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Regulatory T Cells and Foxp3

Alexander Y. Rudensky
Howard Hughes Medical Institute and Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

Summary

Regulatory T (Treg) cells play central role in regulation of immune responses to self antigens, allergens, and commensal microbiota as well as immune responses to infectious agents and tumors. Transcriptional factor Foxp3 serves as a lineage specification factor of Treg cells. Paucity of Treg cells due to loss-of-function mutations of the *Foxp3* gene is responsible for highly aggressive, fatal systemic immune mediated inflammatory lesions in mice and men. Recent studies of Foxp3 expression and function provided critical novel insights into biology of Treg cells and into cellular mechanisms of the immune homeostasis.

Keywords

regulatory T cells; Foxp3; tolerance; inflammation

Introduction

The discovery that a subset of $CD4^+$ T cells expressing IL-2 receptor α-chain (CD25) exhibits potent suppressor activity launched its intense investigation (1). In addition to high amounts of CD25, these cells dubbed regulatory T cells (Treg cells) display increased levels of CD5 and CTLA4 in resemblance of activated T cells (2,3). These characteristic features led to proposition that these cells, rather than being a T-cell sub-lineage with dedicated suppressor function, are but a meta-stable state of chronically activated T cells depriving other cells of the immune system of resources, i.e. growth factors. However, the ability of transferred $CD25^+$ Treg cells to protect mice subjected to day 3 thymectomy from chronic inflammatory lesions suggested that either the effect of their suppressor function is long lasting or that at least some of these cells are long lived or their progeny is persistent (4). Although initial *in vitro* studies suggested that $CD25^+$ Treg cells are 'anergic', i.e. unable to proliferate and produce IL-2 upon TCR cross-linking *in vitro*, $CD25^+CD4^+$ Treg cells were shown to robustly proliferate in unmanipulated lymphoreplete mice (5). Moreover, adoptive transfer of $CD25^+$ Treg cells into lymphopenic mice showed that they proliferate robustly in an MHC class II-dependent manner and their suppressor capacity increases despite a decline in expression of CD25 (6). In contrast to $CD25^+$CD4 Treg cells, TCR transgenic T cells of defined specificity with activation induced expression of CD25 failed to suppress autoimmunity upon adoptive transfer into thymectomized mice (7). These studies suggested that $CD25^+CD4^+$ Treg cells represent a distinct long-lived thymus-derived cell subset with suppressor function.

Correspondence should be addressed: Alexander Y. Rudensky, Howard Hughes Medical Institute and Immunology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA, Tel.: +1 646 888 3109, Fax: +1 646 422 0453, rudenska@mskcc.org.

The discovery of Foxp3 gene and its role in Treg cell differentiation

The aforementioned findings 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 humans leading to a severe multi-organ autoimmune and inflammatory disorder IPEX and similarly devastating widespread lesions in a mouse mutant strain *scurfy* (8-11). Early studies of *scurfy* mice revealed an essential role of T cells in the observed pathologies (12,13). The fact that in both humans and mice only *Foxp3* mutant males but not heterozygote female carriers were affected and the systemic nature of immune mediated lesions were consistent with an idea that *Foxp3* mutations might impair differentiation or function of $CD25^{+}$Treg cells. Indeed, high amounts of Foxp3 mRNA and protein were found in $CD25^{+}$ Treg cells (14-16). Forced expression of Foxp3 in $CD25^{-}CD4^{+}$ T cells using retroviral vectors resulted in acquisition of suppressor function and Treg phenotype, whereas in Foxp3 transgenic mice, $CD8^{+}$ T cells exhibited suppressor function (14-16). Furthermore, transfer of allelically marked bone marrow cells from *Foxp3* knockout and wildtype mice mixed at a 1:1 ratio showed that $CD25^{+}$ Treg cells originated only from Foxp3-suffcieint but not Foxp3-deficient hematopoietic precursor cells in the resulting (Ly5.2 $Foxp3^{ko}$ × Ly5.1 $Foxp3^{wt}$) → $Rag2^{ko}$ chimeric mice which remained as healthy as heterozygote female carriers of the *Foxp3* null allele (14). These results showed that *in vivo* Foxp3 is essential for differentiation of Treg cells and raised a question as to whether lack of Treg cells can fully account for the observed pathology in Foxp3-deficient mice and humans or putative lesions in other tissues and organs resulting from Foxp3 deficiency can contribute to the disease in combination with a Treg deficiency.

