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PREFACE

Regulatory T cells (or Tregs) are a unique subpopulation of T cells with suppressive properties, acting to counter the immunogenic function of other T cells. This function is critical for the prevention of autoimmune disease and also has profound impacts on other aspects of the mammalian immune system, leading to an intensive effort to harness the power of Tregs as a novel therapeutic strategy across multiple immune diseases.

This volume takes a broad and comprehensive look at Tregs in health and disease states. We have expert chapters on the generation of Tregs, with contributions by Sakaguchi, Huehn, Feuerer, and Abramson on the processes by which Tregs are generated in the thymus and peripheral organs such as the gut. Complementing these chapters, we have articles by Gerondakis, van Nieuwenhuijze, and Kallies, which dissect the molecular pathways that control the induction and differentiation of Tregs. Sparwasser and Moser discuss the cellular dynamics Tregs share with Th17 cells and dendritic cells. Finally, we have an assessment of the physiological impact on Tregs in disease, with expert chapters by Takayanagi, Lund, and Walker on the role of Tregs in arthritis, infection, and diabetes.

ADRIAN LISTON

CHAPTER ONE

Transcriptional and Epigenetic Control of Regulatory T Cell Development

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Contents

Abstract

The control of immune responses against self and nonharmful environmental antigens is of critical importance to the immune homeostasis. Regulatory T (Treg) cells are the key players of such immune regulation and their deficiency and dysfunction are associated with various immune disorders, such as autoimmunity and allergy. It is therefore essential to understand the molecular mechanisms that make up Treg cell characteristics; that is, how their unique gene expression profile is regulated at transcriptional and

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epigenetic levels. In this chapter, we focus on the components of molecular features of Treg cells and discuss how they are introduced during their development.

1. INTRODUCTION

Treg cells are a subset of $CD4^+$ T cells, specialized in the maintenance of immune tolerance and prevention of autoimmunity. Treg cells are unique in that their primary function is to suppress aberrant or excessive immune responses harmful to the host by counteracting the effects of conventional T cells. This property of Treg cells is particularly important in the establishment of self-tolerance. Discrimination between self and nonself is required for the immune system to avoid attacking self-tissues and organs and causing autoimmune diseases. Along with deletion of self-reactive T cells during their development and induction of an anergic state in self-reactive T cells in peripheral lymphoid organs, thymic production of Treg cells, and their immune suppression in the periphery are a critical mechanism of self-tolerance. In addition, conventional T cells can give rise to Treg cells under certain conditions, contributing to immune homeostasis in the periphery.

The production of suppressive cells in the thymus was initially noted in experiments where the removal of thymus from neonatal mice led to severe autoimmunity.[1] However, it was not until 1995 that Treg cells were definitively identified by specific expression of the alpha chain of the IL-2 receptor (CD25),[2] which enabled the finding that Treg cells constituted around 10% of $CD4^+$ T cells and clearly demonstrating that they have a critical role in self-tolerance. This was then further confirmed with the discovery of the lineage defining transcription factor Foxp3.[3,4] Foxp3 is essential for the function of Treg cells, as loss-of-function mutations of Foxp3 in either the scurfy mouse strain or IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome leads to severe autoimmunity including Type-1 diabetes (T1D), immunopathology such as inflammatory bowel disease, and allergy accompanying hyperproduction of IgE.[5–7] Furthermore, depletion of Treg cells in adults also leads to similar autoimmune pathology, demonstrating that Treg cells are needed not just for the establishment, but also the lifelong maintenance, of immune self-tolerance and homeostasis.[8]

