# Molecular Mechanisms of Regulatory T Cell Development and Suppressive Function

Jeong M. Kim

*Genentech, 1 DNA Way, South San Francisco, California, USA*



The requirement for regulatory T cells (Treg) to maintain tolerance to self-tissues is evidenced by fatal autoimmune disease that results from genetic deficiencies in Treg cell development or Treg cell depletion *in vivo*. These observations revealed that a normal T cell repertoire harbors self-reactive T cells



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that are kept dormant by Treg cells. In order to prevent auto-reactive T cell activation, Treg cells disarm antigen-presenting cells (APC) through multiple suppressive mechanisms including B7 signaling and sequestration, ATP catabolism, cytolysis, and immunosuppressive cytokine secretion. In addition to APCs, multiple leukocyte subsets are subjected to Treg cell mediated suppression. The acquisition of suppressive activity occurs concomitantly with Treg cell lineage commitment. The identification of molecular cues that guide differentiation of Treg cells versus auto-reactive cells or other $CD4^+$ T cell subsets have been aided by the differential expression of the transcription factor Foxp3 by Treg cells. Foxp3 is the most faithful marker for Treg cells and in its absence, Treg cell development is abrogated. Utilizing Foxp3 expression as a surrogate for Treg cell commitment, factors that promote Foxp3 transcription have provided new insights to Treg cell development at a molecular level.

The recent resurgence of Treg research has been instigated by the identification of CD25 as a marker for Tregs.[1] Prior to this, the very existence of Tregs was questioned due to the relative impurity of fractions containing suppressive activity. Based on CD25 expression, it is now known that Tregs constitute approximately 10% of $CD4^+$ T cells in mice and 5% of the human $CD4^+$ T cell subset. Studies that removed Tregs from the T cell repertoire have revealed a previously unappreciated pool of self-reactive T cells harbored by a normal immune system. Manipulating Treg numbers unleashed pathogenic T cell mediated gastritis, thyroiditis, glomerulonephritis, sialoadenitis, adrenalitis, arthritis, insulitis, and oophoritis. The breadth of target organs and the severity of autoimmunity induced by Treg depletion emphasizes the incomplete nature of clonal deletion of self-reactive T cells during thymic development. Conversely, Treg transfer ameliorates or provides complete protection in mouse models of type 1 diabetes mellitus, inflammatory bowel disease, systemic lupus erythematosus, and multiple sclerosis.[2–5] Although perturbations in Treg numbers or function have been implicated in nearly all autoimmune diseases, single nucleotide polymorphisms in genes that influence Treg dynamics have been detected in type 1 diabetes mellitus and autoimmune thyroiditis, suggesting that Treg impairment can be a primary cause of human disease development.[6–9] The most prominent human disease linked to Treg dysfunction is immune dysregulation polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, which is characterized by early onset of multiorgan autoimmunity.

Positional cloning of the gene responsible for causing the rare autoimmune disorder IPEX identified loss of function mutations in *Foxp3*.[10–12] In parallel, the causative mutation of the mouse analog of IPEX was found in the *Foxp3* gene,

providing a mouse model of Treg deficiency.[13] In mice and IPEX patients with a nonfunctional *Foxp3* allele, Treg cell development is completely blocked. [14–16] Treg cell deficiency is primary for autoimmunity development in *foxp3*$^{-}$ mice because transferring wild-type Tregs prevents multiorgan autoimmunity and lymphoproliferative disorders.[17] The *Foxp3* gene encodes a transcription factor that is selectively expressed by Tregs. In contrast to other Treg cell markers such as CD25, non-Tregs do not express Foxp3 upon standard mitogenic stimulation. Furthermore, ectopic Foxp3 expression in effector T cells partially transfers suppressive activity, and continual Foxp3 expression is required to maintain suppressive activity because conditional Foxp3 deletion in peripheral Tregs abrogates suppressive function.[15,17] Thus, Foxp3 is necessary for Treg cell development, sufficient to initiate suppressive function, and serves as the most reliable marker for Tregs.

Based on the importance of Foxp3 in Treg cell development and its effector function, recent Treg research has focused on identifying Foxp3 target genes, signaling pathways and transcription factors that regulate Foxp3 expression, and the mechanism of Foxp3 activity. This chapter will focus on the developmental origins and suppressive function of Tregs.

## I. Tracing Thymic Treg Development with Foxp3

The developmental origins of Treg cells were examined using Foxp3 expression as a surrogate marker for Treg lineage commitment. Within the thymus, approximately 90% of Foxp3$^{+}$ cells reside within the CD4 single positive (SP) subset.[18,19] More than 80% of Foxp3$^{+}$ CD4 SP cells express low levels of CD24 or HSA, a marker for immature SP thymocytes. Other thymocyte subsets comprise a minor proportion of Foxp3$^{+}$ cells, with CD8 SP constituting 4% and CD4 CD8 double positive (DP) 2% of Foxp3$^{+}$ thymocytes. The remaining unaccounted populations of Foxp3 expressing cells display lower levels of CD4 and/or CD8, suggesting these cells are not bona fide CD4 CD8 double negative thymocytes. Among DP cells, Foxp3$^{+}$ cells express CD69, lower levels of HSA, and higher levels of TCRβ, indicating that Treg lineage commitment is either coincident or occurs after positive selection of thymocytes that recognize self-peptide MHC complexes.[19] Thus, the majority of Treg differentiation occurs late in thymocyte development.

Consistent with Foxp3 expression occurring during the later stages of T cell development, the majority of Foxp3$^{+}$ thymocytes are located within the thymic medulla.[18] In human Treg cell development, medulla-resident cells, including reticular epithelial cells and dendritic cells (DCs), collaborate to promote Foxp3 expression.[20] The medullary determinants that guide Foxp3 expression in CD4 SP cells have not yet been identified. High levels of B7 expression by medullary DCs may contribute to costimulating Treg cell precursors, thus

providing sustained TCR signaling. Consistent with this hypothesis, deficiency of B7.1 and B7.2 or their ligand CD28 causes a dramatic reduction in Treg numbers.[21,22] Although the highest concentrations of thymic Treg cells are detected in the medulla, Treg lineage commitment also occurs in the cortex.[23,24] The contribution of the cortical $Foxp3^+$ cells to the medullary pool of $Foxp3^+$ cells is not known, making the precursor–progeny relationship between $Foxp3^+$ DP and SP ambiguous. It is possible that only a small fraction of $Foxp3^+$ CD4 SP cells are derived from $Foxp3^+$ DP cells may be a possibility. Differences in the nature of peptides presented by cortical and medullary APCs are predicted to generate distinct Treg repertoires, underscoring the importance of the site of Treg cell lineage commitment.

Applying the signal strength model for thymocyte selection, TCR avidities for self-antigens that lie between positive and negative selection are thought to promote Treg cell differentiation.[25–29] Provision of cognate antigens to TCR transgenic thymocytes in "double transgenic" systems induces negative selection of self-reactive T cells and increases both Treg frequency and absolute number. Thus, TCR avidities that minimize clonal deletion while maintaining the quality of TCR signaling are thought to induce Treg cell development. The molecular factors that differentiate self-reactive cells that are culled from the peripheral T cell repertoire from the Treg cells are largely unknown. However, it is hypothesized that Foxp3 expression confers selective survival of thymocytes that react to self-antigens.[30]

In addition to signals derived from the TCR complex, IL-2 receptor signal transduction is required for optimal Treg generation. TCR and IL-2 signaling are thought to occur sequentially as transcription of the IL-2 receptor-α or CD25, the high affinity subunit, is controlled by TCR signaling. Once CD25 is upregulated in a TCR-dependent manner, antigen-independent activation of the IL-2 receptor completes Treg cell differentiation. Kinetic analysis of Treg development in neonates and adult mice has demonstrated that $Foxp3^-CD4^+CD25^+$ SP cells precede $Foxp3^+CD4^+CD25^+$ SP cells.[14,19] Interestingly, a sharp reduction in $Foxp3^-CD4^+CD25^+$ SP cells immediately precedes a wave of $Foxp3^+$ cell production, suggesting that $Foxp3^-CD4^+CD25^+$ SP cells serve as Treg precursors. In support of this "two-step" model of Treg differentiation, $Foxp3^-CD4^+CD25^+$ SP thymocytes are more capable of inducing Foxp3 transcription than their $CD25^-$ counterparts *in vivo* and *in vitro*. Furthermore, TCR sequences derived from $Foxp3^-CD4^+CD25^+$ SP thymocytes largely overlap with $Foxp3^+$ TCRs; this is consistent with the notion that a majority of $Foxp3^-CD4^+CD25^+$ SP cells are poised to express Foxp3 and represent a transitional stage between a non-Treg and Treg cell.[31] Genetic deficiencies in the alpha, beta, or gamma subunits of the IL-2 receptor complex, or Stat5, the transducer for IL-2 signaling, profoundly impair Treg development.[32,33] Conversely, expression of a constitutively active Stat5 transgene enhances Treg cell development.[34]

## A. Genes Regulated by Foxp3

Genome-wide expression profiling of Treg cells with multiple permutations of Foxp3 status has been conducted. Microarray experiments using Treg cells isolated from Foxp3 reporter mice, T effector cells ectopically expressing Foxp3, Treg cells that no longer express Foxp3, and Treg cells that cannot express Foxp3 were designed to identify genes differentially expressed in Tregs and Foxp3 target genes. Combined with Foxp3 chromatin immunoprecipitation (ChIP) to identify genes that associate with Foxp3 protein, a list of approximately 300 Foxp3 gene products has been generated.[35,36] Although differences in methodology and fold-change cutoff yield variable results, reports from multiple laboratories have reached the consensus that Foxp3 serves as a transcriptional activator in three times the number of genes in which it serves as a repressor.

