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Brief Reviews

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# Are Regulatory T Cells Defective in Type 1 Diabetes and Can We Fix Them?

Anabelle Visperas* and Dario A. A. Vignali*,†

**Regulatory T cells (Tregs) are critical regulators of peripheral immune tolerance. Treg insufficiency can lead to autoimmune disorders, including type 1 diabetes (T1D). Increasing evidence in mouse models of T1D, as well as other autoimmune disorders, suggests that there are defects in Treg-mediated suppression. Indeed, whereas Treg frequency in the peripheral blood of T1D patients is unaltered, their suppressive abilities are diminished compared with Tregs in healthy controls. Although expression of the transcription factor Foxp3 is a prerequisite for Treg development and function, there are many additional factors that can alter their stability, survival, and function. Much has been learned in other model systems, such as tumors, about the mechanism and pathways that control Treg stability and function. This review poses the question of whether we can use these findings to develop new therapeutic approaches that might boost Treg stability, survival, and/or function in T1D and possibly other autoimmune disorders.** ***The Journal of Immunology*****, 2016, 197: 3762–3770.**

Type 1 diabetes (T1D), also known as juvenile diabetes, is a chronic autoimmune disorder where a targeted immune response by both T and B cells leads to destruction of insulin producing β cells in the islets of the pancreas (1). T1D is one of the most common chronic diseases of children. Around 70,000 children are diagnosed with T1D each year, a number that is rising by 3–5% each year in developing countries (2). Defects in the control of effector populations is a common culprit in many autoimmune disorders, including T1D (3), and this may be due to dysfunctions in regulatory T cell (Treg)-mediated suppression.

Tregs are either generated within the thymus, known as thymically derived Tregs (tTregs), or in the periphery, known as peripherally derived Tregs (pTregs), where pTreg generation requires TGF-β for their differentiation (4, 5). Although pTregs have been shown to play an important role at mucosal sites and at the fetal/maternal interface (6, 7), we will focus on tTregs, as they are the dominant regulatory population that is impacted in T1D. tTregs arise in the thymus upon high-affinity TCR signals to self-antigens and have a diverse repertoire (8, 9), suggesting that they have broad Ag specificity. tTregs are typically found in lymphoid tissues and can traffic to peripheral tissues during times of inflammation.

Tregs express the transcription factor Foxp3, which is required for their development and function. In the absence of functional Foxp3, humans succumb to a lymphoproliferative disorder known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). Scurfy mice, which have a point mutation in *Foxp3,* develop a similar phenotype and succumb to disease early in life (10, 11). Bone marrow transplantation in IPEX patients and adoptive transfer of $Foxp3^+$ Tregs or T cell–enriched splenocytes into $Foxp3^{-/-}$ or scurfy mice restores normal immune homeostasis, supporting the necessity for Tregs in preventing autoimmune responses (12, 13). pTregs arising from $CD4^+Foxp3^-$ splenocytes have also been suggested to play a role in immune homeostasis, as their TCR repertoire is nonoverlapping with tTregs (14, 15). Of note, splenocyte transfer may also limit the expansion of recipient diabetogenic T cells independently of any impact of tTregs and pTregs (16). Tregs can suppress immune responses by both cell–cell (CTLA4, granzyme B) and soluble factor (TGF-β, IL-10, IL-35, adenosine)–mediated mechanisms (17, 18). These effector functions may become deficient upon Treg instability, which may lead to the development of autoimmunity, in this case T1D.

A two-checkpoint hypothesis has been suggested in the progression of T1D from insulitis to overt diabetes where Tregs play a central role at these checkpoints based on studies performed in mice (19). During the first checkpoint, autoreactive T cells begin entering the islet but are still under Treg-mediated control and therefore insulitic. The transition from insulitis to overt diabetes occurs when Tregs lose their ability to suppress effector cell responses. Is the loss of stability in Tregs a factor in T1D progression from insulitis to overt diabetes? Although many factors, including genetics and environment, contribute to the development of T1D, in this review we focus on the failure of Tregs to control autoreactive T cells and how this may relate to Treg instability. This review summarizes the

*Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and †Tumor Microenvironment Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232

