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# Histone deacetylase inhibitors and transplantation

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## Summary of recent advances

Simply detecting the presence or absence of Foxp3, a transcription factor characteristic of naturally occurring CD4+ CD25+ regulatory T cells (Tregs), now appears of minimal value in predicting the outcome of immunologic responses, since dividing human CD4+ effector T cells can induce Foxp3 without attaining repressive functions, and additional molecular interactions, as well epigenetic events, affect Foxp3-dependent Treg functions in humans and mice. Experimentally, in vivo and in vitro studies show histone deacetylase inhibitors (HDACi) can enhance the numbers and suppressive function of regulatory T cells (Tregs), by promoting Foxp3+ cell production, enhancing chromatin remodeling within Tregs, and inducing acetylation of Foxp3 protein itself. Human studies consistent with a role for HDACi in controlling Fox3-dependent Treg functions are also available. We review these molecular interactions and how they may be exploited therapeutically to enhance Treg-dependent functions, including post-transplantation.

## Introduction

The success of organ transplant (Tx) programs comes with a high price, given the nephrotoxicity, cardiovascular disease, diabetes and hyperlipidemia associated with current immunosuppression [1–3], the significant incidence of post-transplant lymphoproliferative disorders [4,5], and the high rates of graft loss from chronic rejection [6,7]. These shortcomings continue to stimulate efforts to achieve clinical tolerance induction, or at least long-term allograft function despite minimal immunosuppression. Autoimmune and transplant models show the importance of naturally occurring CD4+ CD25+ Foxp3+ Tregs in limiting autoreactive and alloreactive immunity [8–11]. Once Tregs are induced in Tx recipients by costimulation blockade, immunosuppression or other strategies [12,13], they can be adoptively transferred to naïve hosts and exert beneficial therapeutic effects. However, regardless of the numbers of cells transferred into immunocompetent hosts, therapy with Tregs alone does not

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appear to control alloresponses to fully MHC-disparate vascularized allografts. These considerations lead to concern as to whether the presence of Foxp3+ Tregs alone is sufficient to indicate the functional competency of cells to be adoptively transferred, or if adjunctive therapies might enhance their suppressive properties? In addition, in contrast to a general restriction of Foxp3 expression to a subset of CD4 T cells with suppressive functions [14], transient induction of Foxp3 now appears to be a general feature of human T cell activation, including by non-Tregs [15,16]. Moreover, Foxp3 induction and transient expression by human effector T cells (T-eff) does not markedly affect cytokine production or cell proliferation [15], and, unlike in the mouse [17–19], ectopic expression of Foxp3 has not, thus far, enabled human CD4+ T cells to become potent Tregs [20]. Hence, at least for human cells, detection of Foxp3 alone in the context of active immune responses is not a reliable marker of the presence of Tregs, and leading to the question of what else do Tregs need for full function, besides Foxp3 expression?

## Tregs and allografts

### Foxp3 expression post-Tx

Our studies in pediatric renal Tx recipients showed a rapamycin-based protocol had equal or better results than calcineurin inhibitor (CNI)-based protocols [21]. Protocol biopsies from rapamycin-treated recipients, taken during stable graft function, showed multifocal CD4+ Foxp3+ mononuclear cell infiltrates, consistent with a local accumulation of Tregs, whereas infiltrates were not seen in patients receiving CNI therapy. However, our analysis of serial samples from adult renal transplant recipients receiving a CNI-based immunosuppression showed levels of urinary Foxp3 mRNA (i) rose during acute rejection, (ii) were inversely correlated with likelihood of reversibility of a given rejection episode, and (iii) directly correlated with renal function as determined at 6 months post-rejection [22]. Similar findings are now reported by others [23–25]. While differing immunosuppression and clinical parameters can prevent direct comparison of data, it is clearly a concern for immune monitoring studies that elevated intragraft Foxp3 levels can be associated with lack of rejection or the development of acute rejection.

