Published in final edited form as:

Hum Immunol. 2012 March ; 73(3): 232–239. doi:10.1016/j.humimm.2011.12.012.

Nature and Nurture in Foxp3⁺ Regulatory T Cell Development, Stability, and Function

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Abstract

Foxp3⁺ regulatory T lymphocytes (Treg) are critical homeostatic regulators of immune and inflammatory responses. Their absence leads to fulminant multi-organ autoimmunity. This review explores recent studies that have altered our emerging view of the development, stability, and plasticity of these cells. Treg appear not to be a single entity, but a family of immunomodulatory cell types with shared capabilities. On a first level, Treg may alternatively form in response to developmental cues in the thymus as a distinct lineage of CD4⁺ T cells, or adaptively, in response to environmental cues received by mature conventional CD4⁺ T lymphocytes. These two populations bear distinct specificity, stability, and genetic profiles, and are differentially used in immune responses. Secondly, in a manner analogous to the generation of Th1, Th2, and other T cell subsets, Treg may further specialize, adapting to the needs of their immunologic surroundings. Treg therefore comprise developmentally distinct, functionally overlapping cell populations that are uniquely designed to preserve immunologic homeostasis. They combine both an impressive degree of stability and adaptability.

Keywords

Foxp3; Regulatory T Lymphocyte; Cellular differentiation; Immune regulation

1. The Treg Lineage

Lineage: descent in a line from a common progenitor (Merriam Webster)

Tracing one's lineage is important to many who seek to understand their origins and character. The extent to which our complex traits indeed mirror those of our ancestors is uncertain. However, within us, cellular lineages do exist that possess stable, generationally transmitted traits. These traits are the product of self-perpetuating genetic programs that commit a cell and its descendants to a particular fate.

Though not scientifically defined, several common features characterize cellular lineages: 1. Induction by developmental cues of a phenotypic profile in a common progenitor; 2. Maintenance of the profile through the altered expression of one or more genetic regulators; 3. Stabilization of the profile, typically through cell-intrinsic feedback loops or epigenetic modifications; 4. In the absence of terminal differentiation, robust transmission of the profile

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to progeny after cellular division. Conventional CD4⁺ and CD8⁺ T cells are examples of cellular lineages. Their thymic progenitors are imprinted with characteristic transmissible gene expression patterns that maintain a broad range of lineally preserved qualities. Overlaid on these lineage assignments are a variety of additional qualities. Thus T cells can mature into Th1, Th2, Tc1, Tc2, and a host of other subsets which manifest stereotyped effector responses when stimulated by antigen (Ag) [1]. Unlike differentiation into CD4 or CD8 T cells, which occurs developmentally, subset differentiation results from a T cell's integration of local environmental cues so as to optimize the cell's response to a particular immunologic challenge.

CD4⁺ Foxp3⁺ regulatory T lymphocytes (Treg) are a subclass of CD4⁺ T cell receptor (TCR) $\alpha\beta$ ⁺ T cells that are essential to preserve immune homeostasis [2,3]. Absence of Treg or the Foxp3 transcription factor they express leads to the rapid development of fulminant multi-organ autoimmunity. Unlike other CD4⁺ T cell subsets that form extrathymically from conventional CD4⁺ T cells (Tconv), Treg can develop as a separate population in the thymus. Consequentially, Treg are often referred to as a distinct lineage. Yet the extent to which these cells are an independent lineage or a metastable maturation state that is interconvertible with Tconv has been a topic of debate [4,5]. Indeed, Treg show both a high degree of stability with preserved phenotypic and functional properties, as well as an acute sensitivity and adaptability to environmental inputs. Further, Treg differentiation is not restricted to the thymus, where natural Treg (nTreg) are generated. Environmental Ags and extrathymic signals can upregulate Foxp3 in Tconv, converting them into induced Treg (iTreg) [6,7]. Circulating Treg therefore include two populations, one thymically-derived that appears to meet the criteria for a cellular lineage, and a second that forms adaptively and seems not to. In this review we explore the development, stability, and plasticity of these different forms of Treg.

2. TCR specificity and Foxp3 expression in thymic Treg differentiation

TCR α and β chain gene rearrangement and expression in early thymic precursors prompts a series of TCR recognition-dependent events leading to differentiation into CD4⁺ or CD8⁺ T cell subclasses. Expression of T helper-inducing POZ/Kreupel factor (Th-POK) is critical for CD4⁺ T cell differentiation, and its absence is associated with the "helper deficient" mutant phenotype. Runx3, in turn, is required for CD8⁺ T cell differentiation [8,9]. The actions of these factors are further overlaid on a complex landscape of alterations, including in Notch, Gli1, GATA3, Ikaros, and Tox. Ultimately these changes lead to robust lineage assignments.

The *sine qua non* for Treg is the sustained expression of a single transcription factor, Foxp3 [10,11]. Foxp3 can bind the promoters and influence the expression of hundreds of genes [12,13]. Importantly, Foxp3, in a complex with other transcription factors, particularly CBF β and Runx1, can bind its own promoter and support its own expression [14]. Such positive feedback is important in ensuring continued gene expression and lineage persistence. However, Foxp3 expression is not in itself sufficient for Foxp3 transcription. Endogenous Foxp3 is not upregulated in Tconv transduced with a Foxp3 transgene [15], indicating that Foxp3 alone cannot induce its own expression. An evolutionarily conserved element in the Foxp3 promoter, termed the Treg-specific demethylated region (TSDR) is heavily methylated in Tconv but demethylated in Treg, potentially limiting the activity of transduced Foxp3 in this regards [16,17]. Indeed, inhibitors of DNA methylation help stabilize Treg Foxp3 expression whereas germline deletion of the TSDR results in loss of Foxp3 [14,18]. Additional transcription factors not directly induced by Foxp3 are also important for Foxp3 expression [14,19]. Therefore, the presence of Foxp3 defines Treg, but Treg generation is not a simple matter of Foxp3 upregulation.

Foxp3 is also not required for its own transcription. A subset of T cells in mice with a deleted Foxp3 coding sequence still actively transcribe from the Foxp3 promoter [20]. Presumably these cells, which exhibit some properties of Treg but are not suppressive, would have differentiated into Treg if the Foxp3 coding sequence had been present. These “would be” Tregs do show decreased retention of Foxp3 promoter transcription compared wild type Treg when transferred into lymphopenic SCID mice, indicating a role for Foxp3 in persistent gene expression.

For most Treg, Foxp3 is first expressed in a subset of CD4 single positive (SP) cells within the thymic medulla [21]. This SP stage of differentiation is the last maturation stage prior to egress of the newly formed mature T cell. A Treg differentiation program may be induced in these SP cells leading to rapid Foxp3 upregulation or initiate in an earlier precursor with a delay in Foxp3 expression. Some data indicate a multi-stage commitment process that may include priming steps necessary for Foxp3 commitment at early stages of thymocyte development [22]. Supporting a model of earlier Treg commitment, CD28 is required for the expansion of Foxp3⁺ Treg precursors in the thymus [23]. Likewise, transgenic mice in which class II MHC is limited to the thymic cortical epithelium, which interacts primarily with earlier CD4⁺CD8⁺ precursor cells, demonstrate Treg differentiation [24–26].

