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REGULATORY T CELLS
MOLECULAR AND CLINICAL ASPECTS

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Regulatory T Cells – Molecular and Clinical Aspects

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Litterarum radices amarae, fructus dulces.

-Cicero.

To my family,

ABSTRACT

In the immune system's tug of war with cancer, tolerance mechanisms by which the tumor can control anti-tumor immune responses play a central role in determining the outcome. Regulatory T cells (Treg) induced in the thymus or the periphery, represent one such tolerance mechanism that potentially can be exploited by developing tumors. In this thesis, we explore the underlying molecular mechanisms that result in Treg development, and attempt to elucidate the importance of this T lymphocyte subset in urinary bladder cancer.

In humans and mice, the transcription factor FOXP3 is crucial for the function of the Treg subset and maintenance of peripheral tolerance, as illustrated by the lack of functional Treg and autoimmune disorders that result from mutations within this protein. In humans however, clinical studies of this important T lymphocyte subset are obscured by the fact that FOXP3 is transiently induced in conventional T lymphocytes upon activation. Initially, we address the epigenetic regulation of FOXP3 expression, and demonstrate that the committed Treg population has an almost completely demethylated FOXP3 promoter region, whereas conventional non-regulatory $CD4^+$ T lymphocytes are semi-methylated in this region. Furthermore, we study the development of regulatory T cells in mice, and assess the contribution and the impact of adenosine receptor signaling on the T lymphocyte compartment.

Next, the role of the Treg population in urinary bladder cancer is evaluated. First, we investigate the impact of tumor infiltrating $CD3^+$ as well as $FOXP3^+$ T lymphocytes on patient survival. Somewhat surprisingly, we find a positive correlation between both $CD3^+$ and $FOXP3^+$ lymphocytic infiltrates, suggesting that $FOXP3^+$ lymphocytes in this case may not represent a tumor escape mechanism as initially hypothesized. In addition, we observe FOXP3 expression in a subset of tumors, and find that this expression is a negative prognostic factor for survival. To follow up these results, we characterize the T lymphocyte immune response in peripheral blood, lymph nodes and tumor tissue from patients with UBC. We demonstrate that the $FOXP3^+$ fraction of $CD4^+$ T lymphocytes is significantly increased compared to all other locations investigated including macroscopically healthy bladder tissue. Furthermore, these tumor infiltrating lymphocytes express high levels of activation and effector markers, but do not display a demethylated pattern in the *FOXP3* promoter to match its prominent expression. Interestingly, muscle invasive tumors have a lower $FOXP3^+$ fraction at the invasive front compared to non-invasive counterparts. In addition, we observe changes in the cellular immune response dependent on if the patients have received neo-adjuvant chemotherapy or not, both with regard to cell composition and functional reactivity to tumor antigens.

Epigenetic regulation governs the commitment of T lymphocytes to the Treg lineage. The fact that FOXP3 expressing tumor infiltrating lymphocytes in UBC do not display a committed Treg phenotype could potentially explain the differences in reported clinical impact of this population in different cancers and has implications for future immunotherapy.

LIST OF SCIENTIFIC PAPERS

I. Winerdal ME*, Janson PC*, Marits P, Thörn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS One.* 2008 Feb 20;3(2):e1612.
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II. Winerdal ME, Marits P, Winerdal M, Hasan M, Rosenblatt R, Tolf A, Selling K, Sherif A, Winqvist O. FOXP3 and survival in urinary bladder cancer. *BJU Int.* 2011 Nov;108(10):1672-8.

III. Winerdal ME, Krantz D, Rosenblatt R, Zirakzadeh A, Ahlén Bergman E, Winerdal M, Hansson J, Holmström B, Johansson M, Marits P, Sherif A, Winqvist O. Characterization of T lymphocyte Responses in Urinary Bladder Cancer. *Manuscript*.

IV. Winerdal ME, Winerdal M, Krantz D, Yang T, Lindmark E, Marits P, Fredholm B, Ådén U, Carlström M, Winqvist O. Adenosine receptor signaling in T cell development. *Manuscript*.

List of publications not included in the thesis:

Lofgren C, Hjortsberg L, Blennow M, Lotfi K, Paul C, Eriksson S, Albertioni F. Mechanisms of cross-resistance between nucleoside analogues and vincristine or daunorubicin in leukemic cells. *Biochem Biophys Res Commun.* 2004 Jul 30;320(3):825-32.

Janson PC, Winerdal ME, Winqvist O. At the crossroads of T helper lineage commitment-Epigenetics points the way. *Biochim Biophys Acta*. 2009 Sep;1790(9):906-19.

Hu J, Lou D, Carow B, Winerdal ME, Rottenberg M, Wikström AC, Norstedt G, Winqvist O. LPS Regulates SOCS2 Transcription in a Type I Interferon Dependent Autocrine-Paracrine Loop. *PLoS One.* 2012;7(1):e30166.

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CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
BCG	Bacillus Calmette-Guerin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Conserved non-coding DNA sequence
COBRA	Combined bilsulphite restriction enzyme analysis
CP	Central Part
cTEC	Cortical thymic epithelial cell
CTLA	Cytotoxic T lymphocyte antigen
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DN	Double negative
DP	Double positive
ERK	Extracellular signal activated kinase
FACS	Flow associated cell sorting
FOXP3	Forkhead box transcription factor P3
IDO	Indoleamine 2,3-dioxygenase
IF	Invasive Front
IFN	Interferon
IL	Interleukin
IPEX	Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome
MACS	Magnetic-activated cell sorting
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cell
nSN	Non sentinel node
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction

PRR	Pattern recognition receptor
RP	RNA polymerase
SN	Sentinel node
SP	Single positive
T-bet	T-box transcription factor
TCR	T cell receptor
TGF	Tumor growth factor
Th	T helper
TIL	Tumor infiltrating lymphocyte
Treg	Regulatory T lymphocyte
TSDR	Treg-specific demethylated region
TSLP	Thymic stromal lymphoprotein
TUR-B	Transurethral resection of the bladder
TZ	Transitional zone
UBC	Urinary bladder cancer
WT	Wild type

1 INTRODUCTION

The human body hosts an intriguingly complex balance, and ever ongoing battle of sovereignty. Constantly challenged by external pathogens, the immune system has evolved to recognize and eliminate possible threats while sparing and caring for autologous tissues. In this setting, the two main branches of the immune response, innate and adaptive immunity, play integral and complementary parts. By means of their broad specificity but limited repertoire of pattern recognition receptors (PRRs), innate immune cells are constantly vigilant for danger signals (damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs)), ready to quickly initiate a primary immune response upon stimulation (Kono and Rock, 2008). In contrast, T- and B-lymphocytes carry receptors with an almost unlimited potential to recognize foreign antigens, but react slower compared to their innate counterparts. At the intersection of these two responses is the presentation of antigens to T lymphocytes by antigen presenting cells (APCs) (Figure 1); an essential process for the initiation of an adaptive immune response. The context of the antigen presentation determines the outcome of the ensuing T lymphocyte response, where presence of co-stimulatory molecules on the APC in combination with the cytokine milieu facilitates and directs the type of T lymphocyte response.

Since the T lymphocyte receptor repertoire contains approximately in the range of 10^9-10^{15} specificities with potential to theoretically recognize any protein epitope, the distinction of self is essential to avoid autoimmunity. This distinction and thus protection of autologous tissue is ensured by two separate mechanisms – central and peripheral tolerance. For T lymphocytes, central tolerance is molded in the thymus, where T lymphocytes carrying T cell receptors (TCR) with high affinity to self-proteins are deleted (Palmer, 2003) (further discussed in section 1.1). In the periphery, potentially autoreactive T lymphocytes are kept in check by different peripheral tolerance mechanisms (Abbas et al., 2004). The most straightforward mechanism is the concept of ignorance where potentially self-reactive T lymphocytes remain inactive because of low levels of or no accessibility to the antigen. Secondly, in the absence of danger signals, the APC will not upregulate the co-stimulatory molecules required by naïve T lymphocytes for activation. TCR ligation in the absence of co-stimulation leads to anergy or deletion and thus protects from autoimmunity (Redmond and Sherman, 2005). Alternatively, engagement of B7 costimulatory molecules on the APC to cytotoxic T lymphocyte antigen 4 (CTLA-4) on the T lymphocytes instead of its stimulatory counterpart CD28, may also induce T cell tolerance (Perez et al., 1997). Furthermore, regulatory T cells (Tregs) induced peripherally or in the thymus, can regulate the immune response and thus maintain tolerance (Sakaguchi et al., 2010).

Cancer poses a unique threat to the human body in the sense that it is inherently both self and non-self at the same time. Tumor evolution is shaped by the immune system, and successful tumors evade immune responses in various ways, including suppression of the local T lymphocyte responses and induction of tolerance (discussed in more detail in section 1.5). This thesis enacts at the interphase of physiology and tumor pathology, and concerns both the

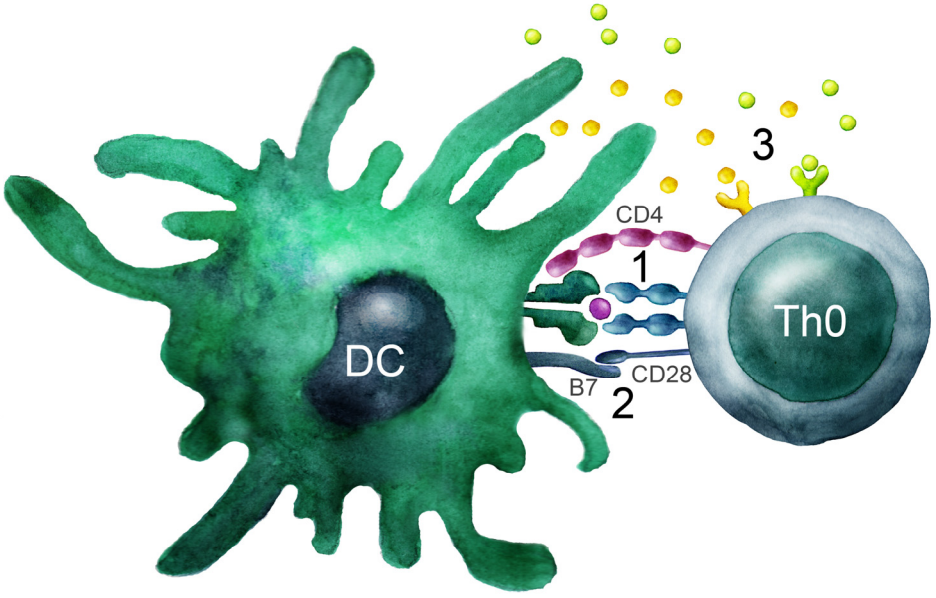

Figure 1. Basic concept of naïve T helper lymphocyte activation. To become activated, a naïve T lymphocyte requires three distinct sets of signals. 1. TCR recognition of a peptide-MHC complex. 2. Costimulatory signals. 3. Inflammatory cytokines.

development and regulation of regulatory T cells (papers I and IV) as well as their role and prognostic impact in urinary bladder cancer (UBC) (papers II and III).

1.1 T LYMPHOCYTE SELECTION AND DIFFERENTIATION

1.1.1 Thymic selection

T lymphocyte progenitors migrate to the thymus from the bone marrow. During their development in the thymus, thymocytes undergo strict selection processes that only permits T lymphocytes with functional but not overtly self-reactive TCRs to exit the thymus. In the end, less than 5% of the initial thymocyte population is selected and allowed to leave for the periphery (Starr et al., 2003). Here follows a brief summary of the selection process for conventional α/β T lymphocytes.

Newly arrived thymocytes are $CD4^-CD8^-$ double negative (DN) and can be further subdivided into differentiation stages based on their expression of the markers CD44 and CD25 (Godfrey et al., 1993). While they migrate though the thymic cortex, thymocytes sequentially pass the $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4) stages (Godfrey et al., 1993; Lind et al., 2001). Rearrangement of the TCR gene starts at the DN3 stage with the expression of the recombination activating genes (RAG 1 and 2), and rearrangement of the TCRβ locus. In a process called β selection, the rearranged TCRβ chain together with a pre-TCRα chain forms a pre-TCR complex, which triggers thymocyte proliferation, induction of the two TCR co-receptors CD4 and CD8, and rearrangement of the TCRα chain locus (Hoffman et al., 1996). $CD4^+CD8^+$ double positive (DP) thymocytes that

successfully rearrange their TCRα chain to form a functional α/β TCR complex undergo positive selection based on their TCR affinity for MHC molecules on cortical thymic epithelial cells (cTEC). Failure to recognize self-MHC results in elimination by apoptosis, while positively selected cells down-regulate CD4 or CD8 expression based on their recognition of major histocompatibility complex class (MHC) I or MHC II respectively and migrate to the thymic medulla (Starr et al., 2003). Interestingly, the positive selection process goes on for several days during which it appears that thymocytes must receive continuous TCR signals with associated activation of the mitogen activated protein kinase (MAPK) pathway in order to pass this check point in development (McNeil et al., 2005; Wilkinson et al., 1995).

Negative selection encompasses the process where T lymphocytes with TCRs that strongly recognize self peptide-MHC complexes are deleted. This is a crucial step in central T cell tolerance, preventing autoreactive T lymphocytes to exit the thymus to the periphery. Medullar thymic epithelial cells (mTECs) as well as thymic dendritic cells (tDCs) are key players at this check point, where presentation of a diverse repertoire of self-antigens on MHC-molecules in combination with co-stimulation drive the negative selection process (Palmer, 2003).