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Address correspondence and reprint requests to Dr. Dario A.A. Vignali, Department of Immunology, University of Pittsburgh School of Medicine, E1052 Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA 15261. E-mail address: dvignali@pitt.edu

Abbreviations used in this article: CNS2, conserved noncoding sequence 2; DT, diphtheria toxin; DTR, diphtheria toxin receptor; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; miRNA, microRNA; mTOR, mechanistic target of rapamycin; mTORC, mechanistic target of rapamycin complex; Nrp1, Neuropilin-1; pTreg, peripherally derived Treg; Sema4a, Semaphorin-4a; T1D, type 1 diabetes; Tg, transgenic; Treg, regulatory T cell; tTreg, thymically derived Treg.



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contributions from other models in understanding what factors are important for Treg stability (Fig. 1). Can we use what has been learned toward stabilizing Tregs in T1D?

*Loss of Treg phenotype and function in T1D and autoimmune diabetes*

Although most studies have reported no differences in the frequency of Tregs in peripheral blood isolated from T1D patients, defects in Treg phenotype and suppressive capacity have been reported (20–24). Unfortunately, most data obtained from T1D patients is from peripheral blood due to the feasibility of obtaining pancreas samples from T1D patients. Therefore, whether Tregs are actively playing a role in limiting β cell destruction or have an altered phenotype or function in the islets during the disease course is unknown. Thus, mouse models of T1D have been employed to investigate disease progression in the islet microenvironment.

The most commonly used model for T1D is the NOD mouse. NOD mice spontaneously develop autoimmune diabetes starting at ∼10 wk of age in females and with increasing incidence over time until ∼25 wk (25). Both diabetes onset and progression are delayed in male NOD mice. Diabetes incidence in females and males is usually ∼80 and ∼30%, respectively. This may be due to differences in the gut microbiome between females and males owing to hormonal differences (26). Other environmental factors, including housing conditions and diet, can also affect the development of autoimmune diabetes (25). Genetic analyses have uncovered susceptibility loci in NOD mice that are known as the insulin-dependent diabetes (*Idd*) loci. More than 40 *Idd* loci have been identified, with the MHC exhibiting the highest linkage with T1D incidence (25, 27). The NOD mouse shares many similarities to T1D in humans, but with some notable differences (25). Nevertheless, the NOD mouse has proven to be a useful model to study the role of Tregs in autoimmune diabetes.

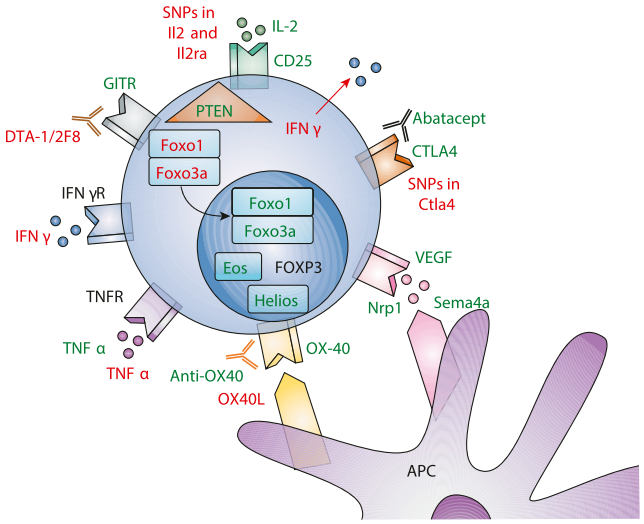


**FIGURE 1.** Mechanisms of Treg stability and instability. IL-2 is critical for Treg stability and maintenance where polymorphisms in both *Il2* and *Il2ra* have been seen in diabetes. Proinflammatory cytokines, including IFN-γ and TNF-α, may alter the Treg phenotype. Many Treg-associated molecules are important for optimal suppressive function, including CTLA4, GITR, and OX-40. Interestingly, agonistic Abs to GITR are detrimental to Treg-mediated stability and suppression. Intracellular molecules, including Helios, Eos, and PTEN, are also key molecules in optimal Treg function. Foxo1/3a localization into the nucleus is necessary to stabilize Foxp3 in Tregs. Green indicates stabilizing signal; red indicates destabilizing signal.

