THE ROLE OF FOXP3 IN AUTOIMMUNITY

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Abstract

FOXP3 controls the development and function of T regulatory cells (Tregs). Autoimmunity is linked to changes in FOXP3 activity that can occur at multiple levels and lead to Treg dysfunction. For example, changes in IL-2 signaling, FOXP3 transcription and/or post-translational modification can all contribute to loss of self-tolerance. As additional pathways of FOXP3 regulation are elucidated, new therapeutic approaches to increase Treg activity either by cell therapy or pharmacological intervention are being tested. Early success from pioneering studies of Treg-based therapy in transplantation has promoted the undertaking of similar studies in autoimmunity, with emerging evidence for the effectiveness of these approaches, particularly in the context of type 1 diabetes.

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Highlights

- Dysregulation of FOXP3 expression can occur at multiple levels in autoimmunity
- Autoimmune SNPs diminish IL-2 sensitivity and FOXP3, resulting in impaired Tregs
- Splicing, posttranslational modification, and subcellular localization regulate FOXP3
- Many new Treg-targeted therapies are being tested in autoimmunity

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Introduction

Forkhead box protein 3 (FOXP3) is the master transcription factor for $CD4^+$ regulatory T cells (Tregs) [1], a cell type that plays a critical role in immune regulation. The essential role of FOXP3 and Tregs in autoimmunity was discovered through studies of humans with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and the scurfy mouse model [2]. IPEX patients and scurfy mice have monogenic mutations in *FOXP3* causing absent or poorly functional FOXP3 protein, a lack of normal Tregs, and the consequent development of multi-system autoimmunity [2].

Following these seminal studies in primary immunodeficiencies, many groups investigated whether changes in FOXP3 and associated changes in Treg numbers or function might also underlie the common polygenic forms of autoimmunity. Indeed there is now ample evidence that FOXP3 can be dysregulated in many ways, leading to altered Tregs that initiate and/or perpetuate autoimmunity. Here we review advances made in our understanding of how FOXP3 regulates autoimmunity, focussing on research in humans in the past 2 years addressing two main questions: 1) How is FOXP3 dysregulated in autoimmunity? and 2) How can FOXP3 be therapeutically targeted to treat autoimmunity?

1. How is FOXP3 dysregulated in autoimmunity?

Autoimmunity is clearly associated with changes in the proportion and/or function of FOXP3-expressing Tregs [3], but there is no dominant mechanism driving these changes. Rather, factors affecting Treg function range from the effects of genetics, to changes in FOXP3-promoting signaling pathways, *FOXP3* mRNA expression, or protein modification, summarized in **Figure 1**. Autoimmunity may be driven by one or more of these mechanisms, ultimately resulting in disrupted balance between Tregs and pathogenic conventional T cells

(Tconvs).

Genomic regulation of FOXP3 expression

In addition to loss-of-function mutations leading to IPEX [2], multiple single nucleotide polymorphisms (SNPs) located in coding or non-coding regions of genes important for FOXP3 are associated with autoimmunity [4,5]. Multiple different SNPs in putative regulatory regions of *FOXP3* are present in children with autoimmunity, allergy or both. Interestingly, children suffering from both autoimmunity and allergy had a distinct genetic profile, with a high prevalence of the 7340C>T SNP, located in the 3' untranslated region of *FOXP3* which could affect mRNA stability [4]. Epigenetic modifications, particularly in the Treg specific demethylated region (TSDR, also known as CNS2) [6], also influence *FOXP3*; however this topic has been comprehensively reviewed [6,7], and will not be further discussed here.

In addition to SNPs in *FOXP3* itself, SNPs in three other loci indirectly affect FOXP3 expression and are associated with autoimmunity: *IL2RA* (CD25), *PTPN2* and *PTPN22* [5,8-12]. All three genes are involved in regulating responses to IL-2, an essential paracrine survival cytokine for Tregs that stimulates a positive feedback loop for STAT5-regulated FOXP3 expression. For example, a Type 1 Diabetes (T1D) associated SNP in PTPN2 results in reduced IL-2-stimulated activation of STAT5 [9], leading to low FOXP3 protein and reduced Treg suppression in conditions of limited IL-2 availability. A similar phenotype of reduced STAT5, low FOXP3 and impaired suppression, is linked to SNPs in *IL2RA* in patients with Primary Sclerosing Cholangitis [11], T1D, and Multiple Sclerosis (MS) [12]. This diminished FOXP3 expression could underlie the finding that T1D Tregs have diminished production of CCL3 and CCL4, two chemokines that are trans-activated by

FOXP3 and are crucial for Treg function [13].

