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FOXP3 and the Regulation of Treg/Th17 Differentiation

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Abstract

CD4 T cell lineages are marked by the signature transcription factor each lineage expresses. For example, regulatory T cells (Tregs) are characterized by expression of FOXP3, which is either induced during thymic development for natural Tregs (nTregs), or in the periphery in the presence of TGF β and retinoic acid for induced Tregs (iTreg). Interestingly, recent work has shown that the signature transcription factor for Th17 cells, ROR γ t, is also induced by TGF β , thus linking the differentiation of the Treg and Th17 lineages. In the absence of a second signal from a proinflammatory cytokine, FOXP3 can inhibit ROR γ t function and drive Treg differentiation. However, when the cell also receives a signal from a proinflammation cytokine (e.g., IL-6), FOXP3 function is inhibited and the Th17 differentiation pathway is induced. Therefore, it is the balance between FOXP3 and ROR γ t function that determines CD4 T cell fate and the type of immune response that will be generated.

Keywords

FOXP3; Regulatory T Cell; Th17

1. Introduction

Tolerance to self antigens is an active process that has a central component and a peripheral component. Central tolerance involves the deletion of autoreactive clones during thymocyte development, whereas peripheral tolerance is achieved largely through three mechanisms: clonal deletion, anergy and suppression. Of these three mechanisms, only suppression has a dedicated set of T cells generated for the specific purpose of controlling the responses of other T cells. This set T cells, referred to as regulatory T cells, is actually comprised of several subsets, including a population of cells referred to natural regulatory T cells (Tregs)^{1–3}. These cells are characterized by the expression of CD25 and the forkhead family transcription factor FOXP3 (forkhead box P3), and have the capability of suppressing the activation of other T cells in a contact-dependent manner^{3–6}.

The importance of this T cell subset, and the role of FOXP3 in its development and function, is highlighted by experiments of nature where mutations in FOXP3 result in fatal autoimmune

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lymphoproliferative disease. The spontaneous scurfy mutation in mice has been shown to be a loss-of-function mutation in the *Foxp3* gene, resulting in a complete loss of Tregs and death at 3–4 weeks of age^{7,8}. Similarly, humans with IPEX (Immune dysfunction/ Polyendocrinopathy/Enteropathy/X-linked) syndrome also have mutations in FOXP3 and display a constellation of symptoms consistent with non-functional Tregs^{9,10}.

While the importance of FOXP3 to thymically-derived Treg (referred to as natural, or nTreg) development and function, less is known as to the role of FOXP3 in the differentiation of other CD4 T cell lineages. As will be described below, a second type of FOXP3+ Treg, referred to as inducible, or iTreg, is present in the periphery of mice and humans. This review will explore the role of FOXP3 in the differentiation of iTreg, and their relationship with Th17 cells.

2. Functional Analysis of FOXP3

The importance of FOXP3 in the development and function of nTregs is quite clear. However, the underlying molecular mechanism by which FOXP3 functions remains to be elucidated. FOXP3 has 3 discernible functional domains, a carboxyl-termination forkhead domain (FKH; a.a. 338–421), a single C2H2 zinc finger (a.a. 200–223) and a leucine zipper-like motif (a.a. 240–261). There is also a domain at the amino terminal region that is somewhat Proline-rich (a.a. 1–193). Some clues as to the function of these domains have come from an analysis of FOXP3 variants containing mutations found in IPEX patients. These patients, as described above, develop a variety of symptoms consistent with a Treg deficit¹⁰. Mutations have been found throughout FOXP3, including in each of the domains described above except for the zinc-finger, demonstrating that these regions of the protein are important for proper function^{10,11}. IPEX mutations in the FKH domain have been shown to affect DNA binding, while 2 separate mutations in the leucine zipper have been found to affect FOXP3 homo- and hetero-dimerization^{12–14}. Further evidence for the importance of the Leucine zipper domain comes from studies of the related FoxP1 and P2, where deletion of this domain abrogated their ability to act as transcriptional repressors^{14,15}. Finally, 3 missense mutations have been found in the amino-terminal domain; the affect of these mutations on FOXP3 function is currently underway.