Treg modulation studies have highlighted their importance in limiting autoimmune diabetes and controlling immune responses in the islet, despite some contradictory observations. Whereas Treg depletion using anti-CD25 (PC61) has been shown to accelerate autoimmune diabetes development in several studies (28–30), one group observed complete protection from the development of autoimmune diabetes (31), perhaps due to the depletion of activated diabetogenic $CD25^+$ effector T cells in addition to $CD25^+$ Tregs as a consequence of late initiation of PC61 treatment (>9 wk). However, mice that lack Tregs due to *Foxp3* deficiency rapidly develop autoimmune diabetes (32). Indeed, temporal depletion of Tregs due to diphtheria toxin (DT) treatment of *Foxp3*–DT receptor (DTR) mice showed strong immune infiltrates in the pancreas 2 wk after DT treatment (33). Of note, NOD.*Foxp3*-DTR mice (*Foxp3* bacterial artificial chromosome transgenic [Tg] DEREG mouse model) do not develop diabetes at an accelerated rate (34). These conflicting observations with two independently generated bacterial artificial chromosome Tg NOD.*Foxp3*-DTR strains may be due to differences in expression and deletional efficiency and warrant further investigation. Interestingly, mice expressing the BDC2.5 TCR transgene (expressed on $CD4^+$ T cells specific for the islet Ag chromogranin A), which are immunocompetent, only develop insulitis (35). However, when the BDC2.5 TCR transgene is expressed on a $Rag^{-/-}$ background, in which $CD4^+$ effector T cells develop but Tregs do not, mice succumb to diabetes rapidly. Indeed, DT-treated NOD.*Foxp3*-DTR mice crossed to BDC2.5 Tg mice also rapidly develop diabetes (33). Collectively, these studies suggest that diabetes onset may be associated with decreased Treg numbers or function.

If humans and mice are not Treg deficient, why do they succumb to T1D and autoimmune diabetes, respectively? What is affecting their functionality? Interestingly, islet-infiltrating Tregs in mouse models still express high levels of Foxp3 but have decreased expression of the high-affinity IL-2 receptor CD25 and survival factor Bcl2 (36). Similarly in T1D patients, Tregs found in PBMCs have low expression of another Treg-associated marker, GITR (37), which is discussed further later in the review. In children with T1D, a higher proportion of Tregs produces the proinflammatory cytokines IL-12 and IL-18, which are also found at increased levels in serum, compared with healthy controls (23). Consequently, this altered Treg phenotype has been implicated in T1D pathogenesis. Thus, when altered Treg numbers and/or function are primary contributors to the development of T1D, boosting either parameter in vivo may provide a therapeutic opportunity.

*Boosting Tregs in mice and humans*

*Pharmacological-based therapy.* Inducing Treg proliferation via multiple pharmacological methods has been proposed and attempted in both the NOD mouse model and in clinical trials. IL-2, which is important for maintenance of Tregs, has been a potential target for Treg therapy (38) (Fig. 1). Although high-dose IL-2 has been used as a therapeutic approach in the treatment of melanoma and renal cancers, low-dose IL-2 in NOD mice can reverse established disease by increasing Treg numbers and function (36, 39). IL-2/anti–IL-2 Ab complexes have also been used to preferentially promote Treg expansion

(40). Modulation of mechanistic target of rapamycin (mTOR) activity with rapamycin has been shown to promote Treg expansion, survival, and function (41). Although no difference in Treg number, proliferation, or cytokine production was seen with rapamycin therapy prior to islet transplantation, Tregs do have increased suppressive capabilities (42). A combinational therapy has also been assessed with the use of IL-2/anti–IL-2 Ab complexes in combination with rapamycin and islet Ag peptide treatment. Treg expansion was observed and mice were protected from diabetes development in both spontaneous and induced models of diabetes (43).