Much as for CD4⁺ and CD8⁺ T cells, Treg development is TCR specificity dependent. T cells from mice transgenic for any of several rearranged Tconv-derived TCR, when bred on a Rag-deficient background so that they cannot rearrange and express endogenous TCR, fail to develop Treg [27–30]. Therefore expression of a Tconv-derived TCR prevents Treg differentiation. On a Rag-sufficient background, allelic non-exclusion of the TCR α chain allows the formation of dual receptor T cells that co-express transgenic TCR $\alpha\beta$ as well as endogenous TCR α paired with transgenic TCR β . This second TCR complex can promote Treg differentiation in a fraction of transgenic T cells. Depending on the efficiency of allelic exclusion and the specific transgene, different numbers of Treg are observed. As additional evidence indicating a developmental role for TCR specificity, several groups have shown that Treg and CD4⁺ Tconv possess distinct TCR repertoires [31–33]. Treg and Tconv TCR sequences show only limited overlap in either the thymus or lymphoid tissue.

The different repertoires of Treg and Tconv endow these cells with distinct functional properties, with a particularly enriched self-specificity among Treg. T cells transduced with Treg TCR show increased activation and proliferation after transfer into syngeneic mice when compared with TCR from Tconv [31]. Spontaneously activated T cells present in Foxp3-deficient mice show repertoire overlap with Treg from Foxp3-sufficient mice, implying that these self-reactive cells would have differentiated into Treg had Foxp3 been available [34,35]. A mouse expressing a Treg TCR transgene showed high levels of thymic deletion, indicating specificity for self Ag [36]. Finally, provision of cognate Ag can promote the thymic development of Treg in TCR transgenic (Tg) mice, indicating that high affinity ligand can prompt Treg differentiation [37]. These findings have led to the hypothesis that a subset of developing thymocytes with an affinity for self Ag intermediate between that required for positive and negative selection develop into Treg [38–40].

Even in the absence of inflammation or disease, lymphoid tissue draining different locations possess Treg with distinct repertoires. Interestingly, these Treg display enriched specificity for regional tissue-restricted Ags [41,42], implying that specificity governs localization. In the setting of autoimmune diseases, responding Treg are also enriched for tissue-specific reactivity [32,43,44]. Though the extent of the anti-self bias among Treg compared with Tconv has been disputed [45], Treg clearly form a predominantly distinct group of cells when compared with other CD4⁺ T cells. Indeed, public Treg-associated TCR sequences can be identified [32,43], indicating a conserved skewing in Treg recognition across individuals.

Importantly, possession of a particular TCR specificity is necessary but not sufficient for Treg differentiation. Whereas the enforced transgenic expression of monoclonal Tconv-derived TCR induces conventional Foxp3⁺ T cell differentiation in the thymus, only a minority of T cells from thymocytes expressing transgenic Treg-derived TCR develop into Treg [36]. An explanation for this came with the production of chimeric mice incorporating variable numbers of hematopoietic or thymic progenitor cells with fixed Treg-derived TCR admixed with unmanipulated progenitor cells [32,46,47]. When only very small numbers of precursors express a monoclonal Treg TCR, the extent of Treg differentiation is proportional to precursor numbers. However, Treg production rapidly saturates as larger numbers of Treg-TCR Tg precursors are added to the wild type cells. The additional transgenic T cells differentiate into Tconv that express the Treg-derived TCR. Therefore, Treg but not Tconv development is quantitatively limited for an individual TCR. Implicitly, niches permissive for Foxp3 induction in thymocytes with a particular specificity are limited, and the default pathway for thymocytes that exceed this niche capacity is formation of Tconv. Specific types of Ag presenting cell types may form these niches. For example, thymic stromal lymphopoietin (TSLP) produced by Hassall's corpuscles within the thymus has been shown to convert migratory DCs into cells capable of upregulating Foxp3 in thymocytes [48].

In summary, Treg possess features of other cellular lineages. Differentiation occurs at a specific developmental checkpoint. Inductive signals, particularly the quality of the TCR's signal to thymic self Ag but also other constitutively available signals, such as through CD28 and CD40, guide development. This leads to the reprogramming of the precursor cell through the expression of an essential transcriptional regulator, Foxp3, which influences the expression of large number of genes. Foxp3 is further stabilized by positively feeding back on its own expression. Epigenetic modifications of the Foxp3 locus helps preserve expression in Treg even after a cell's exodus from the thymus.

3. Adaptive Treg induction

In addition to the thymic route for Treg development, Foxp3 may be induced in Tconv after activation. This has been best defined in *in vitro* cultures where a convergence of signals arising from the TCR, IL2, and TGF β signaling pathways upregulate Foxp3 and lead to iTreg formation [49–51]. TCR and IL2 signals activate transcription factors that bind the Foxp3 promoter and enhancer, including STAT5, NFAT, and CREB/ATF [14,38,52,53]. TGF β mediated Smad signaling may act directly on the Foxp3 enhancer and indirectly by inducing Foxp3 through an E2A and Id3 dependent pathway [52,54]. These essential signals can be further modulated by other stimuli, particularly through retinoic acid (RA) and the aryl hydrocarbon receptor (AHR) [55–57]. Pathways that alter signaling through these mediators also alter iTreg generation. For instance, the bioactive lipid sphingosine-1-P, which prevents cellular egress from lymph nodes, antagonizes iTreg generation in part through its inhibition of TGF β signaling [58].

The mTor signaling pathway, which integrates nutritional status and receptor-mediated signals to regulate proliferation and survival, plays an additional critical role [59]. Inhibition of mTor, through drugs such as rapamycin or through inhibitory signaling molecules such as PD-1, promotes iTreg formation [60,61]. Agents that promote mTor activity, such as persistent signaling through CD28, inhibit their formation. The Foxo transcription factors enhance Foxp3 expression. Inhibition of these as well as epigenetic modifications contribute to mTor's activities [62–65].

Signal timing and intensity, not just signal quality, critically influence Foxp3 induction. Memory T cells are refractory to Foxp3 upregulation, as are naive T cells within 2–3 days after TCR stimulation [50]. Further, delayed addition of TGF β to TCR/IL2 stimulated cells

fails to upregulate Foxp3, indicating that the Foxp3 locus is only transiently accessible to Smad mediated signals downstream of TGF β . The role of TCR signal intensity was studied using monoclonal T cells and Ag ligands of varying affinity that were presented at different density. High affinity ligands presented at low density optimally promoted iTreg formation [66,67]. Increasing the density of low affinity ligands enhanced iTreg generation, though these iTreg were suboptimal and showed poor survival characteristics. Therefore, a fine balance in signal strength, timing, and quality must be present to induce iTreg formation. The extent to which iTreg differentiation requirements correspond to those promoting nTreg generation in the thymus is unclear. Interestingly, whereas CD28 is essential for nTreg formation [68] and for Treg expansion [69], *in vitro*, strong CD28 signaling inhibits iTreg development [70], suggesting that the inductive signals are distinct.

The complex choreography of events necessary for Tconv conversion into iTreg can exist *in vivo*. Isolated Foxp3⁻ T cells transferred into mice can transform into Foxp3⁺ T cells [71]. Transfer studies are made difficult by the need to fully purge nTreg or otherwise account for them in the transferred populations; even small differences in homeostatic expansion rates compounded over time can lead to the appearance of T cell conversion. However, transferred T cells from TCR Tg Rag^{-/-} mice, which wholly lack Treg, are also able to convert to iTreg, definitively verifying the plasticity of this population [72,73]. Several regimens have proven effective in upregulating Foxp3 *in vivo*. These include oral administration of Ag, slow infusion with osmotic pumps or through injection, or directed presentation on immature dendritic cells [74–76]. The role of signal magnitude and quality in Foxp3 induction and persistence [66] suggests that in a heterogeneous population of T cells with variable affinities for a particular Ag, only some reactive cells will receive the balance of signals necessary for iTreg conversion.