Treg cell deficiency fully accounts for inflammatory lesions associated with Foxp3 deficiency

This question was addressed in a series of genetic studies. First, generation and analysis of knockin mice expressing fluorescent reporter proteins under control of the endogenous *Foxp3* regulatory elements showed that Foxp3 protein expression is largely restricted to a subset of T cells with suppressor function (17,18). The majority of $Foxp3^{+}$ cells were found within $CD4^{+}$ T-cell subset. However, relatively small, but readily detectable numbers of peripheral $CD8^{+}$, $CD4^{+}CD8^{+}$ and $CD4^{-}CD8^{-}$ $TCR\alpha\beta^{+}$ T cells expressed Foxp3 and corresponding subsets of Foxp3-positive cells were present in the thymus (17). Although the majority of $Foxp3^{+}$ T cells expressed high amounts of CD25, their CD25-negative counterparts were readily detectable in secondary lymphoid organs and non-lymphoid tissues. Importantly, $Foxp3^{+}$ cells characterized by either high and low CD25 or even lacking CD25 expression exhibited common transcriptional signature and potent suppressor function (17).

Although these results were consistent with the idea that the paucity Treg cells is responsible for the disease in Foxp3 mutant animals, it remained possible that in addition to its abundant presence in Treg cells low level or transient Foxp3 expression in immune cells other than T cells or non-immune cells is equally essential for the immune homeostasis. However, the latter possibility was effectively refuted by the observation that mice subjected to ablation of a conditional *Foxp3* allele in the T cell lineage and in the germ-line were phenotypically indistinguishable, i.e. both strains of mice exhibited T cell-dependent autoimmune disease with identical onset, progression and severity (17).

Furthermore, deletion of self antigen-specific thymocytes as well as activation and clonal expansion of, and cytokine production by peripheral antigen-specific T cells were not affected by the presence or absence of Foxp3 gene (14,17,19,20). In these experiments,

healthy (Ly5.2 *Foxp3*^{ko} × Ly5.1 *Foxp3*^{wt}) → *Rag2*^{ko} bone marrow chimeras were challenged with the virus or bacteria or with a superantigen staphylococcal enterotoxin B. In these mice, allelically marked thymocytes and peripheral T cells containing or lacking functional *Foxp3* gene showed identical responses. Likewise, thymocytes in wildtype mice were deleted to a similar degree by a transgene-encoded cognate ligand expressed in the thymus whereas peripheral T cells from these mice showed identical dose-dependent response to cognate ligand stimulation and dependence on CD28 costimulation (20). Thus, *Foxp3* gene expression was dispensable for cell-intrinsic mechanisms of thymic and peripheral tolerance and of negative regulation of the peripheral T cell responses. Finally, adoptive transfers of Treg cells into 1-2 days old mutant recipients rescued lympho- and myeloproliferative syndrome (14). Together, these results provided a definitive proof that Treg cell paucity accounts for devastating disease associated with Foxp3 deficiency in humans and mice.

However, this notion was challenged by Liu and colleagues (21), who employed staining with Foxp3 polyclonal antibodies and PCR analysis to suggest that thymic, mammary and prostate epithelium widely expresses Foxp3. Furthermore, they proposed that in the absence of Foxp3 in the thymic epithelium early T-cell differentiation is impaired and that the lack of Foxp3 in the thymic epithelium can account for, or largely contribute to the disease in Foxp3 mutant animals (22). In mammary gland and prostate, Foxp3 was proposed to act as a tumor repressor essential to prevent cancer development in these tissues (23,24). In spite of this, several groups using staining of these and other tissues from Foxp3-sufficient and -deficient mice with monoclonal Foxp3 antibodies failed to reveal detectable Foxp3 protein expression in epithelial cells (25,26). Furthermore, deletion of a conditional *Foxp3* allele in thymic epithelium using Cre recombinase driven by Foxn1 regulatory elements did not result in any measurable effect on thymic differentiation or lymphocyte activation and immune-mediated pathology (26). Likewise, deletion of a conditional *Foxp3* gene in mammary epithelial cells using mammary gland epithelium-specific MMTV-Cre did not lead to tumorigenesis or even mammary gland hyperplasia in mice up to 18 month of age (L. Henninghausen and A.R., unpublished observations).