Nonactivating, non–FcR-binding CD3 Abs may currently be the most promising treatment for T1D. More than eight clinical trials have targeted this approach, five of which are using teplizumab, a humanized non–FcR-binding anti-CD3 mAb (44). C-peptide is a byproduct of insulin production and is produced at equimolar concentrations and thus can be used to determine the amount of insulin produced by β cells. Short-term treatment of younger individuals and recent onset patients with teplizumab has shown promising results in 4 y follow-up studies, based on C-peptide levels, with limited toxicity (45–48). Although its mechanism of action is currently unclear, a 2-fold tolerance induction has been suggested through depletion of pathogenic T cells and preservation of Tregs and their function (49, 50). Although the mechanisms of action of all of these therapeutic approaches are different, in all cases the common denominator is increased Treg number and function.

*Cell-based therapy.* As Treg insufficiency may be a key driver of T1D and autoimmune diabetes, increasing the number of Tregs in circulation may overcome this deficiency. Repeated Treg adoptive transfer into neonatal NOD mice can delay the onset of autoimmune diabetes (51), suggesting that Treg number or functionality may be deficient in NOD mice over time, thereby requiring supplementation. Adoptive transfer of prediabetic NOD splenocytes or BDC2.5 TCR Tg effector T cells into immunodeficient NOD mice develop autoimmune diabetes ~14 d posttransfer. Interestingly, disease can be prevented following cotransfer with $>10^6$ polyclonal Tregs or as few as $5 \times 10^4$ BDC2.5 TCR Tg Tregs (34). Adoptive transfer of a low number of dendritic cell–expanded BDC2.5 TCR Tg Tregs into prediabetic NOD mice also blocks diabetes development and can rescue mice with overt diabetes (52). Whereas low numbers of Ag-specific Tregs are able to reverse autoimmune diabetes, adoptive transfer of 10-fold more polyclonal Tregs is not as effective in treating NOD mice therapeutically (53), suggesting that specificity for β cell Ags is critically important for optimal Treg functionality.

In vitro–expanded polyclonal Tregs are currently in clinical trials as a promising alternative to pharmacological-based therapies. Phase 1 clinical trials have been performed in both children and adults with no safety concerns thus far (54–56). Interestingly, some potential efficacy has been observed in children at 4–5 wk follow-up based on C-peptide levels. However, whereas C-peptide levels were increased initially at 1 and 2 y follow-ups, they declined over time. Approximately 25% of the transferred Tregs with a naive/memory-like phenotype were still present in patients at 1 y follow-up based on deuterium incorporation. A similar trial has also been conducted in Poland with promising results. At a 1 y follow-up of 12 children with T1D, increased C-peptide levels and diminished use of insulin were observed in 8 of 12 patients and, remarkably, complete insulin independence was achieved in 2 of 12 patients (55). Whether these observations are durable and can be replicated in phase 2 clinical trials remains to be determined.

Although these initial observations are encouraging, the key challenge is likely to focus on understanding what the primary limitations are for successful, durable responses and can these be overcome with 1) increased Treg numbers, 2) islet Ag specificity, and/or 3) approaches that increased stability, survival, functionality, and longevity. There is a growing consensus that future clinical trails need to focus on the development of Tregs with β cell Ag specificity to maximize 1) islet homing and therapeutic index, and 2) retention of Tregs over time to endure a durable response. Also, is the adoptive transfer of more Tregs the only viable therapeutic approach or could the Tregs that are already present in the patient be "reinvigorated"? Clearly, gaining a greater understanding of the mechanisms and factors that control Treg stability and function will greatly inform future clinical development.

