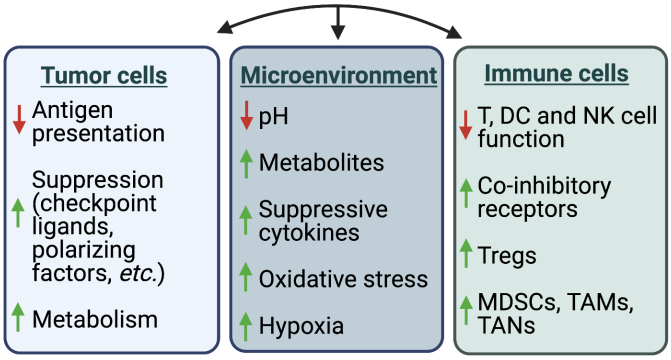

Figure 8. *Examples of tumor escape mechanisms. Modified from Seliger[94].*

Another escape strategy is by hijacking mechanisms which are aimed to dampen the immune response in a normal situation (tolerance) or after clearance of a pathogen (infection). One example is by promoting Tregs, which have important functions in regulating the activity of other immune cells to maintain homeostasis and tolerance. In the context of a tumor, they become tumor-promoting with their wide array of immune-suppressing effects[97]. Another example is by taking advantage of co-inhibitory mechanisms, as mentioned earlier. From the perspective of a tumor, utilizing this available brake/stop-system is a very efficient way to escape. Serving as an example, the expression of PD-1 and its ligands PD-L1/2 is found in many human tumor types[58,98–102]. The PD-1/PD-L1 interaction has been found favorable for tumors not only by inhibiting the PD-1-expressing T cell function, but also by inducing resistance to apoptosis and T cell-mediated killing in the PD-L1-expressing tumor cell[103]. As will be discussed later, intervening with this particular example of tumor-immune escape, called checkpoint blockade, has become one of the most powerful immunotherapeutic approaches available today[104].

The tumor can also interfere with the anti-tumor immune responses by up- or downregulation of other types of receptors and ligands either on themselves or on surrounding cells[94]. The establishment of an immunosuppressive microenvironment also enables tumor escape[90] (***Figure 8***). Both tumor genotype and phenotype are important in shaping the microenvironment[105]. For example, oncogene-driven chemokine and cytokine production is an initial important strategy to recruit and polarize cells to act in favor of the tumor[105]. Examples of such cells are myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and neutrophils (TANs)[105–107]. These cells are not bad guys from the start but are recruited by components in the tumor microenvironment.

Examples of such recruiting chemotactic factors include granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-X-C motif) ligand 8 (CXCL8), chemokine (C-C motif) ligand 2 (CCL2), CXCL5 and CXCL12. Once recruited, the cells are reprogrammed by the pathologic activation occurring in the tumor microenvironment[105–109]. They in turn will contribute to the microenvironment with their own suppressive effects which further supports the immune suppression and escape of the tumor[105–107]. It becomes a positive feedback-loop favoring the tumor. Exploiting the effects of inflammation is a very efficient strategy to amplify the tumor-promoting environment[110] as will be discussed further in section **1.3.5.**

Sadly enough, there is a myriad of escape mechanisms in which components within the immune system will become accomplices in the tumor escape. There are of course many more escape routes than outlined here and the field continuously obtains new knowledge and understanding about these.

1.3.3 The cancer-immunity cycle

The "cancer-immunity cycle" presented by Daniel Chen and Ira Mellman in 2013 is another important model which helps to understand the mounting of a tumor-specific T cell response in seven steps[111] (***Figure 9***). The cycle begins with tumor cells which continuously die and release antigens into their surroundings. The majority of these antigens will be self-antigens expressed by other normal cells and will not elicit an immune response due to induced tolerance. However, there will also be antigens which are expressed specifically by the tumor cells which can be recognized as foreign by the immune system. These include neoantigens (derived from mutations) and germ cell antigens (also known as cancer testis antigens) which are commonly expressed by tumor cells[111].

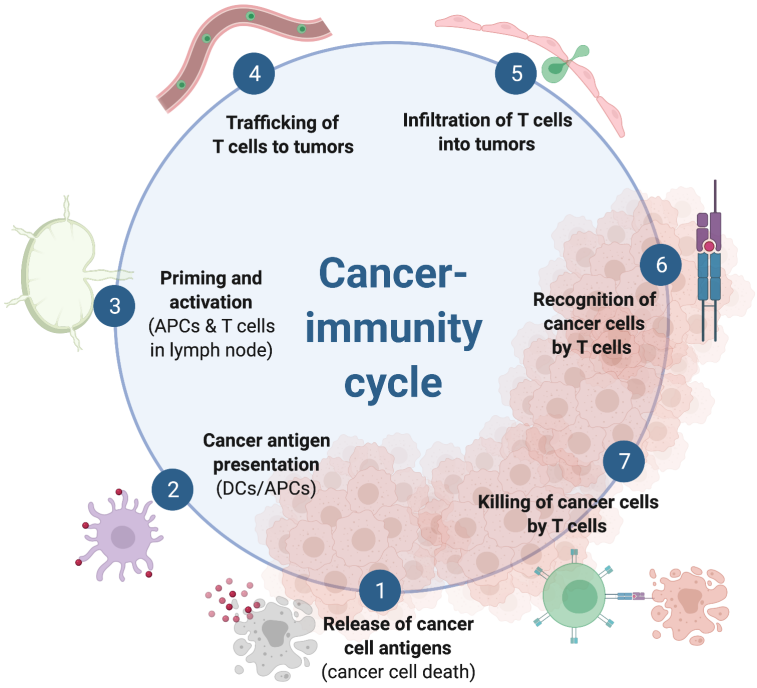

Figure 9. *The cancer-immunity cycle contains seven steps for T cell-driven elimination of tumor cells. Adapted from Chen and Mellman[111].*

The released tumor antigens will be picked up (by phagocytosis for example) by APCs (such as DCs), which are widely dispersed throughout the peripheral tissues to sample the surroundings[29]. The APCs will process the antigens and present them as peptides on MHC class I (introduced earlier as cross-presentation) or class II molecules[29]. The cells will migrate to secondary lymphoid organs (such as lymph nodes) to encounter naïve T cells. T cells which have specificity towards any of the tumor-originated presented peptides will be activated if they also receive proper co-stimulation[17]. The activated T cell will thereafter migrate and infiltrate the tumor. In the tumor, the tumor-specific T cells will recognize the tumor cells carrying the specific antigen and kill them. The killing of tumor cells will cause another round of released tumor antigens and the cycle starts over[111].

Each step of the process provides possibilities for tumor escape mechanisms but also immunotherapeutic approaches to intervene with the escape[111]. This cancer-immunity cycle explains a complex process in a very simplified manner. There is of course a lot more to it.

1.3.4 Immune contexture

In 2007, Franck Pagès and colleagues proposed the importance of immune contexture based on their findings in human colon cancer[112]. In past years, lessons learnt from clinical trials investigating the use of immunotherapy have shed light on additional layers of the cancer-immunity cycle, highlighting the importance of the immune contexture. In 2017, the founders of the cancer-immunity cycle (Chen and Mellman) elaborated their model by proposing three cancer-immune phenotypes and suggested several factors which can help to explain inter-individual differences in observed anti-tumor immune responses[113]. The phenotypes include the immune desert, the immune-excluded and the inflamed phenotype (***Figure 10***). As indicated by the names, these profiles are distinct from one another and have a varying degree of immune involvement[113].

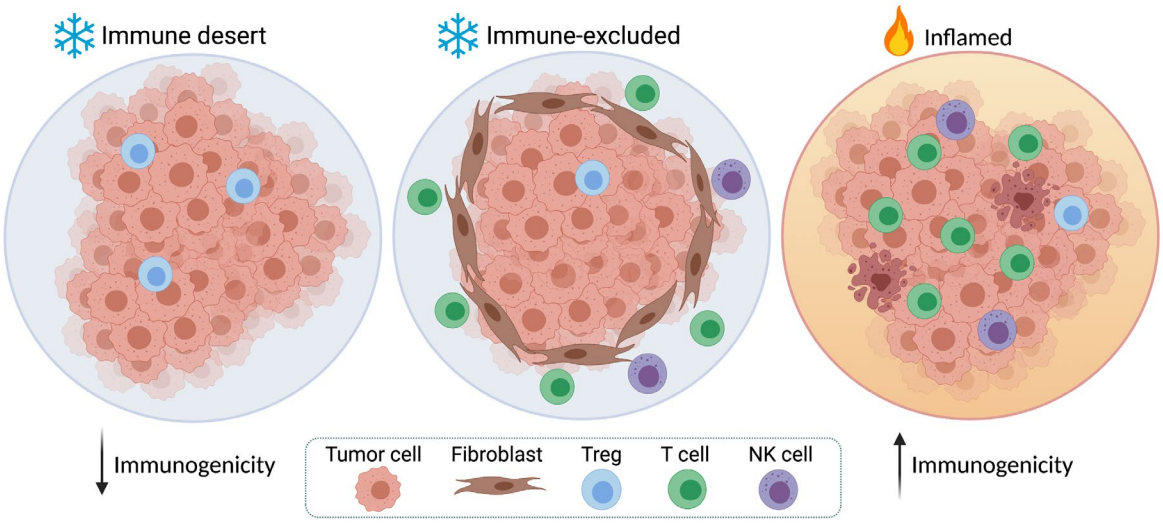

Figure 10. *Schematic of different tumor-immune contexts. The terminology varies but includes the cold/ non-inflamed/non-immunogenic immune desert and immune-excluded phenotypes, while the inflamed/hot phenotype is associated with high immunogenicity.*

Others have used the terms “hot/cold”, “inflamed/non-inflamed” or “immunogenic/non-immunogenic” tumors to describe similar phenotypes. All have in common to reflect differences in tumors with high or low inflammation, T cell infiltration and mutational load[114–116]. Similar to the three cancer-immune phenotypes, a cold/non-inflamed environment is considered immunologically ignorant while a hot/inflamed environment is considered to possess anti-tumor immunity and an abundance of TILs[114] (***Figure 10***). An important identified difference between the phenotypes is the mutational load (number of mutations), and hence the immunogenicity[116]. A high mutational load renders frequent tumor-specific antigens and therefore a higher chance for anti-tumor responses than if the mutation load is low[116]. Different tumors have a varying degree of prevalence of these somatic mutations (mutational load) as presented by Alexandrov *et al*. in 2013[117].

Regardless of the terminology used, the immune context and mutational load are now recognized to affect the possibility of using immunotherapeutic approaches, where the inflamed phenotype is more likely to render a successful outcome due to the high immunogenicity and TIL presence associated with it[105,113]. With the identified importance of the immune context, there are challenges ahead in how to tackle the cold/non-inflamed tumors with immunotherapy[115] as well as finding ways to optimize treatment strategies for hot/inflamed tumors.

1.3.5 Inflammation as a driver of tumor progression

Inflammation has been recognized as an enabling characteristic for tumors to progress[4] (***Figure 1***). Inflammation is an intricate process mainly orchestrated by the innate arm of the immune system[118]. In an acute inflammatory setting, induced by an injury or infectious pathogen for example, the inflammatory process recruits components which form a collaborating cellular and non-cellular network, forming the defense line against the threat. It becomes an environment filled with signaling molecules which quickly help to amplify the response. The physical site of inflammation actually becomes a chaotic battlefield which can harm the surrounding tissue. To restore tissue homeostasis, wound healing and tissue remodeling are important parts of the inflammation process as well as immune-dampening mechanisms. Simply put, in an acute situation the inflammatory process is initiated, amplified and resolved[118].

However, in chronic inflammation, as is the case in tumor-associated inflammation, the amplification phase becomes a problem, and the inflammation process is never resolved. Instead, signals of stress, danger and hypoxia makes the tumor microenvironment an extremely inflammation-amplifying setting[118]. These signals, along with inflammatory mediators such as recruited innate immune cells (as mentioned earlier with MDSCs, TAMs and TANs) and other cell types (such as fibroblasts), contribute further to amplify the inflammatory tumor-promoting environment[119]. These cells maintain and provide the tumor microenvironment with secretion of suppressive soluble factors. Examples of these are pro-inflammatory cytokines IL-1α, IL-6 and IL-10, expression of inhibitory ligands (PD-L1), secretion of growth and angiogenic factors (such as epidermal growth factor and vascular

endothelial growth factor (VEGF)), secretion of reactive oxygen species and nitric oxide, prostaglandins and more[118,120].

These are just some examples of how inflammation can drive and accelerate tumor development. In fact, it is almost as if the inflammation process itself does the job of the tumor. Its recognition as an enabling characteristic in the "Hallmarks of cancer" model is well-deserved. However, inflammation is not a necessity for tumor development as is outlined by the different tumor-immune phenotypes (***Figure 10***).

1.3.6 Tumor-infiltrating lymphocytes and T cell exhaustion

Many human cancers are known to contain tumor-infiltrating lymphocytes (TILs) and increased infiltration is associated with favorable clinical outcome in many cancer types[121–123]. However, the mere presence of TILs does not necessarily ensure an active anti-tumor immune response[124]. The characteristics of TILs between different types of cancer are diverse regarding extent of infiltration, composition, phenotype and functionality[99,125–127]. These factors are affected either directly by the tumor cells or indirectly through the components within the tumor microenvironment, as outlined earlier.

Persistent exposure to antigen, co-inhibitory signaling, presence of immunosuppressive cell types and chronic inflammation can drive the tumor-fighting T cells into a state of exhaustion with resulting loss of functionality[128]. The decreased functional capacity can be manifested as reduced secretion of important effector cytokines (gradual loss of IFN-γ, IL-2 and TNF-α), proliferation and degranulation[128]. The concept of exhaustion was described in mice with chronic viral infection in the late 1990's and since then, the concept has expanded to human viral infections and cancer[128]. Exhausted T cells have a high and sustained expression of multiple co-inhibitory receptors, such as LAG-3, PD-1, TIM-3 and CTLA-4[99,128–130]. Increased number of different co-inhibitory receptors is associated with a more severe dysfunction[84,129]. With tumor cells or suppressive immune cells commonly expressing the ligands, this limits the ability of T cells to control tumor progression[128].

There is currently a lot of exciting research coming out which sheds further light on the distinct status of exhausted T cells[128,131]. Not only are they distinct functionally, but also metabolically, epigenetically and transcriptionally[128]. Several studies have found the exhausted state of T cells to be beyond rescue due to stable epigenetic changes, despite using PD-1 blockade[132,133]. These findings present a massive challenge for current immunotherapies. Also, a recent study identified a large heterogeneity of the exhausted T cell population in both viral infection and cancer by mass cytometry profiling[134]. A follow-up study proposed a developmental hierarchy with four steps of the exhaustive state which introduces opportunities for immunotherapeutic intervention[135]. Lastly, the high-mobility group-protein TOX has been identified as a critical component of terminal T cell exhaustion[136]. TOX acts as a transcriptional and epigenetic regulator of the exhaustive state in both viral infection and cancer[136,137]. Learning more about T cell exhaustion will be key in the continued quest to reinvigorate T cell responses to overcome tumors.

1.3.7 γδ T cells in cancer

The interest for γδ T cells in cancer took off in 2001 when their role in immunosurveillance was first demonstrated by Adrian Hayday and colleagues[138]. They found mice deficient of γδ T cells to be more susceptible to the development of chemically-induced cutaneous malignancy[138]. This became the starting point of a massive interest in the anti-tumor role of γδ T cells.

Today, it is known that their anti-tumor functions in human cancer include exerting cytotoxicity towards tumor cells, being supportive to other cell types (B cells by class-switching[139], DCs by inducing maturation[140], NK cells by expression of co-stimulatory ligands[141] for example), acting as APCs thereby stimulating tumor-directed T cell reponses[142–144] and more[26] (***Figure 11***). The cytotoxicity is initiated by interactions with the TCR[26], NKG2D[145] or DNAM-1[146] for example, and is executed by perforin and granzymes, but also Fas ligand interactions and TNF-α-related apoptosis-inducing ligand (TRAIL, both membrane-bound and soluble)[26,147] (***Figure 11***). One of the attractions with γδ T cells in cancer is their recognition of ligands which are frequently expressed by tumor cells due to being associated to cellular stress (annexin A2[43], MICA/B[148]) and/or metabolism (pAgs[149]), rather than being products of mutation. Many solid tumors express one or more of the ligands for NKG2D (MICA/B and UL16-binding proteins 1-6) which offer numerous ways of recognition, both TCR-dependent and -independent[19].