Site directed mutagenesis has also been used to further define FOXP3 function. Using a GAL4-FOXP3 fusion protein and a reporter construct consisting of ARRE2- and GAL4-binding sites, we have begun to define the regions of FOXP3 that are required for transcriptional regulation¹³. This study showed that the amino terminal 198 amino acids of FOXP3 contains all the sequences required to inhibit transcriptional activation by NFAT; however, it should be noted that the DNA-binding domain for GAL4 contains both nuclear localization and dimerization motifs. Consistent with these studies, Bettelli et al. showed that the amino terminal region of FOXP3 was important for inhibiting transcription mediated by NFAT (nuclear factor of activated T cells)¹⁶. Recently, Wu et al. extended these studies by showing that FOXP3 and NFAT can bind cooperatively to the ARRE2 site on the IL-2 promoter¹⁷. Mutation of the residues in the FKH domain predicted to interact with NFAT abolished its ability to inhibit transcription. Taken as a whole, these data suggest that at least one mechanism of FOXP3-mediated transcriptional repression involves direct contact with NFAT, and subsequent inhibition.

Recent work has begun to define the nature of the protein complexes that associate with FOXP3. Initial studies focused on other transcription factors whose activity was inhibited by FOXP3, including NFAT and NFκB. Both NFAT and NFκB were found to be capable of co-immunoprecipitating with FOXP3¹⁸, and subsequent studies showed that FOXP3 and NFAT bound cooperatively to the IL-2 promoter¹⁷. In the latter study, based on structural analyses, several residues in the forkhead domain of FOXP3 were predicted to interact directly with

NFAT, and subsequent mutagenesis of these residues showed a decrease in the ability of FOXP3 to inhibit IL-2 production¹⁷. More recently, Lee et al. have shown that FOXP3 can interact with phosphorylated c-Jun, and thereby alter its subnuclear localization and inhibit AP-1 DNA binding¹⁹. Finally, FOXP3 has also been shown capable of interacting with Runx1/AML1²⁰. Runx family members (runx1, 2, and 3) are critical for hematopoietic development, and perform a variety of functions in CD4 T cells^{21;22}. Ono et al. found that FOXP3 and Runx1 interacted on the IL-2 promoter in a manner different from FOXP3-NFAT in two ways²⁰. First, the interacting sites were not in either DNA binding domain. Second, the binding sites for each transcription factor on the promoter were physically distant. This suggests the possibility of a tri-partite complex involving FOXP3, NFAT, and Runx1 on the IL-2 promoter.

Using a yeast two-hybrid screening approach, Du et al used the amino terminal region of FOXP3 to screen a library generated from human T_R cells²³. Among the FOXP3-interacting proteins found in the screen was the retinoic acid receptor-like orphan receptor (ROR)- α . Further characterization showed that the interaction was both physical and functional in that FOXP3 was capable of inhibiting ROR α -mediated transcriptional activation. Interestingly, the Δ E2 isoform of FOXP3 did not interact with ROR α , the first demonstration of a function distinction between the two proteins. Also, the forkhead domain was not required for FOXP3 to inhibit ROR α -mediated transcriptional activation, suggesting that FOXP3 is acting as a transcriptional co-repressor in this setting²³.

FOXP3 was found to bind to the AF2 domain of ROR α (also known as helix 12). Within the steroid hormone nuclear receptor family, this domain is functionally important in that it binds transcriptional co-repressors in the absence of ligand, and following a conformational change, binds co-activators after ligand binding^{24;25}. Members of the ROR family have a constitutive 'active' conformation, suggesting that they bind to an endogenous ligand. Consistent with this model, ROR α was co-crystallized with a molecule of cholesterol in its ligand binding pocket²⁶. The binding motif present on the co-activators (members of the Steroid Co-Activator, or SRC, family) required for binding to the AF2 domain is LxxLL, where x is any amino acid^{27;28}. There is a single such motif in FOXP3, in exon 2, providing a mechanistic explanation for the failure of the Δ E2 isoform to bind to ROR α .

The relative importance of each of these interactions in the overall function of FOXP3 remains to be determined. ROR α has been shown to regulate inflammatory responses, so its interaction with FOXP3 may be involved in regulating responses^{29;30}. As described below, the interaction with ROR family members has potential consequences for peripheral CD4 T cell effector differentiation.