Although more studies employing stringent genetic models are needed to explore whether Foxp3 is expressed in cell types other than T cells and its potential role in normal and cancerous tissues, the overwhelming majority of experimental evidence indicates that Foxp3 facilitates differentiation of Treg cells and their paucity is responsible for the disease observed in Foxp3-deficient mice and most likely in IPEX patients. The aforementioned genetic studies provided a definitive proof that the suppressive function of Foxp3-dependent Treg cells is vital for the immune homeostasis.

A critical role for Treg cells in the immune homeostasis throughout the lifespan of normal animals

Since the immune system develops in the absence of Treg cells in Foxp3-deficient individuals, it remained unknown as to whether Treg cell function in adults with a fully developed immune system is not as critical as in newborns. According to the latter scenario, elimination of Treg cells in adults would result in relatively mild immune mediated lesions in comparison to those associated with a congenital Treg deficiency. Additional impetus to this question was given by an observation that Treg cells exhibit TCRs with an increased affinity for self and that in Foxp3-deficient animals activated effector T cells frequently utilize TCRs expressed by Treg cells in wildtype mice (20). This finding raised a possibility that self-reactive T cells, which failed to differentiate into Treg cells in the absence of Foxp3, are the primary cause of the disease in *Foxp3* mutant mice. On the other hand, the documented expression of self-reactive TCR by non-Treg cells and an overlap between self-

reactive TCR repertoire displayed by Treg and non-Treg cells left open a possibility that Treg cell ablation in adults may be as severe as a congenital Treg deficiency in Foxp3-deficient mice (20). These alternative scenarios were explored through generation and analysis of knockin mice harboring human diphtheria toxin receptor (DTR) expressed under control of the *Foxp3* locus. Ablation of Treg cells in *Foxp3*^{DTR} mice induced by chronic administration of diphtheria toxin (DT) starting from birth resulted in a similar disease found in *Foxp3*^{null} mice and death by 4 weeks of age (27). Furthermore, adult healthy *Foxp3*^{DTR} mice on a B6 genetic background resistant to autoimmunity succumb to vast lympho- and myeloproliferative disease within 2-3 weeks upon elimination of Treg cells (27). In the affected mice, massive expansion and activation of diverse immune cell types including granulocytes, dendritic cells (DCs), NK cells, CD4$^+$ and CD8$^+$ T cells, and B cells was observed (27). In contrast to *Foxp3*^{DTR} knockin mice, DT mediated depletion of Treg cells in adult mice harboring a *Foxp3* BAC transgene encoded DTR (DEREG) did not result in a spontaneous autoimmune inflammatory lesions, whereas Treg ablation in DEREG neonates led to a severe disease (28). The latter discrepancy was likely a consequence of incomplete Treg cell elimination in adult DEREG mice due to a BAC transgene inactivation. Within 24-48 h after Treg cell elimination in adult *Foxp3*^{DTR} mice, both DCs and CD4$^+$ T cells showed signs of activation. Importantly, CD4$^+$ T cells of a known specificity for a foreign antigen did not undergo activation upon Treg cell depletion, suggesting that T-cell activation in Treg ablated mice is limited to T cells specific for 'self', which includes genome encoded self, environmental and food antigens and commensal micobiota (27). Furthermore, antibody-mediated depletion of CD4$^+$ T cells when combined with Treg cell ablation in *Foxp3*^{DTR} mice prevented massive lympho- and myeloproliferative disease. However, DC activation manifested in upregulation of costimulatory molecules CD80 and CD86 and increased surface MHC class II expression remained. These findings indicate that in the absence of Treg cells activation of 'self'-reactive CD4$^+$T cells, likely by DCs, fuels massive expansion of cells of the innate and adaptive immune systems. Furthermore, recent detailed analysis of dendritic cell differentiation showed that in normal mice classical DCs proliferate in secondary lymphoid organs and that their differentiation and proliferation is kept in check by Treg cells (29). Consistent with this notion, intravital imaging of Treg cells expressing a transgene-encoded TCR showed stable contacts between Treg cells and antigen-bearing DCs but not with antigen-specific effector T cells (30,31). Although Treg ablation studies failed to definitively identify early cellular targets of Treg cells *in vivo*, they strongly implicated Treg dependent restraint of dendritic cell activation as one of the mechanisms of control of the immune homeostasis by Treg cells. On the other hand, manipulation of dendritic cell numbers through provision of Flt3L or inactivation of Flt3L or Flt3 genes suggested that the size of the dendritic cell population and of IL-2-producing activated effector T-cell subsets control the size of Treg cell population (32,33). Thus, autoregulatory feedback loops maintain a fine balance between suppressive Treg cells, dendritic cell, and activated effector T cells.