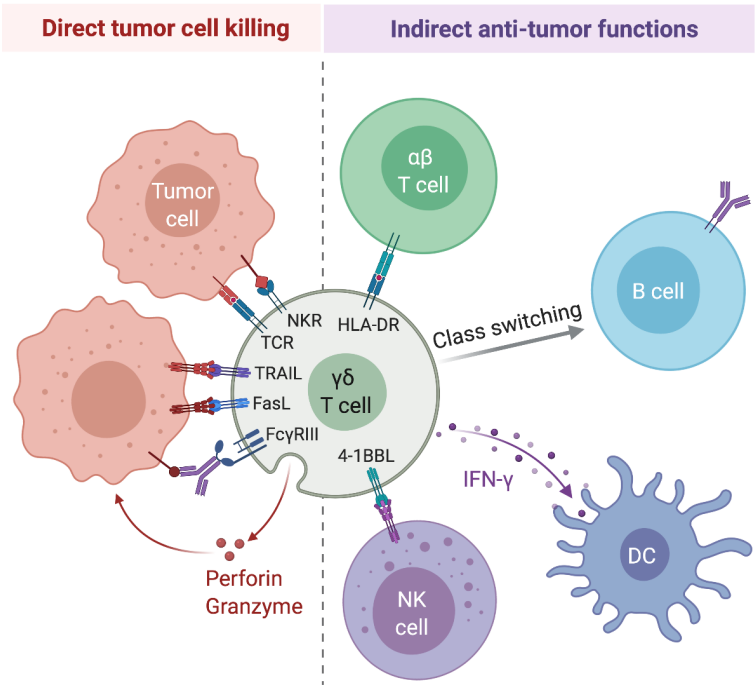

Figure 11. *Anti-tumor effects of γδ T cells. NK cell receptors (NKRs) include several different types of receptors including NKG2D, DNAM-1, etc.*

In 2015, Gentles *et al.* reported in a large meta-analysis covering 25 different tumor types, that γδ T cells were the most prognostically favorable population across 22 different immune cell populations[126]. This study was later questioned by Tosolini *et al.* on the correct identification of certain immune subsets, including γδ T cells, but the authors verified the favorable association of $V\gamma9^{+}V\delta2^{+}$ T cells in numerous cancer types including prostate cancer[150]. Today, there are many additional studies which have identified positive

associations or anti-tumor functions in numerous human cancers including breast cancer[13,151], melanoma[152,153], colon cancer[154], colorectal cancer[10] and gastric cancer[142,155]. These findings, along with other benefits (such as being MHC/HLA-unrestricted and their independence of neoantigens), have made γδ T cells an attractive choice for immunotherapy in cancer[26,156].

However, another side of γδ T cells has also evolved in recent years. In striking contrast, this side involves pro-tumor effects and working in collusion with tumor cells. In 2014, Wu *et al*. reported on the negative effects of IL-17-producing γδ T cells in human colon cancer[157]. Since then, negative associations to survival or pro-tumor functions of γδ T cells have been made in several human cancer types including pancreatic cancer[158], gall bladder cancer[159] and breast cancer[160]. The identified pro-tumor functions of γδ T cells are today centered around the production of IL-17A and its downstream effects[26]. Polarization of γδ T cells into becoming IL-17-producers have been associated with cytokines such as transforming growth factor β (TGF-β), IL-1β, IL-6 and IL-23[161,162]. IL-17-producing γδ T cells have been shown to mediate other immunosuppressive cell types (such as MDSCs[157,163]), suppress T cell responses by acting as Tregs (with production of immunosuppressive adenosine for example)[163], induce angiogenesis[159] and more[26]. They can also express ligands (such as PD-L1) or secrete factors which can induce additional pro-tumor effects[26].

The contradictions in the literature, even among studies of the same cancer type, are intriguing and crucial to learn more about. The opposing reports on γδ T cells in cancer are likely the result of the highly pleiotropic nature of γδ T cells[26]. The high plasticity of human γδ T cells along with differences in factors such as study design, clinical parameters and tumor characteristics (such as tumor microenvironment for example) might also help to explain the different findings[26]. Several studies report on a gradual shift from an IFN-γ-secreting anti-tumor response to a IL-17-producing pro-tumor response with increasing severity of the cancer, as observed in human melanoma[164], squamous cell cancer[165], colon cancer[154] and head and neck cancer[165]. In mice, it appears that the distinction between IFN-γ and IL-17-producing γδ T cells is imprinted early in thymic development[166,167]. However, due to the differences among species along with the high plasticity of human γδ, it is complicated to translate findings from mice to humans. Hence, more studies of human γδ T cells are needed to extend the knowledge on their role and contribution in human cancer.. In mice, it appears that the distinction between IFN-γ and IL-17-producing γδ T cells is imprinted early in thymic development[166,167]. However, due to the differences among species along with the high plasticity of human γδ, it is complicated to translate findings from mice to humans. Hence, more studies of human γδ T cells are needed to extend the knowledge on their role and contribution in human cancer.

1.4 CANCER IMMUNOTHERAPY

"*The history of attempts to immunize against cancer is one of long frustration. As a result of apparent failure during the past half century, it is current consensus that immune mechanisms probably will be of little use in the control of this disease.*" – Richmond Prehn and Joan Main a publication by from 1957 in which they challenged the current view and proposed immunization against cancer possible[168].

The pillars of cancer treatment have traditionally included surgery, radiation and chemotherapy. These are still vital parts of current cancer treatment. However, today there are two additional pillars which have been introduced with success and hold tremendous potential; molecularly targeted therapies and immunotherapy[169]. Molecularly targeted therapies were introduced in the late 1990's with the approval of human epidermal growth factor receptor 2 (HER2)-targeting trastuzumab (commercial name Herceptin®) for treatment of breast cancer[169]. This treatment category includes drugs which target molecules associated with tumor development, such as inhibiting cell growth with HER2-blocking, or inhibiting DNA repair with poly ADP ribose polymerase (PARP)-inhibitors.

Cancer immunotherapy includes approaches which utilize the immune system to overcome the tumor. This pillar in cancer treatment was introduced in the 1980/90's with initial treatments targeting cytokines[170]. Today, examples of immunotherapy include checkpoint blockade, cancer vaccines, adoptive cell therapies and more. With the advancements in tumor immunology in the past two decades, research and clinical translations involving immunotherapy has escalated, especially when it comes to checkpoint blockade[171]. In line with this development, the approvals of immunotherapeutic agents by medical agencies worldwide have also accelerated. Two immunotherapy categories will be reviewed here with an emphasis on checkpoint blockade.

1.4.1 Adoptive cell therapy

Adoptive cell therapy includes approaches in which cells are taken from a patient (or a donor) and are manipulated in some way before administration into the patient. One example is adoptive cell therapy with TILs. This concept was developed and introduced for clinical use by Steven Rosenberg and colleagues in the end of the 1980's[172]. The approach includes isolation of TILs from a tumor biopsy which are expanded *in vitro* and brought up to large quantities before injection into the patient in combination with IL-2 treatment[17]. Various approaches have been developed to boost the efficacy of the treatment, such as performing lymphodepletion of the patient prior to infusion[17].

Another example of adoptive cell therapy is the use of T cells with genetically engineered chimeric antigen receptors (CARs)[173]. This approach combines the intracellular machinery of a TCR with the specificity of an antibody (which is practical since antibodies do not require antigen processing or MHC presentation). The CAR construct is genetically modified to contain desired co-stimulatory domains (CD28 or 4-1BB for example, giving rise to different generations of CAR-constructs) and the recognition of a specific tumor target[17]. An example

of such a target is CD19 which is expressed by all B cells. The use of CD19-directed CAR T cells has been very successful in treating B cell malignancies[173] and this treatment type holds enormous potential. However, a massive challenge for any type of adoptive cell therapy is to overcome the immunosuppressive tumor microenvironment along with the other various escape mechanisms by which tumors can avoid elimination.

1.4.2 Checkpoint blockade

Checkpoint blockade aims to release the co-inhibitory-forced brakes on anti-tumor T cell responses. Today, two such pathways (PD-1 and CTLA-4) are targeted by approved drugs but many more are under investigation. The rapid development from discovery of PD-1 and CTLA-4 in the 1980/90's, development of checkpoint-targeting drugs and translation into the clinic from 2010 and onward has been a success story. Also, the awarded 2018 Nobel Prize in Physiology/Medicine to the checkpoint-pioneers Jim Allison and Tasuku Honjo was an honorable recognition for the impact which this category of immunotherapy has had.

CTLA-4 was the first checkpoint to be explored for immunotherapeutic use[65]. In 2011, ipilimumab (Yervoy®) was approved for use in advanced melanoma by the Food and Drug Administration (FDA, United States) and European Medicines Agency (EMA, European Union). To date (April, 2021) there are seven clinically approved checkpoint-targeting drugs by the FDA and EMA; one targeting CTLA-4, while the remaining six target the PD-1/PD-L1-pathway (two targeting PD-1 and four targeting PD-L1)[174]. An additional three PD-1/PD-L1-targeting reagents have been approved for use by the Chinese National Medical Products Administration, but not yet elsewhere[174]. Emphasis will be made on the PD-1-receptor-targeting agents as this is of primary interest in the work of this thesis.

1.4.2.1 Anti-PD-1 treatment

The interest for anti-PD-1 treatment rapidly increased after the successful results of an initial phase I trial was published in 2010[175]. More clinical trials followed, showing promising results in terms of clinical efficacy in a number of different tumor types at advanced stage[176]. In 2014, the first PD-1-targeting monoclonal antibody (mAb) nivolumab (Opdivo®) was approved in Japan to treat advanced melanoma. Many countries quickly followed and a second PD-1-targeting mAb, pembrolizumab (Keytruda®), was approved by many countries shortly thereafter. Since then, a steady number of indications have been added to the list of approvals along with additional PD-L1-blocking antibodies. Today, there are 19 different cancer types at advanced stage in which the use of PD-1 blockade is approved (including renal cell carcinoma, bladder cancer, head and neck cancer, cervical cancer, and more)[174]. Combination treatment using anti-PD-1 and anti-CTLA-4 (ipilimumab) is approved for numerous of these indications as well.

In 2015, the association between tumor mutational burden and response to PD-1 blockade was recognized in non-small cell lung cancer[177]. More studies corroborated these findings and also identified tumors with high microsatellite instability (MSI) or being mismatch repair

(MMR) deficient (common in colon cancer for example) to be more susceptible to PD-1 blockade[178,179]. Based on these findings, all advanced solid tumors with a high MSI/MMR profile were approved for the use of anti-PD-1 by the FDA in 2017, and in 2020 all tumor mutational burden-high cancers were approved[174]. This provided a groundbreaking shift in the use of PD-1-targeting reagents, where the use of a predictive biomarker rather than tumor origin or histology defines the indication to utilize PD-1 blockade[180]. These findings confirm the importance of immunogenicity and immune contexture as outlined earlier.

The response rate and different outcome parameters are highly variable depending on tumor type and tumor/patient characteristics (prior treatments for example). Generally, the proportion of clinical response ranges from 15-65%[104,181]. The highest efficacy is observed in relapsed/refractory Hodgkin's lymphoma, in which disease-specific genetic alterations are associated with overexpression of PD-L1/2, with an objective response rate of 87% reported in a phase I trial[182].

A recent report on the phase III CheckMate 067-trial, investigating the use of anti-PD-1 (nivolumab) in untreated advanced melanoma patients, can serve as an example of three lessons learnt from past years about checkpoint blockade (***Table 1***). First, monotherapy with anti-PD-1 shows superior clinical efficacy compared to monotherapy with anti-CTLA-4[183]. Second, combination treatment of anti-CTLA-4 and anti-PD-1 shows improved results compared to monotherapy, both when it comes to survival and other outcome parameters[183] (***Table 1***).

Table 1. *Overview of results from the Checkmate 067-trial conducted with advanced melanoma patients[183].*

Outcome parameter	Anti-CTLA-4 (*n*=315)	Anti-PD-1 (*n*=316)	Anti-CTLA-4 & anti-PD-1 (*n*=314)
Objective response rate	19%	45%	58%
Complete response	6%	19%	22%
Progression-free survival	2.9 months	6.9 months	11.5 months
5-year overall survival	26%	44%	52%
Grade 3/4 adverse events	23%	28%	59%

Third, this treatment category has high occurrence of treatment-related severe adverse events. This is especially true for a combination treatment, which is exemplified by the Checkmate 067-trial, in which the percentage of patients who developed the most severe adverse events (grade 3 or 4) was more than twice as high using combination therapy compared to monotherapy[183] (***Table 1***). The onset of autoimmune reactions is common when releasing the important regulatory checkpoint brakes[184]. Also, the spectrum of the potential immune-related side effects is broad and commonly manifests as gastrointestinal problems (such as colitis), endocrinopathies (such as thyroiditis), cutaneous manifestations (such as vitiligo and dermatitis) and hepatitis to name a few[184]. Other less common adverse events, such as myocarditis, encephalitis and pneumonitis, can in worst case cause treatment-related fatality, for which the estimated risk is 0.3-1.3%[184].

This study, along with others corroborating these findings, opens up for both celebration and frustration (***Figure 12***). Celebration because if you put this in a wider perspective, the 5-year overall survival of advanced melanoma patients before the use of targeted therapy and checkpoint blockers was below 10%[185]. In this sense, the results are fantastic. On the other hand, at 5 years there were still at least 48% patients for whom the immunotherapy was insufficient. This is still a high proportion of inadequate responses. Also, for many other cancer types, the results are not as spectacular. There is evidently still much room for improvement.

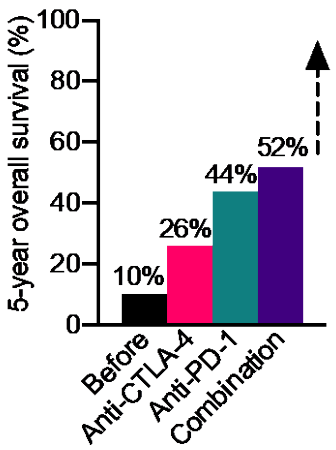

Figure 12. *Overall survival of advanced melanoma patients before the use of targeted therapies and immunotherapy, using monotherapy (anti-CTLA-4 or PD-1) or both. Arrow indicates future direction. Data from Larkin et al.[183] and Robert et al. [185].*

1.4.2.2 The continued development of PD-1 blockade

There are currently over 1200 actively recruiting studies involving PD-1 (clincaltrials.gov, April 2021). This gives an insight into the effort that is currently being made into the investigation of this promising approach. The number of trials has grown remarkably over the last decade and many focus on combination strategies to improve outcome parameters[186]. Despite the associated increased toxicities, we are likely to see more approved combination treatments in the upcoming future. There are many interesting candidates which are being assessed, both immune-related targets such as LAG-3 and TIM-3, but also other components targeting other aspects of the tumor environment such as VEGF or using molecularly targeted therapies such as PARP-inhibitors or traditional chemotherapy[187]. The advantage with combining different treatments is that multiple mechanisms of action are utilized simultaneously. This can lead to increased efficacy and combat problems with primary or acquired treatment resistance.

Checkpoint blockade has provided clinicians with a new powerful toolbox to treat metastasized tumors. Historically, late-stage cancer has had limited treatment options. However, despite being a success story from discovery to clinical application, the story of checkpoint blockade is far from finished. There are many limitations which need to be addressed[188]. Due to primary or acquired resistance, the majority of patients do not have durable responses and there are cancer types in which checkpoint blockade is used with absent or limited response. The results from clinical trials help to increase our understanding of where and why the strategy fails, guiding research of how it can be improved.

There is an urge to understand the mechanisms behind PD-1 blockade and more specifically a need to identify the subsets which are responsible for the therapeutic effect. Most evidence support the need for a pre-existing anti-tumor immunity for successful outcome[104], which the predictive importance of TIL presence and tumor mutational burden supports. Some have proposed PD-1 blockade to lead to enhancement of neoantigen-specific T cells[177]. Jim Allison and colleagues have identified anti-CTLA-4 and anti-PD-1 blockade to affect the TIL populations in different ways[69]. Both treatments have been found to induce the expansion of

exhausted $CD8^+$ T cells but anti-CTLA-4 has also been found to induce expansion of a specific subset of $CD4^+$ T cells[69]. In another study, they found combination treatment to induce expansion of a unique population of activated effector $CD8^+$ T cells, which was not found in either monotherapy[189]. This provides rationale for using combination treatment to increase the chance of positive outcome[69,189].

Other studies have highlighted the limited capacity for PD-1 blockade to rescue terminally exhausted T cells due to stable epigenetic imprints[132,133,190]. However, recent studies have identified the importance of a stem cell-like subset ($PD\text{-}1^+TCF1^+CD8^+$) following PD-1 blockade[191,192]. These two studies propose that PD-1 blockade does not reverse the terminally exhausted T cells, but rather expands this particular subset which is a progenitor subset in the exhaustion phase[191,192]. Others have found PD-1 blockade-induced expansion of $PD\text{-}1^-$ T cell populations[193]. Clearly, much remains to be elucidated about both direct and indirect mechanisms of action of checkpoint blockers. Continued research will be crucial for future optimization of PD-1 blockade.

