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Targeting Treg signaling for the treatment of autoimmune diseases
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Regulatory T (Treg) cells are crucial players in the prevention of autoimmunity. Treg lineage commitment and functional stability are influenced by selected extracellular signals from the local environment, shaped by distinctive intracellular signaling network, and secured by their unique epigenetic profile. Recent advances in our understanding of the complex processes of Treg lineage differentiation, maintenance, and function has paved the way for developing strategies to manipulate these important cells for therapeutic benefit in many diseases. In this review, we will summarize recent advances in our understanding of Treg biology as well as Treg-targeted therapies in the context of autoimmune disease.

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Introduction
Forkhead box P3 (Foxp3)-expressing regulatory T cells (Tregs) are a small subset of CD4⁺ T cells that are vital to immune homeostasis and prevention of autoimmunity in mice and man [1]. Expression of the transcription factor Foxp3 in these cells is essential for their development, maintenance, and function. Treg potency lies in their ability to deploy various immunosuppressive mechanisms depending on the immunological context as well as extending their influence through the process of infectious tolerance [2]. An emerging concept is that Tregs not only control immune responses, but also promote tissue homeostasis by suppressing inflammation and aiding in tissue repair [3]. Moreover, this system is exploited by tumor cells to evade immune surveillance [4]. Thus, changes in Treg number and function underlie many illnesses of the immune system and beyond.

Manipulating Tregs is a new therapeutic strategy for treating various diseases including autoimmunity, transplant rejection, and cancer [5,6]. Elucidating factors influencing Treg homeostasis and function has important implications in understanding disease pathogenesis and identifying therapeutic opportunities. This review will focus on recent advances in how Tregs integrate extracellular and intracellular signaling to control their survival and stability. We will discuss how these new insights can be utilized for the development of new approaches to promote and stabilize Tregs in autoimmunity and transplantation.

TCR, CD28, and IL-2: the essential triad for Treg lineage specification and maintenance
Thymic Treg (tTreg) development is initiated by T cell receptor (TCR) signaling followed by sequential activation of CD25 expression, IL-2 signaling, and then Foxp3 expression [7,8]. tTreg development can be enhanced through the constitutive activation of signal transducer and activator of transcription 5 (STAT5), which is downstream of the IL-2 receptor and directly binds cis elements in the Foxp3 promoter and enhancer to stabilize Foxp3 expression [9]. Indeed, the level of IL-2 in the circulation dictates the size of the thymic Treg compartment [10–12]. In addition to induction of CD25, TCR and CD28 signaling also contribute to establishing and stabilizing the Treg lineage commitment in the thymus by inducing epigenetic and differentiation events in Tregs [10,13–15]. Thus, antigen and IL-2 signaling transmitted via TCR, CD28, and CD25 are essential for Treg lineage specification in the thymus.