Based on gain-of-function activity by ectopic expression, Foxp3 has been dubbed as a "master regulator" of Treg development.[15,17] However, more recent analyses of Treg cells expressing a functionally null ($T_{FN}$) Foxp3 and a closer examination of Foxp3 target genes have challenged this notion.[37–39] $T_{FN}$ were engineered by replacing Foxp3 with GFP expression, permitting tracking and isolation of these cells. Surprisingly, approximately 50% of the Treg gene expression signature is maintained in $T_{FN}$. Corroborating these results, ectopic Foxp3 expression in non-Treg cells reproduces only 40% of the Treg cell transcriptional signature.[38] Although the Foxp3-dependent transcriptional program is essential for Treg effector function, Foxp3 does not serve as a bona fide master regulator in the manner that MyoD and Eyeless determine muscle and eye differentiation respectively.

## B. Foxp3 Binding Partners

The identification of Foxp3 binding partners provided new insights into how Foxp3 mediates transcriptional activation and silencing. The transcription factor Runx1 normally transactivates IFN-γ and IL-2 in effector T cells. However in Tregs, Foxp3 physically interacts with Runx1, preventing Runx1 transcriptional activity at the IL-2 and IFN-γ loci.[40] Similarly, Foxp3 binds to the transcription factors NFAT and ROR-γt to silence their target gene expression.[41,42] In effector T cells, NFAT activates IL-2 transcription and ROR-γt promotes IL-17 expression. In Tregs, NFAT or ROR-γt, when complexed to Foxp3, fails to promote IL-2 and IL-17 transcription, respectively. How Foxp3 binding suppresses NFAT, ROR-γt, or Runx1 transcriptional activity is largely unknown.

Besides binding to and inhibiting gene-specific transcriptional activators, Foxp3 binds to the transcriptional repressors Eos and C-terminal binding protein (CtBP).[43] Eos is a member of the Ikaros family of transcription factors that associates with CtBP to mediate gene silencing. Silencing Eos expression in Treg cells induces the upregulation of normally suppressed Foxp3 target genes including

*Ifng* and *Il2*. Eos was demonstrated to suppress a total of 52 Foxp3 gene products. It remains to be determined if Eos is recruited to NFAT:Foxp3 protein complexes to suppress *Il2* transcription. Extending the Eos:Foxp3 model for target gene silencing, additional transcriptional repressors may associate with Foxp3 to inhibit the expression of genes unaccounted for by the Eos:Foxp3 complexes.

## II. Acquisition of Foxp3 Expression by Peripheral $CD4^+$ T Cells

Naïve $CD4^+$ T cells differentiate into specialized effector cells under the guidance of cytokines during T cell activation. The discovery that TGF-β administration during T cell activation induces Foxp3 expression and suppressive activity raised the possibility that Treg cells can be extra-thymically generated from naïve $CD4^+$ T cells.[44] As an extension of the $T_H1$–$T_H2$ model of helper T cell differentiation, TGF-β serves as the instructional cytokine that activates Smad transcription factors. Licensed Smad family members translocate to the nucleus to bind to DNA elements in the Foxp3 regulatory loci and enhance Foxp3 transcription. Experimentally, activating naïve $CD4^+$ cells in the presence of TGF-β yields T cell cultures comprising up to 90% $Foxp3^+$ cells.[45] TGF-β induced Treg (iTreg) cells share many characteristics with the natural regulatory T cells including *in vitro* anergy, and comparable CTLA-4 and CD45RB expression levels.[46] Additionally, iTregs produce lower levels of IFN-γ and IL-4, confirming previous reports that TGF-β attenuates $T_H1$ and $T_H2$ differentiation, respectively. However, the suppressive quality and the stability of these iTreg cells remain controversial. In some studies, iTregs were as suppressive as thymically derived natural Tregs (nTregs) in inhibiting $CD4^+$ cell proliferation *in vitro* and *in vivo*. Other reports provided evidence for partial acquisition of suppressor activity and unstable expression of Foxp3.

In *in vitro* culture and *in vivo* transfer systems, TGF-β responsiveness is critical for iTreg differentiation. In tissue culture, TGF-β induces expression of the Treg markers Foxp3 and CD25 in a dose dependent manner. *In vivo* conversion of TCR-transgenic $Foxp3^-CD4^+$ effector T cells to Treg is severely compromised in cells expressing a dominant negative TGF-β receptor.[47] Therefore, it is hypothesized that TGF-β responsiveness is essential for peripheral acquisition of suppressive activity.

There are two overlapping mechanisms of TGF-β action during peripheral $CD4^+$ T cell stimulation. First, TGF-β directly promotes Foxp3 transcription through Smad3–Foxp3 enhancer interactions.[48] Additionally, TGF-β indirectly promotes iTreg differentiation by the dampening of TCR signal transduction and the ensuing mitogenesis, which are conditions favorable for Foxp3 induction.[47]

## A. iTreg Cell Generation Is Linked to Suboptimal and Sterile T Cell Stimulation

While nTreg cell development in the thymus is associated with high affinity TCR–MHC peptide interactions, iTreg cell differentiation in the periphery is induced under sub-immunogenic conditions.[47,49,50] In studies that utilized T cell transfer models, the largest induction of Foxp3 in peripheral T cells occurred when the TCR transgenic T cells were primed with low doses of their cognate ligand. *In vitro* stimulation of T cells with TGF-β and low concentrations of anti-CD3 antibodies produced the highest frequency of Foxp3 expressing cells.[51,52] Conversely, increasing TCR-derived signals through deficiencies in negative regulators of TCR signaling such as CTLA-4 and Cbl impaired iTreg generation.[53,54] Suboptimal TCR signaling impairs proliferation and accordingly, Foxp3 expression is highly enriched in peripheral T cells that have undergone the fewest rounds of cell divisions.[47,50] As TGF-β is known to inhibit TCR-dependent expansion, TGF-β partly promotes Treg cell differentiation by limiting T cell proliferation.

Optimal iTreg generation is associated with nonimmunogenic antigen delivery methods such as oral or intravenous injections, peptide pumps, or antibody-mediated DC targeting in the absence of adjuvants.[47,50] Administering LPS with antigen abrogates iTreg cell differentiation, suggesting that inflammatory cytokine environments are incompatible with iTreg differentiation.[50] *In vitro*, the addition of the $T_H1$ or $T_H2$ instructive cytokines, IL-12 or IL-4, respectively, inhibits TGF-β dependent iTreg generation.[55] Conversely, TGF-β antagonizes IL-12 or IL-4 activity in promoting the $T_H1$ and $T_H2$ cell lineage specification.[56,57] Mechanistically, TGF-β impairs $T_H1$ and $T_H2$ development by reducing the expression of T-bet and Gata-3, transcription factors that are required for $T_H1$ and $T_H2$ differentiation respectively.[58–60] Thus, TGF-β induces iTreg generation while simultaneously repressing differentiation of alternate lineages.

The physiological correlate of experimentally derived iTreg cells has not been identified. Teleogically, iTreg cells may arise in response to self-antigens that are not expressed in the thymus in TGF-β-rich environments. One potential anatomical location of iTreg generation is the gut-associated lymphoid tissue. Bacterial antigens derived from commensal microflora and antigens administered orally are tolerated by our immune system through undefined mechanisms. Interestingly, the highest frequencies of $CD4^+$ $Foxp3^{GFP-}$ cells that had acquired Foxp3 expression in lymphopenic recipients were detected in the lamina propria.[61] The APC subset that is responsible for the enhanced Foxp3 acquisition in gut-associated lymphoid tissue was identified as $CD103^+$DCs.[61,62] CD103 is a transcriptional target of TGF-β signaling, suggesting that these DCs were exposed to TGF-β. Not only are $CD103^+$ DCs recipients of TGF-β signals, but they are also expressors of elevated levels of *Tgfb2* and other genes required for TGF-β processing, as revealed by transcriptional profiling experiments. [61] In addition

to TGF-β, $CD103^+$ DCs produce the vitamin A metabolite, retinoic acid, which augments the ability of TGF-β to induce Foxp3 expression.[51,61,62] Although, the physiological relevance of retinoic acid in iTreg generation is controversial, it is widely accepted that iTreg cell differentiation is potentiated by a subset of naturally occurring DCs that are enriched in gastrointestinal tissue.[63,64]

### B. TGF-β Signaling Directly Influences Foxp3 Transcription

TGF-β signal transduction results in the activation and nuclear translocation of the transcription factor, Smad3. Among the multiple transcriptional target genes, active Smad3 binds to a conserved noncoding sequence (CNS) in the *Foxp3* locus that displays enhancer activity in reporter gene assays. Smad3 occupancy at the TGF-β responsive element in the *Foxp3* locus is correlated with Foxp3 transcription and demethylated cytosines within the *Foxp3* promoter. In contrast, effector T cells stimulated in the absence of TGF-β, and therefore Foxp3 negative, show methylated CpG dinucleotides in the *Foxp3* promoter. TGF-β may antagonize the function of a DNA methylase at the *Foxp3* locus or indirectly promote CpG demethylation by recruiting transcription factors to occupy the CpG islands.

