*International Reviews of Immunology*, 33:129–145, 2014

ISSN: 0883-0185 print / 1563-5244 online
DOI: 10.3109/08830185.2013.863303

informa
healthcare

ARTICLE

# *Forkhead box P3:* The Peacekeeper of the Immune System

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Ten years ago Forkhead box P3 (FOXP3) was discovered as master gene driving $CD4^+CD25^+$ T cell regulatory (Treg) function. Since then, several layers of complexity have emerged in the regulation of its expression and function, which is not only exerted in Treg cells. While the mechanisms leading to the highly selective expression of FOXP3 in thymus-derived Treg cells still remain to be elucidated, we review here the current knowledge on the role of FOXP3 in the development of Treg cells and the direct and indirect consequences of *FOXP3* mutations on multiple arms of the immune response. Finally, we summarize the newly acquired knowledge on the epigenetic regulation of *FOXP3*, still largely undefined in human cells.

**Keywords** autoimmunity, epigenetics, IPEX syndrome, regulatory T cells, tolerance

## INTRODUCTION

Primary immunodeficiency disorders (PIDs) are rare genetic diseases of the immune system primarily characterized by recurrent infections and often associated with malignancies and autoimmune manifestations [1]. This dichotomy of overreaction to self-antigens, while lacking a robust response against pathogens suggests that PID patients have a dysregulated immune system, resulting in a decreased ability to distinguish between self and foreign antigens. Autoimmune manifestations associated with reduced number and/or function of regulatory T (Treg) cells, key players in maintaining peripheral tolerance, have been demonstrated in several autoimmune diseases, such as Type 1 Diabetes (T1D) [2], and Systemic Lupus Erythematosus (SLE) [3]. In addition, dysfunction of Treg cells has been detected in several PIDs, such as Adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID) and Wiskott–Aldrich syndrome (WAS), and suggested as the cause of the autoimmune manifestations, further supporting the association of immunodeficiencies and immunedysregulation [4, 5].

The best example of PID with prevailing autoimmune manifestations is the Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome, due to

Accepted 4 November 2013.
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the loss of function of thymus-derived (t) Treg cells [6], the key cell subset that controls immune responses to pathogens and prevents immune responses against inappropriate targets, such as self-antigens or non-harmful antigens [7]. The disease is caused by mutations in the *FOXP3* gene [8], encoding for a T-cell specific transcription factor (TF) belonging to the large forkhead family of TFs. FOXP3 is considered a key player for the specification and function of tTreg cells [7]. Their dysfunction leads to life-threatening autoimmune manifestations in several organs in affected male children [6, 9].

In this review, we summarize the current knowledge on the function of FOXP3 in the regulation of Treg and T effector (Teff) cells, with special attention to the consequences of its dysfunction.



## FOXP3 EXPRESSION IN AND OUTSIDE THE IMMUNE SYSTEM

### Role of FOXP3 in Treg Cells

In the initial studies performed in mice, Foxp3 was reported to be exclusively expressed by $CD4^+CD25^+$ Treg cells and its expression was associated with the acquisition of suppressive properties typical of this T-cell subset [10]. Later studies showed that ectopic Foxp3 expression in Teff cells induced the majority of tTreg cell signature genes [11]. On the other hand, Foxp3 expression *per se* is not sufficient to activate the complete Treg transcriptional program [11, 12] and analysis of *foxp3gfp*ko mice demonstrated that most of the tTreg signature genes are maintained in the absence of Foxp3 expression [13]. These recent findings pointed out that Foxp3 expression is essential, but *per se* insufficient for the development of *bona fide* tTreg cells and paved the way for the identification of additional genes contributing to Treg lineage specification.

Studies in humans confirmed that FOXP3 is expressed by tTreg cells and crucially controls their function and maintenance [7], as also demonstrated by the observation that high and stable expression of FOXP3 in human Teff cells leads to the acquisition of Treg phenotype and functions [14, 15].

### FOXP3 Expression and Treg Cell Plasticity

There is general consensus on the concept that high and constant expression of FOXP3 is required for the stability of Treg cells [16]. Indeed, several evidences demonstrate that loss of FOXP3 leads to diminished suppressive capacity in both human and murine Treg cells [13, 17–20]. In addition to the loss of suppressive function recent studies suggested that loss of FOXP3 expression in Treg cells could switch them from a regulatory fate to the acquisition of pro-inflammatory properties. In murine models it has been proposed that Treg cells can acquire effector functions and become auto-reactive, although the latter phenomenon is still controversial [21]. In an *in vivo* study, thanks to the use of fate-mapping systems, Zhou and colleagues showed that murine "ex-Treg cells" that have lost Foxp3 expression switched to a Teff phenotype, which contribute to the onset of autoimmunity as a consequence of Foxp3 instability [22]. Loss of FOXP3 with acquisition of inflammatory cytokine production after repeated *in vitro* stimulation can also be observed in human Treg cells; several studies indicated that specific subsets of human Treg cells could lose regulatory activity and become effector memory T cells producing IL-17, under specific culture conditions [23–26], or could convert to a prevalent Th2 phenotype [27]. This new concept of Treg plasticity has been proposed as a physiological mechanism to allow efficient response to pathogens or to restrain detrimental immune regulation, but it also suggests a potential dual role, both regulatory and pro-inflammatory, for these cells in a variety of disorders, including autoimmunity, cancer, and infections (reviewed in [21]).

