Natural occurring regulatory T cells: role of transcription factor FOXP3 and new approaches improving Treg-cell based therapy

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Prologue

Research carried out over the past 10-20 years, has greatly expanded our understanding of how T-cell subsets differentiate and acquire a degree of stability that allows them to be considered a distinct T-cell lineage. This is the case for $CD4^+$ regulatory T cells (Treg cells), a class of T cells that develops in the thymus and is essential for maintaining self-tolerance. Treg cells are characterized by the expression of the transcription factor forkhead box P3 (FOXP3), which is essential for the normal development and function of these cells. Although several subsets of T cells with immunosuppressive properties have been identified, $FOXP3^+$ Treg cells have emerged as a dominant cell type. The first indication that cells with regulatory characteristics could exist and be important for self-tolerance came from work by Nishizuka and Sakakara in 1969 in which they observed that neonatal thymectomy of mice 2-4 days after birth resulted in autoimmunity. This autoimmunity could be transferred to immunodeficient mice by adoptive transfer of T cells, and the autoimmunity could be reversed in thymectomized mice by transfer of T cells from wild type mice. Gershon and Kondo formally proposed the existence of "T suppressor" cells in 1970 after observing that T cells could suppress antibody responses. It wasn't until after 1995, however, that intense research into T cells with regulatory properties began, following the discovery by Shimon Sakaguchi that T cells with regulatory capacity highly express the IL-2 receptor alpha chain (CD25), providing a means by which to enrich for and study these cells. Sakaguchi observed that transfer of $CD4^+CD25^-$ T cells into immunodeficient mice resulted in autoimmunity whereas co-transfer with $CD4^+CD25^+$ T cells protected mice from disease. The same year, the dominance of suppression by T regulatory (Treg) cells was demonstrated. Taken together, these pioneering observations have been an auspicious beginning for the role of Treg cells in immune responses. The *Foxp3* gene, identified as the master gene of Treg, was first identified

as a defective gene in the mouse strain Scurfy (X-linked recessive mutant). Foxp3-mutant scurfy mice and Foxp3-deficient mice displayed lethal autoimmune lymphoproliferative disease, which results from a defection of $CD4^+CD25^+$ Treg cells. Similarly, mutations of *FOXP3* gene in humans result in the deficiency or dysfunction of $CD4^+CD25^+$ Treg cells and are responsible for IPEX syndrome, a combination of severe multi-organ autoimmune diseases. Moreover, when Foxp3 was ectopically expressed via retroviral gene transfer, non-Treg cells in mice and in human acquired a Treg cell phenotype. Thus, stable Foxp3 expression is clearly a pre-requisite for the maintenance of the transcriptional and functional program established during Treg cell development. The study of suppressor/regulatory T cells represents one of the major areas of research in immunology and almost every published paper contains some reference or experiment to the involvement of these cells in every model system. Indeed, researches in animal models have demonstrated that adoptive transfer of these cells can be used to treat many auto-inflammatory diseases such as type 1 diabetes (T1D), inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA) and autoimmune gastrtitis (AG). In addition, Treg therapy has been found to be efficacious in controlling allo-responses in the setting of graft versus host disease (GvHD), as well as organ and cell transplantation. Thus the modulation of Treg cells could provide new strategies in creating transplantation tolerance. A key advance for Treg therapy in humans is the finding that Treg cells can be isolated and expanded *in vitro* while maintaining immunoregulatory function. The majority of clinical trials published to date have utilized either fresh, immunomagnetic bead-isolated Tregs derived from adult blood, since clinical grade cell sorting facilities are not widely available, or the same adult Treg cells (or cord blood Treg cells) expanded *in vitro*. However, the requirement to expand Tregs *in vitro*, to achieve a number necessary for a therapeutic benefit, compromises the purity of Treg cells at the end of the culture. Therefore, one of the major goals in Treg therapy

is to preserve the purity and suppressive ability of *ex vivo* expanded Treg preparations limiting their potential to produce pro-inflammatory cytokines, particularly when Treg cells are exposed to an inflammatory environment *in vivo*. FOXP3 expression is a good surrogate to establish Treg purity, but given current expansion techniques, it is unlikely that Treg cultures could be 100% $FOXP3^+$ following expansion. So it will be important to determinate "acceptable" levels of contaminations, from both quantitative and qualitative prospectives. Although many issues and challenges still need to be addressed in this area, the first results of clinical trials applying Treg cells in stem cell transplantation are very encouraging. More new informations will be revealed in the the next few years on the safety and efficacy of *ex-vivo* expanded Treg cells as a result of the multicentre Phase I/II study founded by the European Union FP7 program, "The ONE study".

Given the importance of FOXP3 in Treg cell development and function, it becomes essential to elucidate the mechanisms by which FOXP3 acts in these cells in order to use them as a cellular therapy. Thus, this thesis has two major goals: the first was to undestand the function of FOXP3 on target gene expression, and the second was to develop a protocl for the potential clinical application of FOXP3-expressing Treg cells. Consequently, the thesis has been divided in two parts. The first part contains excerpts from the article entitled "FOXP3 Forkhead Transcription Factor FOXP3 up-regulates CD25 expression through cooperation with RelA/NF-kB" published in PloS ONE, Volume 7, October 2012. Indeed I collaborated with the first author, Cristina Camperio, in performing some experiments of this work coordinated by Prof. Enza Piccolella during my first two years of PhD course in Italy at Department of Biology and Biotechnology "Charles Darwin". The research in the second part of this thesis was conducted under the supervision of Prof. Giovanna Lombardi at Department of Transplantation Immunology and Mucosal Biology, King's College London during my last year of PhD course as foreign student in UK I collaborated with Dr. Cristiano Scottà that

introduced me to the separation and *ex vivo* expansion of Treg cell lines investigating phenotype, stability and function *in vitro*. The article containing the collected results, entitled " Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human $CD4^+CD25^+FOXP3^+$ Treg subpopulation", was written by Dr. Cristiano Scottà and published in Haematologica Journal, December 2012.

Abstract

The immune system requires a network of regulatory mechanisms that enable the host to maintain immune regulation, homeostasis and tolerance. A functionally committed $CD4^+CD25^+FOXP3^+$ T cells subset (Treg cells) have a key role in determining the outcomes of protective immunity to a spectrum of foreign antigens while maintaining tolerance to self-antigens and suppressing excessive inflammation that can cause pathology. The transcription factor forkhead P3 (FOXP3) is highly expressed in Treg cells and it is critical for their suppressive function. The importance of FOXP3 is demonstrated in humans with a severe autoimmunity disease called immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) caused by mutations in *FOXP3* gene. Therefore, there is an increasing interest in manipulating FOXP3 function and/or using $CD4^+CD25^+FOXP3^+$ Treg cells as cell therapy to modify immune responses in cancer, autoimmunity and transplantation. The transcription factor FOXP3 has been shown to regulate negatively some genes such as *Il2* and positively others, such as *Cd25* and *Ctla4*. To better understand the function of FOXP3 as transcriptional activator, the regulation of CD25, the IL-2Rα chain, by FOXP3 was investigated (Part I). Analyzing a regulatory region of *Cd25* promoter, it has found the presence of a κB site and two tandem copies of a non-consensus FOXP3 binding site separated at 5' ends by 19 nucleotides that allow FOXP3 and NF-κB/RelA subunit binding to DNA. The occupancy of the two FOXP3 binding sites in conjunction with NF-κB/RelA binding site occupancy allows FOXP3 to function as a positive activator of *Cd25* gene. Indeed mutations of both FOXP3 binding sites such as mutation of κB site on *Cd25* promoter abolished FOXP3 activatory functions. Moreover, FOXP3 mutation ΔE251, that compromises FOXP3 homotypic interactions, failed to trans-activate *Cd25* promoter, suggesting that both FOXP3 DNA

binding and dimerization are required to trans-activate *Cd25* promoter. So, these findings identify a novel mechanism by which RelA and FOXP3 cooperate to mediate transcriptional regulation of target genes and characterize a region on *Cd25* promoter where FOXP3 dimer could bridge intra-molecularly two DNA sites and trans- activate *Cd25* gene. Then the possibility of using *ex vivo* expanded $CD4^+CD25^+FOXP3^+$ regulatory T cells as cell therapy was investigated (Part II). The ability to isolate and expand human Treg cells is crucial for their use in the clinic and many questions regarding the stability of phenotype and function of the transferred Tregs during inflammation remained unresolved. In this study it has been developed a protocol for the expansion of clinically useful numbers of functionally suppressive and stable human Treg cells. $CD4^+CD25^+FOXP3^+$ Treg cells were expanded *in vitro* with rapamycin (RAPA) and/or all-*trans*-retinoic acid (ATRA) and then characterized under inflammatory conditions *in vitro*. The addition of RAPA to Treg cultures confirmed the generation of high numbers of suppressive and stable Tregs *in vitro*. In contrast, ATRA-treatment generated Treg cells which retained the capacity to secrete pro-inflammatory cytokine IL-17. However, combined use of RAPA and ATRA abolishes IL-17 production and confered a specific chemokine receptor homing profile upon Treg cell preparations. Moreover, the use of purified Treg subpopulations provided direct evidence that RAPA could confer an early selective advantage to $CD45RA^+$ Treg subset, while ATRA favored $CD45RA^-$ Treg subset expansion. So, the expansion of Treg cells using RAPA and ATRA drug combinations provided a new approach for large-scale generation of functionally potent and phenotypically stable human Treg cells, rendering them safe for clinical use. All together, the results reported in this thesis sheds light on the ability of FOXP3 in regulating the expression of CD25 molecule and helps pave the way for use Treg cell therapy in clinic.

Acknowledgements

I am extremely grateful to my supervisor Prof. Enza Piccolella who gave me exceptional guidance and support over the past six years. I am truly fortunate to have had such a rich graduate experience and to have such an inspiring mentor. I thank Prof. Giovanna Lombardi for my time spent at Department of Transplantation Immunology & Mucosal Biology of King's College in London and for the opportunity to continue working. Thanks go to everyone in Piccolella's laboratory past and Lombardi's laboratory present for providing a supportive and collaborative atmosphere. Special thanks go to Dr. Cristiano Scottà for training me as a new graduate student and for being an inspiring model.

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Dedication

“Non sei obbligato a completare l’opera ma non sei nemmeno libero di lasciarla perdere” cit. dalla Torah

Il mio impegno, la mia volontà e la mia tenacia hanno contribuito a portare a compimento non un mero lavoro “scientifico” ma soprattutto un percorso di “crescita” grazie al quale ho maturato la consapevolezza di voler fare la ricercatrice. Questa è la mia tesi di dottorato. E la dedico in particolare alle due persone che hanno sostenuto ed incoraggiato in tutti questi anni le mie scelte: i miei genitori..

1: General Introduction

1.1. Immune tolerance and "suppressor" T cells: history

A hallmark of the adaptive immune system is the generation of diverse immune receptors for the anticipated encounter with rapidly evolving pathogens. This powerful strategy for host defense brings considerable challenges. Because T cell receptors (TCRs) are selected by highly diverse endogenous ligands, i.e. self-peptide-MHC complexes, potentially pathogenic auto-reactive T cells can be generated. Hence, adaptive immunity requires a tightly controlled equilibrium between mounting deleterious responses against self, food antigens and commensal microorganisms (self-tolerance) while at the same time preserving the potential to generate immune responses against pathogens. From an evolutionary point of view, the importance of immune tolerance is proved by multiple non-redundant mechanisms designated to control these two different immunological demands. Negative selection of T cells in the thymus (central tolerance) leads to elimination or inactivation of those T cells reactive to self-antigens above a threshold of affinity for self-antigens-MHC-complexes (Kappler *et al.*, 1987). Nevertheless, although central tolerance is responsible for neutralization of most high-affinity T cells recognizing self, some auto-reactive T cells fail to undergo deletion and they become part of the peripheral T-cell repertoire (Starr *et al.*, 2003). Therefore, in order to prevent the development of autoimmune diseases, the immune system has devised additional check points to limit the activation of mature self-reactive T cells once they have reached the peripheral lymphoid organs. Peripheral tolerance mechanisms provide a second level of control to restrain potentially auto-reactive T cells which escape from the thymus (Wood K.J., 1996). This is important not only because thymic negative selection is stochastic, based on the affinity thresholds, but also because not all self-antigens are

expressed in the thymus. In addition to clonal deletion of auto-reactive T cells, chronic engagement of TCR by self antigens induces a state of long-term hypo-responsiveness in T cells termed “anergy”, characterized by an active repression of TCR signaling and IL-2 expression. Peripheral tolerance is also reinforced by the requirement of simultaneous engagement of TCR by a cognate peptide-MHC complex and the T cell co-stimulatory receptor CD28 by CD80 and CD86 (Lenschow *et al*., 1996). CD80 and CD86 are induced on antigen-presenting cells (APCs) upon the activation of innate immune receptors directly in response to microbial or viral products or through sensors of metabolic changes invoked by microorganisms. However, mechanisms of tolerance operating in a cell-intrinsic manner, termed “recessive tolerance”, and the two-signal requirement for the induction of a productive immune response, appeared insufficient to counter the threat of immune-mediated pathology. The idea of T cell-mediated suppression of immune responses was not new for the immunologists. In the late 1960s, the research by Nishizuka and Sakakura led to the finding that the normal immune system naturally harbors a particular population of thymic derived cells with autoimmune-suppressive activity. They showed that neonatal thymectomy of normal mice between day 2 and 4 after birth resulted in the destruction of ovaries, which they first supposed to be due to deficiency of a certain ovary-tropic hormone secreted by the thymus, hence was called “ovarian dysgenesis” (Nishizuka and Sakakura, 1969). Such destruction of ovaries was not be observed when thymectomy was performed after one week of life indicating that a thymic derived cell subset, arising after 3 days of life, was involved in the prevention of autoimmune oophoritis. Gershon and Kondo demonstrated subsequently that lymphocytes deriving from the thymus in mice could prevent specific immune responses against exogenous antigens and that spleen cells derived from mice that had become tolerant after infusion of T cells, could induce tolerance again when transferred to another mouse. This ability to transfer tolerance using cells has been called "infectious tolerance" by Gershon in

1971 (Gershon and Kondo, 1970). Since these findings by Nishizuka et al. and Gershon et al., intense research has focused on the characterization of suppressor T cells. The studies over the following years showed several types of suppressor T cells: some were antigen-specific, others being nonspecific; some secreted antigen-specific suppressive factors and others nonspecific ones. Moreover, these suppressor populations varied in phenotype and mode of suppression (Green *et al*., 1983). The phenotype of suppressor T cells, assessed by the expression of Lyt-1 (CD5) and Lyt-2 (CD8), was on the most part Lyt-2^+, corresponding to $CD8^+$, while some of them, in particular those suppressing delayed type hypersensitivity, were Lyt-1^+2^- , corresponding to $CD4^+$ cells. $CD8^+$ suppressor T cells expressed the I-J molecule, supposed to be a key suppressor molecule associated with their suppressive function. Active research of suppressor T cells quite abruptly collapsed in the mid 1980s when scrutiny of the mouse MHC gene, by molecular biology techniques, showed no existence of the I-J region, which was assumed to encode the I-J molecule and locate within the MHC gene complex (Kronenberg *et al*., 1983). With this bewildering I-J episode as a turning point, immunologists interest in suppressor T cells rapidly waned, forming, in the late 1980s and early 90s, an atmosphere in which they even shied away from using the word “suppressor T cells” in interpreting suppressive immunological phenomena (Bloom *et al*., 1992). There were also other reasons for this decline in the study; i.e. failure in finding reliable markers for distinguishing suppressor T cells from other T cells; ambiguity in the molecular basis of suppression and difficulty in preparing antigen-specific suppressor T cell clones amenable to fine cellular and molecular analyses. Furthermore, immunologists failed to obtain definitive evidence for anomaly of suppressor T cells as a primary cause of any immunological disease. Then, approaches to immunologic tolerance with new molecular tools, such as TCR subfamily-specific mAb and TCR-transgenic mice, in the late 80s to the early 90s demonstrated clonal deletion, and anergy, as key mechanisms of immunologic tolerance

(Kisielow *et al*., 1988). A culmination of this work came in 1995 when Sakaguchi *et al*. identified a subset of $CD4^+$ T cells expressing high amounts of CD25 (interleukin 2 receptor α-chain) which constituted 5-10% of peripheral $CD4^+$T cells (Sakaguchi *et al*., 1995; Asano *et al*., 1996). These cells were able to prevent autoimmunity upon transfer into day-3 thymectomized mice and, in other experimental models of autoimmunity, inhibit transplant rejection. In addition to their capacity to prevent autoimmunity *in vivo*, these cells were shown to suppress the proliferation of activated $CD4^+$ T and their IL-2 production *in vitro* in co-culture suppression assay (Thornton and Shevach, 1998; Takahashi *et al*., 1998) . This key finding led to denomination of $CD4^+CD25^+$ T cells as regulatory T cells (Treg cells). The presence of a subset of $CD25^+CD4^+$ single-positive thymocytes with suppressive ability indicated that $CD25^+$ Treg cells differentiate in the thymus. Although the discovery of CD25 as a useful marker for the functional analyses of these cells following their isolation from normal naïve animals, several studies revealed that the molecule was not a mere marker for natural Treg cells because of its up-regulation in all activated T cells. The inability to discriminate between tolerance- and inflammation-promoting cells during the immune response impeded the understanding of dominant tolerance and its mechanistic aspects. Furthermore, it was proposed that CD25-expressing Treg cells represented a state of activation of conventional $CD4^+$ T cells and their ability to down-modulate the immune response occurs because of competition for IL-2. The discovery in mice that $CD4^+$ T cells bearing α-chain of IL-2 receptor (CD25) could prevent autoimmunity, led researches in human immunology to look for the human counterpart of murine Treg cells. Human Treg cells were identified by several groups in 2001 confirming that Treg cells had high levels of CD25 and suppressive capacity *in vitro*. Furthermore, it has been subsequently demonstrated that Treg cells not only suppressed the proliferation of responder T cells *in vitro,* but also conferred suppressive capacity to suppressed responder cells. This finding has been

considered the mechanism underlying the “infectious tolerance” described by Gershon 40 years earlier. Together these evidences prompted exploration of genetic mechanisms underlying differentiation and function of Treg cells. These studies were facilitated by the discovery of X chromosome encoded transcription factor Foxp3 and its loss of function mutations in mice leading to a severe multi-organ autoimmune disease (Brunkow *et al*., 2001; Chatila *et al*., 2000). In humans, mutation of *FOXP3* gene resulted in the deficiency or dysfunction of $CD4^+CD25^+$ Treg cells and similarly caused a inflammatory disorder called IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Bennet *et al.*, 2001). The fact that in both humans and mice only *FOXP3* and *Foxp3* mutant males respectively but not heterozygote female carries were affected and the systemic nature of immune-mediated lesions, were consistent with an idea that mutations in this gene could impair differentiation or function of $CD4^+CD25^+$ T cells.

1.2. Role of FOXP3 in the development of Treg cells

1.2.1. FOXP3 expression and Treg lineage commitment

Supporting the role for FOXP3 in Treg cell commitment came from the analysis of $CD4^+CD25^+$ T cell populations in the thymus and peripheral lymphoid organs of mixed bone marrow chimeras generated upon transfer of Foxp3-deficient and allelically marked wild-type bone marrow into T cell-deficient mice. The recipient mice were free of lymphoproliferative disease and immune-mediated tissue lesions, and $CD4^+CD25^+$ Treg cells were generated only from Foxp3-sufficient, but not-deficient precursor cells (Fontenot *et al.*, 2003).This evidence demonstrated therefore that an absolute requirement for Foxp3 in Treg cell differentiation in the thymus. Moreover, retroviral transfer of the *Foxp3* gene into activated peripheral

$CD4^+CD25^-$ T cells also conferred suppressor function and Treg cell surface phenotype (Fontenot *et al.*, 2003; Hori *et al.*, 2003). The level of Foxp3 expression in murine Treg cells is therefore critical for suppressive function, given that experimentally induced reduction in Foxp3 amounts resulted in decreased function. In opposition to mice, FOXP3 expression in humans does not allow the unambiguous identification of Treg cells. As FOXP3 is induced during TCR stimulation in conventional $CD4^+$ T cells, in much the same manner as CD25, there is some debate as to whether the induced $CD4^+CD25^+$ $FOXP3^+$ population is suppressive or anergic (Allan *et al.*, 2007; Gavin *et al.*, 2006; Wang *et al.*, 2007). One further level of complexity compared to murine system has been unveiled with the description of $CD4^+CD25^{hi}$ cells expressing FOXP3 with naïve phenotype (Valmori *et al.*, 2005; Miyara *et al.*, 2009). However, although both in mice and humans the transcriptional factor FOXP3 plays a pivotal role for the development and suppressive ability of Treg cells, its biological function is still incompletely understood.

1.2.2. Requirements for FOXP3 expression

Although various signals inducing FOXP3 have been identified, the precise mechanisms by which the expression of this protein is controlled in Treg cells are not still understood. So far, it has been established that the synergistic action of signaling pathways including signals downstream of TCR, co-stimulatory molecules and cytokine receptors, converge to active *FOXP3* gene locus. However it is unclear the relative contribute of these different signals capable of inducing FOXP3 in thymocytes compared to naïve peripheral $CD4^+$ T cells.

1.2.2.1. TCR signaling pathway

During thymic differentiation, variations in TCR signaling characteristics, such as functional avidity and duration, are central determinants of T cell lineage fate determination and differentiation of specialized T cell subsets. It is not unexpected then, TCR signaling is pivotal for FOXP3 induction and Treg cell lineage commitment. TCR cross- linking, in human cells, triggers proximal signaling events that lead to the binding of the transcription factors Nuclear Factor of Activated T cells (NFAT) and Activator Protein 1 (AP-1) to the *FOXP3* promoter or enhancer regions contributing to its transcription (Mantel *et al*., 2006). Consistent with this view cyclosporine A (CsA), that blocks the calcineurin pathway and NFAT, completely inhibithed FOXP3 expression. In contrast, inhibitors of mitogen-activated protein kinase (MAPK) that affect AP-1, inhibited only partially the induction of FOXP3 .The decrement of FOXP3 expression by CsA in Treg cells suggests that NFAT may be required while AP-1 may be dispensable for Treg production (Mantel *et al*., 2006 ; Wang *et al*., 2006). The requirement for TCR stimulation in *FOXP3* expression and thymic development of Treg cells is illustrated by the failure of TCR transgenic T cells to express FOXP3 in the absence of endogenous TCR rearrangements (Bautista *et al*., 2009). Stimulation of adoptively transferred TCR transgenic T cells demonstrated that peripheral Foxp3 induction is associated with suboptimal activation (increased CTLA-4 and decreased CD28 signaling) and inversely correlates with proliferation (Waldmann *et al*., 2008; Rudensky, 2006). Consistent with these findings, studies *in vitro* have suggested that extensive TCR stimulation is detrimental for Foxp3 expression. In this regard, Sauer et al. showed that a constitutive o prolonged signaling through the PI3K/Akt/mTOR pathway, which is downstream of the TCR, antagonizes the induction of FOXP3 in peripheral T cells. Inhibition of this signaling network conferred *de novo* FOXP3 expression and promoted Treg cell maturation (Sauer *et al.*, 2008). Indeed, PI3K/Akt/mTOR pathway is attenuated in nTreg versus conventional T cells (Bensinger *et al*.,

2004; Walsh *et al*., 2006; Crellin, 2007). Diminished activation of this signaling appears to be important to maintain active levels of FOXO1 and FOXO3. These transcriptional factors bind to the *FOXP3* promoter to facilitate FOXP3 expression (Ouyang *et al*., 2010; Harada *et al*., 2010; Kerdiles *et al*., 2010). When the PI3K pathway is active, FOXO1 and FOXO3 are inactivated by phosphorylation and cannot support *FOXP3* transcription. Collectively, these data suggest that weak TCR stimulation is favorable for the peripheral induction of FOXP3.

1.2.2.2. CD28 co-stimulation

Another important signal required for *FOXP3* gene activation and an efficient generation of Treg cells in the thymus is co-stimulatory signaling through CD28/B7 pathway. This notion is supported by the evidence that CD28-deficient mice contained marked reduction in $CD25^{+}Foxp3^{-}$Treg precursors, suggesting an "early" requirement of CD28 co-stimulation simultaneous to or in relatively close temporal proximity to the presumed initiating TCR signal (Tai *et al*., 2005; Lio *et al*., 2010; Vang *et al*., 2010). Similarly, expression of active STAT5, a target of IL-2 signaling, in $CD28^{-/-}$ mice, improved thymic development of Treg cells. One interpretation of these data is that impaired CD28 co-stimulation leads to lower IL-2 responsible for defective nTreg development and homeostasis (Burchill *et al*., 2008). However, beyond its possible contribution to optimize IL-2 production, CD28 provides an intrinsic signal for Treg cell differentiation program by directly inducing FOXP3 expression (Scottà *et al*., 2008). Generally, CD28 addresses several downstream signaling cascade through distinct SH2- or SH3-domain-bindingg motifs in its cytoplasmic tail that mediate among others interactions with Lck and PI3K pathway respectively. However, the ability of CD28 to support efficient thymic Treg cell generation has been found to not require an intact PI3K-binding motif (Tai *et al*., 2005; Vang *et al*., 2010). Consistent

with this, PI3K-signaling through Akt and mTOR, antagonizes nTreg differentiation by sequestering FOXO1/FOXO3a from the nucleus (Delgoffe *et al.*, 2009; Haxhinasto *et al.*, 2008; Ouyang *et al.*, 2010; Sauer *et al.*, 2008). On the contrary, the Lck-interacting P_{187} YAPP motif in the cytoplasmic tail of CD28 seems to be crucial for thymic Treg differentiation (Tai *et al.*, 2005). Therefore, CD28, in conjugation with TCR stimulation, may generate signals distinct from those elicited by TCR triggering alone. For instance, the activation of the nuclear factor-κB (NF-κB) pathway and NF-κB-regulated genes is a unique feature of CD28 (Tuosto, 2011). Cross-linking of TCR together with CD28 stimulation in T cells, was found to activate nuclear factor-κB (NF-κB), whereas TCR alone was unable to do so (Marinari *et al.*, 2004; Takeda *et al.*, 2008). Thus, NF-κB may be considered the most relevant CD28 biochemical target. Many recent data strongly support the view that NF-κB regulates FOXP3 expression and serves a *bona fide* "lineage-instructing" function (Deenick *et al.*, 2010; Isomura *et al.*, 2009; Visekruna *et al.*, 2010). Consistent with a role of NF-κB activation in Treg development, deletion of upstream components of this pathway, PKCθ, Bcl-10 or CARMA-1, resulted in a significantly reduction of the number of nTreg cells both in the thymus and in periphery (Schmidt-Supprian *et al.*, 2004; Gupta *et al.*, 2008 Medoff *et al.*, 2009; Barnes *et al.*, 2009). Intriguingly, recent report by Long et al. has provided the evidence demonstrating a link between these signaling molecules and NF-κB in regulation of Foxp3 expression. In a transgenic mouse model, NF-κB activity was reduced by mutated non-degradable inhibitor IκBα, which sequesters NF-κB in the cytoplasm of non-activated cells, and both percentage and absolute numbers of $Foxp3^+$ thymocytes were reduced (Long *et al.*, 2009). Furthermore, enhancing NF-κB signal could rescue Foxp3 expression in CARMA-1 deficient mice, indicating that the impairment in thymic Foxp3 expression and Treg development is mediated by defective NF-κB activity. Additionally, transgenic enhancement of NF-κB signaling has been demonstrated to be able to bypass the requirement of TCR

signals to induce FOXP3 expression in the thymocytes and peripheral mature T cells (Long *et al*., 2009). Thus, these findings collectively imply a relationship between these intermediates signaling molecules, PKCθ, Bcl-10 and CARMA-1, and NF-κB in regulation of FOXP3 expression. Although these studies emphasize the importance of NF-κB signaling in nTreg generation, the specific NF-κB family members, required for Treg cell differentiation and their specific mechanisms of actions, remain to be elucidated. NF-κB can be composed as a combination of five related proteins, RelA/p65, c-Rel, RelB, p50 and p52 that form homo-and hetero-dimeric complexes (Natoli *et al*., 2005). Recently, it was demonstrated that c-Rel has a critical cell-intrinsic role in FOXP3 induction by binding to a conserved non-coding sequence (CNS) in *Foxp3* promoter (Ruan *et al*., 2009; Zheng *et al*., 2010). The link between this CNS and c-Rel in Foxp3 induction comes from the evidence that *Foxp3* gene activation of CNS- and c-Rel-deficient thymocytes is significantly impaired (Zheng *et al*., 2010). However, the molecular mechanism of CNS- and c-Rel-mediated Foxp3 induction remains to be elucidated. One possibility is that c-Rel binding to CNS facilitates *Foxp3* locus opening and, consequently, the recruitment of numerous regulators of *Foxp3* gene expression, including factors involved in chromatin remodeling, acetyl-transferases, histone deacetylases and sequence-specific transcription factors (Rao *et al*., 2003). Another interpretation could be that following binding to CNS, c-Rel forms a multifactiorial complex, called enhanceosome, with other factors to control *Foxp3* promoter induction (Ruan et al., 2009). However, whether or not c-Rel enhanceosome formation depends on this CNS is still unclear. In addition, other evidences demonstrated c-Rel binding to another CNS, a regulatory element required for heritable maintenance of Foxp3 expression but dispensable for its induction, suggesting that c-Rel may contribute also to the maintenance of Foxp3 expression (Zheng *et al*., 2010). However, controversial finding concerning the role of other NF-κB family members are present in the literature. For instance, Ruan *et al* (2009). suggested that c-Rel dimerizes with

p65/RelA subunit but not p50 or RelB, consistent with the evidence that p50-deficient mice have normal numbers of nTreg cells (Deenick *et al.*, 2010) However, Long et al. (2009) did not detect a substantial increase in p65 binding in any of potential NF-κB sites in *Foxp3* promoter. Conversely, Piccolella lab recently found that human FOXP3 promoter contains at least three functional and not redundant κB sites for RelA binding that contributes to *FOXP3* promoter activation and its expression (Soligo *et al.*, 2011). However these sites could be not required for c-Rel mediated FOXP3 activation, since their deletion did not modify the effect of c-Rel on *FOXP3* promoter. The reasons for this discrepancy could be various, including different stimulation and cell types. Moreover, a high *FOXP3* DNA conservation between humans and mice was not found, suggesting that murine *Foxp3* promoter could be less dependent on RelA. Altogether these observations, although contrasting for the contribution of NF-κB subunits to *FOXP3* promoter regulation, share the evidence that CD28 signaling is required for Treg differentiation. Most importantly these data shed light on FOXP3 expression is strictly dependent on CD28-mediated NF-κB activation.