At the transcriptional level, TGF-β signaling induces and represses the expression of most Foxp3 dependent transcriptional targets that define the "natural" Treg cell signature. In addition to the canonical Treg cell genes, TGF-β influences the transcription of nearly 2000 gene products, only 10% of which are encompassed within the Treg cell transcriptional signature. Therefore, TGF-β induces the expression of genes beyond the Treg transcriptome, and iTregs are not equivalent to nTregs by gene expression analysis.

## III. Bottom-Up Approach to Analyzing Treg Cell Differentiation

Reliable Foxp3 expression in Treg cells suggests that identifying transcription factors that induce Foxp3 expression will provide new insights into Treg cell differentiation. CNSs within the Foxp3 promoter and within the first intron that could serve as enhancer elements were tested for transcriptional activity.[65] Consensus binding sites for basal transcription factors, including the CAAT, GC, and TATA box were identified −217, −138, and −44 base pairs (bp) upstream of the transcriptional start site, respectively, in the human Foxp3 promoter designated as CNS1.[66] Upstream of these consensus motifs for core transcription factors, evolutionarily conserved nucleotides that conform to AP-1, NFAT, NFκB, and Runx binding sites were identified within CNS1 (Fig. 1). Given the importance of TCR signal transduction in Treg cell lineage

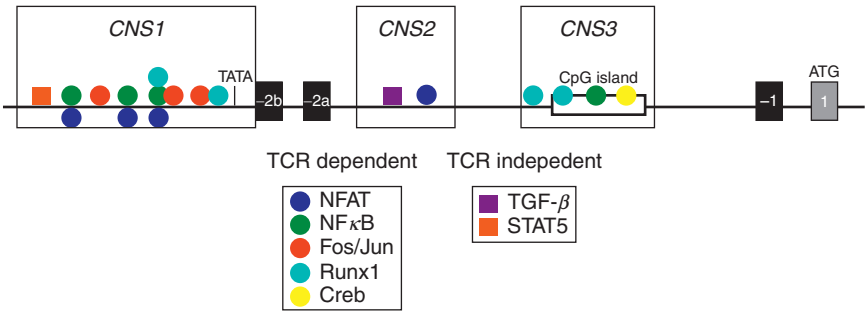


FIG. 1. Schematic map of *Foxp3* CNS 1–3 and its associated transcription factors. The relative location the TCR dependent and cytokine dependent transcription factors are represented as circles and squares, respectively. The CpG methylation island located in CNS3 is designated as a white rectangle.

commitment, it is hypothesized that AP-1, NFAT, and NFκB comprise the TCR response elements within the Foxp3 promoter, similar to the convergence of these transcription factors on the IL-2 promoter to mediate TCR-dependent mitogenesis. In addition to these TCR-derived signals, the Foxp3 promoter is also sensitive to IL-2 administration and harbors a consensus STAT5 binding motif. Although IL-2 and its downstream signaling component STAT5 were known to be required for Treg cell competitive fitness, this finding implicated IL-2 signaling in directly influencing Foxp3 expression.

Two evolutionarily conserved sites found within the first Foxp3 intron were designated CNS2 and CNS3.[65] When sequences derived from either CNS2 or CNS3 were added to the Foxp3 promoter in luciferase assays, reporter activity significantly increased over the Foxp3 promoter alone.[48] As with CNS1, the ability of either CNS2 or CNS3 to augment Foxp3 reporter activity was dependent on TCR engagement. In accordance with this notion, NFAT and NFκB were shown to occupy CNS2, and CREB/ATF and NFκB occupied CNS3 in a TCR-dependent manner. CNS3 also contains a SMAD binding site that is responsible for Foxp3 induction in response to exogenous TGF-β treatment and TCR crosslinking. The evidence and relative contribution of each transcription factor for stable Foxp3 expression are individually discussed below.

## A. NFAT and AP-1

The NFAT family of transcription factors has been shown to bind to the Foxp3 promoter and a TCR-responsive enhancer element in CNS2.[48,66] Three NFAT binding sites reside within 500 bp upstream of the Foxp3 transcriptional start site.[66] Mutations in any of the NFAT binding sites within the Foxp3 promoter result in a twofold decrease in luciferase reporter activity.

Of the four NFAT transcription factors, only NFATc1 binds to CNS2.[48] In contrast, only NFATc2 was shown to bind to the Foxp3 promoter through chromatin IP or nucleotide pull-down assays.[66,67] However, the ability of other NFAT family members to bind to the Foxp3 promoter has not been tested.

In T cells, regulation of NFAT activity is mediated by TCR-induced calcium flux. TCR clustering initiates the activation of tyrosine kinases that eventually phosphorylate LAT, a plasma membrane protein that serves as a key platform for recruiting cytosolic signaling molecules to the cell surface. One key step in the initiation of calcium signaling is recruitment of PLC-γ1 to LAT. PLC-γ1 binding to LAT is regulated by the phosphorylation of tyrosine 136 on LAT. Gene targeted mutagenesis of LAT Y136 to abrogate PLC-γ1 recruitment results in impaired Treg cell development.[68] Once PLC is recruited to LAT and activated, it generates second messengers for calcium release from the endoplasmic reticulum. Calcium release from the intracellular endoplasmic store induces the activation of STIM1 and STIM2, which are essential components that sustain calcium signaling for NFAT activation. T cell specific elimination of STIM1 and STIM2 causes a near complete cell-intrinsic block in Treg cell development.[69] Cytosolic calcium binds to Calcineurin, the target of the immunosuppressant drug Cyclosporine A. Upon calcium binding, Calcineurin licenses NFAT family members for nuclear translocation and transcriptional activity. Inhibiting Calcineurin, and thus NFAT activity, through Cyclosporine A treatment in neonatal mice induces multiorgan autoimmunity that is similar in severity and kinetics to that in neonatally thymectomized mice.[70,71] In hindsight, impaired Treg cell development in the presence of Cyclosporine A may be caused by reduced NFAT activation and thus, NFAT-dependent Foxp3 expression. Additionally, pharmacological inhibition of NFAT activation through Cyclosporine A treatment impairs TCR-induced Foxp3 expression in human $CD4^{+}CD25^{-}$ effector T cells.[66]

## B. Creb/ATF2

CNS3 contains a CpG island and the Creb binding site, 5′-TGACGTCA-3′, which matches the consensus Creb binding motif, 5′-TGAnnTCA-3′.[65] In luciferase reporter assays, disruption of the Creb binding site *in cis* or expression of a dominant negative Creb construct *in trans* abrogates TCR-induced enhancer function.

Creb transcriptional activity is regulated by serine 216 phosphorylation. Multiple kinases including PKA, CamK, and Rsk are capable of phosphorylating and activating Creb, thus invoking the cyclic AMP (cAMP)-, calcium-, and Ras-dependent signal transduction pathways. In T cells, Creb is primarily activated by Rsk2, an upstream kinase in the MAPK pathway, suggesting that Creb activity in Treg cells is governed by TCR-induced signaling via the RAS-MAPK pathway.[72]

## C. CpG Methylation by Dnmt1

Pharmacological inhibition of DNA methyltransferase activity by 5-azacytidine treatment permits Foxp3 expression in non-Treg cells.[65,73] This result was independently confirmed using shRNA mediated reduction of Dnmt1, the major DNA methyltransferase active during cell division and in Dnmt1 deficient T cells.[65,74] Thus, repression of Foxp3 expression in non-Treg cells is mediated by DNA methylation.

Impairments in DNA methylation can influence the expression of multiple gene products that indirectly promote Foxp3 expression. Alternatively, it may directly affect Foxp3 transcription via methylated CpG islands within the Foxp3 promoter and enhancer regions.[65,74] Within the Foxp3 locus, methylated CpG islands have been detected in CNS3 and in the Foxp3 promoter. As expected, these CpG islands are completely demethylated in natural Treg cells. Conversely, the CNS3 CpG island in $CD4^{+}CD25^{-}$ effector T cells is completely methylated whereas the promoter is partially methylated. Artificially methylating the CNS3 CpG island abolishes luciferase reporter activity, suggesting that a demethylated CNS3 is essential for Foxp3 transcription.