Although peripheral Ag exposure can induce iTreg, the majority of circulating Treg are likely thymically derived. This is supported by the distinct repertoires of Treg and Tconv, and the overlap of the thymic and peripheral repertoires of each population [42]. Cellular markers that distinguish iTreg and nTreg could more robustly define their relative quantities and properties, though fully validated markers are lacking. One candidate is the transcription factor Helios. Helios binds to the Foxp3 promoter and supports Foxp3 transcription [77]. Although Helios is present in virtually all Foxp3⁺ thymocytes, it is lacking in 30% of peripheral Treg, and iTreg induced *in vitro* or by Ag feeding *in vivo* lack Helios [78]. Some have questioned the reliability of Helios in distinguishing the populations based on data showing potential signal-dependent upregulation of Helios during iTreg induction [79]. Notably, transcriptional profiles of iTreg and nTreg differ [80], indicating that the genetic programs governing these cell types also differ. It is likely that as additional markers are surveyed, ones able to distinguish the cell classes will be identified.

Considering the dependence of iTreg on environmental factors, including cytokine and TCR signaling provided contextually rather than constitutively available, and the distinct genetic profiles of iTreg and nTreg, it would seem inappropriate to consider iTreg and nTreg a single lineage. Rather, iTreg appear to represent a distinct maturation subset of Tconv, analogous to Th1 or Th2 cells, though with a great deal of functional and phenotypic overlap with nTreg.

4. Stability of Treg

Landmark studies by Sakaguchi and others that identified the role of Foxp3 in immune regulation also indicated that it is a master regulator of Treg function [10,11,81]. T cells transduced with Foxp3 show many of the functions of Treg. They are able to suppress T cell proliferation *in vitro* and T cell mediated autoimmunity *in vivo*. However, many Treg

properties, including *in vitro* anergy, the ability to suppress proliferation of T cells in trans, and IL-2 dependence for survival, are also shared with subsets of Foxp3⁺ cells. For instance, we showed that Foxp3⁺ T cells that are Ag-refractory after stimulation are capable of non-specific suppression *in vitro* and *in vivo* [82–84]. Similar capabilities have been attributed to anergic Tconv [85]. The suppressive mechanisms utilized by Treg, including production of cytokines such as IL10, TGF β , and IL35, expression of CTLA-4, LAG-3, and other downmodulatory cell surface proteins, adenosine production, and direct cytotoxicity, are also utilized by subsets of Tconv [86]. In addition, Foxp3⁺ fluorescent-protein-marker-positive T cells from mice in which Foxp3 in some T cells is replaced with the fluorescent protein are anergic and express Treg-associated proteins [20]. Transcriptional profiling studies analyzing these cells or comparing Treg of different types and from different locations suggest that Foxp3 may not induce a single cellular program [80,87]. Rather it may in part act as a stabilizing factor that helps preserve regulatory function within cells [88].

Though Treg can promote the formation of additional regulatory T cell types [89], the requirement for continuous Treg presence is unequivocal. *In vitro*, loss of Foxp3 in iTreg is associated with a coordinate loss of suppressive activity [50]. *In vivo*, ablation of Treg in adult mice genetically modified to express diphtheria toxin receptor in Foxp3⁺ cells leads to multi-organ autoimmunity [90]. Considering the indispensable and lifelong role of Foxp3 in Treg function, studies of Treg stability have justly used Foxp3 expression as a marker for functional Treg.

Some evidence suggests that Foxp3 expression may not be as stable as critical genetic regulators defining other cellular lineages. In particular, Foxp3 may be downmodulated in response to inflammatory stimuli. Thus TLR2 induced MyD88 signaling was found to depress Foxp3 expression in an IRF-1 dependent manner [91]. IRF-1 binds to the Foxp3 promoter inhibiting transcription. IL6 induces methylation of the Foxp3 enhancer, which may depress transcription [18]. Cytokine induced STAT signaling as well as indirect signaling through TGF β and other pathways influences Foxp3 expression. Indeed, TGF β or IL2 deficiency markedly diminishes Treg numbers [92–95]. Re-differentiation of Treg into Foxp3⁺ effector T cells has been observed in some models. For example, IL6 and IL1 can downmodulate Foxp3 in Treg and promote their conversion into Th17 cells [96]. Loss of DICER, an enzyme required for miRNA formation leads to downregulation of Foxp3 and other Treg associated genes, loss of Treg suppression, and the development of lethal systemic autoimmunity [97]. In one study, Foxp3⁺ T cells were observed to convert into follicular helper T cells in gut Peyer's patches [73]. Lymphopenia may promote loss of Foxp3 in Treg [98]. Therefore, much as iTreg may be induced in physiologic circumstances from Tconv, it is reasonable to consider that nTreg may correspondingly lose Foxp3 and convert into effector types of T cells.

Adoptive transfer studies have more directly examined Treg stability. In one analysis, ~10% of flow cytometrically purified Treg transferred into wt mice converted into Tconv [5]. Features of the Treg, such as the level of CD25 expression, correlated with Foxp3 downregulation indicating that phenotypically distinct populations of Treg with different stabilities normally circulate.

A comparison of Foxp3 stability in *ex vivo* expanded nTreg and iTreg transferred into lymphoreplete mice found that nTreg preserve Foxp3 to a much greater extent than iTreg [50]. Potentially the unstable population of Treg that lose Foxp3 after adoptive transfer are comprised of iTreg. Definitive analyses of the relative stabilities of iTreg and nTreg will be important, but are currently hampered by the lack of definitive subset markers.

Additional tools have been applied to establish Treg stability. In an elegant approach, a bacterial artificial chromosome (bac) Foxp3 promoter-Cre transgene is used to specifically and permanently turn on a floxed fluorescent transgene, YFP, in cells that at any time in their lifecycle express Foxp3 [99]. Enumeration of Foxp3⁺ YFP⁺ cells thereby provides an estimate of the number of cells that expressed and then downmodulated Foxp3, referred to as ex-Treg. In otherwise unmanipulated mice, ~20% of peripheral YFP⁺ cells lack coordinate Foxp3 expression, suggesting that they had previously expressed and turned off Foxp3. Ex-Treg cell numbers are increased in inflamed tissues and autoimmunity, and transfer of β -islet specific ex-Treg T cells lead to diabetes development. Limitations of this approach include the possibility that the Foxp3-Cre transgene does not perfectly mimic native Foxp3 expression and that Foxp3 in ex-Treg is transiently or suboptimally induced leading to YFP expression in cells that have never fully converted into Treg. Indeed, TGF β can transiently induce Foxp3 in naïve T cells destined to convert into Th17 effectors.

