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T Regulatory Cells – Friends or Foes?

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Abstract

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T regulatory cells (Tregs) have been extensively studied in patients with cancer or autoimmunity. These cells hamper the immune system's ability to clear tumor cells in cancer patients. In autoimmune diseases, on the other hand, they are not able to restrain autoreactive immune responses. If we manage to understand Tregs and their role in health and diseases we may be able to develop better immunomodulatory therapies.

Early studies demonstrated that tolerance was maintained by a subset of $CD25^+$ T-cells. CD25 was the earliest marker for Tregs and is still often used to define these cells. Several Treg-associated markers have been suggested throughout the years. However, these markers can be upregulated by activated T-cells as well. The most specific marker for Tregs is currently the transcription factor forkhead box P3 (FoxP3).

In this thesis, we investigated the presence of $CD25^-$ Tregs in patients with B-cell malignancies and in patients with autoimmunity. These cells were identified in both patient groups. Further, patients with B-cell malignancies often have high levels of soluble CD25 (sCD25) in the periphery. In our patient cohorts, the level of peripheral Tregs correlated with the level of sCD25 in patients with lymphoma. Tregs were shown to release sCD25 *in vitro* and sCD25 had a suppressive effect on T-cell proliferation. These data show that Tregs may release CD25 to hamper T-cell proliferation and that this may be an immune escape mechanism in cancer patients.

Previous studies have demonstrated that an increased infiltration of $FoxP3^+$ cells into lymphoma-affected lymph nodes is associated with a better patient outcome. This is in contrast to studies from non-hematological cancers where an increased presence of Tregs is associated with a poor prognosis. Since previous studies have shown that Tregs are able to kill B-cells, we wanted to investigate if Tregs are cytotoxic in patients with B-cell tumors. In the subsequent studies, Tregs from patients with B-cell lymphoma and B-cell chronic lymphocytic leukemia (CLL) were phenotyped to investigate the presence of cytotoxic markers on these cells. FoxP3-expressing T-cells from both patients with CLL and B-cell lymphoma displayed signs of cytotoxicity by upregulation of FasL and the degranulation marker CD107a. Tregs from CLL patients could further kill their autologous B-cells in *in vitro* cultures.

Taken together the studies in this thesis have demonstrated two possible new functions of Tregs in patients with B-cell malignancies and the presence of $CD25^-$ Tregs in both cancer and autoimmunity.

Keywords: Treg, T regulatory cell, FoxP3, CD25, CLL, B-cell lymphoma

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List of Papers

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

I **Lindqvist, CA.,** Christiansson, LH., Simonsson, B., Enblad, G., Olsson-Strömberg, U., Loskog, SIA. T regulatory cells control T-cell proliferation partly by the release of soluble CD25 in patients with B-cell malignancies. *Immunology 2010 May 26 [Epub ahead of print]*

II Fransson, M., Burman, J., **Lindqvist, C.,** Atterby, C., Fagius, J., Loskog, A. T regulatory cells lacking CD25 are increased in MS during relapse. *Autoimmunity 2010 Apr. 7 [Epub ahead of print]*

III **Lindqvist, CA.,** Christiansson, LH., Mangsbo, S., Tötterman, TH., Simonsson, B., Olsson-Strömberg, U., Loskog, SIA. $FoxP3^+$ T-cells in patients with B-cell chronic lymphocytic leukemia express cytolytic markers and kill autologous B-cells. *Manuscript*

IV **Lindqvist, CA.**, Christiansson, LH., Enblad, G., Loskog, SIA. Peripheral T lymphocytes, including $FoxP3^+$ T-cells, exhibit a cytotoxic phenotype in patients with B-cell lymphoma. *Manuscript*

Contents

Abbreviations

3-HAA	3-Hydroxyanthrenilic acid
γc	Common gamma chain
Aa	Amino acid
A2A	Adenosine receptor 2A
AIRE	Autoimmune regulator
AP-1	Activation protein-1
APC	Antigen presenting cell
aTreg	Adaptive Treg
BCR	B-cell receptor
cAMP	Cyclic adenosine monophosphate
CCL22	C-C motif chemokine 22
CD	Cluster of differentiation
CD40L	CD40 ligand
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CSR	Class switch recombination
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
DISC	Death-inducing signaling complex
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FADD	Fas-associated death domain protein
FasL	Fas ligand
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
FoxP3	Forkhead box P3
FSC	Forward scatter
GC	Germinal center
GITR	Glucocorticoid-induced tumor necrosis factor receptor

Gy	Gray
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
iDC	Immature dendritic cell
IDO	Indoleamin 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin 2 receptor
IL-7R	Interleukin 7 receptor
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked
ITAM	Immunotyrosine-based activation motif
iTreg	Induced Treg
JAK	Janus kinase
LAG-3	Lymphocyte activation gene-3
LAMP-1	Lysosomal-associated membrane protein-1
Ly	Lymphocyte antigen
MACS	Magnetic-activated cell sorting
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MAP kinase	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NFAT	Nuclear factor of activated T-cells
NK	Natural killer
nTreg	Natural Treg
PBMC	Peripheral blood mononuclear cell
PE	phycoerythrin
PE/Cy	Phycoerythrin/cyanin
PGE_2	Prostaglandin E_2
PI-3 kinase	Phosphatidylinositol-3 kinase
Pre-B	Precursor B-cell
QUIN	Quinolinic acids
RA	Retinoic acid
RA	Rheumatoid arthritis
ROR	Retinoic acid-related orphan receptor
sCD25	Soluble CD25
SLE	Systemic lupus erythematosus
SMH	Somatic hypermutation
SSC	Side scatter

STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T-cells
TcR	T-cell receptor
Tfh	T follicular helper
TGF-β	Transforming growth factor β
Th	T helper
TNF	Tumor necrosis factor
Tr1	T regulatory cell type 1
TRAIL-DR5	Tumor necrosis factor related apoptosis inducing ligand death receptor 5
Treg	T regulatory cell
TSLPR	Thymic stromal-derived lymphopoietin receptor
tTreg	Thymic Treg

Introduction

Our immune system consists of a complex network of cells and soluble factors all working together to eliminate dangerous intruders such as bacteria, viruses and parasites. The immune system is commonly divided into innate and adaptive immune responses. Innate immune cells respond immediately to intruders by engulfing them and by releasing immunostimulatory substances. Innate immune cells are also necessary to activate cells of the adaptive immune response. Adaptive immune cells are specialized to react to specific antigens whereupon they multiply themselves to create an army of highly specific immune cells. These cells have the capacity to clear infection and to become memory cells that provide us with a life long immunity to the intruder. Hence, the innate immune responses are fast and broad while adaptive immune responses are specific and long lasting. Besides protecting us from infectious agents, the immune system can also protect us from transformed endogenous cells such as cancer cells. The immune system needs to be in balance to protect the surrounding healthy tissues from immune attacks. This balance is mediated by negative feedback-loops by the effector cells themselves, but also through specialized cells that can suppress other immune cells. Immunosuppressive cells are pivotal in restraining the immune system after the infection is cleared but also to stop autoimmune reactions where immune cells are harming healthy tissues. In anti-cancer immune attacks, the presence of suppressive cells is unbeneficial since they hinder the effector cells from killing the cancer cells. In autoimmunity on the other hand, dysfunctional suppressive cells fail to hinder autoreactive immune cells from destroying healthy tissue. This thesis will focus on the immunosuppressive T cells and their importance in B-cell cancers and autoimmunity.

T regulatory cells

The history of T regulatory cells

In the 1970s Gershon and Kondo first described the presence of suppressive T-cells. They found that T-cells could assist B-cells in the antibody production process. However, if the T-cells were pretreated with high doses of antigen they became tolerant and this tolerance seemed to rub of on surrounding T- and B-cells[1,2]. Later that decade, Fujimoto *et al* showed that this type of suppressive T-cells existed in tumor-bearing mice and that they could inhibit anti-tumor immune responses[3,4]. Subsequent studies aimed at classifying these suppressive cells further and both Ly-2^+ (CD8) and Ly-2^-Ly-1^+ ($CD8^-CD5^+$) suppressor cells were identified. $CD8^+$ suppressor cells were the most frequently studied, possible due to the fact that no available anti-mouse CD4 antibody was still available. During the following years, the research interest in suppressor T-cells declined. The fact that no specific markers were available for these cells made it hard to prove their existence (reviewed by Basten and Fazekas de St Groth[5]). In 1995, however, Sakaguchi et al suggested that suppressive T-cells expressed CD25 (IL-2Rα). They showed that by depleting CD25 expressing cells, mice developed autoimmune diseases. The mice also reacted strongly to allogeneic skin graft transplants and this reaction could be hampered by the re-infusion of $CD4^+CD25^+$ cells[6]. During the following years, the interest in suppressive T-cells increased and they were subsequently "rebranded" as T regulatory cells (Tregs)[5]. Until now, suppressive Tregs had only been studied in mice and it was not until 2001 that $CD4^+CD25^+$ cells were identified as suppressive Tregs in humans[7-12].