## D. Runt Domain Containing Transcription Factors—Runx

Runx transcription factors were initially implicated in Treg cell effector function as Foxp3 interacting partners.[40] The Runx transcription factor family is comprised of Runx1 (AML1), Runx2 (AML3), and Runx3 (AML2). For transcriptional functionality, Runx transcription factors have to heterodimerize with their binding partner CBFβ. Since overexpressed Foxp3 can co-immunoprecipitate with all three Runx proteins, definitive analysis of the requirement for Runx family members in Treg cell biology was assessed in mice expressing Cre in T cells or Tregs, and a *CBF*β floxed allele (referred to as $CBF\beta^{F/F}$ mice). Unexpectedly, Tregs isolated from $CBF\beta^{F/F}$ mice expressed lower amounts of Foxp3 protein on a per cell basis, which correlated with a decrease in Foxp3 transcript levels.[75–78] The conditional deletion of Runx1 or RNA silencing in Treg cells phenocopied the decrease in Foxp3 expression observed in $CBF\beta^{F/F}$ Treg cells, indicating that, of the three Runx transcription factors, Runx1 plays a nonredundant role. In contrast, Runx3 deficient Treg cells expressed wild-type Foxp3 levels, while a requirement for Runx2 in Treg cells has not been determined.[75,78]

ChIP with anti-CBFb antibodies revealed that the Runx complex bound to the Foxp3 promoter (CNS1) as well as CNS2 and CNS3.[75–77] Within these Foxp3 regulatory regions, two Runx-binding sites were identified in both CNS1 and CNS2. Mutations in putative Runx-binding sites in either CNS1 or CNS3 did not reduce reporter gene activity in unstimulated cells, but CNS1 mutants

showed impairment of PMA and ionomycin-induced increase in transcription. Within CNS1, the predicted 5′ Runx-binding site overlaps with a NFAT binding site, which is required for optimal Foxp3 expression. Therefore the relative contribution of Runx-binding to CNS1 for Foxp3 transcription is not clear.

Given the limitations of episomal reporter constructs to monitor gene transcription in its native chromosomal context and the dramatic reduction in Foxp3 expression compared to only marginal differences in reporter gene activities, the Runx transcription factor complex was hypothesized to mediate Foxp3 transcription by influencing chromatin dynamics. In support of this notion, ChIP with anti-H3-K9me3, a histone modification associated with gene silencing precipitated DNA segments distributed near the Foxp3 promoter in *CBF*$\beta^{F/F}$ Treg cells at a higher frequency compared to *CBF*$\beta^{WT}$ Treg cells.[76] Thus, the Runx–CBF complex is thought to recruit chromatin modifying molecules to the Foxp3 locus in Tregs.

## E. NFκB/c-Rel

Mice engineered with a genetic deficiency in components of the TCR-dependent NFκB signal transduction pathway, including BCL10, Carma1, TAK1 and IKK2, show a reduction in Treg absolute number and frequency.[79,80] Genetic complementation of Carma1 or TAK1 deficiency with constitutively active IKK-β transgene (IKKEE) restores Treg cell numbers in the thymus and periphery, further validating a role for NFκB in Treg cell development.[81] Additionally, enforcement of NFκB activation during thymic development elevates the frequency of Foxp3$^+$ cells among CD4 SP thymocytes in IKKEE transgenic mice on a wild-type background. In aggregate, these data are compatible with the hypothesis that transcriptional targets of NFκB are necessary for Treg cell differentiation. However, the identification of consensus NFκB binding sites within the Foxp3 promoter and CNS3 suggest that impaired Treg development in mice with attenuated NFκB signaling is due to reduced Foxp3 expression.[81]

Two putative NFκB binding sites at −382 and −327 bp upstream of the Foxp3 transcriptional start site were confirmed through luciferase reporter gene analysis.[67] The relative contribution of each NFκB binding site to Foxp3 expression is not known as comparisons of individual NFκB-binding mutants have not been conducted. However, mutating the 5′ NFκB binding site is sufficient to abrogate c-Rel mediated Foxp3 expression. As both the NFAT and NFκB proteins employ a Rel homology domain for DNA binding, the c-Rel binding domain in the Foxp3 promoter overlaps with the NFATc2 site.[67] Thus, it is plausible that the consensus sequence, TTCC, accommodates both NFAT and NFκB. Indeed, nucleotide pull-down assays demonstrated that Foxp3 promoter sequences are

capable of precipitating both NFATc2 and NFκB. Furthermore, complexes containing both NFATc2 and NFκB were detected in sequential chromatin IP using antibodies to both transcription factors.

From sequence analysis, it is observed that CNS3 contains three potential NFκB docking sites. Although these three sites are separated by approximately 500 bp, c-Rel chromatin IP primarily pulled down the second NFκB binding site that resides within the CpG island.[81] NFκB occupancy at this site correlates with demethylation of the CpG island.[67] Whether c-Rel binding recruits chromatin remodeling enzymes or prevents DNA methyltransferase binding such as Dnmt1 has not been determined.

Among the NFκB transcription factors, c-Rel mediated the largest induction of Foxp3 reporter activity in a transient overexpression system, whereas, p65 yielded a threefold lower signal than c-Rel in PMA and ionomycin treated cells.[81] However, RelB or p50 overexpression does not enhance reporter activity, suggesting that the NFκB binding sequences within the Foxp3 regulatory region display specificity for c-Rel and p65. In agreement with this notion, c-Rel deficiency dramatically impairs Treg cell differentiation.[67] Total thymic cellularity and $Foxp3^{-}CD4^{+}$ SP cellularity were reduced only by 20% in *c-Rel*$^{-/-}$ mice whereas $Foxp3^{+}CD4^{+}$ SP was reduced by 90%, suggesting that Treg cell development is more sensitive to c-Rel deficiency than $CD4^{+}$ effector cell development.

## F. TGF-β/SMAD3

Optimal thymic and extrathymic Treg cell differentiation is dependent on TGF-β signaling.[82] Experimentally, TGF-β is best known for its ability to induce Foxp3 expression by peripheral $CD4^{+}$ T cells when combined with reagents that mimic TCR engagement.[44] Through unclear mechanisms, TGF-β attenuates TCR-induced activation and proliferation. As the acquisition of Foxp3 expression by peripheral T cells is correlated with suboptimal TCR signaling and fewer cell divisions, it was thought that TGF-β indirectly influences Foxp3 induction as a T cell immunosuppressant.[47,52]

A TGF-β response element was identified in CNS2, providing evidence for direct involvement of TGF-β signaling in Foxp3 transcription.[48] As with Stat signaling, TGF-β binding induces phosphorylation and nuclear translocation of the Smad family of transcripton factors. Smad3, but not Smad2 or Smad4, binds to the sequence 5′-AGACTGTCT-3′ in CNS2 that matches the consensus binding motif for the Smad proteins. In the presence of TGF-β, Smad3 binding to CNS2 gradually diminishes over time ($< 24$ h), but imprints Foxp3 expression for longer time periods as acetylated histone H4 is detected at CNS3 and CNS1, 48 h after stimulation. Consistent with this, TGF-β treated T cells have reduced methylated DNA at the Foxp3 promoter.[65] The relative

contribution of Smad3 versus other TCR-induced factors in opening the Foxp3 locus is not known because TGF-β administration alone is not sufficient to induce Foxp3 expression. Interestingly, the CpG island within *Foxp3* CNS3 is completely demethylated in thymically derived Treg cells, but nearly completely methylated in TGF-β induced iTreg cells.[65] It is thought that this difference in the CNS3 methylation status is linked to the unstable nature of Foxp3 expression in iTreg cells. As proposed by Tone and colleagues, it is hypothesized that TGF-β works in concert with TCR-derived signals to enable full CNS2 enhancer activity.[48]

During natural Treg cell development in the thymus, TGF-β signaling is necessary only during the first 5 days of a neonatal mouse's life.[82] Afterwards, Treg cellularity equilibrates to wild-type levels in an adult mouse. Thus, it is envisioned that other signaling arms that promote Foxp3 transcription compensate for this TGF-β RI deficiency. Interestingly, the neonatal thymic architecture provides a suboptimal environment for Treg cell development compared to an adult thymus.[14] Therefore, it can be envisioned that TGF-β signaling and Smad3 signaling are required for Foxp3 transcription when the other Foxp3 promoter occupants are limiting in the neonatal thymus.

## G. STAT5

Six evolutionarily conserved sites that permit STAT5 binding are located in the *Foxp3* regulatory sites, three of which reside in the Foxp3 promoter and three in CNS3.[34,73] Through chromatin IP analysis, bona fide STAT5 binding was detected in the Foxp3 promoter, but not in the CNS3 enhancer region. Consistent with a role for STAT5 in Foxp3 transcription, T cell specific deletion of STAT5 resulted in a threefold decrease in $Foxp3^+$ cells in the thymus and spleen.[34]

STAT5 serves as a signaling unit and transcription factor for cytokine receptors that utilize the common gamma chain (γc). These γc-dependent cytokines include IL-2, IL-7, and IL-15. Although IL-2 has been extensively characterized as a survival factor for Treg cells, it is also implicated in Treg cell development, as Treg specific elimination of the IL-2 receptor reduces thymic Treg cellularity twofold.[32,83,84] Compound elimination of both IL-2 and IL-15 further reduces Treg cell development to levels detected in STAT5 deficient mice.[34] Furthermore, a Foxp3 transgene rescues $IL2Rb^{-/-}$ mice from lymphoproliferative disorders. Thus, reconstitution of Foxp3 expression is sufficient to restore Treg development on an $IL2Rb^{-/-}$ background suggesting that a major function of IL-2 signaling is to promote *Foxp3* transcription.