Polymorphisms of PTPN22 affect multiple signaling pathways [14], including those modulating sensitivity to IL-2 [15], but its role in controlling the strength of TCR activation may be most critical for FOXP3 function. Diminished PTPN22 expression limits Treg differentiation in strong TCR activation conditions, but enhances FOXP3 expression with weak TCR activation [16]. This finding may explain the controversy surrounding whether PTPN22 SNPs are "good" or "bad" for Tconvs versus Tregs, since experimental results would vary significantly depending on TCR stimulation strength. Changes in regulation of TCR signal strength may also be related to the observed requirement for persistent self-antigen and low ERK activity to preserve Tregs in target tissues [17].

It is worth noting that several causal autoimmunity-associated SNPs map to enhancer and super-enhancer-like regions of the genome and are often near, but not within, transcription factor binding sites [8]. Only 10–20% of these noncoding SNPs alter a known transcription factor target motif, indicating that more research is needed to understand how these alleles affect enhancer activity. Since FOXP3 contributes to transcriptional architecture organization [18], it would be of interest to determine whether SNPs in these enhancers interfere with FOXP3-mediated control of short or long-ranging chromosome interactions, and thus affect Treg function.

Regulation of FOXP3 mRNA and protein expression

Tregs are typically enumerated by measuring FOXP3 protein levels at a given time point, but often overlooked are changes in *FOXP3* mRNA splicing and half-life, which have major affects on its function [1,3]. An intriguing study reported that expression of the two main

FOXP3 splice variants in humans (FOXP3a, the full-length isoform equivalent to mouse Foxp3) and FOXP3b (which lacks exon 2 and has diminished repressive activity) is regulated by metabolism [19]. Inhibitors of glycolysis or fatty acid oxidation blocked TCR-induced FOXP3 expression and acquisition of suppressive function by Tconvs. Moreover, impaired in vitro induction of Tregs from patients with MS or T1D was associated with low glycolysis and low expression of *FOXP3a*. Mechanistically, glycolysis inhibition caused increased binding of enolase 1 to the *FOXP3* promoter and TSDR region, inhibiting transcription. The same group showed that *ex vivo* human Tregs express higher levels of various glycolytic enzymes [20], further supporting a role for glycolysis in human Tregs. These findings in human Tregs are contradictory to multiple studies in mice reporting that Tregs preferentially use fatty acid oxidation as an energy source [21]. However, it is important to note that all these studies in mice have used in vitro differentiated Tregs, which may not be fully lineage committed. Moreover, Tregs and Tconvs have different kinetics of proliferation; this is a major confounding factor limiting data interpretation since glycolysis activity changes profoundly depending on the rate of cell division. More studies of metabolism using ex vivo human Tregs that are controlled for measures of cell division are needed to fully understand the impact of glycolysis on FOXP3 expression and Treg function.

Micro RNAs (miRNAs) also regulate *FOXP3* mRNA by binding, cleaving, destabilizing and/or targeting them to stress granules [22]. Activated naïve Tregs from subjects at risk for T1D have increased levels of miR-26a [23], which indirectly impairs FOXP3 function by decreasing expression of EZH2, a histone methyltransferase responsible for repressive epigenetic modifications. Normally FOXP3 and EZH2 associate, leading to repressive methylation at FOXP3-regulated loci [24]; this activity is lost when there is increased miR-26a-induced degradation of EZH2. Accordingly, EZH2-deficient mouse Tregs cannot control

autoimmunity and have defects in FOXP3-mediated gene-expression [25].