1.4.3 DARPin® proteins

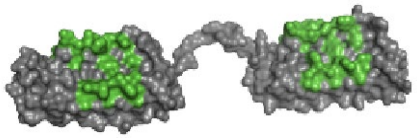

Figure 13. *Example of a DARPin® protein with two modules connected with a linker. The green parts highlight the targeting-binding domain.*

Designed ankyrin repeat proteins (DARPin® proteins) are an alternative to conventional mAbs. This alternative binding protein will be described due to the involvement in **Paper III** and, more specifically, investigation of checkpoint-targeting DARPin® proteins. In 2004, the developers of the DARPin® platform founded Molecular Partners AG, which still owns this technology[194]. DARPin® proteins are small proteins (approximately 15 kDa in its most simple design, compared to a full conventional IgG antibody of about 150-200 kDa) comprising of two caps, one at each end, and a number of variable library modules which make up the target-interacting domain(s)[194] (***Figure 13***). Huge DARPin® protein libraries have been developed using ribosome display as selection method to generate high-affinity DARPin® proteins[194]. The design of the library modules and number of modules are chosen based on the desired target of the DARPin® protein and are fused with the end caps resulting in a highly stable binding agent that can be produced using *Escherichia coli*[194]. DARPin® proteins can be designed to be mono- or multi-specific (targeting one or more targets) as well as being mono- or multi-valent (targeting one or more epitopes). They can be genetically or chemically linked to other small molecule conjugates (including other DARPin® proteins) to, for example, modulate serum half-life[195].

According to the manufacturer, DARPin® proteins have several advantages over conventional antibodies including the smaller size (giving rise to increased tissue penetration), high potency (making them active at lower concentrations), high stability and solubility (prolonging half-life and facilitating the therapeutic use), rapid and simple engineering and a cheap large-scale high-yield manufacturing process[195]. Furthermore, the manufacturer claims DARPin® proteins should display limited immunogenetic properties[196], which can be a problem with murine-derived (and even humanized) antibody parts.

One important difference between DARPin® proteins and conventional IgG antibodies is the lack of effector functions of DARPin® proteins[194]. Conventional antibodies function through either the neutralization of a target or through host effector functions initiated by the Fc region. Such effector functions include activating the complement system and/or binding to Fcγ receptors on effector cells (such as CD16 mentioned earlier). The binding to such Fcγ receptors can result in induced apoptosis of the antibody-bound cell, a process earlier mentioned as ADCC. There are a number of different types of Fcγ receptors and they are expressed by different immune subsets including NK cells, γδ T cells, macrophages and neutrophils[19,197]. In the context of checkpoint receptor blockade, there is however no benefit in having ADCC or complement activation, as the goal is not to lyse the cell to which the therapeutic protein has bound. Different subclasses of antibodies have different structural and functional properties affecting their capacity to act through these host effector functions[197]. The two FDA/EMA-approved PD-1-targeting antibodies nivolumab and pembrolizumab are both of IgG4 isotype and have been modified through a substitution in the amino acid sequence, thereby minimizing the cellular and complement cytolytic activities of the antibodies[175,198–200].

1.5 TWO SELECTED SOLID TUMOR TYPES

The work of this thesis builds on two solid tumor types and this section aims to provide a brief background of the two selected diseases.

1.5.1 Prostate cancer

Prostate cancer is the second leading cause of cancer-related death and the most frequently diagnosed cancer type among men[201]. Fortunately, the majority of diagnosed cases (90%) are discovered at a local or regional stage which has an optimal 5-year survival prognosis of nearly 100%[201]. However, patients with distant disease (*i.e.* metastasis to lymph nodes and/or other organs) have a 5-year overall survival of only 30%[201].

Prostate cancer is clinically staged according to the spread within or around the prostate, referred to as T-stage, ranging from T1-T4 (with different subgroups a/b) where increasing number/letter correlates to increased severity[202]. An additional important grading system is the histology-based Gleason system. Due to different pathology in different areas, the Gleason system builds on two numerical assessments ranging from 3-5. The first assessment represents the severity in the majority of the prostate biopsy and the second represents the worst severity observed in the whole biopsy. The assessments are added resulting in a total Gleason sum, ranging from 6-10 which gives five grades of severity (1-5)[202].

Treatment varies depending on the clinical stage and histology of the tumor, age and preference of the patient and other clinical factors, such as serum levels of prostate-specific antigen (PSA). Men with localized disease, where the cancer has not spread beyond the prostate, might not require any treatment[202]. In fact, for a majority of patients, active surveillance of progression is enough. For others requiring intervention, standard treatment includes surgery and radiation. In advanced disease, metastasis commonly spreads to the

bones and distant lymph nodes, but also liver, thorax and/or brain can be sites for metastasis[203]. At this stage, hormonal therapy with androgen (male sex hormone)-depriving agents and chemotherapy are commonly used treatments[202]. Castration-resistant is a term used for patients with advanced prostate cancer who do not respond to androgen-depriving agents.

At a less severe level, the prostate is a common site for benign transformation and chronic inflammation, especially with increasing age. The benign transformation, called benign prostatic hyperplasia (BPH), commonly develops in the transitional zone of the prostate (while prostate cancer usually has its origin in the peripheral zone) and results in enlargement of the prostate[204]. This non-malignant proliferation of cells can manifest by, for example, obstructing the urethra giving rise to clinical symptoms. Inflammation has been shown to drive the progression of both benign and malignant transformation in prostate cancer, but the link between BPH and prostate cancer remain inconclusive although there are indications of an association[204].

1.5.1.1 Checkpoint blockade in prostate cancer

Checkpoint blockade is not approved for use in prostate cancer and is discussed with ambiguity. Numerous clinical trials have investigated the use of monotherapy with anti-CTLA-4 or anti-PD-1, from which both negative[176,205,206] and positive[207,208] results have been reported (***Table 2***). The largest phase III study, with 799 patients with metastatic castration-resistant prostate cancer receiving radiotherapy with anti-CTLA-4 (ipilimumab) or placebo, revealed no differences in overall survival[206]. A recent phase II study investigating the use of anti-PD-1, reported a modest overall response rate (5% among three combined cohorts), but the authors concluded the response to be durable in a small number of patients[209]. This highlights the need for better tools to identify these responding patients[209]. Offering optimism for the future is the most recent and largest phase II study investigating the use of combination treatment (anti-CTLA-4 and anti-PD-1) in metastasized prostate cancer, which reported a high efficacy but with large differences in the different cohorts (pre- and post-chemotherapy)[210] (***Table 2***).

Table 2*. Results of clinical trials using various checkpoint blockers in prostate cancer. Abbreviations: no data (ND), overall survival (OS).*

Phase	Patients	Treatment	Checkpoint target	Objective response rate	Reference
I	17	Nivolumab	PD-1	0%	Topalian *et al.* 2012[176]
Ib	23	Pembrolizumab	PD-1	17.4%	Hansen *et al.* 2018[208]
II	10	Pembrolizumab	PD-1	30%	Graff *et al.* 2016[207]
II	258	Pembrolizumab	PD-1	3-5%	Antonarakis *et al.* 2020[209]
II	90	Ipilimumab + nivolumab	CTLA-4 & PD-1	10-25%	Sharma *et al.* 2020[210]
III	400	Ipilimumab	CTLA-4	ND, no change in OS	Beer *et al.* 2017[205]
III	799	Radiotherapy ± ipilimumab	CTLA-4	ND, no change in OS	Kwon *et al.* 2014[206]

Prostate cancer has been described to have a relatively low tumor mutational burden[117,179,211], which might limit the immunogenicity of the tumor cells and therefore response to immunotherapy. However, there are prostate cancer patients with reported high MSI, which might benefit from checkpoint blockade[207,210]. Continued investigation of different cohorts and regimens in this cancer type will help to distinguish the patients which are more likely to render response.

1.5.2 Ovarian cancer

Ovarian cancer is the fifth leading cause of cancer-related death among women[201]. Ovarian cancer is comprised of a range of different subtypes with different origins (including ovarian, peritoneal and tubal origin) and is further histologically grouped into five subgroups (high-grade serous, low-grade serous, endometroid, clear-cell and mucinous)[201].

Ovarian cancer is staged according to the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) classification system ranging from I-IV with different substages (A,B,C) where increasing number/letter corresponds to increasing severity of the cancer[212]. Ovarian cancer is often referred to as "the silent killer" due to the common asymptomatic clinical manifestation. This results in that the majority of cases (59%) are diagnosed at an advanced stage (III-IV), where the cancer has spread beyond the pelvic region worsening the prognosis[201]. The 5-year overall survival for these advanced stage patients is only 29%, compared to 48% for patients of all stages[201].

Patients with ovarian cancer commonly develop an accumulation of fluid in the peritoneal cavity within the abdomen called ascites (***Figure 14***). The underlying cause includes changes in the peritoneal membranes, protein concentrations and oncotic pressure[213]. Both presence and volume of ascites has been found to correlate with late-stage ovarian tumors and to a worse prognosis[214,215]. Metastasis primarily occurs to adjacent sites or through the detachment of tumor cells into the peritoneal cavity, using the ascites fluid as transportation, affecting the omentum (***Figure 14***), abdominal wall or any surface of intraabdominal organs (pelvis, other reproductive organs, appendix and/or colon as examples)[216].

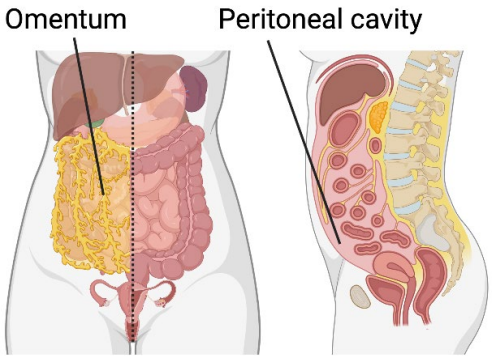

Figure 14. *Anterior view (left) with cross-section to show the omentum, a common site for metastasis, and sagittal view (right) to emphasize the peritoneal cavity, in which ascites commonly accumulates in ovarian cancer.*

Standard treatment includes initial surgical tumor debulking to physically reduce tumor volume and stage the tumor, followed by platinum- and taxane-based chemotherapy. Depending on a number of variables including the effect of initial treatment and histology, additional treatments can include angiogenesis inhibitors (anti-VEGF) and more recently PARP-inhibitors[201,217]. Unfortunately, the majority of patients (70%) will relapse within three years[217]. Cancer-associated antigen 125 (CA-125) is a serum tumor marker which is useful in

disease monitoring to follow the response to treatments (where CA-125 levels should go down) and as surveillance to discover recurring disease[212]. However, CA-125 is not specific for ovarian cancer and should therefore be used with additional diagnostical tools[212]. This is similar to the use of serum-PSA in the monitoring of prostate cancer.

1.5.2.1 Checkpoint blockade in ovarian cancer

Data from clinical trials investigating the use of checkpoint blockade in ovarian cancer has increased in the past couple of years (***Table 3***). The studies have included various cohorts of advanced ovarian cancer patients receiving anti-PD-1 as monotherapy or in combination with other treatments. The reported response rates are relatively low (***Table 3***), in comparison with other tumor types, but nonetheless demonstrating a potential in ovarian cancer. However, reports from three large phase III trials investigating the anti-PD-L1 avelumab puts an end to the optimism. These studies were discontinued due to not meeting primary endpoints of improved progression-free survival/overall survival at interim analysis[218,219] or in the case for the third study, due to the earlier study terminations among other reasons[220].

To balance the pessimistic results are the most recent results on a phase II study involving anti-PD-1 alone or in combination with anti-CTLA-4[221]. As observed earlier in advanced melanoma, the results showed superiority of the combination therapy over anti-PD-1 alone (***Table 3***). These results warrant further investigation of combination treatments in ovarian cancer and highlight that there are differences between different checkpoint blocking agents, where the anti-PD-L1 avelumab has failed to generate durable responses while anti-PD-1 nivolumab and pembrolizumab show some response. Also, ovarian cancer is a heterogeneous group of tumors and the range of results (limited/modest/successful) highlights the need to define patients who have higher chance of responding, similar as for prostate cancer.

Table 3*. Reported response rates in clinical trials using blockers targeting the PD-1/PD-L1 pathway in advanced ovarian cancer.*

Phase	Patients	Treatment	Checkpoint target	Objective/overall response rate	Reference
Ib	26	Pembrolizumab	PD-1	11.5%	Varga *et al*. 2019[222]
II	20	Nivolumab	PD-1	15%	Hamanishi *et al.* 2015[223]
II	376	Pembrolizumab	PD-1	7.4%	Matulonis *et al.* 2019[224]
II	100	Nivolumab ± ipilimumab	PD-1 ± CTLA-4	31.4% combination 12.2% anti-PD-1	Zamarin *et al*. 2020[221]
III	998	Chemotherapy ± avelumab	PD-L1	Discontinued	Merck/Pfizer 2018[218]
III	556	Chemotherapy ± avelumab	PD-L1	Discontinued	Merck/Pfizer 2018[219]
III	-	PARP-inhibitor ± avelumab	PD-L1	Discontinued	Merck/Pfizer 2019[220]

2 RESEARCH AIMS

The general aim of my doctoral research has been to investigate human TILs in two selected types of solid tumors. This has been done to contribute with knowledge about TIL presence, phenotype and functionality to optimize future immunotherapeutic approaches such as checkpoint blockade.

In **Paper I**, the aim was to study the presence and phenotype of TILs in prostate cancer and other relevant reference tissues, including prostate tissue with benign transformation and prostate tissue from healthy controls. The characterization was focused on expression of co-inhibitory receptors on T cells.

In **Paper II**, similar to **Paper I,** we wanted to study the presence and phenotype of infiltrating immune cells in ovarian cancer. Larger amounts of isolated cells allowed us to study the TILs in this cancer type with more detail compared to prostate cancer. Also, enabled by the larger cohort of patients, our aim was to correlate our findings with clinical parameters. Lastly, we performed a pilot on T cell functionality and whether it could be affected by anti-PD-1 treatment.

In **Paper III**, based on our findings in **Paper II**, we wanted to continue to explore the use of anti-PD-1 treatment. We wanted to further investigate the functionality of T cells derived from ascites and tumor tissue of ovarian cancer patients. The aim was to see if we could enhance the functionality using PD-1 blocking agents using conventional and clinically used mAbs (nivolumab and pembrolizumab) and two novel scaffold proteins (DARPin® proteins).

In **Paper IV,** our aim was to contribute with increased knowledge about γδ T cells in ovarian cancer. The ambiguity of the literature about the contribution of this subset in human cancer in combination with the findings in **Paper II**, triggered our curiosity for this subset in ovarian cancer. We aimed to explore the γδ T cells in a thorough manner by assessing their TCR characteristics, phenotype and functionality. Also, we wanted to see if we could find any associations to outcome and determine their contribution in ovarian cancer.

3 MATERIALS AND METHODS

In this chapter, the aim is to provide an overview of the used human materials, ethical perspectives and employed methods. For full details of the material and methods, please view these sections in each individual paper (**Paper I-IV)**.

3.1 HUMAN SAMPLE OVERVIEW

All work in **Paper I-IV** has been done on human-derived material. This has required a number of collaborations with different clinics to collect the patient-derived material needed for the work.

In **Paper I**, we collected peripheral blood and prostate tissue from five patients with prostate cancer (all clinical stage T2). These patients had a radical prostatectomy, a procedure in which the entire prostate is removed. From each of these patients, we obtained two small pieces: one malignant and one non-malignant, provided by a collaborating pathologist. We also collected peripheral blood and prostate tissue from 31 patients with the benign condition BPH, during a surgical procedure called transurethral resection of the prostate (TURP) to remove prostate tissue for symptom relief. Lastly, we collected prostate material from seven deceased donors, which we referred to as healthy reference material for the study. These prostates were also assessed by our collaborating pathologist and some were found to contain pathological findings, as expected due to the high age of some of the donors.

In **Paper II-IV**, we collected material from untreated patients undergoing primary surgery for advanced ovarian cancer (FIGO stage III-IV). We collected peripheral blood, ascites fluid and metastatic tumor tissue from the omentum of these patients at the time of surgery. In total, we used material from 38 patients for the work presented.

In **Paper III** and **Paper IV**, we also used lymphocytes isolated from peripheral blood of healthy blood donors as reference material (**Paper III**) or due to having limited number of patient samples (**Paper IV**).

3.2 ETHICAL CONSIDERATIONS

The work presented in this thesis is fully focused on humans. This type of research is necessary as no animal models can fully mimic the human complex biological context, making the use of human material essential. All work has been approved by the Swedish Ethical Review Authority (previously the Regional Ethical Review Board) in Stockholm, Sweden. The generation of **Paper I-IV** has been completely dependent on the participation of patients and their willingness to donate samples. Their participation is not anything I take for granted and each patient willing to contribute to research has my utmost gratitude and respect. Patient sample collection also puts high demands on clinical collaborators willing to invest time and effort into selecting appropriate patients for the studies, and also the staff working during the sample collection needs to be involved. All of these parts make sample

collection logistically challenging and that is why I am so grateful for all the people enabling our collections.