In a variation of this approach, a Foxp3-eGFP-CreERT2 multicistronic gene was knocked into the Foxp3 locus. Here too, Foxp3 expression encodes a Cre [100]. But the CreERT2 chimeric protein is sequestered until addition of an estrogen analog, tamoxifen. After tamoxifen treatment a floxed transgenic fluorescent marker, here too YFP, is expressed. This system therefore allows a transient, tamoxifen-dependent induction of the YFP marker and thereby assesses the fate of cells that are Foxp3⁺ at a particular time point. In this case, when Foxp3⁺ Treg are induced to express YFP, a high level of stability is found. Even 5–8 months after YFP induction, virtually all of the YFP⁺ cells remain Foxp3⁺. Notably, a small population of Foxp3⁺ YFP⁺ cells (<5%) are observed shortly after tamoxifen treatment, however these fail to expand and decrease over time. Potentially, this population of short lived cells in part corresponds to the “ex-Treg” identified in the study described above where the Cre transgene was constitutively expressed in Foxp3⁺ cells. Interestingly, in this inducible system, Treg expressing YFP also retain Foxp3 expression after infection with *Listeria* or in lymphopenic conditions, indicating that physiologic inflammation or induced homeostatic expansion does not promote Treg conversion into Tconv. Cumulatively, these results demonstrate a high level of stability of Treg, both in terms of Foxp3 expression and long-term survival and maintenance. Nevertheless, some Treg can lose Foxp3 and transform into effector type T cells. The relative representation of iTreg and nTreg in the peripheral circulation remains to be determined, but if endogenously formed iTreg indeed have stabilities as low as *in vitro*-generated iTreg, they likely represent a small fraction of the total Treg population. Although speculative, it is possible that these correspond to a considerable fraction of the unstable Treg population.

5. iTreg and nTreg utilization in immune responses

The findings above indicate dual sources for Treg; a thymic pathway that likely contributes the majority of normally circulating Foxp3⁺ T cells, and an adaptive pathway in which Foxp3 is induced extrathymically.

Both iTreg and nTreg are highly functional in the adoptive immunotherapy of a variety of autoimmune and alloimmune conditions, and often function in similar manners [101–104]. For example, IL10 production is critical for both iTreg and nTreg activity in experimental allergic encephalomyelitis (EAE), a model of Multiple Sclerosis [105]. Yet, considering their differences, it would seem appropriate to consider these cells as separate populations. The extent to which iTreg and nTreg normally participate in immune responses therefore has been a subject of interest.

Several studies have surveyed TCR repertoires to assess the involvement of iTreg and nTreg, and the interconversion between these subsets. In these, TCR sequence, due to its

high level of diversity, serves as a tag to identify individual clones of T cells. By tracking the presence of specific sequences in mice with or without disease, and surveying the overlap between Treg and Tconv sequences, it is possible to define the extent of interconversion between the cell types and hence the formation of iTreg. In two studies, we found limited interconversion between Tconv and Treg in mice with EAE [32,43]. Indeed, analyses of TCR from mice with a fixed TCR α , and focusing on TCR with a specific V β and J β associated with disease, highlighted specific complementarity determining region 3 (CDR3) motifs that were present in TCR that recognized autoantigen, regardless of whether they were Treg or Tconv. Among TCR recognizing autoantigen, a separate CDR3 motif further distinguished Treg and Tconv TCR. This indicates that the Treg and Tconv TCR repertoires in this autoimmune model are ontologically distinct, though possess convergent sequence motifs that enable recognition of a common autoantigen. Interestingly, cloned Treg and Tconv TCR expressed in T cell lines showed similar sensitivities and fine specificities for the autoantigen, implying that the receptor differences did not confer altered Ag-recognition properties.

A study of diabetes, looking at the non-allelically excluded TCR α of a transgenic TCR also failed to find evidence of significant conversion between the Treg and Tconv populations [106]. Studies of Treg responding to tumors have produced more varied results. A carcinogen-induced cancer model did not identify significant interconversion between Tconv and Treg [107]. In contrast, two other studies of T cell tumor responses indicated that iTreg can be induced to tumor Ags [108,109]. Therefore the extent of iTreg induction appears highly contextual.

In the intestinal tract, large numbers of commensal bacteria will provide the bulk of the antigenic content encountered by cells. These Ags may not be well represented in the thymus. Yet guarding against inflammatory responses to them is an imperative for gastrointestinal health. iTreg formation may therefore be important to generate specific Treg, allowing for a flexible response to the large and changing menagerie of commensals the immune system encounters. Indeed, *In vivo*, expression of TGF β and RA in the gut, as well as the presence of a panoply of Ags derived from commensal bacteria provides fertile conditions for iTreg induction [55,110,111]. In support of this, transfer of purified Tconv into Rag^{-/-} mice leads to the development of colitis, but also the conversion of some of these cells into iTreg. Interestingly, *Bacteroides*, *Chlostridia*, and other bacterial spp are able to induce iTreg formation when introduced into germ free animals [111–113]. Therefore iTreg and nTreg may play distinct roles in protecting against immune-mediated pathologies. Depending on location and exposure, the cell types may be alternatively utilized.

6. Functional plasticity of Treg

Emerging evidence indicates that much as Tconv can differentiate into subsets specialized for specific pathogens and hazards, Treg also possess a remarkable degree of functional plasticity. There is logic in this. When effector T cells respond to a pathogen, they secrete or promote the secretion of chemokines that attract like-differentiated cells. For example, Th1 cells express CXCR3 and secrete IFN γ . IFN γ in turn promotes the secretion of ligands for CXCR3, including CXCL9, CXCL10, and CXCL11, which further attract Th1 cells and thereby amplify the response [114–116].

In Th1 inducing conditions, such as infection with *T. gondii* or *M. tuberculosis*, Treg adopt Th1-like properties [117,118]. Tbet, the critical transcription factor for this cell type is induced, and the cells upregulate CXCR3. This occurs in a Stat1 dependent manner in response to IFN γ , already abundant in the ongoing Th1 response. Indeed, many of these

Treg will even produce IFN γ , and this may serve to provide autocrine or paracrine feedback. Importantly, Tbet⁺ Tregs have a heightened capacity for controlling Th1 responses as compared to Tbet⁻ Tregs, and also show activity in a Th1/Th17-biased autoimmune disease, EAE [119]. Neutralizing IFN γ during co-transfer of alloreactive Treg cells along with T effector cells in a model of GVHD resulted in tissue necrosis and graft rejection [120]. Therefore Treg act conservatively, utilizing the same cytokine pathways that promote pathogenicity and effector function to direct immune suppression. Notably, Stat1 stimulation, which may occur through IFN γ or IL27, promotes Foxp3 expression, and so formation of Th1-like Tregs does not appear to indicate a loss of regulatory phenotype [121]. miR146a microRNA in Treg also plays a critical role in promoting Treg-mediated Th1 suppression, and does so by regulating STAT1 signaling [122]. Importantly ablation of Tbet in Tregs leads to uncontrolled Th1 responses [117].

Similar polarization of Treg is apparent in other T cell responses. ROR γ t is the critical transcription factor governing Th17 differentiation. It and Foxp3 are reciprocally regulated during differentiation with TGF β , which promotes iTreg generation, or TGF β and IL-6, which promotes Th17 formation [123]. TCR stimulation in the presence of TGF β or TGF β and IL6 is initially accompanied by a transient expression of both ROR γ t and Foxp3. Interestingly, a significant proportion of extrathymic Treg are found to produce IL17 and express ROR γ t [124–126]. These are also positive for CCR6, an important chemokine receptor expressed by Th17 cells. *In vitro*, IL1 β , IL6, IL21, and IL23 cooperatively promote the generation of IL17-producing Treg.