T-cell development

All T-cells originate from common lymphoid progenitor cells that arise in the bone marrow. The progenitor cells migrate to thymus were they mature before entering the periphery as a T-cell. When entering the thymus, the progenitor cell is negative for most adult T-cell markers. By interacting with the thymic stroma, the precursor cell will start to express their first T-cell surface molecules. Still negative for both CD4 and CD8, the cell will start to express CD44, Kit and at a later stage CD25. The becoming T-cell then

starts so rearrange the genes for the β-chain of the T-cell receptor, whereafter CD44 and Kit are downregulated. The β-chain will pair with a surrogate α-chain called pTα. Cells that fail to generate the β-chain will die, while successful cells will downregulate CD25 and start to proliferate. After cell proliferation, cells express both CD4 and CD8 and will thereafter rearrange the genes for the α-chain. After the cell has assembled the full T-cell receptor complex, it will move from the thymic cortex into the cortico-medullary junction where it will be presented to antigen by thymic cortical epithelial cells. Functional T-cells recognize their antigen in a MHC-restricted manner, which means that the T-cell needs to recognize both the MHC molecule and the peptide that is displayed in that MHC molecule. Because of the thymic transcription factor AIRE (autoimmune regulator), thymic epithelial cells are able to express low levels of self-antigens from organs throughout the body[13].

The T-cell precursor will undergo two types of selection. During the first round of selection (positive selection) the T-cell needs to bind to the MHC molecule. During this step, it is also decided if the T-cell will become a $CD4^+$ or a $CD8^+$ cell depending on if it binds MHC II or MHC I, respectively. The failure of the T-cell to bind any type of MHC will result in death by neglect. Cells surviving the first selection will undergo a second, negative, selection. This is important to eliminate potential autoimmune cells. During this step, cells that bind MHC molecules containing self-peptides will be eliminated.

Scrutinizing the positive and negative selections further, it seems that the same step that rescues the T-cell from death by neglect in the positive selection is also responsible for eliminating the cell during the negative selection. If this simplified model would be true, all T-cells would be eliminated in the thymus. The exact mechanisms involved in the selection processes are not fully elucidated. However, two theories exist. The first is the avidity hypothesis, where the strength of the T-cell receptor signaling upon MHC binding will determine if the cell will live or die. Cells that fail to bind the MHC molecule will die by neglect while cells that bind the MHC:self peptide too strong will die by negative selection. Only cells with the appropriate affinity for the MHC molecule (mediated by an appropriate number of TcRs on the T-cell) will survive[14].

The second theory, known as the qualitative signaling hypothesis[15] describes how the cell will be negatively or positively selected depending on what type of antigen they encounter. The recognition of antagonistic peptides leads to positive selection of the T-cell[16] while recognition of agonistic peptides will result in negative selection[15].

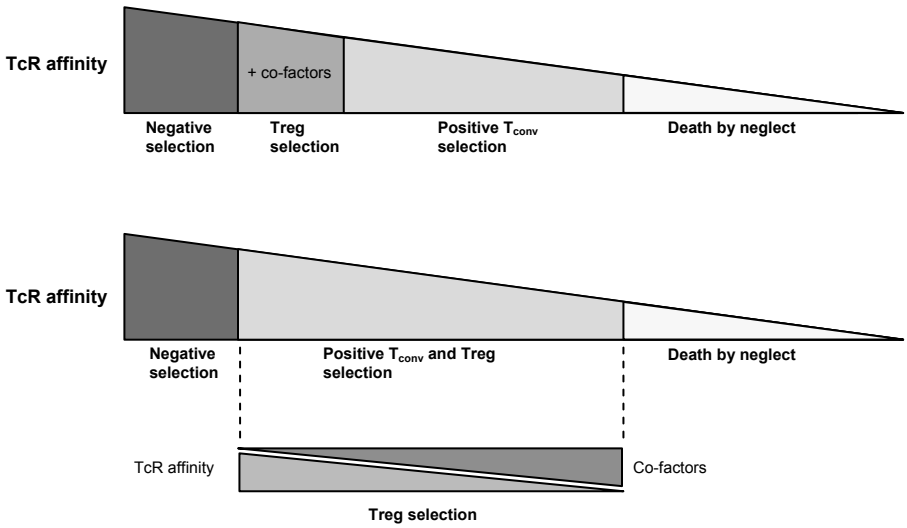

Figure 1. Thymic development of Tregs. Figure modified from Bettini *et al* [17].

Development of T regulatory cells

Tregs can arise either in the thymus or in the periphery. These two cell populations have been indistinguishable from each other since they express similar Treg markers. Recently, however, it was discovered that Helios, a zinc finger transcription factor, was highly expressed in thymic-derived Tregs but not in peripherally-induced Tregs[18,19].

Thymic (tTregs), also known as natural Tregs (nTregs), are selected in the thymus in the same way as conventional T-cells. tTregs have a TcR with high affinity for self antigens. Instead of being deleted, they are kept to control other immune cells with reactivity to self antigens. Several co-stimulatory signals have been suggested to be important for Treg development. These include CD28, CD25 (IL-2Rα), CD40 ligand (CD40L, CD154), glucocorticoid-induced tumor necrosis factor receptor (GITR), thymic stromal-derived lymphopoietin receptor (TSLPR) and signal transducer and activator of transcription factor 5 (STAT5) (reviewed by Bettini and Vignali[17]).

Tregs can also arise in the periphery and are then known as induced (iTregs) or adaptive (aTregs). Tregs are induced from naïve T-cells by antigenic stimulation in combination with factors that are not optimal for effector T-cell generation. These factors include high levels of IL-10, IL-2 and TGF-β[20] as well as inappropriate presentation of antigens by APCs[21]. Retinoic acid (RA) has been shown to induce Tregs even in the presence of inflammatory cytokines[22-24]. Several subsets of iTregs have been described, including $CD4^+$ iTregs, $CD8^+$ iTregs, Tr1 and Th3, all of which will be described further in the section concerning Treg subsets.

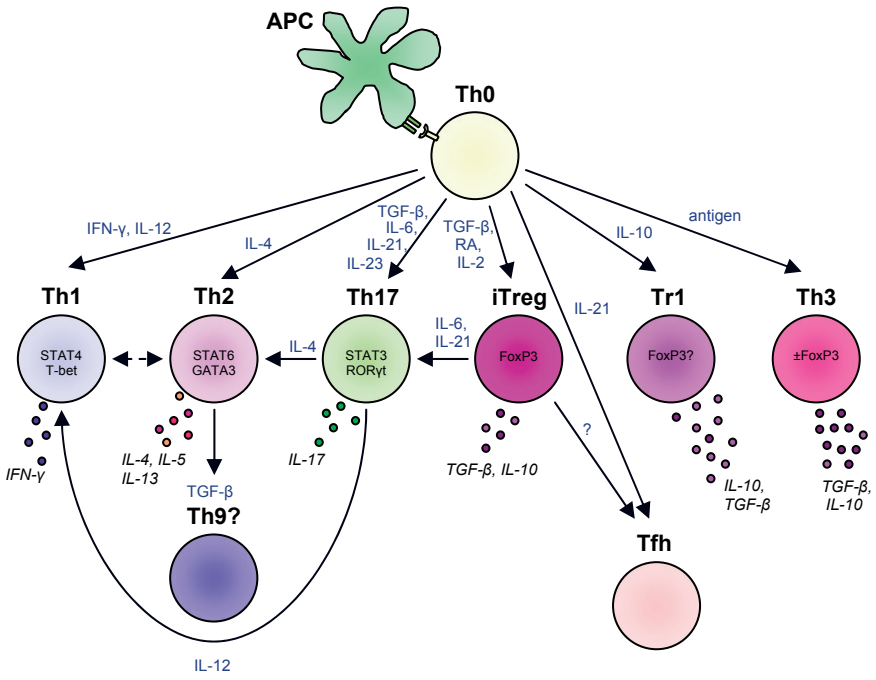

Figure 2. Peripheral development of $CD4^+$ T-cell subsets. Naïve $CD4^+$ T-cells can upon antigen stimulation differentiate into a range of different subsets depending on their surrounding cytokine milieu. Several of the $CD4^+$ T-cell subsets are plastic and can differentiate into a new subtype upon encountering new cytokines. Cytokines shown in blue displays suggested factors for differentiation. Cytokines in *italic* indicate secreted cytokines. The Figure is modified from [25,26].

T regulatory cell markers

Several Treg-associated markers have been described throughout the years. In 1995 Sakaguchi *et al* suggested that CD25 (IL-2Rα) together with CD4 could be used to define Tregs in mice[6]. About six years later, several groups showed that this combination could also be used to define Tregs in humans[7-12]. CD25 is included as a Treg marker in many studies but it is also expressed on activated T-cells[27] and, therefore, it is not specific for Tregs. Other markers that are constitutively or highly expressed on Tregs includes cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)[28-31], GITR[32], lymphocyte-activation gene 3 (LAG-3)[33,34] and Neuropilin-1[35]. However, these markers are also upregulated on conventional T-cells upon activation[36-38].

Today, the transcription factor forkhead box P3 (FoxP3) is considered the most specific marker for Tregs[39-42]. Studies have shown that in humans, FoxP3 can be transiently upregulated in non-regulatory effector T-cells[43-46]. However, since several of these studies[43,45,46] were performed with

an antibody of questioned specificity (PCH101, eBiosciences) it is hard to interpret these results.

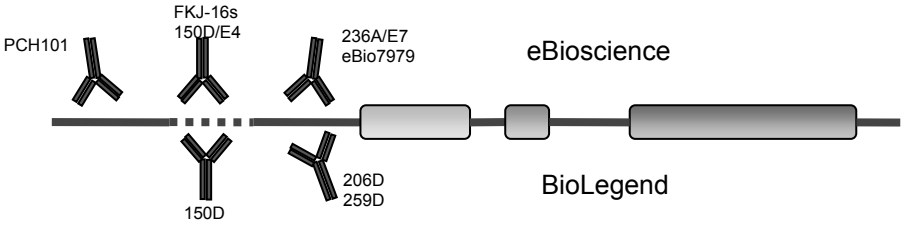

Figure 3. Binding sites of FoxP3 antibodies reactive against human FoxP3. Antibodies located above the FoxP3 gene represent antibodies available at eBiosciences while antibodies below the gene can be purchased from BioLegend. The broken line represents exon 2 which is alternatively spliced in FoxP3b. The Figure is inspired by information from the websites of BioLegend and eBiosciences.

