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FOXP3 AND CTLA-4: HOW ISOFORMS REGULATE IMMUNOLOGICAL TOLERANCE

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FOXP3 and CTLA-4: how isoforms regulate immunological tolerance

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To my family

ABSTRACT

The maintenance of immunological tolerance is vital for preventing the immune system to damage normal tissues and physiological function of the body. CD4+FOXP3+ regulatory T (Treg) cells can suppress immune responses in a dominant manner and are essential for immunological tolerance. Although many pathways and molecules have been attributed to the suppressive function of Treg cells, the exact nature of the Treg cell-mediated suppression program is still elusive.

In this thesis, I aimed to study the role of different protein isoforms, in particular FOXP3 and CTLA-4 isoforms, in the function of Treg cells and uncover the consequences of Treg cell-mediated suppression on dendritic cells (DCs).

In **paper I** the aim was to understand the regulation and functional consequences of FOXP3 isoform expression in chronic inflammatory diseases in human. We found that FOXP3 isoforms were differentially expressed in biopsies from IBD patients. Moreover, the pro-inflammatory cytokine IL-1β promoted the exclusion of FOXP3 exon 7, which favoured the differentiation of naïve CD4+ T cells into Th17 cells.

In **paper II** we generated a mouse model ($Foxp3^{\delta2\delta7}$ mouse) where Treg cells exclusively express an FOXP3 isoform lacking both exon 2 and exon 7 (FOXP3δ2δ7). We found that FOXP3δ2δ7 was unable to confer suppressive function of Treg cells *in vivo*. Homozygote $Foxp3^{\delta2\delta7}$ mice phenocopied $Foxp3$ deficient scrufy mice and died from severe autoimmune disorder starting from 3 weeks of age. The $Foxp3^{\delta2\delta7}$ mouse may provide a useful alternative for studying FOXP3 isoform function in Treg cells *in vivo*.

In **paper III** we investigated the role of CTLA-4 in Treg cell-mediated suppression on DCs. We generated a mouse model ($Ctla4^{ex2fl/fl}Foxp3$-Cre mouse) where Treg cells exclusively express an isoform of CTLA-4 that lacks the extracellular binding domain encoded by $Ctla4$ exon 2. $Ctla4^{ex2fl/fl}Foxp3$-Cre mice were born healthy and only started to display inflammatory lesions in environmental surfaces such as the lung and intestine after 4 months of age. *In vitro* co-culture experiments demonstrated that Treg cells from $Ctla4^{ex2fl/fl}Foxp3$-Cre mice were fully capable of inhibiting the up-regulation of CD80/CD86 on DCs. DCs co-cultured with $Ctla4^{ex2fl/fl}Foxp3$-Cre Treg cells had decreased ability to support effector T cell proliferation. Unexpectedly, we also found that DCs up-regulated PD-L2 when co-cultured with wild type Treg cells in a CTLA-4 dependent manner. Collectively these data suggest that CTLA-4 mediated trans-endocytosis is a dispensable mechanism for Treg cell-mediated suppression and that Treg cell-mediated suppression on DCs is a multi-faceted process involving both CTLA-4 dependent and independent mechanisms.

In summary, these studies showed that it is necessary to investigate FOXP3 and Treg cell function on an isoform basis, and that Treg cell-mediated suppression on DCs is a multi-faceted program that involves both CTLA-4 dependent and independent mechanisms.

LIST OF SCIENTIFIC PAPERS

I. Mailer RKW, Joly A-L, **Liu S**, Elias S, Tegnér J, Andersson J.
   IL-1 β promotes Th17 differentiation by inducing alternative splicing of FOXP3.
   Scientific Reports 5:14674, 2015.

II. Joly A-L*, **Liu S***, Dahlberg CIM, Mailer RKM, Westerberg LS, Andersson J. Foxp3 lacking exon 2 and 7 is unable to confer suppressive ability to regulatory T cells *in vivo*.
   Journal of Autoimmunity, 63:23-30, 2015.

III. **Liu S**, Seitz C, Joly A-L, Klocke K, Wing K, Andersson J.
   Multi-faceted inhibition of dendritic cell function by CD4$^+$Foxp3$^+$ regulatory T cells.
   Manuscript.

   * Shared first authorship

CONTENTS

LIST OF ABBREVIATIONS

$A_{2A}R$	adenosine A_{2A} receptor
APC	antigen presenting cells
ATP	adenosine triphosphate
1/4CTLA-4	CTLA-4 isoform containing exon 1 and 4 only
Bcl-6	B-cell lymphoma 6 protein
BCR	B cell receptor
cDCs	classical dendritic cells
CLP	common lymphoid progenitor
CLR	c-type lectin receptor
CMP	common myeloiderythroid progenitor
CNS	conserved non-coding sequence
CtBP1	c-terminal binding protein 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAMP	danger-associated molecular patterns
DC	dendritic cell
DN	double-negative
DP	double-positive
EAE	experimental autoimmune encephalomyelitis
ES cells	embryonic stem cells
FKH	forkhead
flCTLA-4	full-length CTLA-4 isoform
FOXP3	forkhead box protein 3
FOXP3fl	full-length FOXP3 isoform
FOXP3Δ2	FOXP3 isoform lacking exon 2
FOXP3Δ2Δ7	FOXP3 isoform lacking exon 2 and 7
FOXP3Δ7	FOXP3 isoform lacking exon 7
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA3	trans-acting T-cell-specific transcription factor GATA-3
GFP	green fluorescent protein
GITR	glucocorticoid-induced TNFR-related protein

GM-CSF	granulocyte-macrophage colony-stimulating factor
HAT	histone acetyltransferase
HDAC	histone deacetylases
HIV	human immunodeficiency virus
HIF-1α	hypoxia-inducible factor 1-alpha
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSCs	hematopoietic stem cells
IBD	inflammatory bowel disease
ICOS	inducible T-cell co-stimulator
IDO	indoleamine-2, 3-desoxygenase
IFN	interferon
IgSF	immunoglobulin superfamily
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
JAK	janus kinase
KAT5	Histone acetyltransferase KAT5
LAP	propeptide-latency-associated peptide
LCs	langerhans cells
liCTLA-4	ligand-independent CTLA-4 isoform lacking exon 2
LPS	lipopolysaccharide
MAO	morpholino antisense oligonucleotides
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
Mtb	mycobacterium tuberculosis
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NKT	natural killer T
NOD	non-obese diabetic
Nr4a	nuclear receptor 4a

PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death protein 1
pDC	plasmacytoid dendritic cell
Pdcd1	programmed cell death 1
PD-L	programmed death-ligand
PP1	protein phosphatase 1
PRRs	pattern recognition receptors
PTM	post-translational modification
RORα	RAR-related orphan receptor alpha
RORγt	RAR-related orphan receptor gamma
sCTLA-4	soluble CTLA-4 isoform lacking exon 3
SLE	systemic lupus erythematosus
SMAD3	mothers against decapentaplegic homolog 3
STAT5	signal transducer and activator of transcription 5
RA	rhumatoid arthritis
RLR	retinoic acid-inducible gene-I-like receptor
RUNX1	runt-related transcription factor 1
T1D	type 1 diabetes
T-bet	T-box transcription factor TBX21
TCR	T cell receptor
T_{CM}	central memory T cell
T_{EM}	effector memory T cell
TGF	transforming growth factor
Th	T helper
Tfh	follicular helper T cell
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFRSF	tumor necrosis factor receptor superfamily
Treg	regulatory T cells
TSDR	Treg-cell specific demethylated region

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is a complex defence system with many biological structures and processes that protects an organism from infections and diseases. Generally speaking, the immune system can be divided into two subsystems: the innate immune system and adaptive immune system. The innate immune system appeared earlier in evolution, and can be found in all plants and animals. It provides a quick but non-specific response towards pathogens as a first line of defence. The adaptive immune system emerged later in evolution and can only be found in vertebrates, including both jawed and jawless vertebrates (Guo et al. 2009; Flajnik & Kasahara 2010). Compared to the innate immune system, the adaptive immune system has much higher specificity towards antigens, and it can also create immunological memory after an initial response.

The innate immune system includes physical and chemical barriers as well as cellular innate immune responses. Physical and chemical barriers include epithelial layers of the skin, mucosal surfaces at various environmental surfaces, and soluble substances with antimicrobial activity or low PH value, etc. Cellular innate immune responses rely on phagocytes that can recognize conserved motifs found on the microbes and damage-associated molecular patterns (DAMPs) due to aging, cell death or tissue damages. The receptors that can recognize DAMPs are called pattern recognition receptors (PRRs). There are 4 major families of PRRs, they are toll-like receptors (TLRs), c-type lectin receptors (CLRs), retinoic acid-inducible gene-I-like receptors (RLRs), and nod-like receptors and nucleotide oligomerization domain/leucine-rich repeat-containing receptors (NLRs). TLRs are the first family of PRRs to be discovered and are also the best-characterized PRRs. Today there are 10 functional TLRs identified in human and 12 in mouse. Among these TLRs, TLR1, TLR2, TLR4, TLR5, and TLR6 are localized on the cell surface and recognize microbial membrane components whereas TLR3, TLR7, TLR8, and TLR9 are localized in intracellular vesicles and recognize nucleic acids (Kawai & Akira 2011).

The adaptive immune system has two major components: 1) T-lymphocytes (T cells), including both $CD4^+$ T cells and $CD8^+$ T cells, and 2) B-lymphocytes (B cells). After activation naïve $CD4^+$ T cells can differentiate into many subsets of helper T (Th) cells that can perform effector T cell functions independently or help directing the function of other leucocytes. Naïve $CD8^+$ T cells can differentiate into an effector cell type called cytotoxic T lymphocytes (CTLs). Activated B cells can differentiate into plasma cells that produce antibodies against infectious agents.

A typical immune response starts when a microbe enters the body and encounters an innate immune phagocyte (macrophages, neutrophils or dendritic cells). The microbe is then recognized by PRRs on the phagocytic cells and is engulfed by phagocytes. The recognition of PRRs can induce an innate immune response that results in the killing of the microbe. In

the mean time some phagocytes can also process components of the microbes (antigenic peptides or antigens) to be loaded on the major histocompatibility complex molecules (MHC) on the cell surface for initiating adaptive immunity. These cells are called antigen-presenting cells (APCs). When T cell receptors (TCRs) on T cells recognize MHC-antigen complexes in addition to co-stimulatory signals presented by APCs, they become activated and initiate an adaptive immune response. Activated T cells perform a number of different tasks including direct killing of infected cells by CTLs and/or differentiate into many subsets of Th cells with effector functions to combat the offending microorganism (see section 1.4.3). Naïve B cells recognize antigen with their BCRs and sometimes with the help of Th cells, they get activated and differentiate into plasma cells, which secret antibodies to fight against infection.

1.2   IMMUNOLOGICAL TOLERANCE

The battle between the immune system and pathogens is a battle between "self" and "non-self". Although the immune system needs to recognize diverse types of foreign antigens that could be harmful to the body; it should also avoid miss-identification or overreaction that could disrupt normal physiology by mounting an erroneous or excessive immune response. To keep this delicate balance, an immune response should be tightly regulated so that in certain situations, a state of unresponsiveness of the immune system should be implemented so as not to elicit an immune response to harm "self"; such unresponsive state is referred to as immunological tolerance.

Depending on the origin, immunological tolerance can be classified into central tolerance and peripheral tolerance. Central tolerance is induced in the thymus (for T cells) and bone marrow (for B cells). In the bone marrow, the immature B cells undergo negative selection where most of the autoreactive B cells that recognize self-antigen are deleted by the BCR-mediated apoptotic process of clonal deletion. In the thymus, those highly autoreactive thymocytes are also deleted during T cell development before they mature into immune-competent T cells (see section 1.4.1). Alternatively, some thymocytes that recognise self-antigens relatively strongly, but not strong enough to be negatively deleted, develop into a unique subset of T cells called regulatory T (Treg) cells, which can control potential lymphocyte autoreactivity in the periphery. Peripheral tolerance is induced after fully matured autoreactive T and B cells reach the peripheral tissue, these T and B cells can be rendered unresponsive either by aforementioned Treg cells, or through a state of "anergy" induced by the absence of co-stimulatory signals and/or the presence of co-inhibitory signals.

1.3   THE BIOLOGY OF DENDRITIC CELLS

1.3.1   Antigen presenting cells and antigen presentation

Antigen presenting cells are the bridge between the innate immune system and the adaptive immune system. For a T cell to recognize antigen, it requires the antigen to be processed and loaded on an MHC molecule. There are two classes of MHC molecules: class I and class II. MHC class I molecules (MHC-I) are expressed on almost all nucleated cells and are

specialize in presenting antigens from intracellular locations (endogenous antigens); MHC class II molecules (MHC-II) are restricted to APCs and are specialized in presenting antigens from extracellular spaces (exogenous antigens) that are taken up by APCs. Many cell types can function as APCs, however only a few cell types can express MHC-II as well as deliver co-stimulation signals to T cells, these APCs are referred to as professional APCs, including dendritic cells (DCs), macrophages and B cells.

1.3.2 Dendritic cell subsets

DCs are a very heterogeneous population and the ontogeny of DCs remains ambiguous. All blood cells originate from the same single cell type: the hematopoietic stem cells (HSCs) in the bone marrow. HSCs give rise to two broad progenitor lineages: the common myeloid-erythroid progenitor (CMP) and the common lymphoid progenitor (CLP), both of which can generate dendritic cells.

Historically dendritic cells can be divided into two groups: the classical dendritic cells (cDCs) and the plasmacytoid dendritic cells (pDCs), although neither group is a homogenous population in itself. cDCs are first discovered by Ralph Steinman and colleagues in the late 1970s, and are well-known for their enhanced ability to capture, process and present phagocytosed antigens to T cells. pDCs earned their name for their plasma cell-like morphology in the 1990s. Besides their ability to present antigens, pDCs are specialized in rapid and massive production of type 1 IFN upon stimulation by foreign nucleic acids; therefore they are sometimes considered part of the innate immune system.

