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REVIEW

Transcriptional regulation and development of regulatory T cells

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Regulatory T (Treg) cells are a distinct subset of CD4$^+$ T cells. Instead of triggering adaptive immunity, they suppress immune responses. Small numbers of Treg cells reside within lymphoid organs and peripheral tissues, but their contribution to immune tolerance is so significant that defects in Treg cell function cause catastrophic immune disorders. Since they were first discovered 20 years ago, efforts have been made to understand the differences in developmental processes between Treg cells and conventional T cells that determine the ultimate fate of the overall T-cell population. Transcription factor Foxp3 is crucial for Treg cell differentiation, but it is not the whole story. Owing to recent advances in Treg cell research, we are now on the verge of appreciating the comprehensive mechanisms underlying Treg cell generation. Here, we discuss major discoveries, active study topics and remaining questions regarding Treg cell development.
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INTRODUCTION

The human body is defended by an immune system that responds to invading microorganisms. However, excessive or improper immune responses against self-antigens, innocuous antigens present in food, commensal microorganisms or fetal antigens can have detrimental effects; thus, they have to be constrained. Regulatory T (Treg) cells play a major role in restraining immune responses to maintain immune homeostasis. Since Treg cells are involved in many aspects of immune regulation, they have attracted much attention over the past two decades in terms of their basic mechanism(s) of action and their therapeutic potential. Since the discovery of Treg cells, knowledge about their development and differentiation has increased. Here, we briefly summarize established knowledge and describe recent advancements in the study of Treg cell development.

THE DISCOVERY OF TREG CELLS

Considering the boom in the Treg cell research field at the beginning of the twenty-first century, it is surprising that the earliest evidence of the existence of suppressive T cells goes back to 1969. In Japan, Nishizuka and Sakakura *et al.*[1] found that thymectomizing 3-day-old female mice caused sterility; however, this was not the case for mice aged 7 days or older. The cause of sterility was later found to be a part of autoimmune oophoritis due to a missing cell population derived from the thymus that was produced within the first week after birth.[2] Later reports strongly suggested the presence of T-cell-mediated immune tolerance to autoantigens.[3] When the likely candidate cell type, reported to express a marker called the I–J determinant, was isolated, T cells with an immunosuppressive phenotype became an active research subject.[4] However, despite two decades of study, researchers started to question both the existence of the I-J determinant and that of T cells expressing it. When the I-J determinant was proved to be absent from the expected locus,[5] the field of so-called "suppressor T lymphocytes" collapsed. T cells that could induce immune tolerance were later identified as a subset of CD4$^+$ T cells that constantly express the interleukin (IL-2) receptor α-chain CD25.[6] This cell type was further characterized by the expression of Foxp3,[7,8] a transcription factor already linked to immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX).[9] A detailed history of Treg cell discovery is documented elsewhere.[10]

TRANSCRIPTIONAL REGULATION OF FOXP3 EXPRESSION

As mentioned above, expression of Foxp3 is a distinctive feature of Treg cells. Ectopic expression of Foxp3 alone can induce a suppressive phenotype in conventional T (Tconv) cells.[7] Therefore, stringent regulation of Foxp3 expression is required to maintain homeostasis of T-cell-mediated immune responses. Many years have been dedicated to studying the

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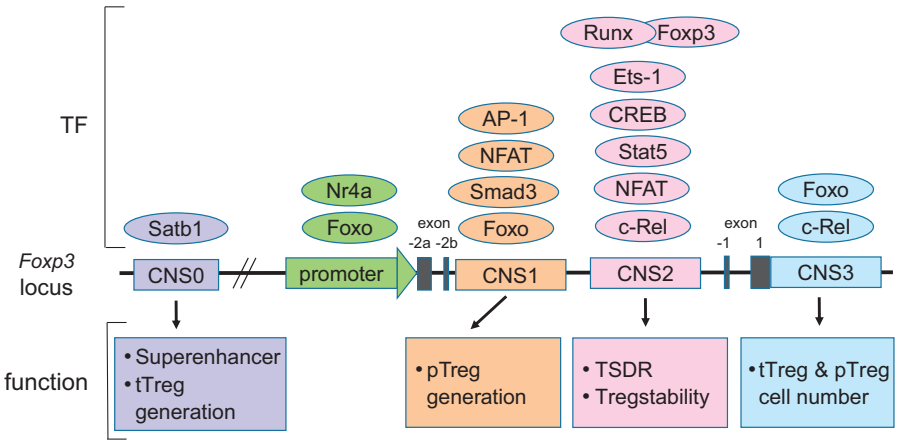

Figure 1 Schematic diagram of transcriptional regulation of the *Foxp3* locus. Regulatory regions of the *Foxp3* locus including the promoter CNS1, CNS2, CNS3, and recently discovered CNS0 are shown. Transcription factors (TFs) binding to each regulatory region and the function of each regulatory region are shown.

mechanisms regulating the *Foxp3* locus (Figure 1), making *Foxp3* one of the most intensively studied genes in recent years.