3. Foxp3 and the control of peripheral T_R vs.Th17 differentiation

Wahl and colleagues showed foxp3 expression could be induced in CD4 T cells stimulated through the TCR in the presence of TGF- β and IL-2³¹. Subsequent studies from a number of groups confirmed these results, and showed that TGF- β signaling was not required for thymic expression of Foxp3, nor was TGF- β signaling or responsiveness required for in vitro suppressive activity of Foxp3+ CD4 T cells. These data suggested that there was an alternative, extrathymic, pathway for Treg differentiation. Support for this model came from studies examining Treg development and function in the gut, where several groups have recently found a role of both TGF β and retinoic acid in the production of gut-specific Treg cells³²⁻³⁴. However, there is some controversy as to the actual role of these TGF- β -elicited T_R cells (referred to as adaptive Treg cells) in vivo. Foxp3 expression in these cells is transient, and is reduced to baseline following removal of TGF- β . Studies of chromatin remodeling at the foxp3 locus suggests that this is due to incomplete demethylation of a specific region of the foxp3 promoter³⁵. These cells function in vitro as suppressors, but there is some question as to their

ability to stably function as Treg cells *in vivo*. Some studies show that, upon transfer, TGF- β -elicited Tregs can function as regulatory cells, while other studies show that they do not perform in a manner that resembles thymically-derived Treg cells^{32;36}. Further work will be required to resolve these contradictory findings.

A new CD4 T cell subset has been identified and shown to be important for autoimmune inflammation in several settings. This subset, known as Th17, is defined by the expression of the cytokines IL-17A, IL-17F, and IL-22^{37–39}. Similar to other Th-subsets, Th17 cells require expression of a specific transcription factor, Ror γ t, for their development⁴⁰.

A link between Th17 cells and iTregs has recently been elucidated. Naïve CD4 T cells can be differentiated into Th17 cells by stimulating through the TCR in the presence of TGF- β and IL-6⁴¹. The finding that TGF- β was required for Th17 differentiation suggested the possibility of a link between iTregs and Th17s. Indeed, TGF- β treatment was capable of inducing both Foxp3 and Ror γ t expression⁴², although, as described above, this treatment led exclusively to Treg differentiation. In a manner similar to that shown for ROR α , Foxp3 was found to be able to associate with Ror γ t and to inhibit its ability to act as a transcriptional activator. In the presence of IL-6, this inhibition was abrogated, and Th17 differentiation was initiated. The effect of IL-6 was not merely to stop foxp3 transcription as foxp3 mRNA was still present in the treated cells⁴².

These data suggest that it is the environment in which activation occurs that determines whether a CD4 T cells will differentiate into a Treg or Th17 cell, and that it is the balance between Foxp3 and Ror γ t. Evidence for an *in vivo* correlate comes from cell fate studies using mice with the capability of ‘marking’ Foxp3-expressing cells. These mice contain an IRES-Cre cassette knocked into the Foxp3 gene, as well as a Rosa26-Flox-stop-YFP gene. Any cell in that animal that expresses Foxp3 will also then express Cre, which in turn will generate a functional Rosa26-YFP gene. These studies show that, at a minimum, 25% of IL-17-producing CD4 T cells in the gut expressed Foxp3 at one time in their life history⁴². These data support the notion that, in the periphery, Treg and Th17 cells can share a common ancestry.

4. Human Treg/Th17 differentiation

In human CD4 T cells the question of what drives the development and persistence of Treg and Th17 cells has led to an examination of differences between mouse and man. However, more importantly have demonstrated the impact of the cytokine milieu on differentiation, and further that the CD4 T cell population has the potential to change based on that milieu. In the case of human Tregs, those cells directly isolated from blood which express FOXP3 have been defined as natural Tregs (nTreg). These cells are characterized as CD4⁺CD25^{bright}CD127^{lo}, FOXP3⁺^{43;44}. In addition, some groups have further differentiated these cells based on surface expression of HLA DR⁴⁵, CD27⁴⁶ and the memory marker CD45RO⁴⁷. However, Treg with similar surface markers and function can also be induced *ex vivo* from CD4⁺CD25⁺FOXP3⁻ cells^{48–50}. These *ex vivo*-derived Treg can be derived from naïve or memory T cells, are FOXP3⁺/CD25⁺/CD127⁻, and suppress *in vitro* in a contact dependent, cytokine independent manner^{48;51;52}.

The signals required to induce Treg in human CD4⁺CD25⁻ T cells has been controversial. However, these controversies frequently reflect differences in the culture conditions, starting population of the cells being induced, and the timepoint at which cells are analyzed. It is clear that activation of CD4⁺CD25⁻ T cells in the presence of IL-2 and TGF β is the most effective method of inducing expression of FOXP3, similar to that seen in mice. IL-2 is required for survival of Tregs as well as for the persistence of FOXP3 expression. Induction of Treg has been described in the absence of TGF β and IL-2 when CD4⁺CD25⁻ T cells are activated, by anti-CD3/CD28, or alloantigen. These systems do not require exogenous IL-2 or TGF β ,

however, in these cultures, the T cell and APC may be producing these cytokines, or serum may contain small amounts of TGF β . In all of these cases it is clear that the character of the stimulation and the cytokine environment have an impact on the induction of FOXP3. It has also become clear that these induced Treg, may not be fully committed to the Treg lineage. Methylation studies of the FOXP3 locus have demonstrated that the FOXP3 promoter of nTreg is fully demethylated, but in those CD4 T cells induced to express FOXP3 upon activation in the presence of TGF β , the FOXP3 promoter is only partially demethylated^{53;54}. These cells when expanded in vitro have the potential to downmodulate expression of FOXP3, and in some cases produce IFN γ or IL-17.

As we extend our understanding of Th17 cell development in humans a similar picture is emerging. As noted above, in mice, Th17 cells require the expression of ROR γ t which can be induced by TGF β and IL-6. This finding highlights the relationship between the fate decision to become a Treg vs Th17 cell. In both cases TGF β is required; however, the presence of the proinflammatory cytokine IL-6 results in the development not of a regulatory cell, but of an inflammatory Th17 cell. In humans the story is beginning to emerge as to what signals are needed to induce IL-17 producing cells. Initial reports indicated that TGF β and IL-6 did not promote the development of Th17 cells in humans. In fact it appeared that TGF β was inhibitory to the development of Th17 cells, and that induction was seen when CD4+CD25RO + T cells were activated in the presence of LPS activated monocytes⁵⁵. A group of recent studies have attempted to dissect the role of antigen presenting cells and cytokines on the development of Th17 cells from either naïve or memory T cells. For example, CD4 memory T cells have been induced to secrete IL-17 when cultured in the presence of IL-1 β and either IL-6 or IL-23⁵⁶. Several different combinations of cytokines have been shown to result in the development of Th17 cells in naïve T cells, including TGF β , IL-1 β , IL-6, IL-21 and IL-23. Although TGF β does not seem to be required under some culture conditions^{57;58}, the possibility that it is present at a very low concentration in the serum, or as cell surface TGF β , makes its role difficult to rule out. On the other hand, TGF β has been shown to be absolutely required for Th17 differentiation in a serum-free system using cord blood-derived CD4 T cells⁵⁹. The importance of IL-1 β in the induction of IL-17 production is unique to human T cells, and may also shed light on the ability of specific APC to assist in induction of Th17 cells, if these cells are producing IL-1 β ⁵⁵. The contrast between the cytokine compartments which promote FOXP3 expression as compared to ROR γ t and IL-17 production, indicate that TGF β is central to both. However, the character of the immune response, regulatory vs inflammatory, is determined by the presence or absence of proinflammatory cytokines such as IL-6, IL-21, IL-23 and IL-1 β .

The plasticity of the T cell compartment allows the immune response to be tailored to the local milieu. This is further emphasized by the recent recognition that Treg themselves may differentiate into IL-17 producing cells, particularly when exogenous IL-1 β , IL-23 or IL-21⁶⁰. Taken together, the CD4 T cell compartment in humans appears to be dynamic, adapting to the requirements and signals of the local environment to ensure an appropriate immune response occurs in response to pathogens. Understanding this delicate balance, will allow us develop therapeutics when the system fails in autoimmunity as well as in response to pathogens.

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