In **Paper I**, material from patients undergoing surgery to remove benign prostatic tissue (TURP) or the entire prostate (radical prostatectomies) is included. The prostate material we obtained would otherwise had been thrown or was redundant for clinical evaluation, hence the collection did not result in any negative effect for the patient. The patients donated peripheral blood samples with the purpose for our research use, and it is our understanding that all collections were done in a satisfactory way.

When material had been collected from the benign and malignant prostate conditions, we needed to know how our findings differed from the normal prostate. For this, we were granted an addition to our original ethical application to collect prostate tissue from deceased transplantation donors. From an ethical point of view, this collection is more controversial compared to the collection of material from living individuals. For the deceased individuals, permission has been obtained for donation of organs both for research use and/or healthcare. This is an important aspect as individuals have the possibility to only donate for healthcare purposes. A large problem with organ donation (regardless of whether for research and/or healthcare) is that the death of the donor is usually tragic, sudden and unexpected, which can mean that the donor has not had the possibility to decide on this matter him- or herself. In those cases, it is the closest family members who take this difficult but important decision. One can only hope that their decision reflects what the donor would have wanted. I am extremely grateful for the contributions from the deceased male donors to our study. This material is unique and a vital part of exploring the context of our findings, comparing diseased tissue with healthy.

For **Paper II-IV**, material from patients undergoing surgery for advanced ovarian cancer is included. The metastatic tumor tissue and ascites material we have obtained would otherwise have been disregarded and thrown away due to excess, therefore not affecting the patient. However, the patients have actively donated peripheral blood for the purpose of our study. The blood donation was in such a small volume that the patients have not risked side effects, aside from possibly bruising. For **Paper IV**, we were also approved an addition to our original ethical application to send samples abroad for NGS analysis we could not perform ourselves. For this purpose, we anonymized samples from eight patients before sending them for analysis.

Importantly, the donation of material from any of the patient groups included in **Paper I-IV** has not put the patients at increased risk. Patient ID has been coded immediately in all described work to ensure patient integrity. Involved clinicians have later on retrieved clinical information such as cancer stage, histology and residual tumor burden for example. This information has been vital to make correlations to our generated immunological findings. It has also been important for the exclusion of several patients (**Paper II**) which did not meet the inclusion criteria. Another ethical aspect to the described work is that the patients themselves have not benefitted from their participation in the studies. However, they were

informed about this aspect and also that could withdraw themselves at any time without affecting their continued treatment and care negatively. It is our understanding, after discussions with our clinical contacts, that patients are generally happy to contribute to further knowledge about the disease which they are affected by. This way, something good can come out of it.

3.3 LABORATORY METHODS

In this part, the aim is to provide a brief overview of the methods used in the presented work. In ***Figure 15***, a generalized schematic is provided for the workflow of the patient samples which will be further described in the upcoming sections.

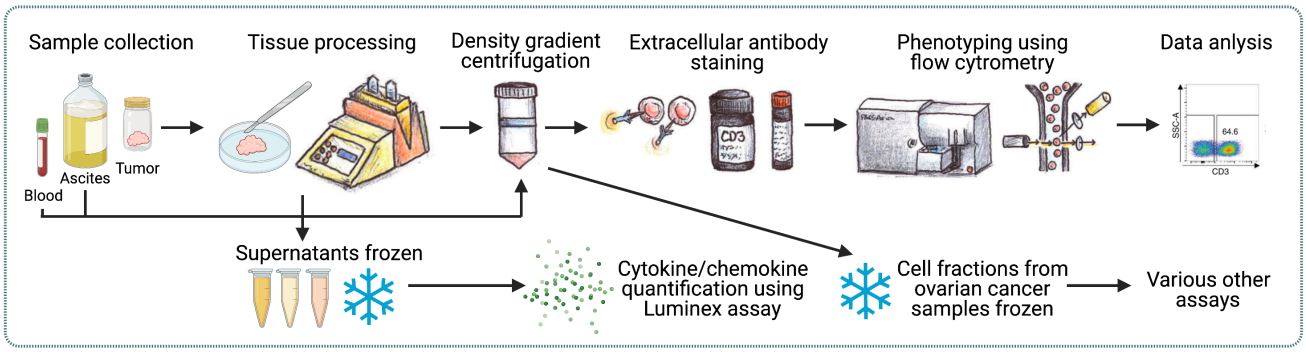

Figure 15. *The workflow for characterizing immune cells from ovarian cancer patients. The workflow is the same for samples from prostate cancer patients, except for saving cell fractions for further assays. Parts of the figure adopted from Norström et al.*[225].

3.3.1 Tissue processing and isolation of mononuclear cells

All work in **Paper I-IV** has been dependent on the initial processing of the collected patient material. We developed the method for prostate tissue[225] but the procedure worked equally well for tumor samples from ovarian cancer (omentum origin). For the tissue processing, we only used mechanical dissociation. Some protocols suggest using enzymatic dissociation, but we have avoided doing so to preserve the integrity of the cells as much as possible. Using a scalpel for manual cutting followed by gentleMACS Dissociator (developed by Miltenyi Biotec) have been very useful ways to increase the yield of cells, and with extensive filtering and washing, the cell suspension can then be placed on a density gradient centrifugation (***Figure 15***). The original procedure of isolating mononuclear cells was introduced in 1968 by Bøyum[226]. This step has been performed to increase the yield of our cell type of interest, the lymphocytes. Materials in a liquid form (peripheral blood and ascites) have also been placed on a density gradient to purify the peripheral mononuclear cells. Density gradient centrifugation takes advantage of different densities of different cell types, and we can remove the big bulk of cells which could interfere in downstream application (such as granulocytes, tumor cells, *etc.*). However, these other cell types still remain to some degree in the cell fraction. The cells can thereafter be used directly in continued downstream applications such as flow cytometry or be frozen until further analysis (***Figure 15***).

3.3.2 Flow cytometry

Flow cytometry is by far the most used method in the generation of **Paper I-IV**. It is a powerful method which enables the detection of both intra- and extracellular markers at the single-cell level[227]. The obtained information can be used to determine the composition of different immune subsets and their phenotype (*i.e.* expression of different receptors/molecules). It is also very useful for intracellular cytokine staining (ICS), to assess the production of cytokines in response to a certain stimulus. In this approach, reagents (such as Brefeldin A and GolgiStop) are added to keep the produced cytokines within the cell by inhibiting secretion.

The principle of flow cytometry is based on detecting fluorochrome-conjugated antibodies which have bound to targets of interest inside or membrane-bound outside individual cells. After cells have been labelled with these specific antibodies, they are acquired on a flow cytometer (***Figure 16***). Through the fluidics system, cells are provided one by one in a stream by hydrodynamic focusing[227]. Each cell is hit by light from different lasers (number is depending on instrument). The light excites the bound fluorochromes (coupled to the antibodies) and light at a different wavelength is emitted. After passing through different optical filters (allowing for light of certain wavelength), different detectors will then receive the emitted light. The detection is amplified and converted into an electrical signal. Depending on the instrument, different number of wavelengths can be detected and hence different number of antibodies can be used. There are also two additional detectors which gather information on other features of the cells beyond fluorescent light, namely size of the cell (by measurement of forward scatter of light) and granularity (side scatter of light).

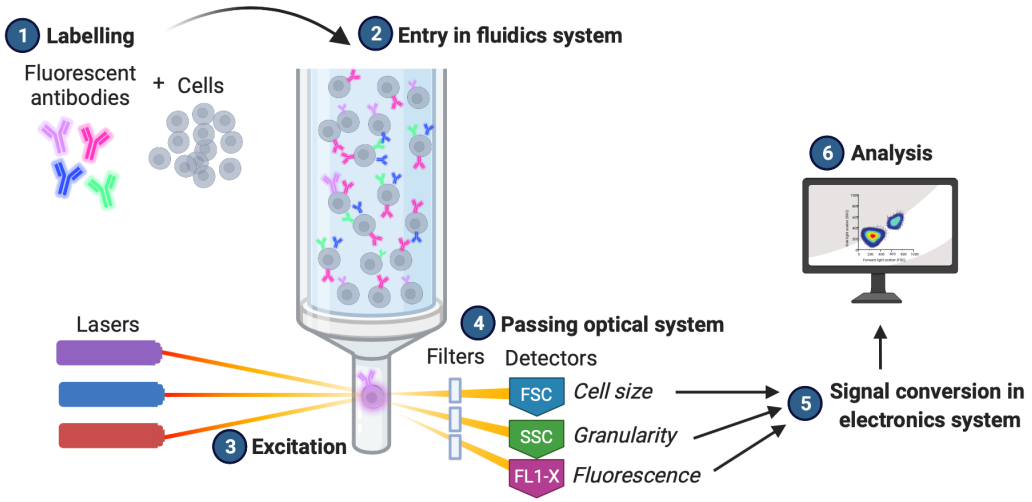

Figure 16. *An overview of the basics of flow cytometry.*

The use of flow cytometry requires the inclusion of several important controls[228]. These controls are necessary to make sure that the instrument is stable (quality controls), to compensate for so-called spectral overlap between fluorochromes (compensation controls), to assess the background (unstained control) and to enable correct data analysis by defining positive and negative populations (fluorescence-minus-one controls). Isotype controls can also be included to make sure that there is no interference from non-specific binding[228]. If

performing stimulation assays, the use of relevant biological comparison controls are also important, such as an unstimulated control to define positive/negative events[228].

The method was introduced in the 1970's and is continuously evolving with regards to instruments, the number of assessable parameters, fluorochrome and antibody availability and more[229]. In the phenotypic work presented in **Paper I-IV**, we have assessed 9-12 parameters on the same cell beyond cell size and granularity. One of these parameters has always been used to stain for viability (by the addition of a DNA-binding dye such as 7-amino actinomycin D) to exclude dead cells, as these might bind antibodies non-specifically and give rise to false positive results[227]. With the remaining parameters, there has been much room to explore the immune composition, immune subsets and subset phenotype in the sample material. We have also used the method to assess the production of cytokines in response to different stimuli (**Paper II** and **IV**).

This method can also be used on specialized equipment to sort cells of interest, referred to as fluorescence-activated cell sorting (FACS). The most common sorting principle is by droplet sorting[229]. The target cell population is defined based on one or several parameters and the cells, provided in individual droplets, are electrostatically sorted into tubes/plates or waste depending on the target criterions[229]. Thereafter, the cells can be used in other applications. This sorting method has been used in **Paper IV** to isolate γδ T cells for FluoroSpot assay, which will be described later on (section **3.3.9**).

3.3.3 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a robust and easy method to detect a single analyte in a biological fluid introduced in early 1970's[230]. It can be done in multiple formats but the sandwich procedure, introduced in 1977[231], is more sensitive[232] and most relevant for the presented work in this thesis (***Figure 17***). With this technique, a plate is pre-coated with mAbs specific for the analyte of interest[232]. After blocking the plate to prevent unspecific binding, the sample is added and allowed to incubate. The analyte will be captured on the plate by binding to the mAbs. Thereafter, a second biotinylated detection mAb is added. The next step is to add enzyme-linked streptavidin, which will bind to the biotin of the detection mAbs. The last step includes adding a substrate which will be cleaved by the enzyme resulting in a color change. This color change is proportional to the amount of analyte in the sample. The plate is analyzed with an ELISA reader (spectrophotometer) which determines the color by measuring the absorbance at a certain wavelength. The absorbance can then be quantified into a concentration by comparing to standard controls of known concentration included in the run[232]. ELISA has been used in **Paper II** and **III** to determine the production of IFN-γ in cell culture supernatants to determine response to different stimuli and PD-1 blocking reagents.

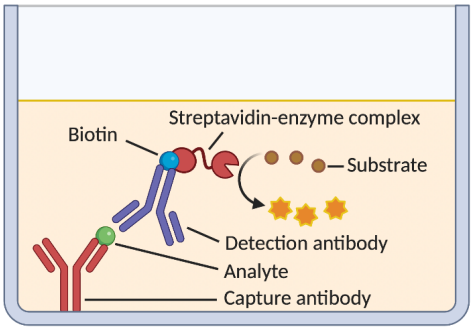

Figure 17. *Overview of ELISA sandwich assay components.*

3.3.4 Luminex multiplex assay

Luminex multiplex assay is a bead-based method building on principles of flow cytometry and enables the detection of multiple analytes in a fluid format such as blood plasma or cell culture supernatant for example. The method was introduced in late 1990's[233] and today builds on x multi-analyte profiling (xMAP) technology, which, according to the developer (Luminex Corporation), offers detection of hundreds of analytes simultaneously depending on instrument and setup. For each assessed analyte, there is one type of bead which is conjugated to mAbs specific for the analyte[234]. Therefore, the number of different beads corresponds to the number of analytes. Each type of bead can be identified by its own unique fluorescent signal. The sample is added to a pool of the beads resulting in binding to the analytes. Thereafter, biotinylated detection mAbs are added which bind specifically to the analytes. Last in line is the addition of fluorescent PE-conjugated streptavidin which will bind to any biotin (*i.e.* to all detection antibodies). This is quite similar to the procedure described for ELISA, except that this happens in solution (rather than to the bottom of wells) and in the end you have a measurable fluorescent product instead of enzyme-substrate reaction. In Luminex, however, information on many more analytes can be obtained as the results are acquired using two lasers; one which helps to identify the analyte (identifying the unique fluorescent signal for each bead), and one which detects the concentration (by the intensity of the PE signal). The inclusion of multiple controls enables quantification of the results into absolute concentrations of the analytes[234].

Multiplexing by Luminex has been used in all four papers included in the thesis. In **Paper I**, **II** and **IV**, concentrations of 26 cytokines and chemokines were determined in blood plasma, ascites fluid and processing supernatants from prostate tissue or metastasized tumor tissue. In **Paper III**, six analytes in supernatants from cell cultures were assessed by Luminex to determine the response to different PD-1 blocking reagents.

3.3.5 T cell stimulation

T cell stimulation can be valuable to track various responses in different settings[129]. In **Paper II-IV**, numerous stimuli have been used to stimulate T cells to assess cytokine production, proliferation or cytotoxicity by ELISA, flow cytometry and/or FluoroSpot. In **Paper II** and **III**, we used OKT3 to artificially stimulate T cells. OKT3 is a monoclonal anti-CD3 antibody, which binds to a subpart of the CD3 in the CD3/TCR complex leading to T cell activation. In addition, in **Paper II** and **IV**, we used phorbol 12-myristate 13-acetate and ionomycin (PMA/I) to stimulate T cells in a shorter time frame. PMA/I activates T cells in a synergistic way by the calcium-induced activation of protein kinase C, which triggers important internal pathways in a TCR-independent way[235].

In **Paper IV**, in addition to using PMA/I, we performed γδ T cell stimulation using the potent pAg HMBPP (stimulating the $V\gamma9^{+}V\delta2^{+}$ subset specifically) and anti-TCRγδ. We also assessed proliferation of γδ T cells in response to anti-CD3 (OKT3), anti-TCRγδ, anti-MICA/B and recombinant IL-15 to determine differences based on origin of the γδ T cells.

The addition of CellTraceViolet enables tracking of proliferation since this dye dilutes as cells proliferate, leading to decreased intensity of the fluorescent signal measured by flow cytometry. Furthermore, we performed killing assays using an ovarian cancer cell line (OVCAR-3) and incubating these cells at various effector:target ratios with γδ T cells or $CD8^+$ T cells. We assessed the effect by IFN-γ production and cytotoxicity (calculated by viability in samples versus controls) measured by flow cytometry.

3.3.6 PD-1/PD-L1 blockade bioassay

In **Paper II**, we assessed the pharmacodynamics of the included PD-1 targeting reagents. Beyond traditional PD-1 binding assessment measured using flow cytometry (for details see Material and Methods of **Paper II**), we used a reporter cell assay in which the capacity of the PD-1-binding reagents was investigated. In this assay, reporter cells (Jurkat T cells) expressing PD-1 and a luciferase reporter triggered by NFAT-activation are incubated with PD-L1-expressing aAPC/CHO-K1 cells[236]. The aAPC/CHO-K1 cells are engineered to stimulate TCRs independently of antigen recognition. The two different cell types are allowed to interact resulting in limited signal, due to the inhibitory effects of the PD-1/PD-L1 pathway. With the addition of different PD-1 or PD-L1-targeting reagents, the inhibition caused by the PD-1/PD-L1 interaction is interfered. This will result in TCR signaling, leading to NFAT activation and the coupled production of luciferace. The resulting luminescent signal is detected and can be compared between different conditions[236].

3.3.7 Magnetic-activated cell sorting

In some applications, it can be useful if the cell population of interest has been purified. This can be done in multiple ways, such as by using FACS for example. A quicker and more gentle method to purify cells is by magnetic-activated cell sorting (MACS)[237]. One of the reasons why this method is faster is because multiple samples can be sorted simultaneously[237]. The MACS method involves magnetic beads which are coupled to mAbs. A cell suspension is incubated and labelled with the beads and thereafter rinsed through a column which is placed inside a magnet. The column helps to amplify the magnetic force and to retain the magnetic beads (and therefore the labelled cells). The labelled target cells are retained in the column, while the unlabeled cells are rinsed and washed away. The target cells are then recovered by removing the column from the magnet and washed into a new suspension. This process is known as positive selection. The purification can also use the principle of negative selection. Using this approach, the non-target cells are labelled and will end up being retained by the magnet. The target cells in this case are unlabeled and therefore pass the magnet and end up in the collected suspension.