1.1.2 T helper cell subsets

Once outside the thymus T lymphocytes circulate the secondary lymphoid organs in search of APCs presenting their cognate antigen. If encountered in the context of co-stimulation and the appropriate cytokines, the naïve $CD4^+$ T lymphocyte is activated as illustrated in Figure 1. The local cytokine milieu caused by the triggering factors, and inflammatory cell composition at the site of activation influences the type of T lymphocyte response that is elicited. The major T helper lineages that have been described include the classical T helper type 1 (Th1) and T helper type 2 (Th2) cells, primarily described as stable lineages involved in promoting cellular and humoral immune responses respectively. With time, new T helper lineages such as the Th17, Treg and T follicular helper (Tfh) have been characterized adding to the complexity of T helper differentiation. Intense studies of the factors that drive the evolution of each lineage have defined transcription factors essential for each subtype as; T-box transcription factor (T-bet) for Th1, GATA3 for Th2, FOXP3 for Treg and Bcl-6 for Tfh (Weinmann, 2014). Interestingly, with the increasing number of T helper differentiation fates, reports describing the co-expression of hallmark cytokines such as IL-17 (Th17) and IFN-γ (Th1) in $CD4^+$ T lymphocytes have emerged (Wilson et al., 2007). There are now also numerous reports of combined expression of the lineage defining transcription factors in various contexts (Oestreich and Weinmann, 2012). With regard to Treg cells, it is interesting to note that the co-expression of other lineage defining transcription factors such as T-bet and Bcl-6, have been implicated in the suppression of their respective type of immune response (here; Th1 and Tfh type responses respectively) (Koch et al., 2009; Linterman et al., 2011). These reports have challenged the established view of T helper lineages as stable final differentiation states, and suggested a more dynamic model of T helper differentiation (Weinmann, 2014). In this setting, the ever changing stimuli that a T cell encounters in combination with its epigenetic and protein phenotype (that is a consequence of its previous history) will finally determine the type of response elicited.

1.2 REGULATORY T CELLS

Regulatory T cells play an integral part in the control of immune responses. The fact that the T lymphocyte compartment contains cells capable of controlling immune responses has been known to the scientific community since the early 1970's (Sakaguchi et al., 2007). At the time generally known as suppressor T cells, this population attracted strong attention during the following years, but was finally abandoned much due to the lack of good phenotypic markers (Sakaguchi et al., 2007). In the mid 1990's however, the field was revived with Sakaguchi's classical report describing the regulatory properties of a subpopulation of $CD4^+$ T lymphocytes that constitutively expressed the α-chain of the interleukin-2 receptor (IL-2Rα, CD25) (Sakaguchi et al., 1995). Since then, this population has been the focus of intense research, and although the knowledge about Treg properties and subtypes has greatly improved, much remains to be elucidated. Here follows a brief summary of main markers, subsets, and mechanisms of action of $CD4^+FOXP3^+$ Tregs dealt with in this thesis.

1.2.1 Regulatory T cell lineage markers

Numerous molecular markers have been suggested for the delineation of the Treg subsets, some of which have already been mentioned. The following subsections will discuss some of the most commonly used markers, with a main focus on the markers used in this thesis.

1.2.1.1 CD25

As mentioned previously, the Treg population was first defined as a subpopulation of $CD4^+$ T lymphocytes that expressed the IL-2 receptor α-chain (CD25) constitutively (Sakaguchi et al., 1995). Together with the β-chain (CD122) and the common cytokine receptor γ-chain (CD132), CD25 forms the high-affinity IL-2R. Interestingly, IL-2R signaling has been shown to be important for the development and maintenance of Treg (Thornton, 2006). In humans, Baecher-Allan et al. showed that only the $CD25^{high}$ population (corresponding to between 1 and 2% of $CD4^+$ T lymphocytes in peripheral blood) correlated with suppressive capacity whereas the $CD25^{low/intermediate}$ cells did not suppress T lymphocyte proliferation (Baecher-Allan et al., 2001). However, CD25 is upregulated in T cells upon activation, and although CD25 expression is subsequently lost again when the stimulus is removed, this fact decreases its specificity and usefulness as a marker in clinical settings.

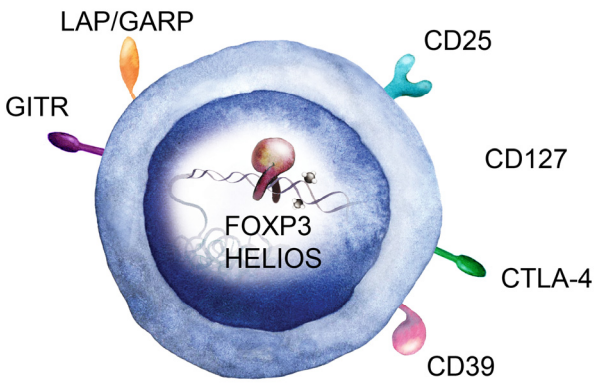

Figure 2. Treg lineage markers. Diagram depicting markers preferentially expressed by Tregs. CD25, CD127, CTLA-4, CD39, FOXP3 and HELIOS are discussed more in detail in the main text. Other markers, such as latency associated peptide (LAP)/GARP and GITR have also been associated with the Treg phenotype, however, a detailed discussion of all Treg associated molecules is not within the scope of this thesis.

1.2.1.2 FOXP3

The transcription factor FOXP3 has been linked to the suppressive phenotype of both human (FOXP3) and murine (Foxp3) Treg populations (Sakaguchi et al., 2010). Its close relationship to the Treg population was first proposed in the Scurfy mouse model, and soon thereafter confirmed by the fact that the human disease Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX) was associated with mutations in the FOXP3 gene (Bennett et al., 2001; Brunkow et al., 2001). In mice, Foxp3 is generally considered to be a specific, required and sufficient factor for Treg development (Fontenot et al., 2003). In humans however, FOXP3 is not as specific since it is transiently upregulated in human $CD4^{+}CD25^{low}$ T lymphocytes upon activation (Walker et al., 2003). Although this expression is associated with hypo-responsiveness and decreased cytokine production (Wang et al., 2007), results have differed regarding the *in vitro* suppressive capacity of the T lymphocytes with induced FOXP3 expression (Walker et al., 2003; Wang et al., 2007), and it seems that a stable FOXP3 expression is required for suppressive function. Indeed, *in vitro* induced FOXP3 expression in conventional human T lymphocytes does confer a regulatory phenotype (Allan et al., 2005; Walker et al., 2005), and the level of suppressive capacity is both time and dose dependent as illustrated by a conditional expression model (Allan et al., 2008).

In further contrast to the mouse setting, humans express different splice variants of *FOXP3* mRNA that lead to the expression of; full length FOXP3 (FOXP3fl), FOXP3 lacking exon 2 (FOXP3Δ2), and FOXP3 lacking exon 2 and 7 (FOXP3Δ2Δ7) (Allan et al., 2005; Smith et al., 2006). Although the FOXP3fl and FOXP3Δ2 isoforms have been reported to confer a Treg phenotype and regulatory activity *in vitro* (Aarts-Riemens et al., 2008; Smith et al., 2006) this does not seem to be the case for the FOXP3Δ2Δ7 isoform that instead appears to exert a dominant negative effect on FOXP3fl upon co-transduction (Mailer et al., 2009). In addition, it has been shown that the exon 2 domain in the full-length FOXP3 protein interacts with the transcription factor retinoic acid receptor-related orphan receptor (ROR) α and ROR γt, suppressing genes related to Th17 development in T lymphocytes, an effect not achieved by FOXP3Δ2 (Du et al., 2008; Ichiyama et al., 2008). Still, the physiological and clinical impact of these three isoforms remains to a large extent unknown.

FOXP3 undeniably plays an essential role for the regulatory function of Tregs in both mice and men. However, the Treg transcriptional profile (in mice) has been demonstrated to be only partly dependent on Foxp3 expression (Hill et al., 2007). This was emphasized in other studies where T lymphocytes lacking suppressive capacity but with similar phenotypes to Treg developed in spite of missing functional Foxp3 (Lin et al., 2007). Together these findings indicate upstream events of Foxp3 expression as important factors in commitment to the Treg lineage.

The transient expression of FOXP3 in activated human T lymphocytes combined with the human heterogeneity with regard to FOXP3 isoforms calls for caution in interpreting human Treg data based on FOXP3 expression alone, especially in clinical inflammatory settings where T cell activation is to be expected.

1.2.1.3 CD127

Low expression of the IL-7 receptor α-chain (CD127) has been suggested as a complementary surface marker for Tregs (Liu et al., 2006; Seddiki et al., 2006). CD127 is downregulated in T lymphocytes upon activation, but whereas effector and memory T lymphocytes resume their expression, $FOXP3^+$ Tregs remain $CD127^{low/-}$. Although not perfect, this makes CD127 especially interesting for *in vivo* studies of immune responses where activated T cells are expected. The study by Liu et al. related CD127 expression to FOXP3 and found that less than half of the $CD4^+CD127^{low/-}$ population was $FOXP3^+$ (Liu et al., 2006). Categorizing the Treg population based on $CD25^{high}$ cells did produce much more pure $FOXP3^+$ populations but left out a considerable number of $FOXP3^+$ Tregs in the $CD25^{int\rightarrow low}$ population (Liu et al., 2006). The authors proposed instead that the combination of the two markers be used for surface based distinction of $FOXP3^+$ cells as the $CD4^+CD25^+CD127^{lo/-}$ population, which leads to both improved purity and yield.

1.2.1.4 CTLA-4

The cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) is another marker that has been used to identify the Treg population. It binds the same ligands as the co-stimulatory molecule CD28 (CD80/CD86 or B7.1/B7.2), but with higher affinity and provides a co-inhibitory signal (reviewed in (Murakami and Riella, 2014)). In contrast to CD28 which is constitutively expressed on most resting human T lymphocytes, CTLA-4 expression peaks within 48h of activation and is only constitutively expressed by the Treg subset (Murakami and Riella, 2014; Walunas et al., 1994). Its surface expression is tightly regulated and in unstimulated cells CTLA-4 is mainly localized to intracellular vesicles that upon T cell activation fuse with the cell membrane to be exposed on the cell surface preferably at the sites of TCR engagement (Linsley et al., 1996). CTLA-4 can interfere with immune activation through various mechanisms including the competitive binding of B7 molecules on APCs, interfering with the intracellular activation signals upon TCR stimulation and induction of regulatory mechanisms such as indoleamine 2, 3-dioxygenase (IDO) production by the APCs (Murakami and Riella, 2014).

1.2.1.5 Helios

In addition to FOXP3, the ikaros family transcription factor Helios has been proposed as a complimentary marker for tTreg (Thornton et al., 2010). This study illustrated that $Foxp3^+$ thymocytes are virtually all double positive for Helios, whereas the peripheral Treg compartment contained only approximately 70% Helios expressing cells. Moreover, they illustrated that *in vitro* induced murine and human iTregs do not express Helios (Thornton et al., 2010). Subsequent studies showed that $Helios^+FOXP3^+$ Treg displayed a more demethylated pattern in their FOXP3 gene compared to $Helios^-$ counterparts, suggesting that these indeed represent a committed Treg lineage (see below) (Kim et al., 2012). However, there have also been reports challenging Helios as a specific marker of tTreg (Akimova et al., 2011; Himmel et al., 2013). In one study, Akimova et al. found that Helios expression contrary to being Treg specific is predominantly associated to activated and proliferating cells (Akimova

et al., 2011). Furthermore, Helios' specificity has been challenged based on the finding of Helios$^-$ Treg expressing markers of naïve T lymphocytes and recent thymic immigrants (Himmel et al., 2013). In contrast to the earlier studies described, none of the latter were able to find a correlation between *FOXP3* methylation status and Helios expression. In conclusion, the fact remains that the peripheral CD4$^+$FOXP3$^+$ Treg compartment can be divided into two subsets based on the expression of the transcription factor Helios, however the functional significance of which at this point remains unresolved.

1.2.2 Regulatory T cell subsets

As a result of the great scientific interest in the Treg field over the past decades, the knowledge has vastly expanded together with the number of described Treg subtypes. In addition to the CD4$^+$FOXP3$^+$ Treg subsets that are the main focus of this thesis, other T lymphocyte subpopulations with regulatory properties have also been described including the peripherally IL-10 induced and producing Tr1 and transforming growth factor β (TGF-β) producing Th3 cells as well as CD8$^+$ Tregs (Buckner and Ziegler, 2004; Niederkorn, 2008). Although of great interest, a detailed discussion of these regulatory populations is beyond the scope of this thesis. Instead, the following sections will focus on Treg subsets defined by their expression of the hallmark transcription factor forkhead box P3 (FOXP3). Due to the immense attention that Tregs have received, the body of litterature on the subject has practically exploded during recent years together with the proposed number of Treg subsets. In an attempt to simplify the nomenclature, a group of leading scientists from different parts of the field suggested the concept of three main subgroups; namely thymus derived Treg (tTreg), peripherally derived Treg (pTreg) and *in vitro* induced Treg (iTreg) (Abbas et al., 2013).

1.2.2.1 Regulatory T cell development

The absolute requirement of the thymus for the development of Treg cells is perhaps best illustrated by animal models such as thymectomy of mice on postnatal day 2-4, which results in abrogated thymic Treg development and autoimmunity (Sakaguchi et al., 2007). Coherent with the timing of this model, under normal conditions in mice, CD4$^+$CD25$^+$Foxp3$^+$ Treg are detectable in the periphery starting from around three days after birth. TCR interactions with MHC-peptide complexes on APCs together with co-stimulatory signals and cytokines are key players in Treg selection (Hsieh et al., 2012). The specificity of the Treg TCR repertoire has been the subject of debate during recent years. However, in general Treg appear to be selected much based on self-reactivity of their TCR, at an intermediate level between that of thymocytes that will mature to conventional T lymphocytes and those that will undergo negative selection due to too high TCR self-affinity (Hsieh et al., 2012).

