Transcriptional regulation and development of regulatory T cells

Wonyong Lee and Gap Ryol Lee

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.

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
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. Later, another intronic enhancer, located directly after exon 1, was found (Figure 1). 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. 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 Treg cell population in gut-associated lymphoid tissue. On the other hand, deleting CNS3 causes a severe reduction in thymic Treg cell population. CNS2, also known as Treg cell-specific enhancer (Figure 1), is another regulatory element named CNS5, and recently discovered CNS0 are shown. Transcription factors (TFs) binding to each regulatory region and the function of each regulatory region are shown.

**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.

The significance of c-Rel was demonstrated by showing that c-Rel deficiency causes a marked reduction in tTreg cell generation. Individual studies suggest different mechanisms for the function of c-Rel during Foxp3 transcription; these include binding and demethylation of CNS2, binding to the promoter followed by formation of a c-Rel enhanceosome over the *Foxp3* locus and binding to CNS3 and triggering Foxp3 induction by T-cell receptor (TCR) and costimulatory signals. 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, 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. 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. 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. Stat5, a protein downstream of IL-2 and other common γ-chain cytokine signaling pathways, targets the Foxp3 locus directly. IL-2 signaling and Stat5 binding to CNS2 protect Treg cell identity from other cytokine signals and maintain heritable transcription of Foxp3. 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. 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 *Cila4* and *Il2ra* at the early stages of
tTreg cell differentiation. 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, 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-kB, Ets-1 and the Runx-Foxp3 complex, all of which induce Foxp3 expression, cannot bind to CNS2 without demethylation, 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. 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). 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. 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. By activating naive T cells in the presence of vitamin C, Ohkura et al. 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 vitamin C. 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. Interestingly, naive 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. 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. 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.

**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. 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. Epigenetic regulation of Treg cell-signature genes is also independent of Foxp3. Demethylation of Treg cell-signature genes Cita4, Il2ra, Tnfrsf18 and Ikek2 is independent of Foxp3 expression, but dependent on TCR stimulation, and is required for the suppressive function of Treg cells. Enhancers of signature genes, in this case Cita4, Il2ra and Ikek2, are acetylated on H3K27 from the pre-Treg cell stage. 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+ 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. 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 autoimmune 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...
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

---

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.
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, 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 complex structures have been studied to verify whether reversed polarity is a common phenomenon in a Treg cell population.

TREG CELL PLASTICITY

Maintaining Treg cell identity is critically important for immune homeostasis, 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, 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. 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. 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. 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 downregulating Foxp3. In human patients of several inflammatory diseases including psoriasis, inflammatory bowel disease and rheumatoid arthritis, Treg cells expressing IL-17 (and IFN-γ), expression. These studies suggest that proinflammatory cytokines cause Treg cell instability by downregulating 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. 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. Rubtsov et al., 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. 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. 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. Despite the relatively short history of Th17 cell research, the impact of this subset on many human diseases (from autoimmune 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), although this is not an absolute necessity for Th17 cells. 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. 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. 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 or hypoxia-inducible factor 1α 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. 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. The mTOR pathway is necessary for proliferation of Treg cells, and certain environmental cues such as toll-like receptor or leptin 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. However, it is not mTORC1 activity, but rather mTORC2 activity, that is regulated by PTEN. 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. Although not shown in that study, Akt is a target of CK2. 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; this contradicts another study that examined the effect of CK2 on Treg cells. 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.

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 lifelong, 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)) 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-allotgen-reactive Treg cells, which are recipient Treg cells that are activated by, and proliferate in, donor tissue. 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.

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.

ACKNOWLEDGEMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant funded by Korean government (NRF-2014R1A2A1A110 52545 and NRF-2017R1A2B3008621).

PUBLISHER’S NOTE

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* 2006; 108: 1571–1579.

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.

15 Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky K et al. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol* 2007; 5: e38.

16 Wierzbicki G, Assemann A, Model F, Turbachova I, Floess S, Liebenberg V et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res* 2009; 69: 599–608.

17 Kitagawa Y, Ohkura N, Kidani Y, Vandenbouwelaer A, Hirota K, Nakamura K et al. Guidance of regulatory T cell development by Stat1-dependent super-enhancer enhancement. *Nat Immunol* 2017; 18: 173–183.

18 Ruan Q, Kameswara V, Tone Y, Li L, Liu HC, Greene MI et al. Development of Foxp3(+) regulatory T cells is driven by the c-Rel enhancerosome. *Immunity* 2009; 31: 932–940.

19 Long M, Park SG, Strickland I, Hayden MS, Ghosh S. Nuclear factor-kappaB regulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. *Immunity* 2009; 31: 921–931.

20 Harada Y, Ely 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.

21 Ouyang W, Beckett O, Ma Q, Paik JH, DePinho RA, Li MO. Foxo proteins cooperate in the differentiation of Foxp3(+) regulatory T cells. *Nat Immunol* 2010; 11: 618–627.

22 Tone Y, Furuzaki K, Kojima Y, Tkocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* 2008; 9: 194–202.

23 Li X, Liang Y, LeBlanc M, Benner C, Zheng Y. Function of a Foxp3 cis-element in protecting regulatory T cell identity. *Cell* 2014; 158: 734–748.

24 Xu L, Kitani A, Stuelten C, McGrady G, Fuss I, Strober W. Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I. *Immunity* 2010; 33: 313–325.

25 Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT et al. Nonredundant roles for Stats in directly regulating Foxp3. *Blood* 2007; 109: 4368–4375.

26 Feng Y, Arey A, Chinen T, van der Veen K, Gasteiger G, Rudensky AY. Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. *Cell* 2014; 158: 749–763.

27 Sekiya T, Kashigawa I, Yoshida R, Fukaya T, Morita R, Kimura A et al. Nr4a receptors are essential for thymic negative selection and immune homeostasis. *Nat Immunol* 2013; 14: 230–237.

28 Alvarez JD, Yasui DH, Nida H, Joh T, Loh DY, Kohwi-Shigematsu T. The binding-mediating protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. *Genes Dev* 2000; 14: 521–535.

29 Polansky JK, Schreiber L, Thelen M, Ludwig L, Kruger M, Baumgrass R et al. Methylation matters: binding of Ets-1 to the demethylated Foxp3 gene contributes to the stabilization of Foxp3 expression in regulatory T cells. *J Mol Med (Berl)* 2010; 88: 1029–1040.

30 Mouly E, Chemin K, Nguyen HV, Chopin M, Mesnard L, Leite-de-Moraes M et al. The Ets-1 transcription factor controls the development and function of natural regulatory T cells. *J Exp Med* 2010; 207: 2113–2125.

31 Josefowicz SZ, Wilson CB, Rudensky AY. Cutting edge: TCR stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* 2012; 37: 785–799.

32 Feng Y, van der Veen J, Shugay M, Putintseva EV, Osmanbeyoglu HU, Diky S et al. A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. *Nature* 2015; 528: 132–136.

33 Arpaia N, Campbell C, Fan X, Diky S, van der Veen J, de Roos P et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nat 2013; 504: 451–455.

34 Furuhashi Y, Obata Y, Fukuda S, Endo TA, Nakagawa T, Takahashi D et al. Commensal microbe-derived butyrate induces the differentiation of CD4(+) regulatory T cells. *Nature* 2013; 504: 446–450.

35 Sugimoto N, Oida T, Hirota K, Nakamura K, Nomura T, Uchiyama T et al. Foxp3-dependent and -independent molecules specific for CD25(+)CD4(+) natural regulatory T cells revealed by DNA microarray analysis. *Int Immunol* 2006; 18: 1197–1209.

36 Hill JA, Feurer M, Tash K, Haxhinasto S, Perez J, Melamed R et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T-cell transcriptional signature. *Immunity* 2007; 27: 786–800.

37 Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* 2007; 445: 766–770.

38 Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Mangiacci VC, Beavo JA et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 2007; 445: 771–777.

39 Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. *Immunity* 2008; 28: 100–111.

40 Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J et al. T cell receptor signal strength in Treg and INKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med* 2011; 208: 1279–1289.

41 Monika H, Sakaguchi S. Genetic and epigenetic basis of Treg cell development and function: from a Foxp3-centered view to an epigenome-defined view of natural Treg cells. *Immunol Rev* 2014; 259: 192–205.

42 Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA et al. Thymic selection of CD4(+)CD25(+) regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2001; 2: 301–306.

43 Legoux FP, Lim JB, Cauley AW, Diky S, Ertelt J, Mariani TJ et al. CD4(+) T cells: tolerance to tissue-restricted self antigens is mediated by antigen-specific regulatory T cells rather than deletion. *Immunity* 2015; 43: 896–908.

44 Malhotra D, Linehan JL, Dileepan T, Lee YJ, Purtha WE, Lu JY et al. Tolerance is established in polyclonal CD4(+) T cells by distinct mechanisms, according to self-peptide expression patterns. *Nat Immunol* 2016; 17: 187–195.

45 Kieback E, Hilgenberg E, Stervbo U, Lambropoulou V, Shen P, Bunse M et al. Thymus-derived regulatory T cells are positively selected on natural self-antigen through cognate interactions of high functional avidity. *Immunity* 2016; 44: 1114–1126.

46 Malchow S, Leventhal DS, Lee V, Nishi S, Socci ND, Savage PA. Aire enforces immune tolerance by directing autoreactive T cells into the regulatory T cell lineage. *Immunity* 2016; 44: 1102–1113.

47 Meredith M, Zemmer M, Mathis D, Benoist C. Aire controls gene expression in the thymic epithelium with ordered stochasticity. *Nat Immunol* 2015; 16: 942–949.

48 Yang J, Silva HM, Trzezak A, Choi Y, Schwab SR et al. Increased generation of Foxp3(+) regulatory T cells by manipulating antigen presentation in the thymus. *Nat Commun* 2016; 7: 10562.

49 Perry JS, Hsieh CS. Development of T-cell tolerance utilizes both cell-autonomous and cooperative presentation of self-antigen. *Immunity* 2015; 42: 141–155.

50 Oh J, Shin JS. The role of dendritic cells in central tolerance. *Immunol Netw* 2015; 15: 111–120.
68 Szurek E, Cebula A, Wojciech L, Pietrzak M, Rempala G, Kisielow P.
66 Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst.
64 Gras S, Chadderton J, Del Campo CM, Farenc C, Wiede F, Josephs TM.
63 Beringer DX, Kleijwegt FS, Wiede F, van der Slik AR, Loh KL, Petersen J.
75 Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N.
62 Scharschmidt TC, Vasquez KS, Truong HA, Gearty SV, Pauli ML.
70 Himmel ME, MacDonald KG, Garcia RV, Steiner TS, Levings MK. Helios+.
59 Yamano T, Nedjic J, Hinterberger M, Steinert M, Koser S, Pinto S et al.
69 Elkord E. Helios should not be cited as a marker of human thymus-derived
1722 S1
1061.
2012; regulatory cells.
differentiates thymic-derived from peripherally induced Foxp3+ T
51x131
482
2007; clonal deletion of thymocytes by circulating dendritic cells homing to
104 Bin Dhuban K, Kornete M, S Mason E, Piccirillo CA. Functional dynamics
121
129
106
199
106
90
199
467.
1908.
1755–1757.
184
433–3441.
106.
260.
1755–106.
345
1492.
1774.
1764.
1538–1586.
1761.
98 Hovhannisyan Z, Treatman J, Littman DR, Mayer L. Characterization of
J Exp Med 2007; 204: 1765–1774.
244–252.
184
1722 –
344
1713–1722 S1–19.
Differences in expression level of helios and neuropilin-1 do not
106.
1720.
104
16
260.
31
1760.
39
106.
106.
1761.
1755–106.
244–252.
31
772–786.
1755.
184
1722 –
497–4997.
107.
1755.
1755.
1755.
1908.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
1755.
106 Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005; 6: 1133–1141.

107 Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006; 441: 235–238.

108 Mangan PR, Harrington LE, O’Quinn DB, Heims WS, Bullard DC, Elson CO et al. Transforming growth factor-beta induces development of the T (H)17 lineage. Nature 2006; 441: 231–234.

109 Das J, Ren G, Zhang L, Roberts AL, Zhao X, Bothwell AL et al. Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. J Exp Med 2009; 206: 2407–2416.

110 Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE et al. Generation of pathogenic TH17 and Treg cells. Nature 2010; 467: 967–971.

111 Waickman AT, Powell JD. mTOR metabolism, and the regulation of T-cell differentiation and function. Immunity 2012; 45: 43–58.

112 Buck MD, O’Sullivan D, Pearce EL. T cell metabolism drives immunity. J Exp Med 2015; 212: 1345–1360.

113 Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 2009; 30: 832–844.

114 Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. J Exp Med 2011; 208: 1367–1375.

115 Dang EV, Barbi J, Yang HY, Jinasena D, Yu H, Zheng Y et al. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. Cell 2011; 146: 772–784.

116 Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. Nature 2013; 499: 485–490.

117 Gerniets VA, Kishotn RJ, Johnson MO, Cohen S, Sijska PJ, Nichols AG et al. Foxp3 and Toll-like receptor signaling balance Treg cell anabolic metabolism for suppression. Nat Immunol 2016; 17: 1459–1466.

118 Procaccini C, De Rosa V, Galgani M, Abanni L, Cali G, Porcellini A et al. An oscillatory switch in mTOR kinase activity sets regulatory T cell responsiveness. Immunity 2010; 33: 929–941.

119 Huynh A, DuFage M, Priyadharshini B, Sage PT, Quiros J, Borges CM et al. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. Nat Immunol 2015; 16: 188–196.

120 Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTK2 to restrain TH1 and TFH cell responses. Nat Immunol 2015; 16: 178–187.

121 Ulges A, Witsch EJ, Pramanik G, Klein M, Birkner K, Buhler U et al. Protein kinase CK2 governs the molecular decision between encephalitogenic TH17 cell and Treg cell development. Proc Natl Acad Sci USA 2016; 113: 10145–10150.

122 Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, Brustolon F et al. Protein kinase CK2 phosphorylates and upregulates Akt/PKB. Cell Death Differ 2005; 12: 668–677.

123 Ulges A, Klein M, Reuter S, Gerlitzki B, Hoffmann M, Grebe N et al. Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo. Nat Immunol 2015; 16: 267–275.

124 Berod L, Friedrich C, Nandan A, Freitag J, Hagemann S, Harmrofts K et al. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. Nat Med 2014; 20: 1327–1333.

125 Feng G, Chan T, Wood KJ, Bushell A. Donor reactive regulatory T cells. Curr Opin Organ Transplant 2009; 14: 432–438.

126 Elinav E, Waks T, Eshhar Z. Redirection of regulatory T cells with predetermined specificity for the treatment of experimental colitis in mice. Gastroenterology 2008; 134: 2014–2024.

127 MacDonald KG, Hoeppli RE, Huang Q, Gillies J, Luciani DS, Orban PC et al. Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor. J Clin Invest 2016; 126: 1413–1424.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/