3.3.8 Next-generation sequencing

Next-generation sequencing (NGS) or high-throughput sequencing as it is also called, is a technique which has revolutionized the field of genomics in the past 15 years[238]. The traditional Sanger sequencing was introduced in 1977[239], and NGS has (as the name indicates) taken this method to the next level by dramatically improving the magnitude, cost

and efficiency of sequencing[238]. One of the key differences which enables this evolution is the sequencing of multiple fragments in parallel rather than one at a time as in Sanger sequencing[240]. Today, there are many available NGS platforms enabling both research and clinical use of this powerful technology. The platforms can vary from one another in numerous ways and there are also variations depending on the application, as the length of region to be sequenced for example[238,240]. However, the majority of available platforms share the same fundamental steps. These include library preparation, clonal amplification, sequencing and data analysis (***Figure 18***).

During the library preparation, DNA (complementary DNA originating from RNA or genomic DNA) from the sample is fragmented into pieces. This can be performed by using enzymes or amplifying the region of interest by polymerase chain reaction (PCR) for example. Small fragments known as adapters and indexes are added to the ends of the pieces. These pieces enable binding and barcoding of the DNA inserts by the sequencing instrument. The DNA fragments can then undergo clonal amplification in which they increase in number while maintaining their adapters and indexes. The sequencing itself can thereafter be done by the concept of "sequencing by synthesis" but there are also other variants. Sequencing by synthesis can be done in different ways but an example is by the complementary binding of fluorescent nucleotides, which are incorporated and detected by the instrument. After sequencing, the vast amount of generated data needs to be put together and analyzed.

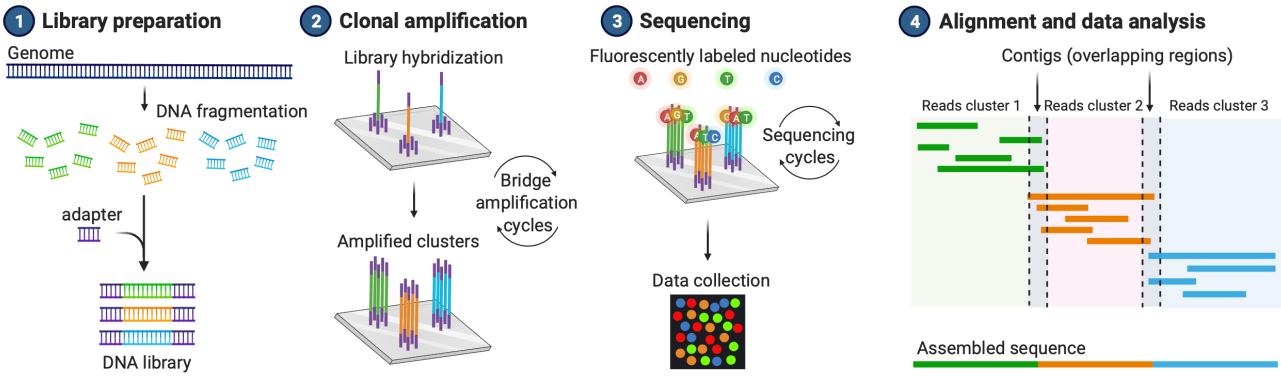

Figure 18. *Overview of NGS using the Illumina system.*

In **Paper IV**, we used NGS to assess the complementarity-determining region 3 (CDR3) region of the TCRγ-chain (TRG) of γδ T cells. Each such sequence is a unique tag of a γδ clone which allows the investigation of clonality but also other TCR characteristics. The company which we used to perform the NGS, Adaptive Biotechnologies, specializes in sequencing of TCRs. For TRG sequencing, their platform (called ImmunoSEQ) uses a synthetic repertoire covering all combinations of the V and J genes to optimize the procedure and reduce bias[241]. Multiplex PCR is used to amplify the region of interest and their platform includes various controls to ensure sufficient quality[241]. For data analysis, we used their in-house software ImmunoSEQ analyzer along with various publicly available packages[242,243] (for full details, see Supplementary Materials of **Paper IV**).

3.3.9 FluoroSpot

The original method, called enzyme-linked immunospot (ELISpot) assay, was first described in 1983[244]. FluoroSpot developed as an extended version of ELISpot in 2003[245]. Using this method, analytes of interest are captured upon the release from cells and detected at the single-cell level with high sensitivity. The method resembles ELISA but with some differences. First, cells are added to a pre-coated plate and allowed to incubate with a stimulus. During this time, plate-bound antibodies at the bottom of the wells will bind any secreted analyte for which they are specific. After the chosen incubation time, cells are washed away while the analytes remain in the plate and are developed in a similar way as in ELISA. Another difference to ELISA (and ELISpot) is that the analyte detection is by fluorescence instead of absorbance. This enables the detection of up to four analytes (at present time) in the same well. The release of analyte from a cell will be seen as a spot. FluoroSpot does not give absolute concentrations as in ELISA, but rather the number of so-called spot-forming units (*i.e.* number of secreting cells). It captures secretion without risk of degradation or consumption of the analytes among cells. With the newly introduced FluoroSpot reader Iris (developed by Mabtech), relative values for secretion between different cells and conditions can be assessed which adds to the quantitative measurements.

In **Paper IV**, we used a triple FluoroSpot to assess secretion of IL-17A, IL-10 and IFN-γ. With this method, we were able to use limited cell numbers and a long-time frame for the incubation. For our purpose, FluoroSpot was valuable to confirm the absence of IL-17A secretion from ovarian cancer-derived γδ T cells.

3.4 STATISTICS

All data in **Paper I-IV** has been assessed using two-sided non-parametrical statistical tests unless stated otherwise (a few instances in **Paper IV** where distribution was checked due to small sample sizes). The choice of treating the data as non-parametric was under the assumption that a normal distribution could not be assumed. The used non-parametrical tests included Mann Whitney U-test for unpaired data and Wilcoxon signed-rank test for paired data when comparing two groups (**Paper III**). When comparing three groups or more, Friedman test for unpaired data and Kruskal-Wallis for paired data have been used. Thereafter, if a significant difference was detected, we have either continued using Mann Whitney U-test followed by Bonferroni correction (**Paper I**), Wilcoxon signed-rank test followed by Bonferroni correction (**Paper I, II**) or Dunn's multiple comparisons test (**Paper IV**). In **Paper I**, the small sample sizes in some of the patient groups made us want to report on the significant findings before applying the Bonferroni correction but clearly stating this to be trends by putting the significance in parenthesis in the figures. Throughout the work described, median values have been provided with interquartile range (IQRs) and minimum/maximum values as these measures are more appropriate when handling data as non-parametric.

For correlation analysis, Spearman's rank coefficient has been used and when plotted, non-linear regression has been applied. Log-rank test has been used to assess differences in overall survival between different groups and results have been plotted using Kaplan Meier survival curves. Univariate analysis has been used throughout, meaning assessing differences between groups looking at one parameter at a time. However, for **Paper II**, a multivariate risk factor analysis, meaning several parameters were assessed simultaneously, was performed using EZR software grouping the different identified risk factors by either number of risk factors (0-4 risk factors or 5-8 risk factors) or based on sample origin (ascites or tumor). In **Paper IV**, analysis of The Cancer Genome Atlas (TCGA) datasets was performed using the publicly available Gene Expression Profiling Interactive Analysis 2 (GEPIA2) web tool to assess the associations between selected transcripts and survival.

Majority of statistical analysis has been performed using GraphPad Prism. In **Paper II**, EZR was also used for statistics and in **Paper IV**, some additional software were used as stated in the supplementary materials of the paper. Significance levels have been set to $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).

4 RESULTS AND DISCUSSION

The field of tumor immunology has rapidly expanded in past decades due to increased understanding of how tumors can avoid elimination by the immune system. The advances have resulted in development of many immunotherapeutic strategies, of which several have successfully been translated into clinical use. These treatments have helped to extend the lives of many patients with advanced cancer diseases. However, there is a vast amount of work remaining, both when it comes to our understanding and in the development of improved future strategies[188].

In the work of this thesis, we wanted to take a step back to cover some of the foundation; to find out more about the presence of TILs in the two selected cancer types. *Which subsets are present? What does their phenotype look like? Which subsets are associated to outcome? What are potential targets for immunotherapy?* All of our work has been performed with the intention of being useful for basic understanding and the development of future immunotherapeutic approaches.

4.1 ASSESSING TILS BY FLOW CYTOMETRY

The traditional way to assess the presence of TILs in solid tumors is by performing immunohistochemical staining on tumor material. This provides a visual overview of present cell types but there are limitations in the amount of information which can be assessed. We wanted to explore the presence of TILs using flow cytometry, which provides much more thorough information on the composition of cell types, subsets and their expression of certain receptors. Increased knowledge about the composition and phenotype of TILs can be useful to determine the suitability of immunotherapeutic targets. Many approaches rely on the expression of certain molecules, co-inhibitory receptors for example, and confirming their presence in different cancer types thereby becomes important.

Before starting to work on TILs, we established a simple, straight-forward method to isolate and characterize lymphocytes from prostate material[225]. For us, it was important to use a method which preserved the phenotype and viability as much as possible, and we therefore excluded the use of any enzymatic digestion. The procedure was optimized using prostate tissue but proved to work equally well for isolating TILs from ovarian cancer-associated tissue (***Figure 15*** in previous chapter). One large difference between the studies of ovarian cancer (**Paper II-IV**) and the study of prostate cancer (**Paper I**) was the amount of tissue we worked with. For ovarian cancer samples, we obtained large tumor volumes from metastasized areas within the abdomen. The situation was different in prostate cancer, where we worked with primary tumor material. This provided a challenge since the amount of material was much more limited. Thankfully, we were able to establish a collaboration with a pathologist to obtain such rare material while maintaining sufficient material for routine histological diagnostics. The limited material and need for logistical solutions are likely reasons for why there are few published studies on TILs isolated from prostate cancer tissue.

In **Paper I**, we found that even in solid tumor types where the amount of malignant tissue for research is limited (median 0.03 grams among our prostate cancer lesions), characterization by flow cytometry is possible. This is good news for all who work with limited material. However, the tumor needs to have an adequate infiltration of T cells to obtain sufficient cell numbers. In the literature, prostate tumors are often referred to as immune deserts or cold tumors, suggesting that they are not abundantly infiltrated with immune cells[188,210]. Despite this, we obtained sufficient T cell numbers from very limited material. We found the prostate cancer lesions to have approximately a 20-fold increase in the amount of present T cells compared to control prostates. This abundance made it possible for us to work with small tumor volumes and still use flow cytometry for characterization. This provides rationale to look into all kind of tumor types, even the ones regarded as cold, to perform flow cytometric phenotyping of immune infiltrates.

4.2 TIL COMPOSITION AND PHENOTYPE

By mapping the immune composition in prostate and ovarian cancer (**Paper I** and **II**), we found all kinds of lymphocytes present at the tumor sites; B cells, NK cells and T cells. In **Paper II**, we also assessed the presence of monocytes in ovarian cancer, which we found to be present in large proportions of the total ($CD45^+$) leukocyte population. The majority of these expressed the myeloid marker CD33, which suggests that these cells could act as tumor-promoting MDSCs. This is speculative as we did not investigate or confirm this further. All continued characterization was performed on T cells.

4.2.1 Mapping the T cell subset landscape

In both prostate and ovarian cancer, we found $CD8^+$ T cells to be the most abundant T cell subset in the tumor (***Figure 19***). The majority of T cells had an effector memory ($CCR7^-$ $CD45RO^+$) phenotype. This is similar to other reports and also in other cancer types, including breast cancer and melanoma[246]. Also, both prostate and ovarian cancer-derived tumors had a substantial proportion of Tregs, based on the commonly used phenotype to describe them, $CD25^{high}CD127^{-/low}$ $CD4^+$ T cells[247] (***Figure 19***). These findings corroborate other reports on abundant presence of Tregs in prostate cancer[248] and ovarian cancer[249,250].

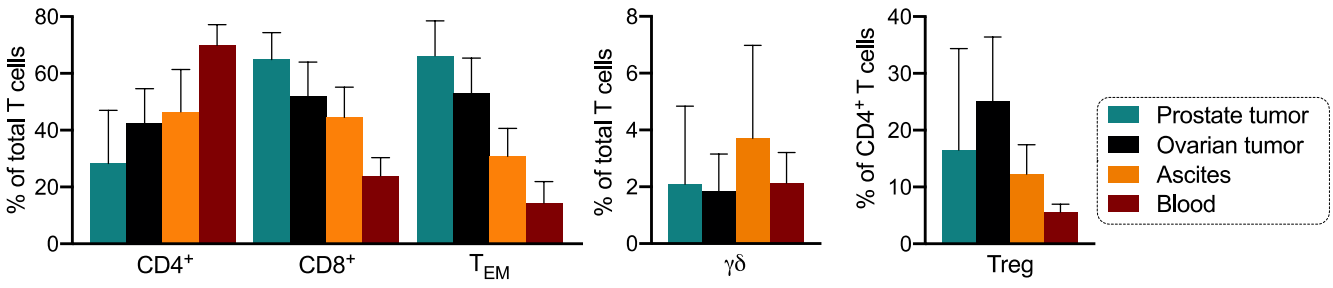

Figure 19. *T cell subsets and their frequencies in prostate cancer samples (from primary malignant site, n=3-5) and ovarian cancer samples: metastasized tumor site (n=35), ascites (n=30) and peripheral blood (n=35). Effector memory (T_{EM}) was defined as $CCR7^-CD45RO^+$ and regulatory T cells (Treg, as $CD4^+/CD25^{high}CD127^{-/low}$). Bars indicate median with IQR. Merged data from* ***Paper I*** *and* ***II****.*

γδ T cells were also found to be present in both tumor types, as well as in ascites of ovarian cancer patients (***Figure 19***). The data on γδ T cells in prostate tumors was excluded from **Paper I** due to not being investigated in all five prostate cancer samples (*n*=3). This points out a large limitation with **Paper I** that I would like to point out early; the limited number of patients which we retrieved material from. The risk with a limited patient number is that it can be difficult to draw conclusions from the findings, as the variation between individuals has not been adequately explored. Nonetheless, the generated data can help to guide continued studies and helps to provide important information on a cancer type where obtaining research samples has its challenges, as mentioned earlier.

The investigation of TILs in the ascites fluid of ovarian cancer patients is intriguing since this is a liquid tumor microenvironment. As seen in ***Figure 19***, we often found the ascites fluid to display an intermediate profile between findings in paired tumor and peripheral blood samples.

4.2.2 Mapping co-inhibitory receptor expression

We profiled the expression of four receptors with known co-inhibitory effects: LAG-3, PD-1, TIM-3 and CTLA-4. For both cancer types, we observed that PD-1 was expressed by the majority of total T cells (***Figure 20***). In prostate cancer, the expression of the other receptors (LAG-3, TIM-3 and CTLA-4) was very low in comparison with the proportions found in ovarian cancer. In contrast, we found TIM-3 to be expressed by a large proportion of the tumor-derived T cells from ovarian cancer patients. This exemplifies that different cancer types can have different profiles, in line with studies of co-inhibitory receptor expression in other cancers such as lung cancer[99] and gastrointestinal stromal tumors[102]. Again, ascites displayed a profile intermediate of tumor and peripheral blood samples.

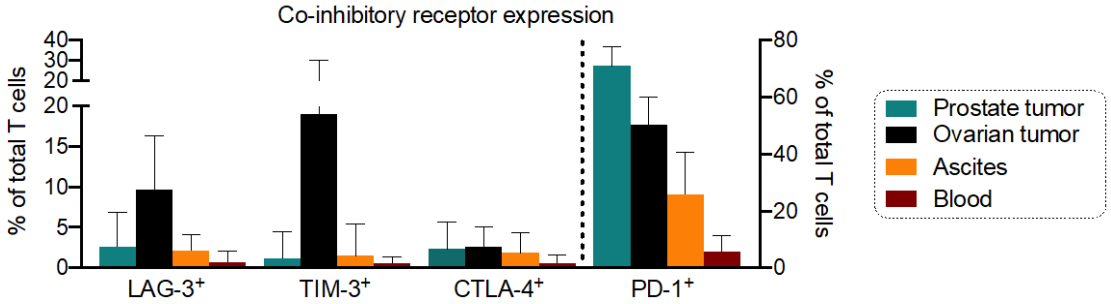

Figure 20. *Expression of different co-inhibitory receptors on T cells isolated from malignant sites of prostate cancer (n=5) or samples from ovarian cancer patients: metastasized tumor site (n=35), ascites (n=30) and peripheral blood (n=35). Bars indicate median with IQR. PD-1 data applies for the right x-axis. Merged data from* ***Paper I*** *and* ***II****.*

In **Paper I,** we were able to obtain relevant reference tissues, which proved to be important. These included prostate tissues with common benign changes and prostates obtained from deceased donors, which we referred to as healthy control prostates. Interestingly, we found that the expression of PD-1 was common in all assessed prostate material, even the control prostates (***Figure 21***). Knowing that our reference material came from deceased donors, some of high age, the prostates were examined by our collaborating pathologist. As expected, some

of the control prostates had histological findings which might have affected the immune infiltration and phenotype. However, two prostates with a normal histology coming from young donors (ages 22 and 36), also presented abundant PD-1$^+$ T cells (marked as filled grey circles in ***Figure 21***). The inclusion of reference tissues was a strength in **Paper I**, while being a limitation in the other papers (**Paper II-IV**), in which we did not have this.