In a publication in Blood in 2007, Tran *et al* suggested that PCH101 is an unreliable indicator of FoxP3 expression in human activated T cells and that it gives a false positive staining on all activated human CD4 T cells[44]. Shortly after, Pillai and Karandikar performed a multipanel staining with different FoxP3-antibodies and found that the antibody clones PCH101, 236A/E7 and 206D stained similar levels of FoxP3 in activated T-cells compared to clone 259D which appeared to have a lower sensitivity[47]. Tran *et al* subsequently demonstrated that FoxP3 was upregulated in the presence of TGF-β, as indicated by both PCH101 and 206D antibody staining. However, in T-cells activated in the absence of TGF-β, only PCH101 stained positive for FoxP3. This study further suggested that PCH101 may bind unspecifically to activated T-cells[47]. Nevertheless, whether or not FoxP3 is upregulated in non-regulatory T-effector cells is still a matter of debate. Considering the above discussed publications and on recommendation from our antibody supplier, we chose to perform our studies with the FoxP3 antibody clone 259D from BioLegend.

A rather recent Treg marker is the IL-7Rα, also known as CD127. This receptor subunit has been used to ease purification of Tregs[48] as FoxP3 negatively correlates with the expression of CD127, leaving Tregs with higher FoxP3 expression a lower expression of CD127[49]. Hence, by sorting $CD25^{high}$ $CD127^{low}$ T-cells, a purer population of FoxP3 positive cells is achieved.

The role of FoxP3 in T regulatory cells

The importance of FoxP3 as a regulator of autoimmunity was first described in the scurfy mouse[50] and later in patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)[51,52] where mutations in the FoxP3 gene results in severe systemic autoimmune disorders. FoxP3 is an important regulator of Treg function and the expression of FoxP3 correlates with the expression of other Treg-associated markers such as CD25 and CTLA-4[39-42].

Human FoxP3 is located on the X chromosome and consists of 11 coding exons. Two isoforms of FoxP3 have been described; FoxP3a and FoxP3b[39,53]. FoxP3b lacks exon 2, which is important for association with the transcription factors RORα[54] and RORγt[55]. For an overview of the two isoforms and their domains, see Figure 4.

FoxP3 functions both as a repressor and an activator for genes associated with T-cell activation (e.g. IL-2, TNF and CD25, CTLA-4, respectively). Rather than functioning alone, FoxP3 forms one or multiple complexes with other transcription factors as well as histone and chromatin modulators. It is believed that the assembly of the different factors is dependent on TcR signaling, co-stimulatory signals and the cytokine milieu (as reviewed by Zhou *et al* [56,57]). Upon TcR and CD28 signaling, NFAT forms a complex with AP-1 to upregulate genes associated with T-cell activation[58]. However, FoxP3 can inhibit NFAT:AP-1:DNA complexes by forming a NFAT:FoxP3:DNA complex[59], thereby inhibiting expression of T-cell activation genes and promoting expression of Treg-associated proteins. It has further been suggested that epigenetic factors such as demethylation of FoxP3 is important for the function of Tregs[60-62].

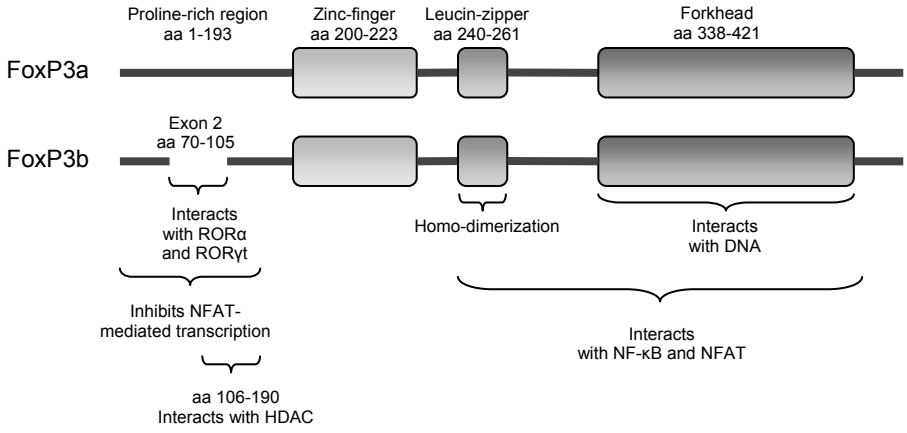

Figure 4. The 2 isoforms of human FoxP3. Human FoxP3 exists in two different isoforms; FoxP3a and the exon 2-lacking FoxP3b. The different FoxP3 domains are indicated in the top of the Figure and suggested functions of these domains at the bottom. The Figure is modified from [63].

The role of CD25 in T regulatory cells

The IL-2R consists of three subunits; the α-chain (CD25), β-chain (CD122) and common γ-chain (γc, CD132). While CD25 is restricted to the IL-2R, the β- and γ-chains are shared with other cytokine receptors such as those for IL-15 and; IL-4, IL-7, IL-9, IL-15 and IL-21, respectively. To form a receptor with high affinity for IL-2 all three subunits are needed. CD25 has the lowest affinity for IL-2 and is incapable of intracellular signaling. It can be found at the surface as a monomer or homodimer. CD25 does not have contact with either IL-2Rβ or γc, suggesting that CD25 is responsible for concentrating IL-2 at the cell surface for presentation to the other two receptor subunits. Further, CD25 changes the conformation of IL-2 and hence stabilizes the receptor complex (as reviewed by Dendrou *et al*[64]). However, IL-2Rβ and γc can form an intermediate affinity receptor that is sufficient for signaling[65]. Upon heterodimerization of the receptor, signaling is initiated through the JAK-STAT, the MAP kinase and the PI-3 kinase pathways, leading to cellular proliferation and inhibition of apoptosis[66].

CD25 has been found to be expressed at high levels on Tregs. Further, Tregs are able to deprive their microenvironment of IL-2[67], possibly due to their high expression of CD25. High doses of IL-2 can induce Tregs and it is also important for their maintenance and survival[68]. Interestingly, the gene for IL-2 is repressed by FoxP3[69] making Tregs dependent on their microenvironment for this cytokine. However, in the absence of IL-2, other γc cytokines such as IL-4, IL-7 and IL-15 can replace the IL-2 functions[69]. Even though most Tregs express CD25 (around 70-80%[26]), the existence of $CD25^-$ Tregs have been reported by us and others[70-72].

T regulatory cell subsets

As previously stated, FoxP3-expressing Tregs can arise both in the thymus and in the periphery. These two subsets have been phenotypically indistinguishable from each other until recently when Helios was found to be highly expressed in tTregs but not in iTregs[18]. These two subsets are otherwise phenotypically similar and it is therefore difficult to study functional differences between iTregs and tTregs.

Other than $CD4^+$ Tregs, $CD8^+$ Tregs from both the thymus and the periphery have been described. $CD8^+$ tTregs from both human and rodents expressed CD8, CD25, FoxP3 and CTLA-4 and acted in a cell-to-cell contact-dependent manner[73-77]. $CD8^+$ human iTregs have been described both in mycobacterial infections[78] and in patients with systemic lupus erythematosus (SLE)[79]. These cells suppressed their target by secretion of CC chemokine ligand 4 (CCL-4) and TGF-β, respectively.

Several subsets of inducible $CD4^+$ Tregs have also been described. Tr1 cells have been suggested to be induced from both human and mouse $CD4^+$ cells by chronic antigen stimulation and high levels of IL-10[80]. Tr1 cells could also be generated *in vivo* by immature DCs in the presence of IL-10[81]. Once activated, Tr1 cells produce large amounts of IL-10 and TGF-β[80] and suppress their target cells in a non-antigen dependent manner. They have been described to express no or low levels of FoxP3[82], and are rather defined for their high production of IL-10. Another inducible Treg subtype that is often defined by its capacity to produce large amounts of cytokines is the Th3 cells. They produce high levels of TGF-β and to a lesser extent IL-10 and IL-4[83]. These cells are induced upon antigen stimulation in the presence of high levels of TGF-β[84]. See Figure 2 for an overview of peripherally-induced Tregs of the CD4 lineage.

T regulatory cell effector functions

Tregs can suppress immune cells in numerous ways. The suppressive mechanisms described below have mainly been investigated in $CD4^+$ tTregs, iTregs, Tr1 and Th3 cells.

Suppression by soluble factors

Tregs can suppress target cells by the secretion of inhibitory cytokines such as IL-10, TGF-β and IL-35.

IL-10 downregulates the expression of costimulatory molecules, adhesion molecules and MHC II on APCs[85-87]. It can also inhibit the release of proinflammatory cytokines which further modulates the stimulatory capacity of DCs and other APCs[88]. Furthermore, IL-10 inhibits the synthesis of cytokines by T-cells and monocytes, and induces long-lasting anergy in both $CD4^+$ and $CD8^+$ T-cells[89,90].