A requirement for Treg cell function is independent on the presence of commensal microbiota

A critical role of Foxp3-dependent differentiation and function of Treg cells in immune homeostasis raised a question as to why numerous cell-intrinsic mechanisms preventing differentiation of self-reactive T cells in the thymus and restraining their numbers and activity in the periphery fail to ensure tolerance to self. Considering that recognition of microorganisms and their products by innate immune sensors displayed by DCs endows them with the ability to activate T cells and that Treg cells control DC activation, it is reasonable to assume that the encounter of cells of the innate and adaptive immune systems with commensal microbiota underlies the need of the dominant suppression *in trans*

afforded by Treg cells. Alternatively, it is possible that regardless of the presence of microbiota 'self-referential' T-cell recognition, i.e. a requirement for certain affinity for self peptide-MHC complexes for thymocyte maturation and for T-cell survival in the periphery, poses a continuous threat of pathogenic self-reactivity that cannot be constrained by 'conventional' cell-intrinsic mechanisms of negative regulation. To explore these possibilities, suppressive capacity of Treg cells differentiating in the presence or absence of commensal microbiota was compared and their role in these two environments was assessed (34). Unexpectedly, Treg cells present in GF and SPF mice were equally potent at suppressing lympho- and myeloproliferative disease and inflammatory lesions caused by effector T cells isolated from Foxp3-deficient mice in a variety of tissues including barrier organs such as skin and lung (34). Thus, the Treg cell subset in GF mice is fully competent and conditioning by commensal microbiota is dispensable for the ability of Treg cells to suppress systemic autoimmunity. Moreover, ablation of Treg cells in GF mice led to comparable systemic inflammatory responses including lymphadenopathy, splenomegaly, T cell and DC activation and expansion, augmented Ig production, increases in CD80 and CD86 expression, and cytokine production including TNF_{α} IFN_{γ} and IL-17 (34). These results suggest that the self-MHC-restricted T-cell recognition combined with basal activity of innate immune sensors poses a threat of inflammatory responses against self that cannot be met by conventional means of negative regulation and requires a dominant mechanism of Treg mediated suppression acting *in trans*.

Role of Foxp3 in Treg cells

Studies discussed above established an essential non-redundant role for Foxp3 in differentiation of Treg cells. However, specific contribution of Foxp3 to differentiation and function of Treg cells remained unclear. This question was addressed through comparison of functional and transcriptional features of cells, which received all necessary signals required to promote Foxp3 expression yet lacked Foxp3 protein, and with those of Treg cells containing functional Foxp3 protein (35). In these studies, analysis of mice harboring a GFP coding DNA sequence knocked-in into the *Foxp3* locus with concomitant disruption of the Foxp3 protein expression revealed that Foxp3 was not necessary for survival of Treg precursors, but was essential for Treg suppressor function (35). Similar results were obtained in studies of Foxp3 reporter mice harboring a truncated Foxp3 protein lacking DNA binding domain (36). It is noteworthy that the amount of Foxp3 protein is critical for Treg suppressor function as indicated by inability of Treg cells to suppress spontaneous autoimmunity in mice with a ~10-fold decrease in Foxp3 protein expression due to alteration in 3′ untranslated region (UTR) of the Foxp3 gene (37). Analysis of transcriptional signatures and functional characteristics of cells expressing *Foxp3* reporter null and functional alleles further showed that some of the features of Treg cells, including increased CD25 and CTLA4, and diminished $IL7R_{\alpha}$ and immune response-promoting cytokine expression, are conferred to their precursors prior to Foxp3 expression likely by TCR and cytokine signaling, but Foxp3 exaggerates this pre-existent pattern and makes it permanent (35). Thus, Foxp3 to a large extent amplifies and stabilizes molecular features of Treg precursor cells, beneficial to their function and maintenance, and reverses features deleterious to Treg cell function. An example of the latter activity is Foxp3-mediated repression of IL-17 expression (35). The latter is accomplished through Foxp3 binding to ROR_{γ} and abrogation of its ability to facilitate IL-17 transcription (38). Comprehensive analysis of transcriptional signatures of Foxp3-expressing and non-expressing cells generated *in vivo* and *in vitro* was fully consistent with these conclusions (39). Furthermore, Foxp3 solidifies Treg cell lineage stability through modification of cell surface and signaling molecules, resulting in adaptation to the signals required to induce and maintain Treg cells. The latter notion is supported by the observation that Foxp3 dependent repression of phosphodiesterase 3B expression results in adaption of Treg cells in chronic TCR and IL-2