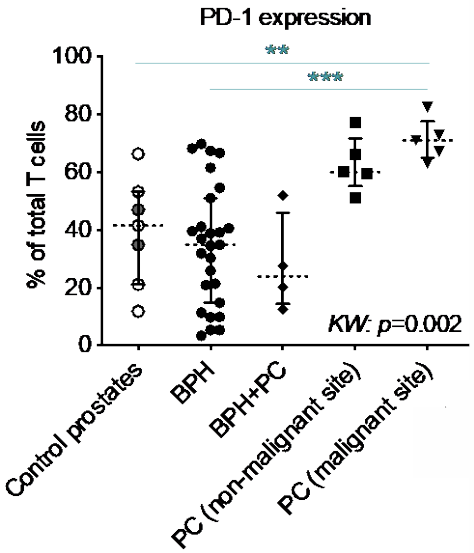

Figure 21. *Expression of PD-1 among T cells in all assessed prostate samples, including healthy control prostates, benign prostatic hyperplasia (BPH), prostate cancer (PC, paired samples from two sites) and BPH with undetected PC (+PC). Median values along with IQR. P-value for Kruskal Wallis (KW) test. Grey filled circles mark control prostates with normal histology. From* ***Paper I***.

Numerous studies report on absent or limited responses using checkpoint blockade in prostate cancer[176,205,206]. Compensatory up-regulation of other co-inhibitory receptors, such as VISTA, can present challenges as shown in prostate cancer[251]. Sfanos *et al.* have previously identified PD-1 expression to be common among prostate cancer-derived TILs[100]. Although PD-1 expression appeared to be more frequent among TILs in prostate cancer compared to other prostate histology (***Figure 21***), we speculated that PD-1 might not be a suitable target for immunotherapy in prostate cancer. Instead, PD-1 might be involved in other aspects of the prostate biology, such as regulating prostate inflammation for example. Based on the limited material, it can also be useful to know that the other assessed co-inhibitory receptors were expressed by a limited proportion of prostate cancer-derived TILs and therefore might not be appropriate targets either to boost T cell responses in prostate cancer. One of the challenges for immunotherapy is understanding the organ-specific contribution to anti-tumor immunity[188,252]. The impact of organ origin, with differences in spatial organization, vascularization and presence of various cell types for example, is discussed to influence tumor progression[252]. Our results support the prostate to be no exception to this. Immune involvement in non-cancerous conditions (such as BPH and prostate inflammation) likely influences the development of prostate cancer in ways not yet fully understood.

4.2.2.1 Co-expression

Due to the identified presence of co-inhibitory receptors in ovarian cancer samples, we assessed the co-expression patterns on CD4$^+$ and CD8$^+$ T cells. Interestingly, we found that a substantial proportion of the T cells (in particular the CD8$^+$ T cells) had multiple expression of these receptors (***Figure 22***). Of the double checkpoint-expressing T cells, the most common receptor combination was PD-1 and TIM-3. This is corroborated by another study, in which they assessed the same set of four receptors on T cells isolated from ascites/tumor and identified the same co-inhibitory hierarchy on CD8$^+$ T cells (PD-1 most abundant

followed by TIM-3, LAG-3 and lastly CTLA-4)[253]. A similar pattern was observed for $CD4^+$ T cells with the exception that they identified CTLA-4 to be abundantly expressed[253], in contrast to our study.

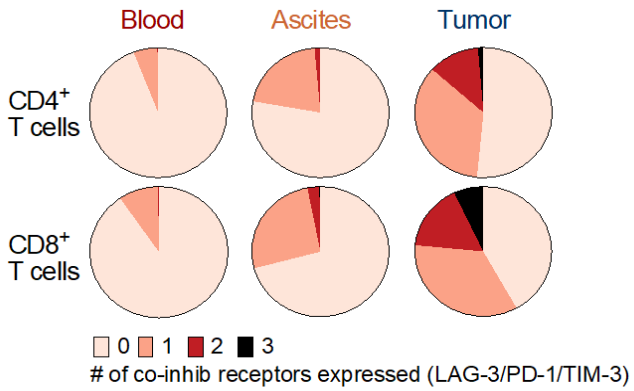

Figure 22. *Boolean gating on median single, double or triple expression of co-inhibitory receptors LAG-3, PD-1 and TIM-3 among $CD4^+$ or $CD8^+$ T cells derived from ovarian cancer patients. From* ***Paper II****.*

Due to the well-established association between multiple co-inhibitory receptor expression and T cell exhaustion/dysfunction[84,99,129,130,254,255], we hypothesized that the tumor-derived T cells are likely limited in their functionality. Later functional assays confirmed this by a reduced ability to secrete cytokines (more on this in section **4.5**).

4.2.3 Other phenotypic traits of TILs

In both prostate and ovarian cancer samples, we found the majority of T cells to express CD69 (**Paper I** and **II**). CD69 is a C-type lectin and traditionally regarded as an early activation marker. However, CD69 is also characteristic of tissue-resident T cells and helps to retain the expressing cell in peripheral tissues[256,257]. Among ovarian cancer samples, we were also able to investigate the expression of the αEß7 integrin CD103, which is another marker associated with tissue-residency and helps to bind the expressing cell to epithelial cells[256]. A large proportion of T cells in the tumor site expressed CD103 and co-expression with CD69 was also frequent, corroborating that these indeed are tissue-resident T cells. These cells have been implicated prognostically favorable in a number of solid tumor types[258], including ovarian cancer[259].

In **Paper II**, we investigated the expression of a number of additional receptors on $CD4^+$ and $CD8^+$ T cells isolated from ascites and tumor sites. In summary, the phenotypic findings suggested that a large proportion of the T cells have been activated (***Figure 23***). Reduced proportions of cells expressing co-stimulatory receptor CD28 along with CD127 (α-subunit of the IL-7 receptor) suggest activation as these receptors downregulate in response to activation. The activation was also supported by the increased proportions expressing OX40 and 4-1BB (however only found for $CD4^+$ T cells), both co-stimulatory receptors which upregulate with activation[48]. Ye *et al.* have also found an increased proportion expressing 4-1BB among TILs compared to T cells from ascites and

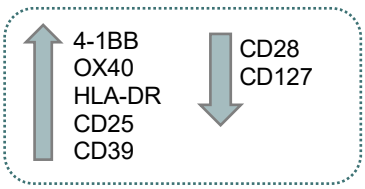

Figure 23. *Changes associated with activation which were found among TILs in ovarian cancer. Summarized from* ***Paper II*** *and* ***IV****.*

blood of ovarian cancer patients[260]. Furthermore, they identified this 4-1BB$^+$ population to be tumor-reactive T cells[260].

Another marker of activation[261], exhaustion[262] and tumor-reactive clones[127,263–265] is CD39, which was expressed by large proportions of T cells in both ascites and tumor in **Paper IV** (especially in tumor, more on this in section **4.6.2.1** and ***Figure 32***). Also, expression of CD25 and HLA-DR are known to be upregulated after activation and we found them to be expressed to a larger degree by T cells isolated from ascites and tumor (CD25 only for CD4$^+$ T cells). We also assessed the phenotype of TILs from prostate cancer and the findings can be found in the results section of **Paper I**. Overall, these are descriptive findings but nonetheless contribute with basic knowledge about TILs in prostate and ovarian cancers. They complement other studies looking into phenotype of human TILs in these cancer types[100,249,253,255,260,265–268].

4.3 INFLAMMATORY MICROENVIRONMENT

As outlined in the introduction of the thesis, the tumor microenvironment is a harsh and chaotic environment for any immune cell trying to act in a tumor-fighting way. In **Paper I**, **II** and **IV**, we analyzed the soluble microenvironment by performing Luminex multiplex assay for 26 different cytokines and chemokines.

In **Paper I**, we assessed the soluble profile of supernatants from processed prostate tissues (***Figure 15***). One striking finding was the abundance of highly pro-inflammatory and chemoattracting IL-6, IL-8 (CXCL8 according to new nomenclature) and monocyte chemoattractant protein 1 (MCP-1 or CCL2) in the control prostates. As mentioned earlier (section **4.2.2**), some of these controls were found to contain histological changes which might explain the presence of these factors. However, these three analytes were also found in high concentrations in the prostates with normal histology from young donors. Again, this points out the complicated immunobiology of the prostate. Inflammation in the prostate is suggested to drive pathologic changes and our findings suggest that this starts early in life.

There were limited cytokines found in the prostate cancer material, but we did observe IL-3 to be present. There are limited studies of IL-3 in cancer, however one study has recognized IL-3 to be released by tumor cells and act pro-tumor by promoting angiogenesis, proliferation and inflammation[269]. Whether this is the case in prostate cancer remains to be further studied. Another interesting finding was the presence of IFN-γ in prostate tissues with benign changes. Speculatively, this could indicate an active immune response, which was not found in control or malignant tissues. Other factors found in these benign tissues included GM-CSF and macrophage inflammatory protein 1-β (MIP-1β or CCL4) which could also reflect ongoing processes.

In **Paper II**, our assessment of soluble factors was performed on supernatants from processing metastasized tumor tissue and ascites fluid. The latter has the benefit of already being in a liquid form, which makes the supernatants directly reflect *in situ*. The supernatants

from tissue processing also reflects *in situ*, but with the addition of liquid (PBS). The Luminex results revealed a highly inflammatory milieu in both ascites and tumor samples of ovarian cancer patients. IFN-γ induced protein-10 (IP-10 or CXCL10) was the most abundant cytokine in all assessed sample types (tumor, ascites and peripheral blood) similar to other studies[250,270]. IP-10/CXCL10 is an important cytokine involved in the recruitment of T cells and NK cells[109]. Similar to the benign prostate samples, we also found IL-6, IL-8/CXCL8 and MCP-1/CCL2 to be present at high concentrations, again indicating a highly inflammatory environment. Interestingly, adipocytes of the omentum have been recognized to be mediators of tumor metastasis to this site through the production of these factors[271]. IL-8/CXCL8 and MCP-1/CCL2 are potent chemotactic factors for the recruitment of MDSCs and neutrophils[109], which can contribute to the suppressive tumor microenvironment. Numerous additional factors were found to be increased in ascites fluid and tumor tissue compared to peripheral blood (see the results section of **Paper II** for details). Our findings are in line with other studies in which ovarian cancer-associated ascites has been analyzed regarding composition of numerous chemokines and cytokines[250,255,270].

4.4 ASSOCIATION TO SURVIVAL

In 2003, George Coukos and colleagues reported on a favorable association between T cells and outcome in human cancer[123]. They found ovarian cancer patients with intratumoral T cells to have a 5-year overall survival of 38%, compared to only 4.5% for patients without tumor-T cells[123]. Since then, many additional studies have identified intratumoral T cells[272] and different T cell subsets (including $CD8^+$ T cells[266,273], Tregs[274] and $CD103^+$ TILs[259]) to be prognostic in ovarian cancer. The majority of these studies have used immunohistochemistry to detect populations of interest.

In **Paper II,** we correlated our phenotypic and soluble findings to several clinical parameters, including tumor stage, residual tumor burden after surgery and outcome of the patients. By using flow cytometry, we were able to assess many parallel markers and could identify prognostic populations with more phenotypic detail than what is possible using immunohistochemistry. We identified eight immune-related factors in ascites and/or tumor, which were prognostic for survival in our cohort (***Table 4***).

Table 4. *The prognostic immune-related factors for ovarian cancer patients identified in* ***Paper II****. Negative association to outcome indicates higher concentrations/proportions → worse prognosis. Positive association to outcome indicates higher levels → better prognosis.*

Immune-related factor	Location	Association to outcome	Risk factor
IFNα2, MIP-1α (CCL3) and MIP-1β (CCL4)	Ascites	Negative	High concentration
$CD8^+$ T cells expressing TIM-3 ($LAG\text{-}3^-PD\text{-}1^-$)	Ascites	Negative	High proportion
$TIM\text{-}3^+$ $CD8^+$ T cells expressing CD127	Tumor	Negative	High proportion
$CD8^+$ T cells lacking PD-1, TIM-3 and LAG-3	Tumor	Positive	Low proportion
$CD4^+$ γδ T cells	Ascites & tumor	Positive	Low proportion

We combined the risk factors and in a risk factor analysis found that having multiple of these was associated with a worse survival (***Figure 24***). We concluded that our findings need verification in a larger cohort but can nevertheless be indicative for future investigations. The individual contribution of each prognostic factor needs to be further explored to obtain insight on how each of these populations or cytokines act in the tumor microenvironment.

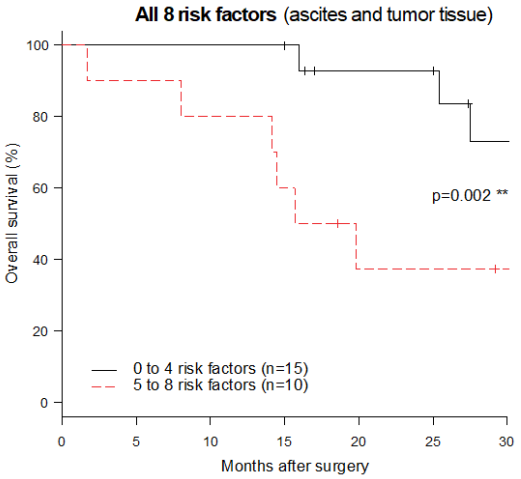

Figure 24. *Results of risk factor analysis of the eight identified risk factors (specified in* ***Table 4****). From* ***Paper II****.*

One limitation of **Paper II** was the heterogenous group of ovarian cancer patients, containing multiple histological subtypes and varying degree of residual tumor burden after surgery for example. These factors can be important for the immunobiology of the patients[266]. In follow-up studies, a more homogenous patient group could be considered, which would potentially highlight additional prognostic immune-related factors.

4.5 T CELL FUNCTIONALITY

In **Paper II**, our findings of frequent single, double and even triple expression of co-inhibitory receptors PD-1/LAG-3/TIM-3 on T cells, caused us to question their functional ability. In a small pilot in **Paper II**, we performed stimulation of tumor-derived T cells by exposing them to PMA/I. These stimuli are commonly used to activate T cells in a quick and powerful T cell receptor-independent pathway[235]. We assessed the production of various effector cytokines and factors using ICS followed by flow cytometry. We found the T cells, both $CD4^+$ and $CD8^+$ subsets, to be functionally capable (by the capacity to produce IFN-γ, IL-2, TNF-α, IL-17 and CD107a). However, when we analyzed the cells based on being $CD4^+$ or $CD8^+$ and PD-1 expression, there were differences in production among the populations. $CD4^+$ T cells expressing PD-1 appeared to be more functional (in terms of having higher proportions producing the analyzed factors) compared to the $PD\text{-}1^-$ counterpart. In opposite, $CD8^+$ T cells expressing PD-1 were less functional compared to $PD\text{-}1^-$ counterparts.

Importantly, when we evaluated the functionality in a longer time frame (48 hours using anti-CD3) and adding the PD-1 blocking mAb pembrolizumab, the amount of IFN-γ was significantly increased compared to control conditions. This revealed that T cell functionality can be boosted using our simple *ex vivo* setup.

4.5.1 PD-1 blockade *ex vivo*

Based on the work in **Paper II**, we entered a collaboration with the biopharmaceutical company Molecular Partners. The aim was to explore their DARPin® proteins targeting PD-1 while also looking at the effects by conventional PD-1 blocking mAbs. In **Paper III**,

we presented the findings of four PD-1-targeting reagents (two conventional mAbs, nivolumab and pembrolizumab, and two novel DARPin® proteins) and their effects on the cytokine responsiveness of ovarian cancer-derived T cells. DARPin-1 consists of a single domain being monovalent for PD-1, while DARPin-2 is comprised of two domains with a linker connecting them, being bivalent for PD-1 (***Figure 25***). These DARPin® proteins are much smaller than conventional mAbs and have several benefits over conventional mAbs (reviewed in section **1.4.3**).