CCR6 is necessary for Treg to suppress EAE disease, a disorder characterized by a prominent Th17 response [127], and in a Th17-driven colitis model, CCR6⁺ Treg, which also produce IL10, preferentially migrate to the colon and provide protection [128]. Treg deficiency in Stat3, a signaling molecule critical for Th17 differentiation, also leads to uncontrolled Th17 responses [129]. Therefore, much as with Th1-skewed Treg, Th17 skewing of Treg appears to play a role in targeting Th17 responses. Though expression of pro-inflammatory genes, such as IL17 may diminish the regulatory activity of such cells, the overall impact of this skewing is likely beneficial. Coordinate expression of homing and other markers may direct these Treg to sites where immunopathology is prominent, and promote a homeostatic response.

The paradigm of Treg subset properties paralleling those of effector cells also extends to Th2 responses. Expression of the transcription factor IRF4 regulates Th subset differentiation. IRF4 deficient mice fail to upregulate GATA3 or generate Th2 cells upon IL4 stimulation [130]. IRF4 further influences cytokine production in differentiated Th2 cells [131]. IRF4 is known to bind to and synergize with NFATc2 and c-maf in promoting IL-4 transcription [132]. Similar to the pattern observed with Th1 and Th17-skewed Treg, IRF4 expressing Tregs are particularly adept at curbing Th2 responses. Conditional deletion of IRF4 in Treg results in a pathologic phenotype characterized by markedly dysregulated Th2 response, IL-4 dependant antibody production, and tissue lesions with increased plasma cell infiltration [133].

A pattern has therefore emerged whereby Treg further mature into Treg subsets specialized for different types of immune responses. Th1, Th17, and Th2-directed Treg have now been identified. The plasticity of mature Treg is, however, incompletely understood. Whether specialized subsets of Treg will also be directed against additional effector cell types, such as Th9 and Th22 cells, and other forms of immune responses will be of interest.

7. Concluding Remarks

Since the identification of Foxp3 as a unique identifier of a regulatory T cell subset nearly a decade ago, studies of Foxp3-expressing Treg have revolutionized our understanding of immune control mechanisms and immune-mediated diseases, and opened a window for a new generation of therapies that act by modulating Treg numbers and function. Treg are now understood to comprise two major constituent groups, nTreg that form developmentally in the thymus and appear to constitute a distinct lineage of CD4⁺ T cells, and iTreg, which form from Tconv in response to environmental cues and display less stability. Within these classes, Treg demonstrate further plasticity, taking on characteristics of the cell types they suppress in order to optimally target specific types of immune responses. These act through a panoply of effector mechanisms to focally modulate immune responses. As our understanding of these crucial peacekeepers of the immune response grows, so does our recognition of their vital interconnectedness with virtually all aspects of immunity and inflammation.

Acknowledgments

This work was supported by the National Institutes of Health Grant R01 AI056153 (to TLG), and the American Lebanese Syrian Associated Charities (ALSAC)/St. Jude Children's Research Hospital (to TLG and ST).

Abbreviations

Foxp3	Forkhead box transcription factor P3
Treg	CD4 ⁺ Foxp3 ⁺ regulatory T lymphocyte
Tconv	conventional CD4 ⁺ T lymphocyte
nTreg	natural Treg
iTreg	induced Treg
bac	bacterial artificial chromosome
YFP	yellow fluorescent protein
Ag	antigen
TCR	T cell receptor
Tg	transgenic

References

1. Jager A, Kuchroo VK. Effector and regulatory T-cell subsets in autoimmunity and tissue inflammation. *Scand J Immunol.* 2010; 72:173–184. [PubMed: 20696013]
2. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol.* 2005; 6:331–337. [PubMed: 15785758]
3. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3⁺ regulatory T cells in the human immune system. *Nat Rev Immunol.* 2010; 10:490–500. [PubMed: 20559327]
4. Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA. Plasticity of CD4(+) FoxP3(+) T cells. *Curr Opin Immunol.* 2009; 21:281–285. [PubMed: 19500966]
5. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural Foxp3⁺ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A.* 2009; 106:1903–1908. [PubMed: 19174509]

6. Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity*. 2009; 30:626–635. [PubMed: 19464985]
7. Horwitz DA, Zheng SG, Gray JD. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol*. 2008; 29:429–435. [PubMed: 18676178]
8. Wang L, Bosselut R. CD4–CD8 lineage differentiation: Thpok-ing into the nucleus. *J Immunol*. 2009; 183:2903–2910. [PubMed: 19696430]
9. Collins A, Littman DR, Taniuchi I. RUNX proteins in transcription factor networks that regulate T-cell lineage choice. *Nat Rev Immunol*. 2009; 9:106–115. [PubMed: 19165227]
10. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003; 299:1057–1061. [PubMed: 12522256]
11. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003; 4:330–336. [PubMed: 12612578]
12. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*. 2007; 445:936–940. [PubMed: 17237761]
13. Marson A, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, Levine SS, Fraenkel E, von BH, Young RA. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature*. 2007; 445:931–935. [PubMed: 17237765]
14. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010; 463:808–812. [PubMed: 20072126]
15. Hori S. Rethinking the molecular definition of regulatory T cells. *Eur J Immunol*. 2008; 38:928–930. [PubMed: 18395863]
16. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang HD, Bopp T, Schmitt E, Klein-Hessling S, Serfling E, Hamann A, Huehn J. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*. 2007; 5:e38. [PubMed: 17298177]
17. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood*. 2009; 114:3727–3735. [PubMed: 19641188]
18. Lal G, Zhang N, van der TW, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J Immunol*. 2009; 182:259–273. [PubMed: 19109157]
19. Ruan Q, Kameswaran V, Tone Y, Li L, Liou HC, Greene MI, Tone M, Chen YH. Development of Foxp3(+) regulatory T cells is driven by the c-Rel enhanceosome. *Immunity*. 2009; 31:932–940. [PubMed: 20064450]
20. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, Rudensky AY. Foxp3-dependent programme of regulatory T–cell differentiation. *Nature*. 2007; 445:771–775. [PubMed: 17220874]
21. Lee HM, Hsieh CS. Rare development of Foxp3+ thymocytes in the CD4+CD8+ subset. *J Immunol*. 2009; 183:2261–2266. [PubMed: 19620303]
22. Pennington DJ, Silva-Santos B, Silberzahn T, Escorcio-Correia M, Woodward MJ, Roberts SJ, Smith AL, Dyson PJ, Hayday AC. Early events in the thymus affect the balance of effector and regulatory T cells. *Nature*. 2006; 444:1073–1077. [PubMed: 17190001]
23. Lio CW, Dodson LF, Deppong CM, Hsieh CS, Green JM. CD28 facilitates the generation of Foxp3(–) cytokine responsive regulatory T cell precursors. *J Immunol*. 2010; 184:6007–6013. [PubMed: 20421644]
24. Bensinger SJ, Bandeira A, Jordan MS, Caton AJ, Laufer TM. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. *J Exp Med*. 2001; 194:427–438. [PubMed: 11514600]
25. Ribot J, Enault G, Pilipenko S, Huchenq A, Calise M, Hudrisier D, Romagnoli P, van Meerwijk JP. Shaping of the autoreactive regulatory T cell repertoire by thymic cortical positive selection. *J Immunol*. 2007; 179:6741–6748. [PubMed: 17982064]