1.2.2.3. Cytokyne-receptor signaling

Cytokine signaling is clearly of paramount importance to Treg differentiation as mice with disrupted IL-2 signaling pathways (i.e. IL-2 or IL-2Rα chain knockout stains) have no Treg cells (Sadlack *et al.*, 1993). By the same token, TGF-β is a critical factor in the peripheral commitment of naïve T cells to the Treg phenotype (Chen *et al.*, 2003; Zheng *et al.*, 2004).

1.2.2.3.1. IL-2R-STAT5 pathway

IL-2 signal transduction is mediated by the JAK-STAT (Janus Kinase-Signal Transducer and Activator of Transcription) pathway involving JAK1, JAK3 and STAT5. Briefly, JAK-STAT pathway is a receptor-coupled signal transduction mechanism linking cytokine-interactions to gene expression. JAK proteins (JAKs) are present in association with the cytoplasmic tails of multimeric cytokine receptors and are activated upon receptor dimerisation (Levy and Darnell, 2002; Boulay *et al.*; 2003). Activated JAKs phosporylate the cytoplasmic tails of associated cytokine receptors, creating docking sites for the SH2 (Src-homology-2) domain of STAT proteins (STATs), and then activate the docked STATs through tyrosine phophorylation. Activated STATs dimerise and translocate to the nucleus to regulate gene transcription by binding to specific promoter regions. STAT5 activation is the main IL-2R-dependent pathway active in Treg cells. The importance of this molecule in Treg biology comes from the evidence that mice deficient in STAT5 exhibit a substantial decrease of Treg cells and largely recapitulate the phenotype of IL-2- and IL-2R deficient mice (i.e. lost of CD25 expression, reduced levels of Foxp3 and impaired number of immature thymic nTreg cells) (Baier *et al.*, 2007; Malek *et al.*, 2002). In addition, STAT5 has also been found to be required for the optimal induction of FOXP3 *in vitro*. The role of this pathway in Foxp3 regulation is further supported by the evidence that at the molecular level, STAT5 directly regulate Foxp3 expression by binding to the *Foxp3* promoter *in vitro* (Yao *et al.*, 2007). As expected, *Stat5* conditional allele ablation in double-positive (DP) thymocytes resulted in a severe reduction in $Foxp3^+$ CD4 single-positive (SP) thymocytes (Burchill *et al.*, 2007; Yao *et al.*, 2007). In complementary gain-of-function studies, expression of a constitutively active STAT5 entailed expansion of Treg cells and rescued Treg cell numbers in the absence of IL-2 (Burchill *et al.*, 2008; Burchill *et al.*, 2003). Nevertheless, the exact mechanism of how IL-2R signaling impinges on Treg differentiation remains to be fully established. Although the role

of IL-2R-STAT5 signaling for the differentiation of Treg cells is well-proved, it is still unclear if STAT5 directly drives FOXP3 transcription, induces changes in the chromatin at the *FOXP3* locus, or instead promotes survival or expansion of Treg cells and/or their precursors. *In vivo*, the quantitative level of IL-2R signaling that leads to Treg maturation is set a low threshold. Therefore, the ability of developing nTreg cells to productively sense low IL-2 is likely important as IL-2 producing cells are rare in the thymus. This unique sensitivity of Treg precursors to minimal IL-2R signaling ensure that key molecules of mature Treg cells, such as FOXP3, CD25 and CTLA-4 are readily expressed (Yu *et al*., 2009). IL-2 is also required for TGF-β mediated induction of FOXP3 in peripheral conventional $CD4^+$T cells *in vitro*. The culture of these cells with TGF-β in the absence of IL-2 does not yield iTreg cells (Zheng *et al*., 2007). This function of IL-2 is not-redundant and cannot be substituted by other γc-dependent cytokines. However, it is still unclear the extent IL-2R signaling is required for iTreg development *in vivo*. Treg cells show, therefore, an obligate requirement, both *in vivo* and *in vitro*, for IL-2 and the structurally-related IL-2 family members, IL-15 and IL-7, for maintenance of FOXP3 expression and suppressive function (Bayer *et al*., 2008; Yates *et al*., 2007).

1.2.2.3.2. Transforming growth factor - β (TGF-β)- pathway

The transforming growth factor *beta* (TGF-β) is a pleiotropic anti-inflammatory cytokine that regulates multiple aspects of T cell development, homeostasis, tolerance and immune responses (Li and Flavell, 2008). Compelling evidence indicate that this cytokine, concomitant with TCR stimulation, induces Foxp3 expression in naïve $CD4^+CD25^-FOXP3^-$ T cells and converts them in $FOXP3^+$ Treg cells (Kretschmer *et al*., 2005; Chen *et al*., 2003; Zheng *et al*., 2004; Bettelli *et al*., 2006) These induced Treg cells (iTreg) are phenotypically and functionally resemble naturally-occurring Treg cells (nTreg) cells *in vitro* (Chen *et al*.,

2003; Fantini *et al.*, 2004; Fu *et al.*, 2004) and are effective at preventing organ-specific autoimmunity in a murine model of autoimmune gastritis (DiPaolo *et al.*, 2007). The notion that TGF-β signal is required for the induction of Foxp3 expression is also supported by the evidence that $CD4^+CD25^-$ T cells deficient in TGF-β signaling could not be converted to $Foxp3^+$ Treg cells *in vivo* or *in vitro* (Liu *et al.*, 2008). In human, although FOXP3 expression is also driven by TGF-β in $CD4^+CD25^-$ T cells (Amarnath *et al.*, 2007; Fantini *et al.*, 2004), the induced $FOXP3^+$ T cells are neither anergic nor suppressive and produce high levels of IL-2 and IFN-γ (Tran *et al.*, 2007). It seems that the conversion of $FOXP3^+$ T cells with Treg phenotype and function is more complex in human and FOXP3 expression alone is not sufficient to confer the regulatory phenotype (Wan and Flavell, 2007; Wang *et al.*, 2007). Both the transcription factors TGF-β-inducible early gene 1 (TIEG1) and Mother Agaist Decapentaplegic 3 (Smad3) bind to promoter regions in *FOXP3* gene and enhance its expression (Tone *et al.*, 2008). In particular, Smad3 binds to the intronic enhancer 1 of *FOXP3* locus and this binding is required for histone acetylation and function of the enhancer (Tone *et al.*, 2008). However, unlike that occurs in nTreg cells, FOXP3 expression in iTreg cells has been shown to be transient both *in vivo* and *in vitro* (Floess *et al.*, 2007; Polansky *et al.*, 2008). This unstable FOXP3 expression maybe correlates with the relative lack of demethylation of CpG-rich regions in *FOXP3* locus in iTreg cells (Josefowicz *et al.*, 2009; Polansky *et al.*, 2008; Kim *et al.*, 2007). The induction of FOXP3 expression by TGF-β is also influenced by many other factors and/or cytokines: IL-2 is involved in the generation of iTreg cells (Zheng *et al.*, 2007); CTLA-4 ligation of CD80 is also required for TGF-β to induce $CD4^+CD25^-$ T cells to express FOXP3 (Zheng *et al.*, 2006); and retinoic acid (RA) has been shown to enhance TGF-β-induced expression of FOXP3 (Sun *et al.*, 2007; Mucida *et al.*, 2007; Coombes *et al.*, 2007; Laurence *et al.*, 2007). In contrast, IL-4 suppress iTerg induction and IL-6 prevents the conversion of naïve $CD4^+CD25^-$ T cells into $FOXP3^+$ Treg cells

switching the differentiation program towards Th17 cells (Hadjur *et al*., 2009; Bettelli *et al.*, 2008).

1.3. Molecular characteristic of transcription factor FOXP3

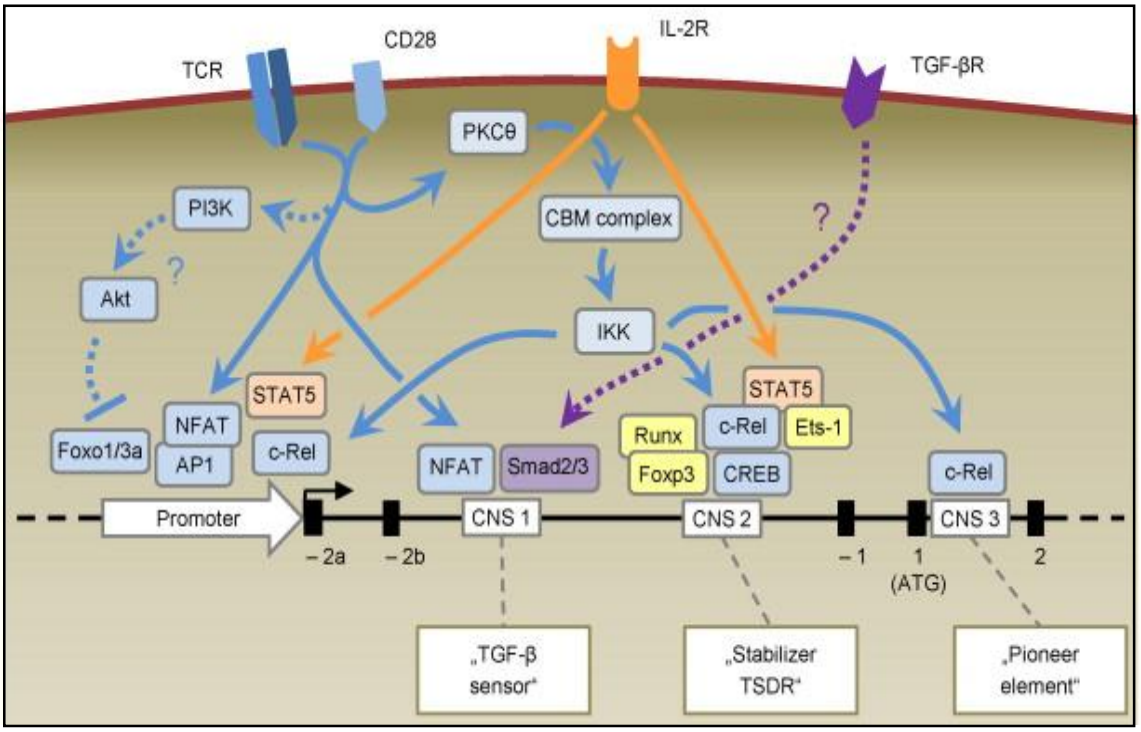
1.3.1. Identification of *FOXP3* gene

The *Foxp3* gene was identified in 2001 as the disease-causative gene in Scurfy mice, which spontaneously developed a fatal autoimmune lymphoproliferative syndrome (Brunkow *et a*l., 2001; Khattri *et al*., 2003; Fontenot *et al*., 2003)). This gene, which was found to map to a gene locus on the X chromosome, was described as a new member of the forkhead/winged-helix family of transcriptional factors (Bennett *et al*., 2000). The *Foxp3* gene is highly conserved between species and mutations in the human *FOXP3* gene, the ortholog of murine *Foxp3*, was found to be the cause of a similar human disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy X-linked) which resemble the diseases observed following depletion of $CD4^+CD25^+$ T cells in rodents (Powell and 1982; Bennett *et al*., 2001; Wildin *et al*., 2001). Mice and humans with a loss-of-function mutation in *Foxp3* and *FOXP3* genes respectively, are afflicted with a fatal, early-onset, T cell-dependent, lymphoproliferative, immune-mediated disorder manifested by diabetes, thyroiditis, hemolytic anemia, hyper-IgE syndrome, exfoliative dermatitis, splenomegaly, lymphadenopathy, and cytokine storm (Gambineri *et al*., 2003). The disease affects only hemizygous mutant males and not heterozygous female carrying *Foxp3* mutations because of random X-chromosome inactivation which ensure that some T cells express a wild-type *Foxp3* allele (Goldfrey *et al*., 1994). Both the mouse and human diseases lack discrete circulating Treg cells, suggesting that *Foxp3* and *FOXP3* are essential for normal Treg cell

development in two species respectively (Hori *et al*., 2003; Fontenot *et al*., 2003). This notion was strengthened by studies in mice which reveled stable expression of high amounts of Foxp3 in $CD4^{+}CD25^{+}$ Treg cells, but not in naïve $CD4^{+}CD25^{-}$ T cells or in activated $CD4^{+}$ T cells (Hori *et al*., 2003; Fontenot *et al*., 2003; Khattri *et al*., 2003). Mutations that inactivate *Foxp3 gene* in mature Treg cells results in a loss over time of suppressor function, characteristic Treg cell surface markers and acquisition of effector T cell properties including production of immune response-promoting cytokines IL-2, IL-4, IL-17, and IFN-γ (Williams and Rudensky, 2007). The identification of heritable mutations in *FOXP3* gene as the cause of IPEX, provided the clearest demonstration to date that, defects in a single gene could constituently disrupt immune homeostasis and lead to severe autoimmunity. They also suggested that this gene is involved in the dominant regulation of immune responses to self-antigens. Therefore, sequencing of *FOXP3* gene is still considered to be the gold standard for diagnosis of IPEX (Gambineri *et al*., 2008).

1.3.2. *FOXP3* locus

Figure 1. *Foxp3* locus in developing thymic Treg cells



Adapted from Klein & Jovanovic. *Seminars in Immunology*, 2011

1.3.2.1. TGF-β-sensitive element (CNS1)

FOXP3 locus, in addition to having a promoter highly conserved between humans, mice and rats (Mantel *et al.*, 2006) contains three proximal intronic conserved non-coding DNA sequences (CNS) that have a pivotal role in regulating *FOXP3* gene. Because the nomenclatures used in various reports differ, I will refer to these elements by their position relative to the *FOXP3* transcription start site (TSS). A second highly conserved "enhancer" region in *FOXP3* locus has been identified as a TGF-β-sensitive element (referred to as CNS1 in Zheng *et al.*, 2010). This sequence, located approximately +2kb downstream of the

promoter, contains binding sites for transcription factors, such as NFAT and SMADs (Tone *et al*., 2008). The increased levels of acetylated histone H4 found in CNS1, account for an accessible state of chromatin in both nTreg and iTreg cells (Marie *et al*., 2006; Tone *et al*., 2008). Moreover, TGF-β-induced chromatin remodeling at this locus could affect the accessibility of the upstream *FOXP3* promoter, as the level of promoter demethylation was found to be slightly increased in TGF-β-treated mouse T cells (Kim *et al*., 2007; Sauer *et al*., 2008). However, the activation-induced opening of the *FOXP3* promoter might have been due to low levels of TGF-β in the culture medium as neutralization of TGF-β completely abrogated the transient FOXP3 expression in human T cells (Tran et al., 2007). This evidence suggests that a different sensitivity to low levels of TGF-β in mouse and human T cells could account for a different activation-induced FOXP3 expression observed in the two species.

1.3.2.2. Treg-specific-demethylated-region (TSDR) or CNS2

The most striking differences regarding the methylation pattern at *FOXP3* locus, have been observed in a third, highly conserved CpG dinucletide-rich region identified both in mouse and human cells. The TSDR (referred to as CNS2 Zheng *et al*., 2010) is located in an intronic region approximately 4.5kb downstream of the TSS. It is fully demethylated in Treg cells and methylated in conventional T cells (Floess *et al*., 2007; Kim *et al*., 2007; Baron *et al*., 2007; Nagar *et al*., 2008). CNS2 has an enhancer activity markedly reduced following methylation (Polansky *et al*., 2008). This evidence is supported by the finding that the transcription factor CREB binds to TSDR when it is demethylated (Kim *et al*., 2007). Taken together, these data suggest a role for DNA methylation in the molecular regulation of FOXP3 expression. TSDR demethylation does not act as an on/off switch, but rather determinates FOXP3 expression stability. This view is consistent with the known role of epigenetic

regulation in Treg cell lineage commitment. TSDR demethylation corresponds with stability of FOXP3 (as in nTreg cells), whereas T cells that express FOXP3 only transiently (TGF-β induced Treg cells and recently activated T cells), have a methylated TSDR (Floess *et al.*, 2007; Polansky *et al.*, 2008). These data are supported by the finding that pharmacological inhibition of DNA methyltransferase-1 (DNMT1) in conventional T cells, using the covalent inhibitor 5-azacytidine followed by TCR activation, results in stable FOXP3 expression and Treg cell phenotype (Kim *et al.*, 2007; Nagar *et al.*, 2008; Polansky *et al.*, 2008).

1.3.2.3. Treg-"pioneer element" CNS3

Worthy of notice, the non coding sequence at +7kb (CNS3 in Zheng *et al.*, 2010), just downstream of the first coding exon, acts as "pioneer element" in generation of Treg cells in the thymus and in periphery. Chromatin modifications, discussed above, at TSDR and TGF-β-sensitive enhancer element, coincide with and directly precede FOXP3 expression (Zheng *et al.*, 2010). However, as acquisition of an activated chromatin state is coincident with FOXP3 expression, it seems unlikely that these regulatory DNA elements would act as the early mediators of chromatin remodeling and *de novo* activation of the *FOXP3* locus. Instead, a distinct regulatory DNA element might act earlier than CNS1 and CNS2 through the reception of appropriate signals. Indeed, CNS3 locus is characterized by chromatin modifications, associated with distal regulatory elements, including H3K4me1 and H3K27Ac, and accessibility, not only in Treg cells actively expressing FOXP3, but also in both thymic and peripheral precursors (Zheng *et al.*, 2010). This evidence suggests that CNS3 is implicated in early regulation of *FOXP3* locus activation. Consistent with this, CNS3 controls the probability of FOXP3 expression among a population of precursor cells (rather than the level of FOXP3 expression) and it is bound by the NF-κB family member, c-Rel (Ruan *et al.*,

2009). Thus, CNS3 represents a developmentally poised regulatory DNA element that function early in *FOXP3* locus activation, likely before the other sequences, CNS1 and CNS2. Identifying additional regulatory DNA elements, transcription factors and chromatin modifying and remodeling complexes and their concerted function with CNS1, CNS2 and CNS3 to promote and propagate the activated chromatin state, will allow for the induction and maintenance of FOXP3 expression.

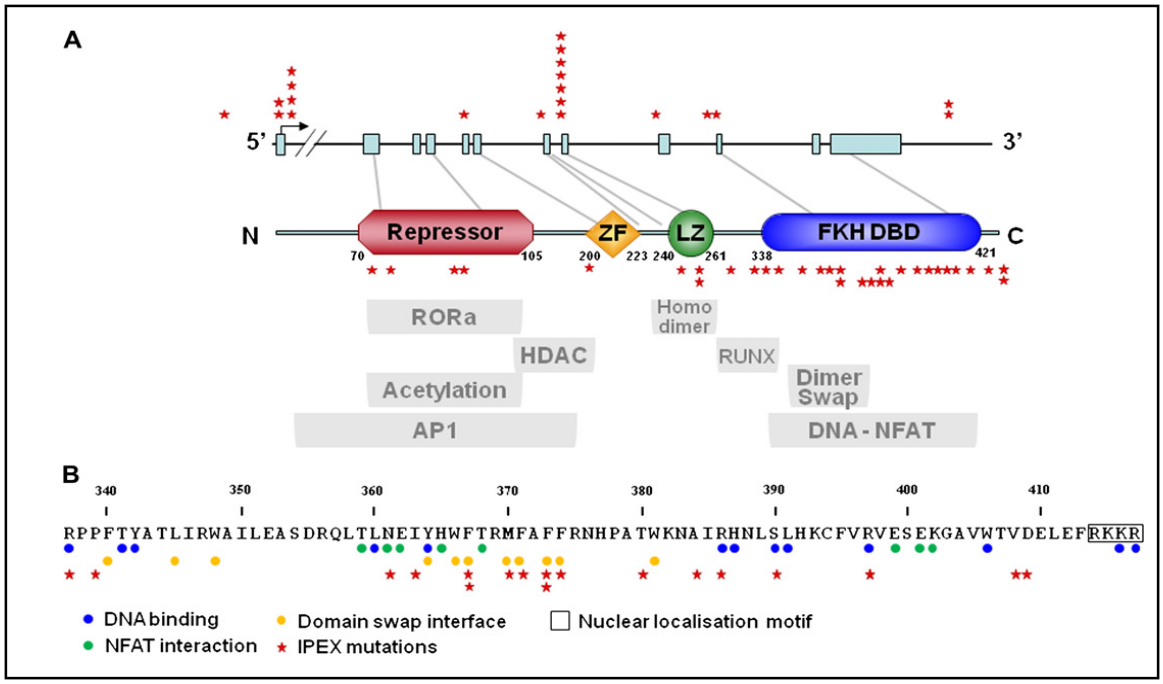
1.3.3. *FOXP3* promoter

The human *FOXP3* promoter, initially characterized by Mantel et al., is located 6.5 kb upstream of the first coding exon, containing six NFAT, AP-1 and three NFkB binding sites and a TATA and CAAT box (Mantel *et al*., 2006; Soligo *et al*., 2011). Treg cells and resting conventional T cells show differences in the epigenetic modifications of *FOXP3* promoter in both humans and mice: the CpG motifs in FOXP3 promoter were found to be hypo-methylated in nTreg cells but almost completely methylated in other T cells and only incomplete demethylation in TGF-β-induced iTreg cells (Baron *et al*., 2007; Floess *et al*., 2007; Kim and Leonard, 2007; Nagar *et al*., 2008). Moreover, *FOXP3* promoter shows a stronger association with acetylated histones in Treg cells, than in conventional T cells, indicative of an "open" chromatin configuration allowing the access by key transcriptional factors such as AP-1, NFAT, NF-κB and STAT5 (Mantel *et al*., 2006; Long *et al*., 2009; Isomura *et al*., 2009; Yao *et al*., 2007). In addition, murine experiments demonstrated that, following *in vitro* activation of conventional T cells, *Foxp3* promoter methylation was increased. This structural effect could restrict the accessibility of the promoter and prevent Foxp3 induction in these cells.

1.3.4. Key structural features of FOXP3 transcriptional factor

Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX)-associated FOXP3 mutations have been identified in both the non-coding and coding sequences of the FOXP3 gene (Bennett *et al.*, 2001; Wildin *et al.*, 2001; Gambineri *et al.*, 2008). Point mutations or small in-frame deletions within the coding region of the gene are the most informative for elucidating structure/function relationships of specific domains of the protein. In humans, FOXP3 is a 431 amino acid (aa) protein consisting of four functional domains: an amino terminal proline-rich region (PRR, aa 1-193), a C2H2 zinc finger domain (aa 200-223), a leucine zipper motif (LZ, aa 240-261), and a carboxyl-terminal forkhead domain (FKH, aa 338-421) (Lopes, 2006). Each of these motifs is involved in the molecular function of FOXP3. Therefore, mutations affecting each domain generate a specific signature of molecular defects.

Figure 2. Schematic diagram of FOXP3 and IPEX mutations along FOXP3 domains



Adapted from d'Hennezel *et al. J.Med. Genet*, 2012

1.3.4.1. N-terminal, PRR domain

The N-terminal domain is involved in various interactions that are critical for multiple aspects of FOXP3 function, including its ability to suppress cytokine production. FOXP3 was originally thought to exclusively repress transcription since it inhibited luciferase activity driven by an SV40 promoter. However, more recent studies have revealed that this factor can also act as an activator of transcription. Chromatin immunoprecipitation combined with microarray studies revealed numerous transcriptional targets of FOXP3, including genes whose expression is down – regulated (i.e. IL-2 and IFN-γ) and genes whose expression is up–regulated (i.e. IL-10, CTLA-4, GITR and CD25) (Chen *et al.*, 2006). The N-terminal region of FOXP3 harbors also a canonical LxxLL motif within a.a. 70-105 that allows FOXP3 to interact with RORα, inhibiting thus RORα -mediated transcriptional activation (Du *et al.*, 2008). RORα is a transcription factor that is critically involved in the regulation of inflammatory Th17 cell development (Cohen *et al.*, 2011). Hence, the fate of a given T cell is highly dependent on factors affecting its internal FOXP3/RORα balance, and the loss of FOXP3 expression is expected to lead to an inflammatory Th17 phenotype. The N-terminal motif of FOXP3 mediates in part the repression of NFAT/AP-1-driven transcriptional programme involving IL-2 and IFN-γ gene expression. Therefore, the interaction of FOXP3 with NFAT/AP-1 complex could affect the maintenance of Treg anergy (Lopes *et al.*, 2006) Amino acids 70-105 of FOXP3 contain a lysine-rich, nuclear export sequence, which renders it differentially sensitive to lysine acetylation (Wen *et al.*, 1995). Lysine acetylation of FOXP3 may represent a pivotal mechanism in regulating its function. Indeed, active and acetylated FOXP3 is preferentially, but not exclusively, bound to chromatin (Samanta *et al.*, 2008). Such acetylation could be mediated by TIP60, a histone acetyltransferase (HAT) that has been demonstrated to associate with FOXP3 (Wen *et al.*, 1995; Li *et al.*, 2007). Therefore, TIP60 may function to enhance the ability of FOXP3 to bind to promoters. Since TIP60 is known to

recruit Class II histone deacetylase 7 (HDAC7) in other transcriptional repressor complexes, the possibility that FOXP3 also interacts with this protein was investigated. Indeed, co-immmunprecipitation studies demonstrated that FOXP3 associates with HDAC7 in human Treg cells (Li *et al.*, 2007). As for TIP60, the HDAC7-association domain is in the amino terminal 1-190 a.a. (Li *et al.*, 2007). Thus, the amino terminal 106-190 a.a are key for the ability of FOXP3 to repress transcription via a mechanism that depends on a tri-molecular complex of FOXP3, TIP60 and HDAC7. These findings suggests that the function of FOXP3 is at least partially mediated by modulating the configuration of chromatin, thereby regulating access to promoter regions for itself and potentially for other transcription factors.

1.3.4.2. Zing-finger domain

Amino acids 200-223 encode a classic C2H2 zinc finger. The role of this domain in the normal function of FOXP3 is unknown. It could be involved in homo-oligomerisation of the protein and mutations that destroy the structure of this motif do not appear to significantly affect the ability of FOXP3 to repress gene transcription from the IL-2 promoter (Lopes *et al.*, 2006).