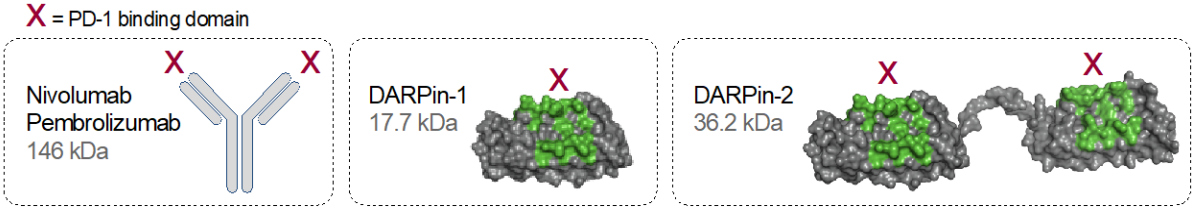

Figure 25. *Overview of PD-1 blocking regents used in* ***Paper III****.*

We first confirmed the dysfunctional cytokine capacity of T cells, by comparing the response to activation by T cells from ovarian cancer patients with T cells from healthy individuals. We used anti-CD3 for 48 hours to stimulate the cells and measured the secretion of IFN-γ by ELISA. The results showed ovarian cancer-derived T cells from all sample sites (in particular from tumor) to have a much more limited capacity to secrete IFN-γ, compared to T cells isolated from peripheral blood of healthy controls. This again confirmed what we had earlier hypothesized, that the T cells had a compromised ability to function.

We continued to investigate the PD-1 antagonists and their ability to induce changes in cytokine production. We first confirmed PD-1 binding and efficient PD-1 blockade in a dose-dependent manner using a PD-1/PD-L1 blockade reporter cell assay. We continued with adding the PD-1-directed compounds to ascites/tumor-derived T cells and found the release of IFN-γ to increase with both conventional mAbs and novel DARPin® proteins (***Figure 26***).

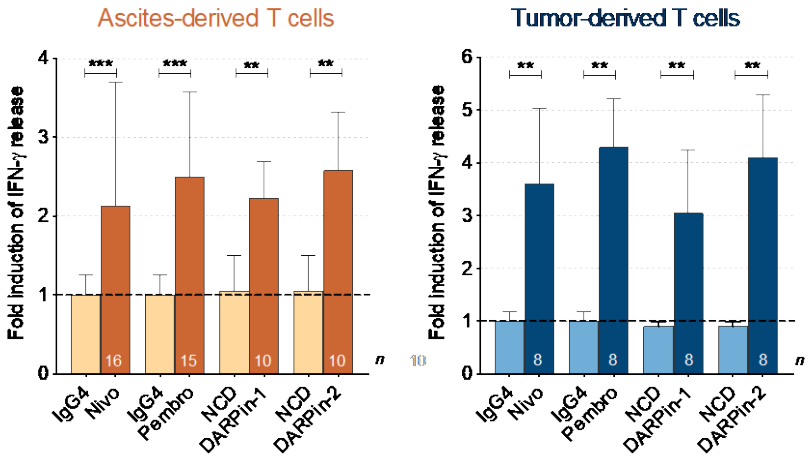

Figure 26*. Fold induction of IFN-γ by T cells isolated from ovarian cancer patients (ascites or metastasized tumor) with the addition of controls (IgG4 or negative control DARPin, NCD) or PD-1 blocking pembrolizumab (pembro), nivolumab (nivo) or DARPin-1/2. Anti-CD3 was used in all conditions and the control for this (only anti-CD3) reflects the dashed line. From* ***Paper III****.*

We observed DARPin-1 to be less efficient compared to the other three reagents which suggests that efficient PD-1 blocking requires bivalency (which is already the case for mAbs). We also used a 6-plex Luminex to assess induced changes on additional important effector molecules. The IFN-γ results generated by ELISA were confirmed with the Luminex

assay and, in addition, we also found increased levels of granzyme B, IL-2, TNF-α, IL-10 and soluble 4-1BB with anti-PD-1 treatment. This showed us that the functional suppression can be reversed using PD-1 blockade.

So, these were good news. However, when we compared the absolute concentrations of IFN-γ produced by T cells based on their origin, we identified large differences. Tumor-derived T cells were found to secrete significantly less IFN-γ compared to ascites-derived T cells; approximately a 10-fold difference (***Figure 27***). This difference in functionality is in line with previous observations when compared to healthy individuals and also findings in **Paper II**, which suggested that tumor-derived T cells have a more pronounced dysfunction based on phenotype (multiple co-inhibitory receptor expression). This presents a tremendous challenge *in situ* and can contribute to understanding of the clinical trials reporting a limited or modest response using PD-1 blockers in ovarian cancer[222–224]. Also, our *ex vivo* cultures do not account for all the components limiting the anti-tumor responses further within a tumor microenvironment.

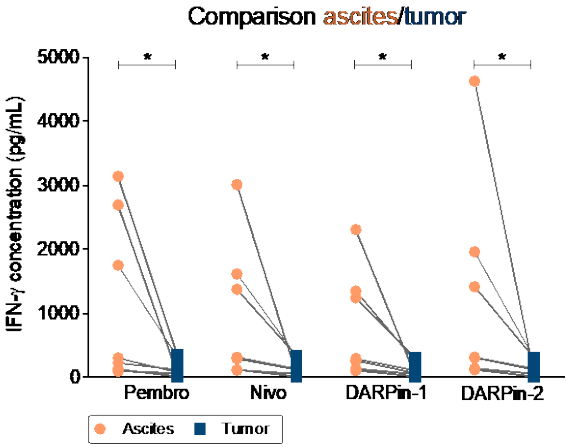

Figure 27. *Comparing the absolute concentrations of IFN-γ released by T cells isolated from seven paired ascites or tumor samples of ovarian cancer patients. From* ***Paper III****.*

There are several limitations with **Paper III**. We normalized the data based on presence of lymphocytes but did not normalize for differences in other cell types. When isolating the lymphocytes from patient material, there will be a varying degree of other cell types which remain. These include APCs and tumor cells for example, which are important for the induced effects of PD-1 blockade. In a recent study, Natoli *et al.* used an *in vitro* tumor cell/lymphocyte co-culture setup to study effects of the anti-PD-1 mAb nivolumab, in which they depleted monocytes to reduce donor variability[275]. They also found the tumor cell to lymphocyte ratio to affect the activation of T cells[275]. We are aware that our approach has an uncontrolled variability between donors. However, since we compared the different PD-1 blockers using the same sample, this does not cause a problem when comparing the responses. However, it could have effects when comparing the sample types, ascites *vs.* tumor, but there are also differences in the PD-1 expression between these sample types, adding additional complexity. Another development of the study could be to look into production and secretion of different cytokines from various subsets using ICS and flow cytometry to understand which cells are responsible for the restored functions using PD-1 blockade.

There are currently large efforts into developing novel delivery technologies (such as nanoparticles for example) to combat limitations of current immunotherapeutics[170]. DARPin® proteins are an example of novel protein scaffolds which hold promise for

improving delivery parameters in cancer immunotherapy, and with our results should be further explored for use in checkpoint blockade.

4.6 γδ T CELLS IN OVARIAN CANCER

There is currently a large ambiguity about the role of γδ T cells in human cancer, where some studies portray them as being tumor-fighting good guys while others as being tumor-promoting bad guys. Based on the large amount of evidence (outlined in section **1.3.7**), it is clear that both exist, and it is crucial to learn more about these contrasting roles in various cancer types, including the gradual shift reported in numerous cancer types. In **Paper IV**, we wanted to investigate this cell type in a thorough manner to unveil their role in ovarian cancer.

4.6.1 TCR characteristics and phenotype

We learned from **Paper II** that γδ T cells are present in both ascites and tumor tissue of ovarian cancer patients. The increased frequencies in ascites were intriguing (***Figure 28***) and in line with observations in an earlier published report[276]. To find out more about the TCR repertoire in blood, ascites and tumor of these patients, we sequenced the CDR3 region on the TRG. This region is unique to a γδ T cell clone, indicating that if the same sequence is found numerous times in a sample, a specific γδ T cell clone has responded to a TCR-engaging antigen and clonally expanded, characteristic of adaptive immunity.

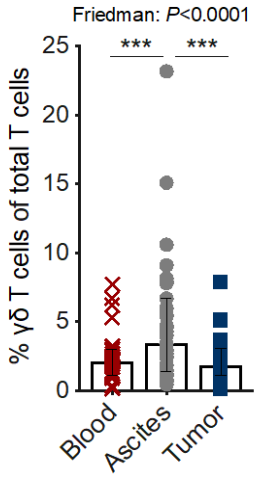

Figure 28. *Frequencies of γδ T cells in paired blood, ascites and tumor tissue of ovarian cancer patients (n=31). From* ***Paper IV****.*

Tree map plots summarize the TCR repertoire findings in a simplified manner (***Figure 29A***). We found the TRG repertoire in tumors to be very diverse (having many different clones present, *i.e.* low clonality), while the ascites repertoire showed less diversity (fewer clones, *i.e.* high clonality) and clonal focusing (occupying larger proportions of the repertoire). Quantification revealed the ascites repertoire to be more focused (less diverse) than in peripheral blood (***Figure 29B***). When assessing different characteristics of the repertoires using the NGS data, the tumor and ascites showed to have distinct repertoires.

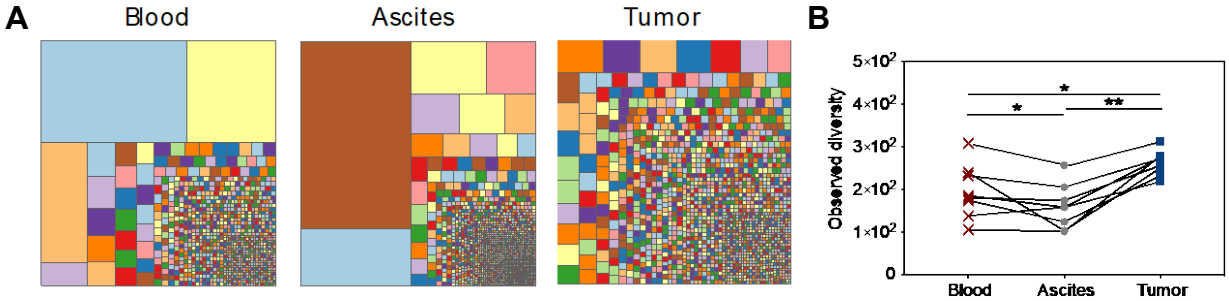

Figure 29. A) *Representative tree map plots of the TRG repertoires found in blood, ascites and tumor of an ovarian cancer patient. Each square represents one clonotype and the size of the square represents the proportion it occupies of the entire repertoire.* ***B)*** *Quantification of observed diversity from eight patients (lines connect paired samples). From* ***Paper IV****.*

To elucidate the repertoires further, we exposed γδ T cells from ascites and tumor to different stimuli and measured their proliferative response. In line with the NGS data, γδ T cells from ascites and tumor responded differently. The ascites-derived γδ T cells displayed enhanced response to anti-CD3, anti-TCRγδ and IL-15, while the tumor-derived γδ T cells were largely unresponsive to these stimuli. Surprisingly, anti-MICA/B resulted in marked responsiveness by tumor-derived γδ T cells, which showed to have a pronounced expression of MICA, unlike their counterparts in ascites or peripheral blood. The underpinning mechanisms of this MICA expression remain to be investigated. Altogether, these results suggest that ascites-derived γδ T cells respond in a clonotypic adaptive-like manner, unlike tumor-derived γδ T cells. This was corroborated by the more frequent $CD27^{-/low}$ phenotype in ascites compared to tumor, a phenotype which has been associated with adaptive-like γδ T cells[277,278].

We also performed a more detailed phenotyping of the major human γδ T cell subsets $V\delta1^+$ and $V\delta2^+$ by flow cytometry. Unfortunately, this was not performed on fully paired samples (blood-ascites-tumor from the same patient), which points out a limitation of **Paper IV**. However, the ascites and tumor samples were always paired. We assessed co-inhibitory receptors PD-1 and TIM-3, tissue-residency markers CD69 and CD103, NK cell-associated receptors DNAM-1 and NKG2D and several chemokine receptors (CCR2, CCR5, CCR6, CCR7, CCR9 and CXCR3). Expression of PD-1 and TIM-3 was frequent among tumor-derived γδ T cells (in particular among the $V\delta1^+$ T cells). Similar to other TILs in **Paper II**, we found the majority of tumor-derived γδ T cells to have a tissue-resident phenotype. Also, the expression of NKG2D was significantly increased among both ascites- and tumor-derived γδ T cells (both $V\delta1^+$ and $V\delta2^+$ subsets), along with expression of CCR5 and CXCR3 compared to peripheral blood γδ T cells. Overall, the phenotyping suggested tumor-derived γδ T cells to have been activated. Together with the NGS data, we suggested the tumor-derived γδ T cells to act mainly through TCR-independent innate-like pathways.

4.6.2 Functionality of ovarian cancer-derived γδ T cells

In the quest to unravel the role of γδ T cells, we needed to look at the cells from a functional perspective to determine their contribution to the surroundings. In the distinction between good and bad, the cytokines IFN-γ and IL-17A have become synonymous with these different roles[26]. Our primary interest was to assess whether the γδ T cells from our samples were active producers of tumor-promoting IL-17A. We assessed their production in response to different stimuli (anti-TCRγδ, PMA/I and HMBPP), in different time frames (4 hours and 48 hours) and with different methods (production by ICS and secretion by FluoroSpot). Regardless of stimuli, time frame or method, we observed very limited (if any) production/secretion of IL-17A from γδ T cells (***Figure 30A***). Instead, we observed production of other important effector cytokines including IFN-γ and TNF-α suggesting an anti-tumor profile (***Figure 30B***). We also observed pronounced production of MIP-1β, which is a sensitive measure for activation[279]. The anti-tumor profile was strengthened by the findings of strong cytotoxic capacity by γδ T cells against tumor cells (***Figure 30C***).

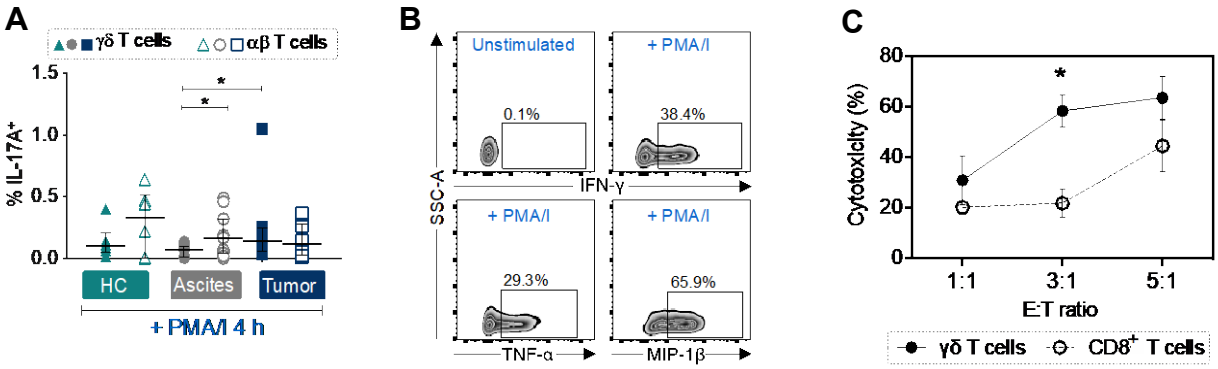

Figure 30. *γδ T cells from ovarian cancer patients have **A)** limited production of IL-17A, and instead **B)** production of important anti-tumor cytokines and **C)** display cytotoxicity towards ovarian cancer cell line OVCAR-3. Parts from **Paper IV**.*

Contrasting our own results, Chen *et al.* have identified a large proportion of γδ T cells in tumors from ovarian cancer patients (mean 35.2% of total T cells)[280]. In addition, they have identified a substantial production of IL-17A by these tumor-derived γδ T cells[280]. They performed a similar stimulation setup as us, using PMA/I to induce cytokine production. Other studies have, in line with our own, found γδ T cells from ovarian cancer patients to have cytotoxic functions and act in anti-tumor manners[276,281,282]. As pointed out earlier, the inconsistencies around the role of γδ T cells in cancer are found in many cancer types, and ovarian cancer is no exception. The reasons for the discrepancies are unknown but likely the result of different patient cohorts and tumor microenvironment for example.

By stimulating T cells *ex vivo*, there is an attempt to mimic what is occurring *in vivo*. A potential problem with this is that the experimental setup (choice of stimulus, stimulation time and protein transport inhibitor for example) can affect the readout differently[283]. Also, different cytokines have different kinetics and consumption rate by other cells. Therefore, it is important to compare samples using the same experimental setup but preferably also use multiple setups in parallel. For us, it was important to use a number of different stimuli when looking into the production and secretion of IL-17A. Since we did not find any substantial production/secretion, it was also important that there have been numerous publications reporting on IL-17A production using the chosen stimuli[157,159,165,280,283].