26. Liston A, Nutsch KM, Farr AG, Lund JM, Rasmussen JP, Koni PA, Rudensky AY. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. *Proc Natl Acad Sci U S A*. 2008; 105:11903–11908. [PubMed: 18695219]
27. Walker LS, Chodos A, Eggena M, Dooms H, Abbas AK. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med*. 2003; 198:249–258. [PubMed: 12874258]
28. Hori S, Haury M, Coutinho A, Demengeot J. Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc Natl Acad Sci U S A*. 2002; 99:8213–8218. [PubMed: 12034883]
29. Larkin J III, Rankin AL, Picca CC, Riley MP, Jenks SA, Sant AJ, Caton AJ. CD4+CD25+ regulatory T cell repertoire formation shaped by differential presentation of peptides from a self-antigen. *J Immunol*. 2008; 180:2149–2157. [PubMed: 18250421]
30. Alli R, Nguyen P, Geiger TL. Retrogenic modeling of experimental allergic encephalomyelitis associates T cell frequency but not TCR functional affinity with pathogenicity. *J Immunol*. 2008; 181:136–145. [PubMed: 18566378]
31. Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity*. 2004; 21:267–277. [PubMed: 15308106]
32. Liu X, Nguyen P, Liu W, Cheng C, Steeves M, Obenaus JC, Ma J, Geiger TL. T cell receptor CDR3 sequence but not recognition characteristics distinguish autoreactive effector and Foxp3(+) regulatory T cells. *Immunity*. 2009; 31:909–920. [PubMed: 20005134]
33. Pacholczyk R, Ignatowicz H, Kraj P, Ignatowicz L. Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity*. 2006; 25:249–259. [PubMed: 16879995]
34. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol*. 2006; 7:401–410. [PubMed: 16532000]
35. Romagnoli P, Hudrisier D, van Meerwijk JP. Preferential recognition of self antigens despite normal thymic deletion of CD4(+)CD25(+) regulatory T cells. *J Immunol*. 2002; 168:1644–1648. [PubMed: 11823492]
36. Dipaolo RJ, Shevach EM. CD4+ T-cell development in a mouse expressing a transgenic TCR derived from a Treg. *Eur J Immunol*. 2009; 39:234–240. [PubMed: 19065648]
37. Simons DM, Picca CC, Oh S, Perng OA, Aitken M, Erikson J, Caton AJ. How specificity for self-peptides shapes the development and function of regulatory T cells. *J Leukoc Biol*. 2010; 88:1099–1107. [PubMed: 20495071]
38. Ohkura N, Sakaguchi S. Regulatory T cells: roles of T cell receptor for their development and function. *Semin Immunopathol*. 2010; 32:95–106. [PubMed: 20179931]
39. Pacholczyk R, Kern J. The T-cell receptor repertoire of regulatory T cells. *Immunology*. 2008; 125:450–458. [PubMed: 19128356]
40. Romagnoli P, van Meerwijk JP. Thymic selection and lineage commitment of CD4(+)Foxp3(+) regulatory T lymphocytes. *Prog Mol Biol Transl Sci*. 2010; 92:251–277. [PubMed: 20800824]
41. Wheeler KM, Samy ET, Tung KS. Cutting edge: normal regional lymph node enrichment of antigen-specific regulatory T cells with autoimmune disease-suppressive capacity. *J Immunol*. 2009; 183:7635–7638. [PubMed: 19923458]
42. Lathrop SK, Santacruz NA, Pham D, Luo J, Hsieh CS. Antigen-specific peripheral shaping of the natural regulatory T cell population. *J Exp Med*. 2008; 205:3105–3117. [PubMed: 19064700]
43. Nguyen P, Liu W, Ma J, Manirarora JN, Liu X, Cheng C, Geiger TL. Discrete TCR repertoires and CDR3 features distinguish effector and Foxp3+ regulatory T lymphocytes in myelin oligodendrocyte glycoprotein-induced experimental allergic encephalomyelitis. *J Immunol*. 2010; 185:3895–3904. [PubMed: 20810983]
44. Korn T, Reddy J, Gao W, Bettelli E, Awasthi A, Petersen TR, Backstrom BT, Sobel RA, Wucherpfennig KW, Strom TB, Oukka M, Kuchroo VK. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med*. 2007; 13:423–431. [PubMed: 17384649]

45. Pacholczyk R, Kern J, Singh N, Iwashima M, Kraj P, Ignatowicz L. Nonspecific antigens are the cognate specificities of Foxp3⁺ regulatory T cells. *Immunity*. 2007; 27:493–504. [PubMed: 17869133]
46. Leung MW, Shen S, Lafaille JJ. TCR-dependent differentiation of thymic Foxp3⁺ cells is limited to small clonal sizes. *J Exp Med*. 2009; 206:2121–2130. [PubMed: 19737865]
47. Bautista JL, Lio CW, Lathrop SK, Forbush K, Liang Y, Luo J, Rudensky AY, Hsieh CS. Intracolon competition limits the fate determination of regulatory T cells in the thymus. *Nat Immunol*. 2009; 10:610–617. [PubMed: 19430476]
48. Hanabuchi S, Ito T, Park WR, Watanabe N, Shaw JL, Roman E, Arima K, Wang YH, Voo KS, Cao W, Liu YJ. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3⁺ regulatory T cells in human thymus. *J Immunol*. 2010; 184:2999–3007. [PubMed: 20173030]
49. Zheng SG, Gray JD, Ohtsuka K, Yamagiwa S, Horwitz DA. Generation ex vivo of TGF- β -producing regulatory T cells from CD4⁺CD25⁻ precursors. *J Immunol*. 2002; 169:4183–4189. [PubMed: 12370347]
50. Selvaraj RK, Geiger TL. A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF- β . *J Immunol*. 2007; 178:7667–7677. [PubMed: 17548603]
51. Davidson TS, Dipaolo RJ, Andersson J, Shevach EM. Cutting Edge: IL-2 is essential for TGF- β -mediated induction of Foxp3⁺ T regulatory cells. *J Immunol*. 2007; 178:4022–4026. [PubMed: 17371955]
52. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol*. 2008; 9:194–202. [PubMed: 18157133]
53. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med*. 2007; 204:1543–1551. [PubMed: 17591856]
54. Maruyama T, Li J, Vaque JP, Konkel JE, Wang W, Zhang B, Zhang P, Zamarron BF, Yu D, Wu Y, Zhuang Y, Gutkind JS, Chen W. Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3. *Nat Immunol*. 2011; 12:86–95. [PubMed: 21131965]
55. Coombes JL, Siddiqui KR, Rancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103⁺ DCs induces Foxp3⁺ regulatory T cells via a TGF- β and retinoic acid-dependent mechanism. *J Exp Med*. 2007; 204:1757–1764. [PubMed: 17620361]
56. Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, O'Shea JJ. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood*. 2008; 111:1013–1020. [PubMed: 17951529]
57. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, Caccamo M, Oukka M, Weiner HL. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature*. 2008; 453:46–47. [PubMed: 18451850]
58. Liu G, Yang K, Burns S, Shrestha S, Chi H. The S1P(1)-mTOR axis directs the reciprocal differentiation of T(H)1 and T(reg) cells. *Nat Immunol*. 2010; 11:1047–1056. [PubMed: 20852647]
59. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4⁺Foxp3⁺ cells. *J Exp Med*. 2008; 205:565–574. [PubMed: 18283119]
60. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009; 206:3015–3029. [PubMed: 20008522]
61. Kopf H, de la Rosa GM, Howard OM, Chen X. Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3⁺ T regulatory cells. *Int Immunopharmacol*. 2007; 7:1819–1824. [PubMed: 17996694]
62. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, Knight ZA, Cobb BS, Cantrell D, O'Connor E, Shokat KM, Fisher AG, Merkenschlager M. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A*. 2008; 105:7797–7802. [PubMed: 18509048]