However, other *in vivo* works suggested that tTreg cells are functionally and phenotypically stable after many cell divisions in inflammatory sites [28, 29], and differences in the *in vivo* outcome (Treg stability vs generation of ex-Treg cells) has been attributed to limitations of the fate mapping systems or of the experimental conditions [30].

More recently, the attention has been shifted away from this controversy by the emerging concept that tTreg lineage commitment is regulated by a complex network of cooperative and counteractive TFs [31]. Treg function can be fine tuned through adoption of inflammatory T helper transcription factor networks, which lead to the generation of Th-like Tregs in both mice and humans [32]. This issue is further discussed in the review by Vent-Schmidt et al.

### Role of FOXP3 in T Effector Cells

In murine T cells Foxp3 expression was originally described as restricted to $CD4^+CD25^+$ Treg cells [10]. More recently, Miyao et al. reported unstable expression of Foxp3 in activated murine Teff cells both *in vitro* and *in vivo*, regardless of the acquisition of classical Treg-related functions [29]. The physiological meaning of heterogeneous Foxp3 expression in murine conventional T cells has not yet been clarified. On the other hand, it has been known for a long time that in humans FOXP3 expression is not restricted to $CD4^+CD25^+$ Treg cells, but it can also be transiently induced in activated $CD4^+CD25^-$ Teff cells, which do not express FOXP3 in the resting state [33, 34]. While the role of *FOXP3* as key regulator of human Treg cells has been widely accepted, its function in $CD4^+CD25^-$ Teff cells remains poorly defined. Also in humans, activation-induced expression of FOXP3 in $CD4^+CD25^-$ Teff cells is transient and does not necessarily result in the acquisition of suppressive properties [33, 34], although it has been associated with suppression of the cytokine production and proliferation of multiple $CD4^+$ T cell lineages [33, 35]. Indeed, FOXP3-deficient Teff cells proliferate more, produce more cytokines than wild type Teff cells, and activation-induced FOXP3 expression is sustained in Th17 cells, suggesting that FOXP3 contributes to the developmental program of human Th17 cells [35].

### Role of FOXP3 in Somatic Cells

Although FOXP3 protein expression was originally reported to be restricted to haematological tissues and in particular to $CD3^+$ T lymphocytes, it has been recently described that FOXP3 expression can occur also in non-immune cells, such as epithelial cells, where it functions as a tumor suppressor and is involved in metastatic spread [36, 37]. The original observation that female mice heterozygous for the mutation of the *foxp3* gene ($foxp3^{sf/+}$) developed cancer (i.e. mammary carcinoma) at higher frequency than wild type mice, led to the recognition of FOXP3 as an X-linked breast cancer suppressor, via repression of the oncogenes *ERBB2/HER2* and *SKP2* [37, 38] both in mice and humans. Subsequent studies demonstrated FOXP3 expression is not specific for breast cancer, where it correlates with metastasis formation [36], but is also expressed by a wide variety of tumor cells, including melanoma [39] and prostate cancer cells [40], confirming the relevant function of *FOXP3* as tumor suppressor gene. However, despite increasing evidence for a role in suppressing transformation of epithelial cells, the physiological functions of FOXP3 outside the Treg cell compartment are still poorly defined.

## IMMUNE DYSREGULATION, POLYENDOCRINOPATHY, ENTEROPATHY, X-LINKED (IPEX) SYNDROME

Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) is a rare autoimmune disease characterized by early-onset systemic autoimmunity.

The distinctive features of the disease comprise: (i) life-threatening enteropathy with refractory diarrhea, usually associated with villous atrophy, (ii) early-onset T1D, and (iii) dermatitis frequently associated with elevated IgE serum levels. Additional autoimmune manifestations comprise autoimmune endocrinopathies, i.e. thyroiditis, cytopenias, including haemolytic anemia, thrombocytopenia, and neutropenia, hepatitis with positive auto-antibodies, and renal disease, either of autoimmune origin or related to the administration of immunosuppressive drugs. As a consequence of lymphoproliferation, splenomegaly and lymphadenopathy can be associated with multi-organ autoimmunity. The clinical condition can worsen with infections, although these latter can often be the consequence of immunosuppressive (IS) therapy, rather than the direct consequence of the mutation itself. In affected males, symptoms typically develop early in infancy, leading to failure to thrive and to premature death, if patients are not promptly treated [6, 9]. The disease is rare, with less than 150 cases described worldwide in the last 10 years [9].

Besides the typical early-onset severe clinical spectrum, both milder forms of the disease and IPEX-like syndromes have been described [6, 41]. The former are characterized either by delayed onset, or better response to IS therapy and milder symptoms, whereas the latter include male and female patients presenting with autoimmune symptoms phenotypically resembling IPEX in the absence of detectable *FOXP3* mutations [6, 41]. Except for rare patients with mutations in *CD25* [42] or *STAT5b* [43], in most of the cases the cause of the IPEX-like syndromes remains unknown. We have recently reported that in at least a subset of these patients autoimmunity may be caused by a quantitative defect of tTreg cells [44], thus indicating Treg cells as the key contributors to disease development in both IPEX and IPEX-like syndromes.