In developing thymocytes, STAT5 is thought to participate in Foxp3 induction in $CD4^+CD25^+Foxp3^-$ SP precursors in the aforementioned "two-step" model for Treg development.[31] In the first step, $CD25^-CD4^+$ SP Treg cell

precursors that express a TCR specificity conducive to Treg cell differentiation are instructed to express CD25 via TCR-derived signals. After acquiring high-affinity IL-2 receptor expression, the $CD25^+CD4^+$ SP Treg cell precursors gain competence to transduce IL-2 signals that are chiefly mediated by STAT5. Therefore, STAT5 is thought to participate in Foxp3 expression in cells that have the TCR-derived components, such as NFkB, NFAT, and CREB, assembled at the Foxp3 promoter.

## IV. Cellular Targets of Treg Cells

### A. $CD4^+$ T Cells

Treg effector function is assessed by two major assays. First, Treg suppressive activity can be tested by cotransferring Treg cells with wild-type $CD4^+$ effector T cells into lymphopenic recipient mice.[1,85] Transferring T cell preparations devoid of Tregs or reduced Treg: T effector cell ratios induces generalized lymphadenopathy, wasting disease, and colitis development in the recipient mice. In this system, pathogenic T cells are the primary mediators of the autoimmunity, suggesting that $CD4^+$ T cells are direct targets of suppression.

Second, suppression of effector T cell proliferation can be assessed *in vitro* by adding Tregs to standard T cell stimulation cultures. As Tregs are anergic in response to TCR crosslinking, and APCs are irradiated prior to culture, measurements of total proliferation reflect effector T cell divisions. *In vitro*, Tregs can directly suppress $CD4^+$ effector T cell proliferation in the absence of APCs.[86,87] Based on these two assays for Treg suppressive function, it was assumed that $CD4^+$ T cells serve as direct targets of Treg suppression. However, the inclusion of APCs in *in vitro* Treg- mediated suppression cultures significantly reduces the ratio of Tregs to T effector cells required to inhibit T effector cell proliferation, suggesting that APCs catalyze $CD4^+$ T cell suppression.[88] Irrespective of the presence or absence of APCs, suppression of effector T cell proliferation is contact dependent and soluble factor independent. However, intravital imaging studies revealed that Tregs rarely come in contact with pathogenic effector T cells, and thus fail to provide supportive evidence that Tregs directly suppress $CD4^+$ effector T cells in the draining lymph nodes.[89,90] Additionally, discordant conclusions reached from *in vivo* versus *in vitro* suppression assays for cytokine involvement reveal the limitations of such tissue culture assays.

### B. Dendritic Cells

An emerging body of evidence suggests that Tregs maintain tolerance to self-tissues by suppressing DC function, numbers, and maturation. *In vitro* observations of DC maturation and cytokine production suggest that Tregs can

directly influence DC function. The culture of immature bone marrow derived DCs or primary $CD11b^+$ DCs with Tregs reduces the expression of the costimulatory molecules, B7.1 and B7.2.[91–93] Additionally, Tregs inhibited the upregulation of B7.1 and B7.2 in response to LPS signaling.[92] This suppression of costimulatory molecule expression was specific to Treg cells because coculturing effector T cells failed to reduce B7.1 and B7.2 expression. Among primary DCs, the $CD11b^+$, but not the plasmacytoid subset was sensitive to Treg cell mediated suppression. Treg cell conditioned DCs were less potent stimulators of conventional $CD4^+$ T cell proliferation than DCs exposed to effector $CD4^+$ T cells, suggesting that Treg cell mediated B7.1 and B7.2 downregulation may contribute to impaired T cell priming.

Similar coculture systems revealed that Treg cells directly influence the DC cytokine profile. DCs modestly upregulated the expression of the proinflammatory cytokine IL-6 and produced low levels of IL-10 in the presence of naïve effector T cells.[93,94] However, IL-10 production is significantly increased when DCs are cocultured with Treg cells. Although Treg cells are known to produce IL-10, DCs were the primary source of IL-10 because *IL-10*$^{-/-}$ DCs and IL-10 sufficient Treg cells failed to reproduce these results. Furthermore, autocrine IL-10 signaling in DCs has been demonstrated to suppress inflammatory cytokine production. Adding Treg cells to DC cultures suppressed LPS-induced production of IL-12p40, TNF-α, and IL-6.[92] DC-derived IL-10 plays an integral role in suppressing inflammatory cytokine production since the administration of anti-IL-10R antibodies reversed Treg cell mediated suppression of IL-12p40, TNF-α, and IL-6 expression.

These results are not unique to Treg and DCs in isolation, as cultures containing unstimulated DCs, effector, and Treg cells were indistinguishable from cultures comprising DCs and Treg cells, suggesting that Treg cells can oppose the DC stimulatory effect of effector T cells in a competitive situation (Fig. 2A). Furthermore, Treg cells suppressed IL-6 production by DCs to levels below those detected in cultures containing DC and effector T cells. Thus, Treg cells were dominant over effector T cells in influencing the cytokine profile of unstimulated DCs. However, IL-6 levels produced by DCs exposed to LPS and effector T cells were not suppressed by the addition of Treg cells, suggesting that Treg cells are not able to suppress DCs in the presence of strong inflammatory stimulation.

In aggregate, these *in vitro* findings indicate that Tregs are capable of impairing the quality of the DC antigen presentation function. *In vivo*, inducible elimination of Tregs in mice engineered to express a toxin receptor in Treg cells corroborated these findings.[95] In these studies, Treg cell ablation increased the absolute numbers of DCs. Similar to germline *Foxp3*$^-$ mice, inducible Treg cell elimination induces lymphadenopathy and splenomegaly increasing the cellularity of all leukocyte subsets[95]. However, the relative

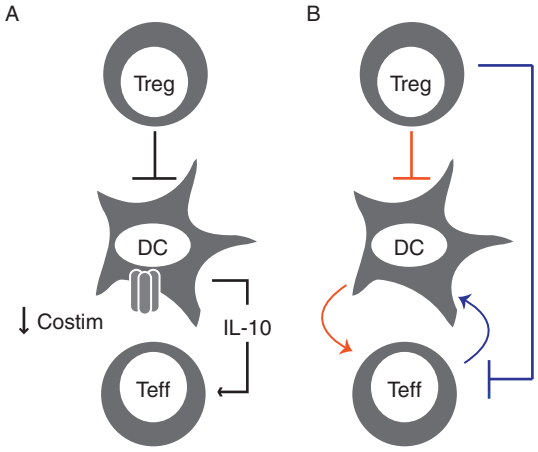


FIG. 2. Celullar interactions involved in Treg mediated suppression. (A) Direct interactions between Treg and dendritic cells induce reduced surface expression of costimulatory molecules and promote inhibitory cytokine production. (B) Dendritic cells integrate suppressive and stimulatory signals from Treg and effector T cells respectively. Two nonmutually exclusive models are depicted as black or gray connectors in the flow chart. In gray, Treg cells directly suppress DCs to indirectly inhibit T effector cell activation. In black, Treg cells directly suppress effector T cells to prevent the expression of DC stimulatory molecules such as GM-CSF and CD40L. The integration of both models results in the Treg mediated suppression of a feed-forward loop between DC and T effector cells.

frequency of DCs was also increased approximately fivefold, indicating that the elevated DC numbers were not solely due to an increase in all leukocyte subsets. Among leukocytes, myeloid cells experienced the greatest expansion. Enlargement of the DC compartment was associated with increased proliferation of committed DC precursors, but not the common myeloid precursor.[96] Treg cell control over DC numbers is dependent on the presence of effector T cells as DC numbers are not elevated in pan T cell deficient mice such as the *TCRb*$^{-/-}$ or *Rag*$^{-/-}$ mice. Furthermore, combining CD4 depletion with Treg cell elimination abrogates DC expansion, suggesting that effector CD4$^{+}$ T cells oppose Treg cell suppression of DCs.[95] Indeed, effector CD4$^{+}$ T cells generated upon Treg cell ablation produce DC modulating factors including GM-CSF, IL-13, and CD40L. Together, these observations evoke a model comprising dendritic, Treg, and T effector cells, with DCs serving as a sensor to integrate the inhibitory signals transmitted by Treg cells and the stimulatory cues transmitted by the effector T cells (Fig. 2B).

Intravital imaging studies that observed the interaction of DCs, Treg cells, and effector T cells support the notion that Treg cells suppress the ability of DCs to prime effector T cells. In a study that compared the migratory behavior of diabetogenic CD4$^{+}$ T cells in Treg cell sufficient or insufficient mice, the

authors observed that the transferred effector T cells tended to cluster around DCs in the pancreatic draining lymph node only in the absence of Treg cells.[90] These descriptive differences were supported by significant reductions in effector T cell displacement and velocity in Treg deficient mice. The physiological consequence of increased effector T cell and DC interactions include a more robust proliferation and increased IFN-γ production by pathogenic T cells. Together, these results imply that Treg cells indirectly affect self-reactive T cell priming and cytokine production by disarming DCs.

An independent study that visualized the dynamics of pathogenic $CD4^+$ T cells in an experimental autoimmune encephalomyelitis (EAE) model largely corroborated the findings from the diabetes prone mice.[89] While the antigen that elicited the above results was unknown and was therefore derived from endogenous sources in the diabetes model, knowledge of the cognate peptide for the pathogenic $CD4^+$ T cells enabled the authors to directly compare cognate peptide presenting or irrelevant DCs in the draining popliteal lymph node. In this analysis, pathogenic T cell velocity and displacement decreased only in the presence of the cognate peptide, suggesting that DCs discriminately suppress effector T cell priming in an antigen-dependent manner.