### *Loss of Treg stability and function*

What is Treg stability, how does this differ from plasticity, and what drives instability? Treg plasticity and stability have been used interchangeably in the past, but they represent two distinct Treg fates. A stable Treg expresses the transcription factor Foxp3, is suppressive, and produces anti-inflammatory cytokines (such as IL-10 and IL-35) and a minimal amount of effector cytokines (e.g., IFN-γ, TNF-α, IL-2) (57). When Tregs exhibit plasticity, they still express Foxp3 and remain functionally suppressive but gain distinct migratory and functional programs that can enhance their capacity to suppress certain Th subsets (58). In contrast, destabilized Tregs lose their suppressive abilities and gain effector functions, while either retaining Foxp3 expression (59) or eventually losing Foxp3 expression and becoming pathogenic "ex-Tregs" in inflammatory environments (60). In both NOD mice and humans with T1D, Tregs are identified based on Foxp3 expression, yet they exhibit defective suppressive activity, suggesting that they may be destabilized. Further analysis of Treg stability has recently been extended to include epigenetic modifications by assessing the methylation pattern of the conserved noncoding sequence 2 (CNS2 or TSDR) in the 5′ untranslated region of the *Foxp3* gene, where tTregs are demethylated at this locus and loss of stability has been associated with remethylation at this locus (61). *Foxp3* CNS2 hypomethylation appears to be important for the binding of key transcription factors, including NF-κB, CREB/ATF, Ets1, and STAT5 (62–64). Methylation studies have expanded to other Treg-associated genes *Il2ra* (CD25), Ikzf4 (Eos), *Ctla4*, and *Tnfrsf18* (GITR), which are also hypomethylated (65).

In addition to epigenetic modifications of target genes, other mechanisms, including microRNAs (miRNAs), may also modulate disease development. These short noncoding RNAs are transcribed and processed via the RNAses Drosha and Dicer to generate mature miRNAs that silence genes either through repressing translation or accelerating transcript degradation (66). Mice with a Treg-restricted deletion of Dicer or Drosha possess unstable Tregs with poor suppressive ability, diminished expression of Treg-associated molecules, increased effector cytokines, and succumb to a scurfy-like disease (67–69).

Similar results have also been seen upon gene silencing of miR-126 in a breast cancer tumor model leading to increased anti-tumor immunity by altering activation of the PI3K/AKT pathway (70). Although miR-155 is not necessary for Treg homeostasis or its suppressive function, its role in down-regulating SOCS1, which increases responsiveness of STAT5, can make Tregs better responders to IL-2, even under suboptimal conditions (71). Treg-specific ablation of the miR-17–92 cluster results in exacerbated experimental autoimmune encephalomyelitis with decreased IL-10–producing Tregs (72) but is not required for thymic generation of Tregs (73). miR-10a is selectively expressed in Tregs, and expression has been correlated to autoimmune disease susceptibility, as the autoimmune-resistant C57BL/6 strain expresses high levels of miR-10a whereas the autoimmune-susceptible NOD strain expresses lower levels (74). These studies suggest that certain miRNAs may be important in maintaining Treg stability and function. Indeed, miR-342, miR-191, and miR-510 are differentially expressed in Tregs of patients with T1D, but whether these are biomarkers or contribute to disease still needs to be further elucidated (75).

Understanding the factors and pathways that control Treg stability would clearly facilitate their therapeutic utilization in T1D, as well as other autoimmune and inflammatory diseases, and potentially in transplantation. Whereas Foxp3 is the master transcription factor that is required for Treg development and functionality, a variety of external signals from cytokines and surface receptors, via intracellular signaling molecules, impinge on Tregs and impact their stability.

### *Factors that impact Treg stability*

*Cytokines.* Several cytokines have a substantive impact on Treg development and function (Fig. 1). IL-2, produced by effector T cells, is necessary for the maintenance and function of Tregs, as they do not make their own autocrine IL-2 (76–78). Most Tregs express the high-affinity IL-2 receptor (*Il2ra*, CD25) that signals via STAT5 (79). Genetically manipulated mice deficient in *Il2* or *Il2ra* phenocopy *Foxp3*-deficient or Treg-ablated mice, yet they still harbor T cells that express diminished levels of Foxp3 (80, 81). Humans with CD25 deficiency also have many of the same symptoms as seen in patients with IPEX (82). IL-2 reverses the anergic, nonproliferative phenotype of Tregs in vitro and promotes their capacity to suppress immune responses (83). IL-2 withdrawal has been shown in vitro to limit Treg suppressive ability (84). Under suboptimal IL-2 conditions, the CNS2 element sustains Foxp3 expression, whereas in its absence, actively proliferating Tregs lose Foxp3 expression at an accelerated rate (85). Genome-wide association studies have identified IL-2 pathway polymorphisms in both T1D (*Il2ra*) and autoimmune diabetes (*Il2*) (86–88). Indeed, reduced IL-2 signaling, via pSTAT5 analysis, has been documented in T1D patients with diminished Treg suppressive capabilities (89, 90). In NOD mice, Tregs have decreased Bcl2 and CD25 expression only in inflamed islets. This may be due to decreased levels of IL-2 in the islet, as low-dose IL-2 treatment increases Treg survival and protection (36). These studies highlight the importance of IL-2 in Treg function and possible defects that might lead to the development of T1D.