TGF-β exists both as membrane bound and in soluble form. Soluble TGF-β is important in the induction of Tregs but neither soluble or membrane bound TGF-β seems critical for Treg functions (as reviewed by Toda and Piccirillo [91]). Transgenic mice deficient of TGF-β exhibited Tregs with similar phenotype and suppressive capacity as Tregs from wild type mice[92]. However, mice deficient in TGF-β exhibited lymphocyte infiltration in multiple organs and eventually succumbed to autoimmune disease[93,94], demonstrating that even if TGF-β doesn't appear to be crucial for Treg function, it has important immunosuppressive properties. TGF-β can inhibit T-cell proliferation by disturbing IL-2 production and can block differentiation of naïve T-cells into Th1 and Th2 cells by interfering with the function of T-bet/STAT4 and GATA3/NFAT, respectively (as reviewed by Li et al[95]).

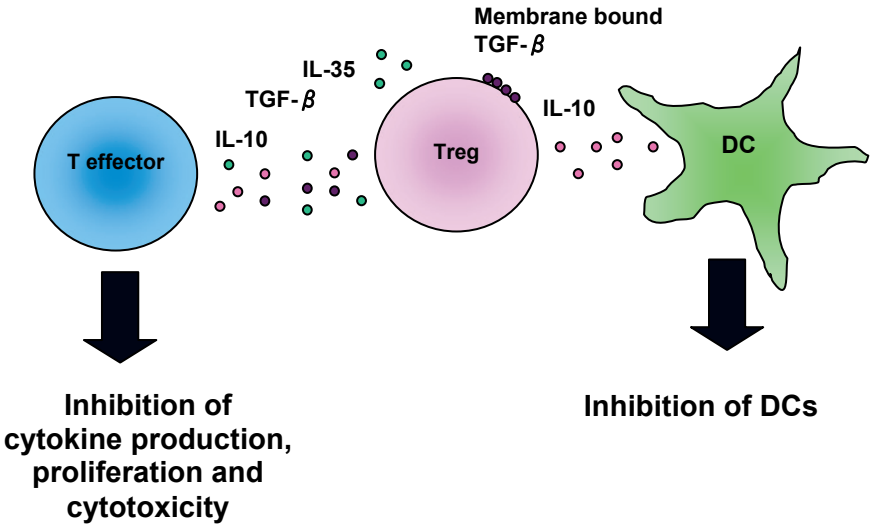

Figure 5. Suppression by soluble factors. Tregs can suppress immune cells by soluble factors, mainly IL-10, TGF-β and IL-35.

IL-35 is highly expressed and secreted by Tregs, but not by conventional T-cells in mice. IL-35 inhibits T-cell proliferation[96] while Tregs cultured in the presence of IL-35 proliferate and produce high levels of IL-10[97].

Suppression of dentritic cells

By interfering with the maturation of DCs, Tregs can indirectly inhibit effector T-cells. To become activated, T-cells need to receive three crucial signals from APCs. Signal one is mediated when the T-cell binds the presented antigen:MHC complex with its TcR. Signal two is received when costimulatory molecules on the APC (CD80 and CD86) interact with CD28 on the T-cell and signal three is mediated by stimulatory cytokines such as IL-12 and IFN-γ.

Tregs can inhibit DC maturation and the immunostimulatory capacity of DCs through LAG-3, which is a CD4 homologue that interferes with ITAM-mediated signalling in the DC[98]. CTLA-4 is important for potent regulatory activity of Tregs *in vivo*[29] and Tregs can induce IDO production by DCs in a CTLA-4 dependent manner[99]. IDO is an enzyme that catabolizes tryptophan into the kynurenine metabolites 3-HAA and QUIN which induce apoptosis in Th1[100] and Th2 cells[101]. IDO can furthermore lead to peripheral generation of Tregs[101,102].

Neuropilin-1 (NRP-1) is constitutively expressed by Tregs but not by other T-cells[35]. Neuropilin-1 has been found to prolong the interaction between Tregs and iDCs[103] which may give them an advantage over naïve

T-cells in modulating the function of DCs. The ligand for Neuropilin-1 in this context is not clear, but it has been suggested to act through homotypic interaction, where Neuropilin-1 on Tregs binds Neuropilin-1 on DCs[104].

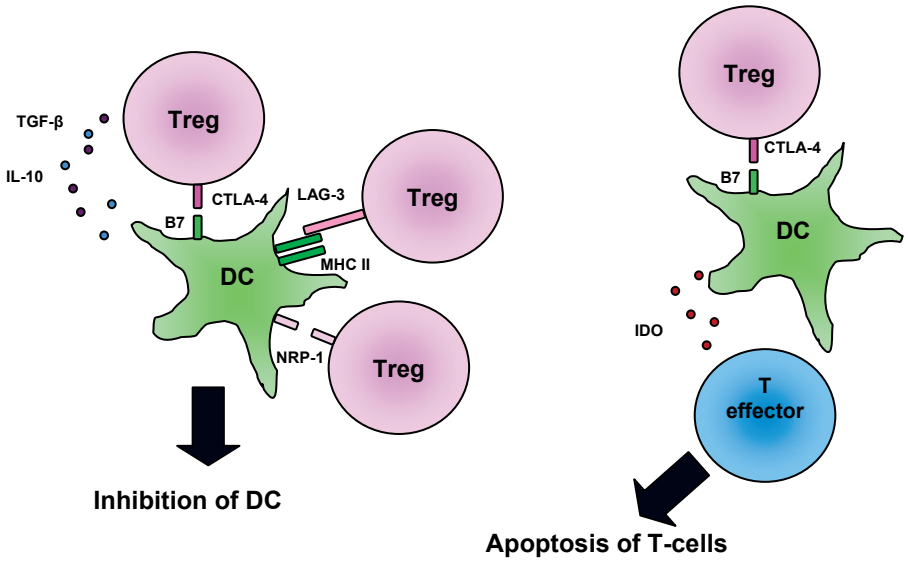

Figure 6. Suppression of dendritic cells. Tregs can suppress the maturation and immunostimulatory capacity of DCs by CTLA-4 or LAG-3 interactions. Tregs can also promote DCs to produce IDO, which can induce apoptosis in T-cells.

Suppression by metabolic disruption

Tregs can disturb the metabolism of target cells in different ways. By expressing the ectozymes CD39 and CD73, Tregs can generate adenosine which can suppress effector T-cells by binding to the adenosine receptor 2A[105-107]. Tregs can also suppress T-effector cells by transferring cAMP into the T effector cell through gap junctions[108]. cAMP inhibits proliferation and IL-2 production by T-cells[109]. Further, human Tregs have been found to release prostaglandin E_2 (PGE_2) which is generated by COX-2[110]. PGE_2 mediates its suppressive function by upregulating the level of cAMP.[111]

Considering that Tregs do not produce IL-2 themselves, probably due to the transcription factor FoxP3 that silence the IL-2 promoter, it is believed that Tregs consume IL-2 from the microenvironment. By depriving its environment of cytokines, Tregs have been shown to induce apoptosis in CD4+ T-cells[67]. In the current thesis we have suggested an additional mechanism of IL-2 deprivation used by Tregs. We proposed that Tregs have the ability to release a soluble form of CD25 (sCD25) which can further inhibit T-effector cells[112].

Tregs have also been shown to deprive T-cells of thiols such as cysteine. T-cells cannot convert cystine into cysteine and, hence, need this amino acid from its environment. DCs create a cysteine rich environment for T-cells[113]. However, Tregs have high contents of intracellular and extracellular thiols and are thought to be competitive consumers of these factors[114].

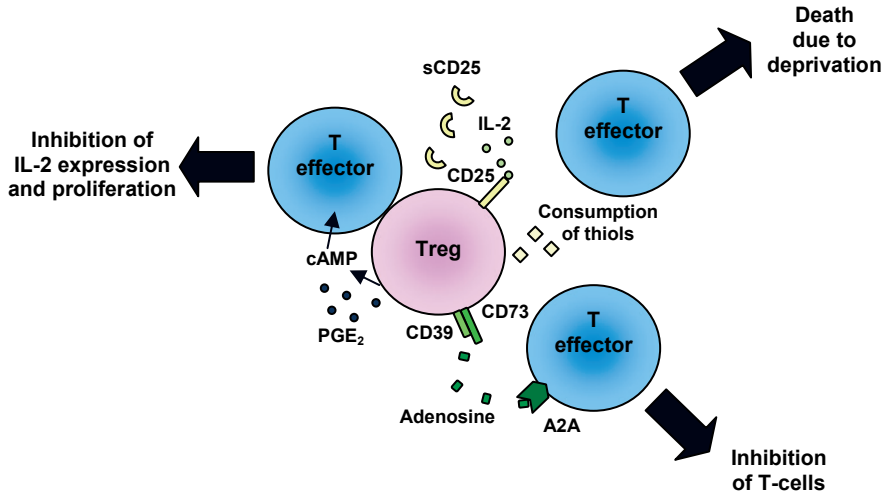

Figure 7. Suppression by metabolic disruption. Tregs can suppress T-cells by consuming factors important for their survival or through adenosine and cAMP.

Suppression by cytolysis

A rather recent finding is that Tregs can kill their target cell by either Fas-FasL interaction[115,116] or through the release of perforin/granzymes[117-122]. Grossman *et al* demonstrated that human tTregs preferentially express granzyme A upon activation in contrast to iTregs which expressed mainly granzyme B. Further granzyme A expressing tTregs were able to kill their target cells at much lower Treg:target ratio than the granzyme B expressing iTregs[118]. Tregs can also kill T-cells by a TRAIL-DR5 pathway[123]. Further, galectin-1 that can induce apoptosis in the target cell is upregulated on Tregs[124]. By killing their target, Tregs control CD4+[118] and CD8+[115,118,120] T-cells, monocytes[118], DC[118], B-cells[116,119,121,125] and NK-cells[120].