1.3.4.3. Leucine zipper domain (LZ)

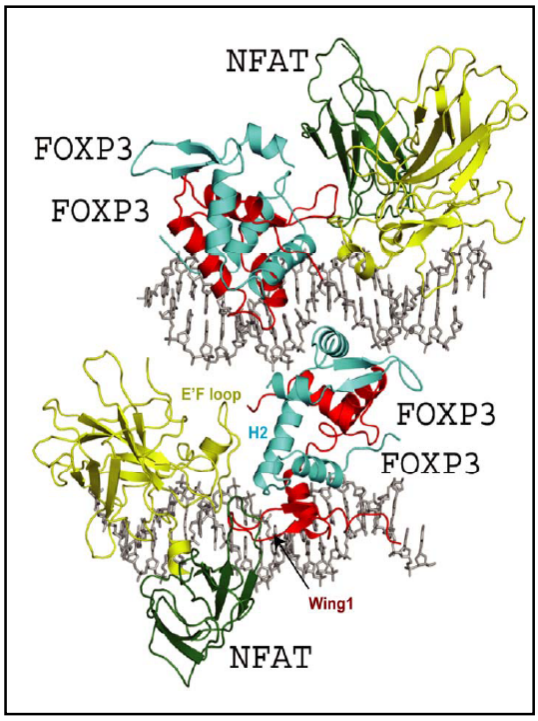
The LZ domain (a.a. 240-261) is necessary to mediate FOXP3 homo-oligomerization and hetero-oligomerization with another member of the FKH family of transcriptional factors, FOXP1. However, this motif is not required in order for FOXP3 to form high molecular weight protein complexes (Li *et al.*, 2007). Several IPEX mutations are found in this short

interval, the most studied of which is the ΔE251 mutant (Gambineri *et al*., 2008; Gavin *et al*., 2006; Wildin *et al*., 2002). Either IPEX-derived or experimental disruption of the LZ domain leads to a loss in transcriptional regulation by FOXP3 and subsequently of suppression by Treg cells (Lopes *et al*., 2006; Chae *et al*., 2006; Mailer *et al*., 2009). In particular, a naturally-occurring splice variant of FOXP3 lacking both exons 2 and 7, which encodes the LZ domain, lacks regulatory properties and has been suggested to play a role in regulating the function of the other FOXP3 isoforms by a dominant negative effect (Mailer *et al*., 2009). Dimerization of FOXP3 ensemble is required for its function as a transcriptional regulator. Indeed, recent evidences have suggested that FOXP3 does not function as a single molecule, but by forming a large supra-molecular complex detected an endogenous FOXP3 complex of more than 500 kD in size in human T cells, and found FOXP3 can exist as homodimers or homotetramers (Li *et al*., 2007). Although the forkhead domain alone (described in details in the next section) may bind to DNA *in vitro*, mutations of the leucine zipper domain disrupting FOXP3 dimerization, can substantially reduce the binding of FOXP3 to promoter regions *in vivo* (Li *et al*., 2007). Finally, the association of FOXP3 with HAT and HDAC proteins is partially abrogated in LZ mutants, and the resulting complex also partly loses its ability to bind DNA. Moreover, the leucine zipper domain is also important for the interaction between histone H5.1 and FOXP3, which cooperatively repress IL-2 transcription in human T cells (Mackey-Cushman *et al*., 2011). Therefore, FOXP3 protein ensemble, as well its DNA binding properties, could be modulated through oligomerization of the leucine zipper region.

1.3.4.4. DNA-binding, FKH domain

The FKH domain spans a.a. 337-421 in the C-terminal region of the protein. Deletion of he FKH domain (truncated after a.a. 327) abrogates FOXP3 ability to bind DNA as measured by various in vitro DNA-binding assays (Schubert *et al.,* 2001) The importance of the FKH domain for in vivo Foxp3 function is demonstrated by the Scurfy mouse in which a two-nucleotide insertion in exon 8 of the Foxp3 gene causes a frameshift and premature termination codon that deletes the FKH domain (Sakaguchi *et al*., 2010). This motif interfaces with the transcription factors NFAT and NF-κB, and targeted mutations of the NFAT binding residues in the FKH domain lead to a loss of IL-2 repression (Wu *et al*., 2006). Recently, Bandukwala and colleagues have solved the crystal structure of the FOXP3/DNA complex demonstrating that a segment of the FKH domain forms a domain-swap interface with the capacity to simultaneously engage two molecules of DNA (Bandukwala *et al*., 2011). Mutations in the domain-swap interface abrogated FOXP3 dimer formation by the FKH domain but did not affect DNA binding. The same authors suggested that FOXP3 exists as a dimer of dimers, wherein a pair of FOXP3 molecules forms a domain-swapped dimer through their FKH domains and the two domains associate with each other through their ZF-LZ regions. The inability of FOXP3 to form a stable dimer was associated with a dramatic loss of FOXP3-mediated suppressive function (Bandukwala *et al*., 2011).

Figure 3. Overall structure of the NFAT1: FOXP3:DNA complex



From Bandukwala *et al. Immunity*, 2011

Predictably, several IPEX mutations directly affect residues that are at domain-swapped interface, including F367C, F367L, M370L, F371C, F373V, F373A and F374C, many of which are associated with severe clinical outcomes (Gambineri *et al.*, 2008; Wildin *et al.*, 2001; Bacchetta *et al.*, 2006; Wildin *et al.*, 2002). However, despite recent insights into the functions of each FOXP3 domain, the role that each plays in mediating and maintaining Treg suppressive capacity under normal physiological circumstances, remains unclear. Indeed, one problem may be that many of the assays used to evaluate FOXP3 function *in vitro* using ectopic expression systems may not be able to discern subtle functional abnormalities that are evident *in vivo* when the protein is expressed at physiological levels.

1.3.4.5.The LZ-FKH loop

The amino acids between the LZ and FKH domains of FOXP3 (a.a. 261-337) but have been shown to be essential for the binding of FOXP3 to RUNX proteins, Runx1, Runx2, and Runx3 (Hu *et al.*, 2006). Most investigations are focused on Runx1 since it is expressed in Treg and Tconv cells and regulates expression from the IL-2 and IFN-γ promoters (Ono *et al.*, 2007). Indeed FOXP3 suppresses Runx1-stimulated IL-2 production in Tconv and Treg cells. A mutant form of FOXP3 unable to bind to Runx1 fails to suppress IL-2 production, demonstrating that interaction is critical for this function. The importance of Runx1 to Treg cell function is further illustrated by the fact that mouse $CD4^+$ T cells transduced with a mutant FOXP3 that cannot bind to Runx1 are less suppressive than cells transduced with wild type FOXP3, and moreover that knockdown of Runx1 in human Treg cells attenuates their suppressive capacity (Ono *et al.*, 2007). Moreover, recent data indicate that interactions between Runx1 and Foxp3 are also necessary for Foxp3-mediated inhibition of IL-17-producing T cells (Zhang *et al.*, 2008). Of note, one of the known point mutations of FOXP3 that causes IPEX occurs in the Runx1-binding region of FOXP3 and causes a late-onset, mild, and spontaneously remitting disease (Bacchetta *et al.*, 2006).

1.3.5. Control of *FOXP3* gene expression by epigenetic modifications

Recently, several groups have observed that epigenetic regulation is crucial for controlling *FOXP3* locus expression. Epigenetic modifications, which can target histones or DNA directly, affect gene transcription by altering the accessibility of distinct DNA regions to transcription factors and other DNA-binding molecules. Histones can be modified by site-specific acetylation and by methylation that are essential for determining the overall

chromatin structure. In addition, CpG motifs in the DNA can be methylated or demethylated. When the CpG motifs are methylated, they are associated with chromatin-remodeling factors, such as methyl-DNA-binding proteins, resulting in chromatin condensation. The opposite occurs following demethylation of these motifs, which induces chromatin relaxation and an increased accessibility of specific transcription factors to target sequences. In mice and humans, distinct regions of *Foxp3* and *FOXP3* locus respectively have a pattern of DNA methylation and specific histone modifications that differ between Treg cells and conventional T cells. As previously described, sequence analysis have revealed three highly conserved non-coding regions in *FOXP3* locus, all of which have been found to be subject to epigenetic modifications and to be involved in regulating *FOXP3* transcription.

1.3.6. Post-translational modifications of FOXP3

Post-translational modifications are essential for the expression, location, stability and function of many functional proteins, including histones, transcriptional factors and other cellular proteins. It is known that FOXP3 can be acetylated, phosphorylated, ubiquitinated, and proteolytically processed. Focusing on lysine residues conserved between mouse and humans in FKH domain, mutations of K383 and K393 have been found to significantly reduce the capacity of FOXP3 to suppress gene target expression, i.e. IL-2, and to confer suppressive ability to naïve $CD4^+$ T cells (Tao *et al*., 2007; Liu *et al*., 2012). However, whether these lysines are the target of acetylation is was not clarified. Other three lysine residues, K31, K62 and K67 have been shown to be acetylated both in mouse and in humans by the histone acetyltransferase p300, recently identified for its ability to acetylate and stabilize Foxp3 protein (Kwon *et al*., 2012). This process can be reversed by the histone deacetylase SIRT1 (van Loosdregtand *et al*., 2010; van Loosdregtand *et al*., 2011). Prevention

of FOXP3 deacetylation by inhibition of SIRT1 decreased FOXP3 poly-ubiquitination and proteosomal degradation (van Loosdregt*et al.*, 2011). These evidences suggested that acetylation of FOXP3 increases its stability and the suppressive function of Treg cells. FOXP3 can be also post-translationally modified and targeted for degradation in the presence of hypoxia-induced factor 1 (HIF1α), although more investigations are required to better understand the mechanism of this process (Dang *et al.*, 2011). Likewise, the significance of FOXP3 phosphorilation is still very limited.

1.3.7. FOXP3 and its interaction partners

Chromatin immunoprecipitation, combined with microarray analyses, revealed numerous transcriptional targets of human and murine FOXP3, including genes whose expression is up-regulated (*Cd25, Ctla4, Gitr*) and repressed (*Il2, Il4* and *Ifnγ)* (Zheng *et al.*, 2007). Several biochemical data indicate that FOXP3 does not function alone, but rather coordinates itself with various transcription factors, such as NFAT, NF-κB, AP-1 and AML1/Runx1, to mediate transcriptional regulation of target genes (Chen *et al.*, 2006; Wu *et al.*, 2006; Bettelli *et al.*, 2005; Ono *et al.*, 2007). Among sequence-specific transcription factors serving as FOXP3-interaction partners, NFAT and Runx1 were proposed to be indispensable for establishing Treg cell transcriptional and functional programs (Wu *et al.*, 2006; Ono *et al.*, 2007). The molecular details of NFAT-FOXP3 interactions were initially provided by crystallographic analysis of tertiary complexes of DNA and DNA-binding domains of NFAT and FOXP2, a close relative of FOXP3 (Wu *et al.*, 2006), and subsequently of similar ternary complexes with the FOXP3 forkhead domain (Bandukwala *et al.*, 2011). Interaction of FOXP3 with NFAT are thought to prevent formation of NFAT-AP1 complexes, required for the expression of immune response –promoting genes in effector T cells, thereby

ensuring their repression in Treg cells. NFAT-FOXP3 cooperation can drive the genomic program required for Treg cell differentiation and function (Wu *et al.*, 2006). Indeed, site directed mutagenesis of predicted NFAT interaction sites in the DNA binding domain of the FOXP3 protein resulted in a loss of its ability to impose the Treg gene signature and suppressor function (Wu *et al.*, 2006). Most investigations are focused on Runx1 since it is expressed in both Treg and T conv cells and regulates expression from the IL-2 and INF-γ promoters (Ono *et al.*, 2007). A mutant form of FOXP3 unable to bind to Runx1 fails to suppress IL-2 production, demonstrating that interaction is critical for this function (Ono *et al.*, 2007). The importance of Runx1 to Treg cell function is further illustrated by the fact that mouse $CD4^+$ T cells transduced with a mutant FOXP3 that cannot bind to Runx1 are less suppressive that cells transduced with wild type FOXP3. Moreover, knockdown of Runx1 in human Treg cells attenuates their suppressive capacity (Ono *et al.*, 2007). The interaction of FOXP3 with chromatin-modifying proteins, such as TIP60, HDACs 7 and 9, shows the mechanism used by this transcription factor to promote o repress target genes. An open chromatin structure is responsible for gene transcription through acetylation of histones 3 and 4 on lysine residues and trimethylation of histones 3 at lysine 4 (H3K4me3). On the contrary, trimethylation of histones 3 at lysine 9, 27 and 20 (H3K9me3, H3K27me3, H3K20me3) marks closed chromatin and represses gene transcription. In regards to this, early studies demonstrated that while FOXP3 binding to promoters that it represses (i.e. IL-2, IL-4 and INF-γ) results in histones 3 at these promoters, promoters that it trans-activates (i.e. GITR, CD25, and CTLA-4) show increased histone acetylation.

1.4. Characteristics of human $CD4^+CD25^+$ $FOXP3^+$ regulatory T cells

As previously described, in mice Treg cells were initially identified based on the expression of CD25hi (Sakaguchi *et al*., 1995). However, different from the mouse, not all $CD4^+CD25^+$ T cells are not regulatory in human since CD25 can be up-regulated in activated T cells. Only ~1-2% of cells with the highest CD25 expression has been shown to be functionally suppressive and, therefore, can be considered "authentic" Treg cells (Baecher-Allan *et al*., 2001; Allan *et al*., 2007). The discovery that the transcription factor FOXP3 was necessary for Treg cell development and function in an entire class of $CD4^+$ T cells sharply focused the spotlight on these cells (Hori *et al.*, 2003). However, the expression of FOXP3 that is specifically expressed by Treg cells in mice, can be induced in naïve $CD4^+$ T cells that are activated *in vitro* (Allan *et al*., 2007; Gavin *et al*., 2006). Thus, although Treg cells express FOXP3, all $FOXP3^+CD4^+$ T cells cannot be considered as suppressive in humans. In addition to CD25 and FOXP3, these Treg cells express several cell surface markers such as CTLA-4, GITR, CD39 and CD73, many of which are also expressed by activated $CD4^+$ T effector cells, and are therefore non Treg- specific. So far, a number of additional markers have been described as putative characterising Treg cells Although they are not perfect, some, however, allow the identification of subsets of Treg cells which may have slightly different functions.

1.4.1. Cytotoxic T-Lymphocyte Antigen- 4 (CTLA-4) and Glucorticoid-Induced TNF Receptor (GITR)

CTLA-4 (CD152), a homolog of CD28, is up-regulated on naïve T cells after stimulation (cell surface levels of CTLA-4 remain limited due to the rapid endocytosis) and suppresses T cell activation upon binding its B7 ligand (Krummel and Allison, 1995). Treg

cells constitutively express CTLA-4 (in the naïve mouse, $CD4^+CD25^+$ cells are only $CD4^+$ cells to express CTLA-4). The origin of its expression on Treg cells is not known. Whether CTLA-4 is required for Treg generation or intrinsic regulatory capacity remains unresolved. However, as the absence or blockade of CTLA-4 is associated with autoimmunity, the signaling through this pathway may be important for Treg suppression function (Takahashi *et al*., 2000; Read *et al*., 2000; Read *et al*., 2006). CTLA-4 up-regulation is also associated with enhanced Treg activity in inflammatory bowel disease models and is required in Ab-mediated therapies for promoting allograft survival (Londrigan *et al*., 2010). As CTLA-4 is also inducibly expressed on human $CD4^+CD25^-$ T cells, it is not a reliable Treg marker.

GITR is a member of the TNF receptor (TNFR) superfamily which is expressed on components of both innate and adaptive immune system, including NK and T cells. Its natural ligand, GITRL, is a member of the TNF superfamily and it is expressed on endothelial cells and APCs (in particular macrophages, DC, and B cells) (Nocentini and Riccardi, 2005). GITR acts as a T cell co-stimulatory molecule upon encounter with GITRL-expressing APC presenting cognate antigen, leading to T cell proliferation and cytokine secretion (Tone *et al*., 2003; Ronchetti *et al*., 2004). Initial enthusiasm that GITR could be a Treg marker, based on its high expression in Treg cells, abrogation of Treg suppression by GITR neutralization (McHugh *et al*., 2002; Shimizu *et al*., 2002), and its induction upon *Foxp3* trunsduction of $CD4^+CD25^-$ T cells (Hori *et al*., 2003; Fontenot *et al*., 2003), collapsed following finding that this molecule can be expressed on non-regulatory $CD4^+$ T cells upon activation (Hanabuchi *et al*., 2006). Furthermore, it has been shown that $GITR^{hi}$ $CD4^+CD25^+$ T cells are not suppressive (Shimizu *et al*., 2002) and that Treg cells from GITR knockout mice are still suppressive (Ronchetti *et al*., 2004).

1.4.2. IL-7 receptor α-chain (CD127)

IL-7 is a member of a cytokine family which includes IL-2, IL-4, IL-9, IL-15 and IL-21 whose receptor utilizes a common γ chain in association with a cytokine specific high affinity α chain, such as CD25 for IL2R and CD127 for IL7R. Although there is some redundancy between the functions of these cytokines (i.e., IL-2 and IL-15), IL-7 is critical for the development and survival of several blood cell types. Mice mutant for IL-7 or either components of its receptor, have major hematopoietic abnormalities and exhibit T cell negative (T^-), B cell negative (B^-), natural killer cell-negative (NK^-) severe combined immunodeficiency (SCID) (Puel *et al.*, 1998). In humans, loss-of-function mutations in IL-7Rα gene have been found to cause $T^-B^+NK^+$SCID. CD127 is constitutively expressed on naïve T cells, lost from the cell surface upon T cell activation and re-expressed by memory T cells (Fuller *et al.*, 2005; Boettler *et al.*, 2006; Huster *et al.*, 2004; Li *et al.*, 2003). Naïve T cell that exit thymus have low expression of IL-2R and IL-5R and they are critically dependent on IL-7 for survival. Recently, several groups showed that the lack of cell surface CD127 can be a useful alternative to CD25 for the delineation and purification of human Treg cells (Seddiki *et al.*, 2006; Liu *et al.*, 2006; Hartigan-O'Connor *et al.*, 2007). Indeed, FOXP3 expression and suppressive ability were enriched in $CD4^+$ T cells expressing low levels of CD127 (Liu *et al.*, 2006; Seddiki *et al.*, 2006). This molecule is a direct FOXP3 target (Liu *et al.*, 2006) and could reflect the mechanism of the inverse correlation between levels of CD127 and FOXP3 (FOXP3 may regulate CD127 transcription following binding to its promoter). However, conventional $CD4^+$ T cells tend to down-regulate CD127 expression after activation. Therefore, CD127 expression alone cannot accurately discriminate Treg cells from activated T cells *ex vivo*. In addition, the combined use of CD127 and CD25 to isolate Treg cells is comprised by the existence of a non-regulatory FOXP3-expressing $CD4^+$ T cell population in the periphery in humans. This population, which expresses low levels of

FOXP3, has low to intermediate CD127 expression but does not suppress effector T cells *in vitro* and produces pro-inflammatory cytokines, including IL-2, IL-17, and IFN-γ (Miyara *et al*., 2009).

1.4.3. CD39 and CD73

Both CD39 and CD73 are ectoenzymes, called ectonucleoside triphosphate diphosphohydrolase-1 (ENTDP1) and 5′-nucleotidase, ecto (NT5E) respectively. The former dephosphorylates extracellular ATP/UTP to ADP/UDP and ADP/UDP to AMP/ UMP (Borsellino *et al.*, 2007), while the latter converts the 5′-monophosphonucleotides into the dephosphorylated nucleotides (adenosine and uridine). In the immune system, extracellular ATP is an indicator of tissue destruction and its concentration is so high that substantial amounts of the nucleotide are released upon cell damage. CD39 and CD73 are expressed by B cells, DCs, all mouse Treg cells, and about 50% of human Treg cells. A complete degradation of 5′-triophosphorilated nucleotides to pericellular adenosine mediates T cell suppression through engagement of adenosine receptors (Huang *et al*., 1997). CD39 may be, therefore, a specific marker of Treg cells (Borsellino *et al*., 2007). In particular, this molecule is predominantly expressed on $CD25^{hi}$ Treg cells (Deaglio *et al*., 2007). CD39 more reliably identifies Foxp3-expressing T cells than CD25, both on a genetic association and on a protein expression level. Removal of extracellular ATP by CD39 may allow Treg cells to enter inflamed regions and permit them to quench ATP-driven pro-inflammatory processes on multiple cell types, particularly DCs. CD39-deficient Treg cells are dysfunctional because they are not anergic and proliferate in response to anti-CD3 and anti-CD28 stimulation in the absence of exogenous IL-2. Moreover, Treg cells from *CD39*null mice have markedly reduced ability to suppress $CD4^{+}CD25^{-}$ proliferation, fail to prevent allograft rejection and develop

spontaneous autoimmune alopecia. This evidence suggests that hydrolysis of ATP and generation of adenosine via CD39 and CD73 represents an important mechanism of immunoregulation Treg-mediated (Bopp *et al.*, 2007).

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1.4.4. Galectins

Galectin are lectins which bind β-galactoside. Using transcriptomic and proteomic assays to identify Treg-markers, two separate groups described expression of members of the galectin family on Treg cells .Galectin-1 is expressed by Treg cells at baseline and upon TCR engagement in both mice and humans. (Garin *et al*., 2007). Galectin-10 was also identified as constitutively expressed by Treg cells (Kubach *et al*., 2007). Antibodies blocking this molecule also abrogated Treg suppression *in vitro*. Interestingly, Treg cells from galectin-1 knockout animals are numerically normal but functionally less suppressive than their wild-type counterparts.

1.4.5. Other Treg markers

A series of other Treg-associated surface markers have also bee described in the literature. They have not been adequately validated and generally identify sub-population of Treg cells as opposed to being quintessential Treg markers in their own right. Of note, HLA-DR is expressed on a subpopulation of Treg cells which account for approximately 20-30% of human $CD4^{+}CD25^{hi}$ T cells (Baecher-Allan *et al*., 2001). HLA-DR^{+} $CD4^{+}CD25^{hi}$ T cells suppress via an obligate contact-dependent mechanism which contrasts them from their HLA-DR^{-} $CD4^{+}CD25^{hi}$ counterparts whose suppressive mechanism also includes the ability to

secrete an inhibitory soluble factor, IL-10 (Baecher-Allan *et al.*, 2006). However, these Treg populations suppress both proliferation and cytokine production by target T cells. The inducible T cell co-stimulator ICOS (CD278) has been described on a subpopulation of both thymic and peripheral $FOXP3^+$ cells (Ito *et al.*, 2008). ICOS is a member of the CD28 and CTLA-4 family of co-stimulatory molecules and it is expressed on the surface of activated T cells where it forms homodimers upon engagement of its ligand, (ICOSL, CD275) playing an import role in cell-cell signaling and immune responses (Sperling and Bluestone, 2001; Dong *et al.*, 2001; Coyle *et al.*, 2000). The expression of ICOS on human Treg cells seems to direct these cells toward different cytokine profiles as $CD4^+CD25^+ICOS^+$ T cells that produce both IL-10 and TGF-β, whereas $CD4^+CD25^+ICOS^-$ T cells produce only TGF-β (Ito *et al.*, 2008). ICOS-expressing Treg cells, therefore, can suppress via contact-independent mechanism. This difference could be due to preferential signaling through ICOS and CD28 respectively (Yoshinaga *et al.*, 1999; .Hutloff et al., 1999)

Other markers described in association with Treg phenotype include: Neuropilin-1 (Nrp-1), a multi-functional protein able to bind class 3 semaphorin proteins and vascular endothelial growth factor (VEGF) family members, which could either activate TGF-β from its latent form on Treg cells or prolong interactions between Treg cells and DCs; the cytotoxicity-associated proteins Perforin and Granzymes A and B which are thought to help suppression through direct death of target cells.

1.5. Thymic and peripheral origins of Treg cells

Similar to all other T cells, Treg cells originate from progenitor cells in bone marrow and undergo lineage commitment in the thymus. A thymic origin for Treg cells was suggested

by the neonatal thymectomy–induced autoimmunity models described above (Sakaguchi *et al.*, 1995). In addition, adoptive transfer of Treg cells from donor animals lacking ovaries, testes, prostate or thyroid glands did not inhibit organ-specific autoimmunity diseases caused by neonatal thymectomy suggesting an antigen- specific imprinting on Treg cells during thymic education. Thymic development of Treg cells requires high-affinity interactions between their T cell receptor (TCR) and self-peptide-MHC complexes presented by thymic stromal cells (Hinterberger *et al.*, 2010). These cells also provide co-stimulatory signals required for Treg cell lineage development as shown by the decrease in the number of Treg cells generated in the thymus following loss of CD40 or CD28 expression. (Salomon *et al.*, 2000; Tai *et al.*, 2005). Other signals that are necessary for Treg cells differentiation include IL-2 and, to a lesser degree, two other cytokines, IL-7 and IL-15 acting through the common gamma-chain (γc) cytokine receptors .Mice deficient in IL-2 or CD25 showed a 50% decrease in the proportion and absolute numbers of $Foxp3^+$cells, whereas loss of IL-7 or IL-15 did not perturb generation of $Foxp3^+$ cells (Vang *et al.*, 2008; Fontenot *et al.*, 2005; Burchill *et al.*, 2007). Based on the role for IL-2 and TCR signaling strength, it has been proposed a "two-step model" for thymic Treg cell differentiation: high functional avidity TCR signals result in CD25 up-regulation and a subsequent increase responsiveness of Treg precusors to IL-2 signals facilitate Foxp3 induction (Burchill *et al.*, 2008; Lio and Hsieh, 2008). Given the known similarities between mouse and human thymocyte development, it is likely that many of these requirements for Treg cells in mice are similar for human Treg cell development. The peripheral pool of Treg cells not only includes those differentiated in the thymus but also Treg cells generated extra-thymically through the "conversion"of naïve precursors in the periphery. The conditions favouring peripheral induction of Treg (iTreg) cells include suboptimal DC activation, presence of appropriate cytokines and subimmunogenic doses of agonist peptide (Gottschalk *et al.*, 2010). Two main subsets of iTreg cells have been described based on the

cytokines they produce and that cause their induction: type 1 regulatory T cells (Tr1), which are induced by IL-10 (Vieira *et al*., 2004), and T helper 3 (Th3) cells, which are induced by TGF-β (Weiner, 2001). Both subsets exert their suppressive activity trough secretion of the same cytokines that are responsible for their induction, IL-10 and TGF-β respectively. Since there are not phenotypic markers to distinguish peripherally-induced (iTreg) from thymically-derived natural Treg cells (nTreg) (molecular differences at the level of epigenetics can distinguish them), the question remains as to whether nTreg and iTreg cells have overlapping or distinct functions. The finding that the requirements for the induction of Foxp3 in the thymus and periphery are distinct, suggests that the function of these two Treg cell subsets, nTreg and iTreg cells, are also distinct. iTreg cells are thought to be an important population in the gut mucosa to maintain tolerance to commensal flora and food antigens (Lathrop *et al*., 2011). In several other physiological setting, these cells are also found, including transplanted tissue allografts, within the tumor microenvironment and chronic inflammatory sites. In all these cases, iTreg cells are thought to be antigen-specific and mediate beneficial effects limiting tissue rejection and inflammation.

1.6. Regulatory T cell and immune-suppression

Treg cells are described as “anergic” due to their poor proliferation in response to TCR ligation in the context of appropriate co-stimulation and, once activated, they can inhibit T cells irrespective for their antigen specificity. Unlike conventional $CD4^{+}CD25^{-}$ T cells, they do not produce IL-2 upon activation. This is the result of proximal block in the TCR signaling that leads to defective receptor-mediated calcium flux and phosphorylation of extracellular signal-regulated kinase (ERK). There are suggestions that the proximal block may be due to impaired CD3ζ chain phosphorylation in response to TCR ligation, although the reasons for

this are unknown (Tsang *et al*., 2006). However, Treg cells have an obligate requirement for TCR activation in order to function and can be induced to proliferate in the presence of exogenous IL-2. *In vitro,* Treg cells have the ability to inhibit proliferation and production of cytokines (i.e. IL-2 and IFN-γ) by responder T cells ($CD4^+CD25^-$ and $CD8^+$) to polyclonal stimuli, as well to down-modulate the responses of $CD8^+$ T cells, NK cells and $CD4^+$T cells to specific antigens. Several mechanisms have been proposed for Treg-mediated suppression including (i) cell contact mediated mechanism; (ii) cytokine mediated mechanism; (iii) cytotoxicity (Sakaguchi *et al*., 2008; von Boehmer and 2005; Shevach , 2009).