### Tregs and immunosuppression

Clinical and animal data indicate CNI but not rapamycin use blocks Treg suppression [26–33]. This may reflect differing effects of these compounds on Foxp3 induction, given multiple NFAT binding sites in the Foxp3 promoter [34]; on Foxp3 function, given cooperative binding of NFAT and FOXP3 [35] and AML1/Runx1 and Foxp3 [36]; on Treg homeostatic peripheral expansion during immune reconstitution post-induction therapy (e.g. with Campath-1H mAb) [29,37]; or the inhibition of IL-2 production by CNI [38,39]. The inhibitory effects of CNI on Treg function mean clinical studies of the numbers of circulating CD4+CD25+ cells post-Tx [40,41], or detection of intragraft Foxp3, have limited significance unless Treg function is also shown to be unaffected [42–45]. In contrast to CNI, corticosteroid or mycophenolate mofetil therapy does not depress Treg function [38,46]. Collectively, these data emphasize the need for more insights into the role of Foxp3 in allograft acceptance through greater knowledge of the downstream consequences of T cell receptor (TCR) signaling for Foxp3 expression and function, as well as the biochemical pathways that control Foxp3-dependent events in Tregs.

## Epigenetic regulation of Foxp3

### Overview of epigenetic regulation

Chromatin organization involves DNA wound around histone octamers that form nucleosomes and are in turn folded into higher ordered structures [47]. Core histones have N-terminal tails extending from compact nucleosomal core particles, and deacetylation of epsilon-acetyl-lysine

residues in these tails affects histone-DNA and histone-non-histone protein interactions. Acetyl groups are added to histone tails by histone acetyltransferases (HATs), comprised of 3 super-families: GNAT (Gcn5-related N-acetyltransferase), MYST (with Myst1/MOF, Myst2/HBO1, Myst3/MOZ, Myst4/MORF and Tip60 as members) and p300/CBP [48,49]. Acetyl groups are removed by histone deacetylases (HDACs); the 18 known HDACs are classified as class I HDACs (HDAC1, HDAC2, HDAC3, HDAC8 and HDAC11) that are mainly localated in the nucleus; class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10) that can shuttle between the nucleus and the cytoplasm; and class III HDACs (SIRT1-7) located in various organelles [50]. Histone acetylation decreases the net basic charge and generally promotes a permissive remodeling, whereas histone deacetylation promotes a repressive chromatin state, though there are exceptions, with histone acetylation in some cases leading to gene repression and histone deacetylation leading to gene activation [51–53]. Lastly, these same HAT and HDAC molecules can affect the functions of non-histone proteins, including transcription factors and other proteins, typically promoting protein stability by preventing ubiquitination and subsequent proteasomal degradation, but acetylation can also increase DNA binding affinity and protein-protein interactions [54].

## Post-translational modifications of Fox family genes

Post-translational modifications of Fox genes include phosphorylation, dimerization, acetylation/deacetylation, methylation and ubiquitination. E.g. Foxo remains transcriptionally active in the nucleus until phosphorylated by Akt [55], leading to the shuttle of Foxo, in association with the protein 14-3-3, from the nucleus to the cytosol [56]. No kinase is yet known to phosphorylate Foxp3 though this is an active area of investigation. In studies of ectopically over-expressed Foxp1, 2 and 4 genes, homotypic and heterotypic interactions were noted suggesting Foxp proteins can dimerize, at least when overexpressed. In addition, association of Foxp1 and 2 with a transcriptional co-repressor protein called C-terminal binding protein (CtBP-1) indicates that multimeric complexes of Fox proteins are possible [57]. Lastly, forkhead proteins often associate with discrete HATs and HDACs. Again in the example of Foxo, Foxo1 is regulated through reversible acetylation catalyzed by the HAT, p300/CBP, and deacetylation by the class III HDAC, hSir-2 (SIRT1), and the acetylation of Foxo1 at Lys-242, Lys-245, and Lys-262 alters its affinity for binding to target DNA and its sensitivity for phosphorylation [52]. While deacetylases can limit acetylation on histones and certain protein lysine residues, phosphorylation of serine or threonine residues on the forkhead domain protein may be permissive for subsequent acetylation/deacetylation events mediated by complexed HATs and HDACs.

## Epigenetic events controlling Foxp3

**Methylation—**Methylation of cytosine in cytosine-phosphate diester-guanine (CpG) islands, which occurs at high density at silenced genes, leads to recruitment of HDACs deacetylases, histone methyltransferase, and chromatin remodeling complexes that promote a closed chromatin conformation [58]. Compared to the extensive cancer literature, little is known concerning DNA methylation and immune functions [59], though methylation of the IL-2 promoter regulates IL-2 production, with the IL-2 promoter being heavily methylated in anergic murine T cells but demethylated in T cells receiving TCR signals and CD28 costimulation [60]. There is now evidence of complete demethylation of CpG motifs, as well as histone modifications, in the Foxp3 locus of murine Tregs but not non-Tregs, whereas developing Foxp3+ thymocytes and Tregs induced by TGF-β in vitro had only incomplete demethylation, despite high Foxp3 expression [61]. In contrast to natural Tregs, TGF-β-induced Foxp3+ murine Tregs lost both Foxp3 expression and suppressive activity upon restimulation in the absence of TGF-β, suggesting that Foxp3 expression must be stabilized by epigenetic modification to allow development of a permanent suppressor cell lineage [61]. Whether cytokines such as TGF-β enhance activation-induced FOXP3 expression, or promote

the recruitment of molecular complexes required for full suppressive activity, is currently under investigation [62–64].

**Acetylation—**We showed that Foxp3 binds to the endogenous IL-2 and IFN-γ loci of murine Tregs or transduced human Jurkat cells only after TCR stimulation, and that activation-induced Foxp3 binding was blocked by CNI use [62], consistent with aforementioned inhibitory effects of CNI on Treg function. TCR ligation increased Foxp3 nuclear translocation, and binding of Foxp3 to the IL-2 and IFN-γ genes induced deacetylation of histone H3, whereas binding of Foxp3 to the GITR, CD25, and CTLA-4 genes resulted in increased histone acetylation. Hence, TCR-induced Foxp3 nuclear translocation can regulate transcription through direct chromatin remodeling, likely involving recruitment of HDACs and repression of certain genes and activation of others. Subsequent studies found that in human CD4+CD25+ naturally occurring Tregs, Foxp3 can be found within a nuclear tripartite complex containing, minimally, as shown by co-immunoprecipitation studies, Foxp3, plus a HAT (TIP60, Tat-interactive protein, 60 kDa) and the class II HDAC, HDAC7 [63]. Foxp3 protein was shown to be acetylated by TIP60, and studies using truncation deletion mutants proved that the region of Foxp3 between amino acids 106–190 was essential for TIP60 and HDAC7 binding, as well as for repression of IL-2 transcription. Moreover, optimal repression of IL-2 required all 3 components, Foxp3, TIP60 and HDAC7. Lastly, a second class II HDAC, HDAC9, was also found complexed with Foxp3 in resting human Tregs, but HDAC9 binding was abrogated upon TCR stimulation, suggesting different roles for HDAC7 and HDAC9 within Tregs.

**Effects of HDAC inhibitors (HDACi)—**Key additional data relevant to understanding the epigenetic regulation of Foxp3 in Tregs came from our murine studies. HDACi administration in vivo increased Foxp3 gene expression, Foxp3 acetylation, thymic Treg production, peripheral Treg numbers and their suppressive functions, and optimal Treg function required acetylation of several lysines in the forkhead domain. In addition to Foxp3, HDACi led to increased Treg expression of CTLA4, PD-1, GITR and IL-10. HDACi therapy enhanced the capability of Tregs to suppress homeostatic proliferation, decreased inflammatory bowel disease through Treg-dependent effects, and in conjunction with brief rapamycin therapy, induced permanent, Treg-dependent, cardiac and islet allograft survival and donor-specific allograft tolerance.

**HDAC9 regulates murine Treg function—**Analysis of HDACs expressed by murine Treg function by real-time quantitative PCR, showed little difference in the expression of class I HDAC (HDAC 1, 2, 3, 8) in non-Tregs (CD4+CD25−) vs. Tregs, whereas class II HDAC (HDAC 4, 5, 7, 9) were mainly expressed by Tregs, especially after TCR activation. TCR activation led to HDAC9 down-regulation in non-Tregs but its 30-fold up-regulation in Tregs, with nuclear export of HDAC9 upon Treg activation. While HDAC9−/− mice had moderately increased numbers of Tregs, HDAC9−/− Tregs expressed more Foxp3 after activation and had increased suppressive function when compared to wild-type Tregs. Transduction of a wild-type Foxp3 construct into HDAC9−/− CD4+CD25− T cells showed enhanced suppressive function compared to Foxp3 retroviral transduction of wild-type CD4+CD25− T cells, further indicating that HDAC9 is a key repressor of Foxp3 function in Tregs.

**Resolving the paradox—**Transfection of cells with Foxp3, Tip60 and HDAC7 provides the basal components for regulation of IL-2 gene transcription in human Tregs [63], and HDAC7 recruitment to a region near the amino terminus of Foxp3 is required for Foxp3-dependent suppression of IL-2. In our studies with murine Tregs, use of a pan-HDACi inhibits Treg HDAC functional activity and yet is associated with enhancement rather than inhibition of Treg functions, as would be expected by inhibition of HDAC7 if the enzymatic activity of the class II HDAC were essential for its action. Our ongoing studies provide a framework for

understanding these apparently contradictory data and resolving this paradox. We propose that the functions of Foxp3 are promoted by acetylation through the action of one or more HATs, and are diminished through Foxp3 deacetylation by one or more HDACs, though additional roles for HDACs may be applicable. A role for acetylation is supported by our mutagenesis studies showing that Foxp3 acetylation is required for optimal murine Treg function, consistent with the human studies, and by our finding that small molecule HAT inhibitors decrease Treg suppressive function. With regard to the HDAC data, class II HDACs can regulate transcription by recruiting an enzymatically active SMRT/N-CoR-class I HDAC complex [65,66], and the deacetylase domain of the HDAC9, for example, can be removed leaving HDAC9 still able to recruit a class I HDAC [67]. Consistent with this, we have found that mice with a targeted deletion of a class I HDAC, HDAC2, have significantly increased numbers of CD4+CD25+ and CD4+Foxp3+ T cells, and that HDAC2−/−Foxp3+ Tregs were twice as potent as WT Tregs in suppressing Teff proliferation, indicating that like class II HDACs, class I HDACs contribute to control of Foxp3+ Treg functions. Moreover, HDACi therapy does not further enhance HDAC9−/− Treg suppressive function over that seen using baseline HDAC9−/− Tregs, suggesting that interruption of either HDAC2 or HDAC9 is sufficient to enhance Treg function. We propose that HDAC9 functions as a tissue-specific deacetylator in Tregs, providing a key on/off switch that functions to assemble large macromolecular complexes with regulatory functions, including additional class I HDACs, HATs, DNMT and SMRT/N-CoR molecules.

In contrast, HDAC7, unlike HDAC9, does not leave the Foxp3 complex upon TCR activation, but its catalytic function should be inhibited by a pan-HDACi such that the role of HDAC7 may involve recruitment of inhibitory complexes such as SMRT and N-CoR [65,66], or additional actions; e.g. protein acetylation or deacetylation can affect protein/HDAC association and promote phosphorylation or dephosphorylation events [68–70]. A working model summarizing our data is shown in Fig. 1.

**Immune monitoring—**Characterization of the acetylation sites central to Foxp3 function will allow generation of antibodies recognizing relevant peptide sequences containing key acetylated lysines vs. deacetylated lysines. Such Abs may allow detection and discrimination of functionally competent Foxp3+ Treg cells in blood and tissues. The feasibility of generating Abs against specific acetylated peptide sequences is demonstrated by the availability of commercial Abs to p53, E2F, PCAF, importin, GATA1, BRCA2, and Rb. Ultimately, knowledge of Foxp3 phosphorylation, nuclear translocation, acetylation and co-association with some HDACs but not others may provide the means for re-energizing the field of immunologic monitoring though the detection of fully competent vs. resting or inactive Foxp3 + Treg cells.

## Conclusions

It is clear that expression of Foxp3 alone is insufficient to define a Treg and that the function of Foxp3+ cells, even classical naturally occurring Tregs, cannot be predicted without knowledge of exogenous (e.g. concomitant drug therapy) and endogenous factors (e.g. TCR stimulation, Foxp3 phosphorylation and translocation, Foxp3 acetylation, assembly of associated macro-molecular complexes that include HAT and HDAC molecules, and nuclear export of HDAC9). Our studies provide insight into control of Foxp3 acetylation and function by specific HDACs, and suggest potential future molecular targets to modulate Foxp3 function in vivo (e.g. HDAC9-selective blockade).

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• of special interest

•• of outstanding interest


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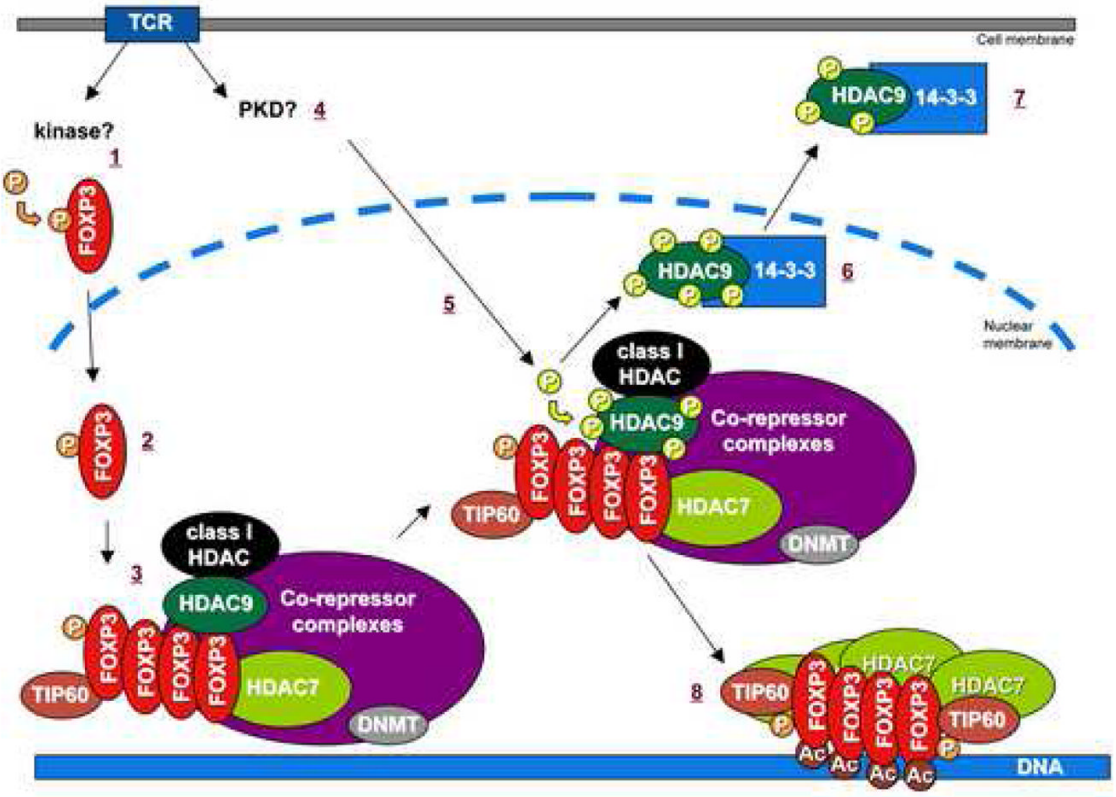


**Figure 1.**

Schematic of events contributing to regulation of Foxp3-dependent functions in Tregs, and highlighting the function of HDAC9 as a key “on/off switch” that binds to, and regulates, Foxp3. **1**) TCR stimulation activates unidentified kinases that phosphorylate (P) Foxp3 and promote its rapid nuclear translocation, though endogenous NLS expression by Foxp3 has led to ongoing, low-level nuclear translocation and accumulation. **2**) Foxp3 translocates to the nucleus and undergoes homo- and hetero-dimerization and higher oligimerization and recruits the HAT, TIP60, and class II HDAC, HDAC7, to a region between 100–200 amino acids from the amino-terminus. **3**) Foxp3 binding to DNA is impaired by its interaction with a second class II HDAC, HDAC9, that acts as a scaffold to recruit and assemble DNMT, class I HDAC (e.g. HDAC2) and very large co-repressor complexes (e.g. SMRT/N-CoR molecules). **4**) TCR activation also generates signals that activate cytoplasmic kinases such as PKD and CaMK. **5**) The latter kinases translocate to the nucleus and phosphorylate HDAC9 (“off switch”), and phosphorylated HDAC9 undergoes a conformational change and dissociates from Foxp3, removing inhibitory complexes and leading to de-repression of Foxp3. **6**) Phosphorylated HDAC9 is exported from the nucleus in conjunction with the exportin, 14-3-3. **7**) In the cytoplasm, phosphorylated HDAC9 is either degraded via the proteasome or may be dephosphorylated and reenter the nucleus to re-establish binding to Foxp3 and limit its action again (“on-switch”). **8**) Upon removal of HDAC9 and its attendant inhibitory complexes, Foxp3 is acetylated (Ac) by TIP60 and binds to the promoter regions of target genes in the DNA.