In the context of inflammation, maintenance of FOXP3 protein expression is crucial for sustained tolerance. In Juvenile Idiopathic Arthritis (JIA) affected joints contain Tregs with high CD25 expression, a demethylated TSDR as well as suppressive function in vitro, yet these Tregs express low levels of FOXP3 [26]. These FOXP3^{lo} Tregs have impaired IL-2R signaling, as judged by low pSTAT5, which is known to reduce FOXP3 mRNA and thus impairs the necessary renewal of at least 50% of FOXP3 proteins every ~10 min. [27]. As discussed below, this constant need for IL-2 signaling forms the basis for a variety of therapies currently being tested in autoimmunity.

In contrast to STAT5, STAT3 negatively regulates FOXP3 transcription by binding to a silencer element and reducing SMAD3 binding [28]. In psoriasis, Tregs seem to have heightened phosphorylated STAT3 and decreased suppressive function, possibly related to high levels of IL-6, IL-21 and/or IL-23 [29]. On the other hand, in vitro downregulation of FOXP3 protein in Tregs from JIA synovial fluid can be rescued by IL-6R-stimulated STAT3 activation [30]. Therefore depending on the context and activity of other signalling pathways, STAT3 may have positive or negative effects on Tregs.

Regulation of FOXP3 through post-translational modifications

FOXP3 protein is regulated through phosphorylation, acetylation and ubiquitination [31]. Here, we focus on studies of post-translational modifications that are linked to autoimmunity. Acetylation of lysine residues normally stabilises FOXP3 protein expression and transcriptional activity; inhibition of this process by histone/protein deacetylases such as Sirtuin 1 (SIRT1) causes loss of FOXP3 expression and impaired Treg function [32]. SIRT1

polymorphisms are associated with autoimmune thyroiditis [33] and T1D [34] suggesting that SIRT1 could affect FOXP3 protein stability in autoimmunity. Indeed, treatment of children who were positive for insulin autoantibodies but non-diabetic with the SIRT1-inhibitor nicotinamide, prevented progression to T1D, although the mechanisms were not explored [35].

FOXP3 can be phosphorylated on Ser, Thr or Tyr residues by cyclin-dependent kinase 2 (CDK2) [36], lymphocyte-specific protein tyrosine kinase (Lck) [37], proto-oncogene serine/threonine-protein kinase (PIM)-1 [38], or PIM-2 [39]. Phosphorylation seems to impair FOXP3 function, leading to reduced transcriptional repression [37,38] and impaired Treg suppression [36,38,39]. Cytokines, such as IL-6, may modulate PIM expression [38], and inhibitors can reduce kinase activity [38,39]. Engineering of a phosphorylation-resistant version of FOXP3 [36] may open possible therapeutic strategies. In contrast, FOXP3 dephosphorylation by protein phosphatase 1 reportedly impairs Treg function. In rheumatoid arthritis, high TNF-α in the synovial fluid drives PP1-mediated dephosphorylation of FOXP3, and this can be reversed by the anti-TNF antibody infliximab [40,41].

2. How can FOXP3 be targeted in autoimmunity?

With more than a decade of evidence that poor FOXP3 expression and Treg function causes or perpetuates autoimmunity, a variety of approaches to reverse these phenomena have been explored. The approaches are broadly classified as cellular or non-cellular treatments, with a combined approach likely being the most effective.

Regulatory T cell therapy in autoimmunity

Definitive evidence from mouse models shows that infusion of Tregs can prevent or treat

autoimmunity, so this strategy is now being tested in humans. The first reports of Treg therapy for autoimmunity were in the context of T1D [42]; 43]. In both studies polyclonal Tregs were infused, with doses ranging from 0.05-28x10^8 cells/Kg, with no safety concerns observed [43]. Tracking Tregs through 6,6-$^2H_2$ glucose labeling revealed that infused cells are present for at least a year, with no evidence for loss of the expected Treg phenotype [43]. These findings contrast to reports of Treg therapy in hematopoietic stem cell transplantation (HSCT) where high levels of circulating Tregs are only detected for 2 weeks [44]. The difference could simply be due to lack of a marker for the infused Tregs in the HSCT trials, but it is also possible that the viability of infused Treg is compromised in lymphopenic and immunosuppressed HSCT patients, who likely have minimal sources of IL-2. An open question is whether Treg therapy in autoimmune patients will require mild pre-conditioning for optimal engraftment, and/or delivery of IL-2 in parallel.