As probably the most effective antigen presenting cell type, cDCs populate most of the lymphoid and nonlymphoid tissues. The phenotypical and functional distinction among different cDC subsets has not been an easy task. Extensive research on DCs has been performed on mouse models, and scientists have grouped cDCs based on both their location and expression of surface markers (Merad et al. 2013). Depending on the expression of surface markers, mouse cDCs can be divided into 3 subsets: the CD8α$^+$ DCs, CD8α$^-$ DCs (or CD11b$^+$ DCs) and Langerhans cells (LCs).

Each subset of cDCs has distinct transcriptome profile, expression of PRRs and immunological functions. For example, CD8α$^+$ DCs are the only cDCs that express TLR3 and TLR11 (Edwards et al. 2003; Davey et al. 2010), they also express high levels of scavenger receptor CD36 (bind to dead cells) (Schulz et al. 2002; Belz et al. 2002), C-type lectin Clec9A (sense necrotic bodies) (Sancho et al. 2009), CD205 (take up apoptotic bodies) (Vremec et al. 2000) and langerin (Flacher et al. 2008). Therefore CD8$^+$ DCs have the unique ability to divert exogenous antigen to a pathway that leads to antigen presentation on MHC-I molecules and activation of naïve CD8$^+$ T cells, this process is called cross-presentation (den Haan et al. 2000; Pooley et al. 2001; Dudziak et al. 2007). On the other hand, CD8α$^-$ DCs are more programmed to prime CD4$^+$ T cells.

1.4 THE BIOLOGY OF T CELLS

1.4.1 T cell development

T cells are developed from CLPs during hematopoiesis. After migrating from the bone marrow into the thymus, lymphoid precursors that are committed to the T cell lineage become thymocytes or immature T cells. These thymocytes then undergo different developmental stages to form mature T cells. In the early stage of T cell development, thymocytes are double-negative (DN) cells lacking both CD4 and CD8 expression on the cell surface. These DN cells then rearrange their T cell receptor (TCR) genes to become either TCRαβ T cells or TCRγδ T cells. The DN TCRαβ thymocytes also turn into $CD4^+CD8^+$ double-positive (DP) thymocytes and are ready for the thymic selection. Two distinct thymic selection processes are required for DP thymocytes to become mature T cells: positive selection selects thymocytes that are capable of binding self-MHC molecules, resulting in MHC restriction; and negative selection eliminates thymocytes that have too high affinity for MHC-self-peptide complexes, resulting in self-tolerance. Once a thymocyte survives the selection processes and makes a decision to be either $CD4^+$ or $CD8^+$ T cell, it leaves the thymus as a fully matured naïve T cell. In this thesis, I mainly focus on the biology of $CD4^+$ T cells.

1.4.2 Treg cell specificity

Although the TCR repertoire of Treg cells is biased towards self, it remains possible that Treg cells can recognize exogenous antigens or become antigen-specific under particular immunological conditions. It has been reported in mice that natural Treg cells can respond specifically to pathogen-derived antigens when repeatedly exposed to *Leishmania major* infection (Suffia et al. 2006). In another mouse model containing a transgenic TCR P25 specific for *Mycobacterium tuberculosis* (Mtb), Treg cell expansion can be observed in the affected lymph nodes after Mtb infection, and transferring Mtb-specific $P25^+$ Treg cells purified from uninfected mice to Mtb infected mice could delay priming of effector T cell in the recipient mice (Shafiani et al. 2010).

1.4.3 T cell activation

A naïve T cell that just left the thymus and is constantly circulating and patrolling the system browsing for their cognate antigens. If the naïve T cell encounters an APC presenting a MHC-peptide complex that it can bind to sufficiently strong, it initiates an activation and differentiation program that produces a broad array of T cell subtypes that can fight against infection, this is called a primary immune response.

For a naïve T cell to activate and differentiate into an effector T cell, three signals are required. Signal 1 is the engagement of antigen-specific TCR with the MHC-peptide complex, which is provided by the APCs that have encountered, internalized and processed antigens. The co-receptor CD4 or CD8 as well as adhesion molecules can further stabilize the

interaction between TCR and the MHC complex to allow long-term cell interactions and signalling. Signal 2 is the contact between a co-stimulatory receptor on the T cell with a co-stimulatory ligand provided by a functional APC. There are positive co-stimulatory receptor/ligand and negative co-stimulatory (or co-inhibitory) receptor/ligand. A positive co-stimulation helps to activate a naïve T cell when needed, while a negative co-stimulation (co-inhibition) fine-tunes a T cell response to maintain peripheral tolerance and reduces inflammation. When signal 1 and signal 2 are present, the T cell is activated and starts to produce cytokines that facilitate cell cycle and proliferation. However, for a T cell to be fully equipped to fight against different types of pathogens and infection, it needs to further differentiate into an effector T cell, which requires assisting cytokines produces by APCs, T cells and many other immune cell types. These polarizing cytokines are sometimes referred to as signal 3. Signal 3 does not only enhance T cell proliferation but also determine which kind of effector T cell a naïve T cell should become.

1.4.4 T cell differentiation and T helper cell subsets

T cells can differentiate into many different functional subsets. After activation, $CD8^+$ T cells differentiate into $CD8^+$ cytotoxic T cells that can kill infected cells in the local tissues. $CD4^+$ T cells become Th cells that orchestrate the activities of other cell types, such as B cells, macrophages and other T cells. Each subset of Th cells is dependent on signalling from distinct polarizing cytokines and expression of a transcription factor (the master gene regulator) that orchestrates transcription of the subset's signature program (Fig. 1). Today many $CD4^+$ T helper cell subsets have been documented, although some subsets are better characterized than others, such as Th1, Th2, Th17, Treg and follicular helper (Tfh) T cells.
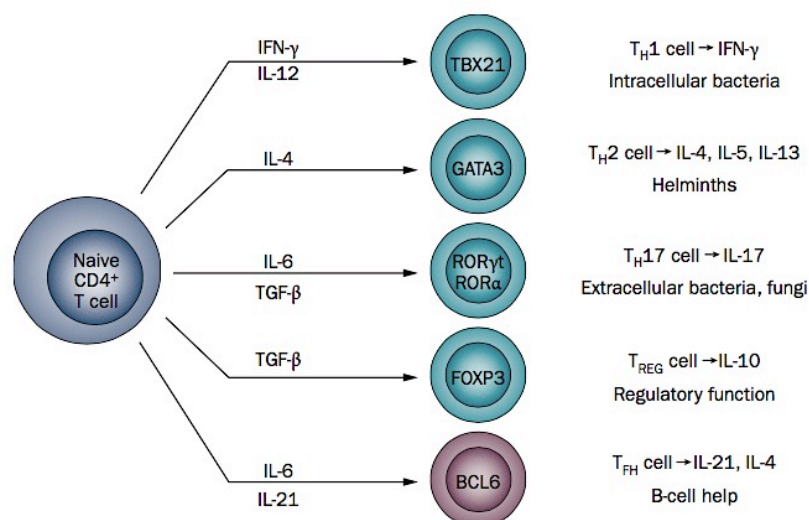

Figure 1: CD4$^+$ effector T cell subsets differentiation. (Craft 2012)

IL-12, IL-18 and IFNγ promote the differentiation of Th1 cells, while IL-4 induces the differentiation of Th2 cells. In the periphery Treg cells can be induced from naïve T cells in the presence of TGFβ, while TGFβ together with IL-6 triggers the differentiation of Th17 cells. On the other hand, IL-6 and IL-21 together activate T cells toward the Tfh lineage.

Different Th cell subsets can also cross-regulate each other. The signature cytokines produced by each subset enhance their own differentiation while inhibit commitment to other helper T cell lineage. For instance Th1 cells produce IFNγ to inhibit proliferation of the Th2 subset, and IL-4 produced by Th2 cells down-regulates the production of IL-12 by APCs, thereby inhibiting Th1 differentiation. In a similar manner, while TGFβ up-regulates both FOXP3 and RORγt, when IL-6 is present, the combined signals inhibit FOXP3 expression and promote the dominant expression of RORγt thereby Th17 differentiation.

1.4.5 T cell memory

After effector T cells clear the pathogens, a majority of them die by apoptosis, leaving only a small number of antigen-specific T cells to become memory T cells. This formation of memory cells is a big advantage of the adaptive immune system because memory cells can live quiescently in the body for a very long time. When they encounter the same antigens again, memory cells can mount a much faster, more robust and more effective secondary immune response. For example, $CD4^+$ memory T cells require much lower dose of antigen for activation and much lower threshold for co-stimulation (Rogers et al. 2000; London et al. 2000; Berard & Tough 2002). Memory T cells can be broadly divided into 2 subsets: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{EM} have already committed to an effector lineage and can respond very rapidly after reactivation. On the other hand, T_{CM} live longer and maintain the capacity to differentiate into many effector T cell subsets after activation. Some surface markers are commonly used to distinguish naïve T cells, effector T cells and memory T cells. These surface markers are listed in Table 1:

Cell type	CD44	CD62L	CCR7
Naïve T cell	Low	+	+
Effector T cell	+	Low	−
Effector memory T cell	+	Variable	−
Central memory T cell	+	+	+

Table 1: Surface markers that are used to distinguish human naïve, effector and memory T cells. (Adapted from Kuby Immunology, 7th edition, by W. H. Freeman and Company, 2013)

1.5 THE BIOLOGY OF CD4⁺FOXP3⁺ REGULATORY T CELLS

An important player for the maintenance of peripheral tolerance is the regulatory T cell population that suppresses immune responses in a dominant manner. Although this population was described as "suppressor T cells" as early as the 1970s (Gershon & Kondo 1970), our understanding of this T cell population only became clearer when Sakaguchi and colleagues identified a population of CD4⁺ T cells expressing the IL-2 receptor α-chain (CD25) that are capable of preventing autoimmune diseases. These CD4⁺CD25⁺ T cells were then termed regulatory T (Treg) cells (Sakaguchi et al. 1995; Asano et al. 1996). A few years later, mutation of the gene *Foxp3* encoding a forkhead/winged-helix transcription factor was identified to be responsible for the fatal autoimmune phenotype seen in scurfy mice (Brunkow et al. 2001) and human immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Chatila et al. 2000; Bennett et al. 2001; Wildin et al. 2001). It was soon revealed that *Foxp3* mRNA and protein were also predominantly expressed in the CD4⁺CD25⁺ Treg cell population and is the defining feature for CD4⁺CD25⁺ Treg cells. Loss-of-function mutation in *Foxp3* gene resulted in developmental and functional impairment in Treg cells; on the other hand, forced expression of *Foxp3* in CD4⁺CD25- T cells conferred a suppressive phenotype similar to that of CD4⁺CD25⁺ regulatory T cells (Hori et al. 2003; Fontenot et al. 2003; Khattri et al. 2003; Wan & Flavell 2005).

To avoid any further confusion regarding studies done in human and mouse, in this thesis, the following simplified nomenclature is used:

Species	Gene	mRNA or protein
Human	*FOXP3 (italicized)*	FOXP3
Mouse	*Foxp3 (italicized)*	FOXP3

1.5.1 Treg cell development

Treg cells can be differentiated intrathymically or extrathymically. Treg cells differentiated in the thymus are termed thymic derived (tTreg) cells. They emerge mostly at the CD4⁺CD8⁻ single positive stage and make up the majority of total Treg cell pool. Sequencing studies in mice demonstrated that Treg cells and naïve T cells have very different TCR repertoire, moreover the TCR repertoire from Treg cells is specifically biased toward self-peptides and is also more diverse than naïve T cells (Hsieh et al. 2004; Pacholczyk et al. 2006).

A TCR with high affinity for a self peptide is required for the development of Treg cells in the thymus (Jordan et al. 2001). This idea is supported by the observation that Treg cells display a higher degree of TCR ζ-chain phosphorylation than effector T cells (Andersson et al. 2007). However, affinity is not the only deciding factor. Other studies suggested that Treg cells selectively utilize a limited antigen niche for the development of tTreg cells (Bautista et

al. 2009). When self-antigen is expressed at low levels, it promotes the development of Treg cells. However when self-antigen is expressed at high levels, it predominantly results in negative selection (Picca et al. 2009). Therefore both antigen affinity and avidity play important roles in the development of tTreg cells.

Alternatively, Treg cells can also be differentiated from naïve CD4$^+$ T cells in periphery *in vivo* (pTreg cells) or induced from naïve CD4$^+$ T cells *in vitro* (iTreg cells). For simplicity both of these two populations are referred to as induced Treg (iTreg) cells in this thesis. iTreg cells are required to uphold immune homeostasis. Mice deficient in a conserved non-coding DNA sequence (CNS1) that is important for *de novo* FOXP3 expression, develop Th2 type pathologies in lungs and intestines (Josefowicz, Niec, et al. 2012).

Other factors such as co-stimulation and cytokine signalling also participate in Treg cell development. These are discussed more in detail in section 1.5.7.1.

1.5.2 Identifying mouse and human Treg cells

Many markers have been suggested in literatures for the identification of Treg population in humans and mice. Unfortunately none of these markers are specific for Treg cells, therefore a combination of different surface and intracellular molecules are commonly used, which I will discuss briefly below.