In addition to severe acute autoimmunity seen in the complete absence of Treg cells, more subtle defects in Treg cell function have been implicated

in the development of a wide range of chronic autoimmune diseases. Partial loss of Treg cell function or reduction in Treg cell numbers has been associated with a range of human autoimmune disorders such as T1D, rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, hepatic disease, and dermatitis (reviewed in Ref. 9). These finding are confirmed in a number of mouse models of autoimmunity. In nonobese diabetes mice, a model of T1D, defective IL-2 signaling is associated with low Treg cell numbers in the pancreas and the development of diabetes. Conversely, treatment with IL-2 expands Treg cells and prevents the development of diabetes.[10] In the case of colitis, transfer of naïve ($CD45RB^{high}$) $CD4^+$ T cells into T cell-deficient mice leads to the development of colitis; while cotransfer of Treg cells is able to prevent the disease.[11] Treg cells also play a critical role in the regulation of humoral immunity and prevention of allergy, as evidenced by the characteristically high levels of IgE production seen in scurfy mice and IPEX patients.[12] Another aspect of Treg cell-mediated suppression of self-reactive T cells is that Treg cells are able to suppress antitumor immune responses. The presence of Treg cells in tumors is often inversely correlated with survival in both mice and humans. This indicates that depletion of Treg cells and targeting of their suppressive functions can be an important tool in antitumor immunotherapy.[13]

A wide range of Treg cell-mediated suppressive mechanisms have been described, suggesting that they may have context-specific roles at different sites.[14] To date, CTLA4, IL-10, TGFβ, ITGβ8, micro-RNA containing exosomes, IL-35, granzyme, perforin, CD39, CD73, and TIGIT have all been demonstrated to have a role in Treg suppressive function. In particular, CTLA4 expression by Treg cells is crucial for Treg cell-mediated immune suppression. CTLA4 downregulates the expression of the costimulatory molecules CD80 and CD86 on the surface of antigen presenting cells, thereby influencing their ability to activate conventional T cells.[15] Treg cell-specific loss of CTLA4 leads to the development of fatal autoimmunity and dysregulated humoral immunity, similar to that seen in scurfy or Treg-depleted mice.[16–18] Further information on the critical role of CTLA4 in humans has been revealed by the finding that haploinsufficiency of CTLA4 leads to a severe autoimmune syndrome, similar to that seen in IPEX, albeit with variable penetrance and age of onset.[19,20]

Another key feature of Treg cells is their inability to produce IL-2, despite their high dependency on IL-2 for survival and proliferation. IL-2 is also a driving factor for conventional T cell proliferation and some effector T cell differentiation. In this competition for IL-2, high expression of the

high-affinity IL-2 receptor even at the resting state gives Treg cells an advantage and IL-2 deprivation by Treg cells from other T cells is one mechanism of immune suppression. Indeed, overexpression of CTLA4 and repression of IL-2 in conventional T cells enable them to behave like Treg cells.[21] Conversely, failure to repress IL-2 in Treg cells is associated with the development of autoimmunity.[22]

These molecular features are regulated at both the transcriptional and epigenetic levels. Foxp3-dependent transcriptional programs, which often involve interaction with other transcription factors, control some Treg cell-type gene expression, while Foxp3-independent epigenetic modifications also contribute to the generation of Treg cell characteristics. There is dynamic cross talk between transcriptional and epigenetic regulation in a cooperative manner, which enables stable maintenance of Treg cell characteristics throughout multiple divisions, regardless of environmental changes. Given the critical and wide-ranging roles of Treg cells in autoimmunity, allergy, infection, and tumor immunology, it is vital to understand the molecular mechanisms underlying the development and maintenance of Treg cells in order to develop more sophisticated strategies to either enhance or dampen the function of Treg cells in clinical settings. Here, we review the current understanding of transcriptional and epigenetic regulation in Treg cells and discuss how these molecular changes occur during Treg cell development.

2. TRANSCRIPTIONAL REGULATION IN TREG CELLS

Treg cells have a distinct gene expression profile. Foxp3 regulates some gene expression directly and others in cooperation with its cofactors, while there is also a set of gene expression that is controlled independently of Foxp3.

2.1 Foxp3-Dependent Transcriptional Regulation

2.1.1 Foxp3 as a Master Regulator

Foxp3 is a transcription factor that is specifically expressed by Treg cells. As its deletion impairs the suppressive function of Treg cells and causes similar autoimmune diseases to Treg cell depletion, Foxp3 is indispensable for Treg cell function and is considered as the master regulator of Treg cells. Indeed, Foxp3 is able to upregulate or downregulate about half of the genes that are overexpressed or underexpressed, respectively, in Treg cells, compared to conventional T cells.[23] Importantly, such transcriptional changes induced by overexpression of Foxp3 in conventional $CD4^+$ T cells are sufficient

to provide suppressive function similar to that of Treg cells.[4] Moreover, overexpression of Foxp3 and certain transcription factors, such as Irf4, Eos, and Gata1, generates almost complete Treg cell-type transcription profile in conventional $CD4^+$ T cells.[24] Taken together, these findings demonstrate that Foxp3, solely or cooperatively with other transcription factors, regulates the majority of gene transcription in Treg cells.

At the molecular level, Foxp3 mainly functions as a transcriptional repressor and contributes to some of the key characteristics of Treg cells.[25,26] The direct targets of Foxp3 are predominantly those that are normally upregulated by TCR stimulation in conventional $CD4^+$ T cells. A large fraction of them are involved in signaling pathways, such as *Zap70*, *Ptpn22*, and *Itk*.[27] Foxp3 also represses the expression of IL-2.[28] This repression and high dependence on paracrine IL-2 enable Treg cells to suppress conventional T cell proliferation by IL-2 deprivation. Furthermore, Foxp3 directly represses Satb1 by binding to its promoter and inducing microRNAs that target Satb1, to prevent the expression of proinflammatory cytokines that are normally produced by effector T helper cells.[29] Thus, one function of Foxp3 is to repress genes that are activated by T cell activation, and Foxp3 targets genes that serve as regulators of many other genes, thereby efficiently maintaining Treg cell characteristics.

Foxp3 is also involved in upregulation some genes. Hallmarks of Treg cells such as *Il2ra*, *Ctla4*, and *Tnfrsf18* are all bound by Foxp3 and positively regulated.[27] However, Foxp3-null Treg cells, analyzed using mouse models that express a fluorescent marker instead of Foxp3, still express these genes, as well as most of the genes upregulated in Treg cells, but at a lower level than in wild-type Treg cells.[30] These findings illustrate the role of Foxp3 in amplification of pre-existing molecular features.

In terms of the regions that Foxp3 binds to, only a subset of Foxp3-bound genes showed differential expression between $Foxp3^+$ and $Foxp3^-$ T cell hybridomas, suggesting that Foxp3 requires cofactors for its transcription.[27] Consistently, many of the Foxp3-binding sites overlap with other transcription factor binding sites.[31] Therefore, Foxp3, as a master regulator of Treg cells, is able to directly regulate some characteristics of Treg cells, but is insufficient for the generation of full Treg cell-type gene expression, which may require other transcription factors and epigenetic regulation.

2.1.2 Foxp3 and Its Cofactors

As with most transcription factors, Foxp3 interacts with a number of other transcription factors: some being general transcriptional regulators and

others being T cell or Treg cell-specific ones. Some of the proteins currently reported to be capable of interacting with Foxp3 are NFκB,[32] NFAT,[22] Runx1,[28] Eos, CtBP1,[33] CBFb, Gata3, Ash2l, Bcl11b, Ikzf3, Foxp1, Smarcc1, Smarce1, Smarca4, Smarca5, Chd4, Hdac2, Rcor1, Lsd1,[34] HIF-1α, IRF-4,[35] p300, TIP60,[36] and Ezh2.[26] Though Foxp3 is likely to exist in a large protein complex, not all these cofactors are always found in the same complex. There are two features determined by the interaction with particular cofactors: effects of binding on target gene transcription and location of Foxp3 binding.

First, Foxp3 can serve as both transcriptional activator and repressor and these modes of action are determined by the recruitment of coactivators or corepressors. For example, human FOXP3 protein is capable of interacting with the coactivators p300 and TIP60 and such interaction promotes the transcriptional activity of FOXP3, while Treg cell-specific deletion of p300 and TIP60 results in loss of Treg function.[36] In contrast, Foxp3 recruits Eos and the corepressor CtBP1 to repress the expression of genes such as Il2. Since IL-2 repression is critical for Treg cell-mediated immune regulation, silencing Eos in Treg cells abrogates their suppressive function.[33] Notably, some of the factors that Foxp3 interacts with, such as Smarca4, Hdac2, and Ezh2 are known as epigenetic regulators, suggesting that Foxp3 recruits these factors to modulate epigenetic features for long-term control of gene expression (discussed in Section 4). Thus, Foxp3 interacts with appropriate cofactors in a locus-specific manner in order to generate Treg cell-type gene expression (Fig. 1).

Second, Foxp3 is dependent on other transcription factors for binding guidance in some loci, meaning that cofactors alter the targets of its gene regulation. Some interactions are fundamentally required for generating Treg cell phenotypes in physiological conditions. For example, NFκB and NFAT transcription factors have been shown to interact with Foxp3 and cooperatively repress the expression of proinflammatory cytokine genes such as *Il2*, *Il4*, and I*fng*.[22,32] Mutations at the interface of Foxp3 and NFAT interaction resulted in the production of IL-2 by Treg cells and failure to prevent the manifestation of type I diabetes.[22] Other interactions are utilized for particular purposes, such as regulation of specific effector T cell subsets during inflammation. For example, during Th2-type inflammation, Foxp3 interacts with IRF4, which is a transcription factor essential for Th2 cell differentiation program, and enables Treg cells to efficiently control Th2-type inflammation.[37] Importantly, in addition to the variety of Foxp3 complexes at different genomic loci, the repertoire of Foxp3–cofactor complexes

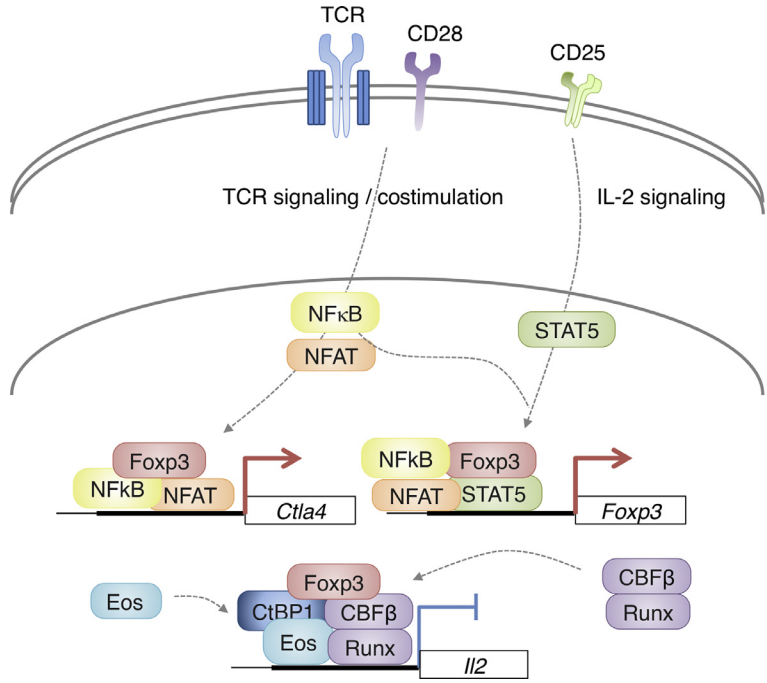

Figure 1 Foxp3-dependent gene expression. Some Foxp3-dependent gene regulation is mediated by the interaction of Foxp3 with transcription factors downstream of TCR/costimulation and IL-2, which are also required for the induction of Foxp3 expression. Others involve the interaction of Foxp3 with T cell-specific or Treg cell-specific transcription factors, such as Runx and Eos.

