REVIEW
Harnessing proteases for T regulatory cell immunotherapy

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The immune system is tightly regulated by a subset of T cells defined as regulatory T cells (Tregs). Tregs maintain immune homeostasis by restraining unwarranted immune cell activation and effector function. Here, we discuss an important but underappreciated role of proteases in controlling Treg function. Proteases regulate a number of vital processes that determine T cell immune responses and some of them such as furin, ADAM (through regulating LAG receptor), MALT, and asparaginyl endopeptidase are implicated in Treg immunobiology. Targeted protease inhibition, using either small molecule inhibitors or gene deficient mice has demonstrated their specificity in modulating Treg function in experimental murine models. These data further highlight the ability of proteases to specifically regulate Tregs but no other T effector lineages. Taken together, it is apparent that incorporating proteases as targets within Treg cell engineering protocols may enable generation of robust Treg cellular therapeutics. These engineered Tregs may possess enhanced regulatory function along with resistance to lineage deviation in inflammatory disease such as colitis and graft versus host disease. Within this review, we summarize research on the role of proteases in regulating Treg function and discuss the translational potential of harnessing Treg function by targeting protease driven regulatory pathways.

Keywords: FOXP3 · immunotherapy · proteases · regulatory T cells

Introduction

Regulatory T cells (Tregs) are a subset of CD4+ T helper cells characterized by the high expression of IL2R (CD25hi), IL7Rlow (CD127), and the Forkhead transcription factor, FOXP3. FOXP3 dictates the regulatory function of Tregs and the stability of FOXP3 protein in Tregs is controlled by a number of environmental stimuli. In this review, we will provide an overview of the plethora of signals involved in the regulation of FOXP3 and highlight the potential of proteases in modulating Treg function.
ABSTRACT

Figure 1. Summary of Treg subsets. Regulatory T cells are generated in the thymus and are denoted as tTregs. In mice, tTregs are marked by surface receptors CD25, NRP1, and CD127. They also express Helios, FOXP3, and possess demethylated Treg Specific Demethylation Region (TSDR). In contrast to mice, human tTregs in the peripheral blood do not express NRP1 (A). In addition to tTregs, a small proportion of Tregs can be generated in the periphery from naive T cells or effector Th cell subsets. These are denoted as pTregs. Subsets arising from naive T cells [nT] usually do not express helper like transcription factors or chemokine receptors. On exposure to effector cytokines, such as IL-4 and IL-12, these cells can upregulate helper like transcription factors such as TBET, GATA3, and RORγT and can be described as “helper-like pTregs.” pTregs can also be generated from CD4+ T helper cells and express FOXP3 along with TBET, GATA3 and RORγT (B). In vitro, Tregs can be generated from naive T cells [nT] or helper CD4+ T cells in the presence of the cytokines IL-2 and TGF-β1 (C). Both pTregs and iTregs possess methylated TSDR and do not express Helios in mice. Receptors either regulated by proteases or protease regulators are denoted in bold.

highlighted by the phenotype of FOXP3 mutations in humans [4,5] and mice [2].

In addition to FOXP3, Tregs (both nTregs and iTregs) can upregulate other Th cell master transcription factors such as TBET, GATA3, and RORγT that mirror and oppose inflammation caused by their respective effector Th cell lineages [6]. Th cell lineages are induced and established when naive CD4+ T cells are activated by specific helper driving cytokines. For example, the cytokine IL-12 in combination with TCR and co-receptor signaling can induce the phosphorylation of signal transducer and activation of transcription protein (STAT)-4. Phosphorylated STAT4 induces the differentiation of Th1 cells by establishing a type 1 program driven by TBET within CD4+ T cells [7]. A similar signaling network results in differentiation of CD4+ T cells into Th2 cells (marked by GATA3) [8] and Th17 cells (denoted by the expression of RORγt) [9]. It is yet unclear whether within the naturally occurring population of Tregs lies a difference in the capacity for tTregs and pTregs to upregulate transcription factors such as TBET and whether upregulation of such transcription factors can affect the regulatory function of tTregs and pTregs. For example, the expression of TBET enhances Treg migration [10,11], suggesting that both tTregs and pTregs might have the ability to express dual transcription factors, but this phenotype is yet to be fully understood. A summary of the various Treg subsets is outlined in Figure 1.