At present, the standard treatment of IPEX syndrome is limited to supportive and replacement therapies associated with the use of multiple IS drugs, with inefficient control of autoimmunity in most of the patients. Among IS drugs, rapamycin is of particular interest since it selectively targets Teff cells and spares Treg cells, which are insensitive to mTOR inhibitors [45]. So far, positive clinical results have been reported in four IPEX patients, with clinical remission in mid-long term follow-up [6, 46, 47]. Although it has yet to be clarified whether rapamycin has the same effect in Treg cells from healthy donors and IPEX patients, the use of such an alternative to calcineurin inhibitors seems to be promising.

Haematopoietic stem cell transplantation (HSCT), is the only curative treatment currently available [6], and the experience in transplanted IPEX patients (recently reviewed in [9]) has taught that the earliest HSCT can be performed, i.e. the earliest autoimmune attack and drug-related damages are prevented, the best the outcome. This observation highlights the critical importance of early diagnosis to guarantee prompt intervention.

Both myeloablative and non-myeloablative conditioning regimens have been used (reviewed in [9]). The non-myeloablative regimens may allow to reduce both post-transplant infectious complications and the toxicity of high dose chemotherapy, to which IPEX patients are very susceptible due to their generally poor clinical conditions.

On the other hand, a non-myeloablative conditioning may increase the risk of a partial chimerism, although we now know that full donor engraftment is not necessary for complete recovery, but rather the engraftment of donor Treg cells is essential to cure the disease [48]. Based on the latter observation, cell/gene therapy approaches designed to selectively restore the Treg cell compartment are currently under investigation by the group of R. Bacchetta at the San Raffaele Scientific Institute of Milan, Italy, as an alternative therapeutic strategy when a suitable donor for HSCT is not available (Passerini et al., manuscript in preparation).

## *FOXP3* MUTATIONS AND IMMUNE DYSFUNCTIONS

### Treg Cell Dysfunction

Mutations of the *FOXP3* gene cause severe autoimmune disease in both humans and mice [8, 49]. As expected based on the pattern of FOXP3 expression in the T cell compartment, the tTreg cell subset is mainly affected by *FOXP3* mutations. Dysruption of *FOXP3* in humans results in loss of suppressive function by Treg cells [41, 50, 51], which is considered the primary cause of disease.

However, it remains unclear how the reported mutations impact on the suppressive function. The domain-specific functional impact of different mutations on FOXP3 protein has been predicted [52] and a study of the model-crystal structure of the FOXP3 forkhead domain has demonstrated that specific IPEX-associated mutations affect formation of dimers without compromising FOXP3 DNA binding [53]. In addition, transduction with point mutant forms of FOXP3 demonstrated incomplete reprogramming of Teff cells into Treg cells [54]. These results are consistent with the observation that the degree of IPEX Treg cell functional impairment can vary from complete abrogation of suppressive function to partial dysfunction [50]. Therefore, it can be hypothesized that either different FOXP3 domains are important for the establishment of suppressive function, or other genes cooperate with FOXP3 to confer appropriate suppressive function. As a result, some mutated variants of the protein could retain residual activity with incomplete impairment of Treg cell function. However, as long as the mode of action of Treg cells is undefined, the molecular mechanisms through which *FOXP3* mutations impair Treg cell function will remain undefined.

In addition to the loss of suppressive function, *FOXP3* mutations are associated with high instability of Treg cells, which upon inflammatory pressure convert from a regulatory to an effector (i.e. IL-17-producing) phenotype, thus potentially contributing to the autoimmune damage [55]. Therefore, stable expression of wild type FOXP3 seems responsible for preservation of Treg identity.

### Teff cell Dysfunction

In humans, FOXP3 is expressed transiently upon activation of Teff cells [33, 34]. Based on this observation it has been hypothesized that *FOXP3* mutations may also directly affect Teff cell functions. In support of this, peripheral blood mononuclear cells (PBMC) from IPEX patients have altered cytokine production, with impairment of Th1-related cytokines and relative skew to a Th2 profile [50, 56, 57]. In addition, we observed an increased proportion of IL-17-producing cells in patients' PBMC [55] and in gut infiltrates (unpublished data). These data suggest that the impairment of FOXP3-dependent Teff cell function may directly contribute to the pathogenic mechanism underlying the disease.

### FOXP3 Mutations and Auto-Antibodies

The impairment in the immune response regulation due to *FOXP3* mutations extends beyond the T cell compartment and indirectly alters humoral immunity. *FOXP3* mutations do not impact central B-cell tolerance, but rather affect the peripheral B-cell tolerance checkpoint, as demonstrated by the accumulation of autoreactive mature naïve B cells in IPEX patients [58]. These results suggest an important role for Treg cells in the maintenance of peripheral B-cell tolerance in humans and highlight an additional *FOXP3* mutation-dependent immune defect in IPEX syndrome.

