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ADAPTIVE IMMUNITY IN UROTHELIAL CANCER

MOLECULAR AND CLINICAL ASPECTS

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About the cover
Micrograph of a bladder tumor infiltrated by light blue T cells, taken in conjunction with paper I of this thesis. The micrograph reached the cover of the “Cancer Immunology Research” journal, vol: 6, issue 5, 2018. Here modified with the journals’ permission.

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Adaptive Immunity in Urothelial Cancer – Molecular and Clinical Aspects

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Oif itlechen terets ken men gefinen a nei'eh kasheh.

-Yiddish proverb

In English: ***To every answer you can find a new question.***

To my beloved family,

ABSTRACT

In the battle between the immune system and cancer, tolerance mechanisms otherwise protective against autoimmunity, are exploited to halt the anti-tumor immune response. In this model, tumors turn distinct parts of the immune system against each other; suppressive cells such as regulatory T cells (Tregs) are hijacked to obstruct effector lymphocytes in their attempt to eradicate the tumor. We explored the effects of this immunomodulation on Tregs, effector T cells (Teff) and B cells in patients with urinary bladder cancer (UBC) and examined what impact chemotherapy has on this process.

Puzzled by our previous finding of tumor-infiltrating Tregs to correlate to a favorable prognosis in patients with UBC, we sought to corroborate our results and ensure that we had not mistaken Teff cells for Treg cells. This was not the case, since we demonstrated tumor-infiltrating $CD4^{+}FOXP3^{+}$ T cells to be phenotypically, functionally and epigenetically stably committed Tregs. In search for a mechanistic explanation to the apparent favorable role of Tregs in UBC, we found this cell population to mediate suppression of the prometastatic factor MMP2, produced by M2 macrophages and UBC cells at the invasive front of the tumor microenvironment (TME). This finding supports the model where Tregs, by controlling inflammation, may benefit patients with inflammation-driven cancers.

In our initial investigation of chemotherapeutic effects on lymphocytes, we found Doxorubicin to enhance the antigen presenting ability of B cells, with a subsequent increased activation of $CD4^{+}$ T cells. This effect was mediated by an increased expression of the co-stimulatory molecule CD86, together with an altered cytokine profile including IL-10 and TNFα. The findings were translatable to the clinical setting, since CD86 expression was increased on circulating B cells of patients treated with Doxorubicin-containing neoadjuvant chemotherapy (NAC). When further evaluating the effects of chemotherapy on the T cell compartment, we changed scenery from peripheral blood to the Sentinel node (SN). $CD8^{+}$ Teff exhaustion was demonstrated to be reduced after NAC treatment, while cytotoxicity was increased. In complete responders (CRs) to NAC, these cells were functionally and epigenetically committed effectors. For $CD4^{+}$ Teff cells, tumor-specific reactivity was observed after NAC. In contrast, Tregs were attenuated by NAC in a dose-dependent manner with decreased frequency and reduced effector molecule expression. Also, CRs had higher Teff to activated Treg ratio, promoting antitumoral T cell activation.

In our further examination of SN T cells we wondered if their proteome was altered by the tumor. We found growth- and immune signaling to be up-regulated in SN Tregs. Most significantly, Interleukin (IL)-16 was identified as central in SN Treg signaling, Furthermore, direct contact with tumoral factors increased Treg IL-16 processing into its bioactive forms and this effect was mediated by active caspase-3.

In conclusion, the adaptive arm of the immune system in the TME is heavily modulated in patients with UBC, where NAC contributes with substantial positive effects on this system. The observed suppression of tumor promoting inflammation by Tregs, manifested by inhibition of M2 macrophage functions, suggests the view of Tregs as a clear-cut negative force in tumor immunity to be a reductionistic and unfortunate vision.

LIST OF SCIENTIFIC PAPERS

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LIST OF ABBREVIATIONS

ACT	Adoptive cell therapy
AIRE	Autoimmune regulator
AIS	Adaptive immune system
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
Breg	Regulatory B cell
CD	Cluster of differentiation
CIS	Cancer in situ
CLP	Common lymphoid progenitor
CNS	Conserved non-coding sequence
CP	Central part
CR	Complete response
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
ECM	Extracellular matrix
EIF2α	Eukaryotic translation initiation factor 2 subunit alpha
FASCIA	Flow cytometric Assay for Specific Cell mediated Immune response in Activated whole blood
FOXP3	Forkhead box P3
HMGB1	High mobility group box-1
ICAM1	Intercellular adhesion molecule 1
ICD	Immunogenic cell death
IDO	Indoleamine 2,3-dioxygenase
IF	Invasive front
IFNγ	Interferon γ
Ig	Immunoglobulin

IL	Interleukin
LFA-1	lymphocyte function-associated antigen-1
MHC	Major histocompatibility complex
MIBC	Muscle-invasive bladder cancer
MMP	Matrix metalloproteinase
NAC	Neoadjuvant chemotherapy
NK cell	Natural killer
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein-1
PRR	Pattern recognition receptors
RC	Radical cystectomy
SN	Sentinel node
STAT5	Signal transducer and activator of transcription 5
TAAs	Tumor associated antigens
TAM	Tumor associated macrophage
TCR	T cell receptor
Teff cell	T effector cell
TGF-β	Transforming Growth Factor β
Th cell	T helper cell
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-α
TNM	Tumor, Node, Metastasis
TPI	Tumor-promoting inflammation
Treg	Regulatory T cell
TSDR	Treg-specific demethylated regions
TURBT	Transurethral resection of the bladder tumor
UBC	Urinary bladder cancer

1 INTRODUCTION

The evolutionary emergence of vertebrate predators coincided with two macroevolutionary events, believed to have contributed to the genesis of the adaptive immune system (AIS) (1). These events occurred approximately 500 million years ago in jawed fish, which equipped them with diversifying antigen receptors on lymphocytes and thus a specific, yet incredibly diverse defense system against a myriad of pathogens. The major advantage of this feature at that time, has been attributed to the extraordinary ability of the AIS to protect offspring.

This advancement, which was enabled through somatic recombination, came at a cost, as it necessitates the generation of tolerance to self to prevent autoimmunity. To this end, regulatory T cells (Tregs), a cell population with the job to maintain immune homeostasis (2), were developed. The implications of this self-tolerance concept on the immune systems' attempt to protect the organism against cancer, is a central theme of this thesis.

The development of cancer, oncogenesis, is initiated by mutations that activate oncogenic drivers (3). This process is accompanied with activation of tumor-promoting genes or inactivation of tumor-suppressive genes, or both thereof. In many cancers, the process of oncogenesis results in an accumulation of mutations due to the selective advantage of cells with an unstable genome as it fosters proliferative fitness. In this way, mutations compile in an accelerating manner, rendering the tumor genome highly diverse. This diversity, again, comes at a price: the further a cancer cell diverges from its normal state, the more prone it is to be recognized as foreign by the AIS.

The notion that cancer may be visible to the immune system, i.e., immunogenic, was considered already by William Coley, a surgeon who linked the occurrence of postoperative infection with improved clinical outcomes for people with cancer. Today, the capacity of the immune system to eliminate tumors is indisputable, owing to the unprecedented success of cancer immunotherapies, including so called ”checkpoint inhibitors” and ”CAR T cells”, which both mediate long-lasting tumor responses in people with a variety of cancers (4, 5).

Yet, far from all people respond to these novel treatments and all tumors that *de facto* arise in immunocompetent organisms are living examples of a failed anti-tumor immune response. The consensus view is that these failures generally result from the capacity of the tumor to escape recognition by the immune system, which the tumor accomplishes by hijacking the otherwise beneficial self-tolerance machinery, including the Treg cell population (6). The recognition that such tumor immune escape is a critical hurdle in cancer immunotherapies, and in tumor immunity at large, has sparked an intense search for strategies to target these escape mechanisms. In this pursuit, standard chemotherapy has risen like a phoenix from the ashes.

The mechanisms behind chemotherapy efficacy are currently being reevaluated, with an increased awareness of their immune stimulatory effects. This insight is the basis for the second central theme of this work, where we explore what impact standard chemotherapy has on the intricate interplay between the tumor and the AIS.

1.1 OVERVIEW OF URINARY BLADDER CANCER

The term "urinary bladder cancer" (UBC) is unspecific and, in strict sense, even comprises metastasized tumors from other primary organs. However, for the sake of simplicity, in this thesis UBC refers to tumors that originate from the transitional epithelium of the urinary bladder, i.e. urothelial urinary bladder cancer.

Fortunately, the knowledge and management of UBC is continuously growing more sophisticated with new diagnostic tools, new UBC subtypes being defined and novel treatment protocols surfacing into the clinic. The following passage is an attempt to, in a condensed format, describe the clinical management and inherent immunological aspects of this malignancy.

1.1.1 Epidemiology, Diagnostic evaluation and Classification

This year, ≈165,000 persons will die from UBC and another 430,000 new cases will appear on the globe (7). This makes UBC the 11th most commonly diagnosed cancer worldwide and the 7th among men, reflecting a strong sex bias with a 3:1 male-female distribution (8). In the European Union, UBC was the 9th most common cause of cancer death in 2012 and the mortality remains high, although a modest decline has been reported for in the industrialized world (7). The incidence rate varies across countries, likely due to differences in exposure to the major risk factors such as tobacco smoking.

More than 90 % of all UBCs are of urothelial origin, and the remainders mainly comprise squamous cell- or adenocarcinomas. At the time of diagnosis, approximately 75 % of the patients present with non-muscle-invasive bladder cancer (NMIBC), which is defined as disease confined to the mucosa (stage Ta and Cancer in situ (CIS)) or submucosa (stage T1). Although these three types of lesions indeed are non-muscle-invasive, T1 and CIS lesions are distinct from Ta lesions since they have a high potential to become invasive (≈50 % of CIS lesions progress if left untreated) (9). Thus, accurate histopathologic assessment and diagnosis is crucial for correct clinical management which differs drastically between Muscle-invasive bladder cancer (MIBC) and NMIBC. The diagnostic work-up includes, but is not limited to, physical examination, imaging and transurethral resection of the bladder tumor (TURBT) with subsequent histological evaluation. In patients with confirmed MIBC, Computer tomography (CT) of the chest, abdomen and pelvis is currently used for staging due to yet insufficient data supporting advantages of FDG-PET/CT (8).

Using the acquired information from these modalities, UBCs are subcategorized according to the Tumor, Node, Metastasis (TNM) system as detailed in **Table I**. The TNM stage is coupled with the histological differentiation grade (according to the World Health Organization classification system) and this categorization guides the choice of treatment. Pre-treatment (*e.g.* cystectomy), the TNM is labeled "clinical TNM" (cTNM) and is partly based on data from the clinical examination whereas post-cystectomy, TNM is termed "pathological TNM" (pTNM), which is based mainly on histopathological data of the excised urinary bladder and lymph nodes.

Table 1: TNM classification of urinary bladder cancer
Modified from the European Association of Urology (EAU) Guidelines on Muscle-invasive and Metastatic Bladder Cancer

T - Primary Tumor		
Tx	Primary tumor cannot be assessed	
T0	No evidence of primary tumor	
Ta	Non-invasive papillary carcinoma	
Tis	Carcinoma *in situ*: "flat tumor"	
T1	Tumor invades subepithelial connective tissue	
T2	Tumor invades muscle	
		T2a Tumor invades superficial muscle (inner half)
		T2b Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue:	
		T3a microscopically
		T3b macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall	
		T4a Tumor invades prostate stroma, seminal vesicles, uterus, or vagina
		T4b Tumor invades pelvic wall or abdominal wall
N - Regional Lymph Nodes		
Nx	Regional lymph nodes cannot be assessed	
N0	No regional lymph-node metastasis	
N1	Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)	
N2	Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)	
N3	Metastasis in common iliac lymph node(s)	
M – Distant Metastasis		
M0	No distant metastasis	
M1	Distant metastasis	

It should be stressed, that there are major ongoing efforts to refine the subgrouping of patients in order to better predict the most suitable treatment for a given patient. In this pursuit, five new distinct MIBC subtypes (luminal-papillary, luminal infiltrated, luminal, basal/squamous, and neuronal) have been identified through next-generation sequencing (NGS) of the whole exome as well as gene expression analysis, an extensive and seminal work provided by The Cancer Genome Atlas Research Network (10). These subtypes do not necessarily reflect the histopathological appearance of UBC, but are rather associated with specific pathway alterations and other biological features.

1.1.2 Treatment Modalities

For Ta tumors, the TURBT itself may be sufficient for complete eradication. However, in NMIBC cases at high risk of recurrence or progression, intravesical treatment with live attenuated Mycobacterium Bovis, often referred to as Bacillus Calmette-Guérin (BCG), is the gold standard. BCG instillation induces a tumor-specific effect with a complete response rate of 55–65% for high-risk papillary tumors and 70–75% for CIS (11). As such, BCG is the most successful cancer immunotherapy at use to date and has proven difficult to excel ever since its first use for UBC in the 1970s (first available as a tuberculosis vaccination already in the 1920s). The mechanism of

action is not fully mapped, however data suggest that the bacterium is internalized into the urothelial cells through macropinocytosis, processed, and presented on major histocompatibility complex (MHC) class II pocket and intracellular adhesion molecule 1 (ICAM-1) (12, 13). Although incompletely understood, it is well substantiated that the effect of BCG is dependent of a T helper (Th) 1 cell immune response. For instance, a T cell requirement was demonstrated in athymic nude mice bearing bladder tumors, who failed to mount an anti-tumor response following BCG instillation (14). These notions emphasize the immunogenic nature of UBC and indicate immunotherapy as a possible approach also in the late stages of this cancer (although BCG specifically is not effective for MIBC).

Further underscoring UBC as an immunogenic malignancy is its pronounced mutational load, potentially raising an abundance of tumor antigens enabling T cell responses. Indeed, checkpoint inhibition has proven beneficial for a subset of MIBC patients and mutation burden was reported to be an independent predictive factor for response to such immunotherapy (15). Finally, the integral role of the immune system in bladder cancer at large is manifested by the well-established relationship between inflammatory settings like schistosomiasis infection (in squamous cell carcinoma) and other chronic urinary tract infections and the development of this malignancy (16).

Immunotherapy against MIBC is a recent progress and, yet, conventional cancer therapies remain the gold standard for this patient category. In essence, following TURBT, fit patients receive cisplatin-containing neoadjuvant chemotherapy (NAC) prior to radical cystectomy (RC) which includes removal of the regional lymph nodes along with the bladder. Despite these efforts, half of the patients will die from their disease within five years from diagnosis (17) and in the presence of lymph node metastases the 3-year mortality is 75% (unpublished data from the Nordic Cystectomy Studies). Thus, there is an urgent need for new and improved treatments.

Meta-analyses of RCTs with long term follow up unanimously demonstrate a 5-8 % survival benefit at 5 years in NAC-treated MIBC patients (17-19). Moreover, patients with complete response (pT0N0M0 stage) to NAC have an absolute risk reduction of 31.1% for death (18). Of note, there is only evidence of a meaningful therapeutic benefit for NAC regimens containing cisplatin combined with at least one additional chemotherapeutic agent. Among the commonly used such regimens is the combination of methotrexate, vinblastine, adriamycin and cisplatin (MVAC). In addition to NAC, pre-operative radiotherapy for operable MIBC may be offered since there is data supporting an increased chance of down-staging, however is not part of the clinical routine in Sweden (19)

Adjuvant chemotherapy is only recommended to pT3/4 and/or pN+ patients and only if no NAC has been given (8). In the case of further metastatic disease, in Sweden, the standard procedure is that additional chemotherapy is used only as a palliative treatment when pN+ patients (or pM+ patients) develop symptomatic progression. For such second-line treatment, the objective response rate is about 10 %.

Because of the dismal prognosis, the dawning of immunotherapy to be used for this patient category has sparked immense enthusiasm. For instance, atezolizumab, was the first checkpoint inhibitor that targets the programmed cell death protein 1 Ligand (PD-L1), to be approved by the US food and drug administration. The approval was limited for use as a second-line treatment and based on a phase II trial demonstrating an overall response rate of 15 % in patients with inoperable locally advanced or metastatic BC refractory to cisplatin-based chemotherapy (15). In patients with high PD-L1 expression, the response rate was 27 %. After demonstrating encouraging response rates and increased survival in the first-line setting for cisplatin-ineligible patients, atezolizumab was granted approval also for this patient category (20). Moreover, additional immune checkpoint inhibitors have been approved for the use in UBC and there are currently numerous ongoing late-stage trials also for other immunotherapies, including adoptive T cell therapy (ACT) and cancer vaccines.

Although immunotherapy against MIBC is promising, many challenges remain to be tackled, as highlighted by the recent phase 3 IMvigor211 trial (21). In the trial, atezolizumab did not meet its primary endpoint of improved survival compared with chemotherapy in the setting of metastatic UBC. One factor that might have influenced this outcome is that the proportion of patients sensitive to checkpoint blockers might be lower than previously appreciated. Hence, the result emphasizes the importance of proper patient selection and the need of better predictive biomarkers. In this pursuit, it appears critical to determine the immune contexture which, simplified, could be defined as the spatial distribution and organization of the leukocyte infiltrate of the tumor (reviewed in (22)). NGS is an additional promising tool for guidance in treatment decisions. For instance, NGS of more than 100 NMIBC tumors demonstrated a correlation between mutations in the *ARID1A* gene and an increased risk of recurrence after BCG treatment (23). Additionally, a single-sample genomic subtyping classifier was able to predict NAC response according to molecular subtype, namely that a basal tumor subtype gained most survival benefit of NAC (24). In sum, the poor prognosis of MIBC justifies the intense research focused on this malignancy, a priority we are now beginning to reap the rewards of, with immunotherapy being a promising modality. Recent technological advances appears useful in the challenge of selecting the patients who will benefit from these therapies.

1.2 AN INTRODUCTION TO THE IMMUNE SYSTEM

The human immune system is a web of intricately interconnected functions with the mutual mission to protect the organism from non-self entities of potential harm. To prevent this safeguarding from inducing damage to self, a solid regulatory arrangement of tolerance makes up a considerable part of this web. Traditionally, the immune system is considered to comprise two major arms; the innate arm which is immediate but non-specific and the adaptive arm which is specific and may acquire memory. Although this view is simplistic, as these arms to a large degree are entwined and inter-dependent, the main principles still hold. The following passages give an introduction to these branches using the example of a skin wound contaminated with extracellular

bacteria. The concluding sections serves to give an in-depth but concise description of immune tolerance.

1.2.1 The innate and adaptive immune response to a dirty wound

Upon contracting a skin wound with concomitant infection of extracellular bacteria, the innate arm, as a first line of defense, will provide resistance through various mechanisms. For instance, antimicrobial peptides will be secreted by tissue-resident innate- and epithelial cells. Membrane disruption and bacterial cell lysis may be exerted by the complement system (a set of plasma proteins that may carry out effector mechanisms of host defense upon activation). Crucially, innate immune cells, such as macrophages, are equipped with pattern recognition receptors (PRRs). These PRRs recognize and bind to pathogen-associated molecular patterns (PAMPs) such as the bacterial component Flagelin and to damage-associated molecular patterns (DAMPs) such as high mobility group box-1 (HMGB1).

Activation of PRRs may induce a series of events. Typically, in a macrophage, the transcription factor nuclear factor-κB (NF-κB) may be activated through its canonical pathway which stimulates the production of a plethora of pro inflammatory factors including cytokines and chemokines. These factors recruit and activate other innate cells such as neutrophils whose influx usually peaks within the first 6 hours of an inflammatory response. Once in the inflamed tissue, neutrophils may kill the bacteria through phagocytosis if provided the proper PRR- and complement receptor signaling. Other factors that may be secreted as a consequence of the activation of this pathway are i) Interleukin (IL)-6 which will stimulate the production of C-reactive protein, an acute phase protein which enhances the activation of the complement system, and ii) Tumor necrosis factor-α (TNF-α), a protein with multiple functions, including preventing bacteria to enter the bloodstream by triggering blood clotting in the local small vessels, occluding them.

Owing to their superior efficiency as antigen presenting cells (APCs) and their high capacity to migrate to T cell zones, dendritic cells (DCs) constitute a critical link between innate and adaptive immunity. In the skin, two types of DCs reside; Langerhans cells residing in the epidermis, and in the dermis, dermal DCs reside. If we envision that the skin wound in this example is rather superficial, LCs will capture the bacterial antigen by means of phagocytic receptors or macropinocytosis. Consequently, a class of PRRs called Toll-like receptors (TLRs) are activated and their signaling results in a “licensing” of the DC. This licensing involves alteration of the DCs’chemokine receptor profile enabling it to leave the skin and enter the lymphatic system where they further mature and acquire the ability to present the antigen to the adaptive arm of the immune system.

The AIS consists of T and B cells. In principle, each of these cells has a receptor specific for only one particular, cognate, antigen. Although these receptors share many features, the nature of the antigens and the mode of recognition differs which reflects the different functions of these two

lineages. While the B cell receptor (BCR) may recognize and directly bind antigens of various kinds such as lipids, polysaccharides and protein, the T cell receptor (TCR) instead recognizes short peptide fragments only from protein-derived antigens presented on a specific MHC pocket expressed on the APC. The MHC molecule is a glycoprotein with a pocket or cleft running across their outermost surface, in which a variety of peptides can be bound. This requirement of a specific peptide-MHC complex, is referred to as MHC restriction as the TCR needs to recognize structures both of the peptide antigen and of the MHC molecule to which it is bound, in order for the T cell to be activated. Such recognition constitutes the first signal out of three required signals for full T cell activation.