signaling and their superior maintenance (35). These results raise a possibility that late acting differentiation factors like Foxp3 direct cell fate decisions by acting in an 'opportunistic' manner by exploiting chromatin landscapes prepared by developmental history of precursor cells rather than drastically changing these landscapes.

This idea is not inconsistent with the results of the genome-wide analyses of Foxp3 target genes in Treg cells or in Foxp3 transfected hybridoma T-cell line using chromatin immunoprecipitation (ChIP) combined with whole genome tiling or custom promoter-enhancer arrays, respectively (40,41). These studies suggested that Foxp3 acts as both transcriptional activator and repressor and that Foxp3 direct targets make up ~10-20% of Foxp3-dependent genes. Foxp3 binding to repressed and activated genes correlated with non-permissive H3K27me3 and permissive H3K4me3 marks, respectively, indicative of epigenetically controlled Foxp3-dependent transcriptional program (40). However, it is possible that relatively few of these marks, if any, are imparted upon Foxp3 binding. Instead, we propose that it is likely that the bulk of Foxp3 target genes are 'prepared' for Foxp3 arrival by other transcriptional factors acting as 'place holders'.

Foxp3 binding-site analysis combined with the assessment of the histone modification status seemed to suggest that Foxp3 expression was necessary to establish Foxp3-dependent transcriptional program during Treg differentiation but was dispensable for its maintenance. Contrary to these expectations, Cre-mediated deletion of a conditional *Foxp3* allele in fully differentiated peripheral Treg cells resulted in a cell division-dependent loss of developmentally established characteristic gene expression pattern and of suppressor function (42). Furthermore, upon loss of Foxp3 'ex-Treg' cells acquired ability to produce immune effector cytokines, i.e. IFN_{γ}, IL-4, and IL-17 and pathogenic potential. Indeed, the 'ex-Treg' cells, upon transfer into lymphopenic recipients without functional Treg cells, caused severe tissue lesions and wasting disease (42).

Stability of Foxp3 expression and of the Treg cell function

These results raised an important issue of phenotypic and functional stability of Treg cells, i.e. whether under physiologic or inflammatory conditions Treg cells lose Foxp3 expression and convert into potentially pathogenic (or even beneficial) effector T cells. A number of recent studies provided varying answers to this question. A notion of plasticity of Treg cell suppressor program came from an *in vitro* study utilizing sorted Treg cells expressing a fluorescent reporter which showed that the exposure to pro-inflammatory cytokines IL-6 and IL-1 leads to Foxp3 down-modulation and production of IL-17 (43). Along the same lines, a recent study suggested that Treg cells suppressor activity is neutralized by IL-6 produced in response to CpG stimulation. Upon CpG stimulation, according to this study, Treg cells become the predominant producers of immune response promoting cytokines and serve a critical role in facilitating cross-presentation by DCs. In this study, Treg cells exposed to inflammatory environment continue to express a GFP reporter, yet Foxp3 protein was undetectable by FACS, yet was detectable by Western blot analysis (44). In contrast to these studies, a relatively small proportion of $Foxp3^{+}$ Treg cells, characterized by somewhat lower levels of CD25, lost Foxp3 expression upon adoptive transfer together with allelically marked 'non-Treg' cells into lymphopenic recipients, whereas $CD25^{hi}$ Treg cells largely maintained Foxp3 expression (45).