In the thymus, Foxp3$^+$ thymocytes are found mainly in the medulla, driving the hypothesis that the Treg lineage is induced mainly in the thymic medulla (Fontenot et al., 2005a). In particular, mTECs expressing the autoimmune regulator (Aire) transcription factor have been implicated in the generation of Tregs, since absence of MHC II on these cells resulted in reduction in the number of Foxp3$^+$ thymocytes in the medulla. Furthermore, the induced expression of a model

antigen in mTECs lead to the generation of Tregs specific for the antigen independently of medullar dendritic cells (Aschenbrenner et al., 2007). However, subsequent studies have shown that also bone marrow derived tDCs are capable of inducing Foxp3 independently of mTECs (Hsieh et al., 2012; Proietto et al., 2008). In addition, although the absolute majority of $Foxp3^+$ thymocytes are found as CD4SP cells in the medulla, a few $Foxp3^+$ cells are also observed at more immature thymocyte stages in the thymic cortex, and Treg development has been observed in mice with MHC II expression restricted to cTECs (Bensinger et al., 2001). $Foxp3^+$ cells are in fact enriched in the late double positive development stage in the cortex, and the blocked migration of thymocytes resulted in accumulation of cortical $Foxp3^+$ thymocytes suggesting that also cTEC may contribute to the induction of Tregs (Liston et al., 2008). In summary, thymic APCs play a crucial part during tTreg development, however, the contributions of each APC subtype is still a matter of investigation.

Although the thymic development of Tregs has been studied intensely in mice, relatively little is known about the mechanisms that drive the development of tTreg in humans (Sakaguchi et al., 2010). Of note, in humans functional Tregs are present already from week 14 of gestation in contrast to the murine setting where Treg development is first detected after birth (Darrasse-Jeze et al., 2005). Moreover, even if many parts of thymocyte development in humans and mice are comparable, the Hassal's corpuscles are unique to the histology of the human thymus. Interestingly, studies have shown that the Hassal's corpuscles produce thymic stromal lymphoprotein (TSLP) that is able to activate thymic DCs. These TSLP activated DCs were subsequently shown to induce FOXP3 expression in CD4SP $CD25^-$ thymocytes (Hanabuchi et al., 2010; Watanabe et al., 2005). Furthermore, in the thymic medulla thymocytes expressing Treg markers were found to co-localize with Hassal's corpuscles and activated DCs, supporting a role for this structure in human Treg development (Watanabe et al., 2005).

1.2.2.2 Role of cytokines in regulatory T cell development

Both in the thymus and the periphery it is clear that the cytokine environment plays a crucial role for Treg development, as demonstrated by the complete abrogation of Treg development in mice missing the common γ-chain, involved in the signaling of several cytokines such as IL-2, IL-7 and IL-15 (Fontenot et al., 2005b). IL-2 signaling in particular has been shown to induce Foxp3 expression, and IL-2 deficient mice or mice missing IL-2 downstream signaling molecules display reduced Foxp3 Treg populations in the thymus and the periphery (Fontenot et al., 2005b; Turka and Walsh, 2008). However, $Foxp3^+$ Tregs do develop in the thymus despite the lack of IL-2 or CD25, and thus it appears that other cytokines signaling through the common γ-chain may play complimentary roles to IL-2 during thymic development.

TGF-β is another cytokine that has been implicated in Treg development, and shown to be important for the peripheral induction and maintenance of $Foxp3^+$ Treg in mice (Marie et al., 2005). However, its contribution to the thymic development of Treg has been debated, and it appears that TGF-β signaling contributes to, but is not absolutely essential for thymic Treg induction (Liu et al., 2008; Marie et al., 2005). Interestingly, the downstream transcription factor of TGF-β, mothers against decapentaplegic homolog 3 (SMAD3) has been demonstrated

to bind a conserved enhancer region within the *Foxp3* gene together with nuclear factor of activated T cells (NFAT) thus promoting *Foxp3* transcription (Tone et al., 2008).

1.2.2.3 Functional Treg subsets

The functional and phenotypical heterogeneity of the human $CD4^+FOXP3^+$ Treg population is well-established (Sakaguchi et al., 2010). Many markers have been suggested to delineate different functional Treg subsets, where one of the most established ones is the naïve and memory T lymphocyte markers, CD45RA and CD45RO respectively (Miyara et al., 2009; Valmori et al., 2005). The $CD45RA^+CD45RO^-$ Treg population, also referred to as naïve or resting Tregs, do not express the proliferation marker Ki-67 *ex vivo*, but readily proliferate, exert suppressive function and convert to $CD45RO^+$ cells upon TCR stimulation (Miyara et al., 2009). This resting human Treg subset is most pronounced in young individuals, decreases with age and has been shown to be relatively resistant to activation induced apoptosis by CD95/CD95L (Fas/FasL) interactions (Fritzsching et al., 2006; Valmori et al., 2005). In contrast, the majority of $FOXP3^+$ $CD4^+$ Treg cells in adults expresses CD45RO *ex vivo*, a population characterized by rapid turnover (Vukmanovic-Stejic et al., 2006), that is functionally suppressive but sensitive to activation induced cell death (Fritzsching et al., 2005; Miyara et al., 2009). The high expression of many markers associated to T lymphocyte activation, such as CD25, CTLA-4 and CD95, within this population makes it difficult to discriminate from activated memory-type T lymphocytes. Interestingly, the level of CD25 and FOXP3 expression seems to delineate two different $CD45RO^+$ populations, where the $CD25^{high}FOXP3^{high}$ cells exhibit greater suppressive function and are less prone to pro-inflammatory cytokine production compared to $CD45RO^+$ cells with lower CD25 and FOXP3 expression (Miyara et al., 2009). These $CD45RO^+FOXP3^{low}$ cells presumably represent, or are at least contaminated by, activated conventional T lymphocytes with an induced transient expression of FOXP3, as demonstrated by the higher methylation status of the *FOXP3* promoter and CNS2 region in this population (Miyara et al., 2009).

In humans, expression of the MHC II molecule HLA-DR also defines a functionally distinct Treg population, where $HLA\text{-}DR^+$ Treg display faster and more potent suppressive responses than their $HLA\text{-}DR^-$ counterparts (Baecher-Allan et al., 2006). Like conventional T lymphocytes, $HLA\text{-}DR^-$ Treg have been demonstrated to upregulate HLA-DR upon activation, however the Treg population appears to stay $HLA\text{-}DR^+$, in contrast to conventional T lymphocytes that only transiently express HLA-DR after stimulation (Baecher-Allan et al., 2006). Thus, it has been proposed that these $HLA\text{-}DR^+$ Treg represent a terminal effector Treg subpopulation in humans (Sakaguchi et al., 2010).

1.2.3 Regulatory T cell stability and plasticity

Epigenetic control is a well-established means of gene regulation within the immune system, and mechanisms such as histone modifications and DNA methylation carefully govern cell fate decisions in developing lymphocytes (Wilson et al., 2005). The uncertain ontogeny of peripheral $CD4^+FOXP3^+$ Tregs, fueled the search for markers that distinguish cells committed

to the Treg lineage from those with only transient FOXP3 expression. Recent years have started to illuminate the underlying epigenetic mechanisms that facilitate FOXP3 expression and thus deepened our understanding of the molecular background to a stable Treg lineage.

Mantel and colleagues first described the FOXP3 promoter region and demonstrated it to be accessible for the transcription machinery in both $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells (Mantel et al., 2006). It was also found to contain binding sites for nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1), transcription factors which are well-established mediators of T cell activation, in agreement with the possibility of FOXP3 transcription in activated $CD4^+CD25^{low}$ T cells (Mantel et al., 2006). In 2006, IL-2 was shown to induce FOXP3 expression in human Natural Killer (NK) cells treated with 5-aza-2'-deoxycytidine, indicating that DNA methylation could be important in the transcriptional control of this gene (Zorn et al., 2006). Indeed, studies by our as well as other research groups soon reported a Treg specific methylation pattern in the *FOXP3* gene (paper I, and (Baron et al., 2007; Floess et al., 2007)). Over the years since, a constantly increasing amount of data has further emphasized the importance of the epigenetic landscape for the stable commitment to the Treg cell lineage, and a number of so called Treg-specific demethylated regions (TSDRs) have been defined in different genes throughout the genome (Morikawa and Sakaguchi, 2014).

To date, four different conserved regulatory elements within the Foxp3 gene have been described; the promoter region and conserved non-coding DNA sequences (CNS) 1-3 (Baron et al., 2007; Floess et al., 2007; Janson et al., 2008; Zheng et al., 2010). During the course of tTreg development in mice, it appears that the epigenetic signature of Tregs is actively imprinted by DNA demethylation starting at the CD4SP stage in the thymus (Toker et al., 2013). Interestingly, paralleling the largely Foxp3 independent Treg expression profile in mice discussed previously, induction of a Treg specific hypomethylation pattern (of *Foxp3* and other Treg associated genes such as *Ctla4* and *Il2ra* (CD25)) is largely unaffected by the absence of functional Foxp3 (Ohkura et al., 2012). Thus, it seems that Foxp3 itself is essential in the execution of the Treg suppressive phenotype and perhaps not so involved in the initial induction of the Treg lineage (Miyao et al., 2012; Ohkura et al., 2012; Samstein et al., 2012).

Establishment of the epigenetic signature of the committed Treg population enables the distinction of uncommitted, transiently FOXP3 expressing cells. This is extremely important especially for the distinction of committed Treg populations in inflammatory settings, as well as for the establishment of future immunotherapies attempting to boost or target the Treg lineage. However, some caution is warranted when attempting to extrapolate mouse data to the human setting. This also applies to FOXP3 and its regulation, since the expression pattern of this gene differs between humans and mice, and the epigenetic regulation of other inflammatory genes such as *Ifng* have been shown to be differentially regulated between these two species (Janson et al., 2009).

1.2.4 Regulatory T cell effector mechanisms

Multiple mechanisms have been proposed for how Tregs exert their suppression on other immune cells including both cell contact dependent and independent modes of action. *In vitro* studies have shown that Tregs require both antigen specific TCR stimulation and co-stimulation to exert suppression, however, once activated the Treg population is non-specific in its suppression (Sakaguchi, 2004).

Figure 3 summarizes major Treg suppressive mechanisms. The secretion of soluble inhibitory factors such as TGF-β, IL-10 and IL-35 can inhibit the local immune response by acting on target cells such as APCs or T cells, and represent a cell-cell contact independent effector mechanism (although both secreted and membrane bound forms of TGF- β have been described) (Schmitt and Williams, 2013; Toda and Piccirillo, 2006). By comparison, cell contact mediated effector mechanisms include surface expression of inhibitory molecules such as CTLA-4 (described above), and LAG-3, a homologue to the TCR co-receptor CD4 that can suppress DC maturation upon interaction with MHC II molecules on these cells (Liang et al., 2008). As mentioned previously, CTLA-4 binds B7 co-stimulatory molecules with higher affinity than CD28 and can thus potentially both block co-stimulation at the same time as it sends negative regulatory signals to the APC (Murakami and Riella, 2014). Moreover, CTLA-4 interactions with APCs has been demonstrated to induce secretion of IDO, an enzyme that catalyzes the local breakdown of tryptophan resulting in metabolic disruption and suppression of T lymphocyte responses (Munn et al., 1999).

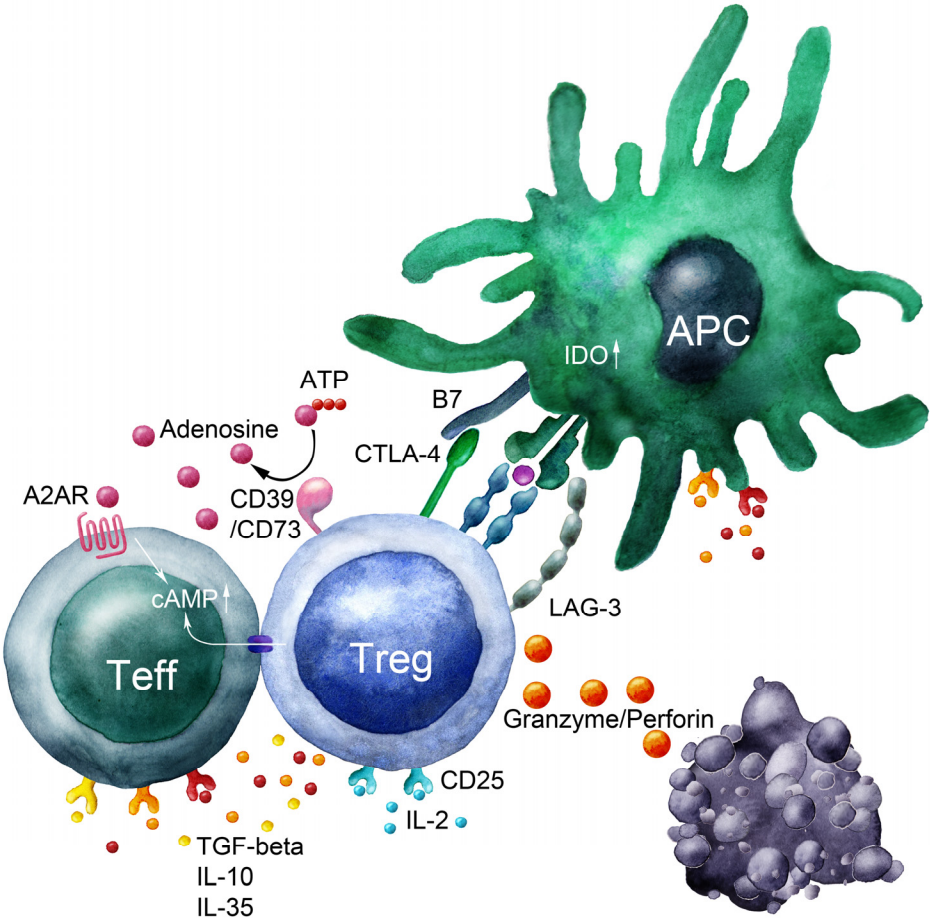

Figure 3. Treg effector mechanisms. Tregs suppress immune responses through several different modes of action, including: Secretion of inhibitory cytokines (such as TGF-β, IL-10 and IL-35), cytokine deprivation, production of pericellular adenosine, direct killing of target cells and immune suppressive effects mediated via antigen presenting cells.