Eventually, the DCs enter the T cell zone of a draining lymph node and if the DCs are exposed to additional common microbial constituents (often called "danger signals") such as unmethylated bacterial DNA, they acquire the ability of co-stimulatory activity. The best characterized co-stimulatory molecules are the B7 molecules which ligate to their cluster of differentiation (CD) 28 surface receptor on the T cell which constitutes the second signal needed for T cell activation. At this stage of activation, the T cell start to proliferate and express the α chain of the IL-2 receptor (also known as CD25) which increases IL-2 signaling leading to an amplified clonal expansion of this T cell. However, in order to mount an adapted response towards the assaulting pathogen, the generated T cell clone must differentiate into one of several functionally distinct T effector cell (Teff) subsets. In this example of an extracellular bacterium, the antigen peptide would be presented on a MHC class II molecule, the class of MHCs presenting exogenous antigens. There are two major T cell populations; those expressing the co-receptor CD8 (i.e. $CD8^+$ T cells) which bind to MHC class I or the co-receptor CD4 (i.e. $CD4^+$ T cells) binding to MHC class II. Thus, only $CD4^+$ T cells are typically activated upon extracellular bacterial infection. Based on their effector functions, $CD4^+$ T cells are, in turn, divided into several subsets. We here consider the secretion of IL-4 from the APC that would support polarization towards the $CD4^+$ Th 2 cells, a subset primarily involved in promoting humoral immune responses towards extracellular pathogens.

A fraction of the newly formed $CD4^+$ Th2 cells would migrate towards a primary follicle of the lymph node – the area where B cells reside. Similarly to DC's, although with some key differences, some B cells would have picked up antigens through their BCR's and presented the processed peptides on their MHC II molecules. Mediated by chemokines such as CCL21 and CXCL13, B cells and T cells then co-localize at the junction between the T cell area and the B cell follicle where they form a primary focus. Here, the previously Th2, differentiates to a T follicular helper (Tfh) cell and may now, through the expression of CD40 Ligand (CD40-L) and IL-21, signal to the B cells to proliferate and differentiate. Remarkably, the Tfh cells will only do so, if it has been activated by an antigen closely associated to the antigen recognized and presented by the B cell – a mode of immune regulation called "linked recognition".

Some activated B cells migrate back into the follicle, accompanied by Tfh cells and form a germinal center where they (the B cells) undergo three processes fundamental for effective

antibody function; somatic hypermutation, affinity maturation and class switching. These B cells differentiate into either memory B cells or plasma cells which will contribute to elimination in the later stage of the infection by secreting versatile antibodies that may i) neutralize the bacteria ii) facilitate phagocytosis by binding and thereby "marking" the bacteria (opsonization) and iii) activate the complement system.

Once the infection is cleared, all these described "on signals" need to be turned off. How this is accomplished, as well as other dimensions of immune regulation, is covered in the following passages. However, as a final note, similarly to the B cells, also a subset of the activated antigen-specific T cells will differentiate into memory cells of different subsets such as CCR7^{+} central memory, CCR7^{-} effector memory and CXCR3^{+} tissue-resident memory T cells which can rapidly be re-activated if we are infected by the same pathogen again.

1.2.2 Two types of receptors, two types of adaptive immunity

B cells, together with their antibodies, are the main contributors to our humoral immunity, as they largely mediate their effects in fluids such as blood and mucosa, particularly in the extracellular space. This niche is possible because the BCR may i) recognize soluble antigens in their native form and ii) be secreted as an antibody and exert various effector functions directly on the pathogen. In contrast, T cells, because of the MHC-restricted TCR, requires a physical cell-cell contact to be activated and subsequently mediate their effects, hence T cells provide cell-mediated immunity.

Albeit these lineage defining differences in how their receptor works, the complex processes in which BCRs and TCRs are generated, are much similar. A basic understanding of these procedures is useful for the comprehension of immune tolerance, a recurring theme in this thesis. So, highly simplified, to recognize the diverse array of antigens an individual may encounter during the course of a lifetime, a broader receptor repertoire than could be encoded in the human genome, considering its limited size (of roughly 3 billion nucleotides), would be necessary. This problem is solved by two main mechanisms: the first is by combinatorial diversity which is the random recombination of multiple gene segments scattered across the chromosomes into one exon encoding the variable region (V-region) of the BCR or TCR. These V-regions encode the antigen binding regions of the heavy- and light-chain for BCR and of the α- and β-chain for the TCR. The second mechanism is junctional diversity; an imprecise incorporation of non-encoded nucleotides in the joints of these gene segments. Together, these processes form the basis for both TCR- and BCR diversity and are enabled through the expression the lymphocyte-specific recombination activating genes (*RAG 1* and *2*). However, the B cell takes advantage of two additional sources of diversity. The first is also of combinatorial character and arises from the many possible different combinations of heavy- and light-chain V-regions that may pair to form the antigen-binding site. Finally, somatic hypermutation, which is the insertion of point mutations in the V-regions of the heavy- and light-chain upon antigen encounter to improve the antigen

recognition and binding capacity of the antibody, but, coincidentally, the potential of receptor diversity also increases.

1.2.3 Central tolerance

As discussed previously, the ability of our AIS to recognize and eliminate an extraordinary diverse array of outer threats comes with the cost of an increased risk to recognize and evoke immune responses against self. Our solution to this challenge, to distinguish between self and non-self, is referred to as central and peripheral tolerance. For T cells, central tolerance is molded in the thymus, whereas for B cells, this process take place in the bone marrow, discussed below.

1.2.3.1 Receptor editing and clonal deletion of B cells

B cells are continuously throughout life developed in the bone marrow. The process starts with a Common lymphoid progenitor (Clp) differentiating into a pro-B cell. At this stage, the heavy chain *locus* is rearranged and those cells that manage to produce the μ isotype receive a survival signal and become pre-B cells. In Pre- B cells, the light chain *locus* is rearranged followed by allelic exclusion which is the process where one of the alleles for both the light-chain and the heavy chain *loci* is "turned off", ensuring that the B cell will only have one antigen specificity. The transition into the state of an "immature B cell" is defined by the pairing of the light chain with the heavy chain, resulting in the expression of Immunoglobulin (Ig) M on the cell surface (as a surface IgM, or sIgM).

The immature B cell is tested for autoreactivity through exposure to self antigens. For self-reactive B cells, depending on how the sIgM interacts with the self antigen, there are four possible outcomes. If the sIgM binds self antigen monovalent or with low affinity, the B cell mature normally and may be self-reactive, however is considered "clonally ignorant", as they are unable to be activated by their ligand. If the self antigen is able to cross-link sIgMs, the immature B cells are rendered unresponsive (anergic) to the antigen and migrate to the periphery, where they remain anergic. In the event of binding multivalent self ligands, for example an MHC molecule, a process referred to as receptor editing is initiated. In short, *RAG* expression continues which enables secondary rearrangement of the light chain *locus* and a new chance to generate an appropriate sequence that does not cause self reactivity upon expression. If this fails, the B cells undergo clonal deletion, in which apoptosis is induced as a means to weed out potentially damaging autoreactive cells from the repertoire.

If receptor editing succeeds, or if there was no self -reactivity in the first place, the immature B cells leave the bone marrow and undergo their final maturation steps in the spleen. Notably, most of the immature B cells will not survive to reach final maturation due to harsh competition to access a spleen follicle which is needed to receive survival signals from a subtype of DCs called follicular DCs. However, those that do survive, either differentiate into mature follicular B cells,

representing the majority of B cells, or marginal zone B cells, predominantly found in the marginal zone of the spleen.

1.2.3.2 Thymic selection of T cells

The life of a T cell begins in the thymus and is preceded by the migration of a Clp cell from the bone marrow. Once reaching the thymus, the Clp may receive notch signaling from a thymic epithelial cell (TEC) which makes it commit to the T cell lineage. Next follows a tightly regulated sequence of events which surround the development of the TCR and includes massive proliferation but also strikingly extensive cell death.

The initial phase of differentiation generates cells that bear distinctive markers of the T cell lineage but do not express the co-receptors CD4 or CD8, *i.e.* double-negative (DN) thymocytes. These give rise to both α:β and γ:δ T cells, however we here focus on the former subset. The DNs migrate through the thymic cortex and sequentially pass through four stages (DN1, DN2, DN3 and DN4) defined according to the expression of the markers CD44, CD25 and Kit. The main events in these developmental stages are related to the rearrangement of the TCR gene *loci* which is initiated at the DN2 stage and continue in DN3 cells. At this stage, a predecessor to the TCR, namely the pre-TCR is assembled by the pairing of the β chain with a surrogate pre-TCR α chain which trigger thymocyte proliferation and blocks further β chain rearrangement. Those cells that fail to produce a successful β chain rearrangement die by apoptosis, making DN3 a first checkpoint in T cell development. In the DN4 stage, rapid cell proliferation occurs, and eventually both CD4 and CD8 co-receptors are expressed, *i.e.* double-positive (DP) thymocytes are formed. Once the DPs cease to proliferate, their α chain *locus* begins to rearrange with the subsequent formation of a functional α:β TCR complex. At this stage, the harsh selection processes are initiated.

Only 2-5 % of all developed thymocytes survive positive and negative selection, highlighting their extensive filter-effect. Although evidence suggests these as quite parallel processes, positive selection is still considered to be first initiated. It encompasses survival- and maturation signals from cortical TECs to those DPs whose newly formed TCRs manage to engage to a self-peptide:self MHC complex. The DPs who fail this enterprise have “useless” TCRs (approximately 90 % in the mouse thymus), with the subsequent absence of intrinsic survival signals and, therefore, die by neglect. The positive selection procedure also coordinates the commitment to either the CD4 or CD8 lineage as TCR recognition of self peptide:self MHC II results in loss of CD8 expression and vice versa. Upon becoming single-positive (SP), the thymocytes express high levels of their TCRs and migrate to the thymic medulla.

As a direct effect of positive selection, all T cells are self-reactive to a certain degree. In the first step towards establishing tolerance to self, a negative selection process ensues in which thymocytes exhibiting a TCR which strongly ligates to the self peptide:self MHC complex, are induced to die by apoptosis. This process occurs throughout thymocyte development, both in the

thymic cortex and in the medulla. Exactly what cells that mediate this death induction in the thymic cortex remain unresolved. However, in the medulla, a bone-marrow derived antigen-presenting cell type known as medullary TECs (mTECs) have been shown to directly induce thymocyte negative selection. A key feature of mTECS is their expression of the *AIRE* (autoimmune regulator) gene (25). AIRE triggers the ectopic expression of genes encoding tissue-specific antigens which, in coordination with thymic DCs, enable a diverse repertoire of self antigens to be presented and strongly self-reacting thymocytes to be deleted (25).

The few thymocytes surviving the selection processes undergo final maturation in the medulla which involves changes in the TCR signaling machinery so that binding to the TCR results in proliferation as opposed to apoptosis - the default mode of a developing thymocyte. The expression of Sphingosine-1-phosphate receptor 1 (S1PR1) marks the final T cell maturation step and enables the emigration from the thymus into the bloodstream.

1.2.4 Peripheral tolerance

Unfortunately, negative selection does not remove all strongly self-reactive T cells. Other mechanisms operating in the periphery are necessary to counteract autoimmunity. Regulatory T cells (Tregs), with their capacity to suppress immune responses, represent a pivotal such tolerance modality (26).

1.2.4.1 Regulatory T cells

Tregs were first identified as a $CD4^+$ T cell subset expressing high amounts of CD25 and capable of suppressing autoimmunity in mice, a pioneering work of Sakaguchi *et al.* in 1995 (2). Since then, a broad heterogeneity within the Treg population has been demonstrated. Even though “generic Tregs” may not exist, some main characteristics are mutual for the vast majority of the to date identified Treg subpopulations; Tregs migrate to inflammatory sites and, by both cell- and non-cell contact dependent mechanisms, are able to suppress other immune cells such as APCs and T eff cells (26). By these means, Tregs mediate peripheral self tolerance, maintain immune homeostasis and protect against autoimmunity.

The potential therapeutic use of Tregs has been implicated in a broad range of diseases, spanning from diabetes to cancer. Ample data support their central role within the tumor-immune interplay, however that topic will be revisited in a later section. Instead, here follows a palette of physiological-centered Treg facets including their origin(s), main markers, subpopulations, epigenetic regulation, plasticity and functionality.

1.2.4.2 Treg cell development

Two origins have been described for Tregs; thymic differentiation from immature T cell precursors into *t*Tregs, which constitutes the major Treg population, and peripheral induction from naïve Teff cells (*p*Tregs). A cardinal feature of *t*Tregs is that, unlike naive Teff cells, they differentiate into a functionally mature and stable cell lineage within the thymus. This process is

a rather recent addition to the T cell fates - and still a matter of intense investigation. It is clear that Treg development involves phenotypically distinct stages with specific transcriptional and cytokine requirements.

Cellular signaling during thymic Treg development

Several lines of evidence indicate the thymic medulla as the main scenery for *t*Treg development. For instance, *t*Treg cell generation is dependent on an intact mTEC compartment (27). Additionally, *t*Treg differentiation is promoted by AIRE-dependent antigens presented by mTECS but also by B cells (28, 29). The above data is derived from mouse studies, knowledge of *t*Treg development in humans is somewhat limited. However, Watanabe *et al.* reported that epithelial cells of Hassall´s corpuscles, structures (located in the medulla) unique to human thymic histology, produce thymic stromal lymphopoietin (TSLP) which promoted thymic DCs to induce differentiation of SP $CD4^{+}CD25^{-}$ thymocytes into distinct forkhead box $P3^{+}$ ($FOXP3^{+}$) Treg subsets (30, 31).

The transcription factor FOXP3 is a distinctive feature of Tregs that is essential, but not entirely vital for Treg development (32, 33). Although $FOXP3^{+}$ cells have been observed at the more immature thymocyte stages (34), diversification to the Treg lineage, is considered to take place subsequent to positive selection from $CD4^{+}$ $CD24^{high}$ SP thymocytes (35-37).

How and which thymocytes selected to become Tregs is, at least in part, determined by the self-reactivity of the TCR. More specifically, the consensus has been that those thymocytes that may differentiate into Tregs are those with a TCR that binds the self peptide:self MHC stronger than would result in differentiation into Teff cells but still with less avidity than would induce negative selection (35, 38). This model is supported by studies using TCR transgenic mice combined with several knockout strains deficient of molecules downstream of TCR and co-stimulation signaling (reviewed in (38)). As a consequence of this selection process, the Treg TCR repertoire is skewed toward recognition of self antigens but, reportedly, is as broad as that of Teff cells (36, 39).

Recent insights have nuanced the view of *t*Treg development as data indicate that, although binding strength is an important factor, the *duration* of the antigen-TCR binding is what specifies the *t*Treg cell fate (36). Supportive to this "hit-and-run model" is the finding that transient, but not continuous, TCR stimulation induces robust FOXP3 expression in CD4 SP T cells (40, 41). Moreover, in thymic precursor cells expressing transgenic Treg-derived TCRs, there is a pronounced intraclonal competition for further differentiation (42, 43), implying that *t*Treg differentiation is facilitated by ligands present in limiting amounts in the medulla.

Although, these studies provide solid evidence for the requirement of proper TCR signaling, the downstream molecules hypothesized to serve as key regulators of Treg-type gene expression (in addition to FOXP3), remain to be identified.

Ample data have delineated a two-step process for the thymic generation of Tregs in which TCR signaling in conjunction with the intermediate binding-avidity to an antigen is the first step (44). This allows transcriptional changes and increases in cell-surface expression of the α-chain of the IL-2 receptor (CD25). Signaling by IL-2, via the intrinsic signal transducer and activator of transcription 5 (STAT5) leads to induction of FOXP3 expression and represents the second step (44, 45). The critical source of thymic IL-2 during Treg development is from other T cells as recently demonstrated in mice by selective deletion of IL-2 in T cells, B cells and DCs (46). The level of IL-2 was demonstrated to be partly regulated by peripherally activated Tregs recirculating to the thymus and sequestering IL-2, thus restricting *t*Treg development through a negative feedback loop (47).

Epigenetic control during thymic Treg development

The establishment of a Treg-specific epigenetic pattern could be added as a third prerequisite for *t*Treg development (35). Given the transcription-factor-dependency of this process, an open chromatin structure is critical for the binding of these factors to their target sequences. Such genome arrangements represent parts of the ever-growing Treg epigenetic landscape. To date, it comprises ~300 Treg-specific demethylated regions (TSDRs) and ~70 regions comprising a subset of enhancers referred to as super-enhancers (SEs) with Treg-specific epigenetic changes. These are typically located in the vicinity of Treg signature genes such as *Foxp3*, *Il2ra*, and *Ctla4,* rendering a positive epigenetic control of Treg-type genes (37). With regards to *Foxp3*, five conserved regulatory elements within the *Foxp3 locus* have been discovered: the promoter region (48) and the conserved non-coding sequences (CNSs) 0-3 (49-52).

Recent studies of stage-specific epigenetic changes in the course of *t*Treg cell development have demonstrated the importance of condensed chromatin to be opened and activated in regulatory elements such as the CNSs and the Treg-SEs. For example, the chromatin organizer Satb1 was found in *t*Treg precursor DP cells to occupy CNS0 and to assort the chromatin, including histone modifications, to a poised state that allows DNA demethylation (49). This Satb1-dependent activity was crucial for *t*Treg development as these cells were absent in Satb1-deficient mice (49). Also, these mice exhibited impaired DNA demethylation at the Foxp3 *locus*, implying that Satb1 prime $Foxp3^-$ Treg precursor for Foxp3 induction.

Intriguingly, DNA demethylation of Treg signature genes such as *Ctla4* and *Il2ra* is largely unaffected by the absence of functional Foxp3. Instead, the DNA demethylation of TSDR’s has been reported to be controlled by Ten-eleven translocation (Tet) proteins 2 and Tet3 which work redundantly and independent of FOXP3 expression but are dependent on TCR-signaling (53, 54).

The fate of becoming a Treg appears to be determined before Foxp3 expression with the enhancer-landscape framed at the early stage, to pave the way for further imprinting of the Treg epigenetic topography including DNA demethylation. This allows subsequent Treg-specific

transcription factors, including Foxp3, to be expressed and by selective binding to their target sequences, they reinforce the Treg cell lineage commitment.

The given question is then: what determines the epigenetic topography in the first place? The main candidate is TCR activation, since TCR engagement with self ligands is required for the demethylation of Treg signature genes as demonstrated in TCR transgenic mice with thymic stromal cells expressing the cognate antigen (53). This framework also fits well with previous findings of TCR-signaling to dictate the first two steps during Treg development (36, 55). TCR-guided differentiation has relevance for Tregs beyond their thymic development, which will be discussed below, but first, a brief overview of markers for Treg identity and function.

1.2.4.3 Regulatory T cells markers

CD25

The Treg population was first defined as a subpopulation of $CD4^+$ T cells constitutively expressing CD25, the high affinity IL-2 receptor α-chain (2). Together with the β- (CD122) and γ-chain (CD132), CD25 forms the high-affinity IL-2 receptor (IL-2R). Mice lacking CD25 or CD122 suffer from massive systemic autoimmunity, which can be reversed by the adoptive transfer of $CD4^+CD25^+$ T cells from wild-type mice (56, 57). It is noteworthy that these mice contracted autoimmunity rather than infection, because it implies a dominant role of IL-2 to act on Tregs and that for Teff cells, IL-2 is somewhat dispensable.

Indeed, IL-2 signaling is essential for the thymic and extrathymic differentiation, maintenance and function of Tregs (44, 58-60). For instance, the central role of IL-2R-signaling-driven activation of STAT5 for the suppressor function of Tregs was demonstrated in mice with conditional deletion of the *Il2ra* and *Il2rb* alleles in combination with induced expression of an active form of STAT5 (61). Through this model system, it was demonstrated that an absence of STAT5 or IL-2R resulted in severe loss of Treg suppressor function beyond what could be attributed to the accompanying decrease in Foxp3 expression.

Baecher-Allan *et al.* showed that only the $CD25^{high}$ population possesses suppressive capacity whereas the $CD25^{low/intermediate}$ cells do not suppress T cell proliferation (62). However, CD25 is generally upregulated in T cells upon activation, limiting its specificity and usefulness as a Treg marker.

Emphasizing the close relation between Tregs and the IL-2R is the markedly lower concentrations required to spark the Treg intrinsic IL-2 signaling machinery and downstream gene activation compared to Teff cells (63). This feature, together with the abundant expression of the cognate receptor, is the basis for the high IL-2 sensitivity of Tregs (64).

FOXP3

Mutations in the FOXP3 gene is associated with the human disease Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX), highlighting the integral role of this transcription factor in the Treg population and in maintaining immune homeostasis at large (65). FOXP3 (in mice labeled Foxp3) is, in both mice and humans, paramount for Treg characteristics with particular importance in orchestrating their suppressive function (26, 32). However, precisely how FOXP3 coordinates this suppression is not fully understood.