## C. $CD8^+$ T Cells

Most of the consequences of coculturing $CD8^+$ T cell with Treg cells are similar to those observed with $CD4^+$ effector T cells. These include *in vitro* suppression of $CD8^+$ T cell proliferation and cytokine production that occur in a cell contact-dependent manner.[97] *In vivo*, suppression of $CD8^+$ T cells has been examined in tumor immunosurveillance and viral infection models.

Immunity to transplanted tumors is significantly augmented in either Treg cell-free conditions or Treg cell depleted environments.[98–100] Enhanced tumor immunosurveillance was associated with increased $CD8^+$ T cell maturation, IFN-γ production, and tumor cell cytolytic activity. The suppression of tumor cell killing is hypothesized to require TGF-β, based on studies that neutralized TGF-β with specific antibodies or impaired TGF-β signaling through dominant negative TGF-β receptor expression.[101,102] TGF-β limits $CD8^+$ T cell cytolytic potential by repressing target cell killing, not the expression of core granule components such as perforin, and granzyme A and B.[101] Thus, it is possible that Treg cells provide a TGF-β environment that prohibits CTL differentiation. However, results obtained from experiments that utilized DN-TGFβR expressing cells or TGF-β neutralization highlight the importance of TGF-β responsiveness but do not describe whether Treg cells provide relevant TGF-β to mediate these effects. In this regard, tumor cells are known to establish an immunosuppressive microenvironment by producing TGF-β.[103]

Depleting Tregs has been demonstrated to be beneficial for boosting $CD8^+$ T cell responses against acute and persistent viral infections. In a HSV-1 infection model, a single injection of CD25 depleting antibodies prior to infection enhanced $CD8^+$ T cell proliferation, IFN-γ production, and cytotoxicity.[104] Interestingly, heightened $CD8^+$ T cell responses were detected at both the acute and memory phases of the anti-HSV response. In another study, acutely depleting Treg cells during secondary infection with *Listeria monocytogenes* impaired $CD8^+$ memory T cell expansion and cytokine production, indicating that Tregs limit the $CD8^+$ recall memory T cell responses.[105] Furthermore, polyclonal $CD8^+$ memory T cells from aged mice proliferated more robustly when transferred in the absence of Treg cells compared to cotransfers with Treg cells, suggesting that Treg cells are capable of reducing steady state levels of an established $CD8^+$ T cell memory population.[106] Thus, Treg cells may impinge on $CD8^+$ memory T cell generation, maintenance, and memory cell reactivation.

## D. NK Cells

In cancer patients and in multiple tumor models in mice, tumor growth has been associated with Treg cell mediated suppression of NK cell activity.[98,107] In mice, Treg cell depletion enhances NK cell mediated clearance of transplanted tumors. In cancer patients, Treg cell numbers are inversely proportional to NK cell activity. Specifically, the absolute number of Tregs was shown to be elevated in gastrointestinal stromal tumor bearing (GIST) patients with comprised NK cell activity compared to GIST patients with normal NK cell function.[108] As in $CD8^+$ T cells, NK cell proliferation, IFN-γ production, and cytotoxic potential are impaired in the presence of Treg cells in tissue culture, indicating that NK cells are direct targets of Treg cell suppressor function.[107,109] Consistent with this, transferring Tregs into T cell deficient but NK cell sufficient mice abrogates NK cell cytotoxicity against tumor cell targets.[110] Furthermore, decreased cytolytic function in transferred mice was associated with reduced expression of NKG2D, an NK cell stimulatory receptor, by the suppressed NK cells. Tumors expressing NKG2D ligands were able to escape NK cell mediated tumor immunosurveillance more efficiently than tumors that did not express the ligands, suggesting that Tregs suppressed NK cell activity in a NKG2D-dependent fashion. In this system, TGF-β deficient Tregs were unable to suppress NK cell cytolytic activity, indicating that Treg derived TGF-β may directly suppress NK tumoricidal activity. In other systems, tumor derived TGF-β down-regulates NKG2D surface expression on NK cells.[110] Therefore, it is possible that regulatory T cell derived TGF-β mediates NK cell suppression by reducing NKG2D expression levels.

## E. B Cells

Genetically or surgically modified mice with a reduced or no Treg compartment exhibit multiple signs of humoral dysregulation. Generalized hypergammaglobulinemia as well as organ specific antibodies are detected in these mice. Additionally, splenic B cells isolated from Foxp3 deficient mice display higher levels of costimulatory molecules, suggesting that B cells possess enhanced antigen presentation function in the absence of Tregs.[111] As Tregs are known to suppress $T_H$ cell activity, B cells may be the indirect cellular targets of Treg cell mediated suppression. However, *in vitro* culture experiments that analyzed B cell responses in the absence of $T_H$ cells suggest that Tregs may directly suppress B cells *in vivo*.[112] To bypass this requirement of $T_H$ cells, human B cells were stimulated with CD40 crosslinking antibodies and $T_H$ derived cytokines, in the presence or absence of Treg cells. The addition of Treg cells to B cell cultures reduced the production of IgG and IgA isotypes from $IgD^+$ precursors in a dose-dependent manner. The reduced capacity to produce isotype-switched antibodies in the presence of Treg cells was associated with decreased activation-induced cytosine deaminase expression in response to CD40 and IL-4 signaling. Therefore, Tregs are capable of inhibiting T-dependent antibody production by impairing class switch recombination.

Tregs have been shown to directly suppress T-independent B cell activation. LPS-induced B cell proliferation is markedly impaired in the presence of an increasing number of Treg cells.[113] This suppression of B cell proliferation is not unique to T-independent mitogenic signaling as proliferative responses to CD40 and IgM crosslinking are also suppressed by Treg cells. Detection of reduced B cell proliferation was correlated with an increase in B cell death in Treg cell containing cultures.[114] Similar to monocytes, DCs, and $T_H$ cells, B cells were shown to be susceptible to granzyme-dependent cell death induction by Tregs. Consistent with the idea that granzyme B mediates target cell killing by murine Tregs, administering DCI, a specific granzyme B inhibitor decreased B cell death. In contrast to another report, perforin-deficient Treg cells displayed reduced cytolytic activity compared to wild-type counterparts. Perhaps perforin is differentially required for B cell versus $T_H$ cell cytolysis.

Intrasplenic Treg and B cell colocalization supports the notion that Tregs are capable of directly controlling B cell numbers *in vivo*. Human Tregs are recruited to B cell zones through the expression of the germinal center associated chemokine receptor CXCR5, after TCR stimulation. In mice, B–Treg cell encounters are facilitated by B cell derived CCL4, which recruit CCR5 expressing Tregs.[113] Indeed, CCL4 neutralization *in vivo*, which is predicted to disrupt B–Treg cell interactions, elevated serum autoantibody titers. Once a B–Treg cell interaction is established, it is hypothesized that Tregs preferentially kill antigen presenting B cells compared to nonspecific B cells. This idea is

supported by results from *in vitro* experiments in which TCR transgenic Tregs were mixed with two populations of allelically marked B cells that were either loaded with the cognate peptide or left untreated.[114] In this competitive situation, antigen presenting B cells were four times more frequently killed than nonpulsed B cells, suggesting that conditions that favor conjugate formation increase the likelihood of target cell lysis. It remains to be determined whether cognate antigen-presenting dendritic and myeloid cells are also preferentially lysed by Treg cells.

## V. Molecular Mechanisms of Treg Cell Suppression

### A. TGF-β

The significance of TGF-β in maintaining immunological tolerance is evidenced by the striking phenotype of TGF-β deficient mice. TGF-$\beta_1$ deficient mice develop fatal lymphoproliferative disease by 3 months of age. Transgenic mice that express a dominant TGF-β receptor under the proximal lck promoter largely recapitulate the autoimmune phenotype of germline *TGF*$\beta_1^{-/-}$ mice, revealing that T cells are a major target of TGF-β mediated immunosuppression.[115] Multiple hematopoeitic and nonhematopoeitic cells produce TGF-β, but T cells are an essential source of TGF-β as T cell specific TGF-β knockout mice succumb to autoimmunity with the kinetics and severity seen in germline TGF-β deficient mice.[116] Among T cells, Treg cells express high levels to TGF-β. The role of TGF-β as a Treg suppressor molecule remains controversial.[86,87,117] As negative results obtained from cytokine neutralization experiments are generally inconclusive, *in vitro* suppression assays were repeated with T cells isolated from mice with genetic deficiencies in either TGF-β production or in responsiveness to resolve the discrepant results.[118] In support of TGF-β having a nonessential role in Treg function, responder T cell proliferation was equally suppressed by *Tgf*$\beta_1^{-/-}$ and wild-type Tregs. Although exogenous TGF-β is sufficient to impair proliferation of unseparated T cells, responder T cells that are insensitive to TGF-β signaling, caused either by a Smad3 deficiency or by the expression of a dominant negative TGF-β receptor, were responsive to Treg suppressor function. Thus, Treg mediated inhibition of conventional T cell proliferation *in vitro* occurs independent of TGF-β activity.