1.5.2.1 *FOXP3: the good and the bad*

Since its discovery, FOXP3 has been viewed as the master regulator and specific marker for Treg cells. However there is now substantial amount of evidence showing that FOXP3 is neither a reliable marker nor the sole regulator of functionally stable Treg population. It has been shown that conventional CD4$^+$ T cells can transiently express FOXP3 under TCR stimulation while confer no suppressive activity both in human and mouse (Gavin et al. 2006; Allan et al. 2007; Tran et al. 2007; Wang et al. 2007; Miyao et al. 2012; Miyara et al. 2009). And functional Treg cells can lose FOXP3 expression in inflammatory or lymphopenic conditions and acquire the ability to produce pro-inflammatory cytokines (Zhou et al. 2009; Yang et al. 2008; Xu et al. 2007; Komatsu et al. 2009; Duarte et al. 2009). In addition, studies also demonstrated significant differences between *bona fide* Treg cells and FOXP3-transduced conventional CD4$^+$ T cell in terms of global gene expression patterns (Sugimoto et al. 2006; Hill et al. 2007). Collectively these findings suggest that although FOXP3 is crucial for the suppressive function of Treg cells, FOXP3 *per se* is not sufficient to define the lineage of functional Treg cells and additional mechanisms/molecules are required.

1.5.2.2 *Other markers used for Treg cell identification*

Given that FOXP3 alone is neither specific nor sufficient for delineating Treg population and the fact that it is an intracellular protein, which is not convenient for isolation of Treg cells *ex vivo*, other surface markers have been added in the panel.

CD25: For murine Treg cells, CD25 is a widely used surface marker for isolation of Treg cells (CD4$^+$CD25$^+$ T cells), however activated conventional CD4$^+$ T cells can also up-regulate CD25, which makes it important to use naïve mice in the study (Sakaguchi et al. 1995). For isolation of human Treg cell isolation, CD25 is an even worse marker. Human CD4$^+$ T cells express a gradient level of CD25 on the surface. Although human CD4$^+$ T cell with the highest expression of CD25 have the strongest suppressive activity (Dieckmann et al. 2001), depending on the gating strategy, the CD4$^+$CD25^{hi} T cell population can get substantial contamination from the activated conventional CD4$^+$ T cells (Baecher-Allan et al. 2001; Allan et al. 2007; Miyara et al. 2009). On the other hand, if a more stringent gate is applied, the risk of missing the FOXP3$^+$CD25^{low} Treg population is also increased.

IL-2: is a marker that is rarely used for identifying Treg cells but may be the most specific marker of all. The main advantage of IL-2 is that Treg cells are unable to produce IL-2 as FOXP3 represses IL-2 production in Treg cells. Therefore a FOXP3$^+$ T cell that is unable to express IL-2 is a *bona fide* Treg cell. However, due to its intracellular location and the requirement for stimulation to induce IL-2 production, it is challenging to use IL-2 as a Treg cell marker in practise.

CD127: also known as IL-7 receptor α-chain, is a useful alternative to CD25. Several studies suggested that Treg cells lack the expression of CD127, which makes it a good marker for isolation of human Treg cells in combination with CD25 (in which case Treg cells are defined as CD4$^+$CD25^{hi}CD127$^-$ cells) (Liu et al. 2006; Seddiki et al. 2006). However conventional CD4$^+$ T cells also down-regulate CD127 expression after activation, which complicates the discrimination of Treg cells *ex vivo* (Mazzucchelli & Durum 2007; Aerts et al. 2008).

CD62L: although not exclusively expressed by Treg cells, when combined with CD25 and CD127, can be useful for distinguishing recently activated T cells (CD4$^+$CD62L^{low}) and Treg cells (CD25^{hi}CD127^{low}CD62L$^+$) (Hamann et al. 2000).

CD45RA and CD45RO: A recent paper suggested to use CD45RO and CD45RA in combination with CD25 to further dissect the heterogeneity of human Treg cells (Miyara et al. 2009). In this study, the CD4$^+$CD45RA$^+$CD25^{low}FOXP3$^+$ population represents naïve or resting Treg cells, which corresponds to murine tTreg cells from the thymus. When these resting Treg cells are activated, they up-regulate FOXP3 and convert to activated/effector Treg cells which are CD4$^+$CD45RO$^+$CD45RA$^-$CD25^{hi}FOXP3^{hi} T cells. Conversely the CD4$^+$CD45RA$^-$CD25^{low}FOXP3^{low} population represents activation-induced FOXP3-expressing cells that transiently express FOXP3 *in vitro* and are not suppressive.

Other markers used for the identification of Treg cells are listed in Table 2. These markers are usually related to Treg cell activation status, localization or suppressive function, and can be very useful for studying different subsets of Treg cells.

Transcription factor	Activation and memory	Homing and origin	Suppressive and effector function	Apoptosis, survival or other
FOXP3	CD45RA CD45RO CD25 HLA-DR Lack of CD127 CD69	CD62L CCR4 CCR6 CCR9 CD103 CD304 CD31 Lack of CD49d	CTLA4 ICOS CD39–CD73 LAP Granzyme B Galectin 1 Galectin 10 TRANCE CD80 and CD86 IL-10 IL-17 CD2 Lack of IL-2	CD27 OX40 CD95 PD1 GITR Galectin 3 GARP MS4A4B IL-1R CD6

Table 2: Treg markers in human and mouse. (Sakaguchi et al. 2010)

1.5.3 Treg cell function

The cellular targets and exact programs of Treg cell-mediated suppression remain controversial. However it has become evident that Treg cells can utilize multiple approaches for their suppressive function (Shevach 2011). Although Treg cells can suppress many cells types, DCs and effector T cells are considered the major targets of Treg cell-mediated suppression.

1.5.3.1 *Modulation of DC maturation and function*

Treg cells can inhibit DC maturation *in vitro* by down-regulating or preventing the up-regulation of co-stimulatory molecules CD80/CD86 on DC surface (Cederbom et al. 2000; Misra et al. 2004; DiPaolo et al. 2007). This effect is largely dependent on cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152) (Wing et al. 2008; Qureshi et al. 2011). Treg cells can also inhibit the production of IL-6 while promoting the production of IL-10 and induction of the immunoregulatory enzyme indoleamine-2, 3-deoxygenase (IDO) by DCs (Veldhoen et al. 2006; Grohmann et al. 2002). In addition *in vitro* and *in vivo* studies have demonstrated that Treg cells can decrease the contact between effector T cells and DCs prior to inhibition of effector T cell activation (Onishi et al. 2008; Tang et al. 2006; Tadokoro et al. 2006).

1.5.3.2 *Modulation of effector T cell function*

Treg cells can inhibit effector T cell proliferation by disrupting the metabolic pathways in effector T cells. For example, IL-2 is an important cytokine for T cell proliferation. *In vitro* experiment in mice demonstrated that Treg cells can inhibit IL-2 production in effector T cells in a cell-contact dependent manner thus limit effector T cell proliferation (Thornton & Shevach 1998). However the role of IL-2 *in vivo* remains controversial since complete IL-2 knockout mice actually die from lymphoproliferative syndrome within 5 weeks of age

(Takacs et al. 1984; Jenkinson et al. 1987; Tigges et al. 1989; Sadlack et al. 1995). Treg cells can also interfere with effector T cell activation by hydrolysing adenosine triphosphate (ATP) into adenosine. This hydrolysis cascade is mediated by extracellular enzyme CD39 and CD73 which are expressed both in human and murine Treg cells (Deaglio et al. 2007; Fletcher et al. 2009; Dwyer et al. 2010; Alam et al. 2009; Kobie et al. 2006). Treg cells from CD39 knockout mouse had a more than 50% of reduction in their ability to suppress effector T cell proliferation (Deaglio et al. 2007). In addition, the accumulation of adenosine can also act via adenosine A_{2A} receptor ($A_{2A}R$) and promote iTreg cells induction (Zarek et al. 2008).

1.5.3.3 *Production of anti-inflammatory cytokines*

Several studies suggested that the production of anti-inflammatory cytokine, such as TGFβ and IL-10, is an essential effector mechanism for Treg cells (Powrie et al. 1996; Nakamura et al. 2001; Fahlén et al. 2005; Asseman et al. 1999; Hara et al. 2001). A cell surface form of TGFβ, propeptide-latency-associated peptide (LAP), expressed by activated Treg cells can also induce Foxp3 expression in naïve $CD4^+Foxp3^-$ T cells in a cell-contact dependent manner, this phenomenon is known as "infectious tolerance" (Andersson et al. 2008). Recently another cytokine IL-35 has also been implicated as a mediator for Treg cell suppression. However the exact role of IL-35 requires further investigation (Collison et al. 2007; Collison et al. 2010; Collison et al. 2012). While Treg cells most certainly can produce anti-inflammatory cytokines, the relative importance of these cytokines in Treg cell-dependent suppression remains to be determined. Both mice with TGFβ deficiency specifically in Treg cells and mice completely deficient in an IL-35 subunit p35 displayed no signs of spontaneous inflammatory disease (Gutcher et al. 2011; Collison et al. 2007). In contrast, mice deficient in IL-10 specifically in Treg cells develop inflammation in skin, lung, colon (Rubtsov et al. 2008).

1.5.3.4 *Target cell killing*

Except for anti-inflammatory cytokine production and modulation of DC and effector T cell functions, Treg cells can also kill target cells by inducing cytolysis and apoptosis. These cellular targets can be DCs, effector T cells, B cells and NK cells. For example human Treg cells express granzyme A and can mediate target-cell killing by granzyme A and perforin via the adhesion of CD18 (Grossman et al. 2004). Murine Treg cells showed similar granzyme-B-dependent suppressive mechanism on effector T cell, B cells and NK cells (Gondek et al. 2005; Zhao et al. 2006; Cao et al. 2007). In addition human Treg cells can kill autologous $CD8^+$ T cells through Fas-mediated apoptosis (Strauss et al. 2009), and murine Treg cells were documented to induce apoptosis of effector T cells through a TRAIL–DR5 (tumour-necrosis-factor-related apoptosis-inducing ligand – death receptor 5) pathway (Ren et al. 2007).

1.5.4 Treg cell: stability vs plasticity

As discussed in section 1.5.2, Treg cells is a highly heterogeneous population that could change their functional, migratory and homeostatic properties depending on the environment (Josefowicz, Lu, et al. 2012). And it has long been debated whether Treg cells are a functionally stable population or if they retain certain plasticity. It is also difficult to address whether such plasticity stems from the possibility that Treg cells can be re-programmed into other cell lineages (lineage plasticity) or they simple acquire other effector T cell characteristics temporarily to facilitate suppressive function in a particular setting (functional plasticity). Regardless, it was evident that the expression and maintenance of FOXP3 is at the centre of this debate, and that induction and maintenance of FOXP3 expression are two separate processes regulated by distinct mechanisms (Zheng et al. 2010).

1.5.4.1 Evidences for plasticity

Initial studies suggested that Treg cells are functionally stable due to the fact that they can maintain their suppressive capacity even after many rounds of proliferation and division (Annacker et al. 2000; Gavin et al. 2002; Fisson et al. 2003; Klein et al. 2003). However under lymphopenic or inflammatory conditions, Treg cells can lose FOXP3 expression and start to produce effector cytokines such as IFNγ, IL-2 and IL-17 (Zhou et al. 2009; Yang et al. 2008; Xu et al. 2007; Komatsu et al. 2009; Duarte et al. 2009). In addition, some murine FOXP3$^+$ T cells can even lose FOXP3 expression upon prolonged TCR/CD28 stimulation or with co-stimulation signals (Vu et al. 2007; Degauque et al. 2008; Gabryšová et al. 2011), similar features have also been observed in human FOXP3$^+$ T cells *in vitro* (Koenen et al. 2008; Hoffmann et al. 2009). Under non-lymphopenic conditions, the instability of Treg cells has also been demonstrated. Using genetically modified fate-mapping mouse model, several groups showed that at least a fraction of FOXP3-expressing Treg cells lose their FOXP3 expression *in vivo* or even become pathogenic (Miyao et al. 2012; Zhou et al. 2009; Bailey-Bucktrout et al. 2013).

In certain conditions, Treg cells can also acquire effector T cell-like features without losing FOXP3 expression. For example, when stimulating Treg cells under Th1 polarizing conditions *in vitro*, some FOXP3$^+$ T cells express T-bet and IFNγ without losing FOXP3 expression (Wei et al. 2009; Dominguez-Villar et al. 2011; Koch et al. 2012; Zhao et al. 2012). These FOXP3$^+$T-bet$^+$IFNγ$^+$ T cells can also be found *in vivo* in human disease conditions such as multiple sclerosis (Dominguez-Villar et al. 2011) or type I diabetes (McClymont et al. 2011) and in mice (Oldenhove et al. 2009; Zhao et al. 2011). FOXP3$^+$RORγt$^+$IL17$^+$ T cells have also been identified *in vivo* both in human and mouse, especially in the intestine (Beriou et al. 2009; Voo et al. 2009; Zhou et al. 2008).

1.5.4.2 Evidences against plasticity

There are also contradictory observations that dispute the notion of Treg cell plasticity. One study using mouse experimental autoimmune encephalomyelitis (EAE) model demonstrated

that myelin oligodendrocyte glycoprotein (MOG)-specific FOXP3$^+$ and FOXP3$^-$ T cells had very different TCR CDR3 sequences and were derived from distinct clones, indicating that these two populations had very limited inter-conversion, if any, during the autoimmune inflammation (Liu et al. 2009). Moreover Rudensky and colleagues also showed that in their genetic fate-mapping mouse model, FOXP3$^+$ T cells are remarkably stable under steady state, and these sorted FOXP3$^+$ T cells did not convert to FOXP3$^-$ T cells under autoimmune conditions in non-lymphopenic host mice (Rubtsov et al. 2010).

1.5.4.3   Concluding remarks

The above-mentioned studies cannot be easily reconciled with each other. However, it is clear that the Treg cell identity is not determined solely by a single factor, for example FOXP3. This notion is further supported by two recent studies where it was shown that FOXP3 together with its cofactors form an interactive network with multiple circuitries and feedback loops, together this interactive network "locks-in" the characteristics of a Treg cell signature (Fu et al. 2012; Rudra et al. 2012). In addition, the epigenetic regulation play an important part in deciding Treg cell fate (Toker et al. 2013). Collectively these findings suggest that the Treg cell identity is shaped through an interactive transcriptional network that can self-perpetuate as a whole.