Inflammatory environments have been shown to destabilize Tregs in many models due to their interaction with or production of proinflammatory cytokines. Although several cytokines may destabilize Tregs, we focus here on those that may be relevant to T1D. IFN-γ is highly expressed in many inflammatory conditions and may limit Treg function. Upon stimulation with IFN-γ in vitro, Tregs downregulate CD25, lose Foxp3 expression, and exhibit limited expansion (91). Under high-salt conditions, Tregs can begin to produce IFN-γ and lose suppressive activity, which can be restored upon Ab blockade of IFN-γ (92). Whether this Treg-derived IFN-γ acts on Tregs or effector T cells still needs to be further elucidated (92). In T1D patients, increases in IFN-$\gamma^+$ Foxp3$^+$ Tregs have been observed in peripheral blood. These cells are predominantly hypermethylated at the CNS2 locus but still exhibit suppressive function (93).

Tregs constitutively express TNFRII, which upon signaling leads to diminished Foxp3 mRNA and protein levels and reduced suppressive actvity. Not surprisingly, patients with active rheumatoid arthritis possess Tregs that express lower Foxp3 expression and suppressive ability, and this could be reversed with anti-TNF (infliximab) treatment (94). In contrast, others have shown the requirement for TNF signaling in the generation of functional Tregs within the thymus and their function in inflammatory settings. In colitis models, expression of TNFRII expression is critical for Treg function (95, 96). Likewise, in NOD mice, TNF receptor deficiency protects mice from autoimmune diabetes and increases Treg-mediated suppression (97).

The role of IL-27 in Treg stability has been quite conflicting. IL-27 has been shown to antagonize pTreg generation (98), but it has been shown to enhance tTreg function in a T cell–mediated colitis model via a Lag3-mediated mechanism (99). In a tumor model, IL-27Rα–deficient mice have decreased Tregs in the tumor microenvironment, suggesting that IL-27 may act indirectly on Tregs via suppressing IL-2 generation by effector T cells (100). Nevertheless, the role of IL-27 specifically on Tregs has yet to be clarified. Increased IL-27 has been documented in autoimmune diabetes, and blockade of IL-27 in NOD mice delays the onset of autoimmune diabetes (101).

Extensive studies still need to be performed to assess whether these cytokines directly impact Tregs before conclusions can be drawn regarding their role in modulating Treg function in T1D and autoimmune diabetes.

*Surface molecules.* Several cell surface molecules have been shown to impact Treg stability and function (Fig. 1). OX40 (*Tnfrsf4*, CD134) is part of the TNFRs and is expressed on Tregs (102), yet its role in Treg-mediated suppression has led to conflicting results both in vitro (103–105) and in vivo. OX40 expression on Tregs may play a role in suppressing inflammatory responses in vivo, as mice with a Treg-restricted deficiency in OX40 retain Foxp3 expression yet develop gut inflammation in a T cell–mediated gut inflammation model (106). Indeed, use of an agonist anti-OX40 (OX86) protects NOD mice from the development of autoimmune diabetes (107). However, disease is inhibited in *Ox40l*$^{-/-}$ mice and neutralizing anti-OX40L–treated NOD mice (108). Whether Tregs are the primary subset responding to OX40L has not been fully addressed, as CD4$^+$ and CD8$^+$ T cells also express OX40 during autoimmune diabetes progression (109).

GITR (*Tnfrsf18*, CD357) is another TNFR family member that is found at high levels on the surface of Tregs (102). Paradoxically, use of an agonist anti-GITR (DTA-1) undermines Treg-mediated suppression and tolerance in tumor models. Decreases in Treg frequency and expression of Foxp3 in intratumoral Tregs have been seen (110). This loss of Foxp3 (and Helios)

expression is mediated by the JNK pathway. Treatment of lung allergy in mice with a JNK inhibitor led to reversal of GITR-induced changes in phenotype and function, resulting in rescue from disease (111). Indeed, accelerated development of autoimmune diabetes has also been seen using a different agonistic anti-GITR Ab (2F8) (112), suggesting that activation of this pathway may be detrimental to Treg stability.