Tissue-specific autoantibodies are detectable very early in IPEX patients, in correlation with the presence of autoimmune response to target organs. In particular, autoantibodies to enterocyte antigens, identified as the 75kDa USH1C protein, also known as harmonin, and the actin-binding 95kDa protein, also known as villin, have

been reported in IPEX patients [59, 60]. We have strong evidence that autoantibodies to harmonin and villin are sensitive and specific biomarkers of IPEX, which correlate with *FOXP3* mutations, and can be used to differentiate IPEX from IPEX-like syndromes [61]. The mechanisms responsible for harmonin and villin autoimmunization in IPEX and the role of these autoantigens in the pathological manifestations of IPEX syndrome are presently unknown, but it cannot be excluded that these antigens could be relevant molecular targets of pathogenic autoimmunity.

### Type 1 Regulatory T Cells in IPEX Patients

Importantly, the presence and function of type 1 regulatory T (Tr1) cells, the major adaptive IL-10-producing Treg cell subset, are not affected by *FOXP3* mutations. Indeed, functional Tr1 cells differentiate independently of FOXP3, thus suggesting that in favorable conditions, Tr1 cells could exert regulatory functions, which might compensate for the lack of tTreg dependent regulation especially in long-term surviving IPEX patients [62].

Based on the above, our current view of the pathogenesis of IPEX syndrome proposes the impairment of Treg cells as a major step, but also includes other events contributing to the maintenance of immune dysfunction, such as auto-reactive Th17 cell expansion, persistence of auto-reactive B cells with autoantibodies production, in the context of an inflammatory environment (Figure 1).

## NATURALLY OCCURRING TREG SELECTION IN THE THYMUS

### Thymic Origin of tTreg Cells

Studies in animal models have shown that a thymus-derived specific subset of $CD4^+$ cells was able to suppress autoimmune reactions and actively transfer tolerance [63–65]. However, the discovery of tTreg cells did not come with a clear understanding of the lineage-specificity and origin of these cells. It is now well established that tTreg cells originate from the thymus and are distinct from other sets of Treg cells that can differentiate from naïve T cells in the periphery (peripherally derived (p) Treg cells) [10, 66]. Several mechanisms have been described to contribute to tTreg cell specification in the thymus, as summarized below.

### TCR/MHC Interaction

tTreg cell specification in the thymus seems to be primarily dictated by the interaction of TCR with the MHC/antigen complexes expressed by thymic epithelium. Data from transgenic mice indicate that a developing T cell is primed for Treg differentiation if the avidity of its TCR for self-antigens is higher than the avidity of conventional T cell TCR [67–69]. The importance of a strong TCR signaling in tTreg differentiation has been highlighted in murine models where a defective expression of molecules downstream the TCR, such as ZAP70, LAT or Pest, results in impaired Treg development [70–72]. In humans, polymorphisms in the PTPN22 gene, which encodes for a negative regulator of TCR signaling, have been linked to autoimmune conditions associated with Treg cell dysfunction, and this phenotype seems to correlate with increased PTPN22 expression and subsequent reduced TCR signaling [73–76].

### Two-Step Model

Based on the known mechanisms of central tolerance, medium/strong TCR engagement in the thymus should lead to T cell death by apoptosis. A mechanism that specifically rescues Treg cells from this pathway has been proposed [77, 78]. According to this two-step model, recognition of MHC/antigen complexes by $CD25^-$ $Foxp3^-$ T cells with high/medium avidity with simultaneous signaling of co-stimulatory molecules, leads