1.5.5   Treg cells in diseases

1.5.5.1   Treg cells in infection

To survive an infection, the host needs to clear the invading pathogens but also limit the extent of the immune response so as not to damage self-tissue. This is where Treg cells come into play as immune regulators. However, the outcome of Treg cell mediated suppression can be either beneficial or detrimental to the host depending on the properties of the invading pathogens as well as the location and phase of infection.

Several studies using immuno-deficient animals demonstrated that Treg cells are needed for controlling the excessive immune response during an infection to limit collateral damage to host tissue (Suvas et al. 2004; Hesse et al. 2004). However, the suppressive activity of Treg cells may also cause unwanted consequences. In deed, too much suppression during an acute infection could hinder the protective immune responses and clearance of pathogens, which leads to either death or the prolonged survival of pathogens and persistence of infection. For example, in patients with chronic HIV infection, removal of CD4$^+$CD25$^+$ T cells enhanced anti-viral response and reduced viral load (Aandahl et al. 2004; Andersson et al. 2005). On the other hand, persistence of infection could also help the host to develop concomitant immunity that protects from reinfection. In a mouse model of persistent *Leishmania major* infection in the skin, after initial recovery from the first infection, Treg cells can suppress the CD4$^+$CD25$^-$ effector T cell function via IL-10 dependent and independent mechanisms, thereby allowing parasite persistence and at the same time maintaining an efficient memory response to *Leishmania major* that protects the host from reinfection.

In summary, it seems that the role of Treg cells in infection is to establish a fine balance between the pathogen and its host that should benefit both. However if such balance were to be displaced, leading to excessive induction of Treg cells or alteration in Treg cell function, it would result in excessive replication of and damage by the pathogen that can overwhelm the host.

1.5.5.2 Treg cells in cancer

Cancer or malignant tumors refers to a group of diseases characterized by abnormal and uncontrollable cell growth with the potential of spreading to other parts of the body that damage normal physiological functions. One of the hallmarks of cancer is the ability to avoid immune destruction (Hanahan & Weinberg 2011). Treg cells may suppress anti-tumor responses since most tumor-associated antigens are derived from self proteins (Kawakami & Rosenberg 1997). On the other hand, Treg cells can also suppress inflammatory responses, which in turn predispose for the development of cancer. Thus the correlation between Treg cell numbers and cancer progression in patients remains ambiguous.

Indeed, increased frequency of Treg cells is present in various types of cancers such as head and neck (Schaefer et al. 2005), lung (Wolf et al. 2003), liver (Ormandy et al. 2005), gastrointestinal tract (Ichihara et al. 2003), pancreas (Hiraoka et al. 2006), breast (Liyanage et al. 2002), ovary (Curiel et al. 2004) and malignant melanoma (Gerber et al. 2014) and the number of Treg cells are negatively related to disease progression in gastric cancer (Sasada et al. 2003), breast cancer (Bates et al. 2006) and ovarian cancer (Curiel et al. 2004; Sato et al. 2005). However, other studies also demonstrated that increased number of Treg cells can associate with good prognosis, for example in head and neck cancer (Badoual et al. 2006), colon cancer (Salama et al. 2009) and Hodgkin lymphoma (Álvaro et al. 2005).

1.5.5.3 Treg cells in autoimmune disorders

An autoimmune disorder occurs when immunological tolerance is broken and the immune system starts to attack healthy body tissue by mistake, this includes diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), type 1 diabetes (T1D), rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). Loss of tolerance due to Treg cells can be reflected in many ways: it could be caused by a reduction in Treg cell number in the circulation or at the site of inflammation, defects in Treg cell suppressive function, inappropriate Treg cell specificities, and/or instability of Treg cell lineage.

For example, decreased Treg cell frequency as well as impaired Treg cell suppressive function were observed in MS patients (Viglietta et al. 2004; Haas et al. 2005; Kumar et al. 2006; Venken et al. 2008). In T1D patients, although Treg cell numbers appeared to be unchanged, those Treg cell had imparied suppressive function (Lindley et al. 2005; Ferraro et al. 2011). The number of Treg cells in IBD patients varies depending on the disease progression (Maul et al. 2005), and reports suggest that some Treg cells may lose their suppressive function and differentiate into Th17 cells (Ueno et al. 2013).

1.5.5.4 Concluding remarks

Many discrepancies exist regarding the role of Treg cells in disease progression and prognosis. This may due to a number of reasons:

1. Human Treg cell population is not an homogenous population, and FOXP3 is not a perfect indicator for the presence of *bona fide* Treg cells (Miyara et al. 2009).
2. It is the number of activated Treg cells, not the number of total Treg cells that matters.
3. It is not given that suppression only occurs within the local tissue, and the number of antigen-specific Treg cells in secondary lymphoid tissues in many studies remain largely unknown.
4. There are a number of technical concerns with the quantification of Treg cells in some studies. The specificity of certain commercial anti-FOXP3 antibodies remain questionable.

To better evaluate the roles of Treg cells in diseases, it is necessary to dissect the functional differences between different subsets of FOXP3-expressing cells in the circulation and in the local tissue.

1.5.6 FOXP3 and its isoforms

The FOXP3 gene is located on the X chromosome. Both human and mouse FOXP3 genes contain 11 coding exons, and the exon-intron boundaries are identical across the coding regions between mice and human (Brunkow et al. 2001). Downstream of the promoter region, the FOXP3 gene also contains multiple evolutionally conserved non-coding sequence regions (CNS1, CNS2 and CNS3), which serve as enhancers that regulate gene expression. The FOXP3 protein consists of a proline-rich N-terminal domain (exons 2–4), a zinc finger and leucine zipper domain (exons 5–7) and a fork-head (FKH) domain (exons 9–11) (Fig. 2).
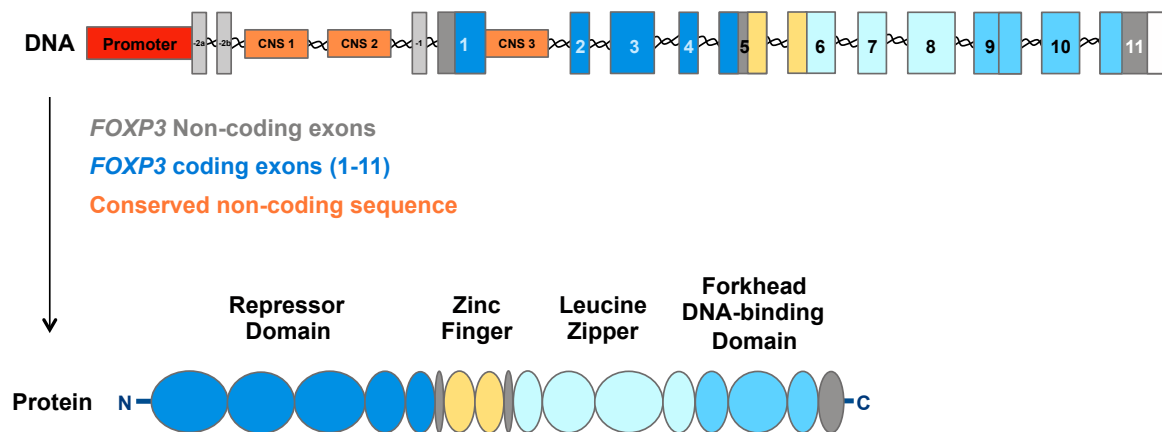

Figure 2: the structure of FOXP3 gene and FOXP3 protein.

1.5.6.1 Requirements for FOXP3 expression

Many transcriptional events govern the expression of FOXP3: TCR signalling, co-stimulation/co-inhibition and cytokine receptors are essential for active transcription of FOXP3 gene (Fig. 3 and Fig. 4).

TCR signalling pathways contribute to FOXP3 expression in both tTreg cells and iTreg cells. In mouse T cells, downstream of TCR signalling, nuclear factor-κb (NF-κB) signals through c-Rel that directly binds the promoter, CNS2 and CNS3 regions to allow *Foxp3* transcription (Isomura et al. 2009; Long et al. 2009; Ruan et al. 2009; Zheng et al. 2010). In addition, the binding of cyclic-AMP-responsive-element-binding-protein (CREB) and activating transcription factor (ATF) to the CNS2 region in *Foxp3* gene after TCR stimulation was also implicated in *Foxp3* expression in the thymus (Kim & Leonard 2007). TCR signalling also induces the expression of the nuclear receptor 4a (Nr4a) family. These factors bind to the *Foxp3* promoter region and induce *Foxp3* expression (Fassett et al. 2012). In human T cells, TCR signalling results in the binding of nuclear factor of activated T cells (NFAT) and activator protein 1 (AP1) to the *FOXP3* promoter and induction of *FOXP3* expression (Mantel et al. 2006).

In addition to TCR signalling, co-stimulation is necessary for FOXP3 gene expression. However, while CD28 stimulation of thymocytes induces the expression of *Foxp3* in the thymus (Tai et al. 2005), strong co-stimulation provided by CD28 impairs *Foxp3* expression and iTreg induction in the periphery (Benson et al. 2007). Unlike in the thymus, the induction of *Foxp3* and iTreg cells in the periphery require co-inhibitory molecules such as CTLA-4 (Zheng et al. 2006).
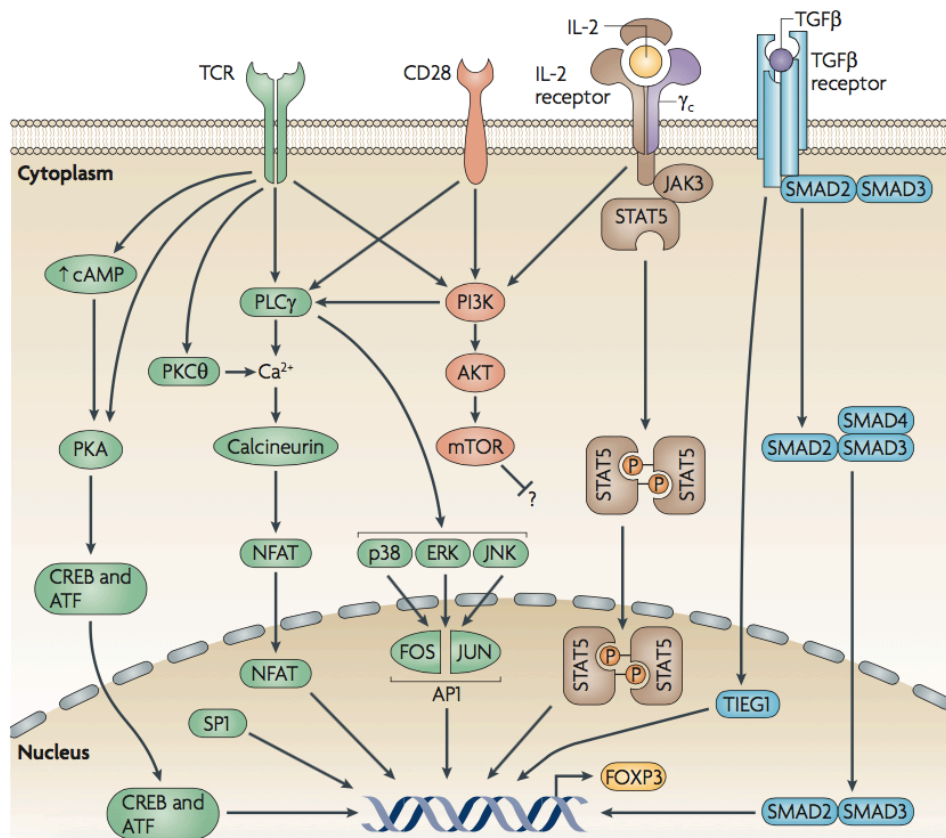
Figure 3: Signalling pathways involved in human FOXP3 expression. (Huehn et al. 2009)
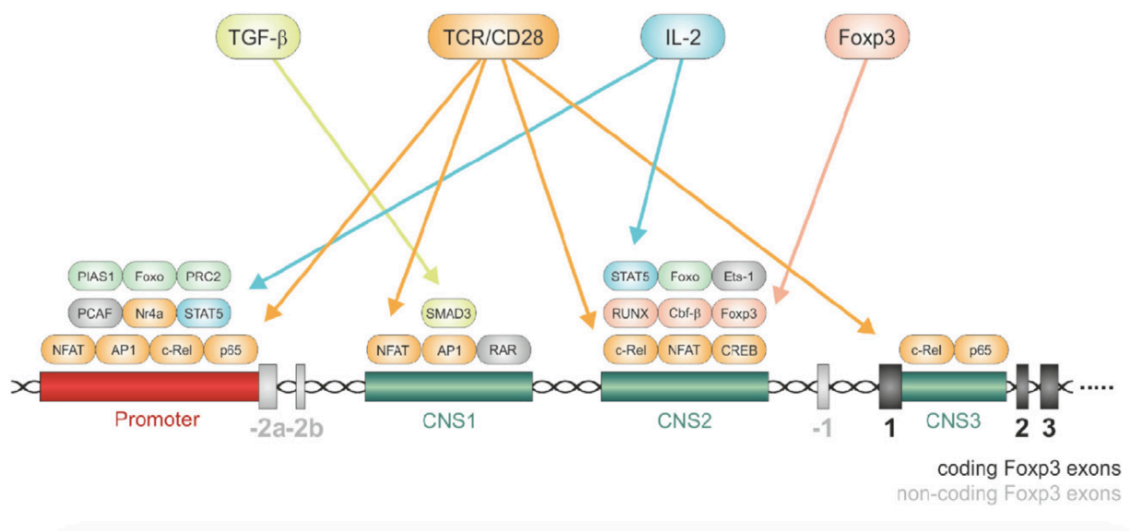

Figure 4: Transcription factors regulating mouse Foxp3 expression. (Huehn & Beyer 2015)

Last but not least, cytokine mediated signalling is also important for FOXP3 gene expression. Treg cells are absent in the mice deficient for the common cytokine-receptor γ-chain, which transmits signals mediated by IL-2 and many other cytokines. However IL-2 signalling is dispensable for the induction of *Foxp3* in the thymus, which means other cytokines are involved in this effect (Fontenot et al. 2005). The signalling cascade following the engagement of IL-2 involves Janus kinase 1 (JAK1), JAK3 and signal transducer and activator of transcription 5 (STAT5), STAT5 binds to both the *Foxp3* promoter and CNS3 which induce and stabilize *Foxp3* gene expression (Yao et al. 2007; Burchill et al. 2007). Although IL-2 is not needed for tTreg cell development in the thymus, it is required for TGFβ-mediated induction of *Foxp3* in iTreg cells (Davidson et al. 2007). In mice TGFβ is important for tTreg cell development as well as the maintenance of *Foxp3* expression and homeostasis of iTreg cells in the periphery (Marie et al. 2005; Liu et al. 2008; Ouyang et al. 2010). The binding of SMAD3 downstream of TGFβ signalling is a prerequisite for the induction of *Foxp3* expression in naïve CD4$^+$ T cells (Tone et al. 2008; Zheng et al. 2010; Schlenner et al. 2012). Furthermore generation of iTreg induced by TGFβ can be further supported by retinoic acid (RA) produced by intestinal DCs (Mucida et al. 2007; Benson et al. 2007; Hill et al. 2008). RA can directly induce histone acetylation at *Foxp3* promoter (Kang et al. 2007), recruiting RA receptors to CNS1 (Xu et al. 2010), or interferes with production of effector cytokine by memory T cells (Hill et al. 2008).