1.6.1. Cell- contact mediated mechanism (i)

Cell-contact dependent mechanism of suppression is based on the evidence that suppression can be abrogated via separation of Treg and effector T cells by a semi-permeable membrane and that TCR signaling is critical to their function (Takahashi *et al*., 1998). A number of mechanisms for Treg cell-mediated suppression *in vitro* has been proposed that appear to primarily affect the function of APCs (Tadokoro *et al*., 2006). Human Treg cells are the only lymphocyte sub-population that constitutively expresses CTLA-4. This notion has raised the possibility that the interaction of CTLA-4 on Treg cells with CD80 and CD86 on DCs is an important pathway for Treg suppressive function *in vitro*. As previously mentioned, selective deletion of CTLA-4 expression provokes systemic autoimmunity. Thus, CTLA-4 deficiency in Treg cells alone is sufficient to cause fatal disease (Wing *et al*., 2008) and maintenance of its expression in activated effector T cells is insufficient to prevent this outcome. There are various mechanisms by which CTLA-4 exerts its role in Treg mediated suppression. CTLA-4 binds to CD80 and CD86 on APCs and down-regulates expression of these B7 molecules (Misra *et al*., 2004; Serra *et al*., 2003), hampering their capacity to

stimulate naïve T cells through CD28 (Onishi *et al*., 2008; Wing *et al.,* 2008). CTLA-4 up-regulates the expression of lymphocyte function associated antigen 1 (LFA-1) thereby augmenting the physical interaction between Treg cells and APCs (Schneider *et al*., 2005). Moreover, ligation of CTLA-4 on Treg cells with CD80 and/or CD86 on DCs could induce the production of the enzyme indolamine 2,3 dioxygenase (IDO) in DCs. IDO catalyzes the conversion of tryptophan into pro-apoptotic metabolites that result in the suppression of effector T cell activation (Fallarino *et al*., 2003). However, there is no clear evidence about the involvement of IDO in Treg function *in vitro* or *in vivo*. It was claimed that Treg cell-mediated suppression of proliferation could be reversed by anti-CTLA-4 or its Fab fragment in *vitro* (Thorton *et al*., 2004). Although very few studies have examined the effects of anti-CTLA-4 *in vivo,* it has been shown that treatment of mice with anti-CTLA-4 abrogates the suppression of IBD mediated by Treg cells (Read *et al.,* 2006). Another cell surface antigen, that may play a role in Treg cell suppression of DC function, is lymphocyte-activation gene 3 (LAG-3, CD233), a CD4 homolog that binds MHC class II molecules with high affinity. Binding of LAG-3 to MHC II molecules expressed by immature DCs induces ITAM-mediated inhibitory signal that suppress DC maturation and immunostimulatory capacity (Liang *et al*., 2008). Because activated human T cells can express MHC class II, Treg cell-mediated ligation of LAG-3 on effectors might also result in suppression.

1.6.2. Cytokine- mediated mechanism (ii)

Although cell contact is required for *in vitro* Treg -mediated suppression, several data *in vivo* show an important role of inhibitory cytokines, like IL-10 and TGF-β, as mediators of Treg activity (Nakamura *et al.,* 2001; Maynard *et al*., 2007) Treg cells isolated from IL-10 knockout mice failed to prevent colitis and homeostatic proliferation of $CD4^+$ T cells from

Rag deficient mice (Annacker *et al.*, 2001). Moreover, blockade of IL-10 receptor and neutralization of TGF-β abolished Treg mediated inhibition of the disease. This evidence suggests that IL-10 production by Treg cells is essential for keeping the immune response in check at environmental interfaces such as colon and lungs (Rubtosov *et al.*, 2008). It also been shown that tumor microenvironment promotes the generation of Tr1 cells which mediate IL-10 dependent immune suppression in a cell contact independent manner. Similarly, another immunosuppressive cytokine secreted by Treg cells, TGF-β, has been suggested to contribute to suppressive activity of these cells. Although stimulated Treg cells express high and persistent levels of TGF-β on their cell surface, the role of this cytokine as a suppressor effector molecule remains controversial. Nakamura et al. first raised the possibility that TGF-β produced by Treg cells is bound to the cell surface, by an unknown receptor, and would mediate suppression in a cell contact-dependent fashion (Nakamura *et al.*, 2001). In their studies this suppression could be reversed by high concentrations of anti-TGF-β. So, they postulated that latent TGF-β, bound to the cell surface of activated Treg cells, is delivered directly to responder T cells and is then locally converted to its active form. It has also recently demonstrated that the major role of the expression of latent TGF-β on the surface of murine Treg cells is to convert $Foxp3^-$ T cells into $Foxp3^+$ Treg cells by a mechanism of infectious tolerance when both populations are activated via their TCRs in a DC-independent manner (Andersson *et al.*, 2008). However, the contribution of TGF-β to suppressive capacity of Treg cells *in vivo* remains controversial. A number of *in vivo* experiments have demonstrated that autocrine production of TGF-β by Treg cells is not essential for their suppressive function. For example, Treg cells from *Tgfb*$1^{-/-}$ mice or from mice expressing a dominant-negative form of TGF-βRII were able to inhibit IBD induced by $Foxp3^-$ T cells *in vivo* (Fahlen *et al.*, 2005). In contrast, mice with a T-cell specific deletion of TGF-β production developed an autoimmune syndrome including IBD (Li *et al.*, 2007). The

differences between these studies are unclear, but may relate to the flora in different animal colonies.

Recently, a new inhibitory cytokine, IL-35, has been shown to be preferentially expressed on Treg cells and contribute substantially to Treg function by directly acting on responder T cells (Collison *et al.*, 2007). IL-35 is a member of the IL-12 heterodimeric cytokine family and constitutes a pairing between Epstein-Barr virus induced gene3 (*Ebi3*) and *Il-12a.* Both *Ebi3* and *Il-12a* mRNA are up-regulated in Treg cells that are actively suppressing effector cells, raising the possibility that cell contact between Treg and T responder cells is required for maximal IL-35 production. Importantly, both *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ Treg cells have substantially reduced regulatory activity *in vitro.* Treg cells from *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ animals also were unable to control homeostatic proliferation and cure IBD (Collison *et al.*, 2007) *in vivo*. Neutralizing antibodies to IL-35 are not available and the nature of its receptor or its cellular distribution remains unknown.

1.6.3. Cytotoxicity (iii)

One other potential mechanism for Treg-mediated suppression of responder T cells is cytolysis of target cells. Activated Treg cells have been shown to express granzyme A and kill target cells in a perforin–dependent, Fas-FasL independent manner (Grossman *et al.*, 2004). Activation of murine Treg cells also results in up-regulation of granzyme B expression. Recent report has claimed that Treg cells kill responder cells by a perforin-independent, granzyme B-dependent mechanisms (Gondek *et al.*, 2005) and that granzyme B-deficient Treg cells had reduced suppressive activity *in vitro.* It has been difficult to demonstrate granzyme B expression by Treg cells *in vivo*. However, Cao et al. demonstrated that 5%-30%

of Treg cells in a tumor micro-environment express granzyme B and these Treg cells are lytic for NK and CTL cells in a granzyme B- and perforin-dependent manner (Cao *et al.*, 2007). Thus, Treg cells in certain contexts can differentiate to become what one termed "cytotoxic-suppressor" cells. Although killing of APCs *in vivo* represents a potentially potent mechanism by which Treg cells can control both primary and secondary immune responses, no studies to date have been able to document Treg cell-mediated cytolysis of DC or B cells in a model *in vivo*. Human Treg cells have been shown to express Fas and FasL in co-cultures containing Treg and responder T cells and activated Treg cells induced Fas- mediated apoptosis in autologous $CD8^+$ cells (Strauss *et al.*, 2009). Activated Treg cells could also induce apoptosis of conventional T cells through a tumor necrosis factor related apoptosis inducing ligand-death receptor 5 (TRAIL-DR5) pathway (Ren *et al.*, 2007). Also galectin-1, which can induce cell cycle arrest and/or apoptosis in T cells, has been shown to be up-regulated by Treg cells upon activation and galectin-1 deficient Treg cells have reduced regulatory function *in vitro* (Garin *et al.*, 2008).

Thus, various *in vivo* and *in vitro* studies suggest several molecules and mechanisms may operate in Treg mediated suppression. One question that arises is how such multiple modes of suppression interact which each other in maintaining immune homeostasis. It is possible that a particular mechanism may play a dominant role under a particular condition, with different mechanism operating in various situations, depending on the local micro-environment in which Treg cells act. Therefore, Treg-mediated or dependent suppressive mechanisms can be categorised into two classes. One is Treg-dependent deprivation of activation signals from responder T cells to keep them in a naïve state; the other is direct intervention by Treg-mediated killing and inactivation of T responder cells and APCs. The former serves in physiological and non-inflammatory states and is responsible for sustaining natural self-tolerance, while the latter acts in inflammatory conditions and maintains immune

homeostasis at local sites (i.e., intestinal mucosa) constantly exposed to invading microbes.

1.7. Maintenance of steady state immune homeostasis

Given that activation, proliferation, and survival of antigen-reactive conventional T cells require several kinds of co-stimuli in addition to antigenic stimulation via TCR, Treg-dependent deprivation or intervention of such co-stimuli can attenuate or suppress immune responses. The deprivation mechanism include: inhibition of CD28 signals (by LFA-1 dependent out-competition of Treg cells over naïve T cells for physical interaction with APCs and by CTLA-4 dependent down-regulation of CD80/CD86 expression on APCs); IL-2 deprivation via CD25 expressed by Treg cells and CD39/CD73-dependent degradation of ATP. In the absence of Treg cells, naïve T cells can repetitively interact with APCs receiving undisturbed CD28, IL-2 and ATP stimuli. This deprivation mechanism controls the survival and function of Treg cells via a negative feedback loop. Since Treg cells themselves are unable to produce IL-2, they are dependent on IL-2 produced by activated conventional T cells. In addition, Treg cells require CD28 signaling for their own peripheral maintenance (Garbi and Hammerling, 2011). Thus, by suppressing conventional T cells activation and APC CD80/CD86 expression, Treg cells also control their own numbers, preventing their excess and ensuring that immune system is still able to mount an effective immune response if challenged. Interruption of this feedback loop at any step may affect immune homeostasis. For effective host defense, however, Treg-mediated suppression should be transiently abrogated when pathogens invade the host. There are many demonstrations that TCR, in the presence of strong co-stimulation via CD28 and/or high concentrations of IL-2, enables responder T cells to overcome the suppression by Treg cells (Takahashi *et al*., 1998; Thornton and Schevach, 1998). This temporary abrogation of Treg-mediated suppression allows the

immune system to respond to pathogens affectively.

While a temporary attenuation of suppression is required for mounting an effective immune response, Treg cells must regain control in high inflammatory conditions in order to prevent immuno-pathology, i.e., tissue-damaging excessive immune responses. In these conditions, the deprivation mechanisms, physiologically in operation, are abrogated by abundant co-stimulation. Therefore, Treg cells produce immuno-suppressive molecules to directly and potently suppress responder T cell activation and proliferation. That is, besides the core mechanism of suppression, cytotoxic or inactivating mechanisms additionally operate to suppress activated conventional T cells by IL-10, TGF-β, granzyme and perforin. Treg cells amplify their expansion and suppressive capacity in a positive feedback loop inducing the differentiation of conventional T cells into T cells secreting respective suppressive cytokines, to effectively contain excessive inflammation. Thus, Treg-mediated suppression mechanisms can be separated in two modes or phases, differentially utilized in non-inflammatory and inflammatory conditions. Exceedance or attenuation of each mode dynamically controls the intensity of immune responses while avoiding collateral tissue damage and sustaining self-tolerance.

1.8. Stability of human $CD4^+CD25^+FOXP3^+$ Treg cells

$FOXP3^+$ Treg cells have been initially viewed as a stable cell lineage highly specialized and fully committed to suppress effector T cells. Therefore, Treg function must be remarkably durable and stable under homeostatic conditions, particularly in evolving inflammatory microenvironments. Recently, the concept that $Foxp3^+$ Treg cells are

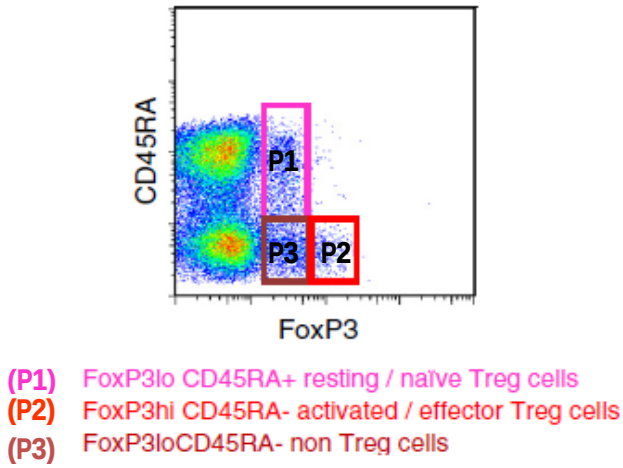
irreversible in their lineage has aroused considerable controversy. Initial studies in mouse showed that up to 20 % of Treg cells could lose Foxp3 expression, particularly at sites of inflammation (Zhou *et al.*, 2009). However, two subsequent studies argued that fully committed nTreg cells are remarkably stable, and that any apparent instability could be related to the presence of a small fraction of un-committed Treg cells or iTreg cells less stable than nTreg cells (Miyao *et al.*, 2012; Rubtsov *et al.*, 2010). In humans, single cell cloning has been used to study the stability of FOXP3 expression (d'Hennezel *et al.*, 2012). Like previous studies in mice, significant heterogeneity was observed in Treg pool, with some cells losing FOXP3 expression and suppressive capacity (Hansmann *et al.*, 2012). These data also called into question the therapeutic applicability of iTreg cells in humans, as these cells are less likely to remain $FOXP3^{+}$ when transferred *in* vivo (Hippen *et al.*, 2011). So, considering the indispensable and lifelong role of FOXP3 in Treg activity (Allan *et al.*, 2007), studies on Treg stability have used FOXP3 expression as a marker for functional Treg cells. Over the last years, many groups have sought to find ways to stabilize the expression of this transcriptional factor. As previously described in the section 1.3.2., epigenetic modifications of the *FOXP3* locus have a major role in controlling *FOXP3* transcription (Floess *et al.*, 2007). In particular, the degree of demethylation of *Foxp3* locus TSDR ensures durable and stable FOXP3 expression resulting in suppressive function of Treg cell population. Whereas nTreg cells are demethylated at this specific region, iTreg cells present an intermediated level of methylation similar to that of effector T cells (Baron *et al.*, 2007; Floess *et al.*, 2007; Polansky *et al.*, 2008). This variation could be one of the reasons for the instability of iTreg cells and, therefore, the paucity of these cells *in vivo* in transplant rejection, even under tolerizing conditions. FOXP3 expression, although instrumental to the development of Treg cells, is not an on- and-off switch to Treg cell functionality. In fact, different levels of this marker influence Treg suppressive activity and, reduced levels of FOXP3 expression result in an

impaired function of these cells. A number of studies coming from the mouse models demonstrated that Treg cells can lose Foxp3 expression in response to specific stimuli. Inflammatory cytokines such as IL-4, IL-6 and IL-21 have been shown to down-regulate Foxp3 expression *in vitro* and contribute to Treg reprogramming *in vivo* (Jin *et al*., 2008, Park *et al*., 2005; Tsuji *et al*., 2009; Zhou *et al*., 2009). By contrast, anti-inflammatory cytokines, such as IL-10, can stabilize Foxp3 expression in both nTreg and iTreg cells, as Treg cells deficient in IL-10 signaling lose Foxp3 expression (Kuhn *et al*., 1993; Rubtossov *et al*., 2008). In humans, $CD4^{+}CD25^{low}$ T cells that express low levels of FOXP3 can readily lose its expression and acquire IL-17-producing ability (Radhakrishnan *et al*., 2008) Recent evidence by Bluestone *et al*. showed that a notable fraction of nTreg cells failed to maintain FOXP3 expression. Such "ex-Treg cells" produce IFN-γ and mediate the development of autoimmune diabetes. Treg cell pool, therefore, seems to contain uncommitted Treg cells that are likely predisposed to lose FOXP3 expression and become effector T cells (Zhou *et al*., 2009). However, Treg cells exhibiting unstable FOXP3 expression do not necessarily acquire effector-like T cell properties. The reasons of this functional re-programming are unknown but could reflect the heterogeneity among human Treg cells.

1.9. Heterogeneity of human $FOXP3^{+}$ Treg cells

As described in the previous section, Treg cells are a heterogeneous pool and only a committed population maintains long-term stable FOXP3 expression and suppression ability *in vitro*. Supporting this hypothesis, Miyara *et al* recently identified three phenotypically and functionally separate populations based on the expression of FOXP3 and CD45RA, namely naïve o resting $FOXP3^{low}$ $CD45RA^{+}$ Treg cells (P1, nTreg cells), effector or activated $FOXP3^{hi}$ $CD45RA^{-}$ Treg cells (P2, eTreg cells) and cytokine-secreting $FOXP3^{low}CD45RA^{-}$

non suppressive Treg cells (P3, non Treg cells). As the expression levels of FOXP3 are proportional to those CD25, eTreg cells (P2) can be defined as $CD45RA^{-}CD4^{+}$ T cells with the highest expression of CD25. This subset can be distinguished from the other $CD4^{+}$ T cells populations because P2 has an expression of CD25 higher than naïve Treg cells (P1) (Miyara *et al.*, 2009). So, $FOXP3^{+}$ Treg cells can be separated according their levels of CD25 and CD45RA only and the analysis of CD127 expression is not indispensable to isolate Treg cells.



Adapted from Miyara *et al*. Autoimmunity Reviews, 2011

Although these cells express FOXP3, are not all suppressive. The difference between non Treg cells (P3) and functional Treg cells (P1 and P2) may be linked to the methylation status of the *FOXP3* gene, which is incompletely demethylated in P3 subset but is completely demethylated in both P1 and P2 subpopulations with suppressive activity. The observation that in patients with active lupus erythematosus $CD4^{+}CD25^{hi}CD45RA^{-}$ T cells are highly expanded (Lin *et al.*, 2007; Bonelli *et al.*, 2008; Zhang *et al.*, 2008; Venigalla *et al.*, 2008; Yan *et al.*, 2008) is an indirect evidence for their non-suppressive status *in vivo*. These findings

indicate that human FOXP3^{+}Treg subsets are distinct and might behave differently *in vivo* and *in vitro.*

1.9.1. FOXP3^{low} CD45RA^{+} Treg cells (P1)

Naïve Treg cells are characterized by their surface expression of CD45RA and their low levels of intra-nuclear FOXP3 (Miyara *et al*., 2009; Ito *et al*., 2008; Fritzsching *et al*., 2006). CD45RA is a phenotypic marker for naïve T cells that have no experienced TCR simulation. However, based on mouse data, Treg cells need to be stimulated continuously by their cognate antigen to be maintained in the periphery (Fisson *et al*., 2003). Therefore, although they express CD45RA, these Treg cells cannot be considered strictly naïve T cells (Miyara *et al*., 2009; Ito *et al*., 2008; Fritzsching *et al*., 2006). Naive Treg (P1) cells proliferate *in vitro* after TCR stimulation and are highly resistant to apoptosis (Miyara *et al*., 2009). On the contrary, FOXP3^{hi}CD45RA^{-} Treg cells (P2) tend to be hyporesponsive and apoptotic in the same conditions. Once activated, naïve Treg cells proliferate, up-regulate FOXP3 expression and convert to CD45RA^{-}CD25^{hi}FOXP3^{hi} Treg cells. This evidence suggests that the human counterpart of mouse thymus-derived nTreg cells is more likely to be the P1 population rather than P3 subset (Miyara *et al*., 2009; Fritzsching *et al*., 2006).

1.9.2. FOXP3^{hi} CD45RA^{-} Treg cells (P2)

As previously mentioned, FOXP3^{hi} CD45RA^{-} Treg cells Treg cells, which seem to derive mainly from FOXP3^{low} CD45RA^{+} Treg subset, have potent suppressive activity and show hypo- responsiveness and high susceptibility to apoptosis following activation *in vitro*

(Baecher-Allan *et al.*, 2001; Dieckmann *et al.*, 2001). In contrast to P1 subset that is in a resting state, CD45RA$^-$FOXP3^{hi} Treg cells can be thought to be an activated and functionally differentiated population of Treg cells. However, these cells need to be further stimulated through their TCR to exert suppression *in vitro* (Baecher-Allan *et al.*, 2001; Levings *et al.*, 2001; Jonuleit *et al.*, 2001). Based on these findings, CD45RA$^-$ FOXP3^{hi} Treg cells are called "effector Treg cell". They are more prevalent in adults and elderly people (Miyara *et al.*, 2009) vs nTreg cells which are enriched in cord blood. Although eTreg cells express CD45RO, a marker of conventional memory T cells, there is no evidence of memory and recall responses by Treg cells in mouse experiments. Indeed, these cells undergo rapid turnover, which is not indicative of a long-lived memory T cell population (Vukmanovic *et al.*, 2001). However, the possibility remains that effector Treg cells may mature to become terminally differentiated suppressor cells that rapidly succumb to cell death.

1.9.3. FOXP3^{low}CD45RA$^-$ non suppressive Treg cells (P3)

These non suppressive T cells that display low expression of FOXP3, may correspond to recently activation-induced FOXP3-expressing cells that transiently express FOXP3 *in vitro* (Allan *et al.*, 2007; Gavin *et al.*, 2006; Tran *et al.*, 2007; Wang *et al.*, 2007). Indeed, although the 5' flanking region of *FOXP3* gene in this Treg subset is highly demethylated, the STAT5-responsive region is poorly demethylated, suggesting that FOXP3^{low}CD45RA$^-$ Treg cells could be unstable in maintaining FOXP3 expression through STAT5 signaling. Moreover, recent evidence showed that activation-induced FOXP3$^+$CD4$^+$ T cells have *FOXP3* gene significantly less demethylated than both P1 and P2 subpopulations (Baron *et al.*, 2007; Janson *et al.*, 2008) and they can produce IFN-γ and/or IL-17 (Miyara *et al.*, 2009).

1.10. Treg cells as cell therapy

The biology of Treg cells has been the subject of much recent interest, in particular with regards to induction of transplantation tolerance. This condition can be defined as a state of unresponsiveness of an immune response to an inflammatory situation such as that generated by the recipient immune response following transplantation (Sagoo *et al*., 2012). Although the primary function of Treg cells is the maintenance of tolerance to self components to prevent development of autoimmune diseases, the presence of Treg cells in tolerated allograft suggests that they play a role in the persistence of transplanted organs. Therapeutic infusion of Treg cells has the potential to induce long-term donor specific tolerance without impeding desired immune responses to pathogens and tumors in transplant patients. Research in animal models has demonstrated that Treg cells can be used to treat many auto-inflammatory diseases such as type 1 diabetes (T1DM), inflammatory bowel disease (IBM), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) and autoimmune gastritis (AG). In addition, Treg therapies have been found to be efficacious in controlling allo-immune responses in settings of graft-versus-host disease (GvHD) as well as organ and cell transplantation in animal models (Trzonkowski *et al*., 2009; Brunstein *et al*., 2010; Di Ianni *et al*., 2011). However, given the small number of Treg cells in peripheral circulation, it is necessary to optimize methods to expand these cells *in vitro*. Therefore, one of the major goals in Treg therapy is to maintain the purity and suppressive ability of Treg preparation after *in vitro* expansion and to limit the potential to produce pro-inflammatory cytokines, such as IL-17, particularly when Treg cells are exposed to an inflammatory environment *in vivo* (d'Hennenzel *et al*., 2012; Hamann *et al*., 2012). Both these limitations may be in part due to the presence of "contaminating" effector T cells within bead-separated Treg preparations. This is not surprising as the culture conditions also favor proliferation of $CD25^{+}$effector T cells, which may contaminate the purified starting

population and outgrowth in culture (Hoffmann *et al*., 2009). Nevertheless, as described earlier, human Treg cells can lose their suppressive capability and convert into IL-17-producing T cells. The ability to expand *ex vivo* Treg cells, while maintaining immunoregulatory function, makes them particularly suitable for adoptive cell therapy to establish clinical transplantation tolerance.

2. Part I: Role of Forkhead Trascription Factor P3 (FOXP3) on *Cd25* gene trans-activation

2.1. Introduction

The central role of FOXP3 in Treg cell identity and function raised the question regarding how much FOXP3 directely affects the transcriptional signature of Treg cells. A direct investigation of the contribution of FOXP3 to transcriptional and functional features of Treg cells was made possible through the analysis of mice harboring a Foxp3 reporter *null* allele (*Foxp3*^{GFPKO}) (Gavin *et al.*, 2007) or a truncated version of the Foxp3 protein lacking DNA-binding domain tagged with GFP (Foxp3ΔeGFP) (Lin *et al.*, 2007). Cells expressing these alleles exhibited some of the phenotypic and molecular characteristics of Foxp3^{+}Treg cells including an inability to proliferate and produce IL-2 in response to TCR stimulation, expression of low amounts of IL-7α chain (CD127) and elevated amounts of CD25, CTLA-4 and GITR, although at significantly lower levels compared with Treg cells. In addition, FOXP3 amplifies and stabilizes the expression of a number of genes transiently up-regulated in activated non-regulatory T cells. Many products of these FOXP3-dependent amplified or stabilized genes in Treg cells (such as CTLA-4 and IL-10) act in a cell-intrinsec manner to limit activation of conventional T cells and serve as negative feedback regulators. FOXP3 enforces repression of the immune promoting-genes normally induced in naïve and effectotr T cells upon TCR stimulation. Thus, the analysis of the FOXP3-dependent trancriptional program invites the question of how many genes are directly regulated by FOXP3. As previously mentioned, in contrast to the early notion that FOXP3 acts as a trascriptional repressor (Bettelli *et al.*, 2005; Schubert *et al.*, 2001; Lopes *et al.*, 2006), more FOXP3-bound genes are up-regulated than repressed in Treg cells. Moreover, the finding that retrovirus-

mediated over-expression of FOXP3 in naive $CD4^+$ T cells from mice is sufficient to recapitulate all of the known features of Treg cells, including suppression of autoimmunity and rejection of transplanted grafts *in vivo*, (Hori *et al.*, 2003; Fontenot *et al.*, 2003) led to a better investigation of how FOXP3 mediates its functions at molecular level. A more thorough understanding of the molecular mechanisms by which FOXP3 activates and represses gene transcription would also be useful for development of therapies to interfere with or enhance these processes.

2.2. Research goal

The high expression of IL-2Rα (CD25) on $CD4^+CD25^+FOXP3^+$ regulatory T cells, suggests that Treg cells are more dependent on IL-2 for survival and function than $CD4^+CD25^-$ T cells. This evidence has stimulated intense research aimed to understand the mechanisms by which FOXP3 regulates transcription of *Il2* and several other genes. Although research carried out over the last years has widely clarified the repressive activity of FOXP3 (Chen *et al.*, 2006), it is still unclear how this transcriptional factor positively can regulate other genes, like *Cd25* and *Ctla4*. Since considerable evidence supports the prediction that CD25 expression is directly regulated by FOXP3 (Hori *et al.*, 2003), the goal of this chapter was to examine the functional responsiveness of the *Cd25* promoter to FOXP3. To this aim a CD25 promoter-luciferase construct has been prepared to clarify the FOXP3 trans activatory function. It has been also investigated whether the effect of FOXP3 on CD25 transcription required a coordinated interaction with NF-κB that it has been identified as the main activator of CD25 expression. However, while the role of NF-κB in *Cd25* gene trans-activation is clear, a direct effect of FOXP3 needed further investigations. In order to clarify the role of both FOXP3 and NF-κB in *Cd25* trans-activation, site direct mutagenesis of FOXP3 and κB

binding site in *Cd25* promoter has been performed. Moreover, given that FOXP3 has been reported to function as a homo-dimer, the impact of an IPEX mutation, that compromises FOXP3 homotypic interactions on *Cd25* trans-activation, has been also investigated.

2.3. Material and methods

2.3.1. List of reagents

- Medium DMEM high glucose (Gibco-BRL, Grand Island, NY)
- PBS (1x): Phosphate buffered saline without Calcium and Magnesium
- Penicillin/Streptomicin (Sigma Aldrich, St Louis, MO)
- L-Glutamine (GIBCO)
- Trypsin 0.05% EDTA (GIBCO)
- Foetal Calf Serum (FCS) (Euroclone, UK)

2.3.1.2. Antibodies.

Anti-FOXP3 (H-190), anti-p65/RelA (C20), anti-HA (Y11), and anti-α tubulin antibodies were purchased from Santa Cruz Biotecnology (Santa Cruz, CA).