It is challenging to obtain therapeutic doses of Tregs. By extrapolating data from mice, the therapeutic dose of polyclonal Tregs is estimated to be 3-5x10^9 Tregs for a 70kg patient [45]. The need to grow billions of cells has led to efforts to improve expansion of Tregs in vitro, with a general consensus that high IL-2 (>1000 U/mL) and mTOR inhibition with rapamycin [46] are needed to stimulate Treg division and limit Tconv outgrowth, respectively. An interesting effect of rapamycin is that it also inhibits the expansion of CD161-expressing effector Tregs, which are poised to produce IL-17 [47]. Since IL-17-expression may actually be beneficial in some diseases, e.g. in IBD where it plays a role in healing of wounded epithelial tissue [48], it may not always be desirable to expand Tregs with mTOR blockade.

In addition to limiting cell numbers, polyclonal Tregs carry the risk of non-specific suppressive side effects. Indeed a transient increase in viral reactivations was observed in

HSCT patients treated with cord blood-derived Tregs [49]. To overcome limitations of polyclonal Tregs, methods to generate antigen-specific Tregs are being explored, including antigen-stimulated expansion [50], TCR transduction [51], and engineering with chimeric antigen receptors (CARs) [52]. All of these strategies should allow for infusion of lower numbers of Tregs since, at least in mice, antigen-specific Tregs are 100 fold more potent than polyclonal cells [53].

We engineered antigen-specific Tregs by CAR-expression, and found this increased Treg potency without compromising phenotype or function in therapy of xenogeneic graft-versus-host disease.[52]. Proof of concept for this approach has also been demonstrated in autoimmunity, with studies of CAR-expressing Tregs in mouse models of inflammatory bowel disease and Experimental Autoimmune Encephalomyelitis [53]. With the success of CAR-T cells for cancer immunotherapy, use of CARs in Tregs promises to be an exciting new direction in cell therapy. Indeed TxCell, a company founded on the basis of Tr1 cell therapy, recently announced efforts to develop CAR Tregs for Lupus Nephritis and bullous pemphigoid (http://www.txcell.com).

In addition to therapy with ex vivo Tregs, Tconvs can be endowed with suppressive function by over-expressing FOXP3, or by culture with immunosuppressive cytokines such as TGF-β. The stability of cells arising from the latter approach, however, is unclear, with epigenetic analysis suggesting that these induced “iTregs” may not be stable in humans [54]. The first application of over-expressing FOXP3, will likely be as gene therapy for IPEX patients [2]. For wider application in autoimmunity, a better understanding of which aspects of Treg function are recapitulated by simple FOXP3 over-expression is needed [55].

Non cell-based Therapies

Because of the complexity and highly personalized nature of cell therapy, strategies to enhance endogenous Treg numbers and function *in vivo* may be preferable to cell infusion approaches. Protocols manipulating IL-2 availability are the most advanced in clinical testing, with other methods that modulate environmental factors to promote FOXP3 expression in early stages of exploration.

Targeting IL-2 signaling. The unique requirement of Tregs for exogenous IL-2, constitutive expression of the high affinity IL-2R, and the association with poor IL-2 response in autoimmunity offers an ideal target for therapeutic manipulation. Whereas high-doses of IL-2 enhance Tconvs in vivo, low doses (1.5-3 x10^6 units/day) seem to specifically stimulate Treg survival/expansion. A trial in T1D found a dose-dependent increase in numbers of $CD4^+$ and $CD8^+FOXP3^+$ Tregs, and increased CD25, GITR, CTLA-4, and pSTAT5 [56] (also Klatzmann et al. in this issue). Encouragingly, at the highest dose, Tconv responses against beta-cell antigens were suppressed in all patients, supporting the initiation of a larger phase IIb trial (NCT02411253). This approach has also had success in the treatment of systemic lupus erythematosus [57], with additional trials of low-dose IL-2 planned in rheumatoid arthritis (NCT02467504), relapsing remitting MS (NCT02424396) and 11 autoimmune/autoinflammatory disorders (TRANSREG study, NCT01988506).