1.5.6.2 *Epigenetic control of FOXP3 expression*

Epigenetic processes are known to play a key role in gene regulation. DNA methylation and histone modification are two major epigenetic mechanisms for establishing and maintaining chromatin structures. Recent studies suggest that FOXP3 gene expression in Treg cells is also under epigenetic control. Treg cells present a distinct DNA methylation pattern and characteristic histone modifications (Ohkura et al. 2012). For example, in both human and mice the CpG sites in the promoter region of the FOXP3 gene are almost completely demethylated in Treg cells while conventional CD4$^+$ T cells have dominantly methylation in the promoter region (Kim & Leonard 2007; Zheng et al. 2010; Floess et al. 2007). Permissive histone modifications were also identified at the *Foxp3* promoter region in Treg cells but not in conventional CD4$^+$ T cells (Rudra et al. 2009; Zheng et al. 2010; Sekiya et al. 2013).

The CNS region in *Foxp3* has non-redundant regulatory functions for *Foxp3* transcription and is also under epigenetic control. CNS1 is a sensor for TGFβ and is important for iTreg cell development. It does not contain any CpG motifs, but enriched permissive histone modifications were found in both tTreg cells and iTreg cells and deletion of CNS1 led to defective generation of iTreg cells (Zheng et al. 2010; Sekiya et al. 2011). CNS2, also known as Treg-cell specific demethylated region (TSDR), is a CpG-rich element downstream of the promoter. Demethylation of CNS2 is critical for the stabilization of *Foxp3* expression in Treg cells (Nagar et al. 2008; Polansky et al. 2008), on the other hand, methylation of CNS2 prevent FOXP3 gene expression in non-Treg cells both in human and mouse (Zorn et al.

2006; Josefowicz et al. 2009). CNS3 is located downstream of the first coding exon. In Treg cells, this region is also enriched in permissive histone modification such as H3K4 mono- and dimethylation even before *Foxp3* was expressed in thymocytes, suggesting that CNS3 may facilitate the opening of *Foxp3* locus in Treg-precursors and is important for the initiation of *Foxp3* expression in both tTreg and iTreg cells. (Zheng et al. 2010).

1.5.6.3 *Alternative splicing and FOXP3 isoforms*

Alternative splicing is a highly regulated biological process that affects gene expression resulting in multiple protein products from a single gene. This process uses precursor mRNA as matrix or occurs co-transcriptionally. During alternative splicing, the spliceosome, a macromolecular ribonucleoprotein complex, selects and recombines certain exons to produce multiple mRNA transcripts that give rise to multiple protein isoforms. Alternative splicing occurs in 95–100% of human genes and approximately 63% of mouse genes (Barbosa-Morais et al. 2012; Merkin et al. 2012), which greatly expands proteome diversity and functions in vertebrates (Nilsen & Graveley 2010; Irimia & Blencowe 2012; Braunschweig et al. 2013).
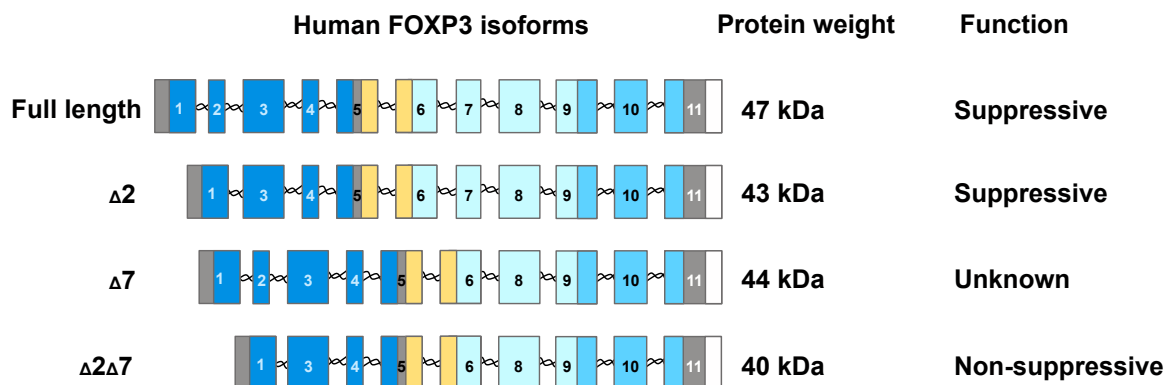

Figure 5: Human FOXP3 isoforms and functions.

So far FOXP3 isoforms have only been identified in humans but not in mice, and in human 4 FOXP3 isoforms have been described (Fig. 5): a full-length isoform that contains all exons (FOXP3fl), an isoform lacking exon 2 (FOXP3Δ2), an isoform lacking exon 7 (FOXP3Δ7) and an isoform lacking both exon 2 and exon 7 (FOXP3Δ2Δ7) (Walker et al. 2003; Kaur et al. 2010; Mailer et al. 2009). The structural differences among human FOXP3 isoforms lead to different biological functions. For example, while FOXP3fl and FOXP3Δ2 confer a suppressive ability to Treg cells *in vitro*, FOXP3Δ2Δ7 inhibit other FOXP3 isoforms in a dominant negative manner (Aarts-Riemens et al. 2008; Mailer et al. 2009). The function of FOXP3Δ7 remains unknown (Kaur et al. 2010).

1.5.6.4   Post-translational modification of Foxp3

Post-translational modifications (PTMs) include phosphorylation, acetylation, ubiquitination, sumoylation, methylation, and hydroxylation. PTMs can regulate the function of many transcription factors (Xu et al. 2012) and PTMs such as ubiquitination, acetylation, and phosphorylation are now known to regulate FOXP3 function (van Loosdregt & Coffer 2014).

Ubiquitination controls the balance between protein synthesis and degradation by adding one (monoubiquitination) or more (polyubiquitination) ubiquitin to the lysine residues of a target protein. Treating murine Treg cells with a pan-deubiquitinase inhibitor can significantly decrease FOXP3 protein level in Treg cells as well as their suppressive capacity (van Loosdregt et al. 2013).

Acetylation is the process where an acetyle group is covalently added to a lysine residue or the N-terminus in a substrate protein. As both acetylation and ubiquitination are restricted to lysine residues, acetylation can inhibit ubiquitination and vice versa. Acetylation of FOXP3 protein has been shown to affect both FOXP3 stability and its DNA binding capacity (Li, et al. 2007; Samanta et al. 2008; van Loosdregt et al. 2010). For example mutation in the lysine residues of FOXP3 FKH domain impaired FOXP3 DNA binding ability and altered transcriptional activity of FOXP3 as well as Treg-associated gene expression profiles (Liu et al. 2012).

Finally, FOXP3 activity can also be regulated by phosphorylation. A recent study demonstrated that phosphorylation at Ser418 in the C-terminal DNA-binding domain of FOXP3 regulates Treg cell suppressive function, and that in rheumatoid arthritis, TNFα induces dephosphorylated of Ser418 thus impair Treg cell function (Nie et al. 2013).

Several extracellular signals can regulate FOXP3 PTMs. For instance TCR stimulation can promote the translocation of HDAC6 to the cell nucleus where it deacetylates FOXP3 thereby increase FOXP3 polyubiquitination and degradation (van Loosdregt et al. 2010; de Zoeten et al. 2011; Beier et al. 2012). On the other hand, TGFβ can stabilize FOXP3 by increasing the expression of histone acetyltransferases p300, p300 acetylates FOXP3 thus prevents FOXP3 from polyubiquitination (Liu et al. 2013; Ghosh et al. 2013; Xiao et al. 2014). Pro-inflammatory cytokine TNFα increases the expression of protein phosphatase (PP1), which can dephosphorylate FOXP3 and decrease FOXP3 DNA binding capacity and transcriptional activity (Nie et al. 2013). In addition, the chemokine CC ligand 3 (CCL3), IL-6 and hypoxia can promote FOXP3 ubiquitination and degradation (Dang et al. 2011; van Loosdregt et al. 2013; Chen et al. 2013).

1.5.6.5   Foxp3-dependent transcriptional programs

Studies on murine Treg cells identified more than 600 genes that consist a typical Treg cell signature, although part of the signature genes are not directly regulated by *Foxp3* but rather co-regulated with *Foxp3* (Hill et al. 2007). Proteomics study revealed that more than 300

proteins interact with FOXP3 and form a complex regulatory network. These interactions are involved in a large number of biological processes such as chromatin modification, chromatin or DNA binding, regulation of transcriptional activity, RNA binding, processing, splicing and metabolism (Rudra et al. 2012).

The 4 functional domains of FOXP3 possess distinct ability to interact with a variety of molecules. The N-terminal domain is responsible for transcriptional repression; the zinc finger and leucine-zipper are important for FOXP3 homo-dimer or tetramer formation; the highly conserved FKH domain has the capacity to bind to DNA (Fig. 6).
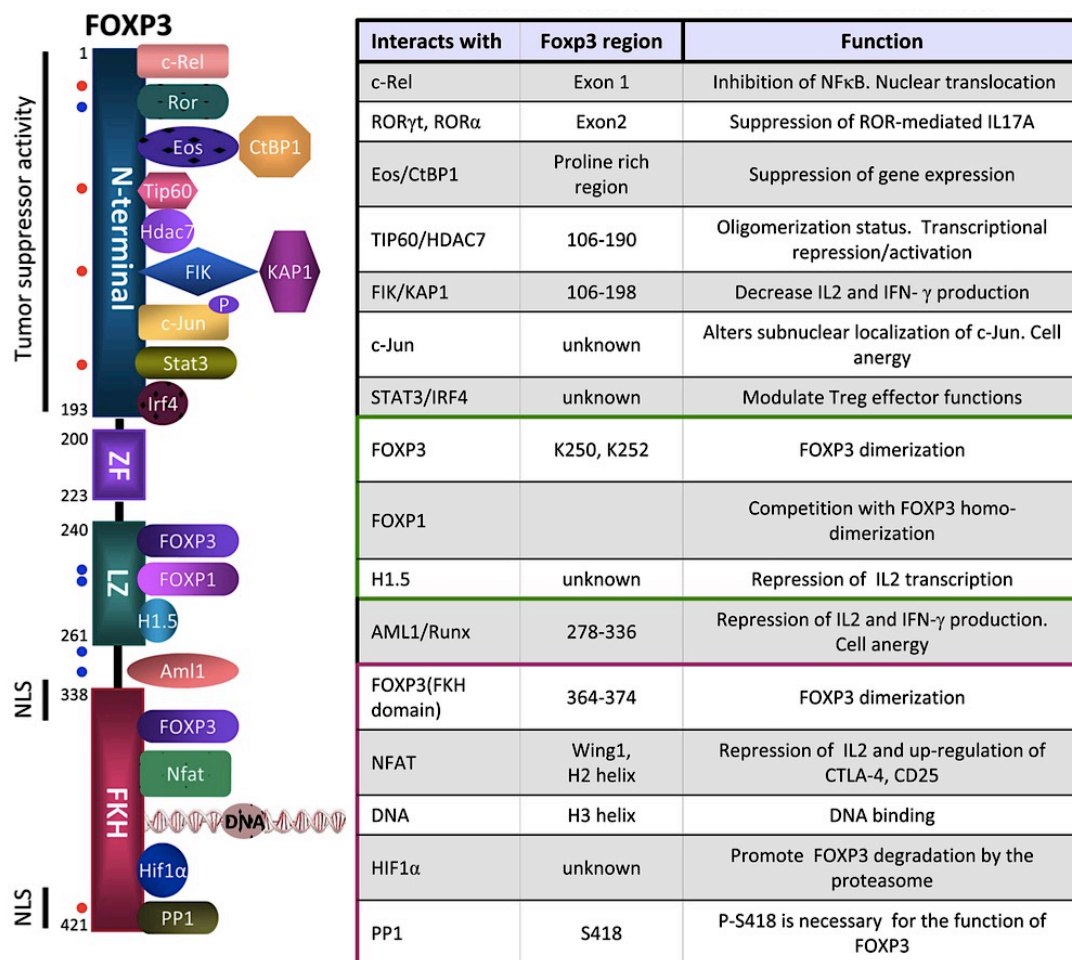

Interacts with	Foxp3 region	Function
c-Rel	Exon 1	Inhibition of NFκB. Nuclear translocation
RORγt, RORα	Exon2	Suppression of ROR-mediated IL17A
Eos/CtBP1	Proline rich region	Suppression of gene expression
TIP60/HDAC7	106-190	Oligomerization status. Transcriptional repression/activation
FIK/KAP1	106-198	Decrease IL2 and IFN-γ production
c-Jun	unknown	Alters subnuclear localization of c-Jun. Cell anergy
STAT3/IRF4	unknown	Modulate Treg effector functions
FOXP3	K250, K252	FOXP3 dimerization
FOXP1		Competition with FOXP3 homo-dimerization
H1.5	unknown	Repression of IL2 transcription
AML1/Runx	278-336	Repression of IL2 and IFN-γ production. Cell anergy
FOXP3(FKH domain)	364-374	FOXP3 dimerization
NFAT	Wing1, H2 helix	Repression of IL2 and up-regulation of CTLA-4, CD25
DNA	H3 helix	DNA binding
HIF1α	unknown	Promote FOXP3 degradation by the proteasome
PP1	S418	P-S418 is necessary for the function of FOXP3

Figure 6: Schematic representation of some FOXP3 binding partners, their site of interactions and function. (Adapted from Lozano et al. 2013)

For example, the Ikaros family member Eos interacts with FOXP3 N-terminal domain, and together with the C-terminal binding protein 1 (CtBP1), form an inhibitory complex that mediates Foxp3-dependent gene silencing in Tregs (Pan et al. 2009). FOXP3 exon 2, which is located in the N-terminal domain, can also interact with and suppress the activity of RORγt

and RORα thus inhibit Th17 differentiation (Ichiyama et al. 2008; Zhou et al. 2008). The N-terminal domain is also known to interact with c-Rel, this interaction can suppress the up-regulation of many cytokines such as IL-6, IL-12, IFNγ and IL-2 (Fraser et al. 1991; Liou & Hsia 2003) and also contributes to the nuclear translocation of FOXP3 (Bettelli et al. 2005; Loizou et al. 2011). In addition to binding to transcriptional factors, the N-terminal domain also interacts with enzymes that regulate FOXP3 PTMs. Histone acetyltransferase (HAT) TIP60 and histone deacetylase HDAC7 both interact with the N-terminal of FOXP3 and this interaction is required for FOXP3-mediated repression (Li et al. 2007).

The zinc finger and leucine-zipper are needed for dimerization of FOXP3 which is required for FOXP3 function as a transcriptional factor (Chae et al. 2006; Song et al. 2012). The leucine-zipper also interacts with histone H1.5 and runt-related transcription factor 1 (RUNXI) which represses the transcription of IL-2 and IFNγ genes (Mackey-Cushman et al. 2011; Ono et al. 2007).

Finally NFAT interacts with FOXP3 FKH domain, this interaction represses the expression of IL-2 and up-regulates Treg markers such as CTLA-4 and CD25 (Wu et al. 2006). Under certain circumstances hypoxia-inducible factor 1 (HIF-1α) can bind to the FKH domain of FOXP3 and promote FOXP3 degradation (Dang et al. 2011).

1.6 T CELL CO-SIGNALING RECEPTOR SUPER FAMILY

The co-signalling molecules belong to the immunoglobulin superfamily (IgSF) and tumor necrosis factor receptor superfamily (TNFRSF). These molecules send stimulatory or inhibitory signals to the T cells to fine-tune T cell activation. Many co-signalling molecules have been identified and extensively studied during the years, among which are CD28, CD80/CD86, CTLA-4, programmed cell death protein 1 (PD-1) and its ligands, inducible T-cell co-stimulator (ICOS), glucocorticoid-induced TNFR-related protein (GITR), etc (Chen & Flies 2013).

1.6.1 CTLA-4 and its isoforms

The CTLA-4 gene is located on human chromosome 2 and mouse chromosome 1, respectively (Harper et al. 1991; Howard et al. 1991; Lafage-Pochitaloff et al. 1990). It contains 4 coding exons for 4 different functional domains: exon 1 encodes the leader peptide, exon 2 the ligand-binding site, exon 3 the trans-membrane region and exon 4 the cytoplasmic tail (Ling et al. 1999). Both the leader peptide and the ligand-binding site form the extracellular part of CTLA-4.

1.6.1.1 CTLA-4 isoforms

CTLA-4 transcripts can form isoforms due to alternative splicing, and different isoforms exist between human and mouse. In human three CTLA-4 isoforms have been described: a full-length isoform that contains all exons (flCTLA-4), a soluble form that lacks exon 3 (sCTLA-4) (Oaks et al. 2000; Huurman et al. 2007) and a third isoform that contains only exon 1 and

exon 4 (1/4CTLA-4). For murine cells, four isoforms have been identified. Except for the three isoforms mentioned before, there is a fourth isoform in murine cells, the ligand-independent CTLA-4 isoform (liCTLA-4), which lacks exon 2 (Ueda et al. 2003).

1.6.1.2 *CTLA-4 expression*

The localization and translocation of CTLA-4 is high dynamic. CTLA-4 is primarily found in intracellular compartments. The cytoplasmic domain contains an intracellular localization motif that may regulate its surface expression and function during T cell activation (Leung et al. 1995). The expression of CTLA-4 in human and mouse naïve T cells is barely detectable. Upon activation, naïve T cells can rapidly express CTLA-4 on the cell surface (Alegre et al. 1996; Jago et al. 2004) and the extent of CTLA-4 expression is determined by the strength of the TCR signal (Egen & Allison 2002). Murine Treg cells constitutively express high level of CTLA-4 both on the surface and intracellularly (Takahashi et al. 2000). Human Treg cells express CTLA-4 intracellular only after activation *in vitro* (Miyara et al. 2009).

Curiously enough, *in vitro* studies on murine T cells suggested that the expression of liCTLA-4 and flCTLA-4 followed a reciprocal pattern in early T cell activation. While liCTLA-4 was found highly expressed in un-stimulated CD3$^+$ T cells, it decreased its expression within 24 hours after TCR engagement and was induced again by 48 hours post-TCR stimulation. On the contrary, flCTLA-4 was rapidly induced within 16 hours after T cell activation and plateaued 24 hours post-stimulation. In addition, the level of liCTLA-4 mRNA in the CD4$^+$CD45RB^{low} cells from diabetes resistant mouse strain was 4 times higher than that of the susceptible strain (Vijayakrishnan et al. 2004).

1.6.1.3 *CTLA-4 function*

CTLA-4 is an important negative regulator of immune responses. It is a structural homologue of the co-stimulatory receptor CD28, but has a stronger affinity to CD80/CD86 than CD28, especially to CD80 (Freeman et al. 1991; Freeman et al. 1993; Harper et al. 1991; Linsley et al. 1991). This interaction is dependent on the extracellular binding domain of CTLA-4 (Linsley et al. 1991; Linsley et al. 1994). Germline *Ctla4*-deficient mice develop lethal immune dysregulation and autoimmunity (Tivol et al. 1995; Waterhouse et al. 1995). In humans, polymorphism in the CTLA-4 gene is associated with many types of autoimmune diseases (Ueda et al. 2003; Gough et al. 2005).

The exact mechanisms how CTLA-4 restrains T cell activation remain controversial. Studies so far suggested that CTLA-4 have both cell-intrinsic and cell-extrinsic functions. As cell-intrinsic functions, the cytoplasmic tail of CTLA-4 can recruit phosphatases SHP-2 and PP2A, which leads to the inhibition of downstream TCR signalling (Marengère et al. 1996; Lee et al. 1998; Chuang et al. 1999; Chuang et al. 2000). Moreover, CTLA-4 can compete with CD28 for the binding of CD80/CD86 in the immunological synapses; this interaction leads to less activation of NF-κB, NFAT and AP-1, and inhibition of IL-2 production and T cell proliferation (Fraser et al. 1999; Olsson et al. 1999; Greenwald et al. 2002; Krummel &

Allison 1996). It has also been shown that the cytoplasmic tail of CTLA-4 is necessary for TCR hypo-signalling in Treg cells (Tai et al. 2012).

The cell extrinsic function of CTLA-4 is mostly attributed to its ability to bind to CD80/CD86. Several studies have suggested that CTLA-4 can down-regulate CD80/CD86 on APCs by either indirect suppression of the APCs (Fallarino et al. 2003; Onishi et al. 2008), possible signalling through CD80/CD86 (Wing et al. 2008), or removal of CD80/CD86 on the surface of APCs by trans-endocytosis (Qureshi et al. 2011; Hou et al. 2015).

The expression of CTLA-4 on Treg cells is also vital for immune homeostasis. CTLA-4 is a target gene of FOXP3 transcriptional regulation (Wu et al. 2006; Zheng et al. 2007). Mice lacking CTLA-4 specifically in Treg cells phenocopy *Foxp3* deficient scurfy mice and die from lympho-proliferative disease early in life (Wing et al. 2008). CTLA-4-intact Treg cells are capable of suppressing autoimmunity mediated by CTLA-4-deficient Teff cells (Kolar et al. 2009; Ise et al. 2010). The discovery of trans-endocytosis by CTLA-4 provided a mechanistic explanation for Treg-mediated suppression via CTLA-4, and some believed that this cell-extrinsic function is essential and sufficient for Treg cell-mediated suppression via CTLA-4 (Walker & Sansom 2015). However, it has been shown that the expression of liCTLA-4 in the diabetes-resistant mouse strain was fourfold higher than that of the non-obese diabetic (NOD) mouse strain (Ueda et al. 2003). In addition, Bluestone and colleagues also demonstrated in a mouse T1D model that liCTLA-4-expressing Treg cells seemed to be suppressive and could reduce diabetes incidence *in vivo* (Stumpf et al. 2013). On the other hand, when a mutation was introduced to the cytoplasmic tail of CTLA-4 so that an amino acid residue (Tyrosine 201) was replaced by a valine that could not be phosphorylated, the suppressive function of Treg cells was impaired while effector T cell function remained intact (Stumpf et al. 2014). This raises further questions about the importance of the cell-extrinsic function of CTLA-4 on Treg cell-mediated suppression.

1.6.2 PD-1 and its ligands

Programmed death-1 (CD279) is another member of the T cell co-signalling receptor super family. Its negative role in immune regulation was discovered by the autoimmune-prone phenotype observed in *Pdcd1*$^{-/-}$ mice (Nishimura et al. 1999; Nishimura et al. 2001). PD-1 has two ligands PD-L1 (B7-H1, also known as CD274) and PD-L2 (B7-DC, also known as CD273). Except for PD-1, PD-L1 can also bind to CD80 (Butte et al. 2007) while PD-L2 does not bind to CD80. However, there are data suggesting that PD-L2 may have another unknown receptor except for PD-1(Wang et al. 2003).

PD-1 can be expressed on T cells, B cells, natural killer T (NKT) cells, activated monocytes, and DCs. Activated effector T cells express PD-1 on the surface, however resting Treg cells highly express PD-1 in the intracellular compartment (Raimondi et al. 2006). PD-L1 is constitutively expressed by murine T cells, B cells, DCs and macrophages, and induced to high level by inflammation. However the expression of PD-L2 is more restricted than PD-L1,

its expression can only be induced on DCs and macrophages (Yamazaki et al. 2002) and some subsets of B cells (Zhong et al. 2007). IL-4, IFNγ and GM-CSF are powerful inducers of PD-L2 (Yamazaki et al. 2002).

Treg cells can promote tolerance by inducing iTreg cells via PD-L1 signalling (Amarnath et al. 2010; Amarnath et al. 2011; Francisco et al. 2009). Some studies in mice have shown that expression and signalling of PD-L1 but not PD-L2 on the dendritic cells are important for the induction of FOXP3$^+$ regulatory T cell from CD4$^+$FOXP3$^-$ T cells (Wang et al. 2008; Amarnath et al. 2011; Francisco et al. 2009) while other groups reported that PD-L1 and PD-L2 are equally important for this effect (Fukaya et al. 2010). Studies using *Pdl2*$^{-/-}$ mouse model suggested that PD-L2 is important for inhibition of T cell activation as well as oral tolerance (Zhang et al. 2006), and it has irredundant functions in tuning Th cells and CTL responses (Shin et al. 2005) as well as promoting anti-tumour immunity in a PD-1-independent mechanism (Liu et al. 2003).

2 STUDY AIMS

The overall aim of this study is to understand how protein isoforms contribute to regulatory T cell function and to define the functional consequences of Treg cell-mediated suppression on dendritic cells.

More specific aims for each paper were:

Paper I -- To understand the regulation and functional consequences of FOXP3 isoform expression in chronic inflammatory diseases in human

Paper II -- To generate and characterize an *in vivo* animal model where we can study the biology of FOXP3 isoforms, specifically the FOXP3Δ2Δ7 isoform

Paper III -- To dissect the functional consequences of Treg cell-mediated suppression on dendritic cells and more specifically, how CTLA-4 contributes to Treg cell-mediated suppression on dendritic cells

3 MATERIAL AND METHODS

3.1 ANIMAL MODELS

3.1.1 Generation of *Foxp3^{δ2δ7}* mouse

A target vector was designed in such a way that a *Foxp3* cDNA lacking coding exon 2 and 7 was inserted after the *Foxp3* translation initiation ATG codon, the TGA stop codon was replaced with a 2A peptide-GFP cassette followed by a double polyadenylation signal (pA) and an FRT flanked Neo cassette. The sequence of the *Foxp3δ2δ7-2A-GFP* cassette was verified using DNA sequencing prior to subcloning into the target vector. The *Foxp3δ2δ7-2A-GFP-2xpA-Neo* cassette was flanked by a 5' homology arm that extended 4.61 kb 5' to the site of the cassette insertion and a 3' homology arm that extended 2.05 kb 3' to the site of the Neo cassette insertion, both of which were derived from a C57BL/6 BAC clone (RP23:54C14). Ten micrograms of the targeting vector was linearized using Not I and then transfected into BA1 (CD57BL/6 x 129/SvEv) hybrid embryonic stem cells by electroporation. After selection with G418 antibiotic, positive clones were screened by PCR and southern blotting analysis. Finally, the Foxp3 knock-in mice were bred with FLP delete mice to remove the Neo cassette, the resulting chimeras were further inbred to obtain mice that have the Neo deletion transmitted in the germ cells, yielding *Foxp3^{δ2δ7}* mice.

3.1.2 Generation of *Ctla4$^{ex2fl/fl}$Foxp3-Cre* mouse

A 8.9kb targeting vector was designed so that the long homology arm extends 6.01 kb 5' to the single LoxP site. The short homology arm extends 2.02 kb 3' to the LoxP/FRT-flanked Neo cassette. The single LoxP site is inserted 249 bp upstream of exon 2 of *Ctla4* gene in intron 1-2, and the LoxP/FRT-flanked Neo cassette is inserted 271 bp downstream of exon 2 in intron 2-3. The designed vector was subcloned from a positively identified C57BL/6 BAC clone (RP23:388N14) by homologous recombination-based technique. The BAC was then sub cloned into a ~2.45 kb pSP72 (Promega) backbone vector containing an ampicillin selection cassette for retransformation of the construct prior to electroporation. A pGK-gb2 LoxP/FRT-flanked Neomycin cassette was inserted into the *Ctla4* gene. The targeting construct was linearized using NotI prior to electroporation into embryonic stem (ES) cells. ES cells were then microinjected into Balb/c blastocysts. To remove the Neo cassette and to obtain *Ctla4$^{ex2fl/fl}$* mice, the chimeras from the ES cells were mated with C57BL/6 FLP mice. The *Ctla4$^{ex2fl/fl}$* mice were then bred with *Foxp3-Cre* mice to yield *Ctla4$^{ex2fl/fl}$Foxp3-Cre* mice.

3.2 ACCQUISITION OF PATIENT SAMPLES

Human peripheral blood and biopsies from affected areas of the rectum and sigmoid colon were obtained from patients diagnosed with Crohn's disease. Disease activity was graded according to the simplified endoscopic activity score for Crohn's disease as previously described (Daperno et al. 2004). A subgroup of patients was treated with anti-TNFα antibodies, infliximab (Remicade®) or adalimumab (Humira®). Anti-TNFα treatment was

administered either as intravenous injections of 5mg/kg infliximab at week 0, 2 and 6, or as subcutaneous injections of 80mg adalimumab at week 0 followed by 40mg adalimumab every other week. The Harvey-Bradshaw Index was used for assessment of patients' response to the treatment (Harvey & Bradshaw 1980). Patients with a decrease of ≥3 points in clinical index activity score were considered as responders to the treatment. The choice of treatment for individual patient was based on clinical evaluations and considerations without any intervention from the study.

3.3 CELL PREPARATION AND PURIFICATION

3.3.1 Isolation of mouse CD4$^+$CD25$^+$ regulatory T cells

Single-cell suspension was prepared from mouse peripheral lymph nodes. CD25$^+$ T cells were enriched by AutoMACS positive selection using anti-mouse CD25-PE antibody and anti-PE MicroBeads. Enriched CD25$^+$ T cells were then stained with anti-mouse CD4-APC antibody and high purity mouse CD4$^+$CD25$^+$ Treg cells were sorted using flow cytometric cell sorting (FACS JAZZ, BD).

3.3.2 Isolation of mouse CD11c$^+$ dendritic cells

Single-cell suspension was prepared from mouse spleens. B cells, T cells and NK cells were depleted with a cocktail of biotin-conjugated antibodies and Anti-Biotin MicroBeads (Miltenyi Biotec). High purity CD11c$^+$ dendritic cells were positively selected from the unlabeled fraction with mouse CD11c MicroBeads (Miltenyi Biotec).

3.3.3 Isolation of mouse CD4$^+$CD25- T cells

Single-cell suspension was prepared from mouse peripheral lymph nodes. CD25$^+$ T cells were depleted using anti-mouse CD25-PE antibody and anti-PE MicroBeads (Miltenyi Biotec). CD4$^+$ T cells were then positively selected from the unlabeled fraction with mouse CD4 MicroBeads (Miltenyi Biotec).

3.3.4 Isolation of human T cells

Single-cell suspension was prepared from human PBMCs using Ficoll-Paque Plus gradient centrifugation (GE Healthcare).

For isolation of human Treg cells, CD4$^+$ T cells were positively selected from PBMCs with human CD4 MicroBeads (Miltenyi Biotec) using an AutoMACS Separator. The enriched CD4$^+$ T cells were then stained with anti-human CD4, CD25, CD127 antibodies and CD4$^+$CD25^{hi}CD127^{low} Treg cells were sorted using a FACSJazz instrument (BD Biosciences).

Naïve CD4$^+$ T cells were enriched from PBMCs by depleting non-T helper cells and memory CD4$^+$ T cells using naïve CD4$^+$ T cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The enriched CD4$^+$ T cells were then stained with anti-human

CD4, CD25, CD45RA and CD62L antibodies and high purity (>95%) CD4$^+$CD25$^-$CD45RA$^+$CD62L$^-$ naïve T cells were obtained by cell sorting using a FACSJazz instrument (BD Biosciences).

3.4 *IN VITRO* CELL CULTURE

Sorted CD4$^+$CD25$^+$ Treg cells were activated with 5ug/ml plate-bound anti-mouse CD3 antibody and 500U/ml recombinant IL-2 (Biolegend) for 3 days and rested in complete medium with 500U/ml recombinant IL-2 for 24 hours. Pre-activated Treg cells were then cultured with purified splenic CD11c$^+$ dendritic cells in a 1:1 cell ratio with 100ng/ml LPS for 24 hours. 24 hours later, CD11c$^+$ dendritic cells were re-sorted from the DC-Treg co-culture using flow cytometric cell sorting, and were either analyzed by flow cytometry or co-cultured again with CD4$^+$CD25$^-$ effector T cells (DC: T cell ratio = 1:10) for 3 days in the presence of 0.25ug/ml soluble anti-mouse CD3 antibody.

3.5 HUMAN TH17 CELL DIFFERENTIATION *IN VITRO*

Sorted naïve T cells were activated with 5ug/ml plate-bound anti-human CD3 and 1ug/ml soluble anti-human CD28 antibodies in X-VIVO 15 serum free medium supplemented with 1% Penicillin-Streptomycin (Lonza) and 300U/ml recombinant human IL-2 (Peprotech). For differentiation towards the Th17 lineage, activated naïve T cells were cultured in addition with 10ng/ml TGFβ, 10ng/ml IL-1β, 25ng/ml IL-6 and 10ng/ml IL-23 (all from Peprotech) for 6 days. Four hours prior to harvesting, cells were re-stimulated with 50ng/ml PMA (Sigma) and 1ug/ml ionomycin (Life Technologies) and treated with GolgiBlocker (BD Biosciences).

3.6 SPLICE-SHIFTING OF HUMAN T CELLS *IN VITRO*

Enhanced FOXP3 exon splicing in human T cells was achieved by using Fluorescein-labeled Morpholino Antisense Oligonucleotides (MAO) with the following sequences (GeneTools):

FOXP3ex1/3: 5'-TGCCCATTCACCGTCCATACCTGGT-3'

FOXP3ex6/8: 5'-AGCTGTGAAATGGCACAAACATGAG-3'

Control: 5'-CCTCTTACCTCAGTTACAATTTATA-3'

Prior to activation, T cells were transfected with 15 μM MAO using the P3 Primary Cell Nucleofector Kit in a Nucleotransfector device (Lonza) according to the manufacturer's instructions.

3.7 EXPANSION OF REGULATORY T CELLS *IN VIVO*

The protocol for *in vivo* expansion of Treg cells using IL-2/IL-2 mAb is as previously described (Boyman et al. 2006). In brief, wild type C57BL/6 mice were injected i.p daily with either 5ug isotype control (Biolegend) or 1ug recombinant mouse IL-2 (Biolegend) mixed

with 5ug anti-IL-2 mAb (Clone: JES6-1A12, Biolegend) for 3 days. Spleens from the injected mice were harvest on day 5 post-injection for FACS staining.

3.8 QUANTITATIVE PCR

Total RNA from T cells was isolated with Trizol (Life Technologies) and cDNA was generated using Vilo cDNA Synthesis Kit (Life Technologies). Amplification was performed with the following protocol: 2 min in 95°C, (15 sec in 95°C, 45 sec in 58°C, 30 sec in 68°C) x 39 cycles with iQ SYBR Green Supermix (Bio-Rad). Gene specific primer pairs are listed below:

FOXP3ex1/2 sense: 5'-CAGCTGCAGCTGCCCACACTG-3'

FOXP3ex1/2 antisense: 5'-GCCTTGAGGGAGAAGACC-3'

FOXP3ex1/3 sense: 5'-CAGCTGCAGCTCTCAACGGTG-3'

FOXP3ex1/3 antisense: 5'-GCCTTGAGGGAGAAGACC-3'

FOXP3ex6/8 sense: 5'-GAGCAGCAGGCA TCA TCCG-3'

FOXP3ex6/8 antisense: 5'-CTGGGAA TGTGCTGTTTCC-3'

Relative gene expression was normalized by housekeeping gene *GAPDH* (*ex vivo* samples) or *HPRT1* (cell culture samples). Primer specificity was confirmed by single peak performances of PCR products in melt curve analysis. Expression of FOXP3 splice variants was adjusted according to individual primer pair efficiency. Total FOXP3 mRNA expression was calculated as the sum of FOXP3ex1/2 and FOXP3ex1/3 and percentage of splice variant expression was calculated as (FOXP3 variant mRNA expression/total FOXP3 mRNA expression)*100.

4 RESULTS AND DISCUSSION

4.1 PAPER I

The key findings in paper I are:

1. FOXP3 isoforms are differentially regulated in pro-inflammatory conditions such as Crohn's disease. Comparing to healthy donors, patients suffering from Crohn's disease had higher expression of FOXP3 isoform mRNA that lacks exon 7 (FOXP3ex6/8) while the expression of total FOXP3 mRNA remained unchanged.

2. The increase in FOXP3ex6/8 mRNA is correlated with disease severity. When patients were successfully treated with anti-TNFα antibody, there was a decrease in the percentage of FOXP3ex6/8 isoform transcripts in the biopsies. The increased splicing of exon 7 was also correlated with IL-17A expression in the biopsies *in vivo*, indicating that pro-inflammatory environment can alter the expression of FOXP3 isoforms.

3. When tested *in vitro*, we found that IL-1β, but not IL-6 or TNFα, was able to promote the splicing of exon 7 in Treg cells in combination with TCR stimulation.

4. When altering FOXP3 isoform expression in naïve T cells *in vitro* in a Th17 polarizing condition, increased expression of FOXP3ex6/8 isoform promoted Th17 differentiation of naïve T cells.

Exon 2 of FOXP3 belongs to the repressor domain, and the removal of exon 2 could disrupt the interaction between FOXP3 and its binding partners. For example it has been known that TGFβ together with other cytokines can regulate the differentiation of Treg and Th17 cells by balancing the expression of FOXP3 and RORγt. FOXP3 can directly bind to and antagonize the function of RORγt thus inhibit Th17 differentiation and the binding of RORγt is largely dependent on exon 2 of FOXP3, although a full inhibition of RORγt also requires the FKH domain (Ichiyama et al. 2008; Zhou et al. 2008). A recent study also suggested that a fully conserved 4-amino-acid motif in the N-terminal region of FOXP3 exon 2 is highly important for FOXP3 function, and that FOXP3 regulates gene expression mostly independent of direct DNA binding (Xie et al. 2015).

These data do not fit with other papers showing that removing exon 2 of FOXP3 did not affect FOXP3 suppressive function. However these functional studies on FOXP3Δ2 isoform were performed in *in vitro* cell cultures often with over-expression of a certain isoform in transfected cells (Aarts-Riemens et al. 2008; Allan et al. 2005; Smith et al. 2006). It is possible that this artificial system does not reflect the true nature of FOXP3 function as such discrepancies have been observed between freshly isolated FOXP3^{+} Treg cells and conventional T cells transduced with FOXP3 (Sugimoto et al. 2006; Hill et al. 2007).

FOXP3 exon 7 is part of the leucine-zipper domain and participates in the dimerization of FOXP3. Mutations within the leucine-zipper domain disrupt FOXP3 dimerization and

significantly reduce FOXP3 binding capability to the promoter regions of its target genes *in vivo* (Li et al. 2007; Lopes et al. 2006; Song et al. 2012; Chae et al. 2006). In addition the acetylation of two lysine residues (K250 and K252) in exon 7 has been proposed to stabilize FOXP3 (Song et al. 2012), however in our hands FOXP3Δ2Δ7 from MAO-treated Treg cells appeared to be stable and maintain DNA binding capacity.

Both FOXP3 exon 2 and exon 7 are associated with Treg-mediated suppressive programs on other gene transcription such as IL-2, the removal of both exon 2 and exon 7 could release FOXP3 repression on other transcriptional factors and promote the expression of IL-2.

All the factors above could potentially explain the loss of suppressive function by FOXP3Δ2Δ7 isoform. More interestingly, we found that IL-1β promotes the exclusion of FOXP3 exon 7. Previous studies have demonstrated that Helios⁻FOXP3⁺ Treg cells down-regulate their suppressive functions in response to IL-1β (Raffin et al. 2013), and Treg cells exposed to both IL-1β and IL-2 differentiate into pro-inflammatory Th17 cells (Deknuydt et al. 2009). These data collectively suggest a role of pro-inflammatory cytokines in alternative splicing of FOXP3 and Treg cell function.

It is not clear how the pattern of isoform expression looks like on a single cell basis. It is possible that each Treg cell is capable of expressing all forms of FOXP3 isoforms simultaneously, and when encountering environmental stimuli, it shifts the isoform expression pattern towards a particular isoform, and the changes in the balance of isoform expression alter Treg cell function. It is also possible that individual Treg cells exclusively express one type of FOXP3 isoforms, thus forming subsets of Treg cells with different functions. However, the flow cytometry data we obtained in FOXP3 isoform staining indicated that the former scenario is more likely. Evaluating FOXP3 isoform expression on a single cell level would be really interesting and would provide refreshing clues into the connection between alternative splicing and immune regulation by Treg cells. In addition, identifying the binding partners of individual FOXP3 isoform can further deepen our knowledge in understanding how FOXP3-dependent and/or independent transcriptional programs regulate Treg cell mediated suppression.

The study on human FOXP3 isoforms is also limited by the methods available today. Flow cytometry is commonly used to measure expression of various surface and intracellular proteins. However currently the antibodies recognizing different FOXP3 isoforms are very limited (Fig. 7). The currently available antibodies can only detect the presence or absence of FOXP3 exon 2, and there is no antibody available that is specifically targeting exon 7. Therefore we can only indirectly visualize the expression of FOXP3Δ2 or FOXP3Δ2Δ7 in flow cytometry and alternative methods for FOXP3 isoform detection are needed.
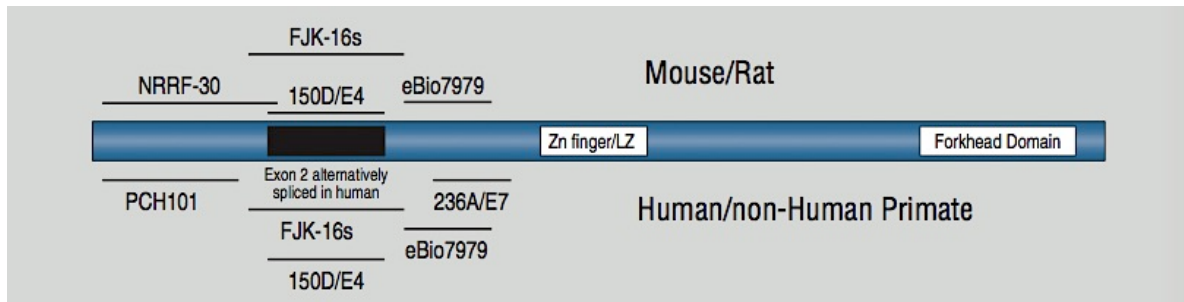
Figure 7: Schematic representation of the FOXP3 protein and the location of the epitopes for each anti-FOXP3 antibody clone. (www.ebioscience.com)

Western blot is another alternative for protein detection. Analysis of FOXP3 isoforms in western blot reveals 3 bands, however the middle band is a composite band of both FOXP3Δ2 and FOXP3Δ7 as they have very similar sizes (43kDa and 44kDa respectively). Therefore it is still difficult to get precise quantification of FOXP3 isoforms using western blot. In addition, FOXP3 contains two R*XX*R proprotein convertase motifs that can be cleaved, resulting in multiple proteins derived from a single FOXP3 isoform (Elhage et al. 2015). Taken all these into account it is almost impossible to get good quantitative data from western blot analysis of FOXP3 isoforms.

Northern blot can be used for inter-molecular comparisons. While challenging to use to analyse patient samples with limited quantity, it may be worthwhile to analyse FOXP3 transcript composition by using northern blot on *in vitro* cultured Treg cells. A concern here is that not only would we identify FOXP3fl, FOXP3Δ2, FOXP3Δ7 and FOXP3Δ2Δ7 but also differences in the transcripts that arise from alternative splicing of un-translated FOXP3 exons.

Due to the limitations presented above and the fact that we could only get access to very limited amount of patient samples in the study, we used quantitative PCR (qPCR) as our main method of detection for the expression of different FOXP3 isoform transcripts. Quantitative PCR has been used extensively to make inter-molecular comparisons of different splice-variants (Shukla et al. 2011; De Arras & Alper 2013) given that the results are compensate for the differential efficiencies of distinct primer pairs. Therefore we believe that comparison of different FOXP3 isoform transcripts using qPCR are valid and in line with several other published studies, although it only reflects the expression of different isoforms on an mRNA level.

RNA sequencing would be an excellent alternative to qPCR. If the experimental conditions are optimized, not only could it help us to quantify isoform expression in Treg cells, it could also be used to identify the differences in transcriptional programs initiated by individual FOXP3 isoforms. These results can be further compared with databases from published

results and would greatly enhance our understanding in the regulation of FOXP3 expression and Treg cell function in general.

We hope that in the future specific antibodies against different FOXP3 epitopes can be developed, in particular an antibody recognizing the neo-epitope generated by combining FOXP3 exon 6 and 8. This would be helpful for checking FOXP3 splicing in subpopulations of Treg cells, including populations that simultaneously express multiple lineage-defining transcription factors such as FOXP3, T-bet and RORγt. In addition, more cost-efficient next generation sequencing techniques, especially on single cell level would vastly enrich our knowledge in the role of FOXP3 alternative splicing in Treg cell biology.

4.2 PAPER II

The key findings in Paper II are:

1. We developed a mouse model with a knockin construct where exon 2 and exon 7 were removed from the Foxp3 transcript, resulting in a mouse that only expresses a mouse version of FOXP3 isoform—Foxp3δ2δ7. While heterozygote female $Foxp3^{\delta2\delta7/X}$ mice appear perfectly healthy, the homozygote males ($Foxp3^{\delta2\delta7/Y}$) died from lymphoproliferative disease starting from 3 weeks of age.

2. Upon closer examination, homozygote males ($Foxp3^{\delta2\delta7/Y}$) phenocopied *Foxp3*-deficient scurfy mice with multi- organ inflammation, splenomegaly, lymphadenomegaly, profound T cell activation, impaired B cell development, autoantibody production and activated APCs.

One concern with this study is that the targeting construct we used to replace the *Foxp3* gene resulted in the deletion of CNS3 in the *Foxp3* promoter, as it is located after the first coding exon. However, since CNS3 deficient mice display normal levels of Treg cells in lymph nodes and only slightly reduced numbers in spleen (Zheng et al. 2010), we consider this impact of CNS3 deletion unlikely in our model. Another worthwhile comparison would be between the *Foxp3*$^{\delta2\delta7}$ mouse and a *Foxp3*$^{\delta2}$ mouse model that only lacks exon 2. This could give us a clue as how removal of different exons in FOXP3 affects Treg cell function *in vivo*.

The reason why we choose mouse models to study FOXP3 function is because FOXP3 gene is highly conserved between species, especially since human FOXP3 confers suppressive function when transfected into murine cells *in vitro*. However, we do not know if similar analogy can be made for FOXP3 isoforms. Alternative splicing is very species-specific (Barbosa-Morais et al. 2012). Although sequencing studies indicated that the exon-intron boundaries are identical across the coding regions of the mouse and human FOXP3 genes (Brunkow et al. 2001), the differences in alternative splicing regulators may lead to completely different isoform patterns in different species. Interestingly, a recent paper suggested that mouse FOXP3 may indeed be alternatively spliced, although the resulting isoform is very different from any of the known human FOXP3 isoforms (Ergun et al. 2013). Despite all these, the FOXP3$^{\delta2\delta7}$ mouse can still be a useful model for studying FOXP3

isoforms *in vivo*. Another alternative would be to use humanized mice where we engraft a human immune system into an adult immune-deficient mouse. It would be interesting to see if alternative splicing in human Treg cells would occur in the recipient mouse the same way as in a human body, and if so, how these isoforms function *in vivo*.

4.3 PAPER III

The key findings in Paper III are:

1. We generated a transgenic mouse model—the *Ctla4^{ex2fl/fl}Foxp3-Cre* mouse where CD4^+Foxp3^+ T cells in these mice exclusively express ligand-independent CTLA-4 that lacks the extracellular domain encoded by *Ctla4* exon 2. The expression of CTLA-4 on other T cell populations was not affected.

2. *Ctla4^{ex2fl/fl}Foxp3-Cre* mice were generally healthy, but slowly developed inflammatory lesions in the lung starting at 4–6 months of age.

3. *Ctla4^{ex2fl/fl}Foxp3-Cre* mice had significantly increased frequency of CD4^+CD25^+ Treg cells in the spleen, and these Treg cells were also highly proliferative. Moreover, in the thymus of young *Ctla4^{ex2fl/fl}Foxp3-Cre* mice there was also increased frequency of Foxp3^+ thymocytes in both CD4^+CD8^+ and CD4^+CD8^− compartments.

4. *In vitro* experiments revealed that Treg cells from *Ctla4^{ex2fl/fl}Foxp3-Cre* mice were just as potent as wild type Treg cells in preventing the up-regulation of CD80/CD86 on activated DCs, and decreasing DCs' ability to support effector T cell proliferation.

5. Interestingly, we also found that DCs co-cultured with wild type Treg cells could up-regulate PD-L2, while Treg cells from *Ctla4^{ex2fl/fl}Foxp3-Cre* mice could not do so. Moreover the up-regulation of PD-L2 was more prominent in the CD8α^+ DCs.

It has long been thought that trans-endocytosis is an important mechanism for CTLA-4 mediated suppression by Treg cells. However, several studies also indicated that this is not the only function of CTLA-4. The overall healthy status of *Ctla4^{ex2fl/fl}Foxp3-Cre* mice demonstrated again that Treg cell mediated suppression via CTLA-4 is not just about trans-endocytosis. However, *Ctla4^{ex2fl/fl}Foxp3-Cre* mice did develop mild inflammation in the environmental surfaces such as the lung and intestine, demonstrating that liCTLA-4 in Treg cells alone cannot function perfectly to maintain immunological homeostasis, This could either be a result of the lack of cell-extrinsic Treg cell-mediated CTLA-4 signalling or insufficient signalling from CTLA-4 into the Treg cell. While liCTLA-4 can signal in *cis*, this signal presumably is weaker than when CTLA-4 binds to CD80 or CD86. This observation has also been implicated in other studies that it requires T cells to express both flCTLA-4 and liCTLA-4 to function properly (Stumpf et al. 2013).

The increased thymic output of Treg cells contradicts a prior study where exons 2 and 3 of CTLA-4 were flanked by LoxP sites, resulting in complete deletion of CTLA-4 in Treg cells

when bred with Foxp3-cre mice (Wing et al. 2008). This discrepancy could be a result of different efficiency in *Ctla4* deletion. Double-positive thymocytes from the *Ctla4$^{ex2fl/fl}$Foxp3-Cre* mice almost completely lacked expression of the extracellular region of CTLA-4, while both single-positive and double-positive thymocytes from *Ctla-4$^{ex2-3fl/fl}$Foxp3-Cre* mice only displayed partial deletion of CTLA-4 in the thymus. Several other studies have addressed the impact of CTLA-4 on thymic development of Treg cells, but their results are contradictory. Those studies utilized mice completely devoid of CTLA-4, and the results might therefore be influenced by indirect changes in the thymic microenvironment, as both cytokines and adhesion molecules modulate thymic selection.

The *in vitro* suppression assay demonstrated that liCTLA-4 on Treg cells were just as potent in preventing DC maturation. This is a strong support for the notion that trans-endocytosis is a dispensable mechanism for Treg cell-mediated suppression. Even more interesting, wild type Treg cells up-regulate PD-L2 on DCs upon co-culture, while Treg cells from *Ctla4$^{ex2fl/fl}$Foxp3-Cre* mice could not. This implies that cell-extrinsic CTLA-4 signalling can initiate an anti-inflammatory program in DCs that transform DCs into a suppressive phenotype.

In support of this notion, a recent paper demonstrated that PKC-η associated with CTLA-4 and was recruited to the immunological synapse of Treg cells and PKC-η-deficient Treg cells displayed defective suppressive ability when co-transferred with effector T cells (Kong et al. 2014). In addition PKC-η-deficient mice developed signs of lymphoproliferative disease with increased numbers of memory T cells and pro-inflammatory cytokines (Fu et al. 2011). Even more interestingly, Sharpe and colleagues have published a paper very recently where a conditional knockout mouse model was generated to delete CTLA-4 in adult mice using tamoxifen (Paterson et al. 2015). To their surprise, the deletion of CTLA-4 in adult mice resulted in resistance to induced autoimmunity (mouse EAE model), which was in part due to a great expansion of Treg cells both in the periphery and the central nervous system. In addition, Treg cells lacking CTLA-4 were able to suppress homeostatic proliferation *in vivo.* An increased expression of IL-10, Lag3 and PD-1 was observed both in conventional T cells and Treg cells after CTLA-4 deletion. When immunizing the CTLA-4 deleted mice for EAE, the percentage of Treg cells was increased in the central nervous system while the level of CD80/CD86 on APCs was unchanged.

Combining with the results from our study, these data suggest that: 1) CTLA-4 has cell-intrinsic function in Treg cells where it probably regulates the homeostasis of Treg cells in the periphery. 2) Trans-endocytosis is dispensable for Treg cell-mediated suppression via CTLA-4. 3) The suppressive program on DCs initiated by Treg cells involved both CTLA-4-dependent and independent mechanisms, and many other factors such as IL-10 and/or other co-inhibitory molecules may play an important role in such processes.

There are still many unanswered questions regarding Paper III. For example, is the up-regulation of PD-L2 dependent on the interaction between CTLA-4 exon 2 and CD80/CD86?

Are there any other molecules being induced in DCs after co-cultured with Treg cells? How much of the Treg cell mediated suppressive program induced in DCs is CTLA-4 dependent, and how much is CTLA-4 independent? To answer these questions, we can utilize DCs from CD80/CD86 knockout mice and compare them with wild type DCs co-cultured with different types of Treg cells. In addition, we are also planning to use RNA-sequencing to define the global changes in transcriptome in DCs that are suppressed by wild type and *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* Treg cells.

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