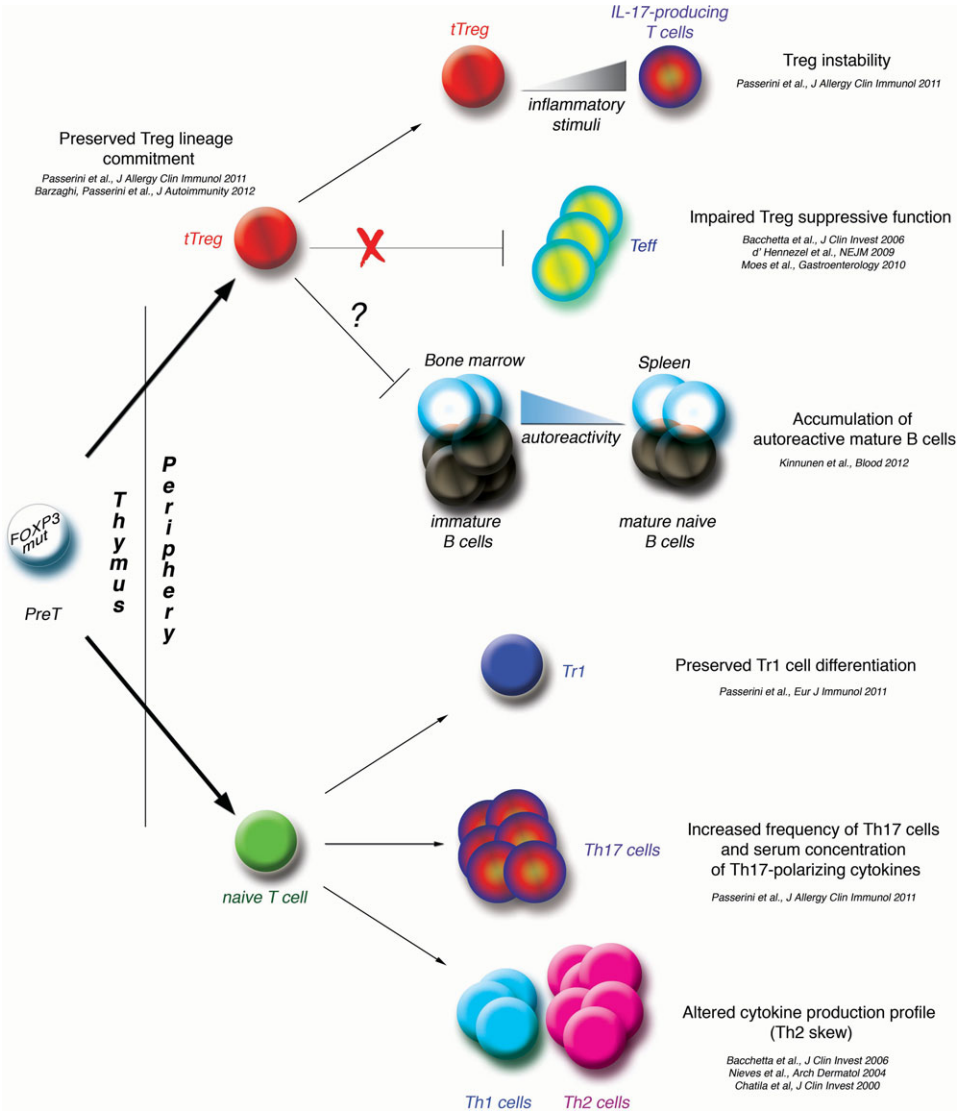


**FIGURE 1.** Pathogenesis of IPEX syndrome. The figure summarizes our current knowledge of the immune pathways directly or indirectly affected or spared by mutations in the *FOXP3* gene in humans. *FOXP3*mut: mutations in the FOXP3 gene; tTreg: thymus-derived regulatory T cells; Tr1: type 1 regulatory T cells; Teff: T effector cells.

to up-regulation of the IL-2 receptor $\alpha$ chain (CD25). The resulting $CD25^{+}Foxp3^{-}$ primed T cells, able to respond to IL-2 and to IL-7 ($\gamma$-chain signaling cytokines), up-regulate the expression of Foxp3 and transduce anti-apoptotic signals, and are thus rescued from death and committed to Treg differentiation ($CD25^{+}Foxp3^{+}$).

### Role of Cytokines

In addition to TCR and $\gamma$-chain signaling cytokines, other external stimuli have been proposed to contribute to Treg cell differentiation and Foxp3 expression in the thymus. TGF$\beta$ is known to activate Foxp3 expression in peripheral naïve T cells and to promote pTreg differentiation in conjunction with TCR signaling [79, 80]. However, ablation of TGF$\beta$ receptor, or of the Foxp3 regulatory element bound by

its downstream signaling molecules, severely reduced peripheral Treg cells in mice, while thymic Treg cells were barely affected [81–84]. These findings suggest that TGF$\beta$ is dispensable for tTreg cell development and its action at the thymic level is currently thought to be rather pro-survival than instructing [85]. On the contrary, the dendritic cell-derived cytokine thymic stromal lymphopoietin (TSLP) has been shown to promote tTreg cell differentiation, but the molecular mechanisms governing this process still need to be clarified [86–90].

### Costimulatory Molecules

tTreg cell development is dramatically impaired in CD28 or CD28 ligands (CD80/CD86) knockout mice, which have reduced PKC activation and subsequent hampered NF-kB pathway [91–97]. The importance of NF-kB pathway in poising Foxp3 expression has been elucidated by a recent study that shows that activated c-Rel directly binds to one of the 4 cis-regulatory non-coding regions conserved in mouse and human, and primes Foxp3 for expression (see Figure 2 and below) [84]. On the other hand, CD28 signaling via the PI3K/Akt pathway induces Foxo1/3 phosphorilation, with consequent sequestration out of the nucleus. Foxo1/3 have been recently identified by three independent studies as key positive factors directly binding to and controlling Foxp3 and the Treg signature gene CTLA4 in murine t- and pTreg cells [98–100] (see below and the review by Vent-Schmidt et al.). How the negative effect of CD28 signaling on Foxo1/3 activity can be reconciled with the positive effect of CD28 signaling on Treg cell development is still debated.