*Ctla4* (CD152) is highly expressed on Tregs and has extensively been studied as an inhibitory molecule important for T cell homeostasis and tolerance (113). *Ctla4*$^{-/-}$ mice succumb to fatal lymphoproliferative disease (114), whereas Treg numbers are increased (115, 116). Results from in vivo models of autoimmunity have been quite conflicting, where *Ctla4*$^{-/-}$ Tregs are suppressive in some instances but not in others (115, 117). *Ctla4* is a susceptibility gene in autoimmune diseases, including T1D, where many polymorphisms have been identified (118–120). Costimulation blockade using anti-CTLA4 (abatacept) has recently been shown in phase II clinical trials to delay the progression of T1D (121), but whether Tregs are playing a direct role needs to be assessed further.

Neuropilin-1 (Nrp1) is an important factor in axonal guidance during embryonic development, but its role in the immune system has only recently been appreciated. Nrp1 is highly expressed on tTregs but is expressed at lower levels in pTregs (122–124). A role for Nrp1 in promoting the stability, survival, and function of Tregs has been suggested (125). Nrp1 on Tregs has been shown to interact with both Semaphorin-4a (Sema4a) and vascular endothelial growth factor. Mice with a Treg-specific Nrp1 deletion had substantially reduced tumor growth in multiple models, suggesting that Treg-mediated suppression of antitumor immunity has been lost (125, 126). Interestingly, these mice did not succumb to autoimmunity and inflammatory disease, and the frequency of Foxp3$^{+}$ Tregs was not altered (125). Stabilization via the Nrp1/Sema4a pathway enhances expression of the survival factor Bcl2 and effector molecules IL-10 and CD73, and limits expression of lineage-associated transcription factors, including T-bet, IFN regulatory factor 4, and retinoic acid–related orphan receptor γt, as well as the proinflammatory cytokine IFN-γ (125). Boosting Treg function by engaging the Nrp1/Sema4a pathway may be a possible therapeutic approach to stabilize Tregs in vivo or prior to adoptive transfer.

*Intracellular signaling molecules.* There are also several intracellular proteins that appear to modulate Treg stability and function by dependently or indirectly modulating Foxp3 function or stability (Fig. 1). Eos (*Ikzf4*), a zinc finger transcription factor, is a member of the Ikaros family of transcription factors and is highly expressed in Tregs. Eos interacts directly with Foxp3 and is necessary for gene silencing (e.g., *Il2*, *Ifng*) while maintaining expression of key Treg-associated genes, including *Ctla4* and GITR (127). Silencing of *Eos* using siRNA does not result in loss of Foxp3 expression but does result in the loss of Treg suppression in a T cell–mediated colitis model and induction of effector cytokines, such as IFN-γ and IL-2 (127). Downregulation of Eos expression is required for the reprogramming of Tregs into helper-like cells that retain Foxp3 expression (128). These Eos$^{-}$Foxp3$^{+}$ Tregs (Eos-liable) exhibit reduced regulatory function and enhanced expression of CD40L, IL-2, and IL-17 (128). Of note, global deletion of Eos in mice does not affect the function or phenotype of Tregs in vivo and in vitro, but it does result in the development of more severe experimental autoimmune encephalomyelitis. This observation was attributed to the function of Eos in effector T cell populations (129).

Helios (*Ikzf2*), another member of the Ikaros transcription factor family, was once thought to distinguish tTregs from pTregs; however, it now appears that Helios expression is highly dependent on Ag stimulation via the TCR (130, 131). Although Helios does not form a complex with Foxp3 or bind to the *Foxp3* locus, it plays an indirect role in supporting Treg stability (132, 133). Mice with a Treg-specific Helios deficiency develop autoimmunity and appear to possess unstable Tregs with diminished Foxp3 expression, increased effector cytokine expression, and reduced suppressive activity (133). Chromatin immunoprecipitation sequencing and pathway analysis of Helios-targeting genes in Tregs highlighted deficiencies in the IL-2Ra/STAT5b pathway, suggesting that Helios may be important in regulating IL-2 signaling and Treg survival (133).