Regulatory elements of the *Foxp3* locus

Comparative genomic approaches involving alignment of human, rat and mouse genomes initially discovered three conserved non-coding sequences (CNSs) on the *Foxp3* locus: a promoter and two enhancers that are positioned within the first intron.[11–13] Later, another intronic enhancer, located directly after exon 1, was found (Figure 1).[14] The *Foxp3* promoter has minimal transcriptional activity, and the mechanism underlying lineage-specific expression of *Foxp3* relies heavily on other *cis*-regulatory elements (Figure 1). Intensive studies of the function of CNSs were undertaken, the most notable being the systemic deletion of each CNS by Rudensky and co-workers.[14] They revealed that CNS1 is largely dispensable for thymic Treg (tTreg) cell development, but is necessary for peripheral induction of Treg cells. Its significance was highlighted when deletion of CNS1 markedly reduced the Treg cell population in gut-associated lymphoid tissue. On the other hand, deleting CNS3 causes a severe reduction in thymic output of Treg cells, indicating its importance in tTreg cell generation. CNS2, also known as Treg cell-specific demethylated region, contains CpG islands that are highly demethylated only in functional Treg cells; its demethylation is considered to be the most definitive marker of commitment to the Treg cell lineage.[15,16] Deletion of CNS2 does not change thymic generation of Treg cells; rather, it affects the stability of Foxp3 expression during proliferation. The most recent discovery in the *Foxp3* locus is another regulatory element named CNS0, which lies on an intron of the neighboring gene 5′ of the *Foxp3* locus (Figure 1).[17] It was found in an attempt to localize Treg cell-specific super enhancers using high-throughput chromatin immunoprecipitation sequencing of acetylated histone H3K27.

Transcription factors binding to regulatory elements

Many transcription factors have been studied for their ability to transactivate the *Foxp3* gene (Figure 1). Among them is c-Rel. The significance of c-Rel was demonstrated by showing that c-Rel deficiency causes a marked reduction in tTreg cell generation.[18] Individual studies suggest different mechanisms for the function of c-Rel during *Foxp3* transcription; these include binding and demethylation of CNS2,[19] binding to the promoter followed by formation of a c-Rel enhanceosome over the *Foxp3* locus[18] and binding to CNS3 and triggering *Foxp3* induction by T-cell receptor (TCR) and costimulatory signals.[14] Foxo family of transcription factors are also involved in regulating *Foxp3* induction. Foxo1 and Foxo3 act redundantly on *Foxp3* transcription by binding directly to the promoters, CNS1 and CNS3.[20,21] T-cell-specific deletion of both genes in mice halves the tTreg cell population and causes a multifocal inflammatory disorder. It was discovered that not only *Foxp3* but also Treg cell-specific genes rely on Foxo transcription factors. Smad3 and NFAT modulate *Foxp3* expression by binding to CNS1 upon transforming growth factor-β (TGF-β) and TCR signaling, respectively.[22] NFAT also binds to CNS2 and mediates formation of a chromatin loop between the promoter and CNS2 of the *Foxp3* locus via a mediator–cohesin complex.[23] AP-1 transcription factors also bind to CNS1 and transactivate *Foxp3* induction, while signal transducer and activator of transcription 3 (Stat3) binding to the CNS2 region silences *Foxp3* transcription.[24] Stat5, a protein downstream of IL-2 and other common γ-chain cytokine signaling pathways, targets the *Foxp3* locus directly.[25] IL-2 signaling and Stat5 binding to CNS2 protect Treg cell identity from other cytokine signals and maintain heritable transcription of *Foxp3*.[26] Nr4a nuclear receptor family members Nr4a1 (also known as Nur77), Nr4a2 and Nr4a3 (the induction of which is proportional to the intensity of the TCR signal) function redundantly to bind the proximal promoter of *Foxp3* to induce transactivation and are thought to translate TCR signaling intensity into a T-cell fate decision via *Foxp3* induction or by triggering negative selection.[27] Recently, chromatin organizer Satb1 was found to bind CNS0 and act as a pioneer factor to activate Treg cell-specific super enhancers of the *Foxp3* gene and other Treg cell-related genes such as *Ctla4* and *Il2ra* at the early stages of

tTreg cell differentiation.[17] Satb1 works by binding to closed chromatin structures and modifies the epigenetic status of the *Foxp3* locus to a poised state, thereby allowing other transcription factors to bind to regulatory elements. Since Satb1 acts not only on Treg cell but also on general thymic T-cell development,[28] it is unclear how Satb1 is induced and binds to Treg cell-specific super enhancers in a Treg cell-specific manner.