Killing mechanisms

T-cells can kill their target cell by two major pathways; through death receptor signaling or through a granule-dependent pathway. Both pathways lead to the induction of apoptosis in the target cell.

T-cells expressing ligands of the tumor necrosis superfamily (TNF) on their cell surface can kill target cells that express the corresponding receptors. One of these members, Fas ligand (FasL, CD95L) can upon ligation cause apoptosis in Fas (CD95) expressing target cells. Upon trimerization of Fas, a death-inducing signaling complex (DISC) containing FADD and FLICE (also known as pro-caspase-8) is assembled.

Next, FLICE is proteolytically cleaved where after active caspase-8 is released from the complex into the cytoplasm where it forms a heterotetramer (as reviewed by Krammer[126]). Caspase-8 then proteolytically activates other downstream caspases such as caspase-3 and 7, which later cleave vital cellular proteins and induces DNA defragmentation which leads to apoptosis of the cell (as reviewed by Strasser et al[127]). Apoptosis induced by Fas can also be mediated through proapoptotic BH-3-only proteins, but since this pathway controls apoptosis of cells other than lymphocytes it is not described in detail here.

T-cells can also kill their target cell via a granule-dependent mechanism. T-cells secrete a family of proteases called granzymes together with perforin, which enables the granzymes to enter the target cell. Several different types of granzymes have been described both in humans and mice, where granzyme A and B are the most abundant.

Upon entry into the target cell, Granzyme A moves to the nucleus where it activates the DNase NM23-H1 by proteolysis and deactivates its inhibitor SET[128]. NM23-H1 then induces single stranded DNA nicks in the chromosomal DNA[128,129] eventually leading to apoptosis of the target cell.

Granzyme B can induce apoptosis in two ways. Either through a direct mechanism where granzyme B directly activates caspase-3[130,131] which subsequently aid the activation of other caspases[132,133] ultimately leading to apoptosis of the cell; or by promoting mitochondrial permeabilization. The latter pathway involves oligomerization of the Bcl-2 family proteins Bak and/or Bax located in the outer membrane of the mitochondria[134]. This results in the escape of intramitochondrial factors such as cytochrome c[134,135] .When it reaches the cytosol, cytochrome c participates in forming a complex called the apoptosome. The assembly of the apoptosome activates caspase-9[136] which activates the rest of the caspase cascade and finally results in apoptosis of the cell. The antiapoptotic protein Bcl-2 can heterodimerize with Bak and Bax and thereby inhibit any further actions by these two death-promoters[137]. Bcl-2 has further been shown to inhibit granzyme B[138-140], but not granzyme A[141]-mediated killing.

CD107a (LAMP-1) is a degranulation marker[142] that correlates with cytotoxic activity of T-cells[121,142,143]. This protein is normally located inside the cell, covering vesicles that contain cytotoxic substances such as

granzymes. Upon degranulation, CD107a is relocated to the surface and can, hence, be used as a cell surface marker for granzyme release (see Figure 8).

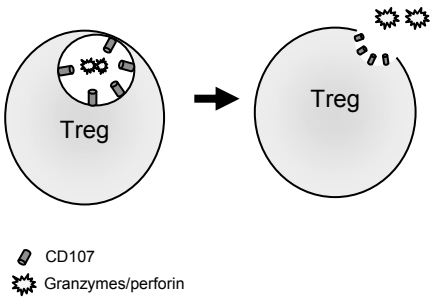

Figure 8. The degranulation marker CD107a. CD107a is normally located inside cytotoxic vesicles inside cells, protecting the cells from its own granzymes and perforins. Upon degranulation, CD107a is mobilized to the plasma membrane and can, hence, be used as a marker for granzyme release.

T regulatory cells in cancer

As early as in the 1950's, Burnet alongside with Thomas suggested that our immune system is able to recognize aberrant self cells and eliminate them before they can form a detectable tumor[144]. However, since cancer is one of the most common causes of death in the Western society, these aberrant self cells must have a way of evading the immune attack. The immunoediting theory describes three phases; elimination, where immune cells initially eradicates the aberrant self cells; equilibrium, where tumor cells that have an enhanced capacity to resist the immune attack arises and; escape, where the resistant tumor cells expand in an uncontrolled manner[145].

It has been shown that tumors have numerous ways by which they can escape immune attacks. By downregulating MHC molecules they avoid killing by cytotoxic T-lymphocytes (CTLs), and by upregulating non-classical MHC molecules such as HLA-G and HLA-E they avoid being killed by NK-cells. Several tumors have the capacity to release immunosuppressive cytokines (e.g. IL-10 and TGF-β) and to activate suppressive pathways in immune cells via CTLA-4, PD-1 and Fas. Furthermore, the tumor microenvironment is beneficial for the maintenance and expansion of Tregs (as reviewed by Croci *et al*[146] and Poggi *et al*[147]).

Tregs are present in the tumor area in most cancer types[148-155]. These Tregs exhibit an immunosuppressive capacity against other immune cells[149-154] and high numbers are commonly associated with a worse outcome for the patients[149,155,156]. Tregs are primed in the same lymph

nodes as the effector cells and later moves to the tumor area by migrating towards a CCL22 gradient released by the tumor microenvironment (as reviewed by Wilczynski *et al*[157]).

Interestingly, patients with B-cell tumors that have an increased infiltration of Tregs into the tumor have a better outcome than those with low numbers[125,158-165]. The reason for this is not clear, however, it has been suggested that the Tregs may regulate the malignant B-cells. As previously discussed, Tregs can regulate normal B-cells through cytolysis[116,119,121,125]. It is therefore possible that they may control malignant B-cells as well. This makes the role of Tregs in B-cell malignancies multifaceted and complex. In this thesis (Paper III and IV) we have attempted to investigate this further.

T regulatory cells in autoimmunity

The most important function of Tregs is the protection it provides against autoimmunity. Cancer and autoimmunity can be considered to be each others opposites. Immunotherapists treating patients with autoimmunity want to activate Tregs to stop the ongoing immune attack against the body's own tissues, while immunotherapists treating patients with cancer wants to block Tregs and promote immune attacks against the self-originating tumor. To develop potent cancer immunotherapy, which is the ultimate goal in our laboratory, it is important to understand the role of Tregs in both cancer and autoimmunity.

Autoimmunity arises when autoreactive immune cells become activated and subsequently injures healthy tissue. There are several types of autoimmune diseases including multiple sclerosis (MS), diabetes mellitus type 1, rheumatoid arthritis (RA), SLE and Sjögren's syndrome. In some of these diseases, autoantibody producing B-cells are the main effectors, while autoreactive T-cells are the main players in others. Patients with MS have an inflammation in the central nervous system (CNS), resulting in demyelination and axonal damage in the brain. The most prevalent immune cell found in MS-affected brains is the $CD8^+$ T-cell. However, $CD4^+$ Th-cells are thought to be the main players in MS since they can both damage the myelin directly by release of proinflammatory cytokines and indirectly by attracting other immune cells such as macrophages/microglial cells, B-cells, mast cells and NK-cells. All these cells contribute to the CNS inflammation which will eventually result in neuronal damage. Even though Th1 cells may be a part of the important $CD4^+$ Th population, it is possible that Th17 cells are even more important since these cells are able to disrupt the brain blood barrier and directly kill neurons *in vitro* (as reviewed by Venken *et al*[166]). Tregs are supposed to protect us against such responses. However, in MS patients these cells seem to fail. Studies have shown that

rather than having a decreased level of Tregs, these suppressive cells seem to be dysfunctional in MS patients (as reviewed by Costantino *et al*[167]). However, studies made by our group have not been able to detect dysfunctional Tregs in the periphery of these patients[168], suggesting that the brain microenvironment may disable Tregs locally.

B-cell leukemia and lymphoma

Malignant B-cells responsible for leukemia or lymphoma formation are believed to arise from different stages of development and differentiation of normal B-cells. Even though leukemia and lymphoma are cancers, it may be easier to study them from an immunological point of view to better understand how and why Tregs could interact with and regulate these malignant cells.

B-cell development

B-cells originate from the same lymphoid precursor as T-cells. In contrast to T-cells which mature in the thymus, B-cell precursors mature in the bone marrow. During the pro-B-cell stage, the precursor rearranges the genes for the heavy chain of the immunoglobulin (Ig), which involves joining of V, D and J segments. During the precursor pre-B cell stage, a surrogate light chain is expressed together with the heavy chain[169]. This complex is called the pre-B-cell receptor (BCR), and its expression is required for continuous B-cell development[170]. Later both the heavy and light chains are expressed on the cell surface, defining an immature B-cell. An estimated 90-95% of the B-cells fail to express the complete form of the receptor and will, hence, undergo apoptosis[171]. Lymphocytes that survive will undergo a selection process to ensure that cells with inappropriate antigen receptors are deleted[172]. If a B-cell recognizes a self-structure it either becomes anergic or succumbs via apoptosis. Approved naïve B-cells will leave the bone marrow for functional maturation which takes place in secondary lymphoid tissues such as lymph nodes and spleen[173].

B-cell activation

When the naïve B-cell encounter its antigen, one of three events may occur. 1) it enters a lymph node where the B-cell proliferates and forms germinal centers (GCs), 2) it differentiates into short lived plasma cells outside the GC or 3) it enters an anergic state.