### Thymic Development of FOXP3-Mutated tTreg Cells

Based on the phenotype of the *Scurfy* mouse, the natural murine mutant of *foxp3*, in which Foxp3-expressing cells are undetectable [49], *FOXP3* has been defined as the lineage determination factor for tTreg cells. According to this assumption, it has been long thought that mutations in the *FOXP3* gene would drastically affect the thymic development of Treg cells in both humans and mice, resulting in the complete absence of circulating tTreg cells. Indeed, data from murine models of Foxp3 deficiency demonstrated that Foxp3 is essential for tTreg cell maintenance in the periphery, but dispensable for their development in the thymus [13, 20]. Using reporter gene transgenic mice, a recent study showed that full Treg cell development is achieved by the concurrent establishment of specific epigenetic changes and the induction of Foxp3 expression. The two processes are independent, since the Treg cell-specific DNA methylation pattern could be established without Foxp3, whereas Foxp3 expression could occur even in the absence of the Treg-specific CpG hypomethylation pattern. Only T cells in which both events occur are developmentally set into the Treg cell lineage [12]. An additional study has highlighted the role of factors other than Foxp3 in tTreg cell development. By computational network inference and experimental approaches Benoist *et al.* defined a set of redundant transcription factors (Eos, IRF4, Satb1, Lef1, and GATA-1) that are necessary to establish and maintain the Treg cell signature and phenotype. These data show that neither Foxp3 nor any of the aforementioned factors alone are sufficient to establish the Treg cell state, which instead needs the activation of expression patterns induced by at least two different trasncription factors [101].

Similarly, we showed that in humans *FOXP3* mutations do not necessarily hamper the accumulation of FOXP3 expressing cells with a Treg-like phenotype in the peripheral blood of patients [6, 50]. Furthermore, a population of genetically imprinted Treg cells, i.e. T cells carrying specific epigenetic modifications in the regulatory regions of the *FOXP3* locus, are present in peripheral blood of IPEX patients, regardless of the type or site of the mutation [44, 55]. Therefore, while the functional impairment of *FOXP3*-mutated Treg cells is undisputed, the identification of circulating cells with

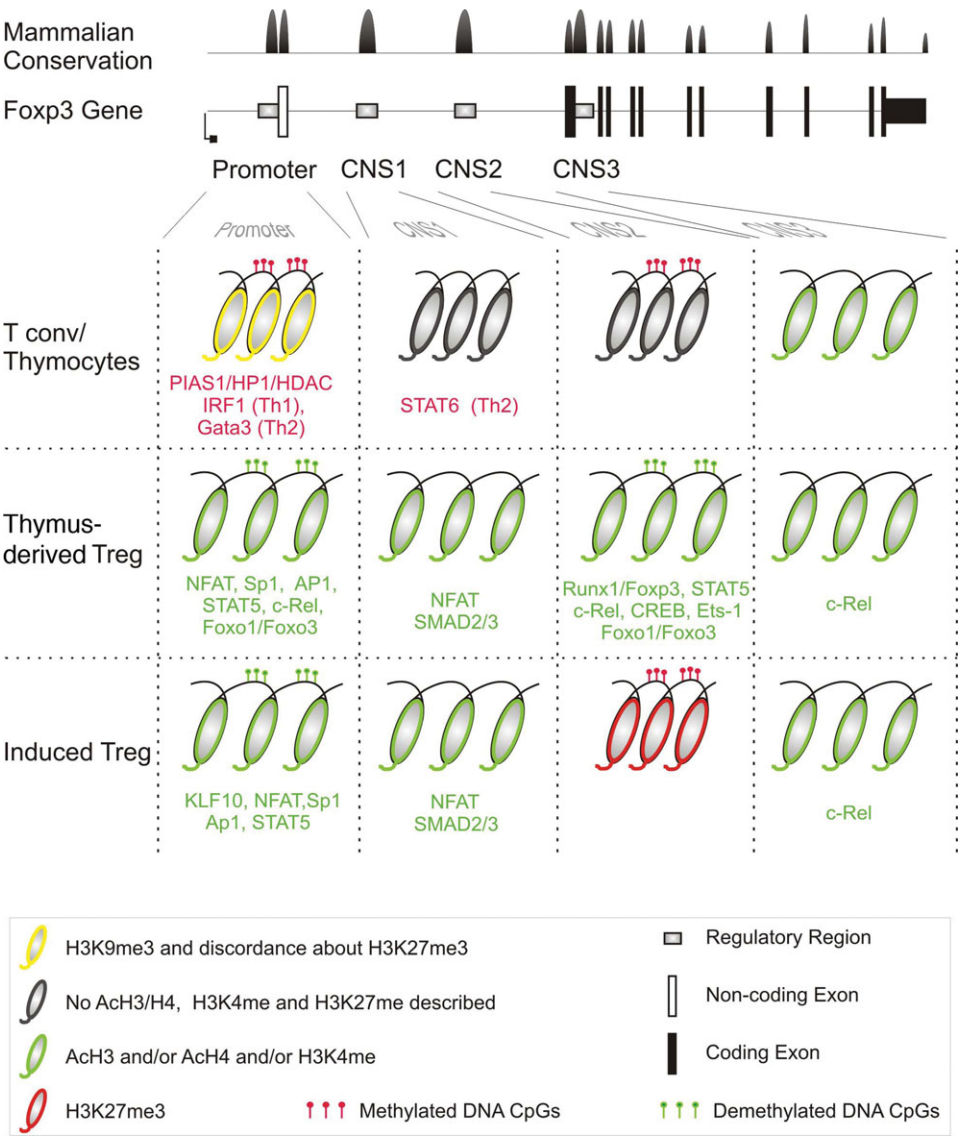


**FIGURE 2.** Regulation of expression of *FOXP3* gene. Upper panels: *FOXP3* gene structure and mammalian conservation of the DNA sequence (modified from UCSC Genome Browser). Lower panels: chromatin status at the level of Promoter, Conserved Non-coding Sequence 1 (CNS1), CNS2, and CNS3 in Thymocytes and Teff, tTreg, and i/pTreg cells. Transcription factors and chromatin modifiers binding to the depicted regions in the depicted T cell population are shown in green or red if exerting positive or negative effect on expression, respectively. For color codes and symbols of chromatin modifications see bottom panel in figure.