IL-2 has a short half-life, which can be prolonged through administration of a cytokine-antibody complex. Careful selection of the anti-IL-2 antibody can allow tailored signaling. For example, the JES6-1 anti-mouse IL-2 antibody lowers the affinity of IL-2 for CD25, favoring signaling to $CD25^{hi}$ Treg cells [58]. IL-2 itself can also be engineered, creating variants that have more or less affinity for the individual receptor chains, allowing

preferential stimulation of Tconvs [59] or, presumably, in the future, of Tregs. An open question is whether these strategies will be feasible in humans due to high CD25 expression on activated human Tconvs.

Alternate approaches to enhance FOXP3 in vivo. Rapamycin (sirolimus) preferentially favors Tregs by blocking Tconv proliferation and promoting *FOXP3* mRNA expression, and is now commonly used as a "Treg sparing" immunosuppressant in transplantation. Its use is also being explored in autoimmunity, with a trial of sirolimus in multi-lineage autoimmune cytopenias showing rapid and long-lasting responses in a majority of children with autoimmune lymphoproliferative syndrome, and encouraging results in those with lupus [60]. Clinical trials are ongoing to test the effect of rapamycin in Crohn's disease patients with stenosis (NCT02675153) or in combination with islet transplantation in T1D (NCT02505893; NCT00679042).

As we learn more about how peripheral Tregs develop naturally, therapies that harness these natural processes are also being explored [61]. For example, Vitamin C can potentiate Tregs by regulating the activity of ten-eleven translocation (TET) enzymes, which demethylate Treg-specific hypomethylated regions, including the *FOXP3* locus [62]. Similarly all-trans retinoic acid, the metabolite of vitamin A, prevents human Tregs from becoming unstable by increasing histone acetylation in the FOXP3 promoter and demethylation of the TSDR [63].

Overall, there are many complementary strategies to enhance Tregs in vivo and it will be important to compare the effectiveness of these relatively simple and low cost approaches to the more complex and costly, but potentially more effective, cell-based therapies.

3. Conclusions and Perspective

How autoimmunity is affected by changes in FOXP3 as it specifically relates to Tregs has been extensively studied, but it is important to note that FOXP3 also has regulatory roles in other immune cells. Activated $CD4^+$ T cells express FOXP3, restraining their cytokine production and proliferation [64], and there are also reports of FOXP3 expression in $CD8^+$ and invariant NKT cells. Whether or not autoimmunity is linked to changes in FOXP3 in non-Tregs is an underexplored area of investigation. Another aspect of FOXP3 that is often over-looked is its subcellular localization [65,66] as well as how changes in expression of FOXP3 isoforms with distinct regulatory functions and/or nuclear export/import sequences [65] are linked to autoimmunity.

Cell therapy with $FOXP3^+$ Tregs, or methods to promote them in vivo show tremendous promise in transplantation [44,67,68], and with the demonstrated safety of these approaches in autoimmunity [42,43,56], there will likely be an expansion of activity in this area. Because of the limitations of measuring FOXP3 expression in humans - i.e. the inability to definitively identify Tregs versus activated Tconvs - an important caveat to all Treg-targeted therapies is the difficulty of assessing success in Treg manipulation. Ways to track the fate of infused, or in vivo boosted Tregs are urgently needed, to understand how long the cells live, where they go, and how their phenotype changes. We recently developed a biomarker test to measure gene expression in Tregs sorted from the blood of children, which can distinguish healthy Tregs from those from subjects with T1D [69]. Use of this signature or other approaches to track changes during Treg-targeted therapy in autoimmunity will help identify the best clinical approaches.

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Figure Legend

Figure 1. ***Layers of FOXP3 regulation***. The expression and/or function of FOXP3 can be affected by multiple molecular mechanisms: from the genome, to the epigenome, to transcription, translation, and protein stability. Through understanding how expression of FOXP3 can be deregulated in autoimmunity, therapeutic approaches to restore normal FOXP3 expression and Treg function are being developed.

Figure

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