In the periphery, mature Tregs continue to depend on TCR, CD28, and CD25 for their homeostasis and function, but their roles appear to be distinct from those in the thymus. Tregs proliferate more than conventional CD4⁺ T cells in steady state in a CD28 dependent fashion, suggesting that Tregs are constantly seeing antigens that drive their cell cycle progression [16,17]. Recently, analysis of Treg subsets in the periphery found that the CD62LloCD44hi effector Tregs (eTregs) were relatively more responsive to TCR stimulation and less IL-2 dependent than CD62Lhi CD44lo central Tregs (cTregs) [18*]. Consistent with the idea that eTregs are TCR
dependent, deletion of the TCR specifically in mature Tregs led to a selective loss of CD62L^{lo}CD44^{hi} eTregs as soon as 9 days after excision of the TCR gene. This suggests that constant stimulation through the TCR is required to maintain this population [19,20]. These TCR-deficient Tregs proliferated less and expressed fewer eTreg molecules such as cytotoxic T-lymphocyte-associated protein 4 (GTLA-4), IL-10, Ebi3, and, correspondingly, conventional T cells became activated to express cytokines. However, this immune activation profile is fairly mild, which is dramatically different from the catastrophic systemic autoimmunity observed after Treg depletion [21]. This is likely because the frequency of Tregs remained normal after TCR deletion and Tregs maintained their responsiveness to IL-2, high levels of Foxp3 expression, and Treg-specific epigenetic profile. Through these observations, it is suggested that the role of TCR signaling in mature Tregs is mainly to activate their proliferation and effector functions, but not for lineage maintenance.
Proliferating Tregs have a tendency to lose their Foxp3 expression and lineage stability in vitro and in vivo in lymphopenic hosts [22–24]. The conserved noncoding sequence 2 (CN2S2) enhancer element, also known as Treg-specific demethylation region, is crucial for safeguarding lineage stability of proliferating Tregs [25**,26**]. However, stimulation via TCR with limited IL-2 leads to a loss of Foxp3 expression in Tregs even in wild type cells with intact CN2S2. CN2S2 has binding sites for both the TCR-triggered transcription factor nuclear factor of activated T-cells (NFAT) and IL-2-induced transcription factor STAT5, providing a transcriptional basis for Treg stability by coordinating TCR and IL-2 signaling. Interestingly, forced expression of constitutively active STAT5 prevented the loss of Foxp3 in CN2S2 deleted Tregs, demonstrating that STAT5 can stabilize Foxp3 expression independent of CN2S2 [25**]. This may be explained by the NFAT-mediated looping between CN2S2 and the Foxp3 promoter, which also has binding sites for NFAT and STAT5 [26**]. In conclusion, TCR-mediated signals are important for mature Treg function but pose a threat to their stability unless they are balanced by IL-2 signaling.

PI3K-Akt-mTOR: a crucial signaling node for Treg development and homeostasis

Phosphatidylinositol 3 kinase (PI3K), protein kinase B (Akt), mammalian target of rapamycin (mTOR) form an intracellular signaling hub common to the TCR, CD28, and IL-2 receptor. PI3K is directly activated when these receptors are engaged, leading to initial activation of Akt by the PH-domain containing protein PDK1 through phosphorylation of threonine 308. Akt is fully activated by additional phosphorylation on serine 473 by the mTOR complex 2 (mTORC2). Akt has many cellular targets, but the Forkhead box O (Foxo) transcription factors and mTORC1 are most relevant to Treg biology. Foxo family transcription factors are crucial for Treg lineage specification [27–29] and are inhibited by Akt. mTORC1 coordinates anabolic activities in cells and inactivates mTORC2, thus limiting further Akt activation. In the thymus, Treg development is enhanced by mutating the p110δ catalytic subunit of PI3K [30] and it is repressed by forced expression of a constitutively active Akt [31], demonstrating a negative role of the PI3K axis on tTreg development. However, deletion of mTOR (thus inactivating both mTORC1 and 2) or individual deletion of mTORC1 or 2 in T cells does not alter thymic development [32], suggesting that the negative effect of PI3K and Akt on tTreg development is mTOR independent and mainly due to their role in Foxo1 inactivation.

In the periphery, this axis controls peripheral Treg (pTreg) generation. Similar to the thymus, Foxp3 induction is favored after T cell activation in the presence of pharmacological inhibitors of PI3K [33]. However, distinct from tTregs, pTreg generation is significantly impacted by mTOR signaling. mTOR-deficient T cells exhibited mild proliferative defects, failed to express effector cytokines, and defaulted to Foxp3 induction after TCR activation. Inhibition of both mTORC1 and 2 was required for this effect [34]. Activation of PI3K is naturally antagonized by phosphatase and tensin homolog (PTEN). PTEN expression is progressively inhibited by stronger TCR stimulation, permitting efficient T cell activation and effector differentiation, an effect mediated by interleukin-2-inducible T-cell kinase (Itk) [35]. Thus, T cells with Itk deficiency fail to down regulate PTEN after activation and favor Foxp3 induction over Th17 differentiation. Similarly, loss of tuberous sclerosis 1 (TSC1), an inhibitor of mTORC1, results in excessive IL-17 production, defective pTreg induction, and severe chemical induced colitis [36]. Lastly, CD5 was found to block PI3K during pTreg induction, making pTregs refractory to destabilization [37]. This integration of signals from surface receptors through the PI3K-Akt-mTOR pathway is depicted in Figure 1. Together, these data support the notion that PI3K and mTOR activity in mature T cells crucially controls the bifurcation between effector versus pTreg cell fates.

In committed Tregs, the PI3K-Akt-mTOR signaling axis continues to be repressed by high expression of PTEN. tTregs constitutively express high level of neuropilin [38,39], which directly binds PTEN and blocks Akt activation during immunological challenge [40]. Treg specific deletion of PTEN disrupted Treg homeostasis, function, and stability [41*,42*]. These PTEN-deficient Tregs lost both Foxp3 and CD25 expression but had a significant increase of mTORC2, but not mTORC1 activities. Additional deletion of mTORC2 in Tregs largely rescues the phenotype in mice with Treg-specific deletion of PTEN, demonstrating the normal function of PTEN in mature Tregs is to keep mTORC2 in check. In fact, intact mTORC1 function is required for Treg function because mice with selective deletion of mTORC1 in Tregs die of multi-organ autoimmune diseases similar to Foxp3-deficient mice [43**]. Mechanistically, mTOR is found to control Treg function in part by regulating metabolic programming. T cells rely on mitochondrial oxidative phosphorylation at rest and switch to glycolysis after activation, a process essential for effector T cell differentiation [44]. In contrast, Tregs preferentially use oxidative metabolism even after activation. An emerging concept is that metabolic input can also dictate T cell fate decision [44]. PTEN-deficient Tregs show exaggerated glycolysis that is thought to contribute to Treg instability [41*,42*]. Additionally, functional defects in mTORC1-deficient Tregs are associated with disrupted lipid biosynthesis [43**]. Thus, the impact of PI3K-Akt-mTOR axis on mature Treg function is far from black and white, while excessive activation of this pathway is clearly detrimental to Treg function as seen in PTEN-deficient Tregs; complete blockade of PI3K impairs Treg function as well [30,45].
Epigenome: a foundation for Treg stability

Treg lineage commitment and maintenance is ultimately secured by their epigenetic traits, which are governed by three complementary elements: histone modification, DNA methylation, and transcription factor binding [46, 47]. Foxp3 binds to many histone-modifying proteins such as TIP60, Histone deacetylases (HDACs), p300, and Enhancer of zeste homolog 2 (Ezh2) to maintain epigenome stability. It is worth noting that Foxp3-mediated epigenetic changes lead to mostly gene repression, rather than activation, which is dependent on histone methyltransferase Ezh2 [48, 49]. This genome wide repression is especially important for maintaining the Treg lineage under inflammatory conditions when activation of effector molecules normally expressed by conventional T cell need to be repressed in Tregs [50]. Although Ezh2-deficient Tregs are phenotypically normal and have unaltered suppressive function in vitro, they lose Foxp3 expression after activation and are unable to control immune responses in vivo. Thus, antigen activation poses a threat to Treg stability and Tregs have intrinsic signaling and epigenetic mechanisms to safeguard their lineage stability.

Manipulating Tregs to treat autoimmune diseases

Elucidating the basic mechanisms underlying Treg biology is the key to manipulating these cells for therapeutic benefit. Changing the balance between effector cells and Tregs is a promising avenue to restore immune homeostasis and treat autoimmune diseases. Experimentally, all the crucial elements in Treg biology described above have been targeted for the purpose of manipulating the balance between Tregs and effector cells and some of these approaches are being actively evaluated in the clinic.

Targeting TCR, CD28, and IL-2 triad

Although both Tregs and effector T cells express TCR and the associated CD3 complex, monoclonal antibodies to CD3 can tip the balance in favor of Tregs and induce long-lasting remission of type 1 diabetes in mouse models [51]. This change of Treg to effector T cell balance is due to higher resistance of Tregs to anti-CD3 induced cell death as well as increased induction of pTregs in the periphery [52, 53]. Interestingly, delayed treatment with anti-CD3 reduced effector T cells and increased the proportion of Tregs in a mouse model of heart transplantation, resulting in long-term graft survival [54]. In humans, anti-CD3 antibodies induce the outgrowth of FOXP3/CD8+ Tregs in vitro and increase IL-10 in the serum in vivo [55]. These encouraging preclinical findings have led to clinical trials with promising results [56-60]. In type 1 diabetes, anti-CD3 treatment improves control of the disease and beta cell function during the first year after onset [56, 57]. However, this therapy does not have efficacy for all patients [58] or in patients with longstanding disease [59].

Targeting CD28 using CTLA4Ig is also effective in changing the Treg to effector T cell balance to prevent immune activation. Although Treg development and peripheral homeostasis depend on CD28, effector T cell differentiation is more sensitive to CTLA4Ig-mediated CD28 blockade; thus, a low dose of CTLA4Ig can block effector differentiation with minimal impact on Tregs [61]. This is also observed in kidney transplant patients treated with belatacept, a high affinity variant of humanized CTLA4Ig [62]. Currently, CTLA4Ig has been approved by the Food and Drug Administration for the treatment of rheumatoid arthritis and for the prevention of kidney transplant rejection [63, 64]. Selectively targeting pathogenic effector cells may be particularly effective for restoring immune tolerance, especially when the pathology arises as a consequence of effector resistance to regulation [65]. In this regard, a CD2-targeting fusion protein, alefacept, has been recently shown to deplete effector T cells while preserving Tregs in type 1 diabetes patients [66]. It is worth mentioning that a form of agonist anti-CD28 was shown to selectively increase Tregs and prevent experimental allergic encephalitis, a model of multiple sclerosis (MS) [67]. When evaluated in a phase 1 clinical trial, TGN1412, the humanized agonist anti-CD28 induced pan T cell activation and severe cytokine storm in healthy volunteers [68]. Therefore, the potential impact on effector cells should be carefully considered when developing drugs that stimulate TCR and CD28. Alternatively, antagonistic antibodies may selectively preserve Tregs depending on the dosing [69].

Owing to their constitutive expression of the high affinity IL-2 receptor and distinct biochemical wiring, Tregs preferentially respond to low-dose IL-2 therapy. This therapy is effective in preventing and reversing type 1 diabetes in mouse models [70, 71]. Low-dose IL-2 therapy has been effective in increasing Tregs in type 1 diabetes [72, 73], GVHD [74], and alopecia areata [75]. In HCV-induced vasculitis, Tregs were induced by IL-2 therapy and 8 out of 10 patients showed clinical improvement [76]. Thus, IL-2 therapy is a promising avenue for increasing Tregs and improving clinical outcomes for patients with autoimmune disease.

However, since many cell types can respond to IL-2, one concern with IL-2 therapy is its Treg selectivity. For example, eosinophilia was observed in patients on IL-2 therapy, and in mouse models, it was found to be mediated by the CD25-expressing type 2 innate-lymphoid cells [77]. Increasing IL-2 dose in a mouse model of type 1 diabetes led to an increase of Natural Killer (NK) cell and cytotoxic CD8 T cells and exacerbation of diabetes [70]. Quantitative measurement of IL-2 sensitivity of
various cell types in human blood showed that Tregs were most responsive followed by CD56hi NK cells and memory T cells [73**,78]. Acute Treg depletion in mice [79–81] led to an increase in NK cells expressing cytotoxic effector molecules. Interestingly, this did not lead to an increase in NK killing of autologous cells, suggesting that NK activation does not contribute to the fatal autoimmunity after Treg depletion [80]. Similarly, anti-CD25 therapy led to a reduction of Tregs in patients with MS, which corresponded with increases of serum IL-2 and CD56hi NK cells, but dramatic disease protection [82,83]. In vitro analysis suggests that the CD56hi NK cells may substitute the function of Tregs and suppress immune responses by killing activated effector cells [84]. Thus, the rise of CD56hi NK cells after IL-2 therapy may actually be beneficial rather than problematic. Nonetheless, ongoing efforts are devoted to improving the safety of IL-2 therapy. One approach to more selectively target Tregs is to mutate the IL-2 molecule to make its binding to its receptor CD25 dependent, which has shown efficacy in a Lewis rat model of MS [85].

### Targeting PI3K-Akt-mTOR axis

A myriad of inhibitors have been developed to target PI3K-Akt-mTOR pathways with the goal of inducing immunosuppression and as therapies for cancer. The most extensively studied inhibitor in the context of Tregs is rapamycin. Initially, rapamycin was thought to be a specific mTORC1 inhibitor but was later found to inhibit both mTORC1 and 2 when used at high concentrations or with prolonged exposure. As discussed above, ablation of both mTORC1 and 2 are required for the preferential induction of pTregs, and mature Treg function crucially depends on mTORC1. In culture, Tregs are more resistant to rapamycin-mediated growth inhibition, thus, rapamycin has been a favored additive to Treg expansion cultures to increase their purity [86]. However, rapamycin does not expand Tregs and has clearly been shown to retard the growth of Tregs in vitro and in vivo [87,88]. In the clinic, rapamycin has been used in transplant recipients as an alternative immunosuppressive agent to the widely used calcineurin inhibitors (CNI). Converting patients from CNI to rapamycin or its analogs has been associated with a rise of Tregs in circulation. However, it is not clear if this effect is mainly a result of decreased use of CNI, which are clearly inhibitory to Tregs, or a direct effect of rapamycin. In type 1 diabetes, mouse studies found that rapamycin and IL-2 combination therapy prevented diabetes [89]. In patients, however, this treatment led to a transient worsening of beta cell function and an increase in both NK cells and eosinophils despite the dramatic rise in Tregs [90]. The negative impact of this regimen in patients was attributed to a direct effect of rapamycin on beta cells. Thus, the effect of rapamycin can be seen on multiple immune and non-immune cells, though its utility in autoimmune diseases is yet to be determined. Additionally, findings from genetic ablation studies in mice suggest that the selective targeting of mTORC2 would be more effective for tipping the balance towards Tregs.

### Targeting epigenetic regulation

Although epigenetic programming is important for safeguarding Treg lineage identity, it is also dynamically regulated, providing an opportunity for pharmacological manipulations. Histone acetylation contributes epigenetic regulation, and the process is balanced by the histone acetyltransferases (HATs) and HDACs. HDAC inhibitors have been extensively explored as anti-inflammatory and immunosuppressive agents. Particularly, inhibition of certain HDACs has been shown to selectively enhance Tregs, although these effects are likely more complicated than just histone acetylation because HDACs have many other cellular targets [91]. Ezh2-mediated repression is essential for Treg stability during antigenic challenge, suggesting that preserving and enhancing Ezh2 function would have an impact for promoting tolerance in the face of autoimmune diseases and inflammation. Much of the pharmacological development targeting Ezh2 focuses on inhibiting the enzyme in cancer cells with the added benefit of destabilizing Tregs. The activity of Ezh2 is naturally opposed by the histone demethylase Jmd3 and UTX. Ablation of Jmd3 in T cells inhibits Th1 and Th17 differentiation and preserves Tregs under Th1 polarizing conditions [92,93]. Thus targeting Jmd3/UTX pathway may be effective for promoting Treg stability.

### Achieving antigen-specific tolerance

Research in animal models shows that antigen-specific Tregs are more effective for controlling organ-specific autoimmune diseases and transplantation rejection when compared to polyclonal Tregs [94–96]. A long-term global increase in Tregs may impair effective immune surveillance against infections and malignancies; therefore, antigen-specific therapies are more effective and safer for organ-specific autoimmune diseases. Self-antigens coupled to killed splenocytes or erythrocytes via chemical crosslinking can inactivate self-reactive effector cells and induce expansion of antigen-specific Tregs in mouse models of MS, type 1 diabetes, and transplant rejection [97–99]. These pioneering studies are just beginning to be translated into the clinic [100]. Various newer experimental approaches have been explored to increase antigen-specific Tregs. For example, apoptotic cells pulsed with peptide have been described to have therapeutic effect in both experimental allergic encephalitis and type 1 diabetes by producing TGFβ and inducing antigen-specific pTregs in vivo [101]. Additionally, CD45 ligation on Tregs resulted in increased antigen-specific Treg-DC interactions and selective expansion of antigen-specific Tregs [102]. Synthetic nanoparticles represent an exciting new therapeutic platform to achieve antigen-specific manipulation of the immune system [103].
Treg cell therapy
Infusion of Tregs is a direct approach to selectively increase Tregs. Several phase 1 clinical trials of Treg cell therapy for the prevention of GvHD [104–106] and one trial in type 1 diabetes have been reported [107]. Currently, more than a dozen Treg cell therapy trials are registered on clinicaltrials.gov website, mostly in GvHD and solid organ transplantations using polyclonal Tregs. Although it is feasible to produce large numbers of alloantigen-reactive Tregs by selective stimulation using allogeneic antigen presenting cells [108], large-scale manufacturing of tissue antigen-specific Tregs for autoimmunity is far more challenging because of their low precursor frequency and the tendency of Tregs to destabilize with repeated in vitro stimulation in an attempt to expand them [22,87]. New technology using chimeric antigen receptor (CAR) engineered T cells is promising for cancer immunotherapy [109], and may have applications for engineering antigen-specific Tregs to combat autoimmune disease. Indeed, engineered Tregs do have utility in mouse models of autoimmunity [110–113]. In addition to therapeutic development, CAR-engineering of Tregs also offers an opportunity for investigating fundamental biology of Tregs by defining the optimal CAR design to preserve Treg stability and function.

Conclusion and future prospects
In the past several years, we have gained deeper mechanistic understanding of the molecular control of Treg development, maintenance, and function owing to genetic tools in mouse models. These discoveries are instrumental for the development of better targeted therapies for alternating the balance between Tregs and effector cells in various disease settings. It is clear that no specific molecule or pathway is uniquely utilized by Tregs and the distinction between Tregs and effector T cells may be quantitative. Tregs may preferentially use a combination of pathways; therefore, combination therapies may be able to be more specific in targeting Tregs with lower and less toxic doses of drugs. Additionally, drugs that are not Treg-specific may be used for ex vivo manipulation of purified Tregs to increase their number while preserving their stability for therapeutic use. In the future, advanced tools for faster and more specific genetic manipulation of human cells [114] will allow us to more directly investigate the crucial molecular pathways of human Tregs, such as engineering better CAR Tregs for achieving antigen specific tolerance.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

This study finds two distinct Treg subsets in the periphery that are either IL-2 responsive and localized primarily to lymphoid organs or TCR/co-stimulation reliant and localize primarily to tissues.


This study ablates the TCR in mature Tregs, finding a crucial role for the TCR in Treg effector function but not lineage maintenance.


This study ablates the TCR in mature Tregs, finding that Treg homeostasis, Treg-specific gene expression, and suppressive function are linked to TCR triggering, while Foxp3 expression and methylation patterns are not.


A Foxp3 intrinsic element, CNS2, is crucial for maintenance of Foxp3 expression after proinflammatory cytokine signaling or when IL-2 is limiting.


A Foxp3 intrinsic element, CNS2, senses TCR/NFAT activation to maintain Foxp3 expression in a proinflammatory cytokine environment, demonstrating a crucial role for epigenetically marked cis-elements in the protection of cell identity.


42. PTEN-deficient Tregs fail to regulate Th1 and B cell responses. These Tregs become unstable, losing CD25 and Foxp3 expression, demonstrating a crucial role for epigenetically marked cis-elements in the protection of cell identity.


This study demonstrates that a CD28 responsive chromatin modifier EzH2 is required for Treg lineage stability and supports the Foxp3 gene-expression program after activation.


114. Schumann K, Lin S, Boyer E, Simeonov DR, Subramaniam M, Gate RE, Halliburton GE, Ye CJ, Bluestone JA, Doudna JA et al.: Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *Proc Natl Acad Sci* 2015, 112:10437-10442. This study was able to successfully use Cas9 ribonucleoproteins (RNPs) to edit the genome of primary human T cells. This establishes Cas9 RNP as a technology for future use in manipulating these cells for many therapeutic applications.