within a cell may vary depending on the differentiation stage of Treg cells and the environmental conditions they are exposed to. In this sense, the balance among Foxp3 cofactors may be an important determinant of what Foxp3 interacts with. When a fluorescent marker is fused to the N-terminus of Foxp3, it impaired the interaction of Foxp3 with HIF-1α and instead recruited IRF4.[35] Consequently, some gene regulation is altered with particular upregulation of IRF4 signature genes, and these mutant Treg cells alleviated rheumatoid arthritis, but exacerbated type I diabetes.[35] The cause of cofactor change may be due to the competition between HIF-1α and IRF4, or due to the alteration in posttranslational modification of Foxp3. Nevertheless, selection of partners for Foxp3 can serve as a molecular switch for Foxp3-dependent transcription and consequent Treg cell function.

The requirement of Foxp3 to interact with its cofactors indicates that these cofactors also need to be expressed in Treg cells for Foxp3-dependent transcription. Interestingly, a large proportion of these cofactors are direct targets of Foxp3.[34] This notion indicates that Foxp3 directly upregulates the minimum targets by itself, and then regulate the rest of the gene expression in cooperation with these Foxp3 targets that now serve as cofactors. Furthermore, some cofactors such as Runx1, NFAT, and Bcl11b are known to promote Foxp3 transcription, suggesting that Foxp3 and some cofactors positively regulate each other to achieve stable gene regulation.[38–40] There are also cofactors that are independently expressed from Foxp3. For example, NFκB and NFAT are transcription factors activated upon TCR/costimulation. The requirement of these factors for Foxp3-dependent transcriptional regulation suggests that Treg cell specification and maintenance requires TCR signaling in addition to Foxp3 expression. In fact, a large part of Foxp3 targets are coregulated by TCR/costimulation and the number of genes regulated by Foxp3 increase dramatically, as Treg cells become activated.[23,25] Consistent with this, genetic ablation of TCR in mature Treg cells results in a loss of 25% of activated Treg cell signature.[41] Therefore, while some cofactors are upregulated by Foxp3, others are independently expressed, possibly under limited conditions in which Treg cell lineage specification occurs.

Finally, there are "quintet" factors that have been shown to redundantly cooperate with Foxp3 to generate most of the Treg-type gene expression: Eos, Gata1, IRF4, Satb1, and Lef1.[24] Notably, these factors and Foxp3 were retrovirally transduced in conventional $CD4^+$ T cells in this experimental setting, suggesting that TCR stimulation required for retroviral transduction may contribute to some of the Treg cell-type transcriptional regulation. However, even so, coexpression of at least one of the quintet factors with Foxp3 enabled the much more efficient induction of the Treg up- and downregulated gene expression profile than the overexpression of Foxp3 alone. Not all of these quintet factors have been shown to physically interact with Foxp3 protein yet, but they are certainly the coregulators of Foxp3-dependent transcription. How they maximize the transcriptional capacity of Foxp3 remains to be elucidated and it is particularly puzzling that two of the quintet factors, Satb1 and Lef1, are downregulated in Treg cells. One speculation is that coexpression of quintet factors and Foxp3 turns on the molecular switch to build and activate the protein complex around Foxp3. The redundancy among quintet factors, despite belonging to different families and having different functions, may be a mechanism to allow the

generation of Treg cell-type gene expression, once Foxp3 is expressed, in various settings where only one of the quintet factors may be expressed.

2.1.3 Foxp3 Posttranslational Modification

For protein interaction and activity of each protein, posttranslational modifications are crucial. Foxp3 is also subjected to such modification. In particular, acetylation of lysine residues is a key determinant of Foxp3 stability and transcriptional activity. Histone acetyltransferases p300 and TIP60, acetylate Foxp3, whereas histone deacetylases SIRT1, HDAC7, and HDAC9 reverse this process.[42] When acetylated, Foxp3 has higher DNA-binding capacity, thereby enhancing transcriptional activity and becomes more resistant to polyubiquitination and proteasomal degradation.[43] This accords with the result that deleting SIRT1 does not have much effect on conventional T cell function and proliferation, but increases Foxp3 expression and Treg cell suppressive activity. These positive effects on Foxp3 function make SIRT1 a promising target for the induction of transplantation tolerance. Indeed, T cell-specific deletion of SIRT1 or administration of pharmacological SIRT1 inhibitors in mice prevented allograft rejection.[44]

Another posttranslational modification that regulates Foxp3 transcriptional activity is the phosphorylation of a serine residue (Ser418 in humans). Lack of this modification results in impaired Foxp3 function as indicated by the failure to repress IL-2 production.[45] Ser418 can be dephosphorylated by protein phosphatase 1 (PP1), and during rheumatoid arthritis, induction of PP1 by the proinflammatory cytokine, TNFα, in inflamed synovium dephosphorylates Foxp3 protein, impairs Treg cell function and contributes to disease pathogenesis. This demonstrates that posttranslational modifications of Foxp3 serve as a key regulator of Treg cell-mediated immune suppression.

2.2 Foxp3-Independent Transcriptional Regulation

Though Foxp3 is the master regulator of Treg cells, Treg cell-type gene regulation also includes Foxp3-independent features.[30,46] This is evident from the fact that Foxp3-null Treg cells retain a large portion of Treg-type gene expression.[30,47,48] This finding can be partly explained by the fact that TCR, IL-2, and TGFβ signaling also regulate the majority of Foxp3 target genes and the number of genes that are solely controlled by Foxp3 is limited.[23] However, there is still a significant fraction (more than 25%) of Treg-type gene expression that is not regulated by Foxp3, TCR, IL-2, or TGFβ signaling.[30,46] Some are regulated by other transcription factors coexpressed in

Treg cells. For example, Foxo1, which is highly expressed and activated by phosphorylation in Treg cells, controls a subset of Treg cell-type gene expression, independently of Foxp3.[49] Others, such as Eos and Helios, are associated with Treg cell-type epigenetic modifications. This suggests that the permissive chromatin status of these genes enables constitutively expressed transcription factors to induce their expression, rather than specifically expressed transcription factors being responsible for their expression.[48]

3. EPIGENETIC REGULATION IN TREG CELLS

To understand the mechanisms of cell type-specific transcriptional regulation, in addition to the activity of transcription factors, the status of target gene loci is another factor that needs to be considered. That is, there are two requirements for the activation of gene transcription: (1) the responsible transcription factors (*trans*-regulatory factors) are expressed and (2) the chromatin configuration of the target gene locus (*cis*-regulatory elements) is permissive so that the transcription factors can bind. The latter is regulated by various epigenetic modifications of chromatin, such as DNA methylation, histone modification, and nucleosome positioning (Fig. 2). These basic criteria need to be met at least at the gene promoters. In addition, such requirements extend to enhancers for stabilizing high gene expression.

Epigenetic modifications of *cis*-regulatory elements have been implicated in lineage determination. There is a close association among cell differentiation, permissive epigenetic modifications at gene loci associated with the cell lineage, and repressive epigenetic modifications at gene loci related to the alternative cell fate. For example, as multipotent progenitors differentiate into common lymphoid progenitors, they show DNA demethylation in lymphoid lineage-specific genes, while undergoing DNA methylation at myeloid lineage-specific genes.[50] These lineage-specific epigenetic modifications are thought to assist irreversible lineage specification by ensuring the stable expression of key regulator genes. This concept is also applicable to Treg cells, which are indeed characterized by distinct epigenetic modifications.

3.1 Stability of the Treg Cell Lineage

The gene expression regulation by Foxp3 and its cofactors is required not only during the Treg cell development but also for their functional maintenance. Ablation of Foxp3 in mature Treg cells resulted in the reversal of Foxp3-dependent gene expression program and consequently these cells lost