EPIGENETIC REGULATION

As a marker of definitive Treg cell lineage commitment, the mechanism underlying CNS2 demethylation has been the center of attention in the quest to understand Treg cell differentiation. The importance of CNS2 demethylation in Treg cell differentiation and function is highlighted by the finding that transcription factors CREB/ATF, NF-κB, Ets-1 and the Runx-Foxp3 complex, all of which induce *Foxp3* expression, cannot bind to CNS2 without demethylation,[11,14,29,30] which results in loss of *Foxp3* expression in Treg cells. Studies using the DNA methyltransferase inhibitor azacytidine and Dnmt1-deficient T cells reveal that the methylation status of the *Foxp3* locus is actively maintained in T cells.[11,31] However, the exact machinery responsible for CNS2 demethylation was not identified until recently. In 2013, researchers found that vitamin C is a critical cofactor for Tet activity (Tet hydroxylates 5-methylcytosine to 5-hydroxymethylcytosine, the first step of CpG demethylation).[32–34] This is also true for demethylation of the *Foxp3* locus in Treg cells, as Tet2 and Tet3 work redundantly during demethylation of CNS2 in the presence of vitamin C.[35,36] Since supplementation with vitamin C alone demethylates CNS2 *in vitro*, demethylation of CNS2 might be a spontaneous reaction during normal Treg cell differentiation *in vivo*, where vitamin C is abundant. This breakthrough could provide a new opportunity for Treg cell therapy because *in vitro* generation of Treg cells showing stable *Foxp3* expression is now more likely.

Permissible histone modification also contributes to *Foxp3* expression in Treg cells. In fact, as in the case of *in vitro*-induced Treg cells, *Foxp3* expression can be achieved without CNS2 demethylation when histone modification is permissive to transcription.[37] By activating naïve T cells in the presence of TGF-β, Floess *et al.*[15] and Ohkura *et al.*[38] found prominent trimethylation of H3K4 on the promoter and CNS1 regions of the *Foxp3* locus, although *Foxp3* expression was transient and subsequently fell in the absence of exogenous TGF-β. Trimethylation of H3K4 is strongly correlated with *Foxp3* expression, and this modification is only visible on the *Foxp3* locus of fully differentiated Treg cells. However, there are other histone modifications on the *Foxp3* locus that are more persistent throughout Treg cell development. From as early as the double-negative 1 stage of thymocyte development, CNS3 shows monomethylation of H3K4 (a poised enhancer marker); this modification can be inherited by subsequent T-cell lineages regardless of *Foxp3* expression.[39] Interestingly, naïve $CD4^+$ T cells lacking *Foxp3* expression maintain the monomethylation of H3K4 poised status of the *Foxp3* promoter region in a CNS3-dependent manner, underlining the importance of CNS3 in peripheral Treg (pTreg) cell development. This could explain the previously held notion of defective *in vitro*-induced Treg cell development in CNS3-KO T cells.[14] Another prevailing histone modification on the *Foxp3* locus is H3K27. In the case of tTreg cell development, H3K27 starts to lose trimethylation status and acquires acetylation on enhancer regions at the pre-Treg cell stage, which implies initiation of enhancer activation.[17] Not only internal cues but also environmental signals affect these modifications. The short-chain fatty acid butyrate, produced by the gut microbiota, promotes H3K27 acetylation on the *Foxp3* locus by inhibiting histone deacetylase during pTreg cell development.[40,41]

FOXP3-INDEPENDENT DIFFERENTIATION OF TREG CELLS

Although the significance of Foxp3 drew much attention, other genes are also regulated in a Treg cell-specific manner. Some of those signature genes are not induced by Foxp3, but are regulated in parallel with the *Foxp3* gene. Many different approaches to screening Treg cell-specific genes on a whole-genome scale have been conducted. Some of these genes are independent of Foxp3 expression.[42,43] Foxp3-deletion studies support the notion that, in the absence of Foxp3, a portion of T cells dubbed "wannabe" Treg cells still maintain a Treg cell-like phenotype.[44,45] Epigenetic regulation of Treg cell-signature genes is also independent of Foxp3. Demethylation of Treg cell-signature genes *Ctla4*, *Il2ra*, *Tnfrsf18* and *Ikzf4* is independent of Foxp3 expression, but dependent on TCR stimulation, and is required for the suppressive function of Treg cells.[38] Enhancers of signature genes, in this case *Ctla4*, *Il2ra* and *Ikzf2*, are acetylated on H3K27 from the pre-Treg cell stage.[17] Based on these studies, it is evident that the developmental process of Treg cells is not entirely dependent on the expression of Foxp3, and that a more comprehensive understanding of Treg cell development is required.

TTREG CELL DEVELOPMENT

Thymocytes undergo positive and negative selection during TCR rearrangement. During these processes, a progenitor Treg cell population emerges that expresses a $CD25^{hi}$ CD4 single-positive phenotype (Figure 2). This population can later induce Foxp3 expression via stimulation by common γ-chain cytokines IL-2 and IL-15.[46] Although Foxp3 is an essential transcription factor for Treg cells, this two-step developmental stage implies that Foxp3 expression is not a prerequisite for Treg cell lineage commitment, but rather an event that follows Treg cell differentiation. The main candidate determinant of the fate of thymocytes is TCR activation.

Before the discovery of Treg cells, clonal deletion of autoreactive T cells was the main hypothesis that explained how T-cell-mediated autoimmunity is prevented, a process termed "central tolerance." However, the importance of Treg cell function in corresponding "peripheral tolerance" has since been demonstrated, and a paradox became obvious: self-recognizing TCR clones must be negatively selected to prevent

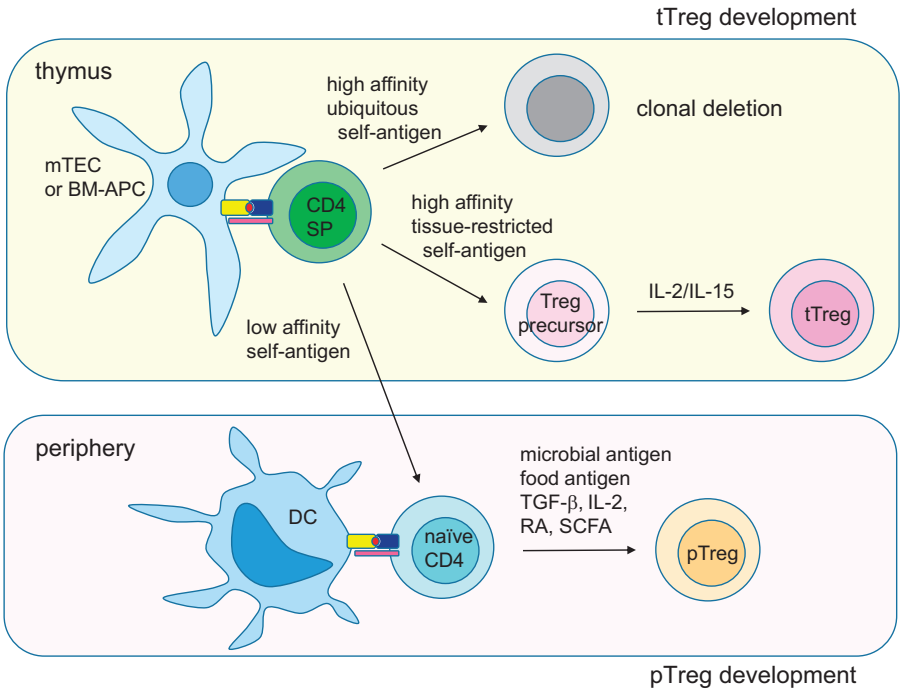

Figure 2 Schematic diagram of Treg cell development. tTreg cells develop in the thymus by two-step process. First, high-affinity tissue-restricted self-antigens presented by medullary thymic epithelial cells (mTECs) or bone-marrow derived antigen-presenting cells (BM-APCs) derive single-positive (SP) T cells into Treg pathway. Second, cytokine IL-2 or IL-15 derives the precursor cells into fully committed tTreg cells. pTreg cells develop in the periphery by environmental antigens such as microbial antigens or food antigens presented by mucosal tissue-resident dendritic cells (DCs). TGF-β, retinoic acids (RAs) and short chain fatty acids (SCFAs) produced in the immunosuppressive environments promote pTreg development.

autoimmunity; however, they have to survive to generate Treg cells specific for self-antigens. This issue remained a mystery until recently.

A Nur77-GFP transgenic mouse experiment showed that, during thymocyte development, Treg cells receive a stronger TCR signal than Tconv cells.[47] Stronger TCR signaling is associated with induction of Treg cell-specific epigenetic changes and gene expression patterns.[48] It has long been known that a Treg cell TCR recognizes a self-antigen with high affinity,[49] which increases the intensity of subsequent signals in the selection process. But how do Treg cells with self-reactive TCR clones evade deletion? Which is more important for determining Treg cell fate: the availability of autoantigen in the thymus or TCR affinity? Legoux *et al.*[50] and Malhotra *et al.*[51] designed elegant experiments to address this issue. They used readily available strains of Cre and fluorescent protein transgenic mice in which expression of the fluorescent protein is either universal or tissue-specific, depending on the promoter activity of the transgenes. They found that T cells reactive with ubiquitously expressed antigens are likely to be deleted, but clones reactive with tissue-restricted peptides are the main sources of Treg cell generation (Figure 2). In medullary thymic epithelial cells (mTECs), tissue-specific antigens are ectopically induced by the transcription factor Aire, and are presented to developing thymocytes at the thymic medulla at low frequency. Some tissue-specific antigens not induced by Aire are ignored. This is also true for endogenous self-antigens.[52] In the absence of Aire, clones that normally differentiate into Treg cells become Tconv cells and induce organ-specific autoimmune diseases.[53] From this point of view, it is noteworthy that Aire-dependent expression of tissue-specific genes is unique to each mTEC, or to each individual mouse.[54] In addition, peptide processing and presentation by mTECs differs between perinatal and adult mice, thereby generating a distinct TCR repertoire on Treg cells in an age-dependent manner.[55] The disparities in Treg cell TCR repertoires caused by altered antigen display might explain different susceptibilities to autoimmune diseases between individuals or at different ages.

Bone marrow-derived antigen-presenting cells (BM-APCs) also participate in T-cell selection in the thymic medulla. For certain TCR clones, tTreg cell development requires Aire-independent antigen presentation by bone marrow-derived antigen-presenting cells.[56] Those APCs, primarily dendritic cells and B cells, take up blood-borne antigens and peripheral tissue antigens and then either migrate to present them within the thymus or acquire antigens from mTECs.[57,58] Another mode of B-cell participation is presentation of ectopic self-antigens along with expression of Aire.[59] It is not clear whether BM-APCs display non-self-antigens derived from the microbiota or environmental antigens in the thymus, and whether this affects negative selection of thymocytes or tTreg cell differentiation. A fluorophore painted onto the skin of mice was found in thymic dendritic cells, implying that migration of peripheral APCs carrying foreign substances to the thymus is

possible.[60] A significant proportion of Treg cell TCRs react with non-self-antigens.[61] Also, tTreg cells accumulate in neonatal skin to induce tolerance against *Staphylococcus epidermidis* colonization.[62] Thus, it is plausible that tolerance to non-self-antigens could, at least in part, be established by thymic selection.

Still, the difference between TCR signals that bifurcate T-cell fate into Tconv or Treg cells is not well understood. Evidence presented so far supports quantitative differences in signaling, but it is possible that the signals are qualitatively variable. An interesting report shows topological differences in a Treg cell TCR–peptide–MHC docking structure. The report shows a 180° rotation of the binding structure between MHC with an insulin peptide and an *in vitro*-induced Treg cell TCR.[63] Inverted orientation of TCR–peptide–MHC binding was also found in other T cells with specificity for epitopes; however, they showed minimal participation in immune responses against cognate antigens.[64] Too few TCR–peptide–MHC complex structures have been studied to verify whether reversed polarity is a common phenomenon in a Treg cell population.

PERIPHERAL TREG CELL DEVELOPMENT

The generation of pTreg cells is even less clear than that of tTreg cells. This is, in part, due to a lack of reliable methods that discriminate pTreg cells from mixed Treg cell populations in peripheral lymphoid tissues. Although the transcription factor Helios,[65] or the surface antigen neuropilin1,[66,67] are suggested to be exclusively expressed by tTreg cells, it is unclear whether they are genuine markers for tTreg cells.[68–70] This makes identifying the origin of pTreg cells troublesome, especially in tissues such as gut, lung or skin in which environmental antigens make direct contact with the immune system and in which tTreg and pTreg cells are thought to coexist. Attempts to tackle this issue by direct TCR sequencing have yielded contradicting results. Some studies of intestinal Treg cells show results that favor peripheral differentiation of major Treg cell populations being influenced by colonic commensal microbiota,[71] while others support a thymic origin.[72] The opposing claims for the origin of major populations of intestinal Treg cells could be derived from the inherent limitations of TCR sequencing because the method uses TCR transgenic mice, which have a relatively small repertoire when compared with that in wild-type mice. In fact, a recent publication notes that it requires a full TCR repertoire to maintain intestinal homeostasis.[73] Other studies that focused on sub-populations of intestinal Treg cells suggest that those Treg cells comprise a mixture of various origins and phenotypes.[74] The exact mechanisms by which certain clones of T cells are driven into the Treg cell lineage by non-self-antigens remain unclear.

Aside from the origin issue, the existence of pTreg cells and the molecular mechanisms underlying their development are well defined (Figure 2). Treg cells can develop from naïve $CD4^+Foxp3^-$ T cells *in vitro* upon TGF-β stimulation.[75] Chronic exposure to antigens in small dosages induces a Treg cell population that is indistinguishable from tTreg cells *in vivo*.[76] This population of Treg cells is also found in mice harboring a chronic *Leishmania major* infection.[77] Owing to the nature of peripheral differentiation induced by non-self-antigens, pTreg cells are assumed to be a main component responsible for immunological tolerance to non-pathogenic substances such as environmental antigens or commensal microbiota. Removal of pTreg cells causes dysregulated immune responses in the gastrointestinal tract and airway,[78] where the mucosal surface is in direct contact with environmental antigens. Under homeostatic conditions, such organs are favorable to Treg cell generation. Gut-associated lymphoid tissue-residing $CD103^+$ dendritic cells produce TGF-β and retinoic acid, both of which promote *de novo* Treg cell induction.[79–81] Also, the microbiota affects Treg cell differentiation. Metabolites of the microbiota, such as butyrate or propionate, boost Treg cell differentiation in the colon.[40,41] A recent study found that Treg cell abundancy in epithelial tissue is increased by $CD11b^+CD103^-CX3CR1^+$ dendritic cells via the TRIF-IFN-β (TIR-domain-containing adapter-inducing interferon-β) pathway.[82] These dendritic cells are suppressed by phosphatidylserine exposed on apoptotic epithelial cells, which binds to CD300a on the dendritic cell surface; this implies that apoptosis of epithelial cells indirectly regulates Treg cell differentiation. This finding may help us understand the mechanism by which barrier surfaces strike a balance between immune tolerance and inflammatory responses. Clonal pTreg cells are thought to be induced mostly by commensal microbiota. Recently, experiments using antigen-free mice fed an amino-acid diet revealed that small intestinal lamina propria Treg cells are induced mostly by dietary antigens.[83] These pTreg cells are relatively short-lived and disappear fast when the supply of dietary antigen stops. It is possible that the reason for the short lifespan of these Treg cells is their conversion into $Foxp3^-CD8\alpha\alpha^+CD4^+$ intraepithelial lymphocytes.[84] The anti-inflammatory property of intraepithelial lymphocytes means that they complement Treg cell-mediated immune suppression.

TREG CELL PLASTICITY

Maintaining Treg cell identity is critically important for immune homeostasis;[37,85–87] for example, if Treg cells are readily converted to Tconv cells, maintaining homeostasis is impossible. Understanding the precise nature of Treg cell stability is crucial for therapeutic applications that use or modulate Treg cells to treat autoimmune diseases, allergies, graft rejection and tumors.[87] Whether Treg cells are phenotypically and functionally stable is a contentious issue.[87–91] Although it was originally thought that Treg cells are quite stable, a number of studies show that some Treg cells lose their identity or are converted into pathogenic effector CD4 T cells under lymphopenic and proinflammatory conditions.[92–97] Yang *et al.*[97] showed that, when *in vitro*-induced Treg and tTreg cells are treated with IL-6 in combination with IL-1 or IL-23 *in vitro*, they are induced to express IL-17 and show defective suppressive activity. Duarte *et al.*[96] showed that half of $Foxp3^+$ cells lose Foxp3 expression when adoptively transferred to lymphopenic mice. This is prevented when $Foxp3^-$ cells are

cotransferred, or IL-2 is injected into the recipient mice. The cells that lost Foxp3 expression produce IL-2 and lose suppressive activity upon secondary transfer to lymphopenic mice. Using bacterial artificial chromosome transgenic mice containing Foxp3-GFP-Cre crossed with Rosa26-YFP mice, Zhou *et al.*[94] showed that a substantial percentage of cells have transient or unstable expression of Foxp3. These "exFoxp3 cells" are more numerous in inflamed tissues under autoimmune conditions. Adoptive transfer of these cells leads to rapid induction of diabetes, suggesting that they have an activated-memory phenotype. Tsuji *et al.*[92] showed that, when $Foxp3^+$ T cells from Foxp3-GFP mice are transferred to T-cell-deficient mice, some cells convert into $Foxp3^-$ cells, migrate into the germinal centers of Peyer's patches and differentiate into T follicular helper cells. Using a *Toxoplasma gondii* infection model, Oldenhove *et al.*[93] showed that Treg cell numbers decline in infected mice. In these mice, Treg cells also acquire T-bet and IFN-γ expression. These studies suggest that proinflammatory cytokines cause Treg cell instability by down-regulating Foxp3. In human patients of several inflammatory diseases including psoriasis, inflammatory bowel disease and rheumatoid arthritis, Treg cells expressing IL-17 (and IFN-γ in some cases) were shown to increase compared with healthy controls.[98–100] Whether these cells maintain suppressive function depends on the context; nevertheless, these results support phenotypic and functional plasticity of human Treg cells.

By contrast, another study showed that Treg cells are very stable and are not easily converted into effector cells.[101,102] Rubtsov *et al.*[101] examined Treg cell stability using tracer mice expressing eGFP-Foxp3-Cre-ER × Rosa26-YFP. In these mice, YFP^+ cells, which once expressed Foxp3 during their lifetime, were chased after tamoxifen treatment. YFP^+ cells hardly lost GFP (Foxp3) expression under homeostatic conditions or under autoimmune inflammatory conditions, suggesting that Treg cells show remarkable stability.

To resolve this apparent controversy with respect to the plasticity of Treg cells, a few models have been proposed. First is the "heterogeneity model" proposed by Hori *et al.*[103] He proposed that Treg cells consist of heterogeneous populations with different degrees of commitment, including fully committed Treg cells and less committed Treg cells. The model proposes that fully committed Treg cells are stable, but less committed Treg cells are unstable. Another model is the "transient flexibility model" proposed by Piccirillo and co-workers[104] In this model, Treg cells are flexible in terms of their phenotype depending on the environment they are in. Under strong inflammatory conditions, Treg cells transiently lose Foxp3 expression and their suppressive properties, but these are recovered after the inflammatory conditions are removed. Thus, further studies should address and resolve the issue. In addition, the key molecular mechanisms that control Treg cell instability under these conditions should be elucidated to fully explain this phenomenon.

METABOLIC REGULATION OF TREG VS TH17 CELL BALANCE

Another subset of $CD4^+$ T cells, discovered later than Treg cells, are T-helper type 17 (Th17) cells.[105,106] Despite the relatively short history of Th17 cell research, the impact of this subset on many human diseases (from autoimmunity to various infections) has attracted much attention during the past decade. One aspect of Th17 cells that ties them to Treg cells is their similarity in terms of differentiation conditions (such as a requirement of TGF-β in the case of Th17 and pTreg cell differentiation),[107,108] although this is not an absolute necessity for Th17 cells.[109,110] However, differentiation results in extreme differences in immunological activity: Treg cells suppress, while Th17 cells promote, immune responses. Initially, the proinflammatory cytokine IL-6 and subsequent Stat3 signaling were found to dictate the fate of these two subsets. Nowadays, more sophisticated mechanisms underlying T-cell fate decisions are being identified: these include cytokines, cellular metabolic pathways, dietary nutrients and the microbiota. Hundreds of articles have been published about the mechanisms underlying maintenance of the Treg/Th17 balance and its influence on diseases. Here, we review how TCR signaling and T-cell metabolism affect Treg cell differentiation.

Upon activation, naïve T cells undergo major metabolic conversion from oxidative phosphorylation and lipid oxidation to glycolysis to meet energy and material demand due to increased proliferation and cell mass. The metabolic mediator mammalian target of rapamycin (mTOR) is closely involved in this transition. mTOR receives and integrates various signals upon cellular stimulation by the environment and regulates metabolic changes and immune responses.[111] The most notable mTOR modulator in T cells is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Activated by costimulatory molecules, PI3K activates Akt and subsequently mTORC1. Phosphatase PTEN reverses PI3K activity and suppresses downstream signaling.[112] The consequence of PI3K/Akt/mTOR activation is upregulation of glucose transporter Glut1 expression and increased glucose consumption to drive anabolic metabolism in T cells.

mTOR activation is required for effector T-cell development, whereas inhibiting the mTOR pathway promotes Treg cell differentiation. In particular, transcription factor hypoxia-inducible factor 1α (induced by mTOR signaling) promotes glycolysis and Th17 cell differentiation, whereas lack of mTOR[113] or hypoxia-inducible factor 1α[114,115] drives cell fate toward Treg cells. Interestingly though, mTORC1 signaling is necessary for proper Treg cell proliferation and function because deletion of Treg cell-specific Raptor, a component of mTORC1, abrogates the suppressive function of Treg cells and eventually leads to inflammatory disorders.[116] New evidence suggests that the mTOR pathway is more strictly regulated in Treg cells than in Tconv cells. One way in which Foxp3 regulates mTORC1 signaling is via toll-like receptors.[117] The mTOR pathway is necessary for proliferation of Treg cells, and certain environmental cues such as toll-like receptor or leptin[118] may enhance mTOR signaling intensity to enrich

Treg cells. However, enhanced mTOR signaling also reduces the immunosuppressive function of Treg cells. In parallel with this, uncontrolled Akt signaling in Treg cells due to Treg-specific PTEN deficiency results in increased glycolysis and loss of Foxp3.[119,120] However, it is not mTORC1 activity, but rather mTORC2 activity, that is regulated by PTEN.[120] Another recent report shows that inhibiting protein kinase CK2 blocks Th17 development and promotes Treg cell differentiation in mice with experimental autoimmune encephalomyelitis; this is due to a defect in Stat3 phosphorylation.[121] Although not shown in that study, Akt is a target of CK2.[122] It is not clear whether CK2 activity affects the PI3K/Akt pathway. It is also interesting to note that the same group reported that CK2 is necessary for proper Treg cell function during suppression of type 2 immune responses in the lung;[123] this contradicts another study that examined the effect of CK2 on Treg cells.[121] Thus, the role of CK2 in Treg cells seems to depend on the context of the immune response, although the exact mechanism(s) is not clear. In Th17 cells, but not Treg cells, glycolysis is linked with *de novo* fatty acid synthesis by acetyl-CoA carboxylase; this is because Treg cells acquire fatty acids from the environment. Disruption of acetyl-CoA carboxylase results in blockade of Th17 cell differentiation and instead promotes Treg cell differentiation.[124]

APPLICATIONS

Understanding the mechanism(s) underlying Treg cell development is a prerequisite for Treg cell-based immunotherapy. Using Treg cells for immunotherapy would enable target-specific immunosuppression; this has clear benefits over nonspecific drug-induced immunosuppression, which can induce side effects such as opportunistic infection and cancer induction. Also, memory Treg cells could provide long-term, hopefully lifelong, tolerance to target antigens, resulting in relatively fewer treatments. Treg cell-mediated immunotherapy could be applied to organ transplantation, autoimmune diseases and allergies, resulting in improved prognoses and fewer side effects. There are more than 60 clinical trials currently registered in the United States examining the utility of Treg cell transfer (or indirect methods of boosting Treg cells (e.g., IL-2)) (https://clinicaltrials.gov) to improve treatment of type 1 diabetes mellitus, graft-versus-host disease, complications arising from organ transplantation, lupus and many other medical conditions. Most of those trials rely on polyclonal Treg cells derived from donors, although such cells are not target-specific. To improve specificity, some trials have utilized donor-alloantigen-reactive Treg cells, which are recipient Treg cells that are activated by, and proliferate in, donor tissue.[125] At the preclinical research stage, several methods have been developed to generate targeted Treg cells. The most recent attempt involves application of a chimeric antigen receptor technique to target Treg cells to specific antigens or more broad alloantigens.[126,127]

CONCLUSION

Treg cell research has progressed at an astonishing pace over the past two decades. Appreciation of the mechanisms underlying Treg cell development has led to the first therapeutic applications involving Treg cell induction. Yet, the fundamental question of how Treg cells enter fates different from Tconv cells (even though they arise from a common progenitor T cell) still remains unclear. Future studies of the processes underlying development of Treg cells should focus on comprehensive analyses of the entire TCR repertoire, intra- and intercellular mechanisms that determine Treg cell fate, and distinct features between thymic and peripheral Treg cell development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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