When the B-cell recognizes its antigen, it internalizes it and (if a protein) presents it on MHC II. T-helper cells, previously primed by DCs,

recognizing the presented antigen can activate the B-cell in the T-cell rich zone of the lymph node. The T-cell provides necessary stimuli for the B-cell, such as CD40L signaling, allowing the B-cell to undergo proliferation. Proliferating B-cells will form a dark zone in the lymph node, pushing naïve B-cells further out to form a mantle zone. Proliferating B-cells in the dark zone undergo somatic hypermutation (SHM), which induces mutations into the Ig with the intention of increasing its affinity for the antigen. However, most SHMs result in BCRs with decreased affinity which in turn results in the death of the B-cell. B-cells with increased affinity for its antigen will be positively selected by follicular T-helper (Tfh) and follicular DCs (FDCs). Some of the positively selected B-cells can undergo class-switch recombination (CSR), altering its soluble Ig isotype from IgM to IgG, IgD, IgA or IgE. Finally, the B-cell will differentiate into memory B-cells or plasma cells and leave the GC microenvironment (as reviewed by Küppers[174]).

Leukemia and lymphoma formation

Both the development and differentiation of B-cells contain steps were the double stranded DNA is broken. During these steps, genetically errors are more prone to occur. Chromosomal translocations are sometimes generated during V(D)J recombination, SHM or CSR which can give rise to a malignant B-cell clone. Different genetical errors acquired by the B-cell can enhance its proliferation, cellular growth and block its differentiation (as reviewed by Shaffer *et al*[175]). Several B-cell lymphomas have translocations of the immunoglobulin and an anti-apoptotic protein, resulting in constitutive expression leaving the B-cell less sensitive to apoptosis. Examples of such translocations include the t(14;18) resulting in Bcl-2-IgH that can be found in the majority of follicular lymphoma (FL) patients and in some patients with diffuse large B-cell lymphoma (DLBCL)[176-178]. Malignant B-cells can also acquire increased levels of anti-apoptotic molecules by interacting with cells in its microenvironment. Chronic lymphocytic leukemia (CLL) patients have a large proportion of CD40L-expressing T-cells in their proliferating centers which may rescue the CLL from apoptosis. T-cells can also secrete cytokines such as IL-4, IFN-γ and IFN-α that upregulate the level of Bcl-2 in CLL cells and thereby rescue them from apoptosis (as reviewed by Caligaris-Cappio[179]). The microenvironment has also been shown to be important for FL and MALT, where the surrounding cells provide the malignant B-cells with proliferation and survival signals (as review by Burger *et al*[180]).

Different putative cell origins of lymphoma and leukemia have been suggested based on the presence or absence of SHM and from gene expression profiling. See Figure 9 for an overview. Some lymphomas, like

follicular lymphoma (FL), seem to have acquired their translocation at a pre-GC stage (during V(D)J recombination)[181,182] but have differentiated into having GC B-cell characteristics.

Several B-cell malignancies have been associated with autoimmune diseases. B-cells from CLL patients have been found to produce autoantibodies[183,184] as have B-cells from FL patients[185] and MALT lymphoma patients[186,187]. Further, several autoimmune diseases increases the risk of developing B-cell lymphoma. Sjögren's syndrome has been associated with MALT lymphoma[188], DLBCL[189-191] and FL[191]; RA with DLBCL[190,192]; and SLE with DLBCL[190].

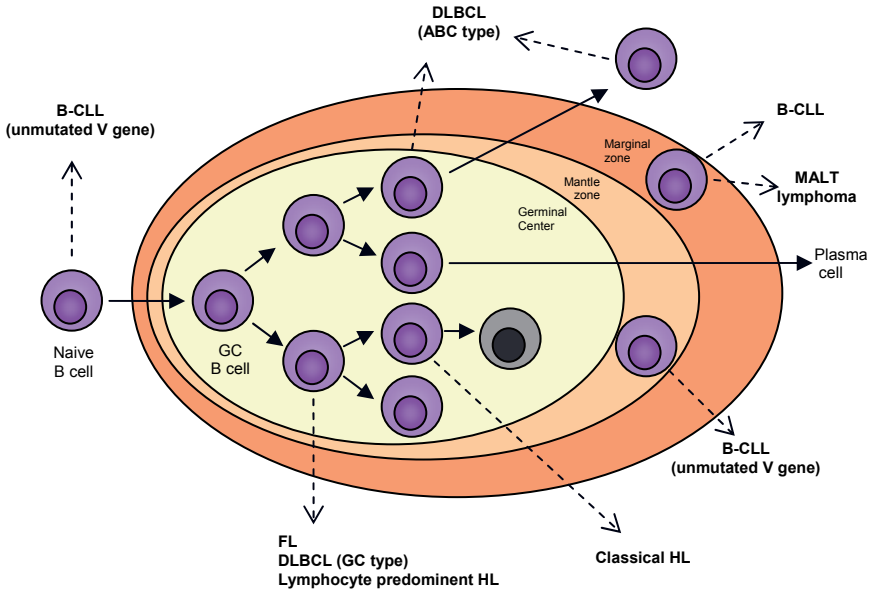

Figure 9. Putative cell of origin for B-cell lymphoma and B-cell chronic lymphocytic leukemia. The Figure shows a germinal centre were the dark zone is located to the left, containing mostly proliferating B-cells; and the light zone to the right where the selection by T-cells and FDCs occur. Arrows with a filled line indicate the normal differentiation of B-cells while dashed arrows demonstrates the putative origin of indicated B-cell malignancies. The black cell represents apoptosis. The Figure is modified from reference [174].

The importance of T-cells and T regulatory cells in the regulation of malignant B-cells

B-cell activation in GCs is supervised by Tfh cells. SHM of the B-cell surface Ig may potentially result in BCRs with high affinity for self antigens. Selection processes ensuring that only B-cells carrying appropriate BCRs are maintained are therefore pivotal. T-cells are involved in this process by killing B-cells with a faulty BCR by Fas-FasL interaction[172,193]. Also Tregs seem to play an important role in B-cell regulation. For several autoimmune diseases with aberrant antibody production, the level or function of Tregs have been found to be decreased[194-198]. Depletion of Tregs in a mouse model of arthritis resulted in a more severe disease and an increase in autoantibody titers[199]. Similar findings could be observed in mice with autoimmune haemolytic anemia[200]. Studies have shown that Tregs may regulate B-cells by interfering with T-cell help in GCs[201] but also by suppressing the B-cell directly[116,119,121,125]. A recent study by Iikuni *et al* demonstrated that Tregs could kill autoantibody-producing B-cells obtained from SLE patients[202], further highlighting the importance of malignant B-cell regulation by Tregs.

Aims of the study

I. To investigate the presence, source and role of sCD25 in patients with B-cell malignancies.

II. To evaluate the level and phenotype of $CD25^+$ and $CD25^-$ Tregs in patients with MS.

III. To evaluate the level of peripheral Tregs in patients with B-cell malignancies such as chronic lymphocytic leukemia and B-cell lymphoma.

IV. To investigate the presence of cytolytic markers on $FoxP3^+$ T-cells in patients with chronic lymphocytic leukemia or B-cell lymphoma.

Study design and Methods

Detailed information about material and methods used in this thesis can be found in Papers I-IV.

Blood samples from patients and healthy donors

Peripheral blood from patients with B-CLL, B-cell lymphoma and MS was collected at Uppsala University Hospital. Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained from buffy coats (Papers I, III and IV) or peripheral blood (Paper I-II) obtained at the blood bank, Uppsala University Hospital (Papers I, III and IV) or from healthy volunteers at Uppsala University (Paper II). Written consent was obtained from all patients in accordance with the Helsinki declaration. All studies were approved by the regional ethics committee in Uppsala.

All blood samples from patients were collected in sodium heparin-coated vacutainers. Plasma was collected the same day as the blood was drawn by shortly centrifuging the tubes. Plasma samples were then stored at -20C or -80C. PBMCs were collected by centrifuging blood diluted in PBS over Ficoll-Paque. PBMCs were stored at -80C until analysis.

FACS analysis (Paper I, III and IV)

Cell samples were acquired using Becton Dickinson's (BD) LSR II at the Rudbeck lab cell analysis core facility (Biovis). Anti-mouse IgG κ compensation beads (BD Biosciences, San Jose, CA) stained with antibodies used in the screenings was used to set up the acquisition protocol. Compensation was performed with help from personnel at the cell analysis core facility. In multi-color staining protocols, fluorochromes with low fluorescence intensity were used to detect strongly expressed antigen (e.g. Pacific Blue on CD3). Surface molecules that are not constantly expressed, such as CD25, FasL and CD107a, were detected using fluorochromes with strong fluorescence intensity (such as PE/Cy7, FITC and PE/Cy5). To eliminate possible sources of errors, such as day-to-day differences in mean

fluorescence intensity (MFI), an equal number of samples from patients and healthy donors were stained and acquired in each run.

Stained acquired cells were analyzed using FlowJo (TreeStar, Ashland, OR). To eliminate as much human error as possible in the analysis of the cells (setting of thresholds etc), all samples for each study was analyzed in the same session.

Cytotoxicity assay (Paper III)

To analyze if Tregs had cytotoxic capacity against B-cells, different cell types were purified using the Miltenyi magnetic-activated cell sorting (MACS) system. PBMCs were purified into B-cells and CD25+ and CD25- Tregs. The purity of each population was measured by CD19 and CD3 plus CD4 staining respectively. For the cytotoxicity assay, B-cells were mixed with each type of Treg at a 1:20 ratio and incubated for 7 hours at 37C. As controls, B-cells were either cultured alone or in the presence of a FasL-blocking antibody and the granule-release inhibitor Concanamycin A. After incubation, cells were stained for CD3 (T-cells) and CD19 (B-cells) and analyzed by flow cytometry. Dead cells (small in FSC and SSC) were eliminated by gating. The ratio of live cells (CD19 to CD3) was subsequently analyzed. To further confirm that we measured killing by cytotoxic mechanisms, cell supernatants from the cultures were collected and analyzed for granzyme A and B by ELISA.