Foxo1 and Foxo3, which are also forkhead box transcription factors, pay a key role in maintaining Foxp3 expression in Tregs (134). Mice deficient in Foxo1 in Tregs succumb to a scurfy-like phenotype by 5 wk. This lymphoproliferative disease is not due to the loss of Treg number but rather to their loss of function (135, 136). This phenotype can be rescued by expression of Foxo1$^{AAA}$, where Foxo1 is insensitive to 14-3-3–mediated cytosolic restriction and is this confined to the nucleus where it can facilitate Foxp3 function. Autoimmunity is further exacerbated by dual deletion of Foxo1 and Foxo3 (136). Foxo1/3 bind directly to the *Foxp3* locus and control promoter activity (136, 137).

The phosphatase PTEN has recently been shown to play a pivotal role in mediating Treg stability. PTEN is an upstream inhibitor of the PI3K/Akt pathway and therefore inhibits mTOR complex (mTORC)1 and mTORC2 activity (138). Upon genetic deletion of PTEN in Tregs, mice have increased levels of autoantibodies, renal pathology, and ongoing age-related autoimmunity. Nevertheless, Tregs are found in high numbers and readily proliferate compared with PTEN-sufficient Tregs. These Tregs are highly activated and express higher levels of ICOS, PD-1, and IFN-γ, decreased levels of CD25, and have a higher proportion of "ex-Tregs" based on the use of lineage-tracing experiments (61, 139). The mechanism of Treg-mediated loss of suppression is via upregulation of mTORC2 activity upon PTEN loss (139). Indeed, inhibition of mTOR in Tregs leads to heightened stability of Foxp3 expression (140), and Treg-specific loss of the mTOR inhibitor tuberous sclerosis 1 results in loss of Foxp3 expression, suppressive functionality, and increased expression of IL-17 (141). Interestingly, Nrp1, which as discussed above promotes Treg stability and function, has been shown to signal via PTEN that in turn limits Akt activity and reduces Foxo phosphorylation and thus nuclear exclusion, thereby promoting Foxp3 activity (125). Taken together, these observations provide a potential causal link between Nrp1, PTEN, and Foxo in mediating Treg stability and function.

## Conclusions

In summary, many factors impinge on Tregs to either promote or undermine their stability, survival, and function (Fig. 1). Some of these pathways are inherent, whereas others are induced or selectively used in inflammatory environments (59). We postulate that a primary driver of autoimmunity may be Treg insufficiency caused by a failure to promote pathways that enforce their stability, survival, and function. In tumors, where

Treg activity is arguably at its most robust, Treg stability is enforced by an Nrp1/PTEN/Foxo axis, and potentially other mechanisms, to prevent effective antitumor immunity. This also appears to protect Tregs from destabilizing forces that may be quite severe given the hostile intratumoral microenvironment, which is hypoxic, acidic, and nutrient and glucose starved. Thus, under normal circumstances Tregs seem to be well adapted to respond to cues from diverse microenvironments to maintain Treg stability and function. However, we posit that genetic, environmental, or contextual factors conspire to undermine these programs that ultimately leads to Treg insufficiency and autoimmunity.

This hypothesis and the information outlined above raise several key questions. First, can we boost Tregs that are already present but appear to exhibit insufficiency? This could be achieved by developing therapeutics that promote utilization of the Nrp1/PTEN/Foxo axis. For example, Sema4a–Ig fusion proteins may act as Nrp1 agonists, thereby promoting Treg stability and function. Alternatively, intracellular delivery of therapies that promote Foxo stability and nuclear translocation may produce a similar Treg stabilizing effect. Second, can we inhibit pathways that lead to instability? Although we need to gain a greater understanding of the factors that promote Treg instability, approaches that limit the factors that are known to drive these processes may be beneficial. The use of blocking Abs against cytokines that can destabilize Tregs may be useful in a manner analogous to TNF-α blockade in rