

Epigenetic mechanisms of regulation of Foxp3 expression

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Regulatory T cells play important roles in the control of autoimmunity and maintenance of transplantation tolerance. Foxp3, a member of the forkhead/winged-helix family of transcription factors, acts as the master regulator for regulatory T-cell (Treg) development and function. Mutation of the *Foxp3* gene causes the *scurfy* phenotype in mouse and IPEX syndrome (immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome) in humans. Epigenetics is defined by regulation of gene expression without altering

nucleotide sequence in the genome. Several epigenetic markers, such as histone acetylation and methylation, and cytosine residue methylation in CpG dinucleotides, have been reported at the *Foxp3* locus. In particular, CpG dinucleotides at the *Foxp3* locus are methylated in naive CD4⁺CD25⁻ T cells, activated CD4⁺ T cells, and TGF- β -induced adaptive Tregs, whereas they are completely demethylated in natural Tregs. The DNA methyltransferases DNMT1 and DNMT3b are associated with the *Foxp3* locus in

CD4⁺ T cells. Methylation of CpG residues represses Foxp3 expression, whereas complete demethylation is required for stable Foxp3 expression. In this review, we discuss how different *cis*-regulatory elements at the *Foxp3* locus are subjected to epigenetic modification in different subsets of CD4⁺ T cells and regulate Foxp3 expression, and how these mechanisms can be exploited to generate efficiently large numbers of suppressive Tregs for therapeutic purposes. (Blood. 2009;114:3727-3735)

Introduction

Foxp3, a member of forkhead/winged-helix family of transcription factors acts as a “master” regulator for the development and suppressive function of regulatory T cells (Tregs). Its constitutive expression is necessary for the suppressive function of Tregs, and mutation or deficiency of *Foxp3* leads to development of autoimmune diseases.¹ In thymus, a subset of CD4⁺CD25⁺ T cells develop into Foxp3⁺CD4⁺ T cells known as natural regulatory Tregs (nTregs). However, in peripheral lymphoid organs, transforming growth factor- β (TGF- β) induces naive CD4⁺CD25⁻ T cells to develop into Foxp3⁺CD4⁺ T cells known as adaptive or induced Tregs. In vitro culture of peripheral naive CD4⁺CD25⁻ T cells in the presence of TGF- β induces expression of Foxp3 and provides a method to generate Foxp3⁺ Tregs ex vivo.² However, nTregs and TGF- β -induced Tregs have different functional characteristics.³ Recent studies suggest that nTregs are more stable compared with TGF- β -induced Tregs, and this may be related to the epigenetic regulation of Foxp3.^{4,6}

Epigenetics is defined by heritable changes in gene expression without changing the DNA sequence of the genome. These gene modifications can take place either in the chromosomal DNA or proteins that are directly linked with the chromosomal DNA. Epigenetic functional units involve either methylation of CpG residues (5' position of cytosine of CpG) or covalent posttranslational modification of histones (Figure 1A-B). In mammals, approximately 60% of CpG dinucleotides are methylated,⁷ and DNA methyltransferases (DNMTs) control DNA methylation of CpG residues and maintenance of methylation during cell differentiation. There are 3 different DNMTs known: DNMT1, DNMT3a, and DNMT3b.⁷ DNA methylation can be divided in 2 types: de novo and maintenance methylation. DNMT3a and DNMT3b are involved in the de novo introduction of methyl group on cytosine residues, whereas DNMT1 is associated with replication machinery to maintain methylation in dividing cells.⁸ However, it has been

reported that, in some CpG dense regions, DNMT1 is not sufficient to maintain the levels of methylated CpG during the replication; and in such somatic cells, the activity of DNMT3a and DNMT3b plays important roles in maintaining methylation.⁹ Methylated CpG recruits transcriptional repressors, such as the methyl-binding proteins MBD and MeCP, and histone deacetylases (HDACs). The HDACs introduce positive charges in the histone tail, which bind tightly to negatively charged nucleic acids and lead to the formation of closely compact nucleosomes that prevent transcription.

Core histone molecules are organized in octamers, consisting of 2 each of H2A, H2B, H3, and H4. The octamers cover approximately 140 DNA bp and form a complex structure called the nucleosome.¹⁰ Specific amino acids of histone tails are targets for several posttranslational modifications, including acetylation, phosphorylation, poly-ADP ribosylation, ubiquitination, and methylation.¹¹ Acetylation of histones in the nucleosome increases their net negative charge, thereby interrupting their interaction with DNA and leading to open chromatin structure of negatively charged DNA (Figure 1B). Thus, open chromatin structure is permissive for recruitment of transcriptional machinery to initiate gene transcription and expression.

Several human diseases are associated with alterations in genome architecture, and genome-wide analysis suggests that epigenetic dysregulation contributes to the severity of these diseases.¹² For example, the T-cell genome of systemic lupus erythematosus (SLE) patients is hypomethylated, resulting in overexpression of integrins, such as LFA-1 (CD11a/CD18).^{13,14} Demethylating drugs can induce overexpression of these same molecules and induce both autoreactive T cells and an SLE syndrome in mice.¹⁵ Demethylation of regulatory elements of the promoter also leads to overexpression of perforin in CD4⁺ T cells and cytotoxic activity in SLE.¹⁶ DNA methylation patterns of

It has been proposed that *Foxp3* Δ exon2 acts as a dominant-negative mutant *Foxp3*.¹ Human Tregs also express a third isoform, which lacks both exon 2 and exon 7 (amino acids 239-260).³⁶ Exon 7 encodes for a leucine zipper motif that acts as a dimerization structural element. However, it has been reported that the absence of exon 7 in natural *Foxp3* Δ 2 Δ 7 does not affect dimerization but abrogates the suppressive function of Tregs.³⁷

Regulation of *Foxp3* expression by *trans*-acting transcription factors

Immediately upstream to the *Foxp3* transcriptional start site (–511 to +176) is a region that possesses several important transcription factor-binding sites (AP-1, NFAT) with features indicative of eukaryotic promoters, including TATA, GC, and CAAT boxes.³⁸ This region acts as a promoter for *Foxp3* transcription and is referred to as the proximal promoter region.³⁸ Other regulatory *cis*-elements are present between noncoding exons (–2b and –1) or far upstream of the transcriptional start site (~5 kb), and these elements are referred to as the intronic enhancers or upstream enhancers, respectively (Figure 1C). Different extracellular stimuli and intracellular signaling molecules control the development and function of Tregs by regulating the transcription of *Foxp3*. For example, IL-2 signaling has an important role in the development and function of Tregs, and IL-2 receptor signaling is generally dependent on STAT5.³⁹⁻⁴¹ Burchill et al identified 11 STAT consensus sites in *Foxp3* (3 at the proximal promoter, 8 between intronic regions), yet only the proximal promoter conserved region shows binding with STAT5 and is dependent on IL-2R β signaling.⁴² Recently, it has been shown that phosphorylation of STAT1 in Th1 cells by IL-27 and IFN- γ leads to *Foxp3* expression, and this is the result of the direct interaction of phosphorylated STAT1 with the *Foxp3* proximal promoter in human CD4⁺ T cells.⁴³ Conversely, IL-27 also inhibits *Foxp3* expression in murine CD4⁺ T cells in a STAT3-dependent manner,⁴⁴ and IL-27 induced murine CD4⁺ T cells to express IL-10 but not *Foxp3*.⁴⁵ These studies suggest that IL-27 is dispensable for Treg generation and that STAT5 but not other STATs are required for *Foxp3* expression.

Small guanosine triphosphate-binding protein superfamily molecules, such as N-Ras or K-Ras, are involved in the control of cell differentiation, proliferation, and apoptosis.⁴⁶ Mor et al showed that inhibition of N- or K-Ras signaling in CD4⁺ T cells leads to induction of NFAT and *Foxp3* expression and enhanced Treg-suppressive function.⁴⁷ However, the mechanism of Ras signaling to regulate *Foxp3* expression is not clearly defined.

In Th1, the IFN- γ -induced protein interferon regulatory factor 1 binds to the *Foxp3* proximal promoter and inhibits *Foxp3* expression.⁴⁸ In Th2, IL-4 inhibits *Foxp3* expression in peripheral naive CD4⁺CD25[–] T cells² by stimulating phosphorylation of STAT6, which binds between exons –2b and –1 (+2459 to +2866 bp) and inhibits TGF- β -induced *Foxp3* expression.^{2,49} TGF- β and IL-4 signaling together induce IL-9 secretion, leading to the newly identified IL-9⁺IL-10⁺*Foxp3*[–] (Th9) subset.⁵⁰

The vitamin A metabolite retinoic acid (RA) inhibits Th17 cells and induces de novo generation of *Foxp3*⁺ Tregs.⁵¹⁻⁵⁵ RA induction of Tregs from naive CD4 T cells may be the result of enhanced TGF- β -driven phosphorylation of SMAD3 along with the inhibition of IL-6 and IL-23 receptor expression.⁵⁶ However, RA also enhances TGF- β -induced conversion of naive CD4⁺ T cells to

Tregs, by inhibiting the CD4⁺CD44⁺ memory T cells that produce *Foxp3* inhibitory cytokines IL-4, IL-21, and IFN- γ .⁵⁷ Kang et al showed that all-*trans*-retinoic-acid induces histone H4 acetylation at the *Foxp3* locus and *Foxp3* expression in naive CD4⁺CD25[–] T cells.⁵¹ RA induces expression of CCR9 and CD103 (α _E subunit of α _E β ₇ integrin) on Tregs, and these Tregs showed increased migration to gut-homing chemokine CCL25.^{51,53} Benson et al reported that, in the presence of TGF- β , CD80/86 knockout dendritic cells (DCs) induce increased *Foxp3* expression compared with wild-type DCs.⁵⁴ The reduced *Foxp3* expression with wild-type DCs can be overcome with the addition of RA to culture.⁵⁴ Furthermore, Coombes et al showed that mucosal CD103⁺ DCs are responsible for TGF- β and RA-induced *Foxp3* expression.⁵⁵ CD103⁺ DCs express high levels of retinal dehydrogenase (*aldh* 1a2) compared with CD103[–] DCs, which is required for the conversion of retinal to RA.⁵⁵ CD103[–] DCs after stimulation with lipopolysaccharide or anti-CD40 produce large amounts of proinflammatory cytokines compared with CD103⁺ DCs.⁵⁵ This suggests that RA together with cytokines and T-cell activation signals provided by different subsets of DCs regulate *Foxp3* expression in CD4 T cells.

Phosphatidylinositol 3-kinase (PI3K) is induced by TCR and CD28 signaling and is required for cell-cycle progression, cell survival, and proliferation. PI3K signaling activates the Akt-mTOR pathway, Akt signaling interferes with *Foxp3* expression, and *Foxp3*⁺ Tregs show reduced Akt phosphorylation.⁵⁸ CD28 signals can interfere with adaptive Treg differentiation by TGF- β ,^{2,54} whereas TCR plus CD28 signals increase *Foxp3* enhancer activity and *Foxp3* expression.⁵⁹ Rapamycin, a chemotherapeutic drug, targets Akt-mTOR signaling and rapamycin-induced differentiation of Tregs and has been used to induce tolerance.⁵⁸ Negative regulator molecules, such as SHIP, a lipid phosphatase, hydrolyze phosphatidylinositol 3,4,5-triphosphate, a second messenger of the PI3K pathway, which in turn inhibits Akt signaling, leading to enhanced differentiation of *Foxp3*⁺ Tregs.⁶⁰ Sphingosine 1-phosphate (S1P) is a natural lysophospholipid, which signals through 5 known G protein-coupled receptors (S1P₁₋₅) and is required for migration of immune cells.²⁸ FTY720, an antagonist, acts as an immunosuppressant by sequestering T cells in lymphoid organs. We showed that receptor S1P₁ causes tissue retention by inhibiting the entry of peripheral T cells to afferent lymphatics.²⁸ Recently, Liu et al showed that the S1P₁ receptor activates Akt-mTOR kinase signaling, thereby inhibiting the development and suppressive function of *Foxp3*⁺ Tregs.⁶¹ Sauer et al showed that PI3K/Akt/mTOR signaling antagonizes *Foxp3* expression by differential modification of histone 3 (H3K4 dimethylation and trimethylation) at the *Foxp3* locus, and continuous TCR stimulation leads to decreased H3K4 dimethylation and trimethylation, and reduced *Foxp3* expression.⁶²

Together, these findings demonstrate that there are several diverse intrinsic and extrinsic signals that regulate *Foxp3* expression and Treg function, yet detailed molecular mechanisms require further investigation.

Role of CpG DNA methylation in Treg development and function

Epigenetic regulation by CpG methylation at specific sites in T cells controls the differentiation of T helper cells.^{24,63,64} Different regulatory *cis*-elements are present in the *Foxp3* locus. There are regulatory elements present upstream of the transcriptional start

site (−600 to −1 bp) at the proximal promoter (Figure 1C). Apart from *trans*-acting factors binding to the proximal promoter, the methylation status of the CpG residues in the proximal promoter region has an essential role in Foxp3 expression. Zorn et al reported that demethylation induced by Aza in human NK cells leads to Foxp3 expression.³⁹ Subsequently, Kim and Leonard reported that 10% to 45% of the CpG sites in the *Foxp3* proximal promoter (−250 to +1) are methylated in naive CD4⁺CD25[−] T cells, whereas all were demethylated in nTregs,⁶⁵ and TGF-β induces demethylation of CpG at this site in CD4⁺CD25[−] T cells. Janson et al showed that this region is approximately 70% methylated in CD4⁺CD25^{lo} cells compared with approximately 5% in CD4⁺CD25^{hi} T cells in humans.⁶⁶ These studies demonstrate that methylation of the proximal promoter is an important regulator of Foxp3 expression.

There are also regulatory *cis*-elements present in between noncoding exons (−2b and −1) that act as enhancers and are defined as intronic enhancers (Figure 1C). This intronic region of *Foxp3* is highly conserved and is responsible for the regulation of *Foxp3* expression. It has been shown that CpG residues in this intronic region (+4201 to +4500) are completely methylated in naive CD4⁺CD25[−] T cells and fully demethylated in nTregs in mice^{5,65} and in human.⁶⁷ Independent studies showed that this region has different levels of demethylation after TGF-β stimulation in mouse^{5,65} and human.⁶⁷ The first intronic CpG containing region (+4393 to +4506 bp, conserved noncoding sequence 3) has decreased methylation of CpG residues after TGF-β signaling, and after TCR signaling has increased binding to the cyclic-AMP response element-binding protein/activating transcription factor, leading to increased Foxp3 expression.⁶⁵ Another evolutionary conserved intronic region between exons −2b and −1 (+2177 to +2198, conserved noncoding sequence 2) binds SMAD3 and NFAT and possesses enhancer activity⁵⁹ (Figure 1C). Because the SMAD3 and NFAT binding sites (CNS2) and the CpG methylation site (conserved noncoding sequence 3) are present in between the same noncoding exons and do not overlap with each other, this suggests that these *cis*-elements may be responsible for the regulation of Foxp3 as a result of different extracellular signaling environments. The interaction of these 2 elements, the signals that regulate them, and the interaction of these signals are areas that require further investigation.

We have shown that there is a CpG island approximately 5 kb upstream of the transcriptional start site, and we refer it as the upstream enhancer. The upstream enhancer (−5786 to −5558 bp) is methylated in naive CD4⁺CD25[−] T cells, activated CD4⁺ T cells, and TGF-β-induced Foxp3⁺ Tregs, but is demethylated in nTregs⁴ (Figure 1C). This region functions as an enhancer. In nTregs, but not TGF-β induced Tregs, this CpG island has acetylated histone 3 indicative of a transcriptionally active site and interacts with the transcription factors Sp1 and TGF-β-induced early 1 product (TIEG1). Conversely, this region is bound by the repressors DNMT1, DNMT3b, MeCP2, and MBD2 in naive CD4⁺CD25[−] T cells, activated CD4⁺ T cells, and TGF-β induced CD4⁺Foxp3⁺ T cells.⁴ Culture of CD4⁺CD25[−] T cells with Aza demethylates the upstream enhancer, leading to positive interactions with transcription factors and markedly increased Foxp3 mRNA and protein expression, and acquired suppressor activity.

TGF-β induces Foxp3 expression in peripheral naive CD4⁺CD25[−] T cells²; however, its activities are very complex in this regard. In addition to TGF-β receptor-induced SMAD3 signaling for Treg generation,⁵⁹ TGF-β signaling may also act via TIEG1 and E3 ubiquitin ligase *itch* in a ubiquitin-dependent

pathway.⁶⁸ Venuprasad et al showed that *itch*^{−/−} and *TIEG1*^{−/−} naive CD4⁺ T cells have less Foxp3 induction after TGF-β stimulation, and these Tregs do not protect from antigen-induced airway inflammation.⁶⁸ TGF-β also inhibits the phosphorylation of ERK leading to inhibition of DNMT expression⁶⁹; and inhibition of DNMT with siRNA or DNMT inhibitors leads to Foxp3 expression in CD4⁺ T cells,^{4,6,65,70} suggesting that inhibition of DNMT activity plays an important role in Foxp3 expression. These CpG methylation epigenetic markers have been used to screen suppressive Tregs derived from the tissues of cancerous or noncancerous patients.^{71,72}

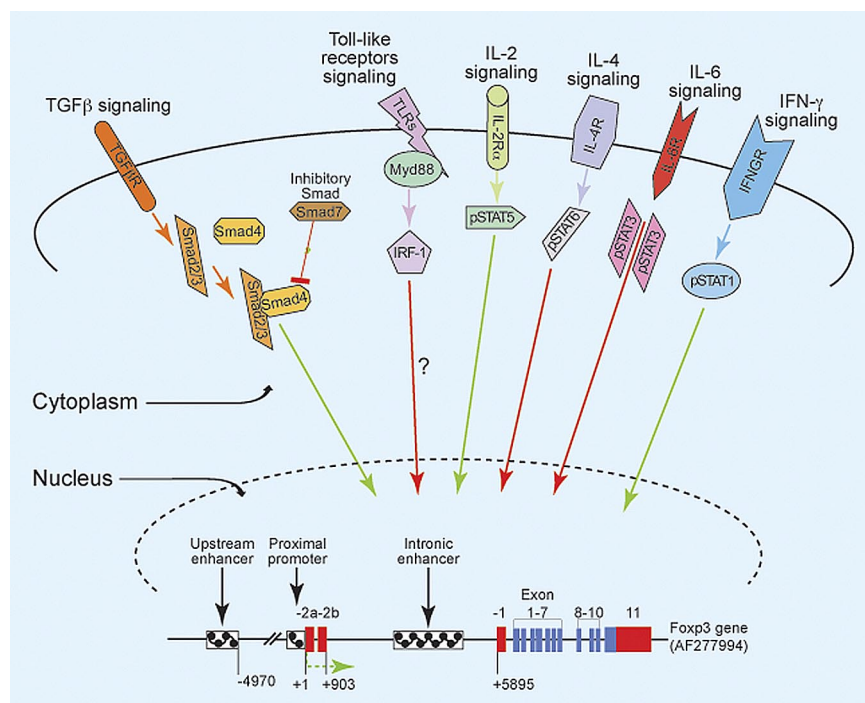
The inflammatory cytokine IL-6 suppresses the development and function of Tregs.^{73–75} IL-6 induces DNMT1 expression and enhances its activity.^{76,77} IL-6 induces STAT3-dependent methylation of the upstream Foxp3 enhancer by DNMT1 in nTregs, leading to repression of Foxp3.⁴ Preactivated CD4⁺CD25[−] T cells or CD4⁺CD25[−]CD44^{hi} memory T cells express very little Foxp3 after TGF-β stimulation.^{4,78} This is probably the result of high levels of DNMT1 activity in these cells^{77,79} because inhibition of DNMT with Aza or deficiency of DNMT1 in T cells leads to Foxp3 expression,⁸⁰ suggesting that regulation of Foxp3 is tightly controlled by epigenetic modification in activated CD4⁺ T cells.

Together, these reports demonstrate that Foxp3 is regulated by complex mechanisms where many extracellular signals control transacting factors as well as chromatin remodeling through covalent modification of CpG DNA. Thus, TGF-β along with inflammatory cytokines has a different effect than TGF-β alone on the development of Tregs. Understanding these signals and their cumulative intracellular effect on Foxp3 *cis*-elements at different T-cell developmental stages will be key for manipulating T-cell responses therapeutically (Figure 2).

Role of HDACs in Treg development and function

Acetylated histone is a marker for open chromatin structure. Acetylation of core histone molecules is catalyzed by histone acetyltransferase, and acetyl groups are removed by HDACs. Histone acetyltransferase acetylates the conserved ε-amino group of the lysine residue at the amino-terminus of the histone tail (Figure 1B). This decreases the overall positive charge, thereby decreasing histone affinity for negatively charged DNA, and providing a platform for the binding of transcription factors to the chromatin template.⁸¹ According to size, subcellular expression, number of enzymatic domains, and structure, HDACs are divided into 4 classes. Class I HDACs (HDACs 1, 2, 3, and 8) are detected in the nucleus and ubiquitously expressed in different tissues and cell lines. Class II HDACs (HDACs 4, 5, 6, 7, 9, and 10) shuttle between the nucleus and cytoplasm and are expressed in a tissue-specific manner. For example, human HDAC4 is more abundant in skeletal muscle, brain, heart, and ovary, but not detectable in liver, lung, spleen, and placenta. HDAC5 is expressed in mouse skeletal muscle, liver, and brain, but not in spleen.⁸² Class III HDACs are composed of NAD⁺-dependent deacetylases SIRT1 to SIRT7. Class III HDACs are structurally unrelated to class I and class II HDACs. They have a unique enzymatic mechanism of action that requires the cofactor NAD⁺ for their activity. HDAC11 is the only member of class IV,⁸³ and its classification is still under debate. Phylogenetic analysis shows that HDAC11 is closely related to HDAC 3 and HDAC8, suggesting that it might be closer to class I than class II. After TCR stimulation, murine CD4⁺CD25[−]

Figure 2. Foxp3 regulation from the various extracellular signals. Red arrow represents negative signal; and green arrow, positive signals for Foxp3 expression.



naive T cells and CD4⁺CD25⁺ nTregs do not show significant differences in the level of class I HDACs, whereas class II HDACs are mainly expressed in Tregs.⁸⁴ Little else is known about the function and distribution of class II HDACs in nTregs, suggesting an area for productive research.

The N-terminal region of the Foxp3 protein is proline rich, which makes Foxp3 different from the other family members Foxp1, Foxp2, and Foxp4, and this region of Foxp3 recruits the corepressor lysine acetyltransferase TIP60. TIP60 acetylates the Foxp3 protein, and in turn acetylated Foxp3 protein binds to its target gene promoter, such as IL-2, to repress its transcription. The N-terminal region of Foxp3 can also recruit the class II deacetylase HDAC7.⁸⁵ During T-cell activation, HDAC7 is recruited to the Foxp3 corepressor complex, deacetylates the Foxp3 protein, and thus inhibits Foxp3 function. It is important to note that HDAC7 can also deacetylate histones in the *Foxp3* promoter and repress transcription. HDAC inhibitors enhance Foxp3 expression in CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells, suggesting that HDACs directly regulate both the Foxp3 gene and protein and enhance the suppressive function of Tregs.^{84,86,87} Another class II deacetylase, HDAC9, interacts with Foxp3 and down-regulates its acetylation. Treatment with the HDAC inhibitor trichostatin A enhances Foxp3 acetylation and Treg function.⁸⁵

TGF-β induces chromatin binding and promoter occupancy of acetylated Foxp3 on the IL-2 promoter. Recently, it has been shown that TGF-β and IL-6 down-regulate the chromatin binding of acetylated Foxp3 to the human IL-2 promoter, and treatment with HDAC inhibitors under these conditions restores the binding of Foxp3 to the IL-2 promoter, suggesting that under inflammatory conditions HDACs play a role in the regulation of Foxp3 function.⁷⁴

Role of histone methylation in Treg development and function

Apart from inheritance of methylated CpG residues in genomic DNA, parental histone molecules can also divide and be transmit-

ted to the progeny cells. Among the 4 types of histones in the nucleosome, H3 and H4 histones are highly conserved and stably transmitted to progeny cells during replication. It has been proposed that methylated histones H3 and H4 act as epigenetic markers. Methylation of lysine residues in histones occurs at positions 4, 9, 27, and 36 in H3 and position 20 in H4. H3 is more heavily methylated and stable compared with H4. H3-K4 trimethylation is specific for the active state of transcription, and H3-K4 dimethylation exists in both active and repressed genes.⁸⁸ These methylated histones recruit methyl-binding proteins (eg, MBD1) and other transcriptional repressors that maintain CpG DNA methylation and regulate the transcription.

TCR signaling controls Foxp3 expression in CD4⁺ T cells. Sauer et al showed that TCR stimulation induces H3-K4 dimethylation and trimethylation after 18 hours of stimulation of naive CD4⁺ T cells at the Foxp3 promoter (−295 to −100 bp) and intronic enhancer (+4505 to +4505, +6144 to +6280).⁶² These regions are also H3-K4 methylated in Tregs.⁶² Continued TCR stimulation leads to loss of these epigenetic marks and subsequent inhibition of Foxp3 expression, suggesting that these epigenetic marks provide a tool for determining the permissiveness for Foxp3 expression in specific cell types. Schmidl et al elegantly analyzed more than 100 differentially methylated regions, including the entire *Foxp3* locus and associated upstream enhancers in human Tregs, and showed that lineage-specific CpG DNA methylation in T cells correlates with H3K4 methylation and its enhancer activity.²³

Stability of Treg

The stability of CD4⁺Foxp3⁺ T cells is an interesting area in Treg biology that has been recently reviewed.^{89,90} nTregs possess demethylated CpG at the *Foxp3* locus and show stable Foxp3 expression, whereas TGF-β induced Tregs show methylated CpG and do not maintain constitutive Foxp3 expression after restimulation in the absence of TGF-β.^{4,5} It has been reported that a fraction of Foxp3⁺CD4⁺ nTregs adoptively transferred into lymphopenic

mice converted into Foxp3⁺ T cells.⁹¹ Under inflammatory conditions, Foxp3⁺ Tregs lose Foxp3 expression and suppressive function in an IL-6–dependent manner.⁹² Yang et al reported that IL-6 and TCR signaling induced down-regulation of Foxp3 expression and led to development of Th17 cells.⁹³ Using Foxp3-GFP-Cre X ROSA26-YFP dual-reporter mice, Zhou et al showed that approximately 10% of YFP⁺ T cells lose their Foxp3 expression over time.⁹⁴ Recently, we showed that IL-6 induces remethylation of CpG DNA at the upstream enhancer and down-regulates Foxp3 expression in nTregs.⁴ It has been reported that Foxp3⁺CD4⁺ Tregs are a very heterogeneous population in mice^{91,95} and humans⁹⁶ and different subsets of Tregs possess different levels of CpG DNA methylation at the Foxp3 locus.⁹⁶ It has been shown that increased methylation of CpG nucleotides at the *Foxp3* locus was linked with less Foxp3 expression, decreased Treg stability, and reduced suppressive Treg function.^{4,71,96} Epigenetic inheritance during the cell cycle is crucial in maintaining chromatin structure in cell lineages.⁹⁷ The extrinsic and intrinsic signals that regulate CpG DNA methylation and perpetuate H3 methylation level at the *Foxp3* locus from one cell cycle to another are not understood and require further investigation. These studies will help us to understand the maintenance of *Foxp3* stability in Tregs.

Pharmacologic agents that alter epigenetically regulated genes

Epigenetic therapy is an emerging field in pharmacology that promises therapeutic agents for the control of various diseases.⁹⁸ Epigenetic drugs can be divided into 2 groups: DNMT or HDAC inhibitors. Some of these inhibitors are shown in Table 1. The prototypic inhibitors 5-azacytidine and Aza were developed as cytotoxic agents and subsequently have been discovered to possess potent DNMT inhibitor activity. These drugs are converted into nucleotide triphosphates and are incorporated in place of cytosine into replicating DNA. Therefore, they are more active in the S-phase of cells. 5-Azacytosine is incorporated into both RNA and DNA, whereas Aza is incorporated only into DNA and is less toxic compared with 5-azacytosine. The disadvantages of azanucleosides are instability in aqueous solutions and strong toxicity. These disadvantages might be overcome by the use of other analogs, such as zebularine, procainamide, 5-fluoro-2'-deoxycytidine, and hydralazine. Procainamide, used to treat cardiac arrhythmias, is a specific inhibitor of DNMT1.⁹⁹ Several natural products derived from tea and sponges, such as epigallocatechin-3-gallate, also show DNMT inhibitory activity.¹⁰⁰ Antisense oligonucleotides complementary to the human DNMT1 mRNA are in clinical trials.¹⁰¹

There are several HDACs inhibitors available, and some are used clinically for the treatment of neurologic disorders and cancer (Table 1). Importantly, the hydroxamate compounds trichostatin A and suberoylanilide hydroxamic acid have been shown to induce Tregs and prolong allograft function in mice.^{84,87,118} Other HDAC inhibitors are not as potent as hydroxamates for enhancing the suppressive function of Tregs.¹¹⁹

Epigenetic regulators as immunotherapy

There are a variety of methods by which Tregs can be generated, although many are laborious, inefficient, and expensive.^{120,121} The stability of Foxp3 expression in Tregs is very important for their therapeutic use. The conversion of antigen-specific Tregs into

Table 1. DNA methylation and HDAC inhibitors

Target/drugs
DNA methylation
Nucleoside analog inhibitors
5-Azacytidine ^{6,22,65}
5-Aza-2'-deoxycytidine (Decitabine) ^{4,6,39,69}
Zebularine ¹⁰²
5-fluoro-2'-deoxycytidine (FCDR) ¹⁰³
Non-nucleoside analog inhibitors
Procainamide ^{4,22,99}
Hydralazine ⁴
Procaine ¹⁰⁴
Epigallocatechin-3-gallate (EGCG) ¹⁰⁰
N-Phthalyl-L-tryptophan (RG108) ⁴
Antisense oligonucleotides
DNMT1 ASO ¹⁰¹
HDAC inhibitors
Hydroxamates
Trichostatin A ⁸⁷
Suberoylanilide hydroxamic acid (Vorinostat; SAHA) ¹⁰⁵
Oxamflatin ¹⁰⁶
Scriptaid ¹⁰⁷
Pyroxamide ¹⁰⁸
M-carboxycinnamic acid bishydroxamide (CBHA) ¹⁰⁹
Cyclic tetrapeptides
Desipeptide (FK228) ¹¹⁰
Apicidin ¹¹¹
Trapoxin-A and trapoxin B ¹¹²
Aliphatic acids
Valproic acid ¹¹³
Phenyl butyrate ¹¹⁴
Benzamides
MS-275 ¹¹⁵
N-acetylaldinaline (CI-994) ¹¹⁵
Electrophilic ketones
Trifluoromethyl ketones ¹¹⁶
α-Ketoamides
Miscellaneous compounds
Depudecin ¹¹⁷

effector T cells will have detrimental effects and limit clinical applicability.¹²² Epigenetic regulation may be an efficient therapeutic strategy for generation of stable Tregs and suppression. In vivo injection of HDAC inhibitors increases Treg numbers and function in mice⁸⁷ and rhesus.¹¹⁸ DNMT inhibitors induce strong Foxp3 expression,^{4,6} but associated cell toxicity as well as induction of Th1 and Th2 cytokines limit its use. To overcome these problems, we cultured naive CD4⁺ T cells with low doses of DNMT inhibitors under TGF-β–induced Treg conditions. After 24 hours, DNMT inhibitor was removed and the T cells further cultured under TGF-β–induced Treg conditions. After this brief exposure, the *Foxp3* enhancer was demethylated and stable Foxp3⁺ Tregs were generated, without indiscriminately activating other gene loci.⁴ These Tregs were more stable than conventional TGF-β–induced Tregs and were able to protect from autoimmune colitis and prolong allogeneic islet survival. It has been reported that prolonged in vitro culture of human Tregs leads to methylation of Foxp3 locus and decreased expression of Foxp3,¹²³ and TGF-β–induced human Tregs are not stable and do not have efficient suppressive function.^{3,4} Using the epigenetic approach, we also generated enhanced Foxp3⁺-suppressive human Tregs that could be used as cell therapy.⁴ Human Foxp3⁺CD4⁺ T cells are very heterogeneous and, based on CD25, CD62L, CD45RA, HLA-DR, and ICOS expression, can be divided in different subsets. Miyara et al reported 3 deferent populations of Foxp3⁺CD4⁺ T cells in the

peripheral blood mononuclear cells of a healthy man.⁹⁶ CD45RA⁺Foxp3^{lo} resting Tregs and CD45RA⁺Foxp3^{hi} activated Tregs show completely demethylated CpG at the proximal promoter (−256 to −16 bp) and more than 85% demethylation of the intronic region promoter (+3824 to +3937 bp), and are suppressive.⁹⁶ However, CD45RA⁺Foxp3^{lo} Tregs show decreased demethylation at the proximal promoter and less than 50% demethylation at the intronic region, secrete cytokines such as IL-2 and IFN- γ , and do not show suppressive function.⁹⁶ These findings suggest that epigenetic mechanisms provide better strategies to differentiate suppressive Tregs, understand the etiology of immunologic diseases resulting from reduced Treg-suppressive function, and help in designing better approaches to generate suppressive human Tregs.

Perspective on epigenetics in Treg biology

In humans, 1% to 3% of total T cells are CD4⁺CD25⁺Foxp3⁺ Tregs. Tregs have been used experimentally for the tolerance induction and the control of autoimmunity.^{2,121,124} These studies suggest that Treg adoptive therapy may provide an effective approach to control alloreactivity. Remaining to be elucidated is the regulation of Foxp3 under inflammatory conditions, such as bone marrow transplantation, where the conditioning regimen leads to enhanced inflammatory responses, or in solid organ transplantation, where inflammation is enhanced by ischemia and reperfusion injury.^{125,126} Recent advances in Treg biology, such as the role of SIP₁ in T-cell migration²⁸ as well as its interference in the suppressive function of Tregs,⁶¹ provide avenues for the development of therapeutic agents. Further studies about Akt-mTOR signaling on epigenetic modification at the *Foxp3* locus may provide a better understanding for the use of chemotherapeutic agents, such as FTY720 and rapamycin in the generation of stable

Foxp3⁺ Tregs. Similarly, the use of immunotherapeutic drugs that inhibit the down-regulation of Foxp3 under inflammatory condition may be potent strategies to enhance the function of Tregs. DNMT and HDAC inhibitors may be candidates as they enhance Foxp3 expression as well as inhibit the effect of the proinflammatory cytokine IL-6 on Foxp3 regulation and function.^{4,74} How epigenetic mechanisms work under different inflammatory and tolerance conditions and regulate Foxp3 expression are fertile areas for further investigation. Understanding what cytokines and surface receptors are involved in the epigenetic regulation of Treg development and function will provide a better approach to generate Tregs for therapeutic use. Global genome-wide analysis of epigenetic markers in Tregs will enrich our ability to design epigenetic therapy. A future challenge is the development of cell type-specific targeting of DNMTs and HDACs to provide better epigenetic therapeutics.

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