In contrast to suppression of *in vitro* proliferation, a requirement for TGF-β has been demonstrated in the aforementioned T cell transfer model of colitis. In this system, effector T cells that expressed a dominant negative TGF-βRII receptor were resistant to wild-type Tregs, indicating that TGF-β responsiveness was necessary to prevent development of colitis. However, *Tgf*$\beta_1^{-/-}$ Tregs

were equally competent as wild-type regulatory T cells in their ability to suppress wild-type effector T cells and colitis. Together, these experiments revealed that although TGF-β plays an essential role in colitis prevention, TGF-β from non-Treg sources could compensate for TGF-β deficiency in Tregs.

## B. IL-10

IL-10 is an immunosuppressive cytokine that inhibits DC maturation and inflammatory cytokine production. Germline *Il10* knockout mice develop severe colitis by three months of age, but are otherwise unremarkable.[119] T cell or Treg specific *Il10* deletion reproduces the kinetics and severity of colitis development seen in germline $Il10^{-/-}$ mice indicating that Treg-derived IL-10 is essential to maintain gastrointestinal homeostasis.[120,121] Besides gut associated lymphoid tissues, generalized lymphadenopathy and T cell infiltration in target organs affected by manipulating Treg numbers are not detected in $Il10^{-/-}$ mice. As predicted by the lack of widespread immunopathologies in $Il10^{-/-}$ mice, suppression of effector $CD4^+$ T cell proliferation is unaffected in the presence of IL-10 neutralizing antibodies.[86]

Consistent with a role for IL-10 in gastrointestinal tolerance, studies that employed the T cell transfer model for colitis confirmed that Treg cell-derived IL-10 is necessary to suppress immunopathology *in vivo*.[122] The severity of colitis induced by wild-type $CD4^+$ effector T cells in the absence of Tregs can be ameliorated by exogenous IL-10 administration. Furthermore, colitis is prevented by equipping transferred effector $CD4^+$ T cells with an *Il10* transgene.[123] Treg cells were demonstrated to be the physiologic IL-10 source *in vivo* because protection from colitis development was abrogated when wild-type effector T cells were cotransferred with *Il10* deficient Treg cells.

Although Treg derived IL-10 is not essential for tolerance induction at most anatomical locations, Tregs promote a tolerogenic state by programming other immune cells to produce IL-10. In a T cell dependent model of asthma, IL-10 plays an essential role in suppressing airway inflammation. The sources of protective IL-10 in this asthma model are the $CD4^+$ effector cells and not the Treg cells. However, Tregs are required to induce IL-10 expression in $CD4^+$ effector cells to prevent airway hyperreactivity.[124] Similar to the role of Tregs in influencing IL-10 production by DCs, Tregs suppress asthma development by redirecting effector T cells to produce to IL-10 and suppressing inflammatory cytokine production.[92,94,125] In summary, Treg derived IL-10 contributes to the maintenance of mucosal homeostasis. In addition to this Treg instrinsic role for IL-10, Tregs instruct other immune cell types to adopt an anti-inflammatory cytokine profile that includes IL-10.

## C. CTLA-4

The immunosuppressive function of CTLA-4 was originally thought to occur in a cell autonomous manner. However, in mixed bone marrow chimeras, the presence of wild-type T cells prevents the activation of CTLA-4 deficient T cells and lymphoproliferative disease development that was detected in CTLA-4 knockout mice.[126] Based on this observation and high CTLA-4 expression levels in Tregs, the role of CTLA-4 in maintaining dominant tolerance was further investigated through a series of CTLA-4 blocking experiments. In the adoptive T cell transfer model of experimental colitis, CTLA-4 blockade did not impair disease development in mice that received only $CD4^+$ effector cells.[127] However, the protective effect of Treg cells in this system was abrogated when CTLA-4 specific antibodies were administered to the recipient mice, suggesting that Treg cells are the primary targets of CTLA-4 inhibition *in vivo*. Importantly, the CTLA-4 specific antibody utilized in these studies does not deplete Tregs.[128] In support of these findings, enhanced effector T cell proliferation was also detected in *in vitro* suppression assays performed in the presence of a CTLA-4 blockade, reversing the anti-proliferative effect of Treg cells. As in *in vivo* transfer studies, CTLA-4 inhibition was specific for CTLA-4 expressed on Treg cells because cultures containing $Ctla4^{-/-}$ effector T cells and CTLA4-sufficient Tregs were sensitive to the addition of anti-CTLA4 antibodies.

As with effector $CD4^+$ T cells, CTLA-4 may function in Treg cells by directly transmitting signals that mediate immunosuppression. In this regard, the cross-linking of CD3, CD28, and CTLA-4 induces the production of the immunosuppressive cytokine TGF-β by unsorted $CD4^+$ T cells.[117] Thus, the suppressor function CTLA-4 on Treg cells may be partially mediated by TGF-β.

Alternatively, constitutive CTLA-4 expression by Tregs may passively suppress effector T cells by outcompeting for B7 binding, as B7 binds ten to twenty times more avidly to CTLA-4 than to CD28.[129,130] In this model, B7 sequestration by CTLA-4 is predicted to limit the provision of costimulatory signals to effector T cells. Therefore, CTLA-4 signaling in Treg cells would not be essential for maintaining its immunosuppressive properties. In support of this hypothesis, the addition of CTLA-4-Ig fusion protein is sufficient to prevent hyperproliferative T cell responses *in vitro*.[131] Furthermore, CTLA-4-Ig administered *in vivo* protects CTLA-4 deficient neonates from lethal autoimmunity and lymphoproliferative disorder.[132] Although these studies suggest that blocking costimulation may be achievable through CTLA-4-Ig administration, predictions based on surface CTLA4 versus CD28 expression levels suggest that cellular CTLA-4 is incapable of saturating B7 molecules.[133]

In addition to functioning as a costimulatory ligand for T cells, an emerging body of evidence suggests that B7 engagement by the CD28 family members initiates a "reverse signaling" cascade in the APCs. In a process that requires

autocrine IFN-γ and STAT-1 signaling, B7 engagement induces indoleamine 2,3-dioxygenase (IDO) expression in macrophages and DCs.[134,135] IDO effects immunosuppression by catalyzing the degradation of the essential amino acid tryptophan into kynurenine. Microenvironments that are depleted of tryptophan have been demonstrated to impair T cell proliferation *in vitro*[136,137] and to limit T cell mediated skin inflammation *in vivo*[134] (Fig. 3A). In contrast to CTLA-4, B7 ligation by CD28 was not capable of inducing IDO expression in APCs,

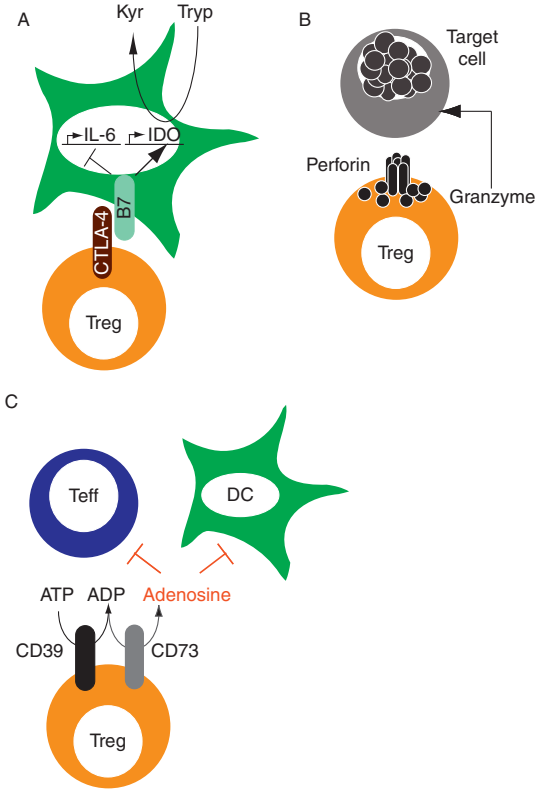


FIG. 3. Tregs inhibit effector T cell activation through CTLA-4, Granzyme, and ATP depletion. (A) CTLA-4 dependent metabolic regulation of effector T cell activation. Treg cells constitutively express high levels of CTLA-4. B7 engagement by CTLA-4 induces the expression of IDO that catabolizes tryptophan to kynurenine. Tryptophan depleted environments impair effector T cell activation. IL-6 production is inhibited by B7 binding to CTLA-4. (B) Treg cells induce target cell apoptosis in a perforin and granzyme dependent manner. (C) Extracellular ATP is converted to adenosine by sequential activities of CD39 and CD73 expressed by Treg cells. Adenosine induces IL-10 production by DCs and attenuates TCR signaling through cAMP generation.

suggesting that IDO expression is dependent on high-affinity B7 interactions.[138] The inability of CD28 to substitute for CTLA-4 in promoting IDO expression was traced to the production of IL-6 in APCs upon stimulation by CD28 but not CTLA-4. Neutralizing IL-6 upon CD28 treatment was sufficient to promote IDO expression, suggesting that IL-6 can overcome the immunosuppressive barrier imposed by CTLA-4 expressing Tregs. Interestingly, IL-6 was demonstrated to make effector T cells refractory to Treg suppression.[139] It is plausible that IL-6 makes effector cells resistant to Tregs by preventing IDO expression.