63. Ouyang W, Beckett O, Ma Q, Paik JH, DePinho RA, Li MO. Foxo proteins cooperatively control the differentiation of Foxp3⁺ regulatory T cells. *Nat Immunol.* 2010; 11:618–627. [PubMed: 20467422]
64. Harada Y, Harada Y, Elly C, Ying G, Paik JH, DePinho RA, Liu YC. Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells. *J Exp Med.* 2010; 207:1381–1391. [PubMed: 20439537]
65. Kerdiles YM, Stone EL, Beisner DL, McGargill MA, Ch'en IL, Stockmann C, Katayama CD, Hedrick SM. Foxo transcription factors control regulatory T cell development and function. *Immunity.* 2010; 33:890–904. [PubMed: 21167754]
66. Gottschalk RA, Corse E, Allison JP. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *J Exp Med.* 2010; 207:1701–1711. [PubMed: 20660617]
67. Oliveira VG, Caridade M, Paiva RS, Demengeot J, Graca L. Sub-optimal CD4 T cell activation triggers autonomous TGF-beta-dependent conversion to Foxp3(+) regulatory T cells. *Eur J Immunol.* 2011; 10
68. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol.* 2005; 6:152–162. [PubMed: 15640801]
69. Golovina TN, Mikheeva T, Suhoski MM, Aqui NA, Tai VC, Shan X, Liu R, Balcarcel RR, Fisher N, Levine BL, Carroll RG, Warner N, Blazar BR, June CH, Riley JL. CD28 costimulation is essential for human T regulatory expansion and function. *J Immunol.* 2008; 181:2855–2868. [PubMed: 18684977]
70. Semple K, Nguyen A, Yu Y, Wang H, Anasetti C, Yu XZ. Strong CD28 costimulation suppresses induction of regulatory T cells from naive precursors through Lck signaling. *Blood.* 2011; 117:3096–3103. [PubMed: 21245484]
71. Liang S, Alard P, Zhao Y, Parnell S, Clark SL, Kosiewicz MM. Conversion of CD4⁺CD25⁺ cells into CD4⁺CD25⁺ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J Exp Med.* 2005; 201:127–137. [PubMed: 15630140]
72. Apostolou I, von Boehmer H. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med.* 2004; 199:1401–1408. [PubMed: 15148338]
73. Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, Hori S, Fagarasan S. Preferential generation of follicular B helper T cells from Foxp3⁺ T cells in gut Peyer's patches. *Science.* 2009; 323:1488–1492. [PubMed: 19286559]
74. Mucida D, Kutchukhidze N, Erazo A, Russo M, Lafaille JJ, Curotto de Lafaille MA. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest.* 2005; 115:1923–1933. [PubMed: 15937545]
75. Verginis P, McLaughlin KA, Wucherpfennig KW, von BH, Apostolou I. Induction of antigen-specific regulatory T cells in wild-type mice: visualization and targets of suppression. *Proc Natl Acad Sci U S A.* 2008; 105:3479–3484. [PubMed: 18299571]
76. Daniel C, Ploegh H, von BH. Antigen-specific induction of regulatory T cells in vivo and in vitro. *Methods Mol Biol.* 2011; 707:173–185. [PubMed: 21287335]
77. Getnet D, Grosso JF, Goldberg MV, Harris TJ, Yen HR, Bruno TC, Durham NM, Hipkiss EL, Pyle KJ, Wada S, Pan F, Pardoll DM, Drake CG. A role for the transcription factor Helios in human CD4⁺CD25⁺ regulatory T cells. *Mol Immunol.* 2010; 47:1595–1600. [PubMed: 20226531]
78. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3⁺ T regulatory cells. *J Immunol.* 2010; 184:3433–3441. [PubMed: 20181882]
79. Verhagen J, Wraith DC. Comment on “Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3⁺ T regulatory cells”. *J Immunol.* 2010; 185:7129. [PubMed: 21127313]
80. Hill JA, Feuerer M, Tash K, Haxhinasto S, Perez J, Melamed R, Mathis D, Benoist C. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity.* 2007; 27:786–800. [PubMed: 18024188]

81. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol.* 2003; 4:337–342. [PubMed: 12612581]
82. Duthoit CT, Nguyen P, Geiger TL. Antigen nonspecific suppression of T cell responses by activated stimulation-refractory CD4+ T cells. *J Immunol.* 2004; 172:2238–2246. [PubMed: 14764692]
83. Inaba H, Geiger TL. Defective cell cycle induction by IL-2 in naive T-cells antigen stimulated in the presence of refractory T-lymphocytes. *Int Immunol.* 2006; 18:1043–1054. [PubMed: 16702166]
84. Inaba H, Steeves M, Nguyen P, Geiger TL. In vivo suppression of naive CD4 T cell responses by IL-2- and antigen-stimulated T lymphocytes in the absence of APC competition. *J Immunol.* 2008; 181:3323–3335. [PubMed: 18714004]
85. Jooss K, Gjata B, Danos O, von Boehmer H, Sarukhan A. Regulatory function of in vivo anergized CD4(+) T cells. *Proc Natl Acad Sci U S A.* 2001; 98:8738–8743. [PubMed: 11438696]
86. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol.* 2008; 8:523–532. [PubMed: 18566595]
87. Feuerer M, Hill JA, Kretschmer K, von BH, Mathis D, Benoist C. Genomic definition of multiple ex vivo regulatory T cell subphenotypes. *Proc Natl Acad Sci U S A.* 2010; 107:5919–5924. [PubMed: 20231436]
88. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity.* 2009; 30:616–625. [PubMed: 19464984]
89. Collison LW, Chaturvedi V, Henderson AL, Giacomini PR, Guy C, Bankoti J, Finkelstein D, Forbes K, Workman CJ, Brown SA, Reh JE, Jones ML, Ni HT, Artis D, Turk MJ, Vignali DA. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol.* 2010; 11:1093–1101. [PubMed: 20953201]
90. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol.* 2007; 8:191–197. [PubMed: 17136045]
91. Lal G, Yin N, Xu J, Lin M, Schroppel S, Ding Y, Marie I, Levy DE, Bromberg JS. Distinct inflammatory signals have physiologically divergent effects on epigenetic regulation of foxp3 expression and treg function. *Am J Transplant.* 2011; 11:203–214. [PubMed: 21219575]
92. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol.* 2007; 178:280–290. [PubMed: 17182565]
93. Bayer AL, Yu A, Malek TR. Function of the IL-2R for thymic and peripheral CD4+CD25+ Foxp3+ T regulatory cells. *J Immunol.* 2007; 178:4062–4071. [PubMed: 17371960]
94. Ouyang W, Beckett O, Ma Q, Li MO. Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity.* 2010; 32:642–653. [PubMed: 20471291]
95. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med.* 2005; 201:1061–1067. [PubMed: 15809351]
96. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, Shah B, Chang SH, Schluns KS, Watowich SS, Feng XH, Jetten AM, Dong C. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity.* 2008; 29:44–56. [PubMed: 18585065]
97. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, Bluestone JA. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med.* 2008; 205:1983–1991. [PubMed: 18725525]
98. Duarte JH, Zelenay S, Bergman ML, Martins AC, Demengeot J. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. *Eur J Immunol.* 2009; 39:948–955. [PubMed: 19291701]
99. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol.* 2009; 10:1000–1007. [PubMed: 19633673]