One caveat associated with studies relying on cell sorting is that a minor contamination of Foxp3-negative cells might lead to their expansion *in vivo* or *in vitro* and cell stress associated with the isolation procedure might facilitate apparent Foxp3 loss or sequestration. These issues were addressed in studies employing irreversible genetic tagging of Treg cells *in vivo* using Cre recombinase expressed under the control of *Foxp3* regulatory elements

combined with a recombination reporter allele. To explore induction and loss of Foxp3 expression one such study employed *Foxp3* BAC transgene driving expression of a GFP-Cre fusion protein and a recombination reporter allele, a ubiquitously expressed *Rosa26* locus harboring YFP coding sequence preceded by a loxP site flanked STOP cassette (R26Y) (46). Studies in these mice showed that some YFP-tagged T cells lacked Foxp3, exhibited features of effector T cells and were able to promote autoimmune inflammation in diabetes-prone NOD mice (46). These results were interpreted as a proof of plasticity of Treg cells and raised concerns with the use of Treg cell transfers in clinic for therapeutic purposes. One caveat with this conclusion was that *Foxp3* BAC transgene driven constitutive expression of GFP-Cre affords continuous labeling of Foxp3 expressing cells including those with low levels of Foxp3 like the $CD25^{lo}$ cells discussed above, as well as cells with transient Foxp3 expression and recently generated Treg cells prior to Foxp3 stabilization (see below). Another recent study employed inducible labeling of a cohort of Treg cells upon tamoxifen treatment of knock-in mice expressing a GFP-ER-Cre fusion protein (containing GFP, Cre recombinase and the ligand binding domain of estrogen receptor) under the control of *Foxp3* locus. The $Foxp3^{GFP\text{-}ER\text{-}Cre}$ allele combined with R26Y reporter allows for YFP tagging of a cohort of differentiated Treg cells and monitor maintenance of Foxp3 expression in these cells. Analysis of these mice revealed remarkably stable maintenance of Foxp3 expression in the progeny of tagged Treg cells under the basal conditions over the lifespan of a mouse, during *Listeria* infection and under irradiation-induced lymphopenia. In addition, in this study Treg cells maintained Foxp3 expression and failed to produce pro-inflammatory cytokines in the context of autoimmune inflammation. In these experiments, double FACS sorted Treg cells expressing diabetogenic BDC2.5 or arthritogenic K/BxN TCRs were transferred into prediabetic or pre-arthritic recipients, respectively, and their ability to produce IL-2, IFN_{γ}, and IL-17 and maintain Foxp3 expression was assessed after the onset of disease in the target tissue or the related lymphoid sites. Although these experiments showed remarkable stability of Foxp3 expression, antibody-mediated neutralization of IL-2 resulted in a moderate decrease in Foxp3 expression in the entire Treg population and the emergence of a minor subset of YFP-tagged cells which completely lost Foxp3 expression. At the same time the entire peripheral Treg population size decreased by more than a third (47). These results were in agreement with the observation that $Foxp3^{+}$Treg cells in mice lacking IL-2 (or $IL\text{-}2R_{\alpha}$ chain) were found in half the numbers of those in wildtype mice and exhibited a similarly decreased amount of Foxp3 protein on per cell basis. Expression of Foxp3 was recovered to wild-type levels upon provision of IL-2 to IL-2-deficient mice (48). Interestingly, a decrease in Treg cell numbers was recently reported during fatal Toxoplasma infection in mice due to a sharp drop in IL-2 amounts. In addition, some Treg cells in infected mice produced IFN_{γ}Provision of IL-2-anti-IL2 immune complexes rescued a loss of Treg cells (49). Although more experiments are needed to evaluate stability of Foxp3 expression and suppressor function of Treg cells in diverse situations of infection and autoimmunity, it is reasonable to assume that there must be a mechanism that ensures stability of Treg function in inflammatory and basal settings.

Mechanisms of Foxp3 induction and maintenance of Treg stability

Considering a central role for Foxp3 in establishing Treg cell function during differentiation and its maintenance in the periphery, recent exploration of regulatory elements within the *Foxp3* locus offered insights into the molecular mechanisms of regulatory T-cell differentiation and maintenance. Analysis of proximal conserved non-coding sequences (CNS) in the Foxp3 locus revealed several intronic elements playing prominent roles in control of Foxp3 expression. One of these elements, CNS3, contains a DNase I hypersensitive site and is enriched in histone H3 monomethylated at position K4 (H3K4me1, a mark associated with enhancer elements) not only in Foxp3 expressing Treg cells, but also in their Foxp3-negative thymic and peripheral precursors (50). NF-κB family

member c-Rel binds to this element and likely facilitates the activity of the *Foxp3* promoter (50,51). Gene targeting studies revealed an essential role for CNS3 in induction of Foxp3 expression in the thymus and in the periphery (50). However, CNS3 deficient mice remain healthy despite a ~5-fold decrease in repertoire of Treg cells in comparison to CNS3-suffcient mice. Consistent with the documented c-Rel binding to CNS3, c-Rel deficiency in thymic precursor cells resulted in a profound impairment in Treg differentiation (50-55). Although the mechanism of CNS3 mediated potentiation of Foxp3 induction remains unclear, it was suggested that c-Rel binding to CNS3 in cooperation with other transcription factors, e.g. NFAT, CREB, p65, and Smad, facilitates formation of c-Rel containing enhanceosome at the *Foxp3* promoter. The experimental evidence in support of this model is limited to temporal changes in c-Rel binding sites within the *Foxp3* locus (51). In addition to c-Rel and other NF-κB family members, CREB/ATF, Ets-1, Foxo1/3, and Stat5 facilitate Foxp3 transcription and bind to its promoter and other regulatory elements within the Foxp3 gene (56-61). The involvement of transcription factors activated downstream of TCR and IL2R signaling pathways is consistent with an elegant two-stage model of generation of Treg cells in response to heightened strengths of TCR engagement and IL-2 signaling (62-65).

Another intronic element dubbed CNS2 (also known as TSDR) contains a CpG island, whose methylation status was correlated with the Foxp3 expression and its stability (66,67). The CNS2 CpGs were demethylated in *ex vivo* isolated Treg cells, whereas in Foxp3-negative peripheral T cells or thymocytes CNS2 was fully methylated and remained so upon induction of Foxp3 in peripheral T cells stimulated in the presence of TGFβ (68). *In vitro* luciferase reporter studies suggested that CNS2 might act as a cryptic promoter by recruiting CREB/ATF and Stat5 (66) as well as Ets-1 (69). Mutation of the corresponding sites resulted in a loss or a marked decrease in transcriptional activity in non-chromatinized reporter assays. Ablation of CNS2 element, however, showed that its non-redundant function lies in heritable maintenance of Foxp3 expression in the progeny of dividing Treg cells. While Foxp3 induction in the thymus and in the periphery was unimpaired in CNS2-deficient mice, Treg cells lacking CNS2 progressively lost Foxp3 expression upon division (50). Furthermore, Foxp3 is recruited to CNS2 with the assistance of Runx1 and its co-factor CBFβ and the binding of Foxp3-Runx1-CBFβ complex confers stability of Foxp3 expression via an unidentified epigenetic mechanism (50). It must be noted that in addition to preserving the active state of the *Foxp3* locus in dividing Treg cells, Runx1-CBFβ complex is also involved in facilitating Foxp3 promoter activity (70,71).

Thus, CNS2 appears to be the first mammalian ‘cellular memory module’ (CMM) analogous to elements involved in the maintenance of hedgehog or Abdominal B expression during *Drosophila* development (50,72,73). Furthermore, proposed role for Foxp3 recruitment to CNS2 and the resulting trans-generational maintenance of Foxp3 expression represents the first description of a feed-forward mechanism enforcing heritable cell lineage identity.

CNS1 (or enhancer 1), the last among the *Foxp3* regulatory elements characterized so far, contains NFAT, Smad3, and RAR_{α} binding sites. Although initially CNS1 was proposed to play a role in Foxp3 induction in the thymus and in the periphery (74,75), analysis of mice deficient in CNS1 revealed that thymic differentiation of $Foxp3^{+}$ cells was unaffected, whereas peripheral Foxp3 induction was severely impaired by CNS1 deficiency (50). Surprisingly, deficiency in peripherally generated Treg cells did not result in detectable tissue-specific autoimmunity in CNS1-deficient mice up to 1 year of age. These results suggested that mechanistic requirements for thymic and peripheral induction of Foxp3 are distinct and that Treg cells generated in the periphery have a non-redundant function likely distinct from that of Treg cells differentiated in the thymus. Future studies of CNS1-deficient mice will likely reveal potential functions of peripherally generated Treg cells.

The results of initial biochemical and genetic analyses of regulation of Foxp3 expression illuminated molecular mechanisms governing generation and function of regulatory T cells and future molecular studies will undoubtedly provide further insights into the biology of regulatory T cells. The uncovered salient features of Treg cell differentiation, including a requirement for Foxp3 expression in fully differentiated Treg cells for their function and for the maintenance of Foxp3 expression during cell division, are likely applicable to other cell lineages. This point is strongly supported by recent demonstration of a need for Pax5 and E2-2 to maintain identity of differentiated B cells and plasmacytoid dendritic cells, respectively (76,77).

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