Although, to a large extent, it has been postulated that all pTreg subsets arise from naive T cells, evidence also exists that effector T helper subsets such as Th1, Th2, and Th17 cells can upregulate FOXP3, which can imprint a suppressive function in these populations [12–16]. Thereby, demonstrating that FOXP3 is the defining factor that dictates regulatory function in Tregs, which arise from naive CD4+ precursor or pathogenic effector CD4+ Th cell subsets [3].

FOXP3 gene structure

Numerous factors control FOXP3 gene expression by directly or indirectly regulating FOXP3 promoter activity and hence Treg function. The transcription of the FOXP3 gene (both its initiation
and maintenance) is highly regulated by conserved noncoding sequence 1 (CNS1), CNS2, and CNS3, which serve as binding sites for a number of transcription factors [17,18]. Each of the CNS regions and the promoter region of FOXP3 in both mice and humans are highly regulated in order to maintain immune homeostasis. The factors that regulate FOXP3 transcription is expanded within Figure 2A and each of these factors can serve as a potential clinical target for boosting Treg numbers.

**FOXP3 protein structure**

Both mouse and human FOXP3 are approximately 47-kDa in size and consists of four domains that are important for its function. These are the N terminal domain, the zinc finger domain, the leucine zipper domain, and the C-terminal Forkhead domain [5,19-21]. On identifying FOXP3 as a critical protein that was responsible for the phenotype noted in both scurfy mice and in human IPEX syndrome, FOXP3 protein expression was characterized in naive CD4⁺ T cells and Tregs in mice and humans. Unlike mice, freshly isolated human Tregs possessed two isoforms of FOXP3 protein (FOXP3 and FOXP3Δ1Δ2). Further, when human naive CD4⁺ T cells were stimulated in the presence of IL-2, FOXP3, and FOXP3Δ1Δ2 was expressed. Taken together, human FOXP3 protein consists of multiple isoforms which is not the case in murine Tregs [22,23]. However, overexpressing exon 2 in human CD4⁺ T cells did not induce functional anergy in human T cells to the same extent as the full isoform [22]. These observations highlight that FOXP3 splice variants may have the potential to regulate the function of human Tregs, but specific function of these isoforms remains unknown. In addition to FOXP3Δ2, other isoforms namely FOXP3Δ2Δ7 has been reported [24,25]. These variants still induce a partial Treg program as noted in overexpression studies and provide circumstantial evidence that lack of exon 7 and
exon 2 may result in dysfunctional Tregs, highlighting another layer of FOXP3 regulation that is not operational in mice.

Transcriptional regulation of FOXP3

FOXP3 transcription is a tightly controlled process, which is regulated by the TCR, co-receptors, and cytokines. Any one of these signals can be manipulated to target Tregs for clinical use and a selected number of the signals are summarized below and all the factors that bind to CNS1, 2, and 3 thereby stabilizing FOXP3 transcription are shown in Figure 2A. The various regulatory elements that control FOXP3 transcription have been reviewed in ref. [26].

TCR stimulation

Tregs require TCR activation by cognate antigen in order to suppress their target cells. TCR signaling also plays a role in FOXP3 transcription by activating NF-κB transcription factors, which bind to the CNS3 enhancer region of the FOXP3 gene and initiate transcription (reviewed in [27]). While NF-κB signal through TCR is required for establishing the Treg program [28,29], prolonged TCR stimulation can alter the hypomethylated Treg specific demethylation region (TSDR) region of FOXP3 by enhancing the activity of DNA methyl transferases (DNMT1) and destabilize FOXP3 [30]. A suitable target within this pathway is a protease called MALT lymphoma translocation protein 1 (MALT1), which will be discussed later in this review, and may play a role in fine-tuning NF-κB signaling. In addition to NF-κB, nuclear factor of activated T-cells (NFAT) NFAT:FOXP3 complexes can also enhance Treg suppressive function by repressing the expression of IL-2 and upregulating cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 [31]) [32]; similarly FOXP3 interaction with AML1 (acute myeloid leukemia-1)/Runx1 (Runt related transcription factor-1) can downregulate IFN-γ [33].

Regulation through CD28, PD-1, and OX40

Activation of the stimulatory co-receptor CD28 is required for stable FOXP3 expression. In mice, iTreg development is severely impaired in the absence of CD28 co-stimulation [34]. In addition to CD28, the expression of numerous co-inhibitory molecules such as CTLA-4 [35], programmed cell death ligand-1(PDL-1 [36]), T cell immunoreceptor with Ig and ITIM domains (TIGIT [37]), and glucocorticoid-induced TNF receptor (GITR [38]) has been reported and studies demonstrate that these co-inhibitory receptors can play a role in either enhancing the function or stability of Tregs. Of note, we and others have identified programmed cell death 1 (PD-1) as a co-receptor that plays an important role in inducing and maintaining FOXP3 expression in murine iTregs[39], Tbet+ iTregs [13] and human Tbet+CD4+ T helper cells [12]. This regulation was first demonstrated by Francisco et al. [39], where PD-1 activation inhibited phosphorylation of protein kinase B (PKB/AKT) resulting in the sequestration of forkhead box (FOXO) protein, which in turn can release FOXP3 to bind its DNA target sequences in iTregs. A similar role has been recently attributed to TNF family receptor member 4 or OX40, whereby it regulates FOXP3 stability through basic leucine zipper transcription factor, ATF-like (BATF) proteins [40]. In Th1 cells and in the presence of iTreg conditions, PD-1 signaling can destabilize phosphorylation of STAT1 and STAT4, key signaling molecules that lie downstream of IL-12 and Interferon-γ (IFN-γ) that drive Th1 effector T cell differentiation and inhibit FOXP3 expression [12]. In human HCV patients, PD-1 activation in pTreg can destabilise STAT5 phosphorylation that is activated downstream of IL-2, a key cytokine for the induction and survival of Tregs [41]. While this study contradicts the idea that PD-1 can maintain FOXP3 expression in Tregs, the observation is restricted to virus infected CD4+ human T cells within an experimental set up, which may not efficiently test FOXP3 stability. In murine chronic viral experimental models, PD-1 induced FOXP3 in pTreg, thereby confirming the importance of PD-1 in maintaining FOXP3 in pTreg populations [7]. However, the molecular mechanism by which PD-1 regulates FOXP3 in antigen primed pTregs is still unclear.

Regulation by cytokines

The best understood cytokines that positively regulate FOXP3 expression in Tregs are IL-2 and TGF-β. Both IL-2 [42–45], through STAT5 phosphorylation, and TGF-β [46–50], through SMAD3 phosphorylation, are essential transcription factors for the induction and maintenance of FOXP3 expression in Treg subsets. Negative regulation of FOXP3 by cytokines also occurs, for example, IL-6 driven STAT3 phosphorylation, which can destabilize FOXP3 transcription by competing with phospho-STAT5 for the same locus on CNS2 [51]. IL-4 driven phospho-STAT6 also competes with STAT5 binding locus within CNS2 and thereby, promoting the loss of FOXP3 expression [18]. These studies implicate direct regulation of FOXP3 transcription by STAT5s and SMADs. However, the effect of these transcription factors in upregulating posttranslational signals, such as ubiquitination, and how they may impact FOXP3 protein is yet to be defined.

Posttranslational regulation of FOXP3 protein

In addition to transcriptional regulation, FOXP3 protein regulation by posttranslational modification has been thoroughly investigated and reviewed in ref. [26]. These studies that are reviewed in ref. [26] and some key studies highlighted here demonstrate that FOXP3 can be regulated through multiple pathways such as phosphorylation [52], acetylation [53], sumoylation [54], ubiquitination [55,56], and proteolytic degradation [13]. The various posttranslational regulators and their position within the FOXP3 protein are summarized in Figure 2B. Within this review, we will discuss the importance of proteases in regulating
FOXP3 expression in Tregs and the clinical relevance of targeting proteases in inducing immune tolerance in disease.

Proteases and Tregs

Reports on furin, A disintegrin and metalloproteinase (ADAM) ADAM10, ADAM17, and MALT1 in Treg biology provide an insight into how proteases regulate Treg function. Harnessing these proteases as a therapeutic target in Treg therapy is an exciting area of research that has been vastly underappreciated. The first report on proteases in Treg function [57] demonstrated that furin, a pro-protein convertase, is upregulated when T cells are activated and cleaves pro-TGF-β1 to release the active protein. Deletion of furin in Tregs resulted in less active TGF-β1 and inhibited Treg function [57,58]. These findings are the first to highlight how Treg surface receptors are regulated by proteases suggesting a new mechanism that maintain Treg suppressor function. In line with this observation is the identification of ADAM10 and ADAM17, which regulate lymphocyte activation gene-3 (LAG-3) surface expression [59]. ADAM10 and ADAM17 are metalloproteases that target and cleave the co-receptor molecule LAG-3 post TCR activation [33]. The expression of LAG-3 is linked to T cell activation and Tregs have been reported to exert their function through this molecule [60]. From this study, one can postulate that inhibiting ADAM10/17 can maintain LAG3 expression on Tregs and enhance Treg function. Further, LAG-3+ Tregs play a major role in regulating innate lymphoid cell-3 function in colitis [61]. Hence, targeting ADAM protein can maintain Treg–innate immune cell communication and regulate mucosal immune tolerance. Such emerging evidence suggests that proteases may play a vital role as therapeutic targets not only in boosting Treg numbers, but also in maintaining immune tolerance in diseases such as autoimmune colitis and graft versus host disease (GvHD).

Other proteases implicated in Treg development include MALT1 that recruits the ubiquitin ligase TNG receptor associated factor 6, which in turn mediates the formation of the IκB kinase complex and promotes NF-κB activation by cleaving RelB [62]. Disruption of this signal in mice resulted in reduced Treg numbers with a concomitant increase in T effector cell numbers, resulting in autoimmune [62]. Similarly, serine protease inhibitor-6 (SpI6), is also involved in regulating Treg function through intrinsic regulatory pathways that control Treg apoptosis. It was noted that Tregs deficient in SpI6 accumulated granzyme B (GrB) activity that resulted in impaired survival and were incapable of protecting mice from GvHD. While both iTregs and nTregs can suppress effector T cells through direct killing via GrB, the mechanism by which Tregs protect themselves from GrB mediated toxicity was unclear. However, the study by Azzi et al. [63] demonstrates that the endogenous serine protease inhibitor SpI6 protects Tregs from GrB mediated intrinsic cellular injury [63]. These reports highlight both intrinsic and extrinsic regulation of Treg function through proteases and depending on the micro-environment, one can harness the extrinsic or intrinsic proteases to regulate Treg function.

Recently, our laboratory has identified a protease called asparaginyl endopeptidase (Legumain/AEP), which can be classified as an intrinsic regulator that negatively regulates Treg stability and function [13]. Legumain or AEP was first identified in the common bean [64], from which it derives its name, subsequently characterized in the trematode Schistosoma mansoni [65] and identified as a protease. AEP is an endo-lysozyme protease and within the mammalian cell system AEP is found in abundance within the kidney and testis; within the immune system it is found in murine and human dendritic cells (DC), B cells, and EBV transformed B cells underscoring its importance in immunity [66–68]. AEP possesses a high degree of specificity for its substrates, cleaving them selectively after asparagine sites at P1 position and less selective at P3-P2 (reviewed in [69]). AEP has been implicated in antigen processing and presentation within DCs. It has also been shown to be important in TLR processing, however cathepsins can compensate for the lack of AEP in performing this task [70]. AEP also processes cathepsin L thereby promoting human Th17 cell differentiation [71]. Recently, a role for AEP in human Th1 cells has been shown where AEP inhibits IFN-γ production in Th1 cells [72]. AEP mRNA transcript was also reported to be expressed in human Tregs [73]. While the function of AEP in human T cells is not clearly delineated, we have fully elucidated the molecular mechanism by which AEP can regulate mouse FOXP3 within iTregs and helper like Tregs (Tbet+iTregs). We have determined that in Tregs, AEP can be activated within the cytosol and the active enzyme can be transported into the nucleus through a nuclear localization signal. On being transported into the nucleus, AEP can directly bind and cleave FOXP3 resulting in the ultimate degradation of both mouse and human FOXP3 protein [13,72]. Blocking AEP can stabilize FOXP3 and enhance Treg function. These data along with previous observations on AEP in immune tolerance have identified a new regulatory axis that can be manipulated in order to boost Treg numbers in disease. However, caution is needed, given the ubiquitous function of proteases within the human immune system. One way of circumventing the disadvantage of targeting proteases using small molecule inhibitors would be to incorporate these as targets for cellular engineering technologies that will be discussed in detail in the section below. For instance, Treg cellular therapeutics that are refractory to AEP mediated regulation may play superior role in inhibiting unwarranted inflammation.

Modulating posttranslational signals for Treg immunotherapy

The current understanding of FOXP3 regulation provides a milieu dependent opportunity in regulating Treg numbers in both autoimmune, alloimmune diseases and cancer. Examples of this success has already been demonstrated in chronic GvHD clinical trials, where the use of low dose IL-2 has shown remarkable success in boosting Treg numbers [74]. The mechanistic reason behind the success of low dose IL-2 can be attributed to the role of STAT5 [75] in stabilizing FOXP3 protein within Tregs. Along
these lines, significant success has also been reported in clinical trials that utilize tocilizumab for treating GvHD [76,77], whereby blocking IL-6 signaling enhances FOXP3 expression by inhibiting STAT3-mediated transcriptional and posttranslational pathways [51,78]. Since both STAT5 and STAT3 compete for same locus within the FOXP3 promoter region, by using targeted cytokine therapies, we can modulate Treg numbers in diseases based on their inflammatory profile.

While it is clear that cytokine therapies can modulate Treg numbers, whether antibodies to PD-1 and other co-receptors harness FOXP3 posttranslational regulation is not known. An in-depth review of the pros and cons of co-receptor blockade in affecting Treg numbers in tumor has been recently published by Tanaka et al. [79] and will not be discussed in detail within this review article. However, it is worth mentioning that PD-1 signaling has been shown to increase the frequency of Tregs in experimental murine models of inflammation [39] and in melanoma [13]. In contrast, in a subset of patients, blocking PD-1 signaling can enhance tumor-derived Treg numbers [80]. Therefore, it is worthwhile to understand whether PD-1 signaling regulates FOXP3 transcriptional or translational pathways, and this understanding may result in identifying patient subsets that may respond better to checkpoint blockade therapy.

There could be a potential explanation for the difference noted in Treg frequency, with PD-1 blockade, in patients versus murine models. This difference could be due to the molecular mechanism associated with PD-1 regulation of FOXP3. We found that PD-1 regulated FOXP3 posttranslational pathway by downregulating AEP, which is a protease that can degrade FOXP3 [13]. One hypothesis is that antibodies to PD-1 do not efficiently block PD-1 signaling (due to enhanced PDL-1/L-2 availability within the tumor micro-environment), thereby still allowing for AEP inhibition and FOXP3 stabilization. This could result in FOXP3 expression being maintained in hyper-proliferating Tregs in some patients and not others. Therefore, one could envision that anti-PD-1 therapies can be stratified by determining the amount of AEP expression in T cells in individual patients prior to treatment. For instance, patients who inherently express low AEP protein in their T cells may generate more proliferative Tregs within their tumors compared to others. AEP, therefore, is a good example of how determining the intrinsic post translational regulation of Tregs by PD-1 can be manipulated for translational purposes. AEP inhibitors have shown efficacy in not only dampening IFN-γ [72] and IL-17 secretion [71] in human T cells, but also in enhancing Treg suppression by maintaining FOXP3 expression. There is precedence for using proteases as targets for immunotherapy in animal models. Two such studies include MALT1 and SERPINB3 (SERPINs or SERin Protease Inhibitors). MALT1 inhibitor mepazine significantly inhibited the function of autoreactive T cells by suppressing their cytokine production while protecting Treg numbers in an experimental murine model of autoimmune encephalitis (EAE) and inhibited disease progression [81]. Similar to MALT1 inhibitor, use of SERPINB3 in an experimental murine model of systemic lupus erythematosus demonstrated the efficacy of these protease inhibitors in both prevention and treatment of systemic lupus erythematosus by significantly increasing Treg numbers with a concomitant decrease in effector Th17 cells [82].

Taken together, these studies highlight the potential for protease inhibitors in specifically enhancing Treg numbers while inhibiting effector cytokines in disease. A similar observation has been made with respect to posttranslational regulatory pathways. One study has reported the use of small molecules that stabilize TIP60-FOXP3 interactions to significantly enhance the treatment of autoimmune diseases, such as colitis and collagen-induced arthritis (CIA), in experimental murine models [83]. Further, use of histone deacetylase (HDAC) inhibitors in clinical trials has shown significant efficacy in enhancing Treg numbers in patients with GvHD [84]. Taken together, these murine and human experiments suggest that there is a plethora of regulatory pathways, which can be targeted to boost immune tolerance in inflammatory diseases.

**Potential advantages and disadvantages of targeting proteases in FOXP3 regulation**

Using small molecule inhibitors that target proteases, which can regulate FOXP3 is an area of investigation that may provide exciting new therapeutics. Nevertheless, given the ubiquitous nature of proteases, caution needs to be applied prior to using such clinical interventions. While animal models and clinical trials have shown encouraging results as summarized above, inhibiting proteases may affect not only critical pathways within the immune system, but also other biochemical processes resulting in unwarranted toxic metabolic and lysosomal dysfunction. One way to circumvent this disadvantage would involve testing small molecules in specific experimental murine models of disease prior to their clinical use. Another option would be to use the knowledge on proteases in cellular engineering technologies. For instance, engineering Tregs that are resilient to protease mediated regulation may result in novel cellular therapeutics beneficial in the treatment of various autoimmune and alloimmune diseases. For instance, engineered AEP resistant Tregs that are refractory to functional plasticity can be administered to patients with severe inflammatory diseases, which are driven by cytokines such as IL-6. These Tregs will not succumb to lineage deviation and will suppress inflammation in diseases such as acute GvHD and colitis. However, the central premise remains that without comprehensively understanding the fundamental biology of proteases in Tregs, their use as viable clinical targets will remain elusive.

**Conclusion and future directions**

In conclusion, we now have an in-depth understanding of how FOXP3 is regulated in Tregs and the use of biologics (such as anti-IL-6 or anti-PD-1 antibodies) along with small molecule inhibitors that can be tailored to reflect this understanding of Treg biology. For instance, considering the cytokine and co-inhibitory signals within the microenvironment prior to selecting biologics may provide tailored therapies to individuals; minimizing treatment-based
toxicity while maximizing efficacy. For example, IL-6 blockade might be beneficial in acute GvHD while low dose IL-2 may be significantly efficacious in other inflammatory conditions. Therefore, while tremendous amount of knowledge has been gained in understanding the post-translational regulation of FOXP3, a clear picture of inter-connecting intracellular proteins that regulate FOXP3 is yet to be systematically mapped out. One can envision that by developing a systematic proteome and phospho-proteome map of Tregs, we can ultimately reveal several important proteases, which can regulate FOXP3 protein stability. This knowledge may ultimately enhance the efficacy of immunotherapeutic regimens while minimizing deleterious side effects.

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