As discussed in Section IV.B of *Cellular Targets of Treg Cell Immunosuppression*, DCs integrate signals derived from contacts with either CTLA-4 bearing Tregs or CD28 expressing T effector cells. As visualized in intravital imaging studies, Treg cells preferentially cluster around DCs in the presence of effector T cells. This phenomenon is dependent on CTLA-4 expression as $Ctla4^{-/-}$ Tregs fail to oust effector T cells when competing for DC access,and provision of CTLA-4 antibodies is sufficient to break Treg-DC aggregates *in vitro*.[140] Based on the higher affinity of CTLA-4: B7 compared to CD28: B7 interactions, and preferential DC access by Treg cells over effector T cells, it is hypothesized that CTLA-4 mediated IDO expression prevails over competing CD28 signals. The importance of CTLA-4 in Treg cell effector function is underscored by the observed phenotype of Treg cell-specific CTLA-4 knockout mice ($Ctla4^{F/F}$). These mice succumb to fatal multiorgan autoimmunity by 7 weeks of age. Autoimmunity is correlated with elevated DC expression of B7 family members, indicating that CTLA-4 expression on Tregs plays a nonredundant role in controlling DC activation.[141] Although essentially all activated $CD4^+$ effector cells in $Ctla4^{F/F}$ produce high levels of CTLA-4, they are incapable of suppressing DC maturation. Therefore, B7 engagement by CTLA-4 cannot be substituted by non-Tregs in DC homeostasis.

Although B7 family members are primarily expressed by professional APCs, it is known that activated T cells also express B7.1 and B7.2.[142–144] To explore the possibility that reverse B7 signaling in T cells may play a role in T cell homeostasis, wild-type Tregs were tested for their ability to suppress B7 deficient effector $CD4^+$ T cells.[145] In this system, $B7.1^{-/-}B7.2^{-/-}$ effector T cells were insensitive to Treg mediated suppression and induced severe gastritis and colitis in the lymphopenic recipients. Additionally, *in vitro* proliferation of $B7.1^{-/-}B7.2^{-/-}$ effector T cells was not suppressed by the addition of wild-type Tregs. Reconstituting $B7.1^{-/-}B7.2^{-/-}$ effector T cells with a B7 truncation mutant lacking the cytoplasmic domain failed to rescue lymphoproliferation, indicating that B7 signaling, and not B7 occupancy, is essential for the suppression. Therefore, the CTLA-4 expressed by Tregs may exert immunosuppressive activity in a B7-dependent manner on both effector $CD4^+$ T cells and APCs. These experiments also provide the best evidence to support the fact that Tregs may directly suppress both effector $CD4^+$ T cells *in vivo*.

## D. Fibroleukin

The immunosuppressive properties of fibroleukin were originally identified in unsorted peripheral blood T cells. Further dissection of human T cell subsets revealed that the CD45RO$^+$ T cell population, which comprises memory and Tregs, primarily expresses fibroleukin.[146] Microarray analyses of genes that are differentially expressed in Tregs confirmed elevated fibroleukin expression levels in Tregs compared to naïve and activated CD4$^+$ T helper cells.[18,147]

T cells express fibroleukin as a soluble secreted molecule that binds to DC and T cells.[148] Exogenous fibroleukin treatment impairs T cell proliferation in APC-containing T cell cultures, suggesting that APCs may also be susceptible to fibroleukin-mediated immunosuppression. Indeed, monocyte-derived DCs exposed to fibroleukin express lower levels of surface B7.1, CD40, and MHC II. Additionally, fibroleukin-conditioned DCs exhibited a decreased capacity to support the proliferation of untreated T cells; whereas a more modest reduction in T cell proliferation was detected when T cells, but not DCs, were pretreated with fibroleukin. However, exogenous fibroleukin administration weakly impairs T cell proliferation in response to concavalin A or plate-bound CD3 and CD28 antibody stimulation, indicating that T cells are also direct targets of fibroleukin immunosuppression, albeit less sensitive to fibroleukin than DCs. Furthermore, fibroleukin-deficient mice show increased number and enhanced maturation of DCs, and this is correlated to increased activity of effector T cells.[149] The clinical outcome of fibroleukin deficiency is restricted to the development of severe glomerulonephritis. Increased protein deposition in the glomeruli is dependent on hematopoietic cells and is correlated to decreased Treg activity, but the requirement of Treg- derived fibroleukin to prevent kidney damage is unclear. These studies demonstrate that fibroleukin is capable of suppressing T cell activation both by reducing T cell priming capacity of APCs and by directly inhibiting the proliferative capacity of effector T cells.

## E. Granzyme Dependent Cytotoxicity

Treg cell mediated inhibition of *in vitro* effector T cell proliferation was demonstrated to require cell-to-cell contact. Although the molecular basis for contact-mediated suppression is largely unknown, recent reports have revealed that Tregs also require cellular contact for target cell killing via the granule exocytosis pathway.[150,151] Granule-mediated cytoxicity is dependent on granzymes, granule resident proteases, which initiate a cascade of apoptosis-promoting cleavage events. As in effector T cells, granzyme expression is induced in Tregs in response to T cell receptor signaling. While granzyme A is primarily expressed by activated human Tregs,[151] granzyme B is the predominant granzyme induced in murine Tregs.[150] Granzyme A and B differ in substrate specificity and the kinetics

of cell death induction, but activated murine and human Tregs comparably induce effector T cell death at 1:1 ratio of regulatory to effector T cells. *In vitro* cytotoxicity was dependent on granzyme function, as suppression of effector T cell proliferation was severely compromised in cultures containing granzyme B deficient Tregs.[150]

Cytolytic granules also contain perforin, which is essential for target cell lysis in $CD8^+$ CTLs and NK cells. The deposited perforin polymerizes on the target cell plasma membrane in a calcium dependent manner and generates holes that were hypothesized to serve as granzyme conduits into the target cell. However, accurate measurements of pores formed by perforin suggest that the diameter of polyperforin channels do not accommodate granzyme passage.[152] Although the exact function of perforin remains unknown, phenotypic similarities in mice deficient in either perforin or granzyme B provide evidence that perforin plays a nonredundant role in targeted cytolysis by lymphocytes. In support of this idea, inhibiting perforin by either EDTA or concanamycin A treatment abrogates target cell killing by human Tregs. In contrast to these findings, perforin deficient murine Tregs were equally suppressive as its wild-type counterparts *in vitro*, suggesting that perforin is not essential for granzyme B dependent target cell lysis in murine Treg cells. These discrepant results may reflect the usage of different granzymes for target cell killing in mouse versus human Tregs. In this regard, granzyme A may be more dependent on perforin for killing that granzyme B. Alternatively, calicium chelators or concanamycin A may not be specific for perforin inhibition, affecting target cell cytolysis independent of perforin function. Human Tregs, additionally, have been demonstrated to kill monocytes, DCs, and activated $CD8^+$ T cells[151] (Fig. 3B). Murine Tregs are also capable of killing B cells *in vitro*.[114]

## F. Extracellular ATP Depletion

Tissue damage and ensuing cell death generate extracellular ATP, which serves as an inflammatory mediator. Tregs express cell surface proteins, CD39 and CD73, which sequentially convert inflammation-inducing ATP to immunosuppressant adenosine. CD39 catalyzes the first step of ATP breakdown to 5′ adenosine monophosphophate (AMP). The ecto-5′ nucleotidase of CD73 cleaves the last phosphate in AMP to generate adenosine. Multiple immune cell subtypes are suppressed by adenosine, most notably T cells and DCs. Adenosine exposure during T cell stimulation attenuates proliferation, cytokine secretion, and cytotoxic activity[153,154] (Fig. 3C). In T cells, adenosine binds to its receptor $A_{2a}$ and exerts its suppressive activity by elevating cAMP concentrations in cells. The second messenger, cAMP, serves as a cofactor for protein kinase A (PKA), which in turn suppresses T cell activation. In DCs, adenosine decreases antigen presentation and increases the expression of the immunosuppressive cytokine IL-10.[155,156] CD39 deficiency significantly diminishes Treg mediated survival of allograft skin transplants. However, $CD39^{-/-}$ Tregs are minimally impaired in suppressing

effector T cell proliferation and *CD39*$^{-/-}$ mice do not display any overt signs of autoimmunity.[153] The CD39-CD73 axis of adenosine generation is not essential for maintaining immune homeostasis in unchallenged mice, but may play an important role in diseases with significant tissue destruction and hypoxia.

## VI. Concluding Remarks

New technologies in gene expression profiling, cell sorting, and mouse engineering have aided the identification of gene products and signaling pathways that are involved in Treg development and suppressive function. Lists of genes differentially expressed by Tregs and Foxp3 target genes have been generated as a result of these advances, but one of the major challenges of Treg research is to assign functional data to these gene products. Most molecules that comprise the Treg suppressive artillery are not unique to Tregs. Thus, the context in which each candidate suppressor molecule exerts its activity needs to be determined. Likewise, hypotheses on Treg development presume that the TCR and cytokine signaling that are common to all T cells are also required for Treg differentiation. Therefore, the unique interplay of common signaling pathways and not novel signaling modules are thought to underlie Treg development. Understanding how common signaling pathways and transcription factors integrate to specify the Treg lineage are likely to provide new insights into how Tregs diverge from effector cells.

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