specific demethylation of the Treg-cell-Specific-Demethylated-Region (TSDR) (see below), demonstrated that wild type FOXP3 is dispensable for thymic development of Treg cell precursors in humans [44, 55]. The lack of functional FOXP3 impairs Treg peripheral maintenance, as demonstrated by the observation that in healthy female carriers of *FOXP3* mutations [102] and in transplanted IPEX patients with low peripheral donor chimerism [48] only Treg cells expressing a wild type FOXP3 are detectable in peripheral blood.

Although the role of FOXP3 during Treg cell development in humans still needs to be clarified, overall these results suggest that the commitment to the Treg cell lineage can occur in the absence of functional FOXP3.

## EPIGENETIC MECHANISMS CONTROLLING FOXP3 EXPRESSION IN TTREG CELLS

### Epigenetics and Lineage Specification

Epigenetics collectively defines the post-translational modifications of DNA and histones (i.e. acetylation and methylation) that are not directly regulated by the underlying DNA sequence. By affecting chromatin conformation and activity of transcription factors, epigenetics plays an essential role in the development and lineage specification of adult tissues, and in particular of the T cell lineage. Recent studies have recognized altered epigenetic regulation as concurrent cause of autoimmune diseases, such as rheumatoid arthritis and SLE, as well as of allergies [103, 104]. Thanks to the development of efficient and unbiased techniques to assess gene expression and histone/DNA modifications at genome level, chromatin landscape in T cells has been shown to be more dynamic than expected, showing bi-valent states and fast changes in response to external stimuli, both during differentiation and activation [103, 105].

### Cis-regulatory Elements at the FOXP3 Locus

Cis-regulatory elements have been mapped in the *FOXP3* gene for binding of several transcription factors, which activate or repress expression (Figure 2). Stable FOXP3 expression, which characterizes tTreg cells, appears to be maintained by epigenetic modifications, primarily composed by DNA methylation patterns and secondarily by histone marks that stabilize chromatin conformation and allow binding of the transcription factors. Conserved Non-coding Sequences (CNS) have been mapped in (i) the promoter region, (ii) and (iii) the first intron (CNS1 and CNS2), and (iv) the intron after the first coding exon (CNS3) and shown to undergo epigenetic regulation.

### CNS3 Regulatory Region

H3K4me1, a marker of active enhancers, is enriched at the CNS3 region of the *foxp3* locus in Treg cell and, at slightly lower levels, in naïve T cells and immature thymocytes [84]. The NF-kB pathway component c-Rel, activated in response to TCR/CD28 engagement, binds the poised CNS3 region and facilitates Foxp3 transcription and Treg differentiation [84]. CNS3 has been accordingly proposed as a *cis*-regulatory element needed for initial activation of Treg expression pattern (pioneer element). Further supporting the priming role of CNS3 in Treg determination and Foxp3 induction, a recent study has shown that the atypical inhibitor of NF-kB IkB(NS) binds CNS3 by interacting with c-Rel and p50 NF-kB and its deficiency in mice leads to impaired t/pTreg cell development [106].

### CNS2 Regulatory Region

While the presence of repressive histone marks on the *foxp3* gene region in non-Treg cells is still debated [107, 108], the DNA methylation status of promoter and CNS2 regions appears to be the main form of epigenetic control acting at the *foxp3/FOXP3* locus. Several studies in both mouse and human showed that a region contained in CNS2 is demethylated in tTreg, but not in *in vitro* induced (i)/pTreg and Teff cells. This region, defined as TSDR, is also adorned by H3 acetylation and H3K4me3 open chromatin marks, at least in the mouse, and can act as enhancer in a luciferase reporter assay [109–111]. Further studies have proposed TSDR demethylation as epigenetic modification needed for Treg lineage commitment, confirmed the tTreg specificity of this region and have shown that the transcription factors recruited to activate