4.6.2.1 CD39 expression

We also looked at the expression of CD39, which proved to be important. CD39 is induced upon activation, and acts as an ectoenzyme, bound on the cell membrane with exposure to the extracellular environment, involved in the generation of immunosuppressive adenosine[261] (***Figure 31***). It has also been shown to deactivate pAgs, impacting the activation of $V\gamma9^{+}V\delta2^{+}$ T cells[284]. It is frequently expressed by many cell types (including Tregs and MDSCs) in a range of different cancers and is tumor-favoring in a number of ways[261,285]. CD39 is also a marker of T cell exhaustion[262] and is expressed by tumor-reactive T cells in various cancer types[127,263,264],

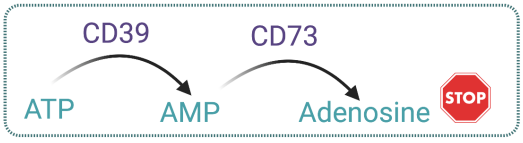

Figure 31. *CD39 is a rate-limiting ectoenzyme in the conversion of extracellular ATP to immunosuppressive adenosine.*

including ovarian cancer[265]. The high abundance of CD39 expression among $CD8^+$ T cells recently reported in ovarian cancer[265] was in line with our own results. We also found a substantial proportion of different T cell subsets to express CD39 (both γδ and αβ T cells) (***Figure 32***). What was particularly interesting to us was the negative association to γδ T cell functionality.

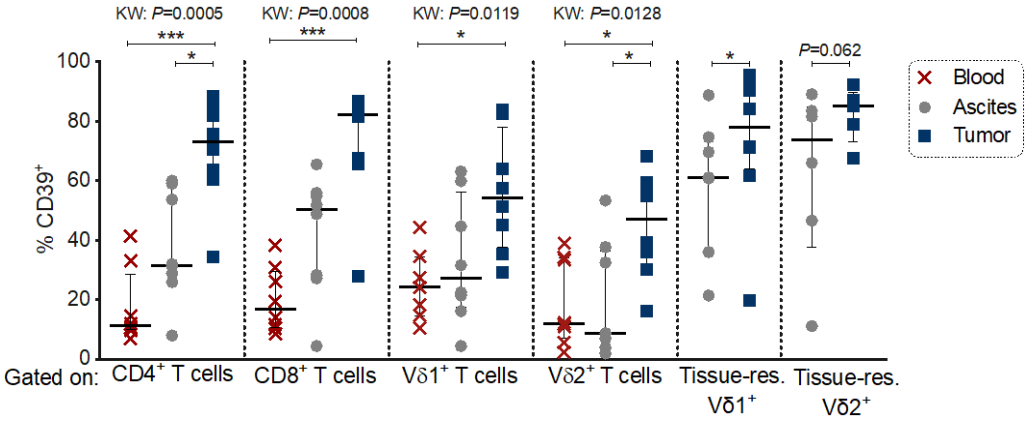

Figure 32. *Expression of CD39 on various T cell subsets in peripheral blood, ascites and tumor of ovarian cancer patients. Expression is also shown on tissue-resident (tissue-res, defined by $CD69^+CD103^+$) $V\delta1^+$ and $V\delta2^+$ T cells. P-values for Kruskal Wallis (KW) test. From* ***Paper IV****.*

4.6.3 Associations to outcome

Our analysis of cytokine production revealed a large range in functionality of γδ T cells. Despite the limited size of the cohort (n=14), we identified a statistically significant association between the γδ T cell cytokine responsiveness and clinical outcome. More specifically, we found the increased production of TNF-α by total γδ T cells and IFN-γ by the $V\delta2^+$ subset to be associated with increased overall survival (***Figure 33A***). The findings were strengthened by a larger cohort retrieved from TCGA, in which data on sequenced RNA from tumor samples of ovarian cancer patients is available. Our use of TCGA exemplifies the strength of such databases being publicly available, along with analysis tools like the GEPIA2.

The results complemented our own and showed a favorable association of both γδ T cells and αβ T cells to outcome (***Figure 33B***). As mentioned earlier (section **4.4**), many before us have identified a positive association between T cells and outcome in ovarian cancer[123]. Our findings on γδ T cells were however novel to current literature and in line with the large study by Gentles *et al.* from 2015, in which they identified γδ T cells to be the most favorable prognostic immune cell population across 25 different types of malingancies[126]. Also, as a recent example, Wu *et al.* reported on prognostically favorable innate-acting $V\delta1^+$ T cells in breast cancer tissues[13].

Our identified link between functionality and outcome was supported by findings in the TCGA cohort, where having high IFN-γ and $V\delta2^+$ subset signature was associated with increased survival (P=0.012). As always, the findings are in need of verification in additional studies, preferentially with a larger patient cohort. Also, the contribution of different γδ T cell subsets need to be further studied.

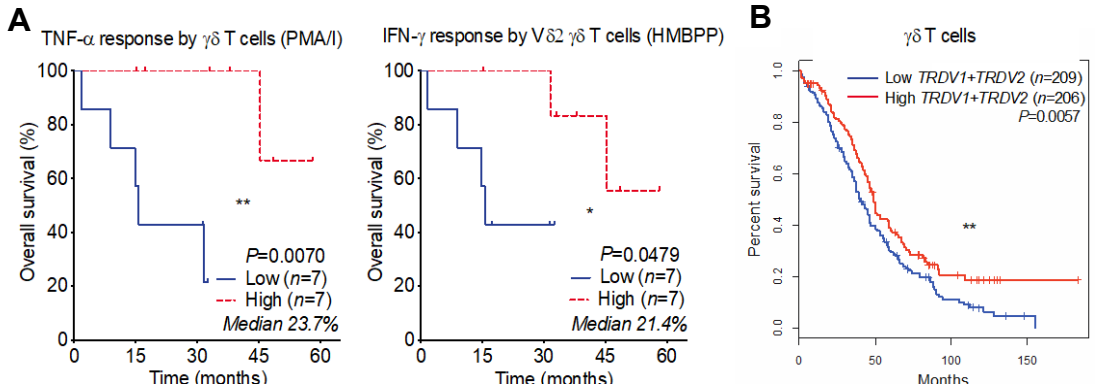

Figure 33. *Associations between γδ T cells and survival of ovarian cancer patients in* ***A)*** *our own cohort linking the cytokine responsiveness of γδ T cells to outcome; and* ***B)*** *in a cohort retrieved from TCGA, in which RNA from tumors has been sequenced and a gene signature for γδ T cells (TRDV1+TRDV2) was analyzed. Parts from* ***Paper IV****.*

Moreover, we looked closer at the impact of CD39 expression on the cytokine production by T cells. In our correlation analysis, we recognized the cytokine responsiveness of γδ T cells to be negatively affected by increased proportion of various T cell subsets expressing CD39. In the analysis of the TCGA cohort, it was recognized that having high expression of CD39 (more specifically the transcript for *ENTPD1* gene, encoding for CD39) negatively impacted the favorable associations of γδ and αβ T cells. This warrants for further studies investigating the impact of CD39 and whether this could be a potential target to increase functionality of γδ T cells. There have been several recent studies reporting on CD39-targeting approaches to improve anti-tumor immunity[286–288] and our results provide another incentive for this continued development. Again, the small size of the study warrants for larger studies but provides new knowledge about γδ T cells in human cancer.

4.6.4 Extended reflections

Taken together, our findings from NGS, stimulations, phenotyping, functionality and associations with survival (both our own and those from the TCGA cohort), suggests that the ascites-derived and tumor-derived γδ T cells display distinct immunobiological features. While ascites-derived γδ T cells appear to act mainly through TCR-dependent adaptive-like manners, TCR-independent innate-like responses are dominant in the tumor. Our results surprised us; we had not thought that the γδ T cell repertoires in ascites and tumor would be distinct from one another.

The results suggest that there are different factors influencing the γδ T cells. Given the shift in understanding about the heterogeneity of tumors and the multi-clonal origin of tumors[289], our results might be a reflection of this. Speculatively, the tumor cells found in the ascites might have different characteristics compared to the tumor cells in metastasized tumor sites. In ascites, tumor cells could have gained (or lost) features enabling them to dissociate into the fluid environment. Ascites is known to play a role in the metastasis of primary ovarian tumors[290–293]. In a metastasized tumor site, tumor cells will have settled and might lose these characteristics and/or adapt others. Thus, tumor cells from different locations might impact γδ T cells differently. This is purely speculation and warrants further investigation.

Another speculation is that they are distinct based on their origin; that the ascites-derived γδ T cells are mainly derived from the peripheral blood while the tumor-derived γδ T cells are mainly derived from tissue-located (omentum) γδ T cells. Based on these different origins, they have different capacities/capabilities to be triggered by tumor cells and react towards them. Future studies investigating the mechanisms of the adaptive *vs.* innate responses of tissue-derived γδ T cells are warranted. There is also a reported heterogeneity among tumor-derived $CD8^+$ T cells in terms of TCR specificity, where a large proportion of cells are considered bystanders and are not tumor-specific[124]. This could also be the case for tumor-derived γδ T cells, however their positive association to outcome suggests that there are clinically significant activities occurring at these sites which warrants further investigation.

5 CONCLUSIONS

To develop efficient immunotherapeutic treatments, it is crucial to learn about the tumor microenvironment and the pro- and anti-tumor features of the immune system in humans. One way to do so is by mapping the immune cells present in tumors and other relevant locations. This is what we set out to do with emphasis on T cells in prostate cancer and ovarian cancer.

In our work about TILs in prostate cancer, we conclude:

- It is possible to isolate and characterize T cells by flow cytometry from very limited material, as little as 0.03 gram of prostate tumor tissue was used in our study.
- T cells were the most common lymphocyte subset in primary cancer lesions, however, other lymhocyte subsets (B cells and NK cells) were also present. The majority of T cells were $CD8^+$ and had an effector memory phenotype.
- There was a limited expression of LAG-3, TIM-3 and CTLA-4 on tumor-derived T cells.
- PD-1 expression was frequent but not unique for tumor-derived T cells; it was also common among T cells from benign transformations and even prostates with normal histology. This was also the case for the abundant presence of Tregs.
- Pro-inflammatory and chemotactic factors IL-6, IL-8/CXCL8 and MCP-1/CCL2 were common in the prostate, even in those with normal histology.

In our work about TILs in ovarian cancer, we conclude:

- T cells were abundant in metastasized tumor tissue and ascites of ovarian cancer patients, but also B and NK cells were present. The majority of T cells were $CD8^+$ and had an effector memory phenotype.
- There was an abundance of Tregs and monocytes (potentially MDSCs) in both ascites and metastasized tumor tissue.
- A large proporiton of T cells from both ascites and tumor were found to have single, double or triple expression of LAG-3, TIM-3 and/or PD-1. Multiple expression was most common for tumor-derived $CD8^+$ T cells. TIM-3 was the second most common co-inhibitory receptor type (after PD-1) while CTLA-4 expression was rare.
- We identified eight immune-related risk factors which were associated with outcome, including both soluble and cellular factors.
- T cells isolated from ovarian cancer patients had a reduced functional capacity in terms of cytokine production. PD-1 blockade restored cytokine production among T cells from ascites and tumor, but the dysfunctional state of tumor-derived T cells was still profound.
- The PD-1-targeting bivalent DARPin® protein (referred to as DARPin-2) had similar effects as conventional anti-PD-1 mAbs, but not the monovalent DARPin-1.

- γδ T cells in ascites and tumor are distinct populations and appear to be acting by different pathways (adaptive-like or innate-like).
- γδ T cells derived from ovarian cancer patients do not produce IL-17A. Instead, they contribute with anti-tumor functions including production of effector cytokines and cytotoxicity.
- γδ T cells are beneficial for prognosis of ovarian cancer patients and their functionality also appears to be associated to outcome.
    - The functionality is negatively affected by CD39, which presents a target to be further studied.

6 POINTS OF PERSPECTIVE

"The more I learn, the more I realize how much I don't know." – Albert Einstein

This holds true for how I feel about the work presented in this thesis. Many additional projects can be initiated from the work presented here. Also, there is so much happening in the field of tumor immunology and immunotherapy, which is very exciting. When I started my PhD studies, not much data was available on the use of checkpoint blockade in neither prostate cancer nor ovarian cancer. Only limited patient numbers and small-scale studies with limited responses were available at the time, which is what made us want to map the immune landscape of these two cancer types.

6.1 IMPLICATIONS FOR IMMUNOTHERAPY

The work presented in this thesis contributes with going back to basics. Finding out what is actually infiltrating into the tumors. The next question is *whether the described work can provide any guidance for future immunotherapy*? Our results are drops in a large sea of available studies. Nonetheless, **Papers I-IV** have contributed with important mapping of certain immune subsets in two types of solid tumors. In this section I will summarize how the findings can help to guide and provide some points of perspective, starting with prostate cancer.

6.1.1 Prostate cancer and immunotherapy

The prostate is an organ in which inflammatory and benign conditions are common. In **Paper I**, we found an abundance of Tregs, pro-inflammatory cytokines and PD-1 expression among T cells, even in prostates with confirmed normal histology. This indicates involvement in the prostate homeostasis and are therefore not specific traits for prostate cancer. However, a larger proportion of the tumor-derived T cells did express PD-1 indicating that this pathway is also an immune escape mechanism of prostate tumors, like many other tumor types. Expression of LAG-3 and the presence of IFN-γ, as well as pro-inflammatory cytokines, in prostates with benign changes could indicate that T cells in this environment are active. Inflammation is discussed to be a driver in the development of prostate cancer, speculatively with a linked decreased functionality of present T cells. This process is complicated and much remains to be learned about the prostate and the link between the development of different pathologies.

Our work offers optimism for other tumor types with limited sample material, or which are considered immunologically cold. A more detailed phenotyping might still be possible! However, our work also suggests a somewhat pessimistic view on additional approaches using checkpoint blockade in prostate cancer, at least the ones targeting PD-1, LAG-3, TIM-3 and CTLA-4, due to the low expression or presence in non-malignant conditions. To combat prostate cancer, for which the prevalence is so high, preventive measures would be very useful, such as preventing the tumor-promoting inflammation for example.

6.1.2 Ovarian cancer and immunotherapy

Ovarian cancer often develops in silence, which results in discovery at an advanced stage where metastasis within the abdomen already frequently has occurred. This results in large amounts of tumor tissue making it more difficult to treat. From a research perspective, this has enabled us to study the TILs to a larger extent compared to our studies in prostate cancer. Due to the common accumulation of ascites in ovarian cancer patients, this has also been an important sample type for us to study as the ascites fluid presents a liquid tumor environment. It has become clear that the ascites has an abundance of pro-inflammatory and chemotactic soluble factors along with all kinds of immune cells. The phenotypic findings in ascites have almost always been an intermediate between the findings in blood and tumor. This also holds true for our functional assessments of the T cells. The ascites presents a more accessible tumor environment, both in the hunt for predictive biomarkers but also in the treatment of patients. The results from **Paper II**, suggested that several immune-related soluble factors and T cell populations from ascites and/or tumor can be prognostic which becomes interesting from an immunotherapeutic point of view. These findings warrant verification in larger cohorts but presents potential targets (of inhibition or boosting).

The findings of **Paper III** suggested the bivalent DARPin-2 protein to induce effects similar to conventional mAbs in our setup. This warrants further investigations and initial *in vivo* work to further elucidate its potential role as a competitor among other available checkpoint blockers. However, the findings also clearly presented the challenges with reinvigorating responses. The flexibility of the DARPin® protein platform would perhaps be more useful in future design of proteins with multi-specific targets and functions to combat additional hurdles in the tumor microenvironment.

With **Paper IV**, we contributed with increased knowledge about γδ T cells in ovarian cancer. However, there is much we still don't know. The findings prompted many new questions. I hope the work can continue to generate more knowledge about γδ T cells in human cancer, because it is surely needed! The enigmatic nature of γδ T cells still remains and future approaches should continue to look into their innate and adaptive characteristics, responses and ways to enhance their anti-tumor capacity. Exploration of CD39 blocking and its effects on γδ T cell functionality is a starting point. Ultimately, if these cells can be used for adoptive immunotherapy or be affected by other types of immunotherapeutic approaches, they provide a powerful tool to combat tumors.

6.2 CONCLUDING REMARKS

The rapid advancement in the field hopefully became clear in the introduction of this thesis, and the past couple of years, during which the work for **Paper I-IV** has been conducted, have been no exception. Increased understanding about mechanisms and factors playing a role for the anti-tumor response, results from clinical trials giving hints into what works and not, the list of new insights is long. Also, high-dimensional technologies including mass cytometry (CyTOF) for example, can contribute with detailed exploration of the TIL landscape in

different cancer types[101,134] and response to different treatments[294]. The mapping of immune cells in solid tumors will continue. In addition, continued studies of γδ T cells in ovarian cancer (and other cancer types) and ways to boost their anti-tumor functionality are needed. The use of γδ T cells in cancer immunotherapy is under exploration by many[19] and it will be interesting to see what upcoming years will generate in terms of knowledge and clinical translation.

7 ACKNOWLEDGEMENTS

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