Results and discussion

Paper I

Previous studies have shown that patients with B-cell malignancies have high levels of sCD25 in plasma[203-206]. Suggested sources of this soluble receptor subunit have been malignant B-cells and activated T-cells[207,208]. However, when we performed phenotyping of Tregs in this patient group, we found that Tregs (defined as $CD3^+CD4^+CD127^{low}FoxP3^+$ lymphocytes) from patients with B-cell lymphoma and CLL contained a lower percentage of CD25 expressing Tregs compared to healthy donors. Tregs are traditionally defined as $CD4^+$ cells with a high expression of CD25[6-9]. However, there are studies that have identified $CD25^-$ Tregs[72]. When further evaluating the CD25 MFI on Tregs, we found that Tregs from B-cell lymphoma patients had a significantly lower CD25 expression compared to Tregs from healthy individuals. This finding made us investigate if Tregs from these patients could shed CD25 into its surrounding microenvironment. Linear regression analysis showed a correlation between the level of Tregs and sCD25 in patients with B-cell lymphoma but not in patients with CLL or healthy individuals. No correlation was found between sCD25 and $CD4^+$ cells in patients with B-cell lymphoma or CLL indicating that the source of sCD25 could be Tregs and not other $CD4^+$ cells. To investigate if malignant B-cells could be a source of sCD25, white blood cell count of CLL patients (that have a lot of malignant B-cells in the periphery) was correlated against sCD25. However, no correlation was found. To confirm that Tregs were a source of sCD25, we purified different cells subsets using the MACS system. Cells were purified into Tregs ($CD4^+CD25^+CD127^{low}$), $CD4^+$ and $CD8^+$ T-cells, monocytes ($CD14^+$) and B-cells ($CD20^+$). Unfractionated PBMCs was used as a control. When cells were cultured without stimulation, Tregs were the largest producers of sCD25. Upon stimulation with OKT-3 and IL-2, the $CD4^+$ cells increased their secretion of sCD25, while Tregs had a similar secretion as when it was unstimulated. It is known that activated T-cells upregulate CD25 upon stimulation and that they can shed this receptor subunit to avoid overstimulation. Whether or not the sCD25 produced by Tregs is due to shedding or if it is secreted as a variant of the membrane bound sCD25 remains to be determined.

Since Tregs seemed to be the greater producers of sCD25 in these patients, we wanted to investigate if this molecule had immunosuppressive

properties. When stimulated PBMCs were cultured with recombinant sCD25 it suppressed T-cell proliferation in a dose-dependent manner. These results suggest that sCD25 indeed have a suppressive capacity. We then assessed the proliferative capacity of T-cells from patients with B-cell lymphoma. Compared to healthy controls, the patients had a somewhat hampered proliferation. However, when patients were divided into those with high plasma levels of sCD25 (>5000pg/ml) and those with low (<5000pg/ml), it was evident that the patients with high levels of sCD25 indeed had a suppressed T-cell proliferation.

In conclusion, a new suppressive function of Tregs includes the secretion/shedding of sCD25 which can inhibit T-cell proliferation.

Paper II

Tregs have had several different definitions through the years. Previous studies have defined them as $CD4^+CD25^+$, $CD4^+FoxP3^+$, $CD4^+CD25^+FoxP3^+$ and lately $CD4^+CD25^+CD127^{low}FoxP3^+$. One of the difficulties in studying Tregs is that no real consensus as to how they should be defined exists. Problems may emerge when results from studies defining Tregs in different ways are compared to each other. This problem was highlighted in this Paper were Tregs were studied by several definitions. PBMCs from patients with MS and age-matched healthy donors were stained for CD4, CD25; CD4, FoxP3 or CD4, CD25, FoxP3. No differences between healthy donors and MS patients were found regardless of Treg definition when MS patients were studied as one group. However, when MS patients were divided into those that were in remission or at relapse differences could be seen. When using the old Treg definition $CD4^+CD25^+$, MS patients in remission had a significant increase in Tregs compared to healthy donors. However, if Tregs were defined as $CD4^+FoxP3^+$ T-cells, it was the relapsing MS patients that had an increase in Treg level. When Tregs were defined as $CD4^+CD25^+FoxP3^+$ T-cells the increased levels were lost in both MS subgroups.

We then identified that it was the $CD25^-$ $FoxP3^+$ cells that varied. The relapsing group had an increased $CD25^-$Treg level compared to healthy individuals.

The intensity of FoxP3 expression has been shown to negatively correlate to CD127 expression[49], hence, $CD127^{low}$ cells have a significantly higher FoxP3 expression compared to $CD127^{high}$ cells (see Figure 1C in Paper III). Since some studies have suggested that FoxP3 may be transiently upregulated in non-Tregs upon stimulation and that true Tregs are those with high FoxP3 expression, CD127 was added as an additional Treg marker. When $CD4^+FoxP3^+$ cells were subgrouped according to CD127 and CD25 expression, both relapsing and remitting patients had the highest level of

Tregs in the $CD127^{low}CD25^{-}$ subgroup. Whether these are true Tregs or recently activated T effector cells are not clear. Interestingly, when FoxP3 MFI was investigated in $CD4^{+}CD25^{+}$ cells, patients at relapse had a significantly higher FoxP3 expression compared to healthy donors. FoxP3 expression in $CD4^{+}CD25^{-}$ T-cells was lower than in $CD25^{+}$ cells and did not differ between patient subgroups or between patients and healthy individuals. To investigate if $CD25^{-}$ Tregs had suppressive properties, $CD4^{+}CD25^{-}CD127^{low}$ Tregs were purified. $CD4^{+}CD25^{+}CD127^{low}$ Tregs were used as a control. Both subgroups of Tregs were irradiated and mixed with stimulated allogeneic PBMCs. $CD25^{-}$ Tregs had a similar suppressive capacity as $CD25^{+}$ Tregs and both Treg-groups expressed FoxP3. These results suggest that $CD25^{-}$ Tregs have similar functions as $CD25^{+}$ Tregs.

In conclusion, $CD25^{-}$ Tregs, which have similar properties as $CD25^{+}$ Tregs, were increased in patients with relapsing MS. These results suggest that not only $CD25^{+}$ Tregs, but also $CD25^{-}$ Tregs should be included in immunoscreenings of these patients.

Erratum Paper II:
Figure 4A “Treg suppression (% proliferation)” instead of “Treg suppression (%)”.

Material and Methods, Tregs separation and suppression: “Both populations were radiated at 25Gy and mixed with stimulated 15µg/ml OKT3 antibodies” Should be: 1.5µg/ml OKT3 antibodies.

Statistical evaluation: “p-values <0.05(*), <0.001(**), and <0.0001(***) were considered significant.” Should be: <0.01(**) and <0.001(***).

Papers III and IV

Since Tregs are able to suppress anti-tumor immune attacks, they are often considered un-advantageous in cancer patients. Indeed, for a majority of patients with non-hematopoietic tumors, an increased level of tumor-infiltrating Tregs is associated with a worse outcome[209]. However, for patients with B-cell lymphoma, an increased infiltration of $FoxP3^{+}$ cells into the tumor has been associated with a better survival[125,158-163]. In several of these publications, the authors have hypothesized that Tregs could have a cytotoxic effect on the malignant B-cells. Tregs are mostly known to suppress T-cell responses. However, it has recently been shown that Tregs can suppress not only T-cells, but also B-cells, monocytes and dendritic cells[119,120,210]. Considering that studies have shown that Tregs can regulate B-cells through perforin/granzymes[121,202] or Fas-FasL

interaction[116], the above discussed hypothesis may be correct. We therefore phenotyped Tregs from patients with B-CLL (Paper III) and B-cell lymphoma (Paper IV) to evaluate the expression of cytotoxic markers on these cells.

When investigating Treg infiltration in tumors by immunohistochemistry, FoxP3 is used as a single marker for Tregs. This is considered to be sufficient since FoxP3 is the currently best known marker for this cell population[40]. When analyzing Tregs by flow cytometry, however, Tregs are often defined by several markers. Since recent studies, including two of our own (Papers I and II) have shown that Tregs do not need to be $CD25^+$, we chose to define our Tregs as $CD3^+CD4^+CD127^{low}FoxP3^+$ lymphocytes. CD127 have shown to be negatively correlated to the intensity of FoxP3 expression[49] and can also ease the purification of Tregs[48]. Little is known about $CD127^{high}FoxP3^+$ cells since this population is hard to separate from $CD127^{high}FoxP3^-$ cells. Considering that this population may belong to the Treg population, we chose to include these cells in our screenings. To ease the discussion, we chose to call these cells $CD127^{high}FoxP3^+$ T-cells.

Patients with CLL had increased levels of both Tregs and $CD127^{high}FoxP3^+$ T-cells. Both of these cell types had an increased expression of CD107a but not FasL. Patients with B-cell lymphoma had similar levels of Tregs and $CD127^{high}FoxP3^+$ T-cells as healthy individuals. Only the $CD127^{high}FoxP3^+$ T-cells displayed signs of cytotoxicity which seemed mediated trough both granzyme release and FasL.