and maintain Foxp3-expression upon TCR/CD28 and $\gamma$-chain cytokine receptor engagement, such as CREB, Ets-1 and STAT5, can bind to this sequence only when it is demethylated [112–115]. The need for CNS2 demethylation for constant Foxp3 expression was further confirmed by data showing enhanced expression of this gene upon knockdown of the DNA methyl-transferase 1 (DNMT1) in Teff cells and stabilization of iTreg phenotype upon Azacytidine-derivatives (Aza, a DNMT1 inhibitor) [111, 112]. The molecular mechanism at the basis of heritable maintenance of Foxp3 expression in tTreg cells has been later revealed as a positive feedback loop mediated by the CpG demethylation dependent binding of a complex formed by Foxp3, Cbf-$\beta$, and Runx1 [84, 116–119]. The discovery of TSDR was also important since it provided a tool to identify tTreg cells. Indeed, since FOXP3 expression is shared by both t- and pTreg cells and activated Teff cells, quantification of TSDR demethylated cells is at the moment the only method to quantify tTreg cell number, especially in humans [44, 120]. Using this quantitative method we demonstrated that in a group of patients with IPEX symptoms the number of tTreg cells was significantly reduced in the absence of *FOXP3* mutation [44]. A recent study in mouse has shown that pTreg can gradually demethylate TSDR in a highly lymphopenic system [12]. It still needs to be defined whether this is a general mechanism for murine and human pTreg and, thus, if TSDR demethylation differentiates between stable and unstable Treg-phenotype rather than between t and pTregs.



### Promoter

A recent study has shown that the SUMO E3 ligase PIAS1 restrains Foxp3 expression by binding the *foxp3* promoter in immature thymocytes and Teff cells, and maintaining a repressive chromatin status by recruiting DNMT3A/B and Heterochromatin Protein 1, eventually inducing DNA methylation and H3K9me3 modifications [121]. These data suggest that during tTreg cell differentiation PIAS1 is removed from the *foxp3* promoter in order to enhance accessibility and binding of the TFs induced by TCR/CD28 and IL2R signaling. Several reports have recently shown an important role of the Foxo1/3 transcription factors in controlling Foxp3 expression in both t- and pTreg cells, by directly binding to the promoter and the CNS2 region at the *foxp3* locus, and regulating Treg cell differentiation [99, 100, 122]. Foxo1 expression is directly regulated by the epigenetic regulator kap1 (also known as Trim28), whose knockout out in mouse T cells induced expansion of peripheral Treg cells *in vivo* and increased ability of naïve T cells to differentiate into i Treg cells [123]. Kap1 has also been shown to control the expression of the PI3K antagonist PTEN in B cells and to bind to *pten* regulatory elements also in T cells, suggesting that it might be further involved in the Foxo1/Foxp3 axis at an upstream level ([124] and F. Santoni de Sio, unpublished data).

### CNS1 Regulatory Region

Foxp3 expression in naïve T cells and pTreg induction in murine cells requires TCR engagement in the presence of TGF$\beta$ [79, 80]. In order to activate Foxp3 in these cells, TGF$\beta$ signaling has been demonstrated to induce histone 4 acetylation and binding of NFAT and SMAD3 to the CNS1 region [80, 125]. While CNS2 demethylation is an exclusive requirement for tTreg cells, CNS1 region is important for pTreg cell function. By classical reverse genetic approaches two independent studies have proposed an E3 ubiquitin ligase ITCH-dependent role for Kruppel-like factor 10 (KLF10, also known as TIEG1) in the opening of the chromatin at the promoter region of *foxp3* in pTreg [107, 126]. Finally, an enhancer element upstream the *foxp3* promoter has been proposed as an important regulatory region for stable Foxp3 expression in the mouse genome

and found to be demethylated, bound by KLF10 and marked by histone acetylation exclusively in tTreg cells [127].

### Post-Transcriptional and Post-Translational Control

Although well beyond the aim of this review, two additional levels of regulation acting on Foxp3 expression need to be mentioned. The first is the post-transcriptional control of the Treg expression pattern mediated by micro RNA and the second includes the post-translational modifications FOXP3 is subject to (i.e. acetylation and phosphorilation). Several studies have indicated an important role of both processes in the control of Treg function [128–130].

## CONCLUSIONS

In the last ten years, since the identification of FOXP3 as key contributor to the maintenance of tTreg identity, much work has been dedicated to the identification of the molecular mechanisms leading from the induction of FOXP3 expression to the acquisition of Treg cell identity. Evidences suggest that during Treg cell development in the thymus functional FOXP3 is dispensable, whereas epigenetic remodeling is crucial for Treg lineage determination. FOXP3 expression becomes fundamental in later stages, being necessary for the peripheral maintenance of tTreg cells. It is now clear that FOXP3 plays an essential role in maintaining homeostasis of the immune system, by allowing the acquisition of full suppressive function and stability of the Treg cell lineage, regulating the production of autoantibodies, and directly modulating the expansion and function of Teff cells. Likely due to its central role in immune regulation, FOXP3 expression in Treg cells is tightly regulated, especially at the transcriptional level by the coordinated action of transcripton factors and chromatin modifying molecules. FOXP3 is therefore a complex molecule reflecting its complex task: preserve peace among the numerous players of the immune response.



## ACKNOWLEDGEMENTS

The authors thank the members of the Italian Study Group of IPEX (www.ipexconsortium.org) for encouragement and support, and Dr. Philippe Begin from Stanford University, for editing the manuscript. The authors are grateful to the patients and their families for their trust and participation in our studies.

### Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

Our work is supported by the Telethon Foundation (Tele 10A4 to R.B.), the Italian Ministry of Health (Grant RF-2009-1485896 to R.B. and GR-2010-2317550 to F.R.S.d.S.), and the Seventh Framework project (FP7) of the European Community (Cell-PID to R.B.).

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