Foxp3 appears to possess two modes of transcriptional regulation in Tregs as several mouse-model studies have demonstrated both positive and negative control of its target genes (66, 67). By its positive, or amplifying, impact on Treg signature genes, Foxp3 acts to stabilize the characteristic Treg gene expression pattern, rather than to *de novo* induce the expression of these genes. This notion is based on the findings that Foxp3-deficient Tregs can express Treg signature genes, albeit at lower levels and that Foxp3 mainly occupies a pre-established epigenetic landscape (33, 68). With regards to its *negative* transcriptional control, this regulatory mode is switched on upon Treg activation which results in a global transcriptional repression (69). Taken together, it has been proposed that the dichotomous nature of Foxp3 gene regulation provides a scheme of how Foxp3 promotes Treg suppressive function, namely by amplifying signature genes like *CD25* and *CTLA-4* while concomitantly repressing genes typically expressed by Teff cells upon activation.

Approximately 5000 and 700 potential target genes for FOXP3 have been identified in humans and mice respectively (66, 70). Among these, there is an overrepresentation of genes encoding for immunoregulatory cytokines, growth factors and, in particular, for key modulators of T cell activation. For example, the protein tyrosine phosphatase 22 (Ptpn22), which mutated forms are associated with numerous autoimmune diseases, is upregulated upon TCR activation in Teff cells but inhibited in Tregs (67, 71).

In contrast to mice, human FOXP3 is not exclusively expressed in Tregs as confirmed by its transient upregulation in $CD4^{+}CD25^{-}$ T cells upon activation (72). However, in these cells, FOXP3 is mainly in the cytoplasm whereas in Tregs, it is predominantly nuclear (73). In agreement, there is controversy regarding the *in vitro* suppressive capacity of cells with transient FOXP3 expression (74). Moreover, human FOXP3 expression is not even restricted to the lymphocyte lineage as its expression has been demonstrated in non-hematopoietic cells of various kinds and in cancer cells of multiple lineages (75).

Despite its non-specificity, in lack of practical alternatives, FOXP3 is often used as a marker for Treg identity. It follows, that data solely based on FOXP3 expression urges careful interpretation, especially in an inflammatory setting where T cell activation is to be expected.

Adding to the heterogeneity of the human Treg landscape, our species express two main isoforms of FOXP3 at similar levels; the full-length protein (FOXP3fl) and the FOXP3Δ2, which lacks

exon 2 (76). A key functional distinction is that the exon 2 domain is necessary for the interaction with the Th17 master transcription factor RAR-related orphan receptor γ (ROR γ) and ROR α. Still, the downstream functional relevance of this, and alternative FOXP3 splicing in general, awaits further investigation (77).

Progresses within the field of immunometabolomics have added another layer to the regulation of FOXP3 expression. For instance, elevated glycolysis is associated with decreased Treg functional stability, in part due to suppression of FOXP3 expression mediated by the metabolic regulator mTOR (78). In contrast, the metabolic Hypoxia-inducible factor 1-alpha (HIF-1α) pathway has been reported to drive FOXP3 expression, which has been suggested as a mechanism for Treg accumulation in the tumor microenvironment (TME), since this pathway is overactive in many tumors (79).

CD127

The expression of low levels of the IL-7 receptor α-chain (CD127) inversely correlates to FOXP3 expression and suppressive capacity in human $CD4^+$ T cells (80). Hence, CD127 is considered a useful complementary Treg surface marker. However, its *complementary* usage should be stressed since $FOXP3^+$ cells are only enriched to the level of ~40 % of the $CD127^{low}$ population (81). Also, CD127 is transiently downregulated in Teff cells early in the course of activation (82). Nevertheless, in a workshop where experts in the Treg field participated and systematically evaluated various Treg markers in both the cancer- and physiological setting, CD127 was validated as a useful marker in both these contexts and suggested to be included in a standardized Treg flow cytometry panel (83). This proposal is in line with other findings of the $CD4^+CD25^+CD127^{lo}$ population to enable both improved yield and purity of $FOXP3^+$ cells compared to the $CD4^+$ $CD127^{lo}$ population (80).

CTLA-4

The expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) peaks within 48h of activation on most resting Teff cells and is only constitutively expressed by the Treg subset (84). In the resting state of Tregs, CTLA-4 is mainly localized to intracellular vesicles which upon T cell activation fuse with the cell membrane to be exposed on the cell surface, preferably adjacent to the TCR (85). CTLA-4 is a key-molecule for Treg suppressive function and activated, suppressive (human) $CD4^+CD25^+$ Tregs harbor higher CTLA-4 expression than its non-activated counterparts (86). On this basis, CTLA-4 is considered a valid Treg effector marker (26). The mechanistic basis for CTLA-4 function is described below in the section designated for Treg effector mechanisms.

1.2.4.4 Functional Treg subsets

There is a vast phenotypical and functional heterogeneity within the Treg population, reflecting the diverse environments and target cells that they regulate. Several markers, and different combinations of these markers, have been suggested to further delineate functionally distinct Treg

subsets (reviewed in (26, 87)). In this endeavor, assessing quantitative shifts in expression of these proteins is preferable to the simple qualitative approach, since Treg-intrinsic molecules linked with immune suppression in general are upregulated from the steady state, rather than expressed *de novo* (87).

Among the earlier markers to be identified for Treg subpopulation delineation, were the naïve and memory T cell markers, CD45RA and CD45RO respectively (88). Upon antigenic stimulation, the naïve $CD45RA^{+}CD45RO^{-}FOXP3^{+}$ population converts to $CD45RA^{-}CD45RO^{+}FOXP3^{+}$ cells. By quantifying the degree of FOXP3 expression, the $CD45RA^{-}CD45RO^{+}FOXP3^{+}$ populations can be further dissected into $FOXP3^{hi}CD45RA^{-}CD45RO^{+}$ cells, referred to as effector Tregs, which are terminally differentiated, highly suppressive and functionally stable, and a $FOXP3^{lo}CD45RA^{-}CD45RO^{+}$ subset, less prone to exert suppression and with the capacity to secrete pro-inflammatory cytokines (88). Still further, distinct functional subsets within the $FOXP3^{hi}CD45RA^{-}$ $CD45RO^{+}$ population have been identified. For instance, the subpopulation with high ectonucleotidase CD39 expression present stronger stability and function under inflammatory conditions compared to their $CD39^{low}$ counterparts (89).

Naïve or resting Tregs do not express the MHC-II molecule HLA-DR but upon activation they do and then remains $HLA\text{-}DR^{+}$. In consistency, $HLA\text{-}DR^{+}CD4^{+}FOXP3^{high}$ cells have been proposed to represent a terminal effector subpopulation of more highly and rapidly suppressive Tregs then their $HLA\text{-}DR^{-}$ counterparts (90). These qualities grant HLA-DR suitable as a Treg activation and memory marker. In contrast, Teff cells only transiently express HLA-DR upon activation.

1.2.4.5 Generation and education of Tregs in the periphery

The generation of *p*Tregs is fringed with even more uncertainties than that of *t*Treg cells. This is in part due to a lack of a marker or method to distinguish *p*Tregs from *t*Tregs wherefore the exact numeral and functional contribution of the *p*Treg population remains in question. The transcription factor Helios or the membrane co-receptor Neuropilin 1 (Nrp1) have been suggested to be exclusively expressed by *t*Treg cells, but consensus has not yet been reached on this matter (91, 92).

Aside from the difficulty to distinguish them, the existence of *p*Tregs and the molecular mechanisms by which they are developed are well defined. Briefly, the conversion of Teff cells to *p*Tregs *in vivo* is favored by long-term exposure to sub-immunogenic doses of non-self antigen such as food antigens presented by mucosal tissue-resident DCs, accompanied with Transforming Growth Factor β (TGF-β), retinoic acids and short chain fatty acids often produced in such immunosuppressive environments (93). For example, gut-associated lymphoid tissue-residing $CD103^{+}$ DCs were demonstrated to produce TGF-β and retinoic acid (94).

*p*Treg cell differentiation is also boosted by metabolites of the microbiota, such as butyrate or propionate and natural antigens from commensal bacteria readily induce *p*Tregs in the colon (95,

96). The central role of *p*Tregs in mucosal tolerance was highlighted by the finding that depletion of these cells caused dysregulated immune responses both in the gastrointestinal tract and airway (97). Their relevance for immune regulation is also manifested by the finding that the *in vitro* suppressive capacity of these cells is equal to that of *t*Tregs (98).

1.2.4.6 Epigenetic maintenance of Treg stability

One definition of Treg stability is the maintenance of sustained FOXP3 expression, suppressive capacity and lack of effector functions (99). A central mechanism underlying this stability is the epigenetic control of the Treg-specific gene network.

Hill *et al.* demonstrated elegantly the critical role of the TSDRs and the insufficiency of FOXP3 to alone maintain the Treg gene signature (100). They found that TCR stimulation of Teff cells conferring a retroviral ectopic expression of Foxp3, represses IL-2 and interferon γ (IFN γ), which do not possess TSDR, but fails to upregulate TSDR-dependent genes such as Ikaros Family Zinc Finger (IKZF) 2 and IKZF4.

The regulatory element CNS2 located within the FOXP3 *locus* contains CpG islands which are highly demethylated only in functional Treg cells. This methylation pattern is considered to be a definitive marker of commitment to the Treg cell lineage (51) and can distinguish a committed Treg from a Teff cell transiently expressing FOXP3 both in human peripheral blood and solid tumor tissue (35, 101). In diligent interrogations, Rudensky *et al*. introduced systemic deletion of each three CNSs (at the time, CNS1-3 were known) and demonstrated deletion of CNS2 to affect the stability of Foxp3 expression during proliferation, pointing out this regions' importance for the heritable maintenance of the active state of the Foxp3 *locus* and, therefore, for Treg lineage stability (60).

Interestingly, variations within CNS2 methylation patterns (10%–60%) have been reported for tumor-infiltrating Tregs (TI-Tregs) (99). Moreover, *in vitro* TGF-β induced Tregs (*i*Tregs) display only a partial demethylation of the CNS2 region (53). In contrast, *p*Tregs exhibit complete demethylation of the CNS2 and stable expression of Foxp3 (102). Hence, methylation status of CNS2 is a useful marker to distinguish committed Tregs (*t*Tregs & *p*Tregs) from transiently FOXP3 expressing Teff cells and *i*Tregs.

We previously showed that hypomethylation at the CpG position -77 of this promoter represents a Treg-specific methylation status (48). Indeed, the transcriptional activity of the *FOXP3* promoter also contributes to the lineage-specific expression of FOXP3, albeit to a lesser extent than the CNSs.

In sum, the Treg-specific epigenetic landscape, installed during Treg development (described above) and inherited through cell divisions, is critical for maintaining long-term Treg lineage stability. In this regard, demethylation at regulatory elements within the *FOXP3 locus* appears to be of particular importance.

1.2.4.7 Treg plasticity

Is it possible for this rigorously regulated and highly differentiated cell population to be reprogrammed into a Teff cell? Considering the therapeutic applications that use or modulate Tregs to treat autoimmune diseases, allergies, graft rejection and tumors, the issue of Treg stability/plasticity is of considerable practical importance. Yet, the topic is ambiguous to unpack, not the least because of the mixed use of nomenclature with regards to *lineage* and *functional* plasticity in the literature. In the following passages Treg *lineage* plasticity refers to Treg loss of one or several of the following traits: sustained FOXP3 expression, suppressive capacity or lack of effector functions. In contrast, *functional* plasticity refers to the capacity to adapt key features to the surrounding environment.

The issue of Treg lineage plasticity is contentious. Briefly, several Foxp3 lineage-mapping studies reported a small population (1–5%) of Tregs that downregulated Foxp3 in response to certain lymphopenic and inflammatory cues, suggesting that Tregs can turn into exTregs. These exTregs exhibit an increased CNS2 methylation compared to its stable Treg counterpart and secretes IFNγ (103). Also, in mouse models of cancer, small populations of exTregs have been observed (99). However, these studies have endured skepticism, some regarding exTregs as a methodological artifact. In humans, Tregs expressing IL-17 (and IFNγ in some cases) have been reported for several inflammatory diseases including psoriasis, inflammatory bowel disease and rheumatoid arthritis (104). The maintenance of the suppressive function in these cells was context-dependent. Taken together, although Treg lineage plasticity remains controversial, published reports suggest that under extreme circumstances, such as strong inflammatory conditions or, on the other extreme, lymphopenic settings, Tregs may lose FOXP3 expression and their suppressive properties.

The capacity to respond to stimuli is a basic criterion for life. The capacity of a cell to respond to environmental cues and self-adapt, may be viewed as an expansion of that notion. Through this perspective, it is easy to conceive that Tregs adapt key features, including their suppressive functions and migratory capacity, to the context they are in. For instance, in response to certain extracellular stimuli, naive Tregs can express Tbet or increase IRF4 or STAT3 activity, which are required for Th1, Th2 and Th17 cell formation, respectively (105-107). The specialized regulatory program determined by each of these transcription factors inflicts a unique gene expression pattern of the Tregs while maintaining expression of core molecular signatures such as Foxp3, CD25 and CTLA-4 expression (37). In a recent report, another such Treg specialization was described, namely that Tregs resident in the follicles of chimeric mice spleens (termed "follicular Tregs") uniquely expressed the transcription factor Bcl-6, similar to follicular helper T cells (108). Bcl-6-dependent CXCR5 expression recruited the follicular Tregs to the germinal center, where they suppressed follicular helper T cells and B cell responses (108, 109).

Specialized Treg populations are also found in non-lymphoid tissues. A well-studied example is visceral adipose tissue (VAT), where Tregs express high levels of PPARγ, a key adipocyte lineage

transcription factor (110). In these VAT-Tregs Foxp3 and PPARγ, together mold a distinct expression pattern, orchestrating the phenotype and function in this tissue-localized Treg subpopulation (110).

A recent advance in our understanding of the molecular basis for how Tregs adapt to their surroundings is the finding that TCR signal intensity shapes activated Treg programs (111), which conforms to the concept of TCR-guidance during Treg development in the thymus.

1.2.4.8 Treg functionality

Recognition of its cognate (self) antigen, paired with co-stimulation, is required for Tregs to exert suppression (112). However, once activated, the suppressor function of Tregs is non-specific and may, via humoral and cell-cell contact mechanisms, target various cell types such as Teff cells, natural killer (NK) cells, B cells, DCs and macrophages, as summarized in **Figure 1** (113, 114).

The contact-dependent mechanisms include surface expression of inhibitory molecules such as CTLA-4, lymphocyte-activation gene 3 (LAG-3), CD39, CD73 and the T cell immunoreceptor with Ig and ITIM domains (TIGIT) whereas the secretion of soluble inhibitory factors such as TGF-β, IL-10, Granzymes, Perforin and IL-35 represent contact-independent mechanisms. One additional principally different mechanism deserves to be mentioned, namely “out-competition”, exemplified by Treg aggregate formation around DC’s and thereby limiting APC access for Teff cells. The paracrine IL-2 loop is a similar example, *i.e.* IL-2 production by Teff cells and consumption by Tregs through extensive CD25 expression (115, 116). All these mechanisms should be viewed as complimentary modalities, utilized by Treg populations in varying extent depending on the contextual setting. The effector mechanisms particularly implied in the cancer setting are addressed in a later section.

CTLA-4 is key for Treg suppressive function and is constitutively expressed by activated and memory Tregs (88). The binding of CTLA-4 on Tregs to its ligands B7-1 (CD80) and B7-2 (CD86) on an APC, activates Tregs to exert suppression. This notion is supported by the observation that blockage of this interaction leads to the failure in Treg activation and suppression (112). In contrast, CTLA-4 on Teff cells competes with the co-stimulatory molecule CD28 at the immune synapse for the binding of their mutual ligands (CD80 and CD86) which provides intrinsic inhibition by disrupting the B7–CD28 axis (reviewed in (17)). As an extension of this mechanism, Tregs may downregulate both CD80 and CD86 on DCs in an extrinsic manner where CTLA-4 (expressed on the Treg), reportedly, binds to such APC-ligands by transendocytosis, targeting them for lysosomal degradation (116, 117).

An additional extrinsic mechanism of CTLA-4 mediated Treg regulation is by reverse receptor signaling to DCs resulting in upregulation of indoleamine 2,3-dioxygenase (IDO) in these cells (118). IDO catalyzes the degradation of the, for Teff cell$_s$, essential amino acid Tryptophan. Both the depletion of Tryptophan and the generated metabolites are suppressive for Teff cells.

Theoretically, such cell extrinsic mechanisms could also be exerted by Teff cells expressing CTLA-4, a subject that remains elusive.

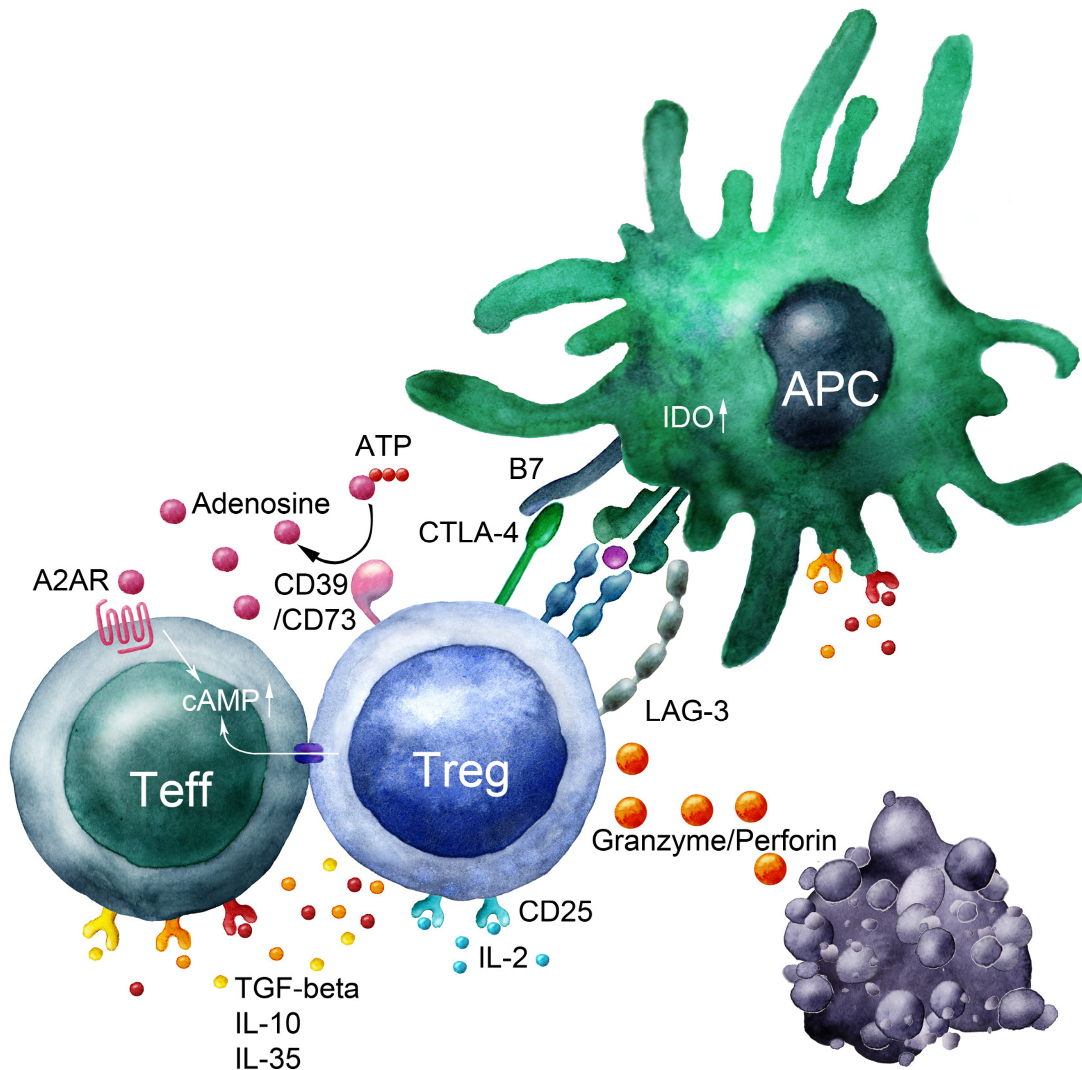

Figure 1: Treg effector mechanisms. Tregs suppress immune responses by using diverse mechanism of suppression including: i) the secretion of inhibitory cytokines such as TGF-β, IL-10 and IL-35 capable of inhibiting Teff cells and DCs, ii) the secretion of cytotoxic agents such as granzymes/perforin and iii) the expression of inhibitory cell surface molecules including CTLA-4 which restrain antigen presenting cells and Teff cells by *e.g.* increasing IDO activity in DC's. The high expression of the high-affinity subunit of the IL2 receptor CD25 diminishes IL-2 levels. CD39/CD73 expression results in elevated pericellular adenosine levels with downstream suppressive effects through the A2A adenosine receptor. Figure courtesy of Malin Winerdal.

As a molecule with such immune inhibitory features, the therapeutic potential of blocking CTLA-4 and thereby releasing an immunological break has been exploited for various cancers to reinforce the patient´s anti-tumor immune response. Blocking antibodies such as Ipilimumab has been in use since 2000, yet the mechanistic basis for their effect is not fully understood, discussed further in a later section (4).

The next wave of co-inhibitory receptor targets being explored in clinical trials includes Lag-3, and TIGIT. These belong to the same class of receptors as CTLA-4, but exhibit unique functions. Lag-3 is a homologue to the TCR co-receptor CD4 and can suppress DC maturation by interacting with MHC II molecules on these cells (119). TIGIT is a direct target gene of FOXP3 and engagement of TIGIT on Tregs leads to an upregulation of the suppressive mediator Fgl2, which confers superior suppressive function to $TIGIT^{+}$ Tregs such as selective suppression of Th1 and Th17 cell responses, which are dominant in driving autoimmune inflammation (120).

The ectoenzyme CD39 exercise Treg effector functions and induce a local anti-inflammatory environment by catalyzing the first and rate-limiting step in the degradation of extracellular ATP, to adenosine (121). CD39 is only constitutively expressed on activated Tregs whereas CD73, which catalyze the second reaction, is expressed on both Tregs and other immune cells. Local adenosine production limits T cell immune responses primarily through engagement of the G-protein coupled adenosine A2A receptor which, in turn, leads to increased cAMP levels in the target cells (122). Tregs have also been described to suppress responder cells by directly transferring cAMP through gap junctions. As an intracellular second messenger, cAMP may trigger a plethora of signaling pathways including the activation of Proteine Kinase A that, via its action on Protein Tyrosine Kinase, initiates downregulation of TCR signaling (123).

1.3 TUMOR IMMUNITY

The idea that the immune system protects the body against tumor development by recognizing and eliminating malignantly transformed cells was formulated in 1957 by Sir Macfarlane Burnet in "The Concept of Immunosurveillance" (124). At the time, the experimental approach was to excise a tumor from an animal and show that, in many cases, the animals could reject a second injection of the same tumor cells. These were the first results to imply the existence of immunosurveillance and tumor immunity. Today, this notion has become tough to question considering the clinical breakthrough for antibodies releasing the negative regulators of immune activation (checkpoint inhibitors), a treatment with unprecedented rates of long-lasting tumor responses in patients with various cancers (4).

A crucial consequence of recognizing the capacity of the immune system to eliminate tumors is that all tumors that *de facto* arise in immunocompetent organisms have been selected to evade the host's immune system. Bearing this in mind, the following chapter discusses some of the key concepts of tumor immunity, with a special focus on Tregs.

1.3.1 Tumor immunogenicity

Tumor immunogenicity refers to properties of the tumor enabling the host to evoke an anti-tumor immune response. In principle, the immunogenicity of a tumor relies on the presence of a tumor antigen (antigenicity) combined with a "damage signal" (adjuvanticity) (125). With regards to antigenicity, tumor antigens may be divided into three broad categories: Tumor associated antigens (TAAs), cancer-germline/cancer testis antigens (CTAs) and tumor-specific antigens

(TSAs) (reviewed in (126)). Simplified, this division is based on *how* tumor-specific these antigens are; TAAs being least specific as they derive from proteins encoded for in the normal genome and hence occur in normal cells but are aberrantly expressed in certain cancers, whereas CTAs are intermediately specific as they normally are only expressed in germ cells (testis and ovary) and trophoblast tissues but are frequent in certain cancer cells. Finally, TSAs are uniquely expressed in tumor cells and may derive from oncoviral proteins or mutations that, when translated, give rise to abnormal proteins bearing neoantigens. Because neoantigens are regarded as altered self, they are not presented in the thymus and thus not subjected to central tolerance, therefore they represent attractive targets for anti-cancer T cell therapies.

Antigenicity is required but not sufficient to evoke an adaptive immune response. In fact, an elevated availability of antigens in the absence of appropriate immunostimulatory signals has tolerogenic rather than activating effects (125). In the context of tumor immunogenicity, such stimulatory signals are mediated by DAMPs released by (or exposed on the surface of) stressed or dying cancer cells. DAMPs alert the host of "danger" by binding to PRRs and thereby, in the presence of an antigen, renders the tumor immunogenic, enabling the initiation of an adaptive anti-tumor immune response (127). For instance, the release of HMGB1 from apoptotic tumor cells enhances DC-mediated antigen presentation via TLR4 signaling (128). The requirements of both antigenicity and adjuvanticity for a tumor to be immunogenic have implications for how a patient responds to chemotherapy, discussed in a later section.

Acknowledging that certain tumors may be immunogenic poses critical questions: why does the immune system so often fail to eliminate immunogenic tumors? Is it because of host- or tumor factors, or a combination thereof? These issues can, at least partly, be understood through the concept of "cancer immunoediting".

1.3.2 Cancer immunoediting

Cancer immunoediting comprises three phases of immune system–tumor interactions during the course of tumor development, namely *elimination*, *equilibrium* and *escape* (129). Compelling evidence for this concept was provided by the demonstration of tumors developed in immunodeficient mice to be highly immunogenic and, in contrast, tumors from immunocompetent mice to display reduced immunogenicity (130). Of note, in some cases, cancer cells may directly, without passing through an earlier phase, enter into either the equilibrium or escape phases.

1.3.2.1 Elimination & Equilibrium

The elimination phase is to a large extent a rephrase of tumor immunosurveillance, where developing tumors and premalignant cells are recognized and eliminated by the immune system before they become clinically apparent. Key components in this phase include molecules and cells of both the innate- and adaptive immune system. For example, in the mouse, type I IFNs

enhance tumor antigen cross-presentation activity of DCs (131) and promotes induction of $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T cells (132).

Rare cancer cell variants may survive the elimination phase and enter the *Equilibrium phase,* better described as a bilateral Darwinian selection process between the immune system and the tumor. In this process, the overall tumor expansion is constrained by immune-mediated tumor cell killing, still there is no *net* tumor cell elimination. Instead, both parties phenotypes are edited but the inherent genetic instability of the tumor eventually favors the emergence of tumoral clones which are not recognizable and *escape* the immune system (129). The equilibrium phase was evidenced for instance by data of immunocompetent mice injected with a low-dose carcinogen, to harbor occult cancer cells for greater than 200 d (133). However, if these mice were depleted of T cells and IFNγ on day 200, tumors rapidly appeared at the site of injection.

Anti-cancer responses by effector $CD8^+$ and $CD4^+$ T cells

The strongest evidence of tumor immunity in humans comes from the plentiful reports of the quantity, quality and spatial distribution of tumor-infiltrating lymphocytes (TILs) to correlate with patient outcome (reviewed in (134)). Indeed, we have previously demonstrated tumor-infiltration of T cells to correlate positively to survival in UBC (135). TILs may correlate positively or negatively to prognosis depending on the particular TIL subset. As a rule, high densities of memory T cells with a Th1 phenotype and a pronounced $CD8^+$ compartment, correlate with favorable prognosis. A description of the behavior of $CD8^+$ and $CD4^+$ T effs and their impact on tumor immunity, here envisioned to occur during the elimination and equilibrium phases, follows below.

Given the wealth of preclinical and clinical data of both these cell types to promote tumor immunity, the underlying cellular mechanism for how $CD8^+$ and $CD4^+$ T cells eliminate tumors, is poorer than one would expect. The anti-tumor properties of activated $CD4^+$ Teff cells are limited by MHC restriction because most tumors do not express MHC class II, preventing $CD4^+$ T cells to recognize them as target cells. In naïve $CD8^+$ T cells, due to most tumor antigens being exogenous, MHC restriction gives another limitation, namely a dependency on cross-priming, a process in which APCs present exogenous antigens on MHC I molecules, otherwise only presenting endogenous antigens.

Once activated, by virtue of their ability to recognize and directly suppress, or even kill, their target cells, $CD8^+$ cytotoxic T cells are considered a potent force in the anti-tumor immune response. When a $CD8^+$ cytotoxic T cells recognizes a tumor antigen-MHC I complex expressed on the tumor cell, it forms an immunological synapse (IS). The IS is stabilized by the binding of lymphocyte function-associated antigen-1 (LFA-1), expressed on the CTL, to the ICAM-1, expressed on the surface of the target cell. This enables the $CD8^+$ cytotoxic T cell to employ a key mechanism by which they kill the target cell, namely the release of perforin and granzymes, where granzyme B is a prominent sort. These cytotoxic molecules are stored in lytic granules. Once the IS is formed, these granules moves along with microtubule organizing centers, which

redistribute themselves towards the IS. A sequence of events follows after which the cytotoxic constituents are degranulated from the $CD8^+$ T cells. Perforin molecules then bind to the surface of the target cell to form pores through which granzymes diffuse into, reaches the cytosol and induces apoptosis.

The critical role of this pathway in tumor immunity is underscored by findings of mutations in the *PRF1* gene to increase the susceptibility to develop various human cancers (136). In congruency, we have previously reported on tumor-induced downregulation of perforin in TME resident $CD8^+$ T cells in patients with UBC (137).

The potential antitumor protective role of $CD4^+$ T effs, has been less obvious compared to $CD8^+$ T cells. Perhaps because most tumors do not express MHC class II. Nevertheless, the exceptions are notable, as tumoral HLA-DR expression correlates to a favorable prognosis (138), and emerging evidence suggest a substantial contribution of certain $CD4^+$ T cell subsets to the anti-tumor immune response. The Th1 oriented subset, which origin from naïve $CD4^+$ T cells previously subjected to priming in the context of *e.g.* IL-12 and induction of the transcription factor T-bet, is the given example. Typically, these cells secrete their signature cytokines IFN-γ and TNFα in response to intracellular pathogens, which activates macrophages and enhances $CD8^+$ T cell responses.

These characteristic Th1 effects also have relevance in the cancer setting. Remarkably, IFN-γ and TNFα may drive cancer cells into a state of senescence and permanent growth arrest in mice, mediated through the $p16^{Ink4a}$/Rb pathway (139). Moreover, these cytokines may enhance tumor immunity by upregulating the expression of MHC I and MHC II molecules on both tumor cells and tumor-resident APCs. Also, Th1 cytokines contribute to the maturation and licensing of DCs (140). Moreover, $CD4^+$ Th1 cells help in recruiting innate cells including natural killer (NK) cells, type I macrophages (addressed below) and eosinophils to tumor sites, where they can act in concert toward tumor elimination (141).

A central feature of $CD4^+$ T cells is their ability to recruit $CD8^+$ T cells and both induce and sustain functional memory $CD8^+$ T cell responses. Indeed, $CD4^+$ T cells have been demonstrated to promote tumor eradication through this feature, mediated partly by their ability to secrete IL-2 (142). Consistently, there are successful examples of the clinical use of $CD4^+$ Teff cells in cancer immunotherapies, including ACT, as we have previously reported for in UBC (143) and others for melanoma (144). Taken together, $CD4^+$ Teff cells, particularly the Th1 subset, reinforce multiple distinct aspects of the anti-tumor immune response, suggesting an underestimated potential of this cell compartment for the use in cancer immunotherapies.

Because of a highly unstable genome, of probabilistic reasons, the equilibrium ensues to an *escape* phase, which unfortunately is the dominating phase at the time of diagnosis. At this stage, an immunosuppressive TME has been established which empowers the cancer cells to rapid growth.

1.3.2.2 Tumor Immune escape

The capacity of a tumor cell to escape immune destruction is considered a "hallmark of cancer"(6) and occur through mechanisms involving tumor cell-intrinsic factors and changes in the microenvironment, where diverse cell types, including immune cells, reside. These mechanisms can be divided into three major categories: i) lack of recognition, ii) lack of susceptibility and iii) induction of immune suppression, discussed below, with focus on the latter category.

Lack of recognition & susceptibility

Multiple means of the cancer cell to disrupt its antigen presentation have been identified. Mutations in the ß2-microglobulin genes are common, resulting in defect, and in case of deleterious mutations, a complete loss of MHC I expression (145). Other examples are mutations or downregulations in the Transporter associated with Antigen Processing protein (TAP) 1 and TAP 2, preventing normal processing and presentation of tumor antigens (146).

Given that antigenicity is a prerequisite for a tumor to be immunogenic, it would be reasonable to presume that disruption of antigen presentation represent a predominant escape mechanism. However, tumors lacking T cell infiltration appear to possess similar levels of antigens as their highly T cell infiltrated counterparts (147, 148). Similarly, within the same patient, metastatic tumor lesions may differ drastically with regards to T cell infiltration (149) and even between sublocations of a single tumor lesion, the T cell contexture may differ (as described in the appended "paper I" (150)). Together, these observations suggest that dominant suppressive mechanisms, through molecular alterations in tumor cell-intrinsic oncogenic pathways amount to a significant category of immune escape mechanisms. Indeed, several such pathways with a known capability to mediate carcinogenesis or cancer progression from within the tumor cells themselves have been found to also mediate immune evasion (reviewed in (151)).

Resistance to cell death is another hallmark of the cancer cell (6) with apoptotic pathways frequently being turned-off or counter regulated, including downregulation of the cell death receptor Fas (6). Tumors may also directly counteract cytotoxicity, exemplified by a recent finding of actin-remodeling in breast cancer cells to reduce susceptibility to NK cell-mediated killing by Granzyme B secretion (152).

Induction of immune suppression – by inhibitory molecules

Tumors may operate on several levels to induce immune suppression. On the molecular level, there is a myriad of suppressive molecules synthesized by the tumor itself including i) IDO, which immunosuppressive effects are mediated by Tryptophan degradation, (as discussed above in the context of Treg effector mechanisms) (153), ii) suppressive cytokines like TGF-β (reviewed in (154)) and iii) suppressive chemokines. An example of the latter is tumoral secretion of the chemokine Cxcl1 which results in the suppression of T cell trafficking into the tumor as demonstrated in a recent study (155). The elevated tumor expression of Cxcl1 was found to be due to an epigenetically altered and more accessible promoter region in these cancer cells. Such

alterations were not observed in corresponding tumors but with a pronounced T cell infiltration (155). Notably, this was true even when comparing two tumors with differential T cell abundance but carried by the same mouse, emphasizing this mechanism as a tumor- rather than a host factor.

Induction of immune suppression – by hijacking immune checkpoints

Another level of induced immune suppression is the hijacking of immune checkpoints. Within 24 hours of activation, T cells express Programmed cell Death protein-1 (PD-1), a transmembrane inhibitory receptor (156). The binding of its dominant ligand, PD-L1, and the subsequent activation of the PD-1 intrinsic pathway down-modulates Teff cell effector functions through dephosphorylation of signaling molecules downstream of the TCR (156). In addition, continuous PD-1 signaling in T cells ultimately induces an epigenetic program of T cell exhaustion (157). Since PD-L1 is broadly upregulated in tissue cells at sites of inflammation, the negative feedback provided by the PD-1/PD-L1 axis, is considered a key immune checkpoint, protecting against autoimmunity and tissue damage, similarly to the CTLA-4/CD80/86 axis (156).

The tumoral hijacking of the PD-1/PD-L1 axis, refers to the altered expression of PD-L1 observed in numerous cancer types, which inhibits the antitumor T cell response (156, 158). The host-protective effect of the checkpoint instead becomes tumor promoting. In agreement, therapeutic PD-1/PD-L1 blockage has proven an efficient immunotherapy in several cancers, similarly to the blockade by Ipilumimab on CTLA-4 (4). Based on the differential kinetics of CTLA-4 and PD-1 expression, CTLA-4 blockage is considered to act at the priming step, mainly in lymph nodes, while PD-1 blockage is thought to release the negative regulation of already antigen-primed T cells within the TME (156).

The response rates for PD-1/PD-L1 blockage varies greatly (15-90 %) across cancer types (4). For urothelial cancers this figure is 15-25 % (15), thus finding biomarkers for response is a research priority. PD-L1 expression alone has not proven reliable for this assignment. The current understanding is that a preexisting antitumor T cell response is necessary but not always sufficient for a significant therapeutic response (4). Because tumoral PD-L1 is most frequently expressed reactively upon a T cell attack, it might be viewed as a surrogate for an evoked T cell response (4, 159). In this setting, co-localization of $CD8^+$ T cells, PD-L1 and PD-1, at the invasive front (IF) of the tumor is associated with response to PD-1 blockade (159).

The expression of PD-1 by Teff cells have been suggested to denote exhaustion. This notion is based on the observation that when Teff cells are repetitively stimulated by antigen (as with cancer), the level of PD-1 expression remains high and the continuous PD-1 signaling in these Teff cells eventually induces an epigenetic scheme of T cell exhaustion (157). However, it is important to keep in mind that this does not mean that PD-1 expression *per se* stands for exhaustion. In fact, since PD-1 expression can be due to recent or ongoing T cell activation, in certain context, its expression may rather mark the cells with the best capacity to become activated and to evoke an anti-tumor response. Furthermore, exhausted $CD8^+$ T cells in the setting of

chronic infection, are capable of re-expansion and to exert protection upon transfer to naïve mice upon re-challenge with acute infection (160). These notions call for caution when interpreting data on PD-1 expression.

Induction of immune suppression – by recruiting Tregs

A third level of induced immune suppression is the exploitation of suppressive cells, including Tregs, by the tumor. This passage will focus on the behavior of Tregs in the TME.

An increased frequency of Tregs have been observed in peripheral blood, tumor tissue and tumor draining lymph nodes in many human cancers (161). Such Treg accumulation is often associated with poor prognosis (162). Experimentally, the involvement of Tregs in tumor immunity was first demonstrated by Shimizu *et al* who found that CD25 depleted mice exhibited tumor rejection and retardation of tumor growth (163). Since then, the negative impact of Tregs in the cancer setting has been well corroborated, both epidemiologically and mechanistically, albeit experimental data is mostly derived from mouse studies.

Given the relative autoreactivity of Treg TCRs, Tregs have been proposed to exhibit an enhanced ability to recognize not only self-antigens but also tumor antigens, since these often are "quasi-self" (162). This notion, together with the observation that Tregs in general are in a more antigen-primed state (as illustrated by higher expression levels of T-cell accessory molecules such as LFA-1), suggests that Tregs are more prone to be activated by a given tumor antigen compared to Teff cells (116, 162). Indeed, Treg clones established from human melanoma recognize TAAs such as gp100, TRP1, and survivin (164). It is not clear if these antigens are exclusively recognized by Tregs or could also be recognized by Teff cells.

Considering that the Treg configuration favors immunosuppression over an anti-tumor attack by Teff cells, it is easy to conceive that tumors apply active means to recruit and exploit Treg functions in its microenvironment. For instance, tumor cells and TME-resident macrophages produce the chemokine CCL22, which mediates trafficking of CCR4 expressing effector Tregs (165). Moreover, according to an evolving model in the field of immunometabolism, signals from the tumor induce a circuitry of glycolysis, Fatty acid synthesis, and oxidation that confers a preferential proliferative advantage to Tregs and thus causes their intra-tumoral accumulation (166). In addition, it has been suggested that a subclass of DCs in the TME may, in a TGF-β-dependent manner, stimulate clonal expansion of Tregs (167).

A variant of Treg plasticity, or tissue-specific adaptations (described above), is observed in tumor-infiltrated Tregs as they display a unique transcriptional machinery and other properties not reported for in Tregs outside of the TME (168, 169). For instance, the suppressive molecule Nrp1 is upregulated and required for the maintenance of intratumoral Treg stability and function but is dispensable for peripheral immune tolerance in mice (170). Also, tumor-infiltrating Tregs (TI-Tregs) display a high tendency to release perforin and granzyme which may induce apoptosis in

NK- and cytotoxic $CD8^+$ T cells (171). Recently, the chemokine receptor CCR8 was found to be upregulated in human breast-cancer-resident Tregs which correlated negatively to survival (135).

It should be noted, that, in contrast to tissue-specific adaptations in the non-cancer setting, the changes observed in TI-Tregs appears to result from an exploitation of this subpopulation by the tumor rather than from an adequate adaptation into a niche with improved capacity. Consistent with this concept, TI-Tregs have been found not only to abrogate tumor immunity but also to employ mechanisms that are directly tumor-promoting. For instance, in a mouse model of breast cancer, Tregs expressed RANKL which promoted metastasis of RANK-expressing cancer cells (172). Also, Tregs have been suggested to stimulate angiogenesis in the TME by secreting VEGFA (173).

The suppressive functions of Tregs normally utilized to protect against autoimmunity have been demonstrated to be employed by Tregs also to suppress tumor immunity. For instance, TI-Tregs exhibit elevated expression of CTLA-4 which mediates several immunosuppressive effects, including interference with DC function (174, 175). In fact, data indicates that the major suppressive function of CTLA-4 in the TME is mediated by Treg-bound rather than Teff cell-bound CTLA-4. These data derive from efforts to clarify the mechanistic basis of how Ipilumimab augments the anti-tumor immune response. In this endeavor, numerous mouse- but also patient analyses, have demonstrated that the main effect of these antibodies is mediated by a preferential depletion of CTLA-4-expressing Tregs through antibody dependent cellular cytotoxicity (95, 96). Initially, blockage of CTLA-4 on Teff cells and thereby unplugging an immune checkpoint was considered the main mechanism, hence the term "checkpoint blockade". Perhaps, this group of drugs will need a new nickname.

Increased levels of extracellular adenosine is considered an immunosuppressive factor in several cancers (176, 177). These elevated adenosine levels have partly been attributed to Tregs as the degradation of extracellular ATP to adenosine is a suppressive mechanism typically employed by these cells. The mechanism is mediated by the ectonucleotidases CD39 and CD73, both highly expressed on TI-Tregs in, for instance, human head and neck cancers (121, 178). Yet, a prerequisite for the generation of extracellular adenosine is the availability of extracellular ATP, which is readily secreted by both tumor- and non-tumor cells during hypoxic- and other stress conditions (reviewed in (179)). The TME is often subverted by such taxing conditions, creating a synergistic Treg-tumor loop which fuels the orchestration of an immunosuppressive environment. A central aspect of this synergism may be the differential metabolic set point of Teff cells and Tregs. The former suggested to be chiefly glycolytic whereas the latter appear to rely also on fatty acid oxidation and thus confers Tregs in the hypoxic TME with both a functional- and proliferative advantage (166).

The above reviewed data gives a one-sided picture of Tregs as suppressors of anti-tumor immunity which could beneficially be targeted to improve clinical outcome in cancer. Intriguingly, a positive correlation of TI-Tregs to prognosis in several cancers, including UBC,

have been reported (135, 180-182). For long, these seemingly paradoxical data have puzzled investigators in the Treg field.

We and others have made attempts to explain these contradictory reports. A first factor to consider, is the large variations in how the Treg identity was determined across studies (due to a lack of consensus in this regard). For instance, many investigators have determined Treg identity primarily based on FOXP3 expression. However, its transient expression also in Teff cells (72) renders these studies prone to methodological artifacts, as convincingly demonstrated in patients with colorectal cancer (CRC) (183). In this study, by using a function-based scheme to classify tumor-infiltrating FOXP3$^+$ cells, it was demonstrated that a fraction of the CRCs were heavily infiltrated with FOXP3$^+$ non-Tregs which secreted inflammatory cytokines. Within the patient group bearing these FOXP3$^+$ non-Tregs, those patients with non-Tregs displaying a high FOXP3 expression had a better prognosis than those patients with low FOXP3 expression in these cells. Thus, FOXP3 expression is non-Treg specific and FOXP3$^+$ T cells is a heterogonous population which can have opposite correlations to prognosis within a single malignancy depending on *the degree* of expression. Most likely, this differential prognostic effect reflects a difference in the contribution to tumor immunity by the two FOXP3$^+$ non-Treg subsets.

While heterogeneity within FOXP3$^+$ T cells might explain the inconsistent data for CRC, this model might not be applicable to other cancers where tumor-infiltration by Tregs correlates positively to survival. These cancers include UBC, as we have previously reported (135), breast, and head and neck cancers (180-182). Because all these cancers are closely linked to chronic inflammation, which reportedly drive rather than protect against tumor progression, we and others have suggested the positive impact of Tregs in these settings, to be attributed to suppression of such unresolved, tumor promoting, inflammation (150, 184).

1.3.3 Tumor promoting inflammation

The link between chronic inflammation and cancer was acknowledged already in the 19th century by Virchow (185). Thereby, this notion of cancer as “a wound that does not heal”, by far preceded the broad acceptance of immunosurveillance as a concept. It is now well substantiated that leukocytes infiltrating a tumor may exert both tumor-*suppressive* and tumor-*promoting* effects. The latter effects are widely implied, in fact, inflammation is considered a principal mechanism by which the hallmarks of a cancer cell are developed (6). Adding to the complexity, tumor-promoting inflammation (TPI) is reciprocal, as inflammation may promote cancer (outside-in) but cancer may also promote inflammation (inside-out).

1.3.3.1 Outside-in

Unlike most healthy tissue, cancer involves continuous cell renewal and proliferation. The several ways by which inflammatory molecules may support such proliferation converge at the level of the transcription factors STAT3 and NF-κB (186). For instance, IL-6 derived from myeloid cells activated STAT3 which increased pre-malignant cell proliferation and inhibited apoptosis in a

mouse model of colitis-associated CRC (187). In addition, activation of STAT3 by TNF, IL-6 and IL-1 caused an upregulated production and activity of matrix metalloproteinases (MMPs) in breast cancer and melanoma cells (188, 189). MMPs are critical for breaching extracellular matrix (ECM) during the epithelial–mesenchymal transition, a central event in the metastatic process (190).

Inflammation may also contribute to carcinogenesis by causing genomic instability and mutagenesis. Mechanistically, inflammatory mediators including TNF, IL‑1β and prostaglandin E2 may interfere with the DNA repair machinery (191). Moreover, reactive oxygen species derived from macrophages and neutrophils, or induced intracellularly in pre-malignant cells by inflammatory cytokines, may directly cause mutations (186, 192).

1.3.3.2 Inside-out

Cancer cells may impact immune cells in numerous ways to favor their own progression, as described in the previous section where cancer immunoediting was discussed. The inside-out model of TPI is an expansion of that concept, where the modulation of the inflammatory response by cancer cells is considered as a major dimension of the overall crosstalk between the tumor and its microenvironment, including stroma and vasculature.

For instance, "cancer associated fibroblasts" (CAFs), are former fibroblasts subverted by the tumor and abundant in the TME with the capacity to promote macrophage recruitment, angiogenesis and tumor progression through NF-kB signaling, in skin cancer models (193). CAFs have also been reported to induce MMP activity in head and neck cancer patients (194).

The tendency of a tumor to tilt the inflammation to its favor is further exemplified by the observation that the anti-tumor T cell response may be skewed by cancer cells from a tumor-suppressive Th1 response, to a tumor-promoting Th2 response (186). In turn, The Th2 $CD4^+$ T cells, by secretion of IL-4, may skew macrophages towards a type 2 differentiation state, a cell subset which potently promotes tumor growth (discussed below). Moreover, tumor cells frequently secrete TGF-β, a cytokine with several suppressive effects, including inhibition of granzymes and perforin expression in $CD8^+$ T cells (195). Lastly, tumor-derived inflammatory mediators, for example prostaglandins, may induce myeloid-derived suppressor cells (MDSCs) which may inhibit an anti-tumor T cell attack by TGF-β secretion or by depleting amino acids central for T cell function (196). Taken together, by secretion of soluble mediators, the tumor creates a distorted network of a wide range of cell types which promotes carcinogenesis and eventually metastatic spread.

1.3.3.3 Tumor associated macrophages

The macrophage compartment is another abundant myeloid cell type in the TME, their accumulation is associated with poor prognosis and treatment failure (197). These tumor-

associated macrophages (TAMs) can contribute to immune evasion and play multiple roles in promoting metastatic spread.

Macrophages have been categorized into two types: the M1 type, which are "classically activated", *i.e.* by IFNγ, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and PRR agonists, express elevated levels of IL-12 and low levels of IL-10, and can be tumoricidal (198). Tumors may corrupt the M1 type and polarize them to M2 macrophages, which happens through stimulation with IL-4, IL-13, IL-10 and M-CSF (198). These molecules may be provided, for instance, directly by cancer cells secreting TNFα (199), or indirectly, by skewing Th1 to Th2 $CD4^+$ T cells, which secrete IL-4 (200). In established tumors, TAMs generally have an M2-like phenotype with low IL-12 expression and high IL-10 expression (197). TAMs facilitate tumor growth and progression by several modules including immunosuppressive effects such as poor antigen presentation because of downregulation of both MHC class I and II molecules (201). Moreover, TAMs secrete CCL22 which attracts CCR4-expressing Tregs to the TME (165), which theoretically creates a positive feedback-loop since Tregs produce IL-10, necessary for M2 polarization.

There is convincing evidence, including intravital imaging, of macrophages to be indispensable for tumor cell migration and invasion (202). Several mechanisms employed by TAMs for this process have been defined: the overexpression and increased activity of MMPs is a key such mechanism (200, 203, 204). MMPs are pivotal for the degradation of normal tissue ECM during the course of metastasis (205). Although stroma and cancer cells also produce these proteinases, TAMs appears to be the predominant source in several cancers including UBC (206). In UBC, high MMP2 expression correlates strongly to decreased survival and the activated form of MMP2 is higher in invasive than in non-invasive bladder tumors (207). Intriguingly, high levels of CCL2, a chemokine which potently stimulates TAM accumulation, correlates with high tumor stage and grade in UBC (208).

Another prometastatic mechanism of TAMs is their secretion of epidermal growth factor, which activates the corresponding receptor on cancer cells resulting in an increased invadopodium formation and subsequent improved motility and invasive potential (209). Lastly, TAMs were demonstrated to stimulate tumor growth through inducing angiogenesis by secreting VEGF in a mouse model of breast cancer (210).

Leukocytes are not evenly distributed within a tumor. This phenomenon has a direct clinical relevance because the density of a given immune cell subset will correlate differentially to prognosis depending on where in the tumor the subset is located (22). The central part (CP) and the IF exhibit particularly distinct immune contextures where the former contains only limited types of immune cells whereas the latter is in general highly infiltrated by a plethora of immune cells, including macrophages (22). At this location, MMPs modulate the ECM, contribute to angiogenesis and cell mobility (211). In agreement, the expression of certain MMPs is most

pronounced at this site in both UBC and gastric cancer, which correlates strongly to higher tumor grade (206, 212).

1.3.4 The sentinel node, a key component of the tumor microenvironment

As discussed previously, immune responses are in general initiated in the lymph node. This notion combined with the hypothesis that immunosuppression would be less pronounced in the tumor-draining lymph node, is the foundation for our groups' focus on this anatomical location, because it represents an alternative source for ACT (143). However, because tumors may induce neolymphangiogenesis, the lymphatic drainage system is frequently deranged which complicates the identification of this node(s). This has been solved by using a method in which a radioactive tracer is injected adjacent and around the tumor (**Figure 2**), which enables to identify the first node of a primary tumor on the direct lymphatic drainage system, referred to as the sentinel node (213). This method has long been established for melanoma and breast cancer and more recently also been introduced for UBC (214). The SN offers a rare opportunity to study the direct communication between the tumor-and the immune system, with access to relevant control nodes, namely non-tumor draining non sentinel nodes (nSNs). In such interrogations, a high degree of immunosuppressive elements, including Treg accumulation, have been reported in the SN relative to nSNs (reviewed in (215)). To improve the efficacy of SN-based ACT, increased knowledge of the dominating escape mechanisms in this compartment is needed.

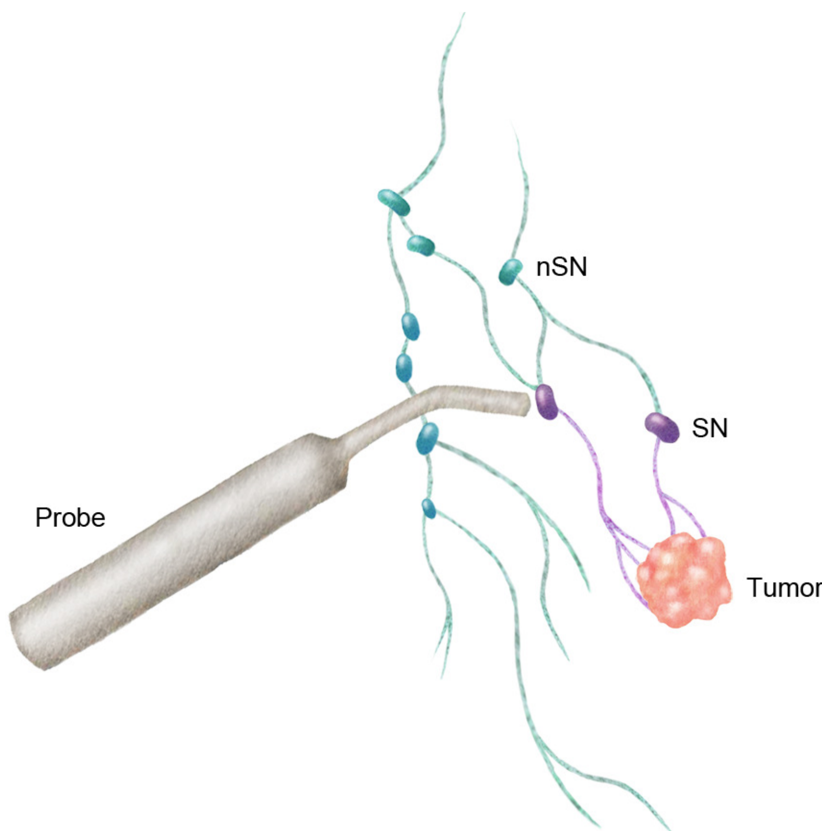

Figure 2. *Sentinel node detection.* Schematic illustration of intraoperative sentinel node detection in urinary bladder cancer. Following peritumoral injection of a radioactive tracer, the first lymph nodes (purple) to drain the tumor are detected by using a probe. Figure courtesy of Malin Winerdal.

1.3.5 Effects of chemotherapy on tumor immunity

Widespread evidence exists that the efficacy of conventional chemotherapy does not only stem from the direct cytostatic/cytotoxic properties, but also relies on positive immunomodulatory effects (reviewed in (22, 216)). The origin of these immune effects may be “On target”, meaning that they result from drug actions on the cancer cells. Such activity boosts the immunogenicity of the tumor through either increasing its antigenicity or its adjuvanticity, or both thereof (216). Alternatively, the immunostimulatory effects of chemotherapeutics may be "off-target," which refers to effects originating from drug actions on the immune system. Off-target effects may be *direct*, i.e. activation of immune effector cells or *indirect*, referring to inhibitory effects on immunosuppressive cells (216). Here follows a brief, T cell-centric, discussion of these immunogenic “side effects” of chemotherapies and how they may be used in conjunction with cancer immunotherapies.

1.3.5.1 On-target - Increased immunogenicity

The triggering of cancer cells to undergo immunogenic cell death (ICD) is the core mechanism by which chemotherapeutics may increase the immunogenicity of a tumor. ICD can involve the activation of both apoptotic and necroptotic (a programmed form of necrosis) pathways resulting in cell death (217). ICD-associated pathways may yield increased antigenicity, for example through exposure of the endoplasmatic reticulum (ER) protein calreticullin on the tumor cell surface. Calreticullin act as a major “eat me” signal to DCs, subsequently supporting the engulfment and transfer of tumor antigens to these APCs (218).

The ICD process is also accompanied by the release of DAMPs such as annexin A1, HMGB1 and ATP, with the former acting on formyl peptide receptor-1 (219), and the latter two binds to their respective PRR; TLR4 and the purinergic receptor P2RX7. These DAMPs potently reinforce the adaptive anti-tumor immune response, exemplified with their capacity to activate the NLRP3 inflammasome, which is particularly versatile, leading to IL-1b secretion and activation of IFNγ-secreting $CD8^+$ T cells. ICD has also been reported to increase tumor antigen cross-presentation and cross-priming of host tumor-specific $CD8^+$ T cells (220).

These ICD pathways have been delineated in mouse models but are supported by human data. Phosphorylation of eukaryotic translation initiation factor 2 subunit alpha (eIF2α) during ER-stress is a marker proposed to be pathognomonic for ICD (221). Low levels of eIF2α phosphorylation in human breast cancer tissue following chemotherapy correlate with a low $CD8^+$ T cell to $FOXP3^+$ cell ratio and with poor responses to NAC (222). Stressing the possible clinical relevance of these pathways, loss-of function polymorphisms in TLR4 and P2RX7 are associated with an increased risk of breast cancer relapse after adjuvant anthracycline-based chemotherapy (128, 223). Similar findings have been reported for TLR4 in head and neck cancer (224).

Non-ICD dependent mechanism favoring antigenicity have also been described, where restored MHC class I expression appears as a main mechanism in both mice and men (225).

1.3.5.2 *Off-target – direct and indirect immunostimulation*

A prerequisite for the "On-target effects" to be of any use, is the presence of immune effector cells with a capacity to be (re)activated and ultimately implement an anti-tumor immune response. We previously reported on a direct positive effect on immune cells upon chemotherapy, namely of cisplatin to augment the immune stimulatory activity of DC differentiating human monocytes, resulting in an increased T cell proliferation (226) and of Doxorubicin to enhance the antigen presenting capacity of B cells (appended paper II). Correspondingly, cyclophosphamide shifts the $CD4^+$ Th phenotype from type 2 to type 1, and promotes durable $CD8^+$ T-cell memory responses capable of eradicating tumors upon rechallenge, in mouse tumor models (227, 228).

There is plenty evidence of chemotherapy to induce indirect immunostimulation, i.e. through inhibition of immunosuppressive circuits, but mostly derived from rodent tumor models. For instance, 5-fluorouracil, cyclophosphamide, gemcitabine, oxaliplatin, paclitaxel and docetaxel have all been demonstrated to deplete circulating or TI-Tregs and/or circulating MDSCs (reviewed in (225)). These findings are supported by patient data comprising various malignancies including non–small cell lung carcinoma, where a reduction of Treg frequency in peripheral blood upon gemcitabine plus cisplatin chemotherapy was reported (229). Moreover, cyclophosphamide, when administered in metronomic doses, selectively depletes Tregs in late stage cancer patients (230). In consistency, breast cancer patients treated with NAC displayed an increased $CD8^+$ T cell to $FOXP3^+$ T cell ratio which was associated with improved recurrence free survival and outperformed conventional predictive factors (231) or predicted NAC response (222).

The fact that chemotherapy-induced alterations of immunosuppressive cells is a relative common finding, together with the tendency of these drugs to increase the Teff cell to Treg ratio, suggest that immunosuppressive cells, at least in certain contexts, are more sensitive to cytotoxic agents compared to Teff cells and thus are selectively depleted. However, given the opposite roles in tumor immunity implied for Tregs, it is reasonable that a Treg-inhibiting effect of chemotherapeutics is not always desirable.

1.3.5.3 *Combining chemotherapy and immunotherapy*

The major effects of chemotherapy on tumor immunity have started to be taken in to account in the effort of developing new immunotherapies but also in the pursuit to develop integrated treatment protocols for already existing chemo- and immunotherapies, striving to avoid antagonistic effects and, in the best of worlds, achieving synergism. One example is an ongoing phase III trial of the TG4010 cancer vaccine containing viral vectors encoding for IL-2 and the tumor antigen mucin-1, and first-line gemcitabine- and cisplatin-containing chemotherapy for advanced non-small-cell lung cancer. Data from the phase 2b part indicate that the combinatory use of TG4010 and chemotherapy improves progression-free survival compared to placebo plus chemotherapy (232).

The limited response rate of checkpoint blockers have sparked an interest to integrate the use of these drugs with immunogenic chemotherapy. A recent study using a novel approach to block PD-L1, namely by using an engineered, locally and transiently expressed "PD-L1 trap" was tested together with oxaliplatin in the setting of murine CRC (233). Indeed, synergistic effects were achieved and with a tendency of fewer adverse effect compared to when using anti-PD-L1 mAb. Moreover, chemotherapy may reportedly sensitize tumors to checkpoint blockade in mouse lung adenocarcinoma because cyclophosphamide/oxaliplatin induces TLR4 signaling which results in tumor influx of $CD8^+$ T cells and, upon checkpoint inhibition, durable tumor control (234). The clinical translation of these findings is emerging. For example, there is an ongoing phase II study where both PD1/PDL1- and CTLA-4 blockade is tested in combination with standard chemotherapy in patients with metastasized CRC, preliminary results are yet to be published (235).

1.3.6 Antigen presenting B cells in cancer

The antigen presenting function of B cells is usually considered, if considered at all, in the context of the $CD4^+$ Th cell-B cell interaction, necessary for isotype switching. However, B cells are professional APCs along with DCs and macrophages and, in certain settings, they have the ability to prime naïve T cells and initiate T cell responses. The following passages will briefly describe this topic and also touch upon the relevance of B cells in the cancer setting.

1.3.6.1 B cells as specialized APCs

Upon capturing an antigen, APCs may internalize it by three means: phagocytosis, fluid-phase pinocytosis, and receptor-mediated endocytosis. In B cells, the last process is favored and carried out by the BCR (reviewed in (236)). Because the BCR has high affinity for a given antigen, B cells can concentrate minute quantities of their specific antigen and present it efficiently. This notion is supported by demonstrations of a direct relationship between BCR affinity and antigen presentation, where B cells may present antigens of concentrations as low as 0.05 nM (237, 238). In contrast, 5000 times higher concentrations were required if the antigen uptake occurred through the fluid phase pinocytosis route.

The binding of the BCR to its antigen induces its internalization through the endocytic pathway (**Figure 3, upper part**) but it also initiates cell intrinsic signaling necessary for mobilizing the antigen processing machinery (239). This coupled mode of action, ultimately favors antigen presentation initiated by the BCR over other means of antigen capturing.

The antigen-containing endosomes acidify as they traffic inside the cell and fuse with lysosomes. In these fused compartments, MHC II molecules are abundant, and here peptide–MHC II complex formation take place. The acidification induces proteolytic degradation of the antigens into peptide fragments. In the vesicular compartments, processed peptide fragments are then loaded on the MHC class II pockets upon which the peptide–MHC II complexes traffic to the plasma membrane. During this process, the high affinity of the BCR to its epitope again has

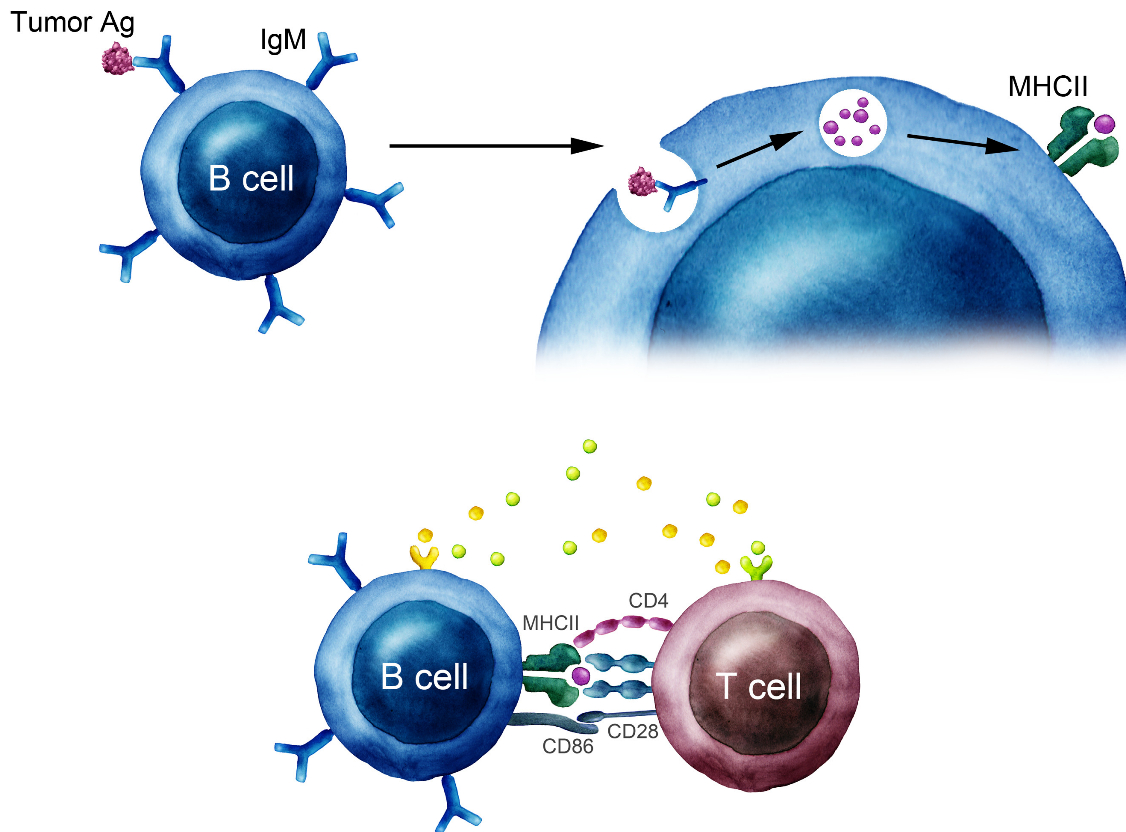

Figure 3. Schematic overview of antigen capture and presentation by a B cell. *Upper part:* Antigen is captured by the B cell receptor and internalized via receptor-mediated endocytosis, processed and loaded on to an MHC class II molecule, forming the MHC class II-peptide complex which traffics to the plasma membrane. *Lower part:* Three signals contribute to complete activation of both B and T cells; 1.) MHC class II-peptide interaction with the T cell receptor complex. 2.) Binding of costimulatory molecule such as CD86 to its CD28 receptor. 3.) Cytokine secretion (green and yellow dots). Figure courtesy of Malin Winerdal.

implications, as this part of the antigen in general is not released, thus not processed and presented (240). In this way, the BCR configuration dictates, or shapes, the immune response.

In addition to expressing the peptide–MHC II complex, other tools such as the upregulation of costimulatory molecules are necessary to activate naïve T cells. This, in turn, requires the binding of CD40 (expressed on the B cell) to its ligand, CD40-L, expressed by *activated* CD4$^+$ T cells. Due to this restriction, the contribution of B cells to prime *naïve* CD4$^+$ T cells have been questioned. However, there is evidence suggesting that B cells are able to elicit CD40-L expression on T cells during their own activation. For instance, when transferring antigen-specific B and CD4$^+$ T cells into histoincompatible lymphocyte-deficient mice, presentation of the antigen by B cells resulted in the priming of naïve CD4$^+$ T cells in a CD40-L dependent manner (241). In a proposed model (236), a strong or durable BCR signal induces a bidirectional cross-talk process between the B cell and naïve CD4$^+$ T cell ultimately resulting in the ability of the B cell to provide all three necessary signals to activate a T cell with the corresponding antigen-specificity (**Figure 3, lower part**); 1) the expression of MHC class II-antigen complex

to which the T cell receptor binds, 2) the expression of costimulatory molecules such as CD86 and CD40 which binds to the CD28 T cell surface receptor and T cell-bound CD40-L respectively and 3) the secretion of cytokines, such as IL-2, IFN-γ or IL-4, which bind to their respective receptors.

1.3.6.2 Conventional and regulatory B cells in cancer

In analogy with the conflicting data on Tregs, some studies report B cells to promote tumor immunity whereas others point on a suppressive, negative role. An example of the former is our previous demonstration of a clonal expansion of B cells in tumor-associated lymph nodes as well as in the tumor of bladder cancer patients (242). In line with these data, TIL-Bs in non–small cell lung cancer patients were recently reported to present antigens to TIL-$CD4^+$ T cells and to affect the phenotype of these $CD4^+$ T cells, as demonstrated in *ex vivo* co-culture assays (243). TIL-Bs appears to preferentially be organized together with $CD4^+$ T cells in tumor-adjacent lymphoid islets, called tertiary lymphoid structures (244). These structures correlate positively with survival in non-small-cell lung carcinoma. Additionally, mouse studies have demonstrated TIL-Bs to produce tumor-antigen specific antibodies (245). Combined, these data indicate a protective role of TIL-Bs also in this malignancy.

On the other side of the coin, a recently defined immune suppressive B cell subpopulation dubbed Regulatory B cells (Bregs) have been suggested to oppose tumor immunity (reviewed in (246)). These cells not only share suppressive effector mechanisms with Tregs, namely the secretion of IL-10, TGF-β and IL-35, they have also been reported to facilitate the generation of Tregs (247). Although the role in humans is not well defined, emerging data indicate the negative role described in the murine setting to be translatable. Breg frequency was reported to correlate negatively to survival in some cancers including tongue squamous cell carcinoma (247). Mechanistically, a tumor-promoting role in hepatocellular carcinoma (HCC) have been described where Bregs were demonstrated to support HCC cell growth via the CD40/CD40-L pathway (248). Taken together, much remains to be learned of the role of B cells in cancer, and their impact on tumor immunity is likely underestimated, considering their sovereignty as APCs in contexts with limited amount of antigen.

2 AIMS OF THIS THESIS

The overarching aim of this thesis was to elucidate how lymphocytes and cancer cells sculpt each other and what effect standard chemotherapy has on this process, with the purpose to identify targets for immunotherapy.

Paper I. To clarify if the tumor-infiltrating $CD4^{+}FOXP3^{+}$ T cell population in urothelial bladder cancer patients confer a stable Treg phenotype and to disclose the role and biological impact of Tregs on this cancer, focusing on tumor-promoting inflammatory pathways.

Paper II. To elucidate the effect of chemotherapeutic drugs on human B cells with a special attention on their function as APC's.

Paper III. To elucidate the effects of neoadjuvant chemotherapy on T cell subsets in the sentinel nodes of MIBC patients with emphasis on phenotype, function and subset composition and their correlation to NAC response.

Paper IV. To explore if the proteome in SN-resident T cells is altered by the tumor and to identify key proteins in SN T cell signaling, focusing on Tregs.

3 MATERIALS AND METHODS

3.1 PATIENTS

3.1.1 Patient characteristics (Paper I-IV)

All papers encompass patients with UBC, prospectively included at one of the collaborating study centers between 2013 and 2017.

In paper I, 52 patients with suspected MIBC were initially included at the time of TURBT. Exclusion criteria were other histopathological malignancy than urothelial, benign neoplasia and/or previous BCG treatment. Six patients were excluded and, thus, 46 patients were included for downstream analysis. Of these, 28 patients had MIBC and 18 had NMIBC. The mean age at the time of diagnosis was 69 years (median 72.5 years) and the male:female ratio was 2.5:1.

In paper II and III, patients were included both at the time of TURBT and at RC. All included patients in these studies had confirmed MIBC. Paper II included 15 patients with an age range of 55-86 and a male:female ratio of 2.75:1. Five patients had been treated with a doxorubicin-containing NAC. The 40 patients included in paper III had a mean age of 69 (median 70.5 years) and a male:female ratio of 3:1. 1-4 cycles of Cisplatin-based NAC was administered to fit patients pre-RC (n=27) except for one patient who received carboplatin-gemcitabine.

In paper IV, five patients with confirmed MIBC and an age range of 58-85 were included.

3.1.2 Surgical methods, sentinel node detection and collection of specimens (Paper I-IV)

At the TURBT, peripheral blood, tumor tissue samples and cold-cup biopsies from macroscopically healthy bladder tissue were obtained. Whenever feasible, tissue from the CP and IF of the tumor were collected separately. SN detection was performed just prior to cystectomy by transurethral injection of 80 Mbq Technetium adjacent and around the tumor border. Thereafter, SNs and non SNs (nSNs) were identified both *in vivo* and *ex vivo*.

Tumor tissue and SNs were immediately immersed in ice-cold RPMI medium in the surgery room, followed by prompt transportation to the laboratory for immediate processing. The overall workflow, including the cell preparation and subsequent flow cytometry analysis, is depicted in **Figure 4**.

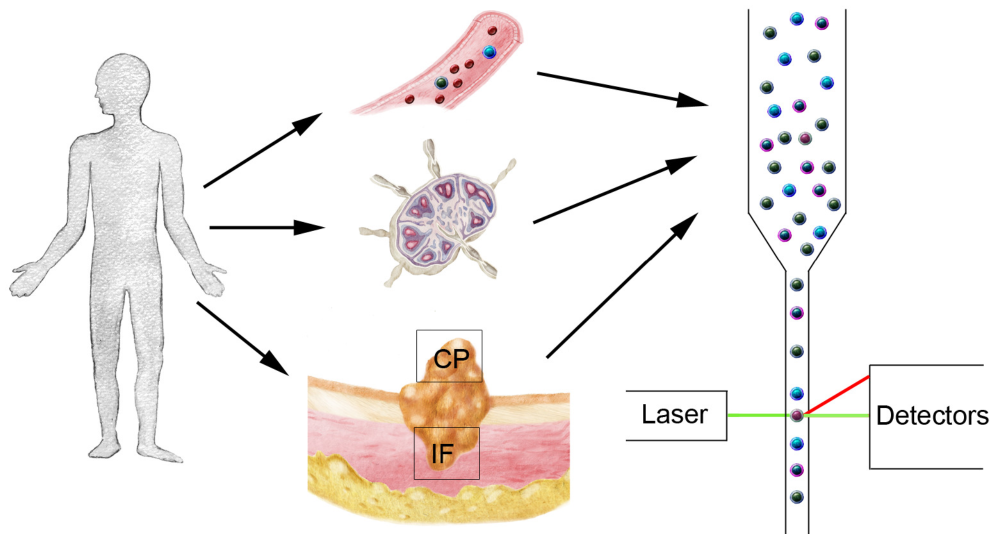

Figure 4. Overall workflow of specimen collection and flow cytometry analysis, paper I-IV. (Left panel) At the time of TURBT or cystectomy, patient specimens were collected including (middle panel, top) peripheral blood, (center) lymph nodes and (bottom) tumor tissue from distinct tumor sublocations, as indicated. From these specimens, lymphocytes were extracted and processed into single cell suspensions, which were labeled with fluorescently-conjugated antibodies and analyzed by (right panel) multicolor flow cytometry. The basic principle of this method is the passage of single cells in a stream to which a beam of laser (green) is directed. The fluorescently labelled cell components are then excited by the laser and emit light at a longer wavelength (red), which reaches a detector, enabling to quantify the amount and type of cells present in a sample. CP= Central part, IF= Invasive front. Figure courtesy of Malin Winerdal.

3.1.3 Patient follow-up (Paper I,III and IV)

In study I, Treg frequency was correlated to Overall survival (OS), calculated from the time of TURBT with a median follow-up time of 3.5 years. In study III, NAC-response was assessed by histopathological staging post-RC and categorized as: complete response, CR, (pT0N0M0), partial response; PR, (pTa/pTis/pT1-N0M0), and no response; NR, (≥pT2-N0M0). In study III and IV, six respectively one patient had nodal dissemination (pN1), as detected by the routine clinical pathological analysis. Due to the small sample size, these metastatic nodes were excluded from all of the immunological status- (paper III) or proteomic analyses (paper IV).

3.2 CELL ISOLATION, PREPARATION AND CULTURE (PAPER I-IV)

In all studies, Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood or buffy coats (paper I, II and IV) by density centrifugation gradient (Ficoll–Paque PLUS Amersham Biosciences). Lymph node leukocytes were extracted (paper III and IV) by gentle homogenization through a 40 µm cell strainer. TILs, were isolated (paper I) by cutting the tumor into small pieces (approximately 1–2 mm in diameter), put into AIM-V medium (Gibco, Life Technologies) with collagenase/hyaluronidase (Stemcell Technologies),

processed in a gentleMACS dissociator (Miltenyi Biotec) and strained through a 40µm cell strainer.

Lymphocyte populations of interest were isolated using magnetic sorting (isolation kits from either Stemcell Technologies or Miltenyi Biotec) alone or in combination with FACS-sorting. All isolated cell populations were confirmed > 90 % pure, using FACS.

In paper I, M2-like macrophages were generated from healthy donor PBMC-derived $CD14^+$ monocytes according to the protocol described by Mia *et al.* (249). Briefly, monocytes were pre-differentiated into macrophages by culture for 6 days in RPMI 1640 supplemented with 50 ng/ml of M-CSF. In parallel, autologous FACS-sorted Tregs were activated *in vitro* using Anti-Biotin beads preloaded with biotinylated CD2, CD3, and CD28 Antibodies (Miltenyi Biotec). At day 6, Tregs were added to the M2-like macrophage containing wells at different Treg:macrophage cell ratios.

In study I and IV, all cell culture steps following cell isolation were carried out in RPMI medium supplemented with 10% fetal calf serum (FCS), 1% glutamine, and 1% penicillin/streptomycin (complete RPMI). In paper II and III, AIM V supplemented only with L-glutamine was used for the adapted Flow cytometric Assay for Specific Cell mediated Immune response in Activated whole blood (FASCIA) experiments.

3.3 IMMUNOLOGICAL EVALUATION

3.3.1 Flow cytometry (Paper I-IV)

3.3.1.1 Surface and intracellular staining

Surface epitopes were stained using combinations of fluorochrome-labelled antibodies. Intracellular FOXP3 and cytokine staining was performed subsequently to surface staining, using the FOXP3 staining buffer kit from eBioscience according to the manufacturer's protocol. For a detailed account on the FACS antibodies used, please refer to the methods section of the respective papers I-IV. As there indicated, isotype- and fluorochrome matched control antibodies were used to define marker positivity. Acquisition of data was performed on a FACS LSR Fortessa II (paper I, III and IV) or on a FACS Aria I (paper II) (BD Biosciences). The flow cytometry data was analyzed using FlowJo X 10.0 7r2 (paper I, III and IV) or FACSDiva (paper II).

3.3.1.2 Sorting (Paper I, III and IV)

Flow cytometry sorting was carried out to isolate $CD4^+$ Teff cells and Tregs for T cell function experiments (paper I and IV), epigenetic- (paper I) and proteomic analysis (paper IV). Also, in paper III, $CD8^+$ T cells were FACS-sorted for epigenetic analysis. All sorting was carried out on a FACS Aria (BD Biosciences). Post sort purity was confirmed at > 90%, using flow cytometry.

3.3.1.3 *Cytokine analyses (paper I, II and IV)*

In paper I, II and IV, isolated lymphocyte populations of interest were stimulated with Phorbol Myristate Acetate (PMA) and Ionomycin. Brefeldin A (GolgiPlug) was added 1 h after stimulation. Cells were harvested 6 h after stimulation for surface- and subsequent intracellular staining following FACS analysis. In paper II, also secreted, extracellular, cytokines were quantified by Luminex 14-plex analysis of the supernatant of cell cultures containing blocked CD86 B-cells or control supernatant.

3.3.1.4 *Phosflow (Paper I)*

Phosflow intracellular staining was carried out according to the manufacturer's protocol (Beckton Dickinson phosflow protocol III for human PBMCs). Briefly, isolated $CD4^+$ T cells were stimulated with IL-2 at indicated concentrations and subsequently fixated and then permeabilized using the recommended buffers. Upon incubation on ice for 30 min, the cells were stained for 60 minutes at room temperature with fluorochrome-conjugated antibodies against pSTAT5, relevant surface molecules, and FOXP3 prior to analysis by flow cytometry.

3.3.2 T cell function assays (I-IV)

3.3.2.1 *Flow cytometric assay of cell mediated immune response in activated lymphocytes (paper II and III)*

For evaluation of cellular activation upon stimulation with tumor antigen, $5x10^5$ cells from peripheral blood (paper II) or SNs (paper III) were cultured with tumor homogenate as described by Marits *et al.* (250). Activated, blasting, cells were identified based on their position on forward- and side-scatter dot-plots. The tumor specific response was quantified by using macroscopically healthy bladder or medium alone as control and calculating a stimulation index (SI) according to the following formula:

$$SI = \left(\frac{T\ cell\ lymphoblasts}{Total\ T\ cells}\right) / \left(\frac{T\ cell\ lymphoblasts\ control}{Total\ T\ cells\ control}\right)$$

3.3.2.2 *Treg suppression assay (paper I)*

Responder cells (Teff cells) were labeled with Carboxyfluorescin diacetate succinimidyl ester (CFSE) and 1 x 10^5 cells/well were co-cultured with Treg cells at indicated ratios in the presence of Treg Suppression Inspector Anti-Biotin beads preloaded with biotinylated CD2, CD3, and CD28 antibodies (Miltenyi; beads:cells 1:1) in 96-well plates for 72 hours. CFSE dilution was measured by flow cytometry.

3.4 EPIGENETICS (PAPER I AND III)

3.4.1 Genomic DNA isolation and Bisulphate conversion

Genomic DNA was isolated from FACS-sorted T cell populations which in turn had been isolated from tumor (paper I), SNs, non-SNs (paper III) and peripheral blood (paper I). The isolation was performed as an on-column step on the EZ DNA Direct- Methylation kit (ZYMO research).

Bisulfite treatment of genomic DNA results in the conversion of unmethylated cytosine residues to uracil, which are recognized as thymines in subsequent PCR amplification. Cytosines that were originally methylated remain intact, thereby enabling distinction between methylated and unmethylated residues (251).

3.4.2 Pyrosequencing

The *FOXP3* target sequences (paper I) and the *IFNG* and *PRF* gene *loci* (paper III) were amplified by PCR of the bisulfite-converted DNA samples using biotinylated primers. Biotinylated PCR products were purified with a Pyromark Q96 Vacuum Workstation (Qiagen), and subsequently pyrosequenced using a Pyromark Q96 ID instrument (Qiagen). Data analysis was performed using Pyromark Q96 software (Qiagen).

3.5 PROTEOMICS (PAPER IV)

Frozen cell pellets were thawed on ice, sonicated and cell debris was removed by centrifugation. 5 µg of protein underwent denaturation followed by tryptic digestion overnight and subsequent clean-up. For a detailed description of these procedures, please refer to the appended paper. 1 µg of the resulting peptide mixture was loaded onto a nano-Ultimate HPLC system with an acetonitrile gradient (Thermo Scientific) in-line coupled to a QExactive orbitrap mass spectrometer (Thermo Scientific). The chromatographic separation of the peptides was achieved using a 28 cm long in-house packed column (C18-AQ ReproSil-Pur®) with an acetonitrile gradient. Tandem mass spectra were extracted using Raw2MGF and the resulting mascot generic files were searched against a SwissProt protein database using Mascot software (Matrix Science Ltd.). Peptides were quantified using the Quanti software (252), which performs label-free quantitation of the ion current for each MS/MS spectrum. It also excludes peptides identified with a Mascot score of 16 or lower, enabling to fulfill the false discovery condition (no more than 1 %).

3.6 WESTERN BLOT (PAPER IV)

Cultured cells were pelleted, washed and incubated in RIPA buffer. Upon centrifugation for 15 minutes at 8000 RPM, the supernatant (protein fraction) was collected and pellets discarded. Protein concentration was measured using BCA assay (ThermoFisher). 12,5 µg protein per well was loaded onto 4-12 % Bis-Tris gels (Life technologies) and gels were run according to manufacturer’s protocol. Proteins were transferred for 45 minutes at 30V to PVDF membranes (Bio-Rad), blocked with 2.5 % BSA in PBS-T for 1 hour and incubated overnight at 4 C^0 with

primary antibodies. All antibodies used were diluted in blocking solution. Please refer to the paper for list and detailed description of the used antibodies.

3.7 QUANTITATIVE PCR (PAPER I AND IV)

Total RNA from macrophages and UBC 5637 cells (paper I) and from T cells (paper IV) was extracted using the RNeasy mini kit (Qiagen). cDNA was synthesized using iScript complementary DNA synthesis kit (Bio-Rad Laboratories). Quantitative real-time PCR (RT-PCR) was performed using qPCR SYBR select mix (Life Technologies) and analysis using the CFX 96 Real Time System (Bio-Rad Laboratories) with GAPDH as housekeeping gene. Relative quantification of the RT-PCR data was calculated using the Pfaffl method. Primer sequences are specified in the respective papers.

3.8 STATISTICAL ANALYSIS

To test if the test data had an underlying normal distribution, Shapiro Wilk´s test or Kolmogorov-Smirnov’s test of normality was used. For such, parametric, data, two-tailed t tests or one-way ANOVA was used to compare groups with approximately equal variances, whereas Mann–Whitney U test or Kruskall–Wallis one-way analysis of variance with Dunn test was used for nonparametric data sets. Repeated-measures ANOVA was used for related samples (paper I), together with Bonferroni or Dunnet multiple comparison's tests. The Kaplan–Meier method was used to estimate survival rates (paper I). Differences were compared using the log-rank test. P values < 0.05 were considered significant.

In paper I, the impact of Treg frequency on gene expression was estimated by standardized coefficients from partial least squares (PLS) analysis. Data from the PLS analysis was plotted against the KEGG pathway for bladder cancer using the free software environment ”R”.

Statistical calculations were carried out with Statistica [StatSoft, Inc. (2013), STATISTICA (data analysis software system), version 12 (www.statsoft.com)] and Prism v.5 software (GraphPad), which also was used to construct graphs.

4 RESULTS AND DISCUSSION

The four articles in this thesis all address the role of lymphocytes in patients with urothelial bladder cancer. The thesis can be divided into two major topics: i) tumor-Treg cross-talk (paper I and IV) and ii) effects of chemotherapy on lymphocytes in bladder cancer patients (paper II and III). The intent with the following sections is to facilitate the readers' understanding of what was studied in the papers and why, as well as the general implications of our findings. For methodological details and more specific discussion of the results, please see the appended papers.

4.1 UNTANGLING A PARADOX – TREGS HOLD BACK THE INVASIVE FRONT (PAPER I)

The starting point for this study was our preceding finding of a positive correlation between Treg tumor-infiltration and prognosis in bladder cancer (135). This was a somewhat controversial finding since, at that time, the view of Tregs as unmitigated suppressors of tumor immunity was rather unchallenged. In the mentioned study, Tregs were defined according to FOXP3 expression status. However, FOXP3 is not exclusively expressed in Tregs but may be transiently expressed upon activation in Teff cells (72). Bearing in mind the inflammatory milieu in the bladder tumor tissue, mistaking activated T effs transiently expressing FOXP3 for Tregs would have been plausible. In paper I, we set out to test this hypothesis.

Patients (n=52) with suspected UBC were prospectively included. We examined and compared T cells extracted from tumor and peripheral blood of patients and of healthy controls, using a broad range of analytical approaches.

We first confirmed $CD4^{+}FOXP3^{+}$ T-cells to be substantially accumulated in the tumor tissue, accounting for over 20 % of the $CD4^{+}$ T cell population, compared to the corresponding 3 % in peripheral blood of healthy donors. In our initial characterization of the tumor-infiltrating $CD4^{+}FOXP3^{+}$ T cell population, we used a set of established markers of Treg memory (CD45RO), activation (HLA-DR and CD69), and effector functions (CTLA-4 and CD39) (26). The two latter markers are key molecules for Treg-mediated suppression in the physiologic setting (26) and suggested to be essential for Treg function in cancers (175). We demonstrated that the fraction of cells expressing these functional markers on the cell surface, as well as the activation marker HLA-DR, was markedly higher among tumor-infiltrating $CD4^{+}FOXP3^{+}$ T cells compared with peripheral blood. The phenotypic analysis thus indicated Treg suppressive function at the tumor site.

The majority of the $CD4^{+}FOXP3^{+}$ and the $CD4^{+}$ $FOXP3^{-}$ tumor-infiltrating T cells, were activated and antigen experienced as reflected by their prevalent expression of CD69 and CD45RO respectively. Intriguingly, also CD39 was abundantly expressed among the tumor-infiltrating $CD4^{+}$ $FOXP3^{-}$ subset and it is tempting to speculate that at least a fraction of these cells represents *p*Tregs which reportedly may lack FOXP3 expression (253).

As a side note, the observed prevalent CD45RO expression among the circulating $CD4^+FOXP3^+$ T cells implies that these cells are actual Tregs as opposed to activated Teff cells transiently expressing FOXP3. This is implied because in adults, the majority of circulating Tregs are antigen experienced - in contrast to Teff cells (88).

To further establish that the tumor-infiltrating $CD4^+$ $FOXP3^+$ T cells were actual Tregs, we investigated functional Treg attributes - which are key to validations of Treg populations. Tregs have a markedly lower threshold for IL2-induced STAT5 phosphorylation compared to other T cell subsets. Thus, assessing pSTAT5 expression upon IL-2 stimulation enables to discriminate Tregs from $CD4^+$ Teff cells. Indeed, using phospho-flow cytometry, we show that IL-2-induced signaling in the tumor-infiltrating $CD4^+FOXP3^+$ cells resembles that of the $CD4^+FOXP3^+$ Tregs in peripheral blood and differ dramatically from the tumor-infiltrating $CD4^+FOXP3^-$ cells.

Next, we demonstrated that the tumor-infiltrating $CD4^+FOXP3^+$ cells bore a Treg-characteristic cytokine profile with negligible production of the pro-inflammatory cytokines IFNγ and IL-2. Moreover, we demonstrated the tumor-infiltrating $CD4^+FOXP3^+$ cells to readily suppress autologous $CD4^+$ Teff cells in a dose-response manner, a hallmark of Treg function. Taken together, these findings provide evidence of the tumor-infiltrating $CD4^+FOXP3^+$ population to functionally act as Tregs in human bladder cancer patients.

As previously discussed, the Treg-specific epigenome is indispensable for commitment to the Treg lineage (253) and is defined by hypomethylation of different regions in key genes or their enhancers, often referred to as Treg cell-specific demethylated regions, or TSDR's. We have previously identified the CpG position -77 of the FOXP3 promoter as such a region (48) and, similarly, Floess and colleagues (51) found the conserved noncoding sequence 2 (CNS2) of the FOXP3 gene to correlate strongly to stable FOXP3 expression. Indeed, in the present study, we found these two TSDR's to be hypomethylated in the tumor-infiltrating $CD4^+FOXP3^+$ population. From this finding, together with the observed stable FOXP3 expression in long-term *ex vivo* cultures, we concluded that these cells are stably committed to the Treg lineage.

Having confirmed that the $CD4^+FOXP3^+$ T cells infiltrating UBC tissue phenotypically, functionally, and epigenetically were Tregs, the seemingly paradoxical prognostic benefit of this immunosuppressive cell population remained to be explained. Since the spatial distribution of immune infiltrates has been noted to differ also within the tumor itself, we examined the IF and the central part CP of the tumor separately. We found that the fraction of Tregs was significantly decreased at the IF compared to the CP in muscle invasive tumors. In patients with non-muscle invasive tumors no such decrease was observed. In agreement, we showed that high Treg frequency specifically at the IF gave a clear survival benefit compared to patients with a low Treg frequency (100 % vs. 25 % 3-year survival). These findings highlight that the impact of Treg frequency on prognosis not only differs between tumor types but also within different parts of the individual tumor. Additionally, the data suggest inhibition of invasion as a protective mechanism for Tregs in MIBC.

To investigate this, we examined the transcriptome of tumor samples from the IF and the CP in conjunction with flow cytometric data on Treg frequency. Since other cancers where Tregs have been reported to be protective are, similarly to UBC, linked to chronic inflammation (180, 181) we focused on factors known to be involved in TPI, such as MMPs.

We identified MMP2 as a gene highly influenced by Treg frequency, using the statistical methods specified in the paper. MMP2 was also, out of the 10 MMPs frequently described in the cancer setting, the MMP with the highest expression at the IF (254). MMP2 was further validated as an invasive factor in UBC. Extracting data from nine previously published independent datasets in the ONCOMINE database, revealed an upregulation of MMP2 in invasive tumors compared to superficial counterparts. In addition, MMP2 expression has been observed in both tumor cells and in TAMs (190, 255). We confirmed this pattern by immunohistochemical co-stainings of MMP2 and FOXP3 in UBC tissue. Moreover, MMP2 staining tended to be scarce in the area surrounding FOXP3^{+} TILs. These findings prompted us to evaluate if Tregs regulate MMP2 expression in tumor cells or in cells mediating TPI such as M2 macrophages. By co-culturing Tregs with either autologous M2 macrophages or the invasive UBC cell line 5637 cells, we demonstrated a dose-dependent Treg-mediated downregulation of MMP2 protein and mRNA expression in both these cell types.

The main limitation of this study is that the sample size for the above described functional assays is small (n ranging from 2-5). Although the results were consistent through the replicates, additional experiments would help to validate these data.

This work describes a novel functional mechanism of Tregs in tumor biology and offers an alternative explanation to the paradoxical benefit of Tregs in certain cancers. Our findings highlight the need for caution in the clinical use of immunotherapies targeting Tregs but also identify the Treg-MMP2 axis as a potential therapeutic target in inflammation driven cancers.

4.2 DOXORUBICIN ENHANCES THE CAPACITY OF B CELLS TO ACTIVATE T CELLS IN UROTHELIAL URINARY BLADDER CANCER (PAPER II)

The potential of conventional chemotherapy to enhance an anti-tumor immune response is being increasingly recognized (reviewed in (216, 225)). Although much remains to be learned, this beneficial "side effect" appears to, in part, depend on an increased antigenicity at the tumor site, elicited by certain chemotherapeutic agents (225). In line with these findings pointing at antigen presentation as a main factor, we have previously demonstrated that chemotherapy treatment during DC differentiation of human monocytes augments the immune stimulatory activity of these cells (226). Since B cells also may present tumor antigens to T cells (236), we addressed this cell type when shifting focus to the adaptive arm of the immune system. Hence, the aim of this study was to elucidate the effect of chemotherapeutic drugs on human B cells with a special attention on their function as APC's.

Fifteen MIBC patients were prospectively included in the study. PBMCs were extracted from blood samples collected at the time of TURBT and at the cystectomy or from healthy donors (buffy coats). B cell antigen presenting function was assessed using multiple cell culture assays.

To evaluate the effect of chemotherapeutic drugs on the ability of B cells to act as APCs, we co-cultured T cells with B cells pretreated with the indicated chemotherapeutics or with untreated control cells. Since tumor specific B and T cells are scarce or absent in peripheral blood, the superantigen SEB, was used to enable MHC class II dependent T cell activation. We observed an increased lymphoblast formation and proliferation of T cells in co-cultures with doxorubicin pretreated B cells, suggesting that doxorubicin enhance the capacity of B cells to activate T cells.

To identify the mechanism for the observed increase in T cell activation, we investigated aspects of all the three signals required for an APC to activate a T cell. To this end, we first analyzed the expression of MHC class II (HLA-DR) and the co-stimulatory molecules CD80/86 respectively on B cells treated with doxorubicin *in vitro*. There was no upregulation of HLA-DR suggesting that signal 1 is unaffected by doxorubicin. However, the CD86 expression was elevated in treated B cells, implying that doxorubicin reinforce co-stimulation, *i.e.* signal 2, providing additional help for activating T cells, as we could demonstrate.

We further demonstrated a causative link between the observed increase in CD86 expression and the increased $CD4^+$ T lymphoblast formation, since CD86 blockage restored T cell activation, thus corroborating the notion of doxorubicin to potentiate immune responses via signal two reinforcement. Moreover, in our cytokine profiling, we disclosed that doxorubicin decreased the secretion of IL-10 and TNF-α, cytokines with immunosuppressive effects, suggesting an effect also on signal three. In addition, we proposed a thwarting effect of doxorubicin on both immunosuppressive Tregs and Bregs as we noted a decrease in IL-2 and of intracellular IL-10. This finding conforms to a nature paper, where Shalapour *et al.* reported that $CD8^+$ T cell dependent anti-tumor effects of chemotherapy were potentiated by the elimination of mouse Bregs (256). Finally, CD86 expression was increased on circulating B cells of patients treated with doxorubicin-containing NAC, establishing an *in vivo* relevance of our findings.

In sum, we show that doxorubicin can enhance the antigen presenting ability of human B cells and this effect is mediated by increased expression of a co-stimulatory molecule and an altered cytokine profile. Our findings are in line with our previous reports on cisplatin to augment the immune stimulatory activity of DC differentiating human monocytes (226) and of Antigen-dependent clonal expansion of B cells infiltrating human bladder tumors (242).

The multiple assays utilized strengthen this study and the conclusions drawn. However, we do acknowledge some limitations. For instance, the respective cell types were pretreated with chemotherapeutics separately and then co-cultured with an untreated cell type. Naturally, this is dissimilar to the *in vivo* situation and in hindsight, additional samples with mixed cells during treatment would have added value to the study. However, the *in vivo* increase of CD86 expression in NAC treated patients supports the mechanisms being operative also in patients. Moreover, the study size of fifteen patients with an incomplete sample set warrants for larger studies to validate the findings.

Effective tumor antigen presentation is crucial in order to evoke a clinically relevant anticancer immune response. Thus, if the here reported effects could be maximized, for example by refining doxorubicin containing chemotherapy regimens, patient outcome may be improved. Perhaps most significant, our data could be useful in the major efforts currently devoted to develop combinatorial regimens that harness the immunostimulatory effects of chemotherapy to boost immunotherapy. In this endeavor, detailed knowledge of what effect chemotherapy has on the immune system is fundamental.

Our results suggested an indirect effect of doxorubicin on Tregs that warrants further investigation, preferably on Treg frequency and function since we observed cytokine alterations crucial for these aspects. Moreover, to further support therapeutic targeting of B cell-mediated T cell activation, studies addressing the effect of doxorubicin on B cells' impact on T cell activation *per se*, *i.e.* on the magnitude of the intrinsic T cell activation signaling pathway, would be of value.

To conclude, our data show that chemotherapeutic drugs can enhance the antigen presenting ability of human B cells, which is useful knowledge in the pursuit of improving patient response to cancer therapy, may it be chemotherapy alone or in combination with immunotherapy.

4.3 NEOADJUVANT CHEMOTHERAPY REINFORCES THE ANTI-TUMOR T CELL RESPONSE IN UROTHELIAL URINARY BLADDER CANCER (PAPER III)

At the time when this study (paper III) was initiated, the notion that certain chemotherapies have immunostimulatory effects was well substantiated. However, somewhat surprising, only a few studies had described such effects on T cells in the setting of human solid cancers. One such example was a report of cisplatin to both increase the Teff cell to Treg ratio and to reduce Treg immunosuppressive activity in peripheral blood of non-small cell lung cancer patients (257). These results were congruent with our findings of chemotherapy to elicit B cell-mediated effects on both Tregs and T effs (paper II). Shifting focus to the T cell arm of the AIS, we also changed scenery from peripheral blood to the SN. This is a key site for the induction of tumor-specific Teff responses, a concept which is fundamental for the groups' overarching research idea.

On that basis, we aimed to elucidate the effect of NAC on the composition and phenotypes of T cells from the SN. We further determined how these effects correlate to pathological NAC response and to the number of received treatment cycles.

Patients (n=40) diagnosed with MIBC were prospectively included. Out of these, 27 patients underwent NAC and 13 patients were NAC-naïve. T cells from SNs and peripheral blood were characterized by multicolor flow cytometry and epigenetic analysis by pyrosequencing. Cell reactivity was investigated using an adapted FASCIA protocol.

We found, that in NAC-patients, the expression of the exhaustion marker PD-1 was markedly decreased in both $CD8^+$ and $CD4^+$ SN-Teff cells. FACS-analysis further demonstrated that in

$CD8^+$ Teff cells, the activation marker T-bet and the cytotoxic molecules granzyme B and perforin were all increased by NAC in a dose-dependent manner and these effects were most pronounced in patients with CR. In addition, CR-patients exhibited functionally committed $CD8^+$ T cells, marked by hypomethylation in the *PRF1* and *IFNG* gene *loci*. These findings indicate that NAC induces $CD8^+$ T cell cytotoxicity in a dose-response manner and accord with observations in mice where chemotherapeutic agents have been demonstrated to induce $CD8^+$ T cell dependent eradication of prostate cancers and lung adenocarcinoma (234, 256).

In our evaluation of total SN T cell ($CD45^+CD3^+$ cells) frequency, an increase was observed in NAC patients, mainly reflecting a significant increase in the $CD4^+$ T cell subset. The fraction of PD-1-expressing $CD4^+$ Teff cells was decreased in the unstratified NAC group, similarly to the $CD8^+$ T cells. However, when stratifying for response to NAC, NAC-responders had an increase in PD-1-expressing $CD4^+$ Teff cells, whereas in non-responding (NR) patients, PD-1 expression was nearly absent in the corresponding cells. These findings prompted us to further characterize the PD-1^+ cells, which almost exclusively were $CD45RO^+$ (mean = 94% $CD45RO^+$ cells), demonstrating their antigen experience.

In line with our findings from paper II, we found that NAC-treated patients displayed a greater anti-tumor reactivity in the SN-$CD4^+$ Teff cells compared to the NAC-naïve group when stimulating SN-derived lymphocytes with autologous tumor homogenate.

With regards to Tregs, we confirmed an accumulation in SNs compared to PBMC, previously described for other solid cancers (161). However, when stratifying for treatment, NAC-treated patients exhibited decreased Treg fractions in the SN compared to NAC-naïve patients. Additionally, this decrease followed a dose-dependent pattern as we observed significantly lower number of Tregs in patients receiving ≥3 NAC-cycles vs. 2 cycles. Our phenotypic characterization of the Treg subset, disclosed a reduced SN-Treg suppressive activity in NAC patients, marked by a decreased expression of HLA-DR, PD-1, and the inhibitory molecule CD39. In agreement, the decrease of $CD39^+$ or $CD69^+$ Tregs was most pronounced in patients with CR. Altogether, these findings indicate that NAC-treatment has an opposite effect on Treg frequency and function, compared to $CD8^+$ and $CD4^+$ Teff cells. This is in accordance with a noticeable amount of reports on chemotherapies to abrogate immunosuppressive cellular networks in patients with various malignancies including non–small-cell lung- (257), pancreatic- (258) and colorectal cancer (216). The many reports on this type of indirect immunostimulation, suggest that suppressive cells may be more sensitive to cytotoxic drugs than immune effector cells.

Finally, we investigated if the relation between SN bulk $CD4^+$ Teff cell frequency and the frequency of Treg subpopulations correlated to NAC-response. The bulk $CD4^+$ Teff cells/bulk Treg ratio did not correlate to NAC-response. However, when relating $CD4^+$ Teff frequency specifically to Tregs expressing the suppressive marker CD39 or the activation marker CD69, we observed that patients with CR displayed an increased ratio of $CD4^+$ Teff cells to the number of Tregs expressing these markers. The data suggests that clinical outcome is better predicted based on phenotypical data rather than the sole frequency of T cell subsets in the TME. Such

data also serve as a reminder of the complexity of tumor immunology research, comprising a plethora of immune cell subsets that presumably stand in constant dynamic phenotypical change in response to cues in the TME, enabled by a postulated high degree of plasticity.

In aggregate, we revealed considerable stimulatory effects of NAC on the phenotype, function and epigenetic regulation of SN T cells in MIBC patients. These effects were both direct, *i.e.* stimulation of immune effector cells, and indirect, *i.e.* inhibition of immunosuppressive cells.

As acknowledged in the appended paper, the major limitation of this study was that NAC-naïve patients were not NAC-eligible, and thus was not a fully matched control group. Another limitation is that the other commonly used, Gemcitabine-Cisplatin, NAC regimen and its putative immune effects, were not investigated (except for in one patient), limiting the applicability of the data.

This report, of NAC to promote SN anti-tumor T cell immunity, urge for novel refined chemotherapy protocols, aiming to maximize these effects in the treatment of MIBC. A tailored approach may be successful, as indicated by the notable inter-patient variability with regard to these effects within the NAC group. The exact predictive markers that could be used for tailoring chemotherapy regimens remain to be identified. In this effort, the markers and adapted FASCIA method used in this study might be useful. Adapted protocols to optimize the use of NAC alone would also pave the way for the development of protocols which yields synergistic effects of NAC and immunotherapy in combination.

Furthermore, we hope that this study – with data on both PD-1 expression and tumor-specific reactivity, might be used in the pursuit to find biomarkers that predict response to checkpoint inhibitors. On this subject, we noted in our data that overall, the reported positive T cell effects were nearly absent in patients with no histopathologic response. Additionally, in these (NR) patients, PD-1 expressing $CD4^+$ T effs were absent. We imagine that, collectively, these results reflect a reduced immunogenic effect of NAC that follows with the limited cytotoxic effect of the chemotherapy in NR-patients. In turn, this lack of immunogenicity, hinder $CD4^+$ T eff activation as well as exhaustion, explaining the absence of PD-1 expression.

Along such lines of thoughts, strategies to increase patient response to checkpoint blockers has been developed and proven successful on a pre-clinical level. For instance, Pfirschke *et al.* demonstrated that immunogenic chemotherapeutics can induce a T cell response through increased adjuvanticity mediated by enhanced TLR4 signaling, and that this response can be harnessed to sensitize lung adenocarcinomas to immune checkpoint therapy (234).

To clinically apply such knowledge, the main tumor-specific factors determining patient response to chemotherapy (and other standard cancer therapies with immune effects) and the concomitant tumor-immunogenicity needs to be identified for individual cancer types and subtypes. If the key factors would be identified and pharmacologically targeted, a boosting effect could, hypothetically, be achieved for all ”on target” immune effects of cancer therapies. Naturally, this is an ongoing, challenging, endeavor throughout the field of tumor immunology.

4.4 IL-16 PROCESSING IN SENTINEL NODE TREGS IS A FACTOR IN TUMOR IMMUNITY (PAPER IV)

The presence of immune suppressive circuits in the SN is well known. In this context Tregs have been proposed to play a central role, but the suppressive signaling pathways employed by this population are poorly understood likewise the mechanism utilized by tumor cells to instigate such SN immune tolerance. We have demonstrated the SN as a promising source to harness T cells for the use in ACTs (143) and detailed understanding of inhibitory networks would enable optimization of such treatment. Furthermore, our findings in study III did imply a negative role of SN Tregs as opposed to the protective role for tumor-derived Tregs demonstrated in study I.

These somewhat conflicting findings and incomplete understanding framed the initial idea for this study that aimed to explore if the proteome in SN-resident T cells is altered by the tumor and to identify key proteins in SN T cell signaling, focusing on Tregs. To this end, MIBC patients were prospectively included along with healthy donors. At cystectomy, SNs, non-SNs (nSNs) and peripheral blood samples were collected. Proteomics, flow cytometry and immunoblotting assays were conducted.

In our initial analysis, we found that protein expression is generally increased in both SN and nSN derived Tregs compared to peripheral blood whereas for Teff cells the opposite was found, with a reduced protein expression in SN Teff cells. These findings suggest a selective stimulatory effect of tumor-derived factors on Tregs and an inhibitory effect on T effs in tumor associated lymph nodes. Alternatively, this could be explained by a physiological higher immune regulating activity in lymph nodes than in peripheral blood. Network enrichment analysis enabled clarification on this matter as we found that growth- and immune signaling pathways are specifically up-regulated in SN Tregs, suggesting an active role of these cells in the SN of MIBC patients. This finding is in congruence with a recent report of pre-metastatic, immunosuppressive, changes in regional lymph nodes of prostate cancer, demonstrated in a mouse model (259) and is also in line with studies of other malignancies demonstrating a suppressive impact and increased frequency of Tregs in the SN (260, 261).

Based on centrality analysis, we identified the cytokine IL-16 to be central in SN-Treg specific signaling and an increased IL-16 protein expression was further validated by flow cytometry. We were quite surprised by this finding, considering the infrequent reports of IL-16 function in Tregs. However, a few functions of IL-16 relevant to Tregs have been previously described and include expansion of $CD4^{+}CD25^{+}$ T cells in long-term cultures with IL-2, *de novo* induction of FOXP3 and preferential induction of the migration of $CD4^{+}CD25^{+}CTLA\text{-}4^{+}$ T cells.

IL-16 is mainly produced in T-cells. It is produced as a precursor protein that, following cleavage by Caspase 3, yields two biologically active proteins; a mature form comprising the 14 kDa C-terminal part which is secreted as a tetramer, and an N-terminal part, referred to as pro-IL-16 (262). Mature IL-16 is a CD4 co-receptor specific ligand which is secreted during inflammatory responses, and it has been suggested to act as a growth- and differentiation factor

and as a chemoattractant. In contrast, pro-IL-16 is a growth repressor that translocate to the nucleus. In addition to these physiological functions, IL-16 has been implicated in several malignancies. Kovacs *et al.* reported on increased levels of IL-16 in sera of patients with various late-stage cancers, including bladder cancer (263). Moreover, IL-16 polymorphisms have been linked to several types of cancers, such as cutaneous T cell lymphoma and CRC (264).

In our attempt to further delineate IL-16 SN Treg function, we probed for the two cleavage products by immunoblotting and found a considerably higher ratio of mature IL-16 to precursor IL-16 in these cells. This finding was replicated in a controlled system in which Tregs were cultured with tumor supernatant. Since IL-16 processing only occurs upon activation of Caspase-3 in $CD4^+$ T cells (262), these findings indicated such an impact of a tumor-derived factor on SN Tregs. Indeed, when assessing Caspase-3 activation in Tregs cultured with tumor supernatant, we observed an increase compared to the medium control.

In summary, we here profiled the proteome of SN Tregs and found growth- and immune signaling to be up-regulated. Most significantly, IL-16 was predicted to be central in SN Treg signaling, a finding validated in subsequent experiments. Furthermore, direct contact with tumoral factors increased active Caspase 3 and concomitant alterations in IL-16 processing.

This is, to our knowledge, the first study to map the Treg proteome in human lymph nodes. Naturally, since only two patients were profiled and the sample size was small also in the subsequent experiments, the study should be considered as a proof of concept. We demonstrate that mass spectrometry can be a useful tool to identify what pathway changes that have occurred in TME-resident immune cell subsets. For this approach to be clinically applicable, proteome changes in relevant immune cell types would need to be mapped in large-scale proteomic studies on MIBC patients, with a long-term follow-up. With these points addressed, this approach could be used to i) identify predictive biomarkers for response to already existing cancer immunotherapies, ii) to counteract resistance development to immunotherapies and iii) to identify novel proteins that could be targeted in order to circumvent tumor immune escape which would increase the efficacy of immunotherapies like the SN-based ACT developed by our group.

We propose that IL-16 represent such a target, albeit this notion remains to be validated in a larger patient series. Future studies also need to identify the tumor-derived molecule(s) that activate Caspase-3 and address their downstream effects and impact on Treg function.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

In patients with urinary bladder cancer, the tumor-immune system interaction is active, with a clearly altered lymphocyte infiltrate (summarized in **Figure 5**). This finding aligns with the consensus view of this malignancy to be immunogenic and suitable to target with immunotherapy. During the last decade, immunotherapy has proven an effective therapeutic approach in various cancers but the rapid pace of both clinical and preclinical studies within the field has also helped to define three major remaining challenges: i) To evoke a directed immune response against a (non-tolerogenic) neoantigen, ii) To render this immune response sizeable and iii) durable.

It is being increasingly recognized that standard chemotherapy may significantly enhance anticancer immune responses in people with cancer (225). This thesis supports this notion, since substantial stimulatory effects of NAC on the phenotypes and functions of SN T cells and on circulating B cells in patients with advanced bladder cancer were observed. Furthermore, our finding of an increased tumor-specific reactivity after NAC treatment imply an increased exposure of neoantigens and thus relates to the pursuit of overcoming the first challenge, where an optimized NAC regimen appears beneficial. This idea fits with the common mechanistic model of the positive immune effects to partly result from an increased tumor immunogenicity, which chemotherapeutics elicits by inducing a certain manner to die, namely through a so called immunogenic cell death (ICD) (225).

Our observation that patients without tumor downstaging did not demonstrate these positive immune effects imply a linkage between these effects and the direct cytotoxic effect of NAC on the tumor cells. It is key to resolve the causal relation here, because if these tumors are refractory to chemotherapy because of a lack of stimulatory immune effects and not vice versa, it would suggest that the cancer cells have developed intrinsic resistance mechanisms to specifically escape the immunogenic effects of chemotherapy. Such scenario is plausible, given the recent recognition of cancer cell intrinsic oncogenic pathways to frequently involve immune escape mechanisms (151). An obvious such candidate to examine in a future study would be ICD pathways, where mutations in, for instance, eIF2α, hypothetically could constitute such an oncogenic, “escape through drug resistance” mechanism. Theoretically, targeting of such pathways could extend the beneficial immunogenic chemotherapy effects and perhaps turn non-responders to responders.

The increased tumor immunogenicity induced by chemotherapy can be harnessed through the combinatory use with checkpoint blockade (234). Such ongoing efforts of developing combinatory treatment protocols represent promising approaches to overcome the second challenge. This thesis has contributed also in this endeavor. Specifically, we hope our data on PD-1 expression and tumor-specific reactivity may be useful in the effort of finding biomarkers predicting who would benefit from combination therapies. On a more general level, we have demonstrated a profound context-dependency of lymphocyte function in the TME. It appears that a given immune cell may play different roles even within a single tumor and its microenvironment, in which leukocytes are irregularly distributed. The spatial distribution,

together with an array of factors regarding the organization of the leukocyte infiltrate of the tumor, adds up to the ”immune contexture”. This is a useful concept because, if properly assessed, it yields information relevant to prognosis and may predict response to immunotherapies. Our findings of the Treg role to differ between the tumors’ central part, invasive front and sentinel node, advances the framework of this concept. In the sentinel node, a negative role was indicated by our observation of a shifted balance favoring Teff cells over activated Tregs in complete responders to NAC. We also observed a sentinel node-specific alteration of the Treg proteome, where IL-16 signaling was prominent, suggesting proteomic analysis of separate sentinel node cell populations as a useful complementary tool for the exploration of the immune contexture. Taken together, these findings may contribute in the early efforts of crafting a platform to provide guidance in treatment decisions on personalized immunotherapies.

A critical question within the field of oncoimmunology is: What are Tregs regulating in cancer? This thesis provides the answer that, in UBC tumor tissue, Tregs suppress tumor-promoting inflammation, which may at least in part explain the favorable prognosis associated to Treg tumor-infiltration in this cancer. Perhaps most importantly, the finding gives a more nuanced comprehension of the role of Tregs in tumor immunity at large which hopefully will provide guidance in the clinical use of immunotherapies targeting these cells (which include checkpoint blockers) in people with cancer (162). In inflammation-driven cancers, boosting the Treg-MMP2 axis rather than Treg depletion appears as a potential therapeutic target.

Although the responses to checkpoint blockade are often durable, complete tumor eradication is only rarely seen (4). This fact highlights the importance of the third challenge – to induce long-lasting memory responses, establishing a sizeable pool of potent tumor antigen specific T cells residing in the TME. This might be achieved by further additional drug combinations targeting distinct aspects of tumor immunity, including drugs counteracting T cell exhaustion. A promising such attempt is IDO-inhibition combined with PD-1 blockade in an ongoing trial for patients with MIBC (265).

The complexity of the immune system is both a blessing and a curse for our species. To constrain the immunosubversive effects casted by tumors, the “onco” and “immunology must be brought closer together, for us to get the full picture of this intricate interplay. Along those lines, examining the immune contexture of the emerging bladder cancer taxonomies, which are based on molecular footprints (266), would provide knowledge of the basic cellular mechanisms of cancer immunity, and perhaps also point on targets enabling to nip tumor immune evasion in the bud.

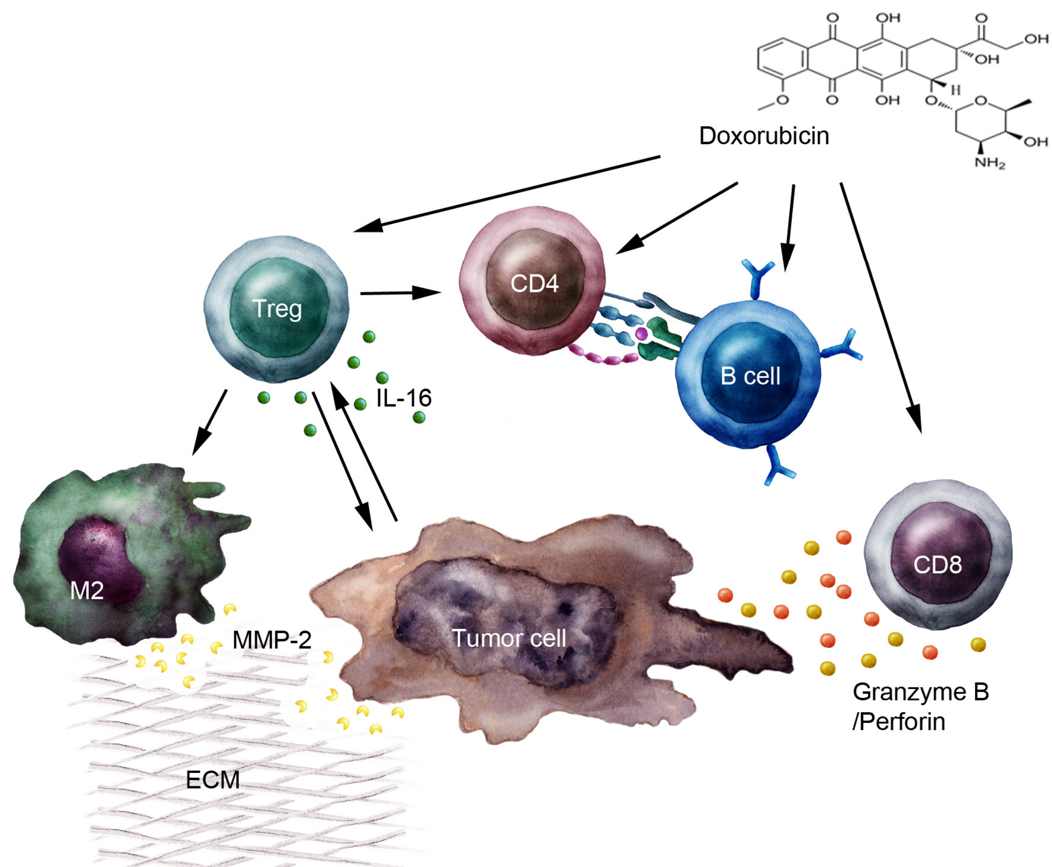

Figure 5. *Thesis summary*. The major findings from each of the included papers are illustrated. Arrows indicate the direction of the demonstrated effects exerted by a cell type or by Doxorubicin-containing chemotherapy. In paper I, we found Tregs to suppress tumor invasiveness through inhibiting the production of the ECM-degrading enzyme MMP2, in both M2 macrophages and UBC cells. Paper II uncovers a stimulatory effect of chemotherapy on the antigen presenting ability of B cells, mediated partly through an increased co-stimulation and with a subsequent increased activation of $CD4^+$ T cells. In paper III, we demonstrated that chemotherapy treatment promotes T effector functions in the sentinel node, manifested by an increased tumor-specific reactivity in $CD4^+$ Teffs and, in $CD8^+$ Teffs, by an increased expression of the cytotoxic Granzyme B and Perforin molecules and a decreased exhaustion. Paper IV reveals a substantial tumoral impact on the proteome of sentinel node Tregs with an altered IL-16 signaling and processing, mediated by tumor factors inducing caspase-3 in these cells. Figure courtesy of Malin Winerdal.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Tesen om att immunförsvaret har förmågan att skydda mot tumörers utveckling kan spåras ända tillbaks till 1800 talet, men tog lång tid att vetenskapligt bekräfta. Idag är existensen av detta viktiga skydd helt uppenbar, inte minst på grund av ett flertal nya immunterapier som under de senaste decennierna haft en succéartad framgång. Dessa behandlingar ger varaktiga skydd, ibland till och med botar patienter, mot cancrar där det tidigare inte fanns några medel att ta till. Hur kommer det sig då att tumörer ändå, i så pass stor omfattning, utvecklas och att så många drabbas av cancer? För att förstå det måste man först förstå hur immunsystemet försvarar oss mot denna sjuka.

Förenklat kan man säga att cancerceller utvecklas till följd av förändringar i sin arvsmassa, DNA. Dessa förändringar kan ge upphov till så kallade *tumörantigen*, det vill säga proteiner som till viss del är kroppsfrämmande, vilket innebär en möjlighet för immunförsvaret att känna igen, och i många fall eliminera dessa förvandlade celler. Dessvärre har många tumörer utvecklat strategier för att undvika igenkänning eller för att på andra sätt undslippa immunförsvarets attack Dessa strategier, som gemensamt brukar kallas *escape-mechanisms*, eller *flyktkmekanismer*, är en del av svaret på frågan. Den givna följdfrågan blir då: Hur åstadkommer tumören denna igenkänningsflykt? För att förstå det måste man först förstå hur immunsystemet gör för att särskilja kroppsegna från kroppsfrämmande celler.

Det är en stor utmaning för immunsystemet att, å ena sidan vara ett effektivt skydd mot yttre faror som exempelvis bakterier, medan å andra sidan inte angripa kroppsegna vävnader och orsaka så kallad autoimmunitet. För att lösa denna uppgift, att upprätthålla tolerans, används bland annat Regulatoriska T celler (Tregs). Tregs är en typ av immunceller som reglerar tolerans genom att hämma andra typer av immunceller, exempelvis T effektor celler (Teffs) och B celler. Samtliga dessa celler tillhör det specifika adaptiva immunsystemet. En strategi som tumörer använder för att undslippa en immunattack är att ”tjuvkoppla” Tregs, som då, i stället för att skydda mot autoimmunitet, skyddar tumören från det övriga immunsystemet, det vill säga den (tumören) ”lurar” immunsystemet att den är kroppsegen.

I det här arbetet avhandlas den intrikata interaktionen mellan tumören och nämnda celltyper i patienter med urinblåsecancer (UBC). Vi studerade även vilken påverkan kemoterapi, ibland benämnt som ”cellgifter”, har på denna process.

Det första arbetet tog avstamp i en föregående studie där vi sett att tumör-infiltrerande Tregs korrelerade till en gynnsam prognos vid UBC. Det var något förvånande eftersom Tregs generellt anses vara negativa för prognos enligt ovan beskrivna logik. Vi hade använt proteinet FOXP3 för att identifiera Tregs, vilket inte är en fullständigt specifik markör. För att bekräfta att vi inte hade misstagit Tregs för en annan celltyp, gjorde vi en djupgående analys av tumör-infiltrerande, FOXP3-uttryckande celler. Vi kunde visa på att dessa celler uppfyllde karaktärsmässiga, funktionella och epigenetiska (vilket kort kan beskrivas som förändringar på men inte i DNA) definitioner på Tregs, vi hade alltså klassificerat dem korrekt. Som nästa steg återstod då att kunna förklara varför Tregs är gynnsamma för prognos vid UBC. En ledtråd

gavs från andra rapporter med motsvarande resultat för vissa andra, specifika cancertyper. Gemensamt för dessa cancrar är en uttalad inflammation, som anses kunna driva på cancerutveckling. Vi fokuserade därför på inflammatoriska signalvägar och fann att Tregs kunde hämma produktionen av MMP2, ett enzym som kan öka tumörens invasiva förmåga, i både inflammatoriska celler (makrofager) och i UBC-celler belägna vid den invasiva fronten av tumörens närmiljö. Vår förklaringsmodell är därför att Tregs, genom dessas anti-inflammatoriska effekt, kan gynna patienter med inflammatoriskt drivna cancrar.

Tvärtemot en vanlig uppfattning om att kemoterapier är förödande för immunsystemet, finns det nu en stor medvetenhet kring att deras effektivitet delvis beror på en positiv effekt på immunsystemets attack mot tumören. I vår föresats att undersöka effekterna av kemoterapi på celler tillhörande det adaptiva immunförsvaret, började vi med att undersöka B celler i perifert blod i friska donatorer, men även i patienter med UBC. Vi fann att doxorubicin, en standard kemoterapi för ett flertal cancertyper, har en stimulerande effekt på B cellers förmåga att presentera ovan nämnda tumörantigen till Teff celler. Detta ledde till att dessa Teff celler i större utsträckning aktiverades och initierade en attack specifikt riktad mot tumören. Ett sätt för immunceller att kommunicera med varandra är genom att utsöndra cytokiner, vilka är signalmolekyler som kan ha antingen en stimulerande eller hämmande effekt. I denna studie fann vi att doxorubicin minskade B cellers produktion av hämmande cytokiner, vilket indikerar även en indirekt positiv effekt på T cellsaktivering, genom att skapa en mer stimulerande cytokinsignalering.

I våra fortsatta undersökningar kring immuneffekter av kemoterapi fokuserade vi på T celler och bytte från att undersöka blod till att istället undersöka lymfknutor i tumörens närmiljö, fortfarande från patienter med UBC. Vi kunde då se att, precis som i föregående studie, så ökade kemoterapi $CD4^+$ Teff cellers tumörspecifika aktivering även i lymfknuta. $CD8^+$ Teff celler, en cytotoxisk subtyp av T celler, det vill säga celler med kapacitet att direkt döda tumörceller, fick en ökad sådan cytotoxisk profil och uppvisade i lägre utsträckning tecken på utmattning. När vi specifikt tittade på de patienter som svarade allra bäst på kemoterapi, såg vi att deras $CD8^+$ Teff celler, uppvisade tydliga tecken, bland annat epigenetiska sådana, till att potent kunna eliminera cancerceller. Däremot var situationen annorlunda, faktiskt helt motsatt, vad gällde Tregs. Vi observerade att ju fler kemoterapikurer en patient fått, desto lägre andel Tregs fanns i lymfknutorna och, det fåtal som fanns där, visade tecken på nedsatt funktion, jämfört med patienter som inte fått kemoterapi. Slutligen, när vi undersökte sammansättningen av de olika typerna av immunceller, visade det sig att de som svarat bäst på kemoterapi hade den mest gynnsamma cellkompositionen för en effektiv immunattack mot tumören.

I avhandlingens sista arbete fortsatte vi att undersöka T celler i tumör-associerade lymfknutor, men vi undersökte nu hela proteomet, det vill säga samtliga proteiner som vid undersökningstillfället uttrycks i den givna cellpopulationen. Vi undrade om tumören påverkade proteomet i Tregs och jämförde därför proteinuttrycket i Tregs från tumördränerande lymfknutorna med Tregs från lymfknutor som inte dränerar tumören. Det visade sig att proteiner som är involverade i direkt immunologiska funktioner var ökade och,

mest förvånande, att cytokinen IL-16 var en central signalmolekyl i Tregs från tumördränerande lymfknutor. Eftersom det endast finns ett fåtal rapporter om funktionen av IL-16 i Tregs, gjorde vi ytterligare analyser och kunde då se att direkt kontakt med tumörfaktorer ökade modifieringen av IL-16 molekylen till sin aktiva form. Slutligen kunde vi identifiera ett enzym, caspase-3, som vi tror förmedlar denna modifiering. Vidare studier behövs för att utröna den funktionella betydelsen av dessa fynd.

Sammanfattningsvis påvisar dessa studier utpräglade förändringar av det adaptiva immunsystemet i tumörens närmiljö i patienter med UBC. På denna process, har kemoterapi en tydligt positiv effekt. Våra fynd, av bland annat nya tumörbiologiska funktioner av Tregs, ger ökade detaljkunskaper inom det onkoimmunologiska fältet som förhoppningsvis kan användas för att hitta nya strategier att frigöra det immunsvar som lamslagits av tumören.

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