To assess if Tregs from patients with B-cell malignancies could kill autologous B-cells, Tregs and B-cells from CLL patients were purified and co-cultured. Since CLL patients have their cancer largely in the periphery, PBMCs are a good source of malignant cells in these patients. Both $CD25^+$ and $CD25^-$ Tregs from these patients were able to kill the malignant B-cells. Further, Tregs released both granzyme A and B into the cell culture supernatant, which was analyzed by ELISA. The purity and functionality of Tregs were measured by flow cytometry and Alamar Blue assay, respectively. These results indicated that there was no difference in Treg suppression between $CD25^+$ and $CD25^-$ Tregs or between Tregs from healthy individuals or CLL patients. Further, both subgroups of Tregs expressed FoxP3.

These results indicate that Tregs may have a dual function in patients with patients with B-cell malignancies. Since Tregs express cytolytic markers and are able to kill malignant B-cells they seem beneficial for this patient group. However, they are also able to suppress T-cell responses. It is possible that two different immune responses exist in these patients. Both leukemia and lymphoma are cancers; hence, an anti-tumor immune response should exist against the malignant cells. This immune attack is probably mediated largely by T-cells, which can be suppressed by Tregs. In this sense, the presence of

Tregs should be unbeneficial. However, it is also possible that the Tregs are trying to regulate the malignant B-cells.

Future studies using larger cohort of patients are needed to investigate if Tregs express cytotoxic markers in the tumor microenvironment and if these cytotoxic Tregs are associated with patient survival.

Conclusions

I. The plasma level of sCD25 was increased both in patients with CLL and in patients with B-cell lymphoma. One source of sCD25 in B-cell lymphoma patients seems to be Tregs. Purified Tregs released sCD25 and sCD25 had an immunosuppressive capacity when tested *in vitro*. These data suggest that Tregs mediate T-cell suppression by the release of sCD25.

II. The level of Tregs was increased in relapsing MS patients. Interestingly, it was the $CD25^-$ Tregs that were elevated. $CD25^-$ Tregs expressed FoxP3 and had a similar suppressive capacity as $CD25^+$ Tregs. These results conclude that not only $CD25^+$ but also $CD25^-$ Tregs need to be considered when evaluating Tregs in health and disease.

III. The level of peripheral Tregs was elevated in patients with CLL, but not in patients with B-cell lymphoma.

IV. Tregs from patients with CLL had a $CD107a^+$ cytotoxic phenotype. Occasional patients with B-cell lymphoma also had Tregs with a cytotoxic phenotype, but their Tregs seemed to use FasL as an effector molecule. When purified Tregs from CLL patients were cultured with autologous B-cells, they killed the B-cells. These data support the theory that Tregs may be able to regulate malignant B-cells.

Future perspectives

Scrutinizing the literature on B-cell lymphomas and CLL it becomes clear that these cancers are not just any cancer. Cancer immunologists often have a preconceived view that increased levels of Tregs in cancer patients are unfavorable since they inhibit anti-tumor immune responses. We initially shared this view. However, after reading several papers claiming that an increased level of $FoxP3^+$ cells in lymphoma-affected lymph nodes are associated with a better outcome we had to change our opinion on this matter.

As discussed in this thesis, Tregs are able to kill B-cells. Hence, we performed a study investigating the phenotype of peripheral Tregs in patients with B-cell lymphoma or CLL. These studies could be improved by 1) investigating larger cohorts of patients. Our cohort of B-cell lymphoma patients is small and diverse. Collection over a longer period of time or in cooperation with other hospitals would allow for the collection of more patients with the same type of lymphoma. This would make it possible to perform correlation assays to determine the impact of cytotoxic Tregs for these patients. 2) investigating tumor-affected lymph nodes in patients with B-cell lymphoma. Even though Tregs in peripheral blood may give a hint about what is happening in these patients, investigating Tregs in the tumor would be very interesting. Are Tregs ($FoxP3^+$ cells) expressing cytolytic markers (FasL, Granzyme A/B) and are they in close contact with malignant B-cells? Or are they rather in contact with the Th-cells which provide support for the malignant B-cells?

Studying the impact of cytotoxic Tregs in CLL patients may be harder. Most CLL cells can be found in the peripheral blood. However, these cells are hard to culture *in vitro* since they undergo apoptosis. Further, CLL patients have proliferation centers where malignant CLL cells can be found in close contact with FDCs and T-cells. The importance of cytotoxic Tregs in these patients should therefore ideally be studied in an *in vitro* system where at least part of the microenvironment is present.

The main focus of our laboratory is to develop immunotherapies against cancer. Our approach is to equip the patient's own T-cells with a chimeric artificial receptor targeting CD19 and thereafter transfusing the genetically enhanced T-cells back to the patient. These T-cells will then ideally migrate to the malignant B-cells, recognize CD19 expressed by the malignant B-cells

and subsequently eradicate them. This type of therapy is often inhibited by the presence of Tregs. Before adoptive transfer of therapeutic T-cells, Tregs are often depleted. It would be interesting to investigate if these patients would benefit from Treg depletion or if it would rather worsen their disease.

Summary of the thesis in Swedish

Populärvetenskaplig sammanfattning på svenska

Att immunförsvaret skyddar oss mot bakterier och virus känner förmodligen de flesta till, men att det också kan skydda oss mot cancerceller kanske inte är lika känt. Immunförsvaret är uppbyggt av flera olika celltyper och lösliga ämnen som alla samarbetar och kompletterar varandra för att ge oss ett så bra skydd som möjligt. När immunförsvaret reagerar för starkt kan autoimmunitet uppstå. Autoimmunitet är när immunförsvaret av misstag angriper kroppens egen vävnad och därmed skadar den. För att skydda oss mot autoimmunitet har immunförsvaret inbyggda spärrar som hindrar immuncellerna från att reagera för starkt. Det finns till exempel specialiserade celler som har förmågan att stänga av de immunceller som reagerar felaktigt. Dessa specialiserade celler kallas ofta suppressiva och där ingår T regulatoriska celler (Tregs). Arbetena som ingår i denna avhandling har studerat Tregs hos patienter med cancer i immunförsvaret (leukemi och lymfom) samt hos patienter med autoimmunitet (multipel skleros, MS).

Immunologer har länge studerat immunförsvarets betydelse i cancer. En viss cell i immunförsvaret, T-cellen, tros vara speciellt viktig i vårt försvar mot cancer då den kan döda cancerceller. Cancer börjar med att en normal cell i kroppen får fel i sitt DNA som den inte kan reparera. Cellen börjar då bete sig annorlunda än friska celler. Den svarar inte på stoppsignaler från kroppen och den kan börja tillverka nya proteiner som på olika sätt hjälper cancern att växa sig större. Eftersom cancercellen en gång varit en frisk cell så liknar den på många sätt sin friska motsvarighet. Detta gör att om T-celler börjar döda cancerceller så börjar andra delar av immunförsvaret att motarbeta attacken eftersom det är kroppsegen vävnad som angrips. Denna hämning av immunförsvaret brukar till stor del utföras av Tregs. De flesta cancerpatienter som har många Tregs i tumörområdet har dålig prognos.

Hos många cancerpatienter beter sig immunförsvaret ganska lika mot tumören. Det har gjort att man lätt drar alla cancrar ”över samma kam” när det gäller immunförsvarets betydelse hos cancerpatienter. När cancern sitter i immunförsvaret blir det hela däremot mer komplicerat. Vad händer i kroppen vid en sådan cancer? Beter sig immunförsvaret på samma sätt som i andra cancrar? D.v.s. finns det T-celler som försöker döda cancercellerna?

Det är mycket möjligt, men med tanke på att cellerna i immunförsvaret normalt sett samarbetar med varandra och även reglerar sig själva och varandra så blir förhållandet vid leukemier och lymfom mer komplext.

Jag har intresserat mig av en typ av cancer som uppstår i B-celler. B-celler är de celler i immunförsvaret som producerar antikroppar (när vi vaccinerar oss aktiveras dessa celler). När andra grupper har studerat patienter med lymfom som utgörs av B-celler har de sett att dessa cancerpatienter lever längre om de har mycket Tregs i sina tumörer. Detta är helt tvärtemot patienter som har cancer som inte kommer från immunförsvaret (t.ex. hudcancer). Vad detta beror på vet man idag inte. Man vet att Tregs kan reglera flera olika immunceller, däribland B-celler. Det har därför spekulerats att Tregs kanske dödar de sjuka B-cellerna. Hittills har det inte funnits några bevis för att detta händer i dessa patienter. I studierna som ingår i denna avhandling har vi undersökt Tregs från patienter med leukemi och lymfom. Dessa celler uppvisar tecken som tyder på att de har försökt döda andra celler. Om man tar ut patienternas sjuka B-celler och odlar dem tillsammans med Tregs från samma patient dör de sjuka B-cellerna. Våra resultat tyder på att Tregs i patienter med leukemi och lymfom dödar B-cellen, alternativt att de dödar en cell som är nödvändig för den sjuka B-cellens överlevnad.

När man undersöker celler i immunförsvaret identifierar man dem oftast efter vad de har på sin yta (och ibland också vad de har inuti sig). Tregs är svåra att studera eftersom forskare inte är helt överens om hur de ska identifieras. Hittills har de flesta kommit överens om att ett protein som kallas FoxP3 är utmärkande för Tregs. De flesta som studerar Tregs inkluderar också CD25 som en markör för Tregs. I manus I och II har vi visat att Tregs inte behöver vara CD25 positiva. Vi har även visat att Tregs kan utsöndra CD25. Lösligt CD25 kunde hämma T-celler. Vi anser därför att detta är en ny mekanism som Tregs har för att kunna hämma immunförsvaret.

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