100. Rubtsov YP, Nieuwehuis RE, Josefowicz S, Li L, Darce J, Mathis D, Benoist C, Rudensky AY. Stability of the regulatory T cell lineage in vivo. *Science*. 2010; 329:1667–1671. [PubMed: 20929851]
101. Mekala DJ, Alli RS, Geiger TL. IL-10-dependent infectious tolerance after the treatment of experimental allergic encephalomyelitis with redirected CD4+CD25+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2005; 102:11817–11822. [PubMed: 16087867]
102. Zheng SG, Meng L, Wang JH, Watanabe M, Barr ML, Cramer DV, Gray JD, Horwitz DA. Transfer of regulatory T cells generated ex vivo modifies graft rejection through induction of tolerogenic CD4+CD25+ cells in the recipient. *Int Immunol*. 2006; 18:279–289. [PubMed: 16415106]
103. Weber SE, Harbertson J, Godebu E, Mros GA, Padrick RC, Carson BD, Ziegler SF, Bradley LM. Adaptive islet-specific regulatory CD4 T cells control autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo. *J Immunol*. 2006; 176:4730–4739. [PubMed: 16585566]
104. Allan SE, Broady R, Gregori S, Himmel ME, Locke N, Roncarolo MG, Bacchetta R, Levings MK. CD4+ T-regulatory cells: toward therapy for human diseases. *Immunol Rev*. 2008; 223:391–421. [PubMed: 18613849]
105. Selvaraj RK, Geiger TL. Mitigation of Experimental Allergic Encephalomyelitis by TGF- β Induced Foxp3+ Regulatory T Lymphocytes through the Induction of Anergy and Infectious Tolerance. *J Immunol*. 2008; 180:2830–2838. [PubMed: 18292504]
106. Wong J, Mathis D, Benoist C. TCR-based lineage tracing: no evidence for conversion of conventional into regulatory T cells in response to a natural self-antigen in pancreatic islets. *J Exp Med*. 2007; 204:2039–2045. [PubMed: 17724131]
107. Hindley JP, Ferreira C, Jones E, Lauder SN, Ladell K, Wynn KK, Betts GJ, Singh Y, Price DA, Godkin AJ, Dyson J, Gallimore A. Analysis of the T-cell receptor repertoires of tumor-infiltrating conventional and regulatory T cells reveals no evidence for conversion in carcinogen-induced tumors. *Cancer Res*. 2011; 71:736–746. [PubMed: 21156649]
108. Kuczma M, Kopij M, Pawlikowska I, Wang CY, Rempala GA, Kraj P. Intratumoral convergence of the TCR repertoires of effector and Foxp3+ CD4+ T cells. *PLoS ONE*. 2010; 5:e13623. [PubMed: 21049016]
109. Fourcade J, Sun Z, Kudela P, Janjic B, Kirkwood JM, ElvHafnawy T, Zarour HM. Human tumor antigen-specific helper and regulatory T cells share common epitope specificity but exhibit distinct T cell repertoire. *J Immunol*. 2010; 184:6709–6718. [PubMed: 20483736]
110. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. 2007; 204:1775–1785. [PubMed: 17620362]
111. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A*. 2010; 107:12204–12209. [PubMed: 20566854]
112. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov II, Umesaki Y, Itoh K, Honda K. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*. 2011; 331:337–341. [PubMed: 21205640]
113. Lyons A, O'Mahony D, O'Brien F, MacSharry J, Sheil B, Ceddia M, Russell WM, Forsythe P, Bienenstock J, Kiely B, Shanahan F, O'Mahony L. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. *Clin Exp Allergy*. 2010; 40:811–819. [PubMed: 20067483]
114. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000; 100:655–669. [PubMed: 10761931]
115. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, Glimcher LH. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. *Blood*. 2005; 106:3432–3439. [PubMed: 16014561]
116. Clark-Lewis I, Mattioli I, Gong JH, Loetscher P. Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. *J Biol Chem*. 2003; 278:289–295. [PubMed: 12417585]

117. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol.* 2009; 10:595–602. [PubMed: 19412181]
118. Oldenhove G, Bouladoux N, Wohlfert EA, Hall JA, Chou D, Dos SL, O'Brien S, Blank R, Lamb E, Natarajan S, Kastenmayer R, Hunter C, Grigg ME, Belkaid Y. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity.* 2009; 31:772–786. [PubMed: 19896394]
119. O'connor RA, Leech MD, Suffner J, Hammerling GJ, Anderton SM. Myelin' reactive, TGF-beta-induced regulatory T cells can be programmed to develop Th1-like effector function but remain less proinflammatory than myelin-reactive Th1 effectors and can suppress pathogenic T cell clonal expansion in vivo. *J Immunol.* 2010; 185:7235–7243. [PubMed: 21084662]
120. Sawitzki B, Kingsley CI, Oliveira V, Karim M, Herber M, Wood KJ. IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *J Exp Med.* 2005; 201:1925–1935. [PubMed: 15967822]
121. Ouaked N, Mantel PY, Bassin C, Burgler S, Siegmund K, Akdis CA, Schmidt-Weber CB. Regulation of the foxp3 gene by the Th1 cytokines: the role of IL-27-induced STAT1. *J Immunol.* 2009; 182:1041–1049. [PubMed: 19124747]
122. Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, Yoshimura A, Baltimore D, Rudensky AY. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell.* 2010; 142:914–929. [PubMed: 20850013]
123. Ziegler SF, Buckner JH. FOXP3 and the regulation of Treg/Th17 differentiation. *Microbes Infect.* 2009; 11:594–598. [PubMed: 19371792]
124. Ayyoub M, Deknuydt F, Raimbaud I, Dousset C, Leveque L, Bioley G, Valmori D. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. *Proc Natl Acad Sci U S A.* 2009; 106:8635–8640. [PubMed: 19439651]
125. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, Bover L, Hanabuchi S, Khalili J, Marinova E, Zheng B, Littman DR, Liu YJ. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A.* 2009; 106:4793–4798. [PubMed: 19273860]
126. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, Hafler DA. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood.* 2009; 113:4240–4249. [PubMed: 19171879]
127. Villares R, Cadenas V, Lozano M, Almonacid L, Zaballos A, Martinez A, Varona R. CCR6 regulates EAE pathogenesis by controlling regulatory CD4+ T-cell recruitment to target tissues. *Eur J Immunol.* 2009; 39:1671–1681. [PubMed: 19499521]
128. Kitamura K, Farber JM, Kelsall BL. CCR6 marks regulatory T cells as a colon-tropic, IL-10-producing phenotype. *J Immunol.* 2010; 185:3295–3304. [PubMed: 20720211]
129. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, Rudensky AY. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science.* 2009; 326:986–991. [PubMed: 19797626]
130. Lohoff M, Mittrucker HW, Prechtl S, Bischof S, Sommer F, Kock S, Ferrick DA, Duncan GS, Gessner A, Mak TW. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A.* 2002; 99:11808–11812. [PubMed: 12189207]
131. Ahyi AN, Chang HC, Dent AL, Nutt SL, Kaplan MH. IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *J Immunol.* 2009; 183:1598–1606. [PubMed: 19592658]
132. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J Exp Med.* 2002; 195:1003–1012. [PubMed: 11956291]
133. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, Corcoran L, Treuting P, Klein U, Rudensky AY. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature.* 2009; 19:458:351–356.