2.3.1.3. Plasmids.

FOXP3 WT expression plasmid was generously provided by Dr A Rao (La Jolla Institute for Allergy and Immunology, La Jolla, CA). FOXP3ΔE251 expression plasmid. was kindly provided by Dr SF Ziegler (Benaroya Research Institute, Seattle, WA). HA-tagged p65/RelA and p50 expression vectors were kindly provided by Dr G Natoli (Department of Experimental Oncology, European Institute of Oncology, Milan, Italy). pGL4-CD25 promoter luciferase reporter vector was generated as described below. Plasmid DNA was purified using

Plasmid DNA Purification kit (NucleoBond Xtra Midi; Macherey-Nagel). Purified DNA was than diluted in dH_2O to 0.5-1 µg/µl.

2.3.2. Cloning of the *Cd25* promoter, and construction of mutant constructs

The human *Cd25* promoter containing –546 bp from transcription start site (TSS) was amplified by PCR using CD25 promoter sequence-specific primers from position –546 to + 163. The genomic DNA extracted from $CD4^+$ T cells of a healthy donor was used as a template. The CD25 promoter amplicon was cloned into the pGL4 basic vector (Promega) to generate the pGL4-CD25 promoter luciferase reporter vector. The FOXP3 and κB binding site mutants were derived from pGL4-CD25 promoter luciferase construct by substituting five nucleotides within the consensus-binding sites by PCR. Primers used to generate the individual constructs are listed in Table 1.

name	sequence and oligo	purpose
CD25prom	CGGGGTACCCCAGGGCACTGTGGTGAAATG CGGGGTACCCCAGGGCACTGTGGTGAAATG	Cloning
FOXP3[3]	-1□5 CCAGCAATTGAACTTGAAAAAAAAAACCTG CCAGCAATTGAACTTGCCCGGAAAAACCTG	Mutation
FOXP3[2]	-14□ ACCTGGTTTGAAAAATTACCGCAAACTATA ACCTGGTTTGCCCGGTTACCGCAAACTATA	Mutation
FOXP3[1]	-134 GAAAAATTACCGCAAACTATATTGTCATCA GAAAAATTACCGCCGCGGATATTGTCATCA	Mutation
κB	-2□□ CCTTCAACGGCAGGGGAATCTCCCTCTCCT CCTTCAACGGCAAAAGAATCTAACTCTCCT	Mutation

Table 1. Primers. Primers CD25 introduced a restriction enzyme recognition site for *KpnI* or *XhoI*. The primers used for mutational analysis are also shown. In the upper lane, FOXP3 and κB binding sites are evidenced in gray and indicated by position. The primers used for mutational analysis are shown in the lower lane and the underlined letters denote mutated

nucleotides.FOXP3 ^{23} mutant was obtained by site-directed mutagenesis using FOXP3^{2} and FOXP3^{3} primers.

2.3.3. Hek 293 cell line

The human embryonic kidney cells (Hek 293) were kindly provided by Dr M. Crescenzi (Istituto Superiore di Sanità Rome, Italy). The cells were cultured in DMEM supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL), L-Glutamine (4mM) and 10% FCS. When the cells reached a confluence of 90-100%, the medium was removed from the culture and the cells were washed with 10mL of PBS (1x). To detach the cells from the culture dishes (10 cm), 2mL of pre-wormed trypsin 0.05% EDTA was added and left for 2 minutes until all the cells rounded up. The trypsin activity was neutralized by adding fresh DMEM containing 10% FCS. In a new petri-dish, the appropriate volume of fresh medium was added to keep the cells at 3 x 10^5-5 x 10^5/mL. The petri-dishes were kept at 37 ºC under humidified air containing 5% CO_2 until the next splitting day which usually was 2 days from the first splitting day.

2.3.4. Cell electroporation and luciferase assay

HEK293 cells were plated at 3 x 10^5/ mL in petri-dishes (10 cm) to obtain 70% confluent layer after 24 hours. 2 x 10^6 cells were then transfected by electroporation using 5 µg of total DNA in 450 µl of DMEM supplemented with 20% FCS. For the co-transfection, pGL4-CD25 promoter luciferase reporter vector was mixed at ratio 1:2 with FOXP3 WT, FOXP3ΔE251, HA-tagged p65/RelA and p50 expression vectors. The pGL4 empty vector was added to the preparation to reach the final DNA concentration. Electroporation was

performed in 0.45-cm electroporation cuvettes (Gene pulser; BioRad, Hercules, CA) at 960 μF and 250V. The cells were then incubated at 37 °C and 5 % CO_2 and after 24h luciferase activity was measured according the manufacturer's instruction (Promega). Luciferase activity, determined in triplicates, was expressed as fold induction over the basal activity of cells transfected with empty vectors and/or with pGL4-CD25 promoter luciferase construct after normalization to protein concentration. All co-transfections were performed in the presence of pEGFP expression plasmid (Clonotech). On overage, about 35% of the cells expressed the transfected plasmids, as determined fluorescence-activated cell sorter (FACS) analysis of green fluorescent protein (GFP) co- trasfected cells.

2.3.5. Immunoblotting

The expression of RelA and FOXP3 encoding plasmids was analyzed by immunoblotting after 24h from the transfection. An aliquot of each Hek293 cell preparation was centrifuged, re-suspended in 30 μl of Lysis buffer (1% NP-40, 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM, EDTA, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 1 mM Na3VO4 and 50 mM NaF) for 30 minutes in ice. The lysate was centrifuged for 10 min at 14000 x g for removal of unlysed cells and the protein concentration of supernatant was determined by a BioRad protein assay (BioRad, CA, U.S.A.). Samples were boiled in he presence of SDS sample buffer. Proteins (30 μg for each sample) were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were than incubated with anti-FOXP3 anti-HA primary antibodies over night at 4º and after incubation with horseradish peroxidase-labeled goat anti-rabbit or horseradish peroxidase-labeled goat anti-mouse (Amersham), developed with the enhanced chemiluminescence detection system (Amersham). Blots were than normalized with anti-tubulin.

2.3.6. Bioinformatics

Human *Cd25* promoter sequence was obtained from GenBank with the accession number NG_007403. The FOXP3 binding sites were identified by using the online pattern matching application developed by Dr A Cabibbo, University of Rome Tor Vergata, freely accessible at: http://immuno.bio.uniroma2.it/cabibbo/toolies/oligo_finder/.

2.4. Results

2.4.1. Characterization of the FOXP3 binding sites on *Cd25* promoter

Accumulating findings suggest that FOXP3 regulates CD25 expression by acting as transcriptional activator (Chen *et al*., 2006; Fontenot *et al*., 2003). However, no evidence so far demonstrated the presence of FOXP3 binding sites on *Cd25* promoter. To investigate this possibility, we first analyzed the region -340 +80 of human *Cd25* promoter containing two important positive regulatory regions, PRRI (–276 to –244) and PRRII (–137 to –64), that together are required for mitogenic stimulation of CD25 (Kim et al., 2006). As depicted in **Figure 1A**, sequence analysis of *Cd25* promoter confirmed the presence of a NF-κB binding site at position -267 and showed two tandem copies of the sequence 5'-TGAAAAA-3' at positions -165 and -146. The sequence 5'-TGAAAAA-3' matched the complement of FOXP3 binding sequence 5'-TGTTTCA-3' in the *Il2* promoter except for the substitution at position 2 of G with T (Wu *et al*., 2006) **(Figure 1B)**. Interestingly, the two identified sequences were separated at 5' ends by 19 nucleotides, a distance very close to two helical repeats in the B-form DNA. Since it has been demonstrated *in vitro* that FOXP3 FKH domain can simultaneously bind two distinct FOXP3 binding sites separated by a flexible 19-base single-stranded DNA linker (Bandukwala *et al*., 2011; Thomas *et al.*, 2005), we hypothesized that the sequence 5'- TGAAAAA -3' could represent a putative FOXP3 binding site in *Cd25* promoter.

Figure 1A.

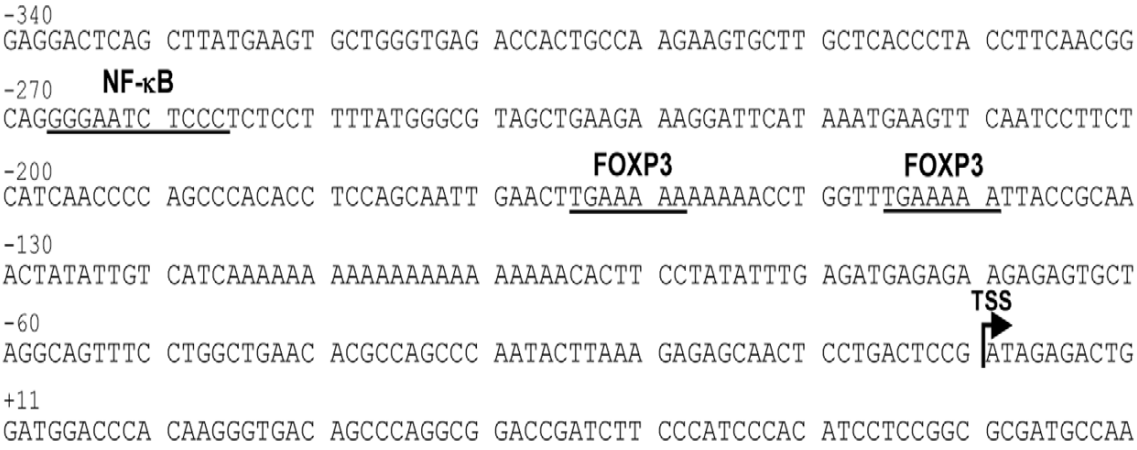


Fig. 1A. Putative FOXP3 binding sites in *Cd25* promoter. The partial human Cd25 promoter sequence (-340 +80) is shown (GenBank accession nº NG_ 007403). The TSS is indicated by broken arrow. The underlined regions represent the κB binding site (-267) and the two putative FOXP3 binding sites (-165 and -146).

Figure 1B.

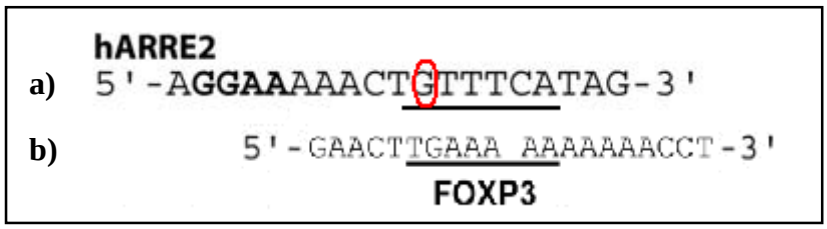


Fig. 1B. Putative FOXP3 binding sequence in *Cd25* promoter matches the complement of FOXP3 binding sequence in *Il2* promoter except for the presence of G (red circle) instead of T. Fragments of *Il2* (a) and *Cd25* (b) promoters are indicated. Non consensus FOXP3 binding sites are underlined. *Il2* ARRE2 site is adapted from Wu et *al*. Cell, 2006.

2.4.2. RelA and FOXP3 synergize to trans- activate *Cd25* promoter

The NF-kB family contains five members: RelA (p65), RelB, c-Rel, NF-kB1 (p50 and its precursor p105), and NF-kB2 (p52 and its precursor p100). These proteins form homo- and heterodimers and share a conserved N-terminal Rel-homology domain. NF-κB binding sites have been identified in the PRRI region of *Cd25* promoter and the classical NF-κB p65-p50 complexes have been described as the main activators of CD25 transcription (Algarte *et al*., 1995). The previous finding that the two putative FOXP3 binding sites mapped downstream NF-kB binding site (-267) on *Cd25* promoter, suggested the possibility that FOXP3 was able to function as co-activator of *Cd25* gene in association with NF-κB/RelA. Moreover, a recent work from Piccolella laboratory showed that the expression of FOXP3 NF-κB/RelA-mediated in CD28-stimulated $CD4^+CD25^-$ T lymphocytes correlated with CD25 expression on the membrane, highlighting a link between FOXP3 and CD25 (Soligo *et al*., 2011). Starting from these evidences, the trans-activatory function of FOXP3 on *Cd25* promoter in the presence or absence of NF-κB/RelA subunit has been analyzed. To this aim, a new CD25 promoter-luciferase construct as described in section 2.3.2. of Material and Methods has been prepared. Expression vectors encoding NF-κB/RelA, FOXP3 and FOXP3 mutant ΔE251 (FOXP3ΔE251), an IPEX mutation in the leucin zipper domain that abrogates the FOXP3 ability to homodimerize (Lopes *et al*., 2006), have been used to analyse the trans- activation of *Cd25* promoter. HEK 293 cells were co-transfected with the CD25 construct and RelA or FOXP3 or FOXP3ΔE251, or RelA and FOXP3 or NF-κB/RelA and FOXP3ΔE251 expression vectors. After 24h, luciferase reporter gene activity was measured such as NF-κB/RelA and FOXP3 protein levels. The result in **Figure 2A** shows that although the trans-activation of *Cd25* promoter was dependent on NF-κB/RelA, the presence of FOXP3 expression vector alone did not affect *Cd25* promoter activity. However, the co-expression of NF-κB/RelA and FOXP3 resulted in a synergic effect on *Cd25* gene activation. The ability of FOXP3 mutant

ΔE251 (FOXP3ΔE251), an IPEX mutation in the leucin zipper domain that abrogates the FOXP3 ability to homodimerize (Lopes *et al*., 2006) to trans -activate *Cd25* promoter was also tested. As shown in the Figure 2A, the mutant FOXP3ΔE251 encoding vector was not transcriptionally functional either alone or in association with NF-κB/RelA (Figure 2A, column 4 and 6 respectively). This result suggests that FOXP3 functions as a positive activator of *Cd25* gene only in conjunction with NF-κB and mutations that disrupt FOXP3 oligomerization, compromise FOXP3 function. Furthermore, the effect of the NF- κB p50-p65 heterodimeric complex, the main form of NF- κB regulating CD25 expression (Algarte *et al*., 1995) on the activation of the CD25 construct in the presence or absence of FOXP3 was also tested. To this purpose, the endogenous NF- κB trans-activation over-expressing NF- κB /p50 subunit was induced. The result in **Figure 2B** confirms the ability of NF- κB p50-p65 heterodimeric complex to trans -activate CD25 construct and shows that the activity of FOXP3 on *Cd25* promoter is equally dependent on the overexpression of either p50 or p65 NF- κB subunits (Figure 2B, column 4 and 6 respectively).

Figure 2.

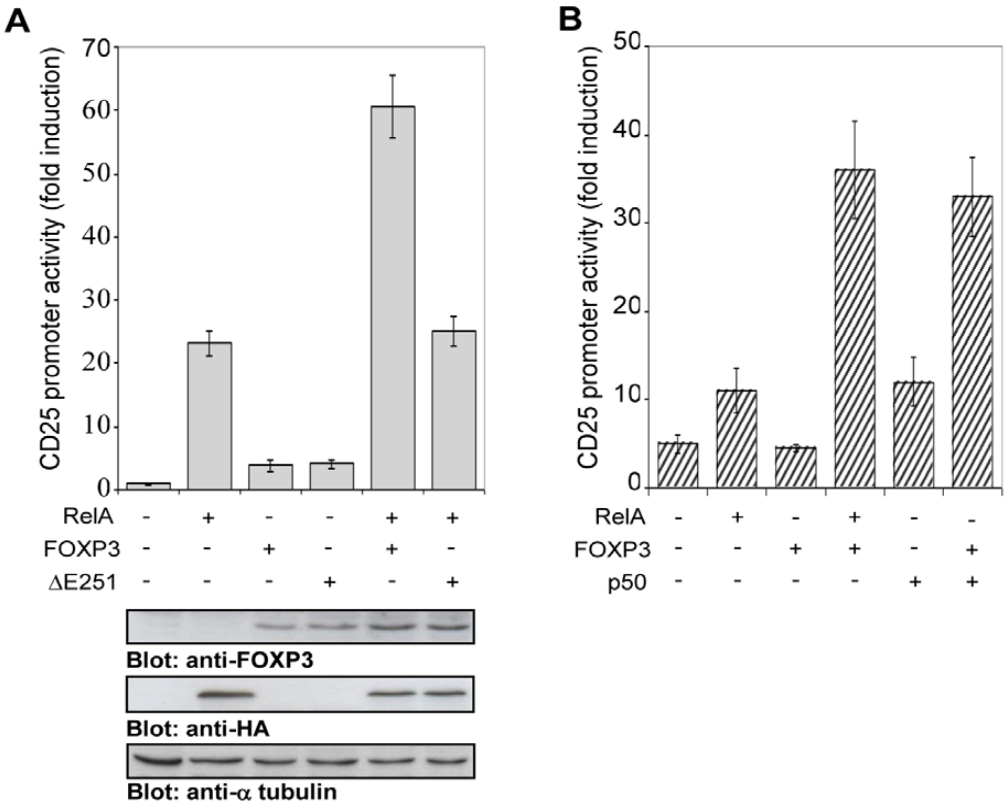


Fig. 2. FOXP3 and RelA synergize to trans-activate Cd25 promoter in HEK 293 cells. HEK 293 cells were transfected with CD25 promoter luciferase reporter vectors and cotransfected with HA-tagged RelA or FOXP3 or FOXP3DE251 **(A)** or p50 **(B)** expression plasmids, where indicated. Data are the mean (+) SD of luciferase light units normalized for Renilla luciferase of the same sample.Results are representative of five independent experiments performed in triplicate. An aliquot of each sample (A, lower panel) was analysed by immunoblotting with anti-FOXP3, anti-HA and anti α tubulin Abs.

2.4.3. Effect of κB and FOXP3 binding site mutagenesis in *CD25* promoter

To better understand the functional role of FOXP3 and NF-κB/RelA on *Cd25* gene activation, the experiment of Figure 2A upon site direct mutagenesis of FOXP3 and κB binding sites in *Cd25* promoter was repeated. To this aim, four different constructs as described in Table 1 of Materials and Methods, including the sequence 5'-GCAAACT-3' at position -134 that presented a correspondence of the first six nucleotides with a FOXP3 consensus site, were generated. Hek cells 293 cells were then co-transfected with the mutant constructs in the presence of RelA or RelA and FOXP3. In these experiments the levels of activation mediated by RelA and FOXP3 were put in relation to those mediated by RelA alone in the culture. The result of **Figure 3**, shows that the mutation of –134 sequence did not modify the CD25 reporter gene activation. However, when the two FOXP3 binding sites were progressively mutated, a significant decrease of the reporter gene activities was obtained. Indeed the mutation of both –165 and –146 sequences brought the level of activation to that obtained by RelA alone in culture, suggesting that both binding sites on *Cd25* promoter can act co-ordinately to exert trans- activation of *Cd25* promoter. However, the loss of the luciferase activity of CD25 promoter was only obtained by mutation of κB site at position –267, suggesting that FOXP3-dependent activation of the *Cd25* promoter is first dependent on the RelA transcription factor complex. Therefore, NF-κB/RelA is the principal activator of *Cd25* promoter and the binding to its κB site is required to mediate the FOXP3 activatory function. Collectively, these data strongly suggest that the positive transcriptional activity of FOXP3 on *Cd25* promoter could be dependent on the binding of RelA to its κB site and on the interaction between FOXP3 and NF-κB/RelA that could favour FOXP3 binding to the two tandem copies of the sequence 5'-TGAAAAA-3' with consequent trans activation of *Cd25* gene.

Figure 3.

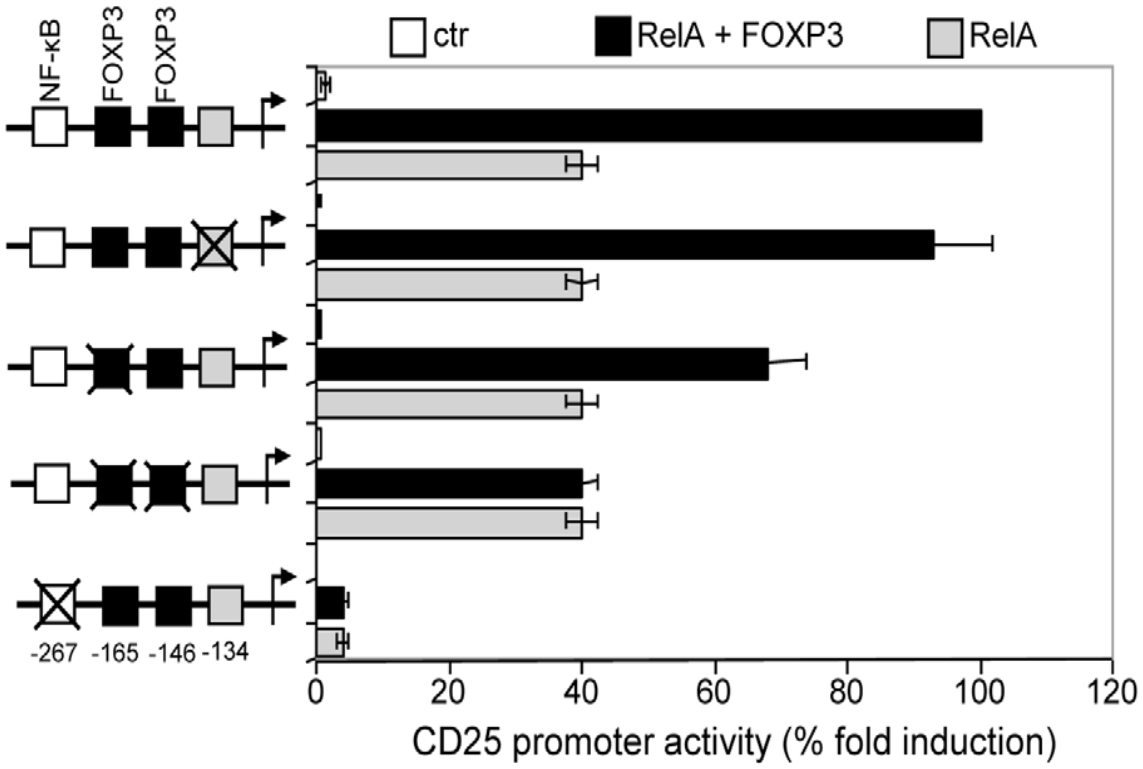


Fig. 3. The binding of FOXP3 to two non-consensus binding sites arrayed in tandem concurs to the trans activation of *Cd25* promoter. Four different constructs of human Cd25 promoter region were generated by mutation in a site-directed manner as indicated. Both wildt ype and mutated luciferase reporter vectors were transfected into HEK 293 cells. Where indicated, the cells were cotransfected with HA-tagged RelA and FOXP3 expression plasmids. Data are the mean 6 SD of luciferase light units normalized for Renilla luciferase of the same sample. Effect of mutagenesis is shown as percentage relative to wild-type construct, cotransfected with HA-tagged RelA and FOXP3, and arbitrarily set to 100%.

2.5. Discussion

Investigation of the role of activation-induced FOXP3 has been overshadowed by the prominent role of FOXP3 in Treg cell function and by the fact that this phenomenon was thought to occur in humans but not mice. FOXP3 regulates expression of several cell-surface markers characteristic of Treg cells by acting as either a transcriptional repressor or activator. Nevertheless, the mechanisms by which FOXP3 acts need further investigations. Thus, the research presented in the first part of this thesis aimed to investigate how FOXP3 activates target gene expression, analyzing in particular the mechanisms that could account for a direct regulation of CD25 expression. Considerable evidence supports the prediction that CD25 is directly regulated by FOXP3. Ectopic expression of FOXP3 in $CD4^+CD25^-$ naïve T cells has been shown to convert these cells to $CD4^+CD25^+$ T cells (Fontenot *et al*., 2003; Hori *et al*., 2003). Moreover FOXP3 can be recruited on specific binding site on *Cd25* promoter after T cell activation and mediate histone acetylation of the involved region (Chen *et al*., 2006; Wu *et al*., 2006). Given that CD25 is normally up-regulated in all activated T cells, regardless of whether they express FOXP3, and the difficulty of analysing *in vitro* signal transduction pathways that specifically target FOXP3 to *Cd25* promoter, this issue has still to be definitively demonstrated. Previous studies have shown that a promoter/enhancer region of *Cd25* gene, spanning the nucleotides –276 –64 relative to the major transcription initiation site, controlled at least in part CD25 expression in T cells (Lin *et al*., 1990). This critical region contains an NF-κB binding site at position –267 –257 (Algarte *et al*.,1995) and the occupancy of this site by various Rel/NF-κB is required to trans activate *Cd*25 in conventional $CD4^+$ T cells. Sequence analysis of *Cd25* promoter allowed the identification of two non-consensus FKH sites (5'- TGAAAAA -3') at positions –165 and –146 down-stream of the known κB-binding site at position –267. Moreover, by using a reporter construct composed of

the promoter/enhancer region of *Cd25* gene, the results of this thesis have not only confirmed the role of NF-κB factors as potent activators of *Cd25* promoter in conventional $CD4^+$ T cells, but have also showed FOXP3 as a direct activator of *Cd25* gene. However, the capacity of both RelA and FOXP3 to regulate *Cd25* gene raises a question: how to reconcile the role of RelA and FOXP3 in the regulation of CD25 expression in Treg. The reported data demonstrating that the simple bind of FOXP3 to *Cd25* promoter is not sufficient to activate CD25 expression, support the hypothesis that FOXP3 could acquire activatory function in a cooperative complex with RelA, and both RelA and FOXP3 function as direct activator of *Cd25* promoter in Treg. Therefore the complex FOXP3-RelA-DNA could represent a minimal complex required to stimulate *Cd25* gene in Treg as the complex FOXP3-NFAT-DNA has been described as a minimal complex required to inhibit *Il2* gene in the same cells (Chen *et al*., 2006; Wu *et al*.,2006) although with different requirements for the interaction FOXP3-RelA-DNA and FOXP3-NFAT-DNA. Indeed, FOXP3 and RelA interact on sites that are separated in *Cd25* promoter, while FOXP3 and RelA interact on 'composite' DNA elements with adjacent binding sites for FOXP3 and NFAT in *Il2* promoter (Wu *et al*., 2006). However, the presence of NFAT binding site located at approximately –650 and –585 upstream of the promoter region in the mouse *Cd25* gene, suggests that additional transcription factors could be linked to FOXP3 and assemble complex larger than FOXP3-RelA-DNA to up-regulate CD25 expression. It has been proposed that the degenerate nature of FOXP3 binding sites *in vivo* (Marson *et al*., 2007; Zheng and Rudensky, 2007) may reflect the contributions of additional co-factors at specific loci (Koh *et al*., 2009). Therefore large-scale 'ChIP-chip' assay have been used to identify DNA elements likely to bind FOXP3 *in vivo*, either alone or in complex with transcriptional partners (Marson *et al*., 2007; Zheng and Rudensky, 2007). FOXP3 binding *in vitro* has been observed only on consensus sites, and EMSA experiments confirm that both the DNA-binding FKH domain and intact leucin-zipper domain, which

mediates homo-multimerization of FOXP3 are required for DNA binding (Koh *et al*., 2009) supporting the view that FOXP3 can bind DNA with high affinity as at least a dimer (Bandukwala *et al*., 2011; Li *et al*., 2007). With respect to this, the two non-consensus FKH sites (5'- TGAAAAA -3') identified were separated at 5' ends by 19 nucleotides. This FOXP3 binding region, where two FOXP3 molecules, bound to the two distinct binding sites, could dimerize, presents the requirements necessary to allow the binding and stabilization of FOXP3 to its binding Indeed, although the identified FOXP3 target sequences are not consistent with a FKH consensus motif (Koh *et al*., 2009; Sadlon *et al*., 2010), these sites could become permissive *in vitro* to FOXP3 bind at least in two distinct modes: through post-translational modifications of FOXP3 and through the distinctive preference of FOXP3 for tandem sequences in DNA. The reported data highlight also the importance of dimer formation for transcriptional regulation by FOXP3 of target genes. Indeed it has been found that FOXP3 ΔE251, a FOXP3 mutant that compromise homotypic interactions (Lopes *et al*., 2006) poorly associated with the *Il2* promoter in human T cells and was less efficient at repressing IL-2 transcription (Li *et al*., 2007). In this thesis it has been demonstrated that FOXP3ΔE251 fails to upregulate CD25 expression induced by RelA. Therefore, this evidence suggests that the homodimerization of FOXP3 is crucial for the binding of FOXP3 to DNA but is not implicated in mediating inhibitory or activatory functions. In conclusion, the reported results have provided novel information on the mechanisms by which RelA and FOXP3 cooperate to mediate transcriptional regulation of target genes. Moreover it has been identified a region on *Cd25* promoter where FOXP3 dimer could bridge intra-molecularly two DNA sites and trans activate *Cd25* promoter. Together these studies contribute to the larger body of literature that demonstrates the crucial role of FOXP3 in immune cell function. A better understanding of how FOXP3 mediates its functions at both cellular and molecular level will be of utmost importance to harnessing the power of Treg cells for therapies and

manipulating both Treg and Tconv cells in diseases settings.

3. Part II: Development of methods to expand stable and suppressive human $CD4^+CD25^+FOXP3^+$ Treg cells

3.1 Introduction

Given the concerns regarding the stability and function of *ex vivo* expanded Treg cells, several purification and expansion protocols have been tested during the past years. For example, the application of tolerance permissive drugs has been shown not only to enhance Treg expansion and function *in vitro* but also to increase the purity of these cells by eliminating non-Treg cells present in the starting population. However, while is possible to examine the effect of specific agents on Treg cells *in vitro,* extrapolation of results to the in vivo setting is problematic because of the difficulty in identifying true dose comparisons. So, careful consideration will need to be given to the immunosuppressive drug regimen.

3.1.1. Rapamycin (RAPA)

Rapamycin (RAPA) is an mTOR (mammalian target of rapamycin) kinase inhibitor which is downstream of phosphatidylinositol 3-kinase (PI3-K), a signaling molecule activated by CD28 or IL-2 receptor engagement in T cells (Thomson *et al.,* 2009). IL-2 receptor engagement activates both PI3-K and Janus kinase-STAT pathways. Whereas PI3K-mTOR pathway is attenuated in Treg cells, Janus kinase-STAT pathway remains intact, suggesting that these cells preferentially signal through the latter and may be resistant to mTOR inhibition (Zeiser *et al.,* 2008). This notion is supported by genetic ablation and cellular experiments that demonstrate mTOR deficiency or addition of rapamycin favors the

outgrowth and function of Treg cells (Battaglia *et al.*, 2005; Delgoffe *et al.*, 2009). Consistent with these *in vitro* observations the use of rapamycin in mouse transplant models was found to promote expansion of suppressive Treg cells (Coenen *et al.*, 2007; Gao *et al.*, 2007; Kopf *et al.*, 2007). Interestingly, Valmori et al. demonstrated that rapamycin can also induce suppressive functions in conventional human $CD4^+$ T cells (Valmori *et al.*, 2006). Therefore, it cannot be excluded that this drug can both expand Treg cells and induce them from human $CD4^+$ T cells. It has recently shown that rapamycin is able not only to increase the number of Treg cells, but also to enhance their donor-specific suppressive capability *in vivo* (Monti *et al.*, 2008). So, there is substantial evidence that rapamycin favors Treg expansion and function and, by suppressing effector T cells, tips the balance from an aggressive towards a more protective type of allo-response.

3.1.2. All trans retinoic acid (ATRA)

Retinoic acid, a derivative of vitamin A, promotes differentiation of naïve human and murine T cells into Treg cells (Mucida *et al.*, 2007; Mucida *et al.*, 2009) and, more recently, has been shown to increase *in vitro* Treg function (Wang *et al.*, 2009). Two non-mutually exclusive mechanisms have been proposed to account for the ATRA-promoted induction of suppressive T cells. Some data indicate that ATRA augments TGF-β signaling (Benson *et al.*, 2007; Xiao *et al.*, 2008), while other studies report that ATRA suppress the ability of memory T cells to block the induction of FOXP3-expressing Treg cells (Hill *et al.*, 2008). Suppressive T cells generated *in vitro* by ATRA are attractive immunotherapeutic agents because of their ease generation and their potent function in murine models of autoimmunity, but concerns remain regarding their stability. It has been shown that ATRA is capable to skew the Treg-Th17 axis of naïve T cell differentiation toward the former (Mucida *et al.*, 2007). Addition of

ATRA inhibits development of Th17 cells from naïve precursors in a skewing environment TGF-β and IL-6. The mechanism of this effect is not completely understood but it is established to be independent of STAT signaling (Elias *et al.*, 2008). ATRA may inhibit IL-6 receptor α chain expression, enhance Smad3 activity in response to TGF-β and/or directly interact with the *Foxp3* promoter (Xiao *et al.*, 2008). In regards to this, selective retinoic acid receptor α (RAR α) gene deletion in animals' models has been showed to decrease Foxp3 expression in Treg cells (Hill *et al.*, 2008). This evidence suggests that ATRA may favor FOXP3 stability and *ex vivo* expansion of highly suppressive Treg cells. However, as ATRA plays an important role in T helper cell fate decisions inducing both effector T cell activation and differentiation to Th1 and Th17, its use for Treg expansion warrants further investigation.

3.2. Research goal

The use of expanded $CD4^+CD25^+FOXP3^+$ regulatory T cells as a cellular therapy is an attractive approach for autoimmunity disease and for limiting immune responses to allograft after transplantation. The major challenge of clinical trials is deciding the basis on which Treg cells should be isolated to maintain pure populations after *in vitro* expansion. Purity is an issue because of potential out-growth of non-Treg cells that could contribute to rejection or cause autoimmunity. Thus, the goal of this research was to investigate the effect of tolerance permissive drugs, Rapamicyn (RAPA) and all-*trans* retinoic acid (ATRA), on the expansion of human $CD4^+CD25^+FOXP^+$ regulatory T cells *in vitro*. I wanted to examine whether the treatment with these drugs modified the expression of distinct molecular markers on expanded Treg cells, including chemokine receptors, and whether they affected the purity and the suppressive function of *ex vivo* expanded Treg cells. Even if extremely pure populations can be obtained, there is a great debate over the stability of Treg cells. Indeed, one factor to

consider is that different subpopulations of $CD4^+CD25^+FOXP3^+$ regulatory T cells may be more stable than others. In order to clarify the different effect of drug treatment on Treg plasticity, the behavior *in vitro* of three phenotypically and functionally Treg subsets described by Miyara *et al* was studied. In particular it has been investigated whether RAPA and/or ATRA treatment differently selected the expansion of a particular Treg subset. It has been also investigated whether drug treatment affected the function of these Treg subpopulations by analysing the potential of these cells to release pro-inflammatory cytokine.

3.3. Material and methods

3.3.1. List of reagents

3.3.1.1. Medium and solutions

- Medium X-Vivo 15 (Lonza, Switzerland)
- Dulbecco's PBS (1x): Phosphate buffered saline without Calcium and Magnesium (PAA, Austria)
- Staining buffer: PBS (1x) with 0.5% fetal calf serum (FCS) (SeraQ, Sussex, UK) and 0.4% EDTA (ethylenediamine tetra-acetic acid) 0.5M (Gibco)
- Freezing solution: 10% DMSO and 90% FCS

3.3.1.2. Other reagents

- Lymphocyte (PAA, Austria).
- miniMACS $CD4^+CD25^+$ T Regulatory Cell Isolation Kit (Miltenyi-Biotec, UK)
- Duo-Set ELISA kits from R&D (R&D system)
- Dynabeads Human T activator anti CD3/CD28 beads (Invitrogen)
- Mouse pan-IgG magnetic beads (Dynal Biotech, Norway)
- Carboxyfluorescein diacetate, sucinnimydil ester (CFSE) (Molecular Probes, USA)
- Dimethyl sulfoxide (DMSO) (Sigma)
- Foetal Calf Serum (FCS) (SeraQ, UK)
- Penicillin and Streptomicin (Sigma Aldrich, St Louis, MO)
- Human AB serum (HS) (Biosera, UK)
- Bovine serum albumin (BSA) (Sigma)

- Phorbol Myristate acetate (PMA, Sigma)
- Ionomycin (Sigma)
- Monensin (eBioscience).
- Rapamycin (RAPA) LC-Laboratories, USA)
- All-*trans* retinoic acid (ATRA) (Sigma-Aldrich, USA)
- IL-2 (Proleukin-Novartis, UK)

3.3.2. Flow Cytometry

3.3.2.1. Reagents

The following mouse monoclonal antibodies, direct against human antigens conjugated to fluorescein isothiocyanate (FITC), phycoerytrin (PE), allophycocyanin (APC), a tandem fluorochrome that combines PE and a cyanine dye (PE-Cy7 or PE-Cy5) and peridinin-chlorophyll-protein complex (PerCP) were used: CD3-FITC, CD4-FITC (both Sigma-Aldrich); CD25-PE (4E3; Miltenyi-Biotec,UK); CD62L-FITC (Greg-56; Invitrogen,UK); CD152-PE (14D3), CD127-PE-Cy7 (eBioRDR5), GITR-APC (eBioAITR), ICOS-PE-Cy7 (ISA-3), CD39-FITC (eBioA1), Integrin α4/CD49d-PE (9F10), CD161-APC (HP-3G10), IFN-γ-FITC (4S.B3), IL-17-PE (eBio64DEC17) and FOXP3-FITC (PCH101 and 236A/E7) (all eBioscience, USA); CD27-PE (M-T271) and Integrin β7-PE-Cy5 (FIB504) (both BD-Bioscience, UK); CLA-FITC (HECA-452) and CCR4-PerCP-Cy5.5 (TG6/CCR4) (all Biolegend, USA) and appropriate isotype controls from mouse or rat were used. Prior to use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions. Flow cytometric data were acquired on a FACScalibur flow cytometer

running CellQuest software (Becton Dickinson) and analyzed subsequently with FlowJo software (Treestar Inc, Ashland, OR).

3.3.2.2. Intracellular cytokine staining (ICS)

For ICS, cells were stimulated with PMA and Ionomycin for 5 hours at 37 ºC in the presence of Monensin at final concentration of 50ng/mL, 1 μM and 2μM respectively. Cells were then washed twice with PBS, once with 1% FCS/PBS and blocked on ice for 15 minutes with 50% FCS in PBS. Permeabilization was carried out by resuspension in PBS 1% FCS/0.1% saponin (Sigma). Monoclonal antibodies against human cytokines IFN-γ and IL-17 and isotype controls were then added in a staining volume of 30 μl and incubated for 30 minutes at 4 ºC in the dark. Cells were then washed once with PBS 1% FCS/0.1% saponin and resuspended in PBS. Flow cytometry was carried out within 2 hours of staining.

3.3.2.3. Surface and FOXP3 Intracellular staining

After harvest, cells were washed and surface stained with the above listed mAbs for 15 minutes at 4°C in the dark. Appropriate isotype control antibodies were used for each sample. Following staining, cells were examined by flow cytometry.

FOXP3 staining was performed using the eBioscience human FOXP3 staining kit. Briefly, cells were surface stained if required, washed once in PBS and then vortexed and incubated in 1mL of Fixation/Permeabilization solution for 30 minutes at 4 ºC in the dark. Cells were then washed twice with Permeabilization buffer 1x and incubated with anti-human

FOXP3 antibody for 30 minutes at 4 ºC in the dark. The cells were washed once with Permeabilization buffer 1x and resuspended in the same solution for FACS analysis.

3.3.3. Enzyme-linked ImmunoSorbent Assay (ELISA)

IL-17 and IFN-γ in supernatants were analyzed by an indirect sandwich ELISA, using purified and biotinylated anti-IL-17 and anti-IFN-γ (BD Pharmingen, USA). ELISA for human IFN-γ and IL-17 was carried out using the kit from R&D system. Nunc Maxisorb ™ plates were coated overnight at room temperature (RT) with 100 µl/well anti-IFN-γ and anti-IL-17 antibodies at final concentrations at 4µg/mL in PBS. Plates were then washed three times with 0.5% Tween Sigma (Sigma) in PBS and blocked for one hour at rtp with 300µl/well freshly made 1% BSA in PBS. Plates were washed three times as before and blotted to dry before addition of 100µl/well of samples and standards in duplicate. Standards curves were prepared by serial two-fold dilutions of the manufacturer's standards in 1% BSA in PBS from a top concentration of 1000 pg/mL to15.63 pg/mL and one concentration of 0pg/mL (diluent alone). Plates were incubated for two hours at RT and then washed three times as before. 100µl/well detection antibodies biotinylated anti-IL-17 and anti-IFN-γ were added at final concentrations of 75ng/mL and 175ng/mL respectively in PBS/1% BSA and incubated for two hours at rtp. Plates were then washed three times with 0.5% Tween/PBS and 100µl manufacturer's streptavidin-HRP (horseradish peroxidase) diliuted 1 in 20 (in 1%BSA/PBS) was added to each well and incubated at rtp for 20 minutes. Plates were again washed three times and blotted to dryness before addition of 100µl substrate solution (TMB) (Zymed, USA) to each well. A further incubation for 20 minutes at RT in the dark was followed by addiction of 50µl 0.5M sulphuric acid (H_2SO_4) to each well to terminate the reaction. Optical density at 450nm was measured on a Bio-Tek EL800 automatic plate reader

(Wolf Laboratories, UK). The concentration of each cytokine was calculated from standard curves constructed from the optical densities of the known concentrations.

3.3.4. Cell culture

3.3.4.1. PBMCs separation

Human buffy coats, coming from healthy donors, were provided by the National Blood Transfusion Centre (South Thames, Tooting, UK). Diluted blood 1 in 7 with PBS was layered onto 15mL of Lymphocyte followed by centrifugation at 2000 rpm at 20ºC for 20 minutes with slow acceleration and no brake. Peripheral blood mononuclear cells (PBMC) were derived by harvesting of interface cells followed by washing with PBS three times at 2000 and 1800 rpm twice respectively for 10 minutes at 4 ºC at normal acceleration and deceleration.

3.3.4.2. Isolation of $CD4^+CD25^+$T cells

Different techniques have been used for the isolation of human regulatory T cells.

3.3.4.2.1. Isolation using Magnetic activated cell sorting

$CD4^+CD25^+$ regulatory T cells were isolated using miniMACS $CD4^+CD25^+$ T Regulatory Cell Isolation Kit. The kit contains a cocktail of biotinylated antibodies and anti-biotin micro-beads for depletion of non-$CD4^+$ T cells, and CD25 micro-beads for subsequent

positive selection of the $CD4^+CD25^+$ regulatory T cells. PBMC were incubated with 90µl Staining buffer and 10µl of biotin antibody-cocktail /10^7 cells for 10 minutes followed by 15 minutes incubation with anti-biotin micro-beads at 4 ºC. The biotin antibody-cocktail contained biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a (glicophorin A). The cells were then washed once with Staining buffer (centrifugation 1800 rpm for 5 minutes at 4 ºC) and resuspended in 500µl Staining buffer/10^8 cells. An LD MACS separation column was prepared by single rinse with 2mL Staining buffer and placed on a MACS separator magnet. The resuspended cells were passed over the column and the negative fraction (unbound $CD4^+$ cells) eluted with a series of two x 1mL Staining buffer washes.

The eluted cells were then centrifuged and resuspended in 90 µl Staining buffer and 10 µl of $CD25^+$ micro-beads/10^7 cells and incubated in dark for 15 minutes at 4 ºC. The cells were then washed once with Staining buffer (centrifugation 1800 rpm for 5 minutes at 4 ºC) and resuspended in 500 µl Staining buffer /10^8 cells. The cells were then passed through an MS column (prepared by single rinse with 500 µl Staining S buffer) and the negative selection ($CD4^+CD25^-$ responder T cells/Tres) eluted with a series of three x 500 µl Staining buffer washes. Aliquots of these cells were cryopreserved and used as autologous responder cells in suppression assays (sections 3.3.5 and 3.3.6). The column was the removed from the magnet and the CD25-microbeads positive fraction, $CD4^+CD25^+$ regulatory T cells, eluted with 1mL of Staining buffer and firm column pressure from the plunger. $CD4^+CD25^+$ T cells were analyzed by flow cytometry following staining with anti-human CD4 and anti-humanCD25 antibodies. Their purity was between 90-98%.

3.3.4.2.2. Separation of $CD4^+$ T cells using Dynabeads

3.3.4.2.2.1. *Antibodies*

The following mouse anti-human CD marker monoclonal antibodies were purchased for cell purification and enrichment procedures: CD8 (3B5), CD14 (B-A8), CD16 (B-E16), CD19 (B-C3), CD56 (B-A19), CD33 (4D3) (Invitrogen, USA), CD235a (GA-R2), TCR γ/δ (IIF2, Becton-Dikinson (BD), CA).

3.3.4.2.2.2. *Enrichment of $CD4^+$ T cells*

$CD4^+$ T cells were enriched from human PBMC by negative selection using an antibody cocktail. Cells were incubated in 5mL MACS buffer containing antibodies against CD8 (6 µl/10^8 cells), CD14 (7 µl/$10^{\wedge 8}$ cells), CD16 (7 µl/10^8 cells), CD19 (18 µl/10^8 cells), CD33 (3 µl/10^8 cells), CD56 (3 µl/$10^{\wedge 8}$ cells), TCR γ/δ (7 µl/10^8 cells), CD235a (7 µl/10^8 cells) for 30 minutes at 4 ºC on a roller. Cells were then washed with MACS buffer (centrifugation 1800 rpm for 5 minutes at 4 ºC) and resuspended in 5mL X-Vivo 15 medium supplemented with 5% HS containing mouse pan-IgG magnetic beads. Beads were used at 1µl/10^6 cells and washed twice with 5mL PBS in a magnet before addition to cells. A passage through a magnet to remove unwanted cells was fallowed by incubation for 30 minutes at 4 ºC on a roller. Isolated $CD4^+$ T cells were incubated over night at a final concentration of 5 x 10^6 cells/mL in X-Vivo 15 medium supplemented with 5% (HS) at 37 ºC in 5% CO_2.

3.3.4.3. Cell sorting of human Treg subpopulations

Fluorescence activated cell sorting of subpopulations of $CD4^+$ T cells were carried out using a 3-laser FACS-Aria high-speed cell sorter (BD Biosciences). For each cell sort, single positive and unstained controls were used to set up the gates and instrument compensations. Cell staining of 200 x 10^6 cells was carried out in 10mL MACS buffer for 20 minutes at 4 ºC on a roller, followed by a single wash, resuspension in 10mL MACS and cell sorting. $CD4^+$ T cells, enriched from human buffy coats as previously described (section 2.2.2.), were stained with monoclonal antibodies conjugated to fluorescence dyes directed against specific cell surface markers. To sort Treg subpopulations, $CD4^+$ T cells were stained with anti-CD4 (FITC), anti-CD25 (APC) and CD45RA (PE) antibodies to sort them into $CD4^+CD25^+CD45RA^+$, $CD4^+CD25^+CD45RA^-$ and $CD4^+CD25^{hi}CD45RA^-$ T cells.

3.3.5. Cryopreservation of cells

PBMCs and $CD4^+CD25^-$ responder T cells were centrifuged at 1800 rpm for 5 minutes. The pellet was resuspended in freezing solution at final concentrations of 50x10^6 and 10x10^6/mL respectively. Cells were transferred to a -80 ºC freezer for a minimum of 24hours. The cryovials were then stored into liquid nitrogen tanks.

3.3.6. Suppression assay

For functional assay, cryopreserved responder T cells (Tres cells) were thawed and labeled with CFSE as follows. Cells were washed twice in PBS, before being stained with 2.5 μM CFSE in PBS at $10x10^7$/mL. After 3 minutes at room temperature in the dark, the reaction

was stopped by washing three times the cells with X-Vivo 15 supplemented with 5% HS. The cells were then resuspended in the same medium at the required concentration. In order to quantify differences in susceptibility to suppression, Tres cells, labeled with CFSE, were cultured alone or in co-cultured at different ratios with Treg cells treated with or without RAPA (100nM) and/or ATRA (2μM). In each case, the number of Tresp cells was kept constant ($1x10^5$) and the number of Treg cells titrated down (Treg:Tres ratio= 1:1, 1:3 and 1:10) in X-Vivo15 supplemented with 5% HS. The cells were stimulated with anti-CD3/CD28 beads in U-bottom 96-well plates and incubated at 37°C, 5% CO_2 for 5 days. After harvest, qualitative CFSE dilution was assessed by flow cytometry on day 5 through acquisition of fluorescence in FL1 channel. For qualitative estimation of proliferating precursors, 20μl of counting beads (Cytognos, Spainn) was added to each FACS tube. The number of acquired cells in each CFSE peak was multiplied by the ratio of acquired to added counting beads prior analysis. For concurrent estimation of apoptosis, cells were also stained with 7-Amino-actinomycin D (7-AAD) (Beckton Dickinson) for 3 minutes at room temperature prior to flow cytometry. The percentage of suppression was calculated based on the proliferation of responder T cells alone compared with the proliferation of cultures containing responder and suppressor cells.

3.3.7. Expansion of Treg cell lines

Healthy human $CD4^+CD25^+$ T cells, purified as described in section 3.3.4.2., were plated at $1x10^6$/ml in X-Vivo 15 supplemented with 5% HS with or without RAPA (100nM) and/or ATRA (2μM). RAPA (100nM) final concentration was used according to previous reports (Battaglia). Different doses of ATRA (0.002-2μM) were investigated and 2μM chosen for *in vitro* Treg expansion because of its strong biological effect on Treg phenotype. Cells

were initially stimulated with anti-CD3/CD28 beads at a bead: cell ratio of 1:1. IL-2 (1000IU/mL) was added at day 2 post-activation and replenished every 2 days. Beads were removed by magnetic adherence every 7 days post-activation and fresh anti-CD3/CD28 beads (1:1 ratio), RAPA, ATRA and IL-2 (1000 IU/ml) were added. Cells were cultured for between 3 or 5 weeks prior to use.

3.3.8. *In vitro* Treg stability assay

Fresh ($5x10^5$), untreated, RAPA and/or ATRA treated Tregs were activated with anti-CD3/CD28 beads at a bead:cell ratio of 1:1 and cultured for 5 days in X-Vivo 15 medium supplemented with 5% human serum with the following cytokine cocktails: Cocktail-A IL-2 (10 IU/ml), IL-1β (10ng/ml), IL-6 (4ng/ml) and TGF-β (5ng/ml, all R&D-Systems, USA); Cocktail-B IL-2 (10 IU/ml), IL-21 (25ng/ml, Cell-Sciences, USA), IL-23 (25ng/ml, R&D) and TGF- β (5ng/ml, R&D). Cells cultured in complete medium supplemented with IL-2 (10 IU/ml) were used as control.

3.3.9. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad software Inc., USA). Parametric and non-parametric data were calculated as the mean±s.d. and median (interquartile range, IQR) respectively. For comparison of parametric and non-parametric data, t-test, one- or two-way ANOVA with Bonferroni test comparison and Kruskal-Wallis test were used where appropriate.

3.4. Results

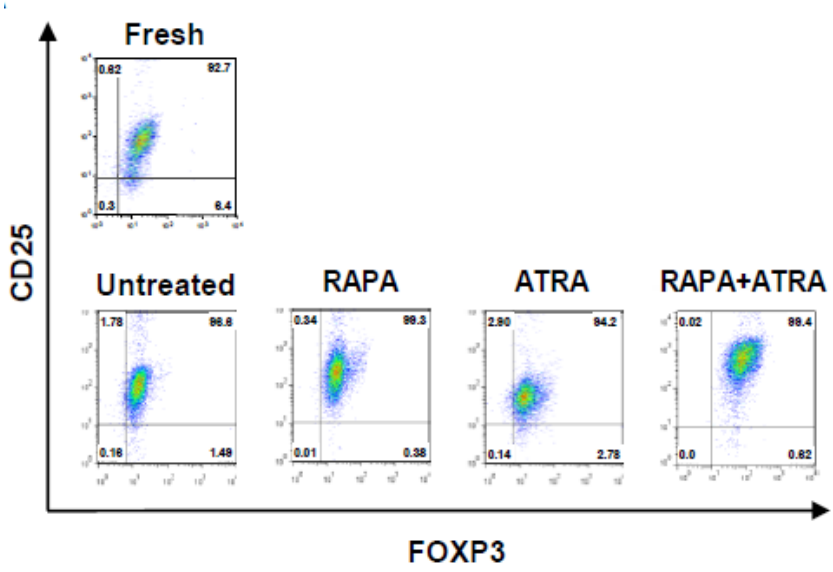
3.4.1. RAPA and/or ATRA treatment do not modify FOXP3 expression in human Treg cells

It has been observed that Treg cells isolated by magnetic bead based-enrichment of $CD4^{+}CD25^{hi}$ lose FOXP3 expression when expanded *ex vivo* (Hoffmann *et al.*, 2009) Although FOXP3 in the human system is not exclusively expressed by Treg cells, but it can be also up-regulated by activated non Treg cells, the evaluation of the percentage of $FOXP3^{+}$ cells is still the best way of controlling the purity of these cells (Morgan *et al.*, 2005; Ziegler, 2006). In order to work towards an optimal protocol for the expansion of bead-separated Treg cells, we tested the effects of two drugs on *ex vivo* purified human Treg cells. RAPA and more recently ATRA have been shown to enhance human $CD4^{+}CD25^{hi}FOXP3^{+}$ T cells expansion *in vitro* (Battaglia *et al.*, 2005; Golovina *et al.*, 2011). Thus, the effects of RAPA and ATRA on human Treg cell phenotype with regard to FOXP3 and CD25 expression were first analyzed and compared. Polyclonal Treg cell lines, with a purity of more than 90% for FOXP3-positivity on isolation, were generated as described in Material and Methods. These cells were activated using anti-CD3/CD28 beads and IL-2 (1000 IU/mL), in the absence (untreated) or presence of RAPA (100nM), ATRA (2μM) or a combination of both drugs (RAPA+ATRA). Phenotypic analysis of fresh isolated Treg cells or Treg cell lines cultured for 28 days under different conditions **(Figure 1A)** showed no significant differences in the percentage of CD25 and FOXP3 expression. However, the mean fluorescent intensity (MFI) of CD25 expression was highest following exposure of Treg cell lines to RAPA alone or in combination with ATRA **(Figure 1B)**. In contrast, FOXP3 MFI was mostly unchanged by the addition of RAPA alone or RAPA plus ATRA. As the different Treg cell lines maintained high levels of FOXP3 expression, it follows that the treatment of these cells with RAPA, ATRA or the combination

of both drugs could help to maintain Treg phenotype during *ex vivo* expansion.

Figure 1.

A



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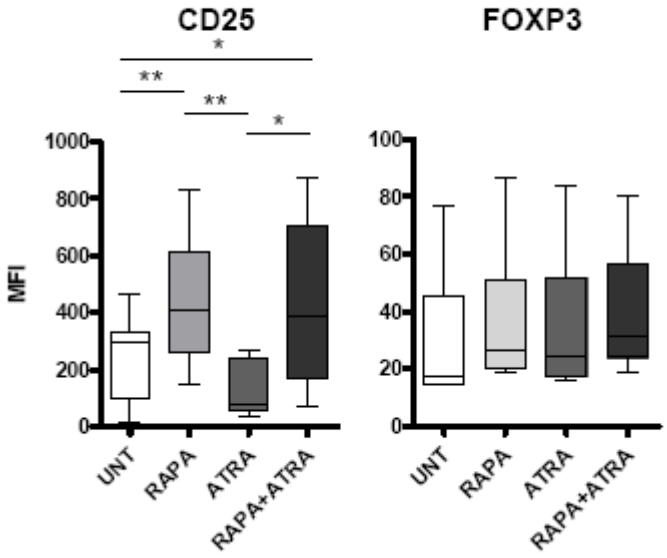


Fig. 1. CD25 and FOXP3 expression of untreated, RAPA-, ATRA- and RAPA+ATRA-treated human Treg cells. (A) Expression of indicate molecules was determined in both fresh cells, untreated or drug-treated Treg cell lines. The cells were stimulated with anti-CD3/CD28 beads and IL-2 (1000 IU/mL) for 28 days in the presence or absence of drugs (RAPA 100nM and/or ATRA, 2μM) and analyzed by flow cytometry. Representative FACS dot plots from 7 independent experiments of FOXP3 and CD25 co-expression are shown. **(B)** CD25 and FOXP3 expression calculated as mean fluorescent intensity in Treg cell preparations. Asteriks indicate a significant difference compared to untreated cells (UNT) and

between different Treg drug treatment *p<0.05, **p<0.01.

3.4.2. Human Treg cells are expanded in the presence of RAPA and/or ATRA.

As human Treg cells represent only 5-10 % of the circulating $CD4^+$ T cells, their potential use as a therapy is limited. In order to reach therapeutic cell numbers, isolated Treg cells need to be expanded. Thus the effects and mechanisms of action of RAPA, ATRA and the combination of both drugs on the expansion rate of human bead-enriched Tregs, were analyzed and compared. These cells were cultured with anti-CD3/CD28 and IL-2 (1000 IU/mL), in the absence (untreated) or presence of RAPA (100nM), ATRA (2μM) or both (RAPA+ATRA). Treg cells were harvested weekly for up to 28 days and the total cell number measured. Analysis of the population doublings **(Figure 2A)**, demonstrated that cell expansion reached a peak between the second and third re-stimulation. The same analysis showed that the mean expansion after 28 days of culture for 10 Treg lines was comparable between different culture conditions although some variability was observed between Treg preparations derived from different donors **(Figure 2B)** This result suggests that RAPA, ATRA and the combination of both drugs can promote *ex vivo* human Treg proliferation.

Figure 2.

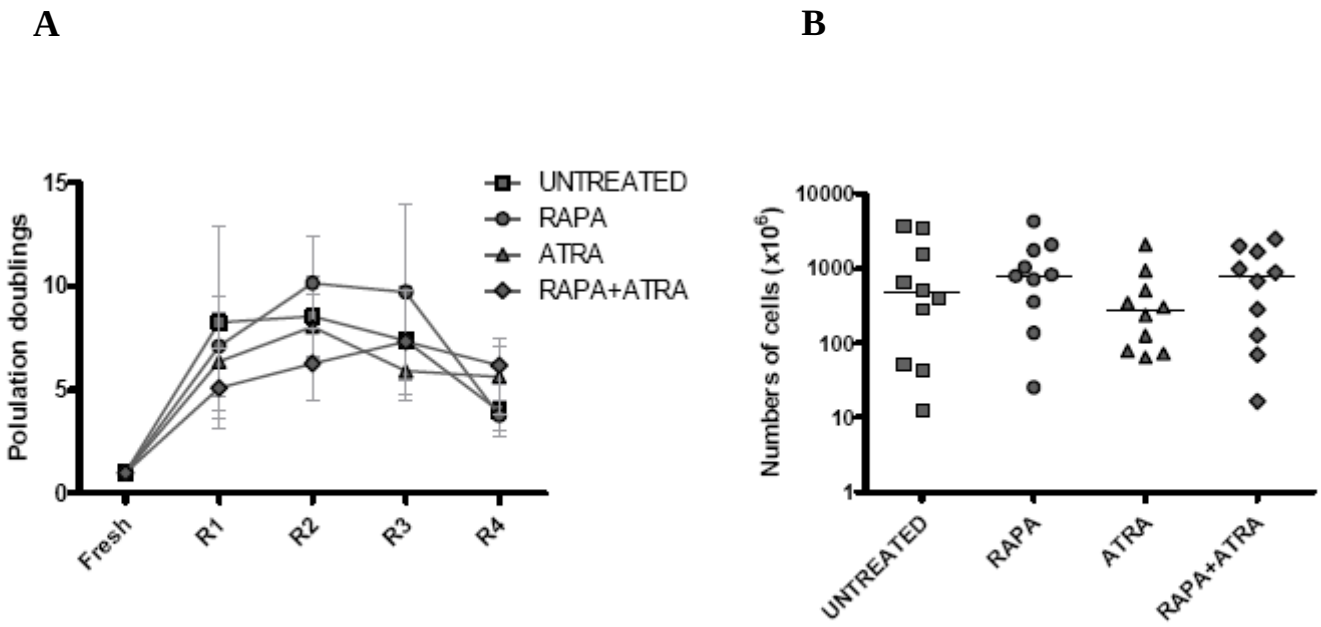


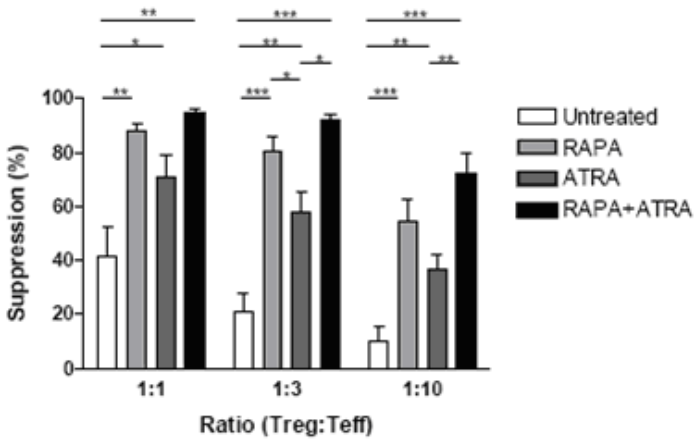
Fig. 2. Expansion of RAPA-, ATRA- and RAPA+ATRA-treated Treg cells *in vitro*. (A) Treg cell lines were stimulated with anti-CD3/CD28 beads and IL-2 (1000 IU/mL) in the presence or absence of drugs Rapa (100nM) and/or ATRA (2μM) and cultured for 28 days. Cell counts were performed at weekly intervals (R) throughout culture period. The *in vitro* expansion of 10 different Treg cell lines was calculated as populations doubling. **(B)** Expansion rates of 10 different Treg cell lines cultured as described above. Mid lines indicate median expansion.

3.4.3. ATRA and RAPA treatments affect Treg suppression function *in vitro*

To compare the regulatory function of each Treg cell line preparation, responder $CD4^+CD25^-$ T cells were stained with CFSE as described in Material and Methods and stimulated with anti-CD3/CD28 beads in the presence or absence of expanded Treg lines (after 4 re-stimulations, d28) at varying ratios. After 5 days of co-culture, the ability of the different Treg cell lines to suppress a polyclonal stimulus was determined by CFSE dilution by flow cytometry. As depicted in **Figure 3A**, RAPA- and RAPA+ATRA-treated Treg cells had the greatest suppressive activity on Teff proliferation, followed by ATRA-conditioned Tregs. In comparison, Treg cells expanded in the absence of ATRA and RAPA had a significantly reduced suppressive ability. This evidence suggests that Treg treatment with RAPA, ATRA or the combination of both drugs affects positively their function *in vitro*. However, following adoptive transfer of *ex vivo* expanded Treg cells, it is possible that these cells may revert to an 'untreated' Treg phenotype in the prolonged absence of the conditioning drug used for expansion. To verify that our expanded Treg cells do not lose their ability to function *in vitro*, Treg suppressive function was re-evaluated following two additional weeks of culture in the absence of drugs. As shown in **Figure 3B** each Treg line tested uniformly maintained high suppressive activity following drug withdrawal from cell cultures. These data indicate that our Treg cell lines preparations "starved" of RAPA, ATRA, and RAPA plus ATRA are stable in their function suppressing effector T cell proliferation *in vitro*.

Figure 3.

A



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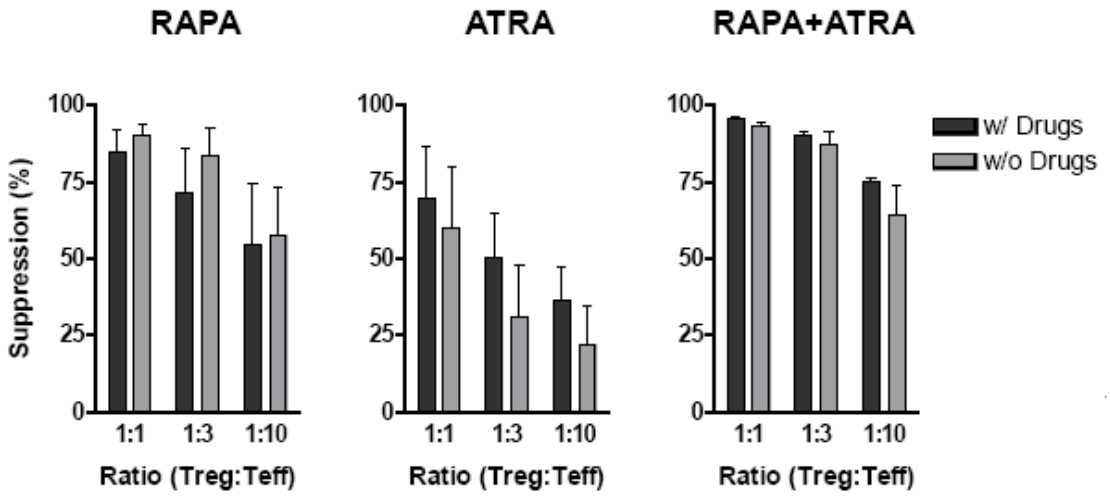

Fig. 3. Effect of RAPA, ATRA and RAPA+ATRA on Treg cell line suppressive ability *in vitro*. (A) Suppression of Teff cell proliferation by Treg cell lines cultured for 28 days in the presence or absence of drugs was determined by CFSE dilution. Treg cells were co-cultured with CFSE labeled Teff cells at the indicate ratios of Teff : Treg cells and stimulated with anti-CD3/anti-CD28 beads. Five days later, CFSE dilution was determined by flow cytometry. **(B)** Suppression of Teff cell proliferation by 28-day Treg cell lines that were either kept in the presence of drugs (w/) or "starved" of RAPA, ATRA and RAPA+ATRA (w/o) for a further 2 weeks. Bar graphs show mean ± s.d. of 9 independent experiments. Data are expressed as average percent of suppression of proliferation of Teff cells alone. Asterisks indicate significant differences in suppressive ability of Treg preparations compared to untreated cells and between different Treg drug treatments *$p<0.05$, **$p<0.01$, ***$p<0.001$

3.4.4. RAPA and ATRA treatments confer distinct phenotypic signatures on Tregs

Human Treg cells have been shown to be a heterogeneous population in the expression of cell surface markers. In order to investigate if RAPA, ATRA and the combination of both drugs were effective in promoting phenotypic signatures on these cells, the surface expression of several molecules expressed by Treg cells after 28 days of culture was evaluated. Flow cytometric analysis in **Figure 4A** shows that all Treg preparations maintained low levels of CD127 whose expression has been reported to be inversely correlated with human Treg suppressive function *in vitro*. Another surface marker that appears promising to identify Treg cells is CD62L. CD62L (L-selectin) is a member of the selectin adhesion molecule family and is required for lymphocyte homing to peripheral lymph nodes (Butcher and Picker, 1996). Transcriptional loss of CD62L is frequently used as a marker to distinguish naïve and antigen experienced T cells in the blood and lymphoid organs during immune responses (Bradley *et al*., 1991). The application of this paradigm to Treg cells is problematic as very little is known about antigen experience of these cells. However, recent data showed that Treg $CD62L^+$ cells possess superior regulatory activity *in vivo* (Ermann *et al*., 2005). Therefore, it has been analyzed whether the percentage of Treg cells expressing this molecule differed between our Treg preparations. It has been found a high percentage of Treg cells expressing CD62L in RAPA- and RAPA plus ATRA-treated Treg cells in comparison with untreated or ATRA-treated Treg cells. Interestingly in the same Treg culture conditions, RAPA- and RAPA plus ATRA-treated Treg cells, a high percentage of cells expressing CD27 was observed. This molecule is a member of the TNF-receptor superfamily and it has been shown to be correlated with regulatory activity in Treg cells expanded from $CD4^+CD25^+$ T cells (Duggleby *et al*., 2007). Like CD62, is CD27 is present on both naïve $CD4^+CD25^-$ T cells and Treg cells before activation. However this molecule is down-regulated in conventional T cells after activation

while it is maintained in a very suppressive Treg cell subset. Flow cytometric analysis in **Figure 4B** confirms previous data showing high percentages of Treg cells co-expressing CD62L and CD27 molecules in RAPA- and RAPA plus ATRA-treated Treg preparations. Lower expression of the same markers were expressed in both untreated and ATRA-treated cells. To notice, the co-expression of these two molecules by Treg cells has been associated with high suppressive ability both *in vitro* and *in vivo* (Issa *et al*., 2010; Koenen *et al*., 2008; Koenen *et al*., 2005; Nadig *et al*., 2010). Based on this evidence, these results suggest that either RAPA alone or RAPA in combination with ATRA could selectively induce the expression of markers involved in Treg activity.

Figure 4.

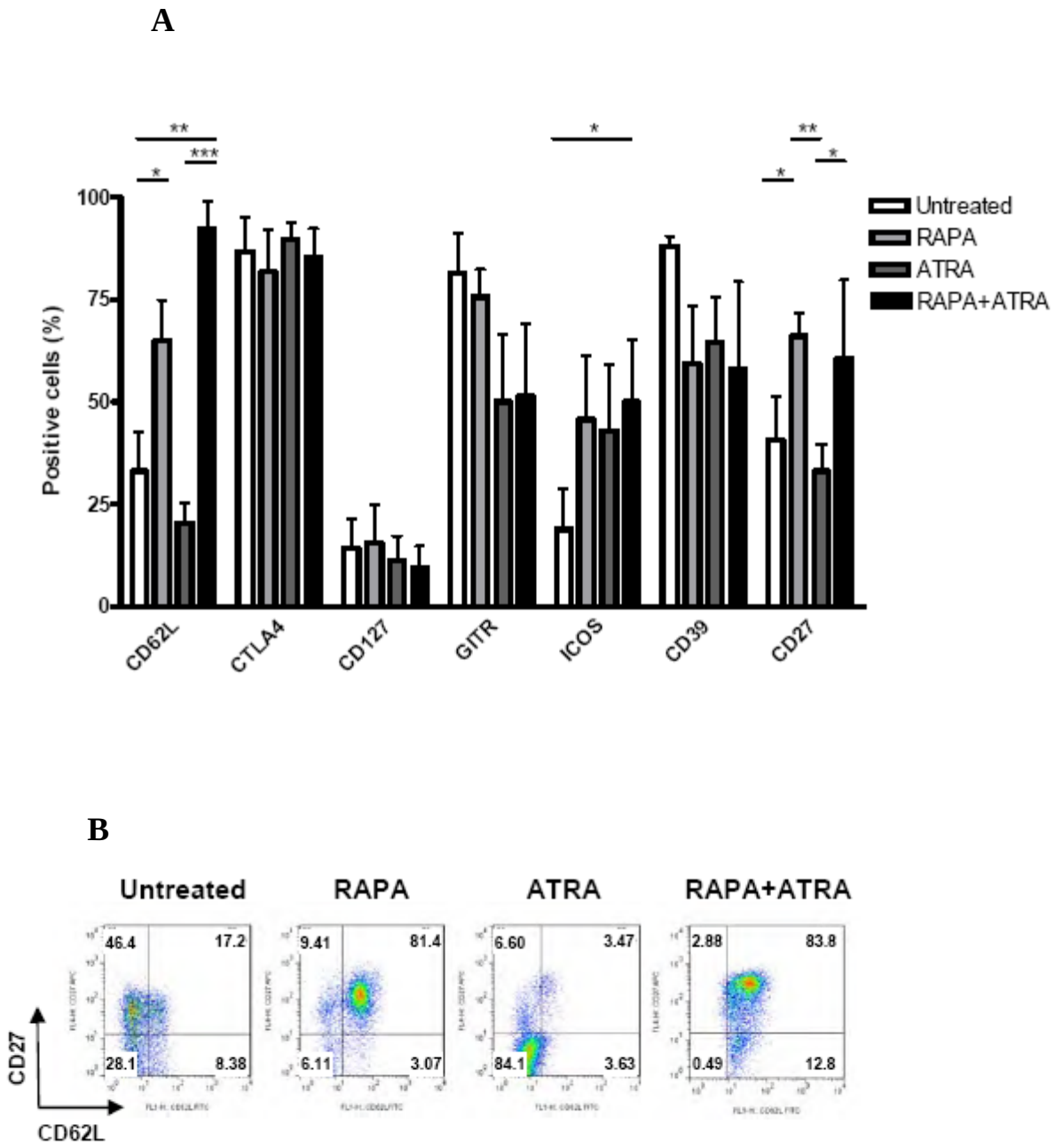

Fig. 4. Phenotypic analysis of untreated, RAPA-, ATRA- or RAPA+ATRA-treated Treg cells. (A) Percentage of expression of indicate molecules was calculated after 28 days of Treg cell culture. Bar graphs show mean ± s.d. of 7 independent experiments. Asterisks indicate significant differences compared to untreated cells and between different Treg drug treatment *p<0.05, **p<0.01, ***p<0.001. **(B)** Flow cytometry analysis of CD27 and CD62L co-expression on differently treated Treg cell lines is shown. Dot plots depicted are from 7 independent experiments

3.4.5. RAPA and ATRA augment the expression of homing receptors on Treg cells

Migration of Treg cells to secondary lymphoid organs is critical for limiting activation of immune cells in response to alloantigens and is important for hematopoiesis and prevention of GVHD after allogenic transplantation. Human Treg cells have been shown to express a set of homing receptors for their trafficking from the blood circulation to peripheral tissues and for their subsequent migration to draining lymph nodes (Kim, 2006). Therefore, It has been investigated whether the expression of different homing receptors of Treg cells could be affected by drug treatments. After 28 days of culture, CLA, CCR4, CCR9 and integrin $\alpha_4\beta_7$ expression in Treg preparations was analyzed by flow cytometry. The expression of CLA and CCR4 is important for skin homing while CCR9 and integrin α4β7 are related to the liver and gut homing trafficking. As shown in **Figure 5** a high percentage of Treg cells co-expressing CLA and CCR4 in RAPA treated Treg cells was observed, whereas ATRA-treatment resulted in high percentages of cells co-expressing liver and gut homing receptors CCR9 and integrin $\alpha_4\beta_7$. Finally, Treg cells treated with both drugs showed the combined homing characteristic features of Treg cells stimulated by each drug. These results highlight that the treatments with RAPA and ATRA could preferentially induce the expression of different homing receptors. As migration receptors on Treg cells are highly informative in assessing Treg behavior *in vivo*, this evidence has important relevance for the use of these two drugs in the treatment of diseases with very well defined target organs.

Figure 5.

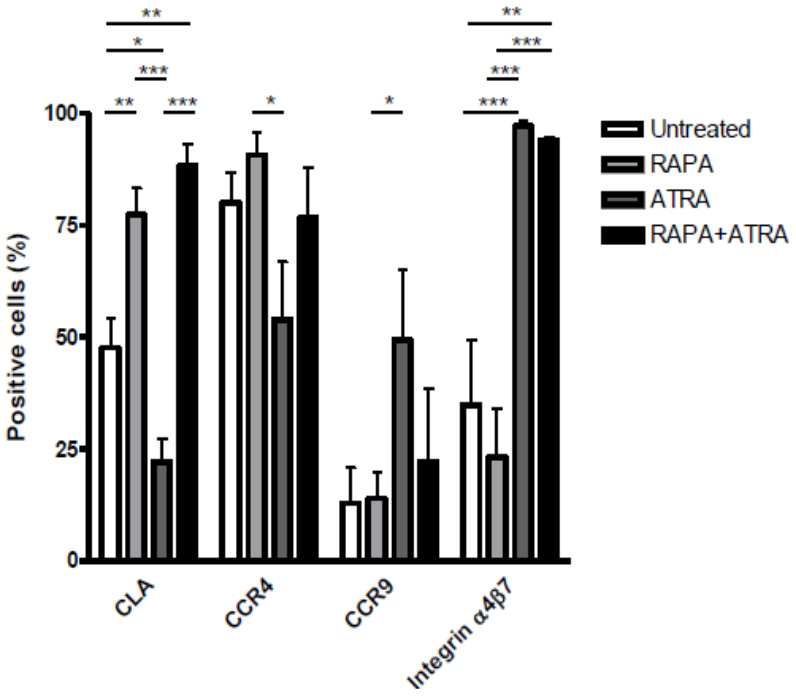

Fig. 5. Expression of homing receptors on Treg cell lines. Untreated, RAPA-, ATRA- or RAPA+ATRA-treated Treg cells at day 28 of culture were analyzed by flow cytometry for the expression of the reported molecules. Data are expressed as percentage of positive cells for each molecule. Bar graph depicts the mean ± s.d of 7 independent experiments. Asterisks indicate significant differences compared to untreated cells and between different Treg drug treatment *p<0.05, **p<0.01, ***p<0.001.

3.4.6. Treg cells expanded in the presence of RAPA, but not ATRA, do not produce inflammatory cytokines

Treg cells were initially believed to be highly specialized and fully committed to suppress effector T cells proliferation at all times. However, their lineage programs exhibit unexpected plasticity. They can lose FOXP3 expression with a resultant loss of immunosuppressive function and acquisition of a pro-inflammatory phenotype (Hori, 2010). Treg cells are potential immunotherapeutic tools and the capacity to produce pathogenic cytokines in an inflammatory environment is an undesirable option. In order to evaluate the stability of bead-purified Treg cells expanded in the different conditions described above, the production of two inflammatory cytokines, IL-17 and IFN-γ, following RAPA- and/or ATRA-treatment was measured. Supernatants from 28 day cell culture were analysed for the presence of IL-17 and IFN-γ by ELISA. Data showed that ATRA-treated Treg cells produced levels of IL-17 and IFN-γ comparable to untreated Treg cells **(Figure 6A)**. In contrast, Treg cultures expanded with RAPA or RAPA plus ATRA showed a strong decrease of these cytokines. This result suggests that the presence of RAPA may help to preserve the stability of *ex vivo* expanded Treg cells in order to assure safety when they are re-stimulated with alloantigen even in an inflammatory milieu. To investigate this possibility, we measured IL-17 production from our Treg preparations cultured in the presence of pro-inflammatory cytokines that favour the skewing to inflammatory Th17 lineage. In this regard, two different cytokine cocktails, cocktail A (IL-1β, IL-2, IL-6 and TGF- β) and cocktail B (IL-2, IL-21, IL-23 and TGF- β), reported to induce Th17 conversion, have been used. The analysis of supernatants after 7 days of culture in the absence of treatments showed significant increase of IL-17 production in supernatants of freshly isolated Treg cells, untreated and ATRA-treated Treg cells in the presence of both cocktails **(Figure 6B)**. On the contary, no IL-17 secretion in RAPA– and RAPA plus ATRA-treated Treg supernatants in response to the cocktails was detected (Figure

6B, bottom left and bottom right), ATRA-treatment in combination with pro-inflammatory cytokines and in particular of cocktail B, significantly increased the production of IL-17 by Treg cells (Figure 6B, bottom middle). This result confirms the previous findings empathizing the role of RAPA or the combination RAPA plus ATRA to ensure *in vitro* the stability of Treg cells even in an inflammatory context. These data also confirm that ATRA-treatment fails to protect Treg cells against Th17 conversion favoring IL-17 production by Treg cell preparations.

Figure 6.

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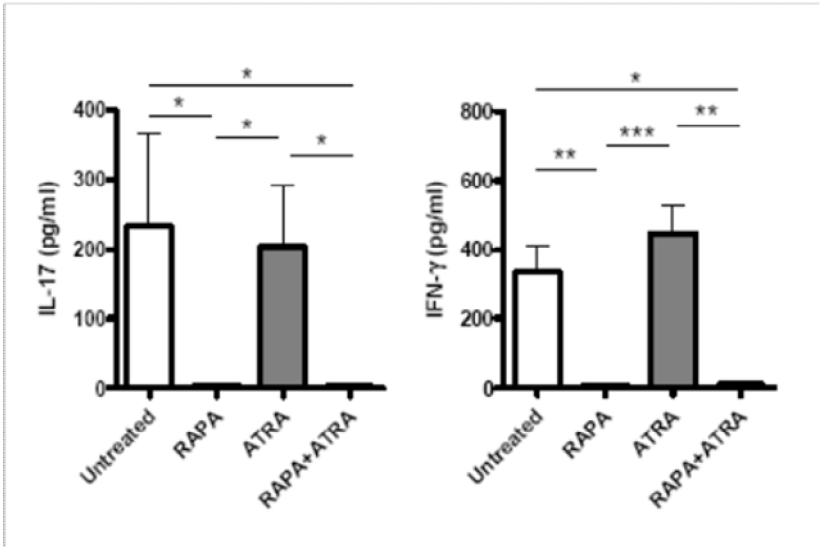

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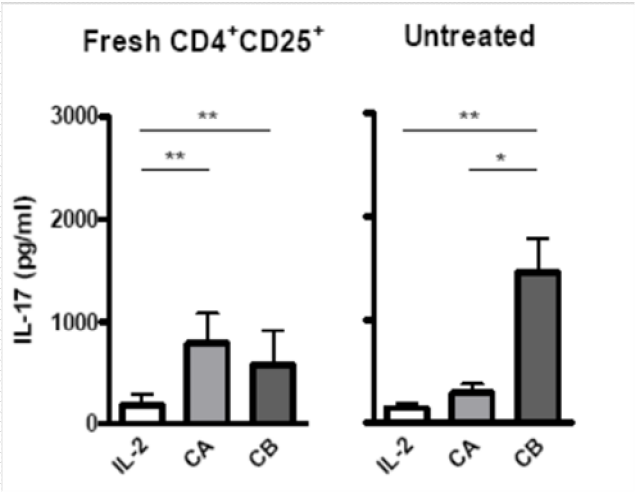


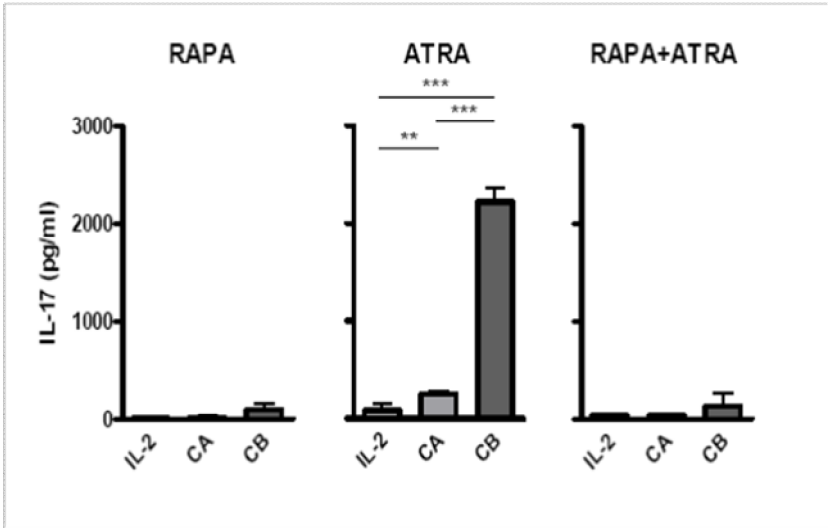


Fig. 6. Effect of RAPA, ATRA or RAPA+ATRA on cytokine production by Treg cell lines. **(A)** Treg cells were stimulated with anti-CD3/CD28 beads and IL-2 (1000 IU/mL) in the presence or absence of drugs and cultured for 28 days. Supernatants of Treg preparations were collected after 7 days of culture in the absence of drugs.IL-17 and IFN-γ production was measured by ELISA. Bar graphs show the cumulative mean ± s.d. from 5 independent experiments. **(B)** Treg stability in the presence of pro-inflammatory milieu was valuated measuring IL-17 concentration in fresh $CD4^+CD25^+$ T cell and Treg cell culture supernatants. IL-17 concentration was measured after 1 week of culture in the absence of drugs and in the presence of only IL-2 (10 IU/ml) or 2 different cytokines cocktails. Cocktail A (CA): IL-2, IL-1β, IL-6, TGF- β. Cocktail B (CB): IL-2, IL-21, IL-23, TGF- β. Graphs show pooled mean ± s.d. from 3 independent experiments Asterisks indicate significant differences in cytokine production compared to untreated cells and between different Treg drug treatment. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.4.7. ATRA-treatment promotes $FOXP3^+CD161^+$ Treg expansion *in vitro*

To better characterize the nature of IL-17-producing Treg cells, the expression of CD161 was analyzed. This molecule has been found to be correlated with the ability of T cells to produce IL-17, and it has been described as a marker for Th17 cells (Cosmi *et al.*, 2008; Maggi *et al.*, 2010). CD161 interacts with CLEC2A, a molecule that belongs to the C-type lectin domain family 2 (CLEC) (Rosen *et al.*, 2005). CLEC2A is selectively expressed in the skin where Th17 cells migrate in the course of chronic inflammatory disorders (Spreu *et al.*, 2007). Thus, CD161 expression could allow the trans-endothelial migration of Th17 cells into

tissues. Treg cell lines were cultured as previously described in the presence of absence of RAPA (100 nM), RAPA plus ATRA (100 nM and 2 μM respectively) and different concentrations of ATRA alone. As illustrated in **Figure 7**, the percentage of CD161-expressing Treg cells cultured with increasing concentrations of ATRA was higher in comparison with untreated Treg cells. On the contrary, in RAPA-treated Treg cells the proliferation of this subset was significantly suppressed. The difference in CD161 expression between RAPA and ATRA-treated Treg cells, reached statistical significance when ATRA was used at the highest concentration (2μM). Surprising, in the presence of both drugs, the percentage of $CD161^+$ Tregs was similar to that found in cultures treated with ATRA alone. Thus, the combination of ATRA and RAPA in Treg preparations showed that the addition of both ATRA and RAPA has an additive effect inhibiting IL-17 production and favouring CD161 expression. The latter evidence also suggests that IL-17 release and CD161 expression are controlled by two molecular pathways differently affected by RAPA.

Figure 7.

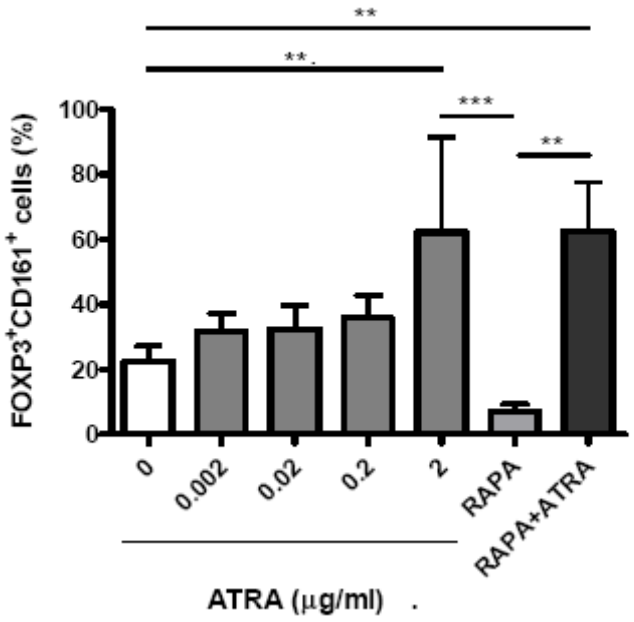


Fig. 7. Percentage of CD161-expressing Treg cells cultured with increasing concentrations of ATRA. The percentage of expression CD161 molecule was valuated on Treg cell preparations by flow analysis. Treg cell lines were cultured with RAPA (100 nM) RAPA+ATRA (100 nM and 2 µM respectively) or with different concentrations of ATRA. Bar graph shows cumulative mean ± s.d. from 3 independent experiments. Asterisks indicate significant differences in CD161 expression compared to untreated cells and between different Treg drug treatments. **p<0.01, ***p<0.001

3.4.8. RAPA-treatment favors selectively naive Treg subset expansion *in vitro*

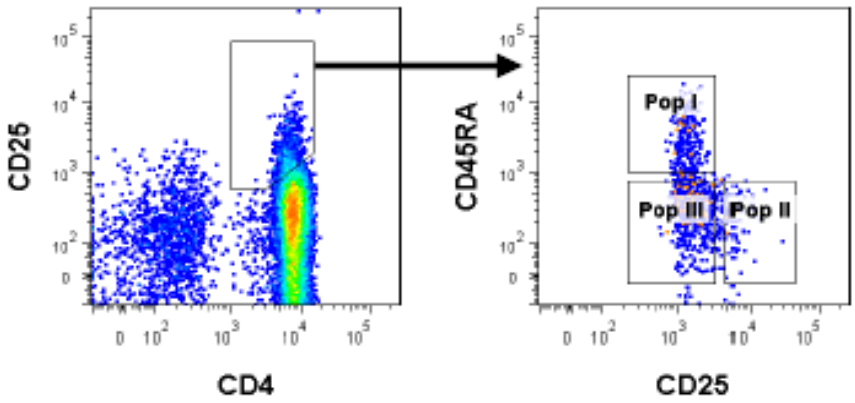
All the data presented so far have been obtained with bead-separated Treg cells expanded under different conditions. Human $FOXP3^+$ Treg cells are phenotypically and functionally heterogeneous including suppressive and non-suppressive T cells. The subpopulations described by Miyara et al, resting $FOXP3^{low}$ $CD45RA^+$ Treg cells (P1, nTreg cells), effector or activated $FOXP3^{hi}$ $CD45RA^-$ Treg cells (P2, eTreg cells) and cytokine-

secreting FOXP3^{low}CD45RA^{-} non suppressive Treg cells (P3, non Treg cells), have been shown to behave differently *in vitro* and *in vivo* upon activation (Miyara *et al*., 2009). Based on this evidence, the effect of RAPA, ATRA or the combination of both drugs on proliferation of these subpopulations was tested. CD4^{+} T cells were enriched from human PBMC as described in Materials and Methods and then sorted according their levels of CD25 and CD45RA **(Figure 8A)**. Treg subset proliferation was monitored for 28 days of culture in the presence or absence of drugs. P2 did not expand at all within any of the *in vitro* culture conditions (data not shown), while P1 and P3 proliferated in the presence of the different drug treatments **(Figure 8B-C).** It has been observed also that during the first week of culture P3 subset grew more slowly both in the presence and in absence of dugs than P1 subset expanded under the same drug treatments **(Figure 8C)**. After the first re-stimulation (R1) P1 and P2 Treg proliferation was comparable in all the culture conditions and reached a peak around the third re-stimulation (R3). The graphs suggest also that while ATRA-treatment tended to promote P3 expansion without affecting P1 subset, RAPA seemed to favor P1 proliferation.

To better investigate the influence of the drugs on the two Treg subsets, P1 and P3 population doubling after the first re-stimulation (R1) was evaluated. As depicted in **Figure 8D**, cell-sorted Treg subsets showed expansion rates comparable to the "whole" Treg cells described above in the section 3.4.2. Interestingly, this result confirms the suspicion showing that while P1 Treg expansion was affected by the presence of RAPA, ATRA alone enhanced only P3 subset proliferation. In culture containing both the drugs, the proliferative effect of ATRA on P3 subset was overcome by RAPA resulting in a selective P1 Treg expansion. As P1 Treg cells have been described as the most stable of the subpopulations, this evidence suggests that the effect mediated by RAPA to enhance the expansion of a specific Treg subset rather than whole FOXP3^{+} T cells, may be a promising strategy for the management of various immunological diseases.

Figure 8.

A



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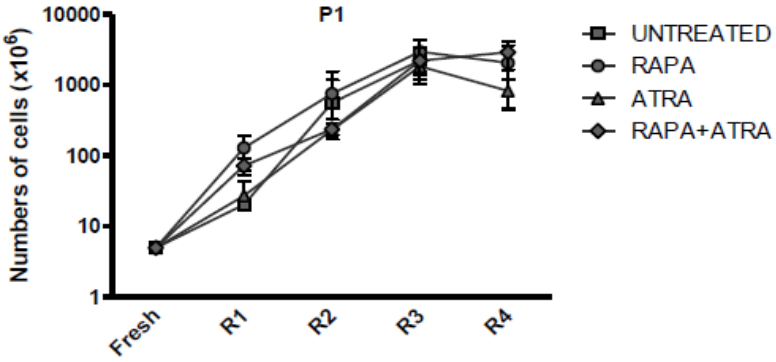

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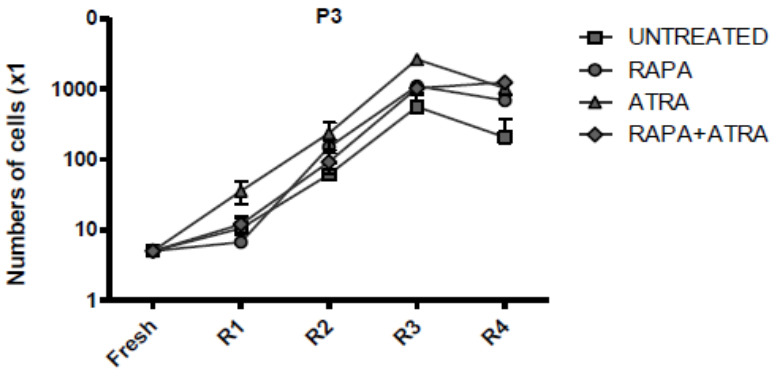

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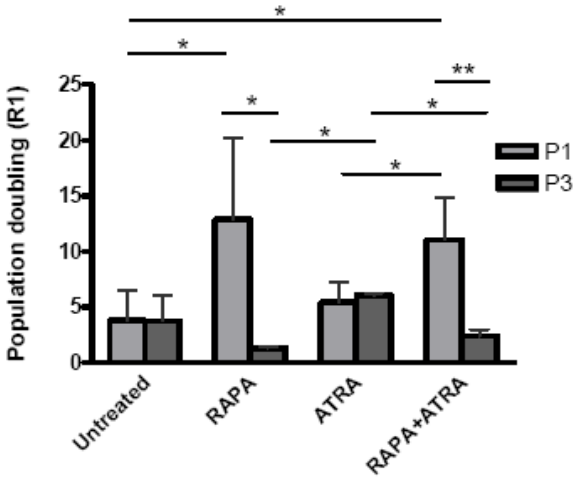

Fig. 8. Treg subsets expansion in the presence of different treatment. (A) Gating strategy for Treg subsets cell sorting is shown. $CD4^+$ T cells were isolated for human PBMCs prior to sorting on a BD FACSAria. **(B-C)** P1 (B) and P3 (C) cell count was performed at weekly intervals (R) throughout 4 rounds of stimulation. Cumulative data from 5 different P1 and P3 Treg subpopulations are shown. **(D)** Bar graphs depict the *in vitro* expansion of RAPA-, ATRA- or RAPA+ATRA- treated P1 and P3 Treg lines after the first round of stimulation (R1). Cumulative data from 5 different P1 (B) and P3 (C) Treg lines are expressed as population doubling. Asterisks indicate significant differences compared to untreated cells and between P1 and P3 Treg subseets. *$p<0.05$, **$p<0.01$.

3.4.9. Only RAPA protects Treg subsets from pro-inflammatory cytokines production

The next step was to understand whether the inhibitory effect of RAPA on pro-inflammatory cytokines production observed in "whole" RAPA- treated Treg culture, was due to a selective effect of the drug on P1 subset. To this aim, an ELISA was performed to measure IL-17 and IFN-γ production in P1 and P3 subpopulations following RAPA- and/or ATRA-treatment. Supernatants of P1 and P3 subset were collected after 4 rounds of stimulation and IL-17 and IFN-γ production was measured by ELISA. Cytokine analysis revealed that while P1 did not produce IL-17 in any of the culture conditions (**Figure 9A**), untreated and ATRA Treg cells derived from P3 subset produced large amounts of IL-17. The same analysis showed that the production of this cytokine was inhibited only by the addition of RAPA both in P1and P3 cultures. No significant differences among the other culture

conditions of P3 for IFN- γ production were observed. **(Figure 9B)**. Although not statistically significant, P1 Treg cells showed some IFN-γ production particularly following treatment with ATRA. These findings were further confirmed looking at IL-17 and IFN-γ production on a per cell basis by intracellular staining. Both subpopulations, cultured in the presence or in the absence of drugs, were stimulated and stained for intracellular IL-17 and IFN-γ cytokines as described in Material and Methods. A higher percentage of IFN-γ $^{-/}$ IL-17^{+} P3 Treg cells in ATRA preparations in comparison to the other culture conditions was observed **(Figure 9C).** On the contrary, no IL-17^{+} P1 Treg cells were found in the cultures containing ATRA although this drug increased the percentage of IFN-γ P1 Treg cells. However, both the percentage of P1 and P3 Treg cells expressing IL-17 and IFN-γ was reduced by RAPA as observed above with regard to the absolute concentrations of the two cytokines in ELISA. These results suggest that ATRA as single agent, in comparison to RAPA, is unable to promote the generation of a stable Treg subpopulation. Based on this evidence, only RAPA or the combination of both drugs could be a potential avenue to prevent pro-inflammatory cytokines production. Since the presence of ATRA alone favoured IL-17 production by "whole" Treg cells and in particular by P3 subset, in according to the same assumption previously described, CD161 expression in subset preparations was investigated,. As depicted in Fig 6D, the cytokine profile of both P1 and P3 correlated very well with the expression of this marker. In all the culture conditions, P3 subset contained the highest percentage of cells expressing CD161 molecule, which was decreased when P3 was cultured with RAPA. In contrast, P1 subpopulation showed a lower number of cells expressing CD161 and again RAPA was able to further decrease it. Together, these data confirms that P1 subset is more stable than P3 subset *in vitro* emphasizing the pivotal role of RAPA in this process.

Figure 9.

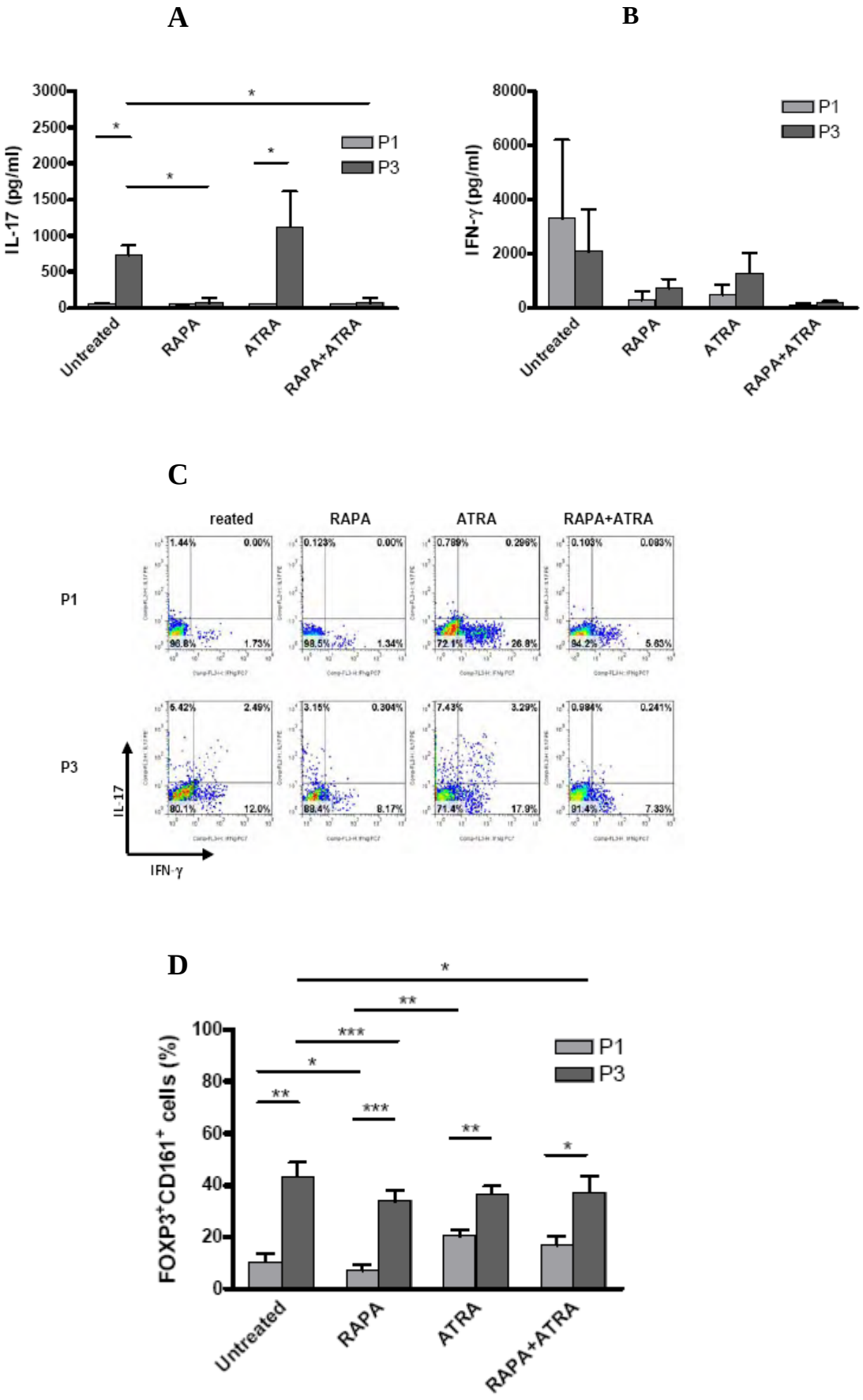
A
B
C
D
IL-17 (pg/ml)
IFN-γ (pg/ml)
P1
P3
Untreated
RAPA
ATRA
RAPA+ATRA
reated
RAPA
ATRA
RAPA+ATRA
IL-17
IFN-γ
FOXP3+CD161+ cells (%)

Fig. 9. Treg subsets stability and CD161 expression in the presence of different treatment. (A-B) Comparison of IL-17 (A) and IFN-□ (B) production by ELISA in 7 days supernatants of P1 and P3 Treg lines. **(C)** The percentage of IL-17^{+} and IFN-□ cells in CD25^{+}FOXP3^{+} P1 and 3 cultured with different treatments was measured by intracellular staining. **(D)** Bar graph shows a comparison of CD161 expression in P1 and P3 Treg lines. All data are from Treg cell lines after 4 rounds of stimulation. Dot Plots are representative of 5 independent experiments. Asterisks indicate significant differences compared to untreated cells and between P1 and P3 Treg subseets. *p<0.05, **p<0.01.

3.4.10. The more stable P1 phenotype results in a potent suppressive activity *in vitro*

Given that P1 Treg cells have been shown to be *in vitro* more suppressive than P3 subset (Miyara *et al*., 2009), the function of each Treg subset after drug-conditioning was tested. To this aim, a suppression assay as previously described for *in vitro* expanded "whole" Treg cells was performed. P1 consistently showed a tendency to have superior suppressive abilities independent of the culture treatment, compared to P3. Interestingly, statistically significant differences in the suppressive function of these subsets were observed when P1 cultures were treated with ATRA alone. **(Figure 10)**. Thus, this result confirms the inherent differences of suppressive activity between P1 and P3 subpopulations reported in the literature. Moreover, as in the presence of ATRA the function of P1 subset was significantly higher, one could suppose that this drug exerts a positive effect on expanded P1 Treg function.

Figure 10.

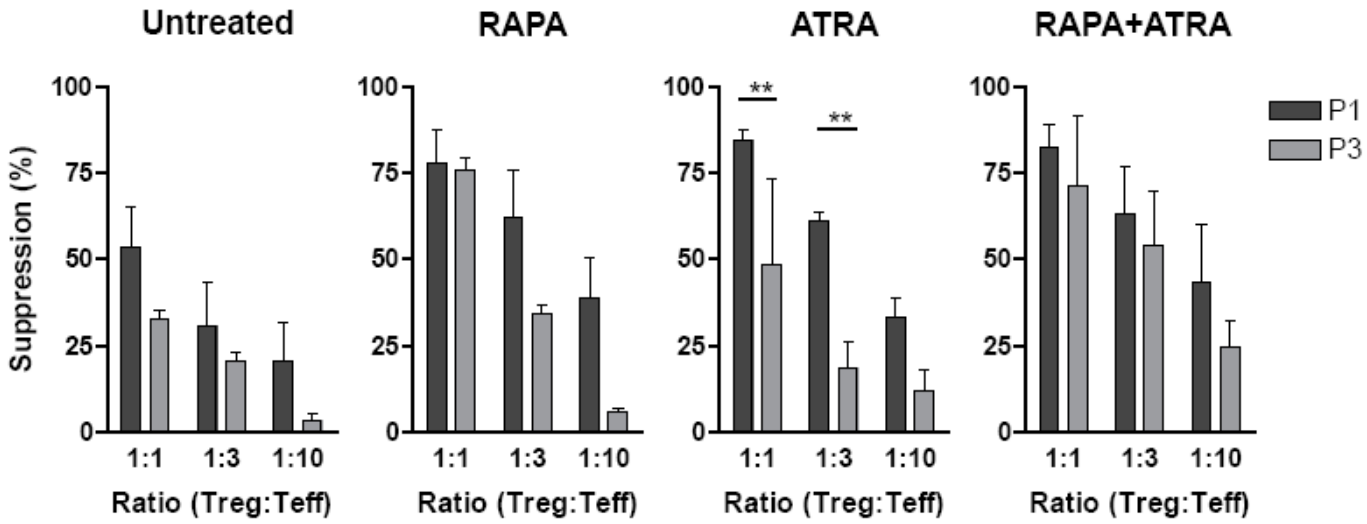

Fig. 10. Treg subsets suppressive abilities in the presence of different treatments. A comparison of P1 and P3 suppressive abilities at different ratios of Treg:Teff after 4 rounds of stimulation in the presence or absence of drugs. from 5 independent experiments Data from 5 independent experiments are expressed as average percent of suppression of proliferation of Teff cells alone. Statistical analysis shown only on significantly different data. **p<0.01.

3.5. Discussion

Of particular interest is the potential for the $CD4^+CD25^+FOXP3^+$ Treg cells to be used as a cellular therapy in the context of organ transplantation for the induction of tolerance (Lombardi *et al.*, 2011). However, the small number of $CD4^+CD25^+FOXP3^+$ Treg cells accessible in the peripheral circulation means that for cellular therapy is necessary to expand clinically sufficient numbers of Tregs *in vitro*. So, the second part of my PhD project was to optimize a Treg expansion protocol to maintain a pure, suppressive, and stable Treg population, with well defined Treg homing characteristics for *in vivo* Treg targeting to specific sites. So far much work has gone into developing *in vitro* methods to expand Treg cells. Methods employed to stimulate $CD4^+CD25^+FOXP3^+$ Treg cells include anti-CD3/anti-CD28 coated beads as well as cell-based artificial antigen presenting cells expressing co-stimulation molecules and/or Fc receptors. In addition to stimulus through TCR and CD28 co-stimulation, Treg cells require also exogenous IL-2. Adding Rapamycin to the culture has been shown to preserve Treg purity and allow selective Treg expansion. Indeed RAPA gives a proliferative advantage to Tregs by affecting basic signal pathways such as Akt/mTor, which is not essential for Tregs (Peter *et al.*, 2010). Notably, rapamycin- expanded Treg cells retain suppressive capacity *in vitro* and, when tested in a GVHD model, were more effective than natural Tregs expanded under conventional conditions. Other studies have shown that ATRA is also a suitable treatment for the induction of adaptive Tregs (Mucida *et al.*, 2009). Indeed, recently ATRA has been described as a treatment to expand human nTregs (Golovina *et al.*, 2011). The molecular pathway by which ATRA favors the expansion of Tregs is not completely clear, but it is thought to induce chromatin decondensation recruiting histone acetyltransferases and transcription machinery to the FOXP3 promoter (Kang *et al.*, 2007; McGrane *et al.*, 2007). However, due to its multi-faceted effects on gene transcription, ATRA has also been shown to affect T cell fate by contributing to Th1/Th17 differentiation (Hall *et*

al., 2011). In this thesis it has also identified significant differences between untreated, RAPA- and ATRA-treated Tregs in terms of homing receptor expression. Although others have previously reported that Tregs express multiple homing receptors (Hirahara *et al.*, 2006; Zhang *et al*., 2009), such as CD62L (Hoffmann *et al*., 2006; Baecher-Allan *et al*., 2001, Iellem *et al*., 2003), CCR4 (Hirahara et al., 2006; Iellem *et al*., 2003, Lee *et al*., 2005), CLA (Hirahara *et al*., 2006; Iellem *et al*., 2003, Booth *et al*., 2010) and CCR7 (Iellem *et al*., 2003, Hoffmann *et al*., 2004), this is the first study in which the expression of homing receptors was evaluated on human Tregs during *ex vivo* expansion with drug conditioning. The migration of these Treg preparations *in vivo* is now under investigation. The major concerns in Treg therapy is to prevent the induction and expansion of IL-17-producing cells, which could arise from Teffs contaminating the Treg preparations (Cosmi *et al*., 2010), and/or $FOXP3^+$ Tregs converting to IL-17-producing cells (Koenen *et al*, 2008). The reported data clearly show that RAPA has an inhibitory effect on the development of IL-17-producing cells, both *in vitro* and *in vivo*. This is consistent with previous descriptions in the literature of the inhibitory effect of RAPA on Th17 cells (Tresoldi *et al*., 2011). In contrast, the data of this thesis show that ATRA maintained the expansion of IL-17 and IFN-γ producing $FOXP3^+$ Tregs. The effect of ATRA on Th17 cells has been controversial. Some *in vitro* studies have shown that retinoic acid induces pro-inflammatory T cell responses while others demonstrated that ATRA strongly inhibits Th17 polarization *in vitro* (Mucida *et al*., 2007; Elias *et al*., 2008; Hippen *et al*., 2011). Furthermore Hall and collaborators have demonstrated *in vivo* that in mice fed with a vitamin A deficient diet, robust Th17 and Th1 responses are generated (Hall *et al*., 2011). The general conclusion is that the effect of retinoic acid is very much dependent upon its concentration and the inflammatory environment in which the immune response takes place (Wang *et al*., 2009; Hall *et al*., 2011; Uematsu *et al*., 2008). This study has demonstrated that ATRA expanded Treg highly suppressive phenotype, but at the same time did not prevent IL-

17 secretion. Notably, isolation of $CD25^{+}CD45RA^{+}$ (naïve) T cells (P1) yields Treg cells with a grater suppressive capacity than total $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells. The reason of this became clear when Miyara et al. examined subpopulation of human $FOXP3^{+}$ T cells and discovered that the $CD25^{+}CD45RA^{-}FOXP3^{low}$ T (P3) population is not suppressive *in vitro*, contains IFN-γ and IL-2 producing cells, and also could contain Th17 precursors (Miyara *et al*., 2009). After three weeks of in *vitro* expansion, $CD127^{-}$ Treg cells became methylated at the TSDR while $CD45RA^{+}$ expanded Treg cells remained de- methylated. Moreover, the $CD127^{-}$ Treg cells that lost FOXP3 expression were $CD45RA^{-}$ suggesting that naïve Treg cells may represent the most stable population for expansion. So this result obtained with drug conditioning of "whole" Tregs was tested on Treg cell subsets sorted and cultured with RAPA and/or ATRA. It has demonstrated that the presence of RAPA provided an initial proliferative advantage to P1, while the same drug had an inhibitory effect on the P3 subset. In contrast, ATRA conditioning favored expansion of cells in P3. This finding leads to the hypothesis that when the two drugs were used with "whole" Tregs, RAPA expands P1 to the detriment of P3, while ATRA allows the expansion of cells within the P3 that retain IL-17 secretion capacity. It is noteworthy that analysis of CD161 in general confirms this hypothesis by showing a higher percentage of Tregs expressing this marker in P3. However when P3 is cultured with the combination of RAPA and ATRA, the same percentage of Tregs expressed CD161 while IL-17 production was reduced, suggesting that these two parameters do not necessarily correlate. In addition it is also not clear why the percentage of Tregs expressing CD161 molecule increased when "whole" Treg are treated with ATRA but not when the single subpopulations are treated with the same drug. This result may be explained by the influence of the two subpopulations on each other when "whole" Tregs are treated with ATRA and needs further investigation. In conclusion, this study has identified a clinically applicable protocol to obtain and expand human immunomagnetic bead-separated $CD4^{+}CD25^{+}$ Tregs. These additional

data performed *in vitro* describe how the treatment with RAPA and ATRA can affect Treg phenotype, function and plasticity during cell expansion. Moreover it has been demonstrated directly for the first time that one of the ways that RAPA stabilizes Tregs is by selecting and expanding the subpopulation of functional, no plastic naive Tregs (Miyara *et al.*, 2009; Hoffmann *et al.*, 2006). In contrast, the effect due to ATRA can be explained by the fact that this drug favors the expansion of P3 that contains cells that retain the capacity to produce IL-17. Although these findings further emphasize that Treg therapy with bead-separated Tregs requires RAPA for the expansion of functional and stable Tregs, the selection of additional drug, such as ATRA, can be based on the type of organ or tissue targeted. So, the Treg expansion protocol here reported suggests that the combination of ATRA and RAPA may be an ideal culture condition to expand Tregs for their use in the induction of tolerance.

4. General Conclusion

The *sine qua non* for Treg stability and function is the sustained expression of the transcription factor FOXP3. Here FOXP3 plays a key role in tolerance by mediating the ability of Treg cells to suppress immune responses including controlling autoreactive T cells circulating in the periphery and limiting responses to the infections. Unfortunately, we do not know yet how exactly Treg cells suppress immune responses, though several mechanisms have been proposed, and we understand even less about how FOXP3 might orchestrate these mechanisms. So, a better knowledge of FOXP3 activity in Treg cells would be extremely useful either to boost or inhibit their function for maintaining immune homeostasis. Of particular interest is the potential for $CD4^+CD25^+$ $FOXP3^+$ Treg cells to be used as cellular therapy in preventing graft rejection and inducing tolerance. Compelling evidence from animal models demonstrated the efficacy of Treg therapy proividing a strong framework for testing Treg therapy in humans. However, the low frequency of Treg cells accessible in the peripheral circulation led to the need to expand ex *vivo* these cells. Therefore, the two researchs presented here aimed both to investigate the molecular mechanisms by which FOXP3 activates gene transcription and to optimize a protocol that can reliably produce large numbers of clinical grade, highly pure and stable suppressive $CD4^+CD25^+$ $FOXP3^+$ Treg cells. Together these studies contribute to the large body of literature that demonstrates the importance of FOXP3 in immune cell function and the prospect of using Treg cells as therapy in various transplant models. Although much work is still to be done in this regard, there is now ample evidence to support the translation of this approach to the clinical arena.

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