In addition, several distinct mechanisms have been described whereby Tregs can inhibit T lymphocytes through the elevation of intracellular cAMP levels in the responder cells. Firstly, Tregs can induce a local anti-inflammatory environment by the production of pericellular adenosine generated from ATP by CD39 (nucleoside triphosphate diphosphohydralase-1 (NTPDase1)) on the Treg cell surface and CD73 (ecto-5'-nucleotidase) on Tregs or other cells in the local environment (Antonioli et al., 2013b). The adenosine signaling system is complex and has several implications in immune regulation (see also section 1.3). Local adenosine production limits T lymphocyte immune responses primarily through engagement of the adenosine A_{2A} receptors that leads to increased cAMP levels in the target cells (Ohta and Sitkovsky, 2001). Moreover, human iTreg have been shown to express cyclooxygenase-2 (COX-2) that result in prostaglandin E2 production dependent elevation of intracellular cAMP and suppression of immune responses (Mahic et al., 2006). Strikingly, Treg have also been described to suppress responder cells by direct transfer of cAMP through gap junctions (Bopp et al., 2007).

FOXP3 in itself has been shown to repress IL-2 expression, coherent with the fact that $FOXP3^+$ Treg do not express this cytokine (Schubert et al., 2001). They are however, inherently dependent on IL-2 signaling for survival and maintenance (Maloy and Powrie, 2005) and given their constitutively high expression of CD25, the local consumption of IL-2 depriving other T lymphocyte subsets of this growth factor, has been suggested as a contributing effector mechanism (Pandiyan et al., 2007).

Finally, Tregs have also been demonstrated to directly kill their target cells by a variety of mechanisms involving either apoptosis inducing ligands such as FasL (Janssens et al., 2003) or granzyme/perforin secretion (Grossman et al., 2004a; Grossman et al., 2004b). Taken together, the above mechanisms most probably represent complimentary pathways utilized by Treg populations under different conditions to control immune responses, and mirror the complexity of the Treg phenotypes described in various contexts.

1.3 ADENOSINE RECEPTOR SIGNALING

The purine adenosine is ubiquitously present throughout the body as an important player in cell metabolism. Its extracellular concentration is highly dependent on the metabolic state of the tissues and influenced by various stimuli such as stress, hypoxia and inflammation (Fredholm, 2007). The importance of adenosine receptor signaling has been demonstrated in multiple both physiological and pathological contexts ranging from cardiovascular effects and temperature regulation to cell differentiation, migration and immune regulation (Fredholm, 2007). Adenosine signals through four 7-transmembrane G-protein coupled receptors namely the adenosine A_1, A_{2A}, A_{2B} and A_3 receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) (Figure 4). The $A_{2A}R$ and $A_{2B}R$ signal predominantly through Gs whereas the A_1R and A_3R couple predominantly to Gi proteins thus resulting in elevated or decreased intracellular cAMP levels respectively (Fredholm et al., 2011). In addition, all four receptors have been shown to affect MAPK signaling pathways, including extracellular signal activated kinase 1 (ERK1), ERK2, p38, and c-jun N-terminal kinase (JNK) (Schulte and Fredholm, 2003b). In terms of adenosine

concentrations required for signaling, A_1, A_3 and A_{2A}Rs are thought to be able to signal already at physiological concentrations, whereas A_{2B}Rs require higher adenosine levels to induce intracellular changes in cAMP level (Fredholm, 2007). In contrast, A_{2B}Rs have been shown to have the lowest threshold for MAPK signaling, suggesting that this pathway may be the major contributor to A_{2B}R mediated effects under physiological conditions (Schulte and Fredholm, 2000).

In the immune system, the immunosuppressive effects of the A_{2A}R are well-established. The A_{2A}R is expressed on immune cells from both myeloid and lymphoid lineages and its importance in immune regulation is demonstrated by the exaggerated inflammation displayed by $A_{2A}R^{-/-}$ mice (Ohta and Sitkovsky, 2001; Ohta and Sitkovsky, 2014). Interestingly, also $A_{2B}R^{-/-}$ mice display a dysregulated immune response, and these findings are mirrored in murine tumor models by a lower susceptibility to tumors in both $A_{2A}R^{-/-}$ and $A_{2B}R^{-/-}$ mice (Ohta et al., 2006; Ryzhov et al., 2008). Activation of A_{2A}Rs on the different immune subsets has been shown to inhibit T and NK lymphocyte responses, and A_{2B}R signaling promotes alternative activation of APCs (Antonioli et al., 2013a). Furthermore, it appears that adenosine signaling also plays a role in T lymphocyte homeostasis and differentiation, where A_{2A}R stimulation is involved in maintenance of peripheral T lymphocytes (Cekic et al., 2013), and both A_{2A}R and A_{2B}R stimulation have been implied in Treg induction in mice (Ehrentraut et al., 2012; Ohta et al., 2012).

Although the A_{2A}Rs are most abundantly expressed by T lymphocytes and the most well-studied of the adenosine receptors in this cell type, expression of all four receptors have been reported on conventional T lymphocytes as well as Tregs (Cekic et al., 2013; Ehrentraut et al., 2012). Still, the function of A_1R and A_3Rs in the immune system has mostly been studied in myeloid derived cell types, where adenosine signaling has been linked to diverse functions such as degranulation, adhesion, migration and antigen presentation (Burnstock and Boeynaems, 2014).

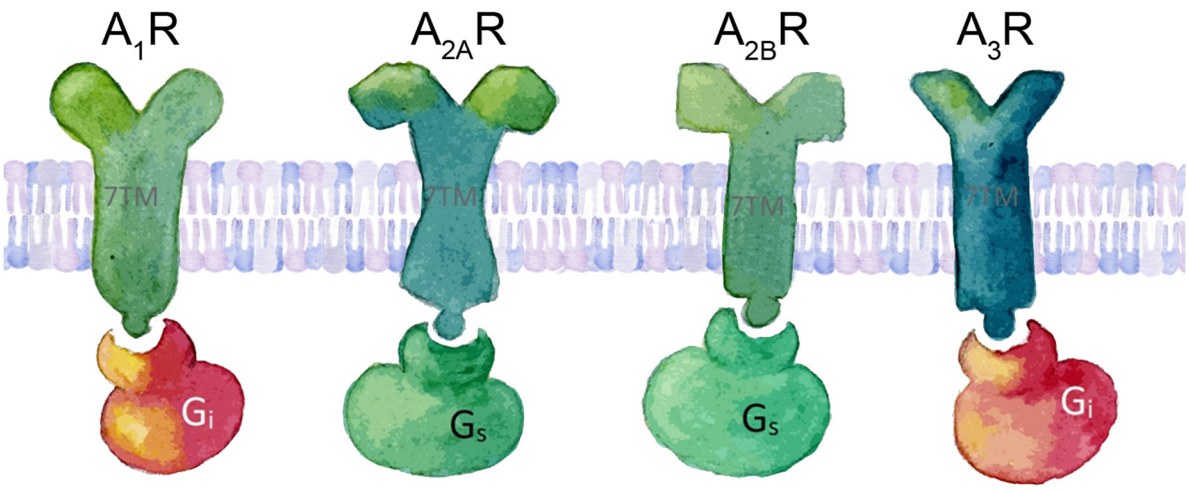

Figure 4. The four types of adenosine receptors: A_1, A_{2A}, A_{2B} and A_3 receptors, and the preferentially associated G-protein to each receptor.

1.4 THE CONCEPT OF CANCER

Cancer as a disease has captivated the scientific community for generations. Intense research has resulted in a deepened understanding of the traits that characterize human malignancies, first summarized by Hanahan and Weinberg in 2000 (Hanahan and Weinberg, 2000). Their theory stipulates six basic hallmarks of cancer that are acquired during the multistep progression of human tumors, namely: (1) Independence from exogenous growth signals, (2) resistance to antigrowth signals as well as (3) apoptosis, (4) limitless proliferative ability, (5) angiogenesis induction and (6) invasion/metastasis capability (Hanahan and Weinberg, 2000). The multistep cancer evolution theory is supported by pathological data from several tumors where an evolution from premalignant lesions to invasive tumors is observed (Foulds, 1954). In addition, the theory is further strengthened by epidemiological evidence from solid tumors, such as e.g. bladder cancer, with an age related incidence where between seven and eight separate evolutionary events have been inferred (Renan, 1993). These tumor specific changes are acquired through a variety of different molecular changes, enabled by genetic instability and DNA mutations in the tumor cells. Consequently, throughout the process of tumor development, tumor cells acquire a protein signature that separates them from their normal tissue progenitors. Such foreign proteins can potentially elicit an anti-tumor immune response by which the body is able to recognize and eliminate altered cells. The field of tumor immunology has vastly expanded over the past decades, and in a recent update of the Hallmarks of Cancer theorem, tumor immune evasion is now included as an emerging hallmark (Hanahan and Weinberg, 2011).

In the context of tumor development however, it is interesting to note the ambiguous role that inflammation plays where the inflammatory process as such has been shown to promote tumor progression in many tumors (de Visser et al., 2006). Indeed, inflammation facilitates most if not all of the basic characteristics of cancers, and epidemiological data link chronic inflammatory conditions to the development of different kinds of tumors in humans (Elinav et al., 2013; Hanahan and Weinberg, 2011). Myeloid cells of the innate immune system have been widely implied in promotion of tumor progression through mechanisms such as production of reactive oxygen species, metalloproteinases, a diverse palette of chemokines and cytokines as well as suppression of adaptive immune responses (de Visser et al., 2006; Elinav et al., 2013; Gabrilovich et al., 2001). Of note, even though lymphocyte infiltration is a positive prognostic indicator in many tumors (Galon et al., 2006; Lipponen et al., 1992; Zhang et al., 2003) and adoptive immunotherapy of expanded T cells has proven promise in the clinical treatment of patients (Dudley et al., 2005; Karlsson et al., 2010), the adaptive immune system has also been implied to promote tumor progression under certain circumstances (Alizadeh et al., 2013; DeNardo et al., 2009). In summary, it appears the range of immune effects on tumors can span from tumor cell elimination to promotion of tumor growth and invasion. Understanding the cellular and molecular pathways that shift the response in either direction will help us exploit these mechanisms in the design of future immunotherapies of cancer.

1.5 TUMOR IMMUNITY

Cancer immune surveillance – the idea that cells of the immune system continuously circulates, monitors and protects the body from developing cancers – as a means to eliminate tumors is now widely accepted (Dunn et al., 2004). Studies on IFN-γ and perforin in mice revealed the importance of these key immunological substances to prevent the development of both spontaneous and chemically induced tumors, providing a basic proof for the hypothesis of tumor immune surveillance (Shankaran et al., 2001; Street et al., 2001). In humans, tumors are often infiltrated by T lymphocytes, the presence of which correlates positively to disease outcome (Galon et al., 2006; Lipponen et al., 1992; Zhang et al., 2003), and tumor antigen-specific functional T lymphocytes have been observed in human cancer patients (Guckel et al., 2006). The concept of immunoediting and its "three E's" comprises: 1) Elimination, which basically encompasses the concept of immune surveillance, 2) equilibrium, a process where the immune system interacts with the tumor, contributes to the evolutionary selection of tumor cell clones and may serve to "edit" the tumor phenotype, and finally, 3) escape, where tumor cells manage to evade the immune system and grow in otherwise apparently immunocompetent hosts (Dunn et al., 2004).

1.5.1 Immune escape

Tumor immune escape encompasses multiple mechanisms by which tumors manage to elude the immune system and involves diverse immune cell types. T lymphocyte responses are evaded mainly by avoiding recognition or by disabling the effector T cells (Teff), each of which is executed through a number of different mechanisms, including induction of tolerance, altered antigen presentation, immunosuppressive microenvironment, co-inhibition, and/or involvement of regulatory cell populations (Rabinovich et al., 2007).

1.5.1.1 Induction of tolerance

A major obstacle in mounting an immune response against tumors is that not all tumor antigens are tumor specific, i.e. some are also expressed by normal tissues. Thus the immune system may fail to recognize the cancer as foreign and be rendered unresponsive, which has indeed been shown in both $CD4^+$ and $CD8^+$ T lymphocyte populations (Lee et al., 1999; Rabinovich et al., 2007; Staveley-O'Carroll et al., 1998).

A major mechanism of tolerance induction is mediated through APCs, where especially dendritic cells, play a central role in the development of immune responses against cancer, but also contribute to the control of immune responses (Rabinovich et al., 2007; Steinman et al., 2003). DC antigen capture and presentation of antigen in the absence of an inflammatory environment fails to trigger DC maturation and expression of co-stimulatory molecules, rendering the DCs incapable of eliciting a robust antigen-specific response. Studies of cancer patients revealed decreased numbers of differentiated DCs, but an accumulation of immature DCs with reduced capacity to activate T cells (Pinzon-Charry et al., 2005). Many different pathways have been implicated by which DCs may suppress anti-tumor immune responses (Ma et al., 2012). Such mechanisms include expression of IDO (Munn et al., 2004) and arginase

(Liu et al., 2009), secretion of immunoregulatory cytokines (Shurin et al., 2013) and expression of inhibitory molecules such as programmed death ligand 1 (PD-L1) (Mu et al., 2011). Thus DCs are very much implied in tumor escape mechanisms, however, a more in-depth description of DC subtypes and their involvement in tumor escape are beyond the scope of this text.

1.5.1.2 Altered antigen presentation

Genetic instability is a hallmark trait of cancer cells (Hanahan and Weinberg, 2011), and forms the basis for the production of cancer-specific antigens that the body can recognize as foreign. How then, is it that tumor cells manage to hide these changes from the immune system? One classical way of tumor immune evasion is the defective presentation of antigens through the MHC I pathway. Downregulation of MHC I expression has been observed in many forms of human cancers, where the most common mechanism for total loss of expression is mutations in or deletion of the ß2-microglobulin genes (Hicklin et al., 1999; Rabinovich et al., 2007). Alternatively, a variety of different mechanisms can work to downregulate the transcription of MHC I genes, or affect the antigen processing through the transporter associated with antigen processing (TAP) and to some extent proteosomal subunits low molecular mass polypeptide (LMP) 2 and 7 (Hicklin et al., 1999).

1.5.1.3 Importance of the tumor microenvironment

The tumor microenvironment and its molecular interactions between the tumor and immune as well as other cells in the tumor stroma are central in both development and progression of tumors. Of note, chronic inflammation in tumor tissue sometimes not only fails to elicit an adequate response but can also promote cancer development (de Visser et al., 2006). Tumors release inhibitory factors that affect both the innate and the adaptive immune system, recruit stromal cells and regulatory cell populations such as Tregs and myeloid derived suppressor cells that during recent years have been attributed increasing importance in the development and progression of solid tumors (Hanahan and Weinberg, 2011).

The enzyme IDO (discussed briefly in previous sections) is one mediator of T cell suppression that has been linked to cancer, where it can be expressed not only by immune cell populations but also by the tumor itself (Uyttenhove et al., 2003). IDO catalyzes the rate-limiting step in the degradation of the amino acid tryptophan, and is expressed by a variety of cell types in response to inflammatory signals, within the immune system normally by APCs in response to external stimuli (Munn et al., 1999). The depletion of tryptophan and the generation of immunosuppressive metabolites, result in direct T cell suppression as well as enhancement of local Treg function (Munn and Mellor, 2007). IDO expression has been observed in human tumors as well as tumor-draining lymph nodes, and is associated with poor prognosis (Brandacher et al., 2006; Ino et al., 2006; Munn et al., 2004; Okamoto et al., 2005).

Many cytokines have been implicated in tumor immune suppression, of which TGF-ß is one of the most prominent. This cytokine has a dual role in cancer development as it inhibits proliferation of normal cells, but its expression is associated with immunosuppression in cancers; TGF-ß promotes the generation and maintenance of Tregs, but can also directly

suppress T and NK cell anti-tumor immune responses (Wan and Flavell, 2007). Besides TGF-ß, several other suspected microenvironmental molecular culprits have been implicated in tumor immune escape, including IL-10 and prostaglandin E2 (Rabinovich et al., 2007).

Moreover, the relatively hypoxic environment within the tumor can in itself suppress immune responses, e.g. through the accumulation of local adenosine. The recruitment of activated Tregs to the tumor tissue can further increase adenosine levels through the action of the membrane bound enzymes CD39 and CD73 as discussed previously, and contribute to immune suppression by adenosine receptor signaling (Antonioli et al., 2013b). Interestingly, many tumors have also been shown to express these enzymes (Antonioli et al., 2013b; Bastid et al., 2013), which have been linked to higher stage tumors and poor prognosis (Kunzli et al., 2011; Stella et al., 2010). Furthermore, the metabolism of ATP to adenosine may also contribute to tumor immune escape by the decreased engagement of ATP receptors such as $P2Y_2$ and $P2RX_7$ on APCs and thereby reducing the activation of these cells (Bastid et al., 2013).

Yet another way to achieve immunosuppression is through the engagement of negative co-stimulatory pathways. CTLA-4, discussed previously, is one such molecule that can be expressed by tumor cells and has been shown to induce apoptosis in target cells (Contardi et al., 2005). Furthermore, the interactions of programmed death receptor 1 (PD-1) and programmed death receptor ligand 1 (PD-L1) have also been implied in this context, where PD-L1 expression on tumor cells show a strong inverse correlation to patient survival (Blank and Mackensen, 2007). Of note, the importance of these pathways has been emphasized during recent years with the successful development of immunotherapies such as ipilimumab and Nivolumab, targeting CTLA-4 and PD-1/PD-L1 pathways respectively (Hodi et al., 2010; Philips and Atkins, 2015).

1.5.1.4 Regulatory T cells and FOXP3 in human cancers

The induction and/or recruitment of $CD4^+$ Tregs by tumors represent a possible means of tumor immune escape. In concordance with this hypothesis $CD4^+$ Tregs have been observed to increase in the peripheral blood of patients with several types of cancers, and accumulate in tumor tissue and draining lymph nodes (Nishikawa and Sakaguchi, 2010). It is at present unclear whether these cells represent a non-specific increase in the Treg pool, expansion of tumor-specific tTreg or pTreg cells, although antigen-specific Treg clones have been described (Wang et al., 2004). Interestingly, cancer resection has been reported to normalize the elevated Treg levels, indicating that the increase of the Treg population is indeed caused by the cancer (Kono et al., 2006). In tumor draining lymph nodes, one study demonstrated increasing numbers of Tregs the closer the proximity of the tumor (Kawaida et al., 2005). Intratumoral localization of $FOXP3^+$ Tregs has also been shown in several tumor types, but whether these cells are activated T helper cells with transiently induced FOXP3 expression or committed Treg remains to be determined (Adeegbe and Nishikawa, 2013). Indeed the known heterogeneity of human Treg populations is mirrored by the clinical prognostic implications of tumor infiltrating $FOXP3^+$ Tregs, which range from poor to good in a wide collection of studies in different human tumors (reviewed by (deLeeuw et al., 2012)). For example, Curiel and colleagues

demonstrated that ovarian tumor infiltrating Tregs were linked directly to reduced survival, and also illustrated that the chemokine CCL22 secreted by ovarian tumors was responsible for Treg recruitment through the interaction with CCR4 on these cells (Curiel et al., 2004). In contrast, the presence of tumor infiltrating $FOXP3^+$ cells is associated to improved survival in other types of cancer such as colorectal cancer (Salama et al., 2009) and as we have shown urinary bladder cancer (Winerdal et al., 2011). Studies have even reached opposite conclusions regarding the same cancer location; as different papers have reported good, neutral or poor prognostic claim for $FOXP3^+$ TILs in e.g. oral and gastric cancer, and this general diversity appeared independent of antibody clone used or quantification method (deLeeuw et al., 2012). The molecular subtype of tumor could possibly contribute to this diversity, as exemplified by breast cancer where the prognostic significance of $FOXP3^+$ TIL differs between estrogen receptor positive and negative tumors (Mahmoud et al., 2011). It would thus seem that both location and type of tumor influences the clinical impact of $FOXP3^+$ TIL.

Interestingly, recent studies have shown that tumors can also express FOXP3 (Hinz et al., 2007; Karanikas et al., 2008). This expression correlated to IL-10 and TGF-β (Karanikas et al., 2008), and co-culture of naïve T cells with FOXP3-expressing tumor cells from pancreatic carcinoma inhibited T cell proliferation (Hinz et al., 2007). Interestingly, although FOXP3 has been shown to function as a tumor suppressor *in vitro* e.g. by the repression of c-Myc expression (Wang et al., 2009), it is generally associated to bad clinical prognosis and higher risk of metastasis (Triulzi et al., 2013). Different models have been proposed to explain this apparent discrepancy: Tumor related mutations within the FOXP3 protein could abrogate the tumor suppressor effect, FOXP3 could play a dual role by limiting proliferation but supporting tumor progression, or FOXP3 expression could putatively bestow the tumor with immunoregulatory properties, fostering immune escape (Triulzi et al., 2013). Of note, at least the first and last of these alternatives are not mutually exclusive. In conclusion, it appears that FOXP3 is involved on more than one side in tumor-immune interactions, and still much remains to be elucidated regarding its biological implications.

2 AIMS OF THE THESIS

The overall aim of this thesis was to study regulatory T cells in both the physiological context and the pathological cancer setting, in order to better understand their natural characteristics, illuminate their impact disease progression and find potential ways to target them in immunotherapy. The specific aims of each paper were:

Paper I. To map the relationship of *FOXP3* promoter methylation and FOXP3 expression in Treg and conventional $CD4^+$ T helper lymphocytes in order to assess whether this may be used as a marker of committed human Tregs.

Paper II. To investigate the impact of $CD3^+$ and $FOXP3^+$ tumor infiltrating lymphocytes as well as tumor FOXP3 expression on survival in urinary bladder cancer.

Paper III. To examine how the T lymphocyte responses vary with tissue location and with therapy in patients with urinary bladder cancer.

Paper IV. To study the impact of adenosine receptor signaling on T lymphocyte development in general and Treg development in particular in adenosine receptor knockout mice.

3 MATERIALS AND METHODS

Here follows a summary of the materials and methods used. For more detailed descriptions, please refer to the respective papers (I-IV).

3.1 PATIENTS

3.1.1 Patient characteristics (Paper I-III)

In paper I, only buffy coats from male healthy blood donors were used in the methylation analyses to avoid the risk of artefacts due to random X-chromosome inactivation in women.

Paper II and III both encompass urinary bladder cancer (UBC) patients. Paper II is a retrospective study of 37 UBC patients with muscle invasive disease that underwent radical cystectomy at Karolinska University Hospital between the years 1999-2002. The mean age of the included patients was 67 (median 69, range 46-81) at the time of the diagnostic TUR-B and 11 out of 37 patients in this study were female. At the time, neoadjuvant chemotherapy was not routinely given to this group of patients, however, 10 of the patients received postoperative chemotherapy, a fact which was accounted for in the study analyses.

In comparison, paper III is a prospective study of 32 UBC patients with suspected muscle invasive disease at the time of inclusion at one of the collaborating study centers (Umeå University Hospital, Sundsvall-Härnösand County hospital, Gävle Hospital, Falun Central Hospital, Enköping Hospital and Uppsala University Hospital) 2013-2014. The mean age of the included patients in this study was 69.2 years (median 70, range 55-87) and 10 out of 32 patients were female. 7 out of the 15 patients that underwent cystectomy received neoadjuvant chemotherapy prior to cystectomy according to the MVAC (Methotrexate, Vinblastine, Adriamycin and Cisplatinum) routine.

3.1.2 Sentinel node detection and surgical methods

In study III, patient tissue samples were received both at the initial transurethral resection of the bladder (TUR-B) and at cystectomy. At the TUR-B, peripheral blood and cold-cup biopsies from macroscopically healthy bladder tissue were received in addition to tumor samples. Whenever possible, separate tumor fractions were taken from the central part (CP), transitional zone (TZ) and invasive front (IF) of the tumor. At cystectomy, sentinel node detection was carried out preoperatively as previously described (Sherif et al., 2001) by transurethral injection of the radioactive tracer Nanocoll® around the edge of the tumor area, and sentinel nodes (SNs) were identified after excision of the main specimen both *in vivo* and *ex vivo*. An example of a lymph node map from a cystectomized patient in the study is shown in Figure 5. In addition to the tumor-draining sentinel lymph nodes (SNs), non-draining lymph nodes (nSNs), peripheral blood, tumor and macroscopically healthy bladder tissue samples were collected at the time of cystectomy whenever possible.

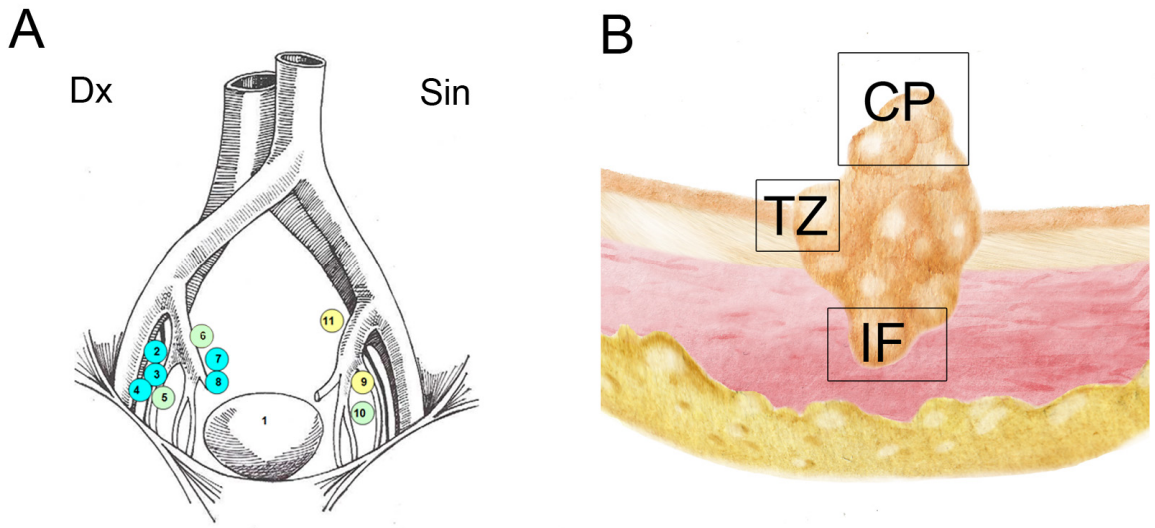

Figure 5. (A) Detection map from a cystectomy in the study. Numbers indicate different specimens: 1: The bladder. 2-4, 7, 8: Sentinel nodes. 9, 11: Non Sentinel nodes. (B) Schematic image of the different tumor sublocations investigated in the study. CP = Central Part, TZ = Transitional Zone, IF = Invasive Front

3.1.3 Patient follow-up

In the retrospective study II, primary and secondary endpoints were overall survival and progression-free survival respectively, where disease progression was defined as local recurrence, appearance of distant metastases or pathologically enlarged lymph nodes in patients that had been regarded as free of disease after cystectomy. The prospective design of study III in combination with the relatively short follow-up time of the patients included, limits the interpretability of any results based on the clinical follow-up parameters at this stage. Therefore the results in this paper are based solely on the clinical parameters at the time of surgery and do not take follow-up into account.

3.2 MICE (PAPER IV)

Five separate mouse genotypes were studied in paper IV: In addition to wild type C57BL/6 (WT), adenosine A_1 receptor knock-out (Johansson et al., 2001), adenosine A_{2A} receptor knock-out (Chen et al., 1999), adenosine A_{2B} receptor knock-out (Csoka et al., 2007) and adenosine A_3 receptor knock-out (Salvatore et al., 2000) animals, all on C57BL/6 background were used. All experiments were approved by Stockholms Norra Djurförsöksetiska Nämnd and carried out in accordance with local institutional guidelines.

3.3 CELL PREPARATION AND CULTURE (PAPER I, III AND IV)

In the human studies, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (paper I) or heparinized peripheral blood (paper III) by density gradient centrifugation (Ficoll-Paque PLUS, Amersham Biosciences). Tissue infiltrating immune cells from tumor and macroscopically healthy bladder tissue were extracted with a GentleMACS homogenizer in AIM-V medium (Gibco, Life technologies) containing Collagenase/Hyaluronidase (StemCell

Technologies). Lymph node leukocytes were extracted by gentle homogenization through a 40µm cell strainer in AIM-V medium. Murine cells from thymi, lymph nodes and spleens were extracted by gentle homogenization and filtration through a 70µm cell strainer.

Magnetic cell sorting (MACS) in combination sorting of immune cell subpopulations on a FACS Aria Cell sorter was used to isolate lymphocyte populations of interest (paper I and III). All sorted cell populations were confirmed >95% pure by FACS.

In paper I, cells were cultured in AIM-V or RPMI 1640 supplemented with 10% human serum, 180U rIL-2, 100µg/mL streptomycin, 100U/mL penicillin and 2mM L-glutamine (all additives from Sigma) and anti-CD3/CD28 Dynabeads (Invitrogen) or anti-CD3 and anti-CD28 antibodies were used to stimulate the cells. In paper III, AIM-V alone was used as culture medium, only L-glutamine was added, and cells were stimulated with tumor extract as described previously (Marits et al., 2006).

3.4 IMMUNOLOGICAL EVALUATION

3.4.1 Flow cytometry (Paper I, III and IV)

For surface staining, leukocyte cell populations of interest were labelled with antibodies as indicated in the respective materials and methods section of paper I, III and IV. Intracellular staining for FOXP3 (paper I and III) or Foxp3 and Helios (paper IV) was performed using the Foxp3 staining buffer set from eBioscience according to the manufacturer's protocol. Acquisition of flow cytometry data was performed on a FACSCalibur or FACSAria (paper I) alternatively an LSRForstessa II (paper III and IV) (all from BD Biosciences). Data analysis was performed with Cell Quest Pro or FACSDiva (paper I) or FlowJo X 10.0.7r2 (paper III and IV) software.

3.4.2 Functional T lymphocyte assays (Paper I and III)

3.4.2.1 Proliferation assays (Paper I)

The suppressive capacity of isolated Treg populations was assessed by co-culture with $CD4^+CD25^-$ responder cells at Treg:Tresponder ratio 1:1-1:4. The cells were stimulated with plate bound anti-CD3 and soluble anti-CD28 antibodies and proliferation evaluated by $[^3H]$Thymidine incorporation after 4-5 days of culture. Alternatively, proliferation was evaluated by dilution of carboxyfluorescein succinimidyl ester (CFSE) staining in the responder population.

3.4.2.2 Flow cytometric assay of cell mediated immune response in activated lymphocytes (Paper III)

In order to assess the responsiveness of cells isolated from the different locations to tumor antigens, $5x10^5$ cells from peripheral blood or lymph nodes were cultured in U-bottomed 96-well plates with tumor homogenate prepared as described in paper III. As control for tumor specific reactivity macroscopically healthy bladder tissue homogenate was used, and in

addition 5µg/mL of pokeweed mitogen or medium only were included as positive and negative controls respectively.

3.4.3 Immunohistochemistry (Paper II)

Serial, 4µm sections of paraffin embedded tumor samples were mounted on glass slides and incubated at 60ºC for 1h before the sections were deparaffinized in xylene and subsequently rehydrated in an ethanol dilution series. Heat induced epitope retrieval was performed in a microwave oven in Citrate buffer pH 6.0 (for CD3 staining) or Tris-EDTA buffer pH 9.0 (for FOXP3) for 20 min. Blocking was performed with diluted serum from the host species of the secondary antibody (goat). Mouse anti human CD3 antibody (clone PS1; Vector Laboratories, dilution 1:200) and mouse anti human FOXP3 (clone 259D; BioLegend, dilution 1:100) were used as primary antibodies, diluted in PBS with 1% bovine serum albumin and 0.05% Tween-20. Subsequently, the VECTASTAIN Elite ABC kit (Vector Laboratories) was used according to the manufacturer's instructions. Biotinylated goat anti-mouse IgG (Vector Laboratories) was used as a secondary antibody (dilution 1:200). In parallel, stainings without the primary antibody were performed as negative control and sections of human spleen were used as positive control. All washing steps were done in PBS with 0.05% Tween-20. Slides were developed in 3, 3' diaminobenzidine solution (Vector Laboratories) and counterstained with Mayer's hematoxylin before mounting in Pertex solution (HistoLab).

For analysis, three separate photographs of high power fields (X40) of randomly selected tumor areas were taken in corresponding areas of consecutive slides for CD3 and FOXP3 stainings respectively. The number of $CD3^+$ and $FOXP3^+$ tumor infiltrating lymphocytes (TILs) was quantified, and in addition, FOXP3 expression in tumor cells was defined as positive if >25% of the tumor cells were positively stained. Immunohistochemical evaluation was performed by two independent researchers blinded to clinicopathological data.

3.4.4 Polymerase chain reaction (PCR) (Paper I)

For quantitative real time PCR, total RNA was extracted using the RNA Mini kit (Bio-Rad) alternatively TRIZOL reagent (Invitrogen) according to the manufacturers' protocols. Conversion to cDNA was performed with the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed with iQSYBR Green Supermix (Bio-Rad) on an iCyclerIQ Real Time Detection System (Bio-Rad), and data analyzed with iCycler IQ™ Optical System Software Version 3.1 (Bio-Rad). The reliability of different commonly used housekeeping genes has been questioned, especially with regard to their stability in activated cell populations (Bas et al., 2004; Radonic et al., 2004). With this in mind, we chose *RNA polymerase II* (*RPII*) as a housekeeping gene since it has a documented stability in many cell types including lymphocytes (Radonic et al., 2004). Expression levels were normalized to the housekeeping gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For regular PCR, ThermoPol reaction buffer and Taq DNA polymerase (New England Biolabs) were used on a MyCycler Thermal Cycler (Bio-Rad). Primers, with sequences specified in paper I, were obtained from Cybergene.

3.4.5 DNA methylation analysis (Paper I and III)

3.4.5.1 Bisulphite sequencing (Paper I)

DNA from sorted cell populations was isolated with the DNeasy Blood & Tisse Kit (QIAGEN), and bisulphite conversion of all non-methylated cytosine bases in the DNA to uracil was carried out with the EZ DNA Methylation Kit (ZYMO Research) according the manufacturers' protocols. Subsequently, the *FOXP3* promoter region was amplified by PCR and the PCR product cloned into a pCR®4-TOPO® vector using a TOPO TA Cloning® Kit (Invitrogen). DNA from individual bacterial clones was sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with T3 and T7 primers according to the manufacturer's instructions, and run on a 310 Genetic Analyzer (Applied Biosystems). Sequence data was analyzed with ABI Prism Sequencing Analysis Software 3.7 (Applied Biosystems).

3.4.5.2 Combined bisulphite restriction enzyme analysis (COBRA) (Paper I)

Bisulphite converted DNA, prepared as described above, was used as template to amplify the *FOXP3* promoter region with an unconjugated forward primer and a 5' 6-FAM conjugated reverse primer. The PCR product was treated with the restriction enzyme Aci1 in excess, which would cleave the PCR product only if the -77 cytosine in the FOXP3 promoter in original template was methylated and thus conserved through the bisulphite conversion. The digested DNA product was isolated with a phenol-chloroform extraction and run on a 310 Genetic Analyzer (Applied Biosystems) where fragment length was analyzed. Gene Scan v3.7 application software (Applied Biosystems) was used to analyze the data.

3.4.5.3 Pyrosequencing (Paper III)

DNA isolation and bisulphite conversion was carried out with the EZ DNA methylation Direct kit (Zymo Research). The *FOXP3* target sequence was amplified with biotinylated primers (Thermo Scientific and Biomers.net) and pyrosequenced on a PSQ96 Pyrosequencer (Qiagen) with Pyromark Gold 96Q reagents (Qiagen) and sequence primers. PyroMark CpG Software 1.0.11 (Qiagen) was used to analyze the resulting data sequences.

3.5 BONE MARROW TRANSFER EXPERIMENT (PAPER IV)

Bone marrow cell suspensions in sterile PBS were prepared from donor knockout mice and $5x10^6$ cells injected in the tail vein of irradiated (900 rad) WT C57BL/6 recipients. Recipient mice received prophylactic antibiotics (neomycin sulphate) for two weeks after the transplant. Resulting immunophenotypes were evaluated after 8 weeks.

3.6 STATISTICAL ANALYSIS (PAPER I-IV)

Categorical data (paper I) was analyzed with Fisher's exact test. Survival data was related to categorical predictor variables with the log-rank test and Cox's proportional hazards model (paper II). Shapiro-Wilk's test of normality was used to evaluate if a particular data set was parametric or not (paper III and IV). For parametric data, two-sided T-test or one-way ANOVA

was used, whereas the Mann-Whitney U test or Kruskall-Wallis ANOVA with Dunn's test was used for non-parametric datasets (paper III and IV). Statistical calculations were done in Statistica (StatSoft, Inc. (2013). STATISTICA (data analysis software system), version 12. www.statsoft.com) or GraphPad Prism (Version 5.04). P-values <0.05 were considered significant.

4 RESULTS AND DISCUSSION

4.1 *FOXP3* PROMOTER DEMETHYLATION REVEALS THE COMMITTED TREG POPULATION IN HUMANS (PAPER I)

In the years preceding this study, many Treg markers had been suggested, several of them useful in an unchallenged setting but less specific in inflammatory environments as they were also upregulated in conventional $CD4^+$ T lymphocytes upon activation as discussed in section 1.2. When FOXP3 was first discovered as the master transcription factor of the Treg lineage it was initially considered a specific Treg marker in both mice and humans (Sakaguchi et al., 2007). However, before long, reports came of transient FOXP3 induction in human activated conventional $CD4^+$ T lymphocytes (Walker et al., 2003; Wang et al., 2007) making them impossible to distinguish from Treg based on their protein expression alone. This prompted us to ask whether Treg may be distinguished from conventional $CD4^+$ T lymphocytes by the epigenetic signature of the *FOXP3* gene. We identified a CpG rich region within the conserved *FOXP3* promoter region and hypothesized that differential methylation of these sites may account for the differential stability of FOXP3 expression in $CD4^+$ T lymphocytes.

Human $CD4^+CD25^{hi}$ Tregs displayed a demethylated *FOXP3* promoter (1.4%±0.95% SEM methylated) in contrast to $CD4^+CD25^{lo}$ T lymphocytes, which were partially methylated (27.9%±7.1%), and to B cells which were fully methylated (93.8%±1.7%). This was in concordance with parallel study by Kim et al. describing the methylation status of the murine *Foxp3* promoter (Kim and Leonard, 2007). In addition, the promoter appears to have a more accessible chromatin configuration in Tregs compared to conventional T lymphocytes, as indicated by increased histone acetylation in this region (Mantel et al., 2006), further strengthening the role of epigenetic control over FOXP3 transcription.

Furthermore, we examined the methylation status of the FOXP3 promoter in sorted $CD25^{hi}$ and $CD25^{lo}$ cell populations during the course of activation of $CD4^+CD25^{lo}$ T lymphocytes (Paper I, Figure 6A). Interestingly, there was an initial difference in methylation status in the sorted populations during early activation, but over time this difference leveled off at approximately 60% demethylation. One could speculate that the initial difference in methylation between the CD25hi and CD25lo populations during activation may reflect a small contaminating resting $CD45RA^+$ Treg subset in the starting population. This population has a demethylated *FOXP3* promoter region, lower CD25 and FOXP3 expression, but higher proliferative capacity than their $CD45RA^-$ Treg counterparts (Miyara et al., 2009; Valmori et al., 2005), and could potentially respond faster to activation than conventional $CD4^+$ T lymphocytes completely lacking CD25 expression at the start of the culture. At later time points, both the $CD25^{hi}$ and the $CD25^{lo}$ population are more likely to consist of cells of mixed origin, possibly explaining why the initial difference gradually disappears. Still, the *FOXP3* promoter in $CD4^+CD25^{hi}$ T lymphocytes sorted *ex vivo* remained demethylated and these cells stayed $FOXP3^+$ throughout the culture. In contrast, although a limited decrease in *FOXP3* promoter methylation was observed in the stimulated $CD4^+CD25^{lo}$ T lymphocytes, it remained partially methylated corresponding to the transient FOXP3 expression observed in these cells. Of note, murine

$CD4^+CD25^-$ T lymphocytes reportedly increase their promoter methylation upon TCR stimulation in the absence of exogenous TGF-β, possibly preventing the induction of Foxp3 expression in these cells (Kim and Leonard, 2007), and potentially contributing to the differences in FOXP3 expression pattern observed in human and murine conventional $CD4^+$ T lymphocytes.

In addition, we investigated the effects of various culture conditions such as addition of TGF-β and/or IL-10, on *FOXP3* promoter methylation. Of note, no additional effects of these substances on promoter demethylation were observed. In contrast, addition of TGF-β during activation of murine naïve $CD4^+$ T lymphocytes has been demonstrated to induce demethylation of the murine *Foxp3* promoter as well as partial demethylation of the CNS3 intronic region (Floess et al., 2007; Kim and Leonard, 2007). These results could indicate that TGF-β is more proficient at inducing a Tregs in mice consistent with *in vitro* findings (Chen et al., 2003; Tran et al., 2007). However, they may also reflect different responsiveness to low levels of TGF-β in humans versus mice, as the neutralization of this cytokine in the culture medium reportedly blocks the induction of transient FOXP3 expression in naïve human $CD4^+CD25^-$ T lymphocytes (Tran et al., 2007).

The unique FOXP3 promoter methylation profile in Tregs compared to conventional $CD4^+$ T lymphocytes suggested that a demethylated pattern is a prerequisite for stable FOXP3 expression and suppressive phenotype. This was one of the first reports regarding the epigenetic regulation of FOXP3 transcription. Since then, with expanding knowledge and technological advances within the field of epigenetics, extensive mapping of the chromatin landscape and methylation pattern in Treg versus conventional $CD4^+$ T lymphocytes has deepened our understanding of the epigenetic control of the Treg lineage (Morikawa and Sakaguchi, 2014). Although most studies have been performed on mice, it is now clear that epigenetic modifications are crucial in Treg development, and currently represent the most specific marker of commitment to the Treg lineage available (Sakaguchi et al., 2013).

4.2 FOXP3 AND SURVIVAL IN URINARY BLADDER CANCER (PAPER II)

Urinary bladder cancer (UBC) is a malignancy highly associated to inflammation during its development and also harbors great potential for immunotherapeutic strategies as illustrated by the long-standing tradition of successful BCG treatment of superficial UBC (Herr and Morales, 2008). In study II, the prognostic significance of tumor infiltrating $CD3^+$ and $FOXP3^+$ lymphocytes as well as tumor expression of FOXP3 was evaluated in a retrospective material of 37 patients with muscle invasive UBC.

In accordance with previous studies in UBC and other malignancies, the presence of $CD3^+$ tumor infiltrating lymphocytes (TIL) in the tumor was a positive prognostic factor for survival in our study (Galon et al., 2006; Lipponen et al., 1992; Zhang et al., 2003). This supports the notion that T lymphocytes are important players in the anti-tumor immune response to UBC and validates further studies to better understand how to potentiate this response in patients.

Somewhat to our surprise, FOXP3$^+$ TIL were also associated to a better prognosis. In other malignancies, the results vary as to the prognostic significance of this population where some report a negative association to prognosis (e.g. hepatocellular cancer) and others positive correlations (e.g. colorectal cancer) (Gao et al., 2007; Salama et al., 2009). The fact that FOXP3 is induced upon activation in conventional T helper lymphocytes could be one explanation for this apparent paradox, but it is also conceivable that anti-inflammatory effects may be beneficial to the patient in contexts where the inflammation as such acts to promote tumor progression (deLeeuw et al., 2012).

In addition, we observed a distinct expression of FOXP3 in tumor cells in a subgroup of the patients (17/37) (Figure 6). Coherent with reports from other malignancies, this expression was not strictly confined to the nucleus, but also observed in the cytoplasm in some cases (Merlo et al., 2009). We found a long-term negative correlation between FOXP3 expression in tumor cells and survival, a finding that did not seem to be affected by the subcellular localization of the FOXP3 protein. This negative prognostic impact of FOXP3 expression by tumor cells has also been noted in several other forms of cancer, but the mechanisms behind this remains unclear (Triulzi et al., 2013).

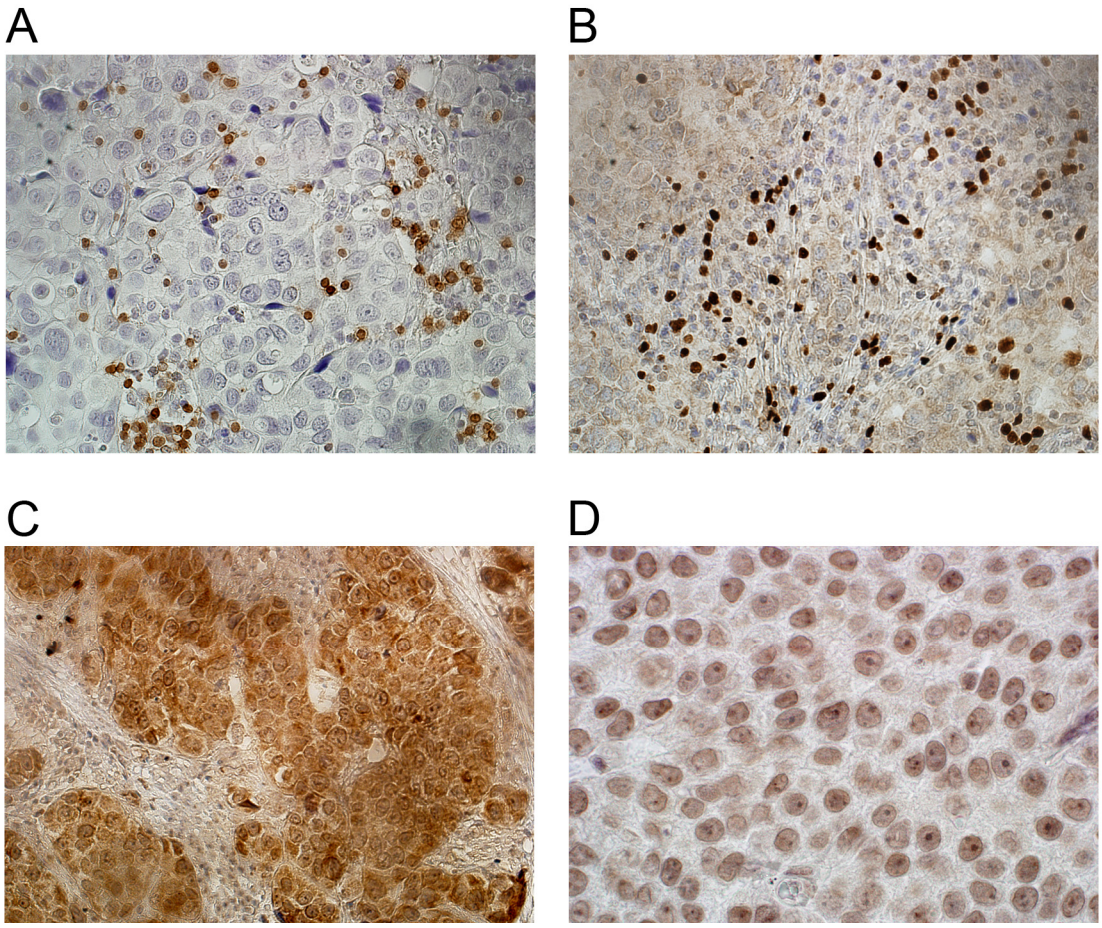

Figure 6. Immunohistochemical evaluation of UBC tumors. (A) Tumor infiltrating lymphocytes expressing (A) CD3 or (B) FOXP3 (40x magnification). (C) 40x magnification of FOXP3 expression in tumor cells. (D) 100x magnification of FOXP3$^+$ tumor cells with nuclear staining.

In conclusion, tumor infiltrating T lymphocytes are indeed important for patient outcome in urinary bladder cancer, as both $CD3^+$ and $FOXP3^+$ TIL were positively correlated to survival. The negative impact of tumor FOXP3 expression in urinary bladder cancer warrants further investigation to elucidate the biological impact of this expression on the tumor cells themselves as well as the consequent interactions with the immune system.

4.3 ANTI-TUMOR IMMUNE REPONSES ARE SHAPED BY LOCATION AND THERAPY (PAPER III)

As study II indicated a clear role for T lymphocytes in determining the prognosis of the disease in UBC, we set out to investigate the T lymphocyte immune response in more detail in a prospective study. In order to illuminate the effects of tumor-immune interactions on the T cell compartment at multiple levels, study III examines the immunophenotype of T lymphocytes in peripheral blood, tumor draining lymph nodes (sentinel node, SN), non-draining lymph nodes (non sentinel node, nSN) as well as tumor and macroscopically healthy bladder tissue.

We demonstrate that the $FOXP3^+$ fraction of $CD4^+$ T lymphocytes was significantly increased in TIL populations as compared to all other locations. In addition, these $CD4^+FOXP3^+$ TIL T lymphocytes displayed high levels of the Treg effector markers surface CTLA-4 and CD39 as well as the activation and memory markers CD69, HLA-DR and CD45RO. Interestingly, this activated phenotype was to some extent mirrored by both $CD4^+FOXP3^-$ and $CD8^+$ TIL T lymphocytes. Furthermore, methylation analysis of tumor $CD4^+$ TIL revealed a highly methylated *FOXP3* promoter region despite the pronounced FOXP3 expression in these cells, in contrast to the $CD4^+$ T lymphocyte population in peripheral blood where FOXP3 expression seemed to correlate better with the methylation level observed. Just as the previous study, these results indicate that the $FOXP3^+$ TIL populations may represent an activated T lymphocyte subpopulation rather than committed Tregs. It is possible that the differing prognostic impact of $FOXP3^+$ TILs in human cancers could relate to this finding (deLeeuw et al., 2012). Active recruitment of Treg to the tumor has been implied in other forms of cancer such as ovarian cancer, and were then associated to negative prognosis (Curiel et al., 2004). Notably, a recent study of tumor infiltrating Tregs in head and neck cancer, where this population also has been linked to worse prognosis (Sun et al., 2012), described elevated levels of immunosuppressive markers which correlated to more potent suppressor activity of these cells (Jie et al., 2013). Future studies comparing the epigenetic commitment and suppressive capacity of this subset in cancers with differing prognostic impact may help decipher the role of $FOXP3^+$ TIL in humans.

Since the immune infiltrates in tumors have been noted to differ also within the tumor itself (Fridman et al., 2012), we examined different tumor sublocalizations at the initial TUR-B. Interestingly, the invasive front (IF) of the tumor stood out with higher $CD8^+$ T lymphocyte fraction compared to the central part (CP) or transitional zone (TZ) of the tumor. Furthermore, the IF also had a lower $FOXP3^+$ fraction of $CD4^+$ T lymphocyte in patients with muscle invasive disease. Thus again, higher FOXP3 appears to associate to better prognosis (lower

stage). Whether this association is linked to FOXP3 as an activation marker or directly to some immunomodulatory effect of these $FOXP3^+$ cells remains to be investigated.

In addition to direct anti-tumor effects, chemotherapy is known to potentiate immune responses by the induction of immunogenic cell death in the tumor (Krysko et al., 2012), and furthermore, different chemotherapeutic agents have been demonstrated to exert direct immunostimulatory effects (Maccubbin et al., 1992; Markasz et al., 2008). Interestingly, patients that had received chemotherapy in this study had higher lymph node T lymphocyte fractions compared to patients that had not received such therapy. Moreover, the neoadjuvant treated patients displayed higher T lymphocyte anti-tumor reactivity in their lymph nodes, as assessed with a flow cytometry based assay evaluating the proportion of blasting T lymphocytes to certain stimuli. Of note, a corresponding difference was not observed in the peripheral blood from these patients, in agreement with the hypothesis that the draining lymph nodes are the most probable sites for an anti-tumor response to be elicited.

The prospective study design limits the conclusions that can be drawn from clinical parameters at this time due to at present relatively short follow-up time (3-18 months). In the future, it will be interesting to follow the disease in these patients and correlate clinical outcomes to the immunological parameters investigated.

We conclude from this study that anti-tumor T lymphocyte responses are shaped by the environment in the different T lymphocyte compartments investigated, where T lymphocytes in lymph nodes and tumor display higher levels of both activation and effector suppressor markers. The greater anti-tumor reactivity in regional lymph nodes following chemotherapy suggests a future for therapeutic combinations of chemotherapy with adoptive T cell based immunotherapy of cancer.

4.4 ADENOSINE RECEPTOR SIGNALING AFFECTS THE DEVELOPMENT OF TREG AND CONVENTIONAL T LYMPHOCYTES (PAPER IV)

Given the recognized pronounced effects of adenosine receptor signaling in both tumor pathology and the immune system at large, we were interested in examining the impact of these receptors on the T lymphocyte compartment in mice, in order to set the base line for future studies in murine tumor models. Thus, in study IV, the influence of each individual adenosine receptor on the central and peripheral T lymphocyte compartments was evaluated in adenosine receptor knockout mice ($A_1R^{-/-}$, $A_{2A}R^{-/-}$, $A_{2B}R^{-/-}$ and $A_3R^{-/-}$).

Immunophenotyping was performed on thymus, spleen and lymph nodes, and compared to WT controls. In the thymus, there was an overall effect of knocking out any one of the four adenosine receptors, resulting in decreased fractions of CD4 and CD8 single positive thymocytes. The most pronounced effects were observed in $A_{2B}R^{-/-}$ mice, which displayed a prominent reduction in cell yield from the thymus as well as alterations in relative and absolute numbers of thymic subpopulations. Previous studies in mice have implicated a role for $A_{2A}R$ signaling in the induction of apoptosis in DP thymocytes, but interestingly also depicted an $A_{2A}R$ independent role for adenosine signaling in inhibition of TCR induced activation of

thymocytes (Apasov et al., 2000). Furthermore, although the $A_{2B}R$ has the highest adenosine concentration threshold for induction intracellular of cAMP signaling, it has been shown to affect MAPK-signaling already at physiological adenosine concentrations (Schulte and Fredholm, 2000; Schulte and Fredholm, 2003a). In relation to the pronounced thymic phenotype of $A_{2B}R^{-/-}$ mice, it is thus interesting to note that MAPK-signaling is essential during thymic selection and development of thymocytes (McNeil et al., 2005; Palmer, 2003). Since all four adenosine receptors can affect signaling via the MAPK pathway (Schulte and Fredholm, 2003b), it is possible that alterations in the balance of these receptors may explain their apparent non-redundant functions in thymic selection in our study. Interestingly, BM transfer to lethally irradiated WT hosts did not restore normal SP thymocyte frequencies, indicating that the changes observed were not attributable to the radioresistant stromal cells.

The thymic $CD4^+Foxp3^+Helios^+$ Treg population was also addressed and not surprisingly found to be decreased in number, given the reduced frequency of CD4SP thymocytes in all of the knockouts. Only the $A_1R^{-/-}$ animals stood out with a reduced fraction of $Foxp3^+Helios^+$ CD4SP thymocytes, which normalized after bone marrow transfer to WT hosts, suggesting that adenosine A_1R signaling in the thymic stromal cells may account for this part of the $A_1R^{-/-}$ phenotype. This is in line with the fact that most studies describe very low expression level of the A_1R in T lymphocytes (Bours et al., 2006).

In the periphery, again the $A_{2B}R^{-/-}$ animals stood out with reduced splenetic cell yield and T cell fraction as well as an altered CD4/CD8 ratio. The peripheral Treg compartment was also affected in these animals with the $Foxp3^+Helios^+$ fraction of $CD4^+$ T lymphocytes found to approximately double of that found in WT mice. Of note, we observed a distinct $Foxp3^-Helios^+$ $CD4^+$ T lymphocyte population, particularly in the spleen of $A_{2B}R^{-/-}$ mice. In light of, the marked reduction in thymic cell count and reports linking Helios expression to activation and proliferation (Akimova et al., 2011), one could speculate that this population represents a peripheral expansion of T lymphocytes to compensate for the reduced thymic output. Interestingly, after bone marrow transfer to WT hosts, $A_{2B}R^{-/-}$ T lymphocytes displayed normal CD4/CD8 ratio and peripheral T lymphocyte fractions, as well as an almost normalized $Foxp3^+Helios^+$ Treg fraction. This would suggest that adenosine $A_{2B}R$ signaling in the WT environment is largely able to rescue the peripheral T lymphocyte phenotype of $A_{2B}R^{-/-}$ T lymphocytes, although the reduction in CD4SP thymocytes remained.

It is clear that adenosine signaling contributes to the control of peripheral T lymphocyte responses via Treg dependent and independent mechanisms (Antonioli et al., 2013a). This study illustrates that also the thymic development of T lymphocytes is affected by abrogation of either one of the four adenosine receptors. In light of these findings, a more detailed examination of the functional consequences of adenosine receptor deletion in both Treg and conventional T lymphocyte subsets would be of great interest.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Tregs are vital in the maintenance of peripheral self-tolerance and play a pivotal role in cancer immunology as demonstrated by the prognostic significance of these cells in several forms of cancer. The intriguing, and at first glance contradictory findings regarding the prognostic impact of $FOXP3^+$ tumor infiltrating T lymphocytes, underscore the heterogeneity of this population and the importance of considering the whole immune contexture of the tumor (Fridman et al., 2012). Transient expression of FOXP3 in human activated T lymphocytes as well as the existence of FOXP3 splice variants in humans that are not present in mice deepen the complexity further and highlights the necessity to validate findings from murine models in humans.

Our studies suggest that in the case of urinary bladder cancer, tumor infiltrating $FOXP3^+$ $CD4^+$ T lymphocytes do not represent committed tTreg, but rather a $CD4^+$ conventional T lymphocyte population with induced expression of FOXP3. Still, FOXP3 expression in these cells is associated to high surface expression of effector Treg markers such as CD39 and CTLA-4, which indicates that these cells do harbor suppressive function. Since urinary bladder cancer development has been linked to chronic inflammation (Thompson et al., 2014), it may be that the positive prognostic impact associated with $FOXP3^+$ TILs relates to suppression of this inflammatory response. Alternatively, given the methylated state of the *FOXP3* promoter in these cells and the transient expression of FOXP3 upon activation in conventional $CD4^+$ T lymphocytes, one could speculate that the observed FOXP3 expression mirrors a higher level of activation and thus anti-tumor responsiveness. Ongoing studies in our laboratory are currently addressing the functional suppressor capacity and expression profile of these tumor infiltrating Tregs. In light of the expanding view on T helper lineage plasticity (Weinmann, 2014), it will also be of great interest to assess the cytokine profile and possible expression of transcription factors other than FOXP3 within this cell population.

Increased understanding of the mechanisms that govern stable FOXP3 expression and a suppressive Treg phenotype, is instrumental for the development of future therapies targeting the Treg population. Multiple murine studies have illustrated the value of Treg depletion to augment anti-tumor immune responses in different cancer models, and in humans there are currently clinical trials underway that target this population by various mechanisms (Nishikawa and Sakaguchi, 2014), which stresses the importance to advance knowledge concerning the clinical impact and implications of Tregs in cancer. Furthermore, the same issues become of importance at the opposite end of the clinical spectrum, when attempting to harvest and expand Tregs for immunotherapy of autoimmunity. The evaluation of stable Treg commitment as opposed to the expression of FOXP3 in activated effector T lymphocytes, assessed by e.g. methylation analysis, is likely to be essential for the development of optimal expansion protocols.

As demonstrated by us and others (Jie et al., 2013), both Treg and conventional T lymphocyte populations appear to upregulate immunosuppressive markers in the context of the tumor. With this in mind, it is interesting to note the successful introduction of therapeutics designed to target co-inhibitory pathways such as ipilimumab (Hodi et al., 2010). However, the limited response rate combined with the autoimmune adverse effects of these treatments leave room for improvement (Weber et al., 2012). Since many pathways converge to elevate adenosine levels within the tumor, which in turn has been associated with immune suppression, modulation of adenosine signaling pathways represent a promising area of therapeutic development. Such approaches include CD39/CD73 inhibitors and $A_{2A}R$ antagonists that potentially could tip the balance between Treg and T effector cells in favor of an anti-tumor immune response (Bastid et al., 2013; Ohta and Sitkovsky, 2014).

The immune effects of different conventional therapeutic regimens such as chemotherapy should also be considered in the evaluation of anti-tumor immune responses, as illustrated by study III in this thesis. Other studies have noted direct immunostimulatory effects of certain chemotherapeutic drugs in addition to their direct anti-tumor effects and promotion of immunogenic cell death (Krysko et al., 2012; Maccubbin et al., 1992; Markasz et al., 2008). In the clinical setting, further characterization of each of these different routes is essential to allow optimization of therapeutic protocols designed to promote the anti-tumor effects combined with the pro-immune effects as much as possible.

In addition to immunotherapies aiming to enhance the anti-tumor immune response *in vivo*, multiple studies describing adoptive cell therapy of cancer have been reported (Dudley et al., 2005; Karlsson et al., 2010; Qian et al., 2014; Sherif et al., 2010). Current adoptive immunotherapeutic strategies with *in vitro* expanded anti-tumor T lymphocytes have shown much promise but are cumbersome and still have a limited response rate in patients, possibly in part due to immune escape mechanisms employed by the tumor. It is tempting to speculate that the combination of adoptive T cell therapy with immunomodulatory strategies such as those discussed above may represent ways to overcome this local immune suppression.

The immune system hosts great potential in the fight against cancer. Recent years have seen the emergence of many successful immunotherapies and undoubtedly more are still to come. All the same, a lot remains to be elucidated in the intricate interplay between tumor and immune cells. Further clarification of the immune contexture of human tumors will pave the road for new immunotherapeutic strategies and optimization of current clinical treatments to promote anti-tumor immunity.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

I människokroppen pågår en ständig kamp för överlevnad där immunförsvaret kämpar för att försvara kroppens egna vävnader mot olika typer av faror såsom bakterier och virus. Samtidigt är förmågan att särskilja kroppens egna celler av yttersta vikt då oförmåga att upprätthålla tolerans mot dessa kan leda till immunattack mot kroppsegna organ, så kallad autoimmunitet. Cancerceller har förvärvat förändringar i sin arvsmassa, DNA, som dels ligger till grund för sjukdomsutvecklingen, men som också betyder att dessa celler uttrycker proteiner som till viss del är kroppsfrämmande. Dessa förändringar kan kännas igen av immunförsvaret som i det bästa scenariot identifierar förändrade celler redan på ett tidigt stadium och eliminerar dessa innan någon klinisk sjukdom hunnit bryta ut. Framgångrika tumörer utvecklar därför olika mekanismer för att undvika igenkänning.

T celler är centrala spelare i både det specifika adaptiva immunförsvaret mot cancer och i upprätthållandet av tolerans mot kroppens egna vävnader. De så kallade regulatoriska T cellerna är essentiella för balansen i kroppens immunförsvar och kontrollen av det inflammatoriska svaret. Dessa celler karaktäriseras till stor del baserat på deras uttryck av proteinet FOXP3. I människor kompliceras dock studierna av dessa celler av att vanliga aktiverade T celler också visat sig kunna uttrycka detta protein. Den första studien i denna avhandling handlar om regleringen av FOXP3 uttryck i T celler. Här studerar vi så kallad epigenetik, vilket handlar om förändringar av kroppens arvsmassa som kan påverka proteinuttrycket i cellen, men som inte påverkar själva DNAsekvensen. En sådan förändring är tillägg av metylgrupper på DNAt (metylering). Generellt kan man säga att gener med en hög grad av metylering är mindre aktiva än ometylerade motsvarigheter. I vår första studie visar vi att regulatoriska T celler som kontinuerligt uttrycker FOXP3, har en ometylerad konfiguration i början av genen som kodar för detta protein, till skillnad från de vanliga T cellerna som är halvmetylerade i detta område. Som kontrast är B celler, som inte alls uttrycker FOXP3 helt metylerade. Vår slutsats från denna studie var att metyleringsgraden av *FOXP3* genen kan förutspå ett kontinuerligt eller övergående uttryck av proteinet och således kan användas som en markör för stabila regulatoriska T celler.

I nästa steg undersökte vi vilken effekt tumörinfiltrerande regulatoriska T celler har på den kliniska prognosen vid urinblåsecancer. Intressant nog fann vi att både T cellsinfiltration generellt och T celler som uttryckte proteinet FOXP3 förutspådde bättre prognos för patienterna. Detta kan tyckas motsägelsefullt då regulatoriska T celler generellt anses trycka ned det tumörspecifika immunförsvaret och således borde vara associerade till sämre prognos. I nuläget har dock många rapporter publicerats där betydelsen av tumörinfiltrerande regulatoriska T celler undersöks i olika former av cancer och det är tydligt att den prognostiska betydelsen kan variera alltifrån god till dålig. Orsakerna till detta är i nuläget inte helt klarlagda, men det tycks som om både tumörtyp och lokalisation kan spela en roll. Det har spekulerats att den goda prognoskopplingen i vissa cancerformer har att göra med kontroll av den kroniska inflammation som annars kan agera som en drivande faktor i tumörutvecklingen, alternativt skulle detta FOXP3 uttryck kunna vara en reflektion av allmän T cellsaktivering i

konventionella T celler. Intressant nog noterade vi även ett uttryck av FOXP3 i tumörceller hos en andel av patienterna. Detta uttryck var kopplat till sämre klinisk prognos, men de bakomliggande mekanismerna till detta är fortfarande oklara.

För att vidare undersöka hur vanliga och regulatoriska T celler blir påverkade av tumören i urinblåsecancer, undersökte vi dessa cellpopulationer från blod, lymfknutor, och tumör med flödescytometri. De tumörinfiltrerande T cellerna generellt, men särskilt de regulatoriska T cellerna uppvisade en mycket hög aktiveringsgrad och uttryckte höga nivåer av proteiner som är aktiva vid nedreglering och kontroll av immunsvar. Intressant var också att vi fann skillnader i sammansättningen av de tumörinfiltrerande cellerna i olika delar av tumören, där den invasiva kanten stod ut i jämförelse med centrala delar av tumören. Vi fann att patienter med muskelinvasiva tumörer hade lägre andel FOXP3 uttryckande T celler vid den invasiva kanten jämfört med ytligt växande tumörer. Intressant nog visade sig de tumörinfiltrerande T cellerna vara till hög grad metylerade i sin *FOXP3* gen, vilket tyder på att dessa celler inte representerar en dedikerade regulatoriska T celler utan snarare T celler med ett aktiveringsrelaterat uttryck av FOXP3. Dessutom noterade vi en tydlig effekt av genomgången cellgiftsbehandling på immunsvaret mot tumören, där denna behandling tycktes potentiera förmågan av T celler från tumördränerande lymfknutor att reagera på tumörproteiner.

En metod som regulatoriska T celler utnyttjar för att kontrollera immunreaktioner är produktion av adenosin som i sig är immunhämmande i närmiljön. Det har visat sig att vissa tumörer också aktivt producerar adenosin, som kan hjälpa dessa att kontrollera ett eventuellt immunsvar mot tumören. För att studera vilka effekter adenosin har på T celler använde vi oss av så kallade knockoutmöss som saknar endera av de fyra definierade receptorerna för adenosin. I dessa möss karaktäriserade vi sedan T cellernas utveckling från deras utbildning i thymus (brässen) till deras sammansättning i mjälte och lymfknutor. Vi fann att adenosinsignalering tycks bidra till T cellsutveckling med både T cellsberoende och oberoende mekanismer. Vidare studier behövs för att utvärdera den funktionella betydelsen av dessa fynd.

Sammanfattningsvis har dessa studier påvisat en epigenetisk signatur hos mänskliga regulatoriska T celler som kan användas för identifiering av denna population i patienter. Genom studier av denna cellpopulation hos patienter med urinblåsecancer har vi börjat kartlägga en del av de mekanismer som påverkar T cellssvaret och fortsatt prognos hos dessa patienter. Studier av T cellsutvecklingen i möss som saknar specifika adenosinreceptorer har belyst betydelsen av adenosinsignallering i thymus. Bättre förståelse för hur tumören interagerar med kroppens immunförsvar i patienter kan i kombination med grundläggande studier av hur regulatoriska system i kroppen fungerar, leda till nya behandlingsstrategier och sätt att väcka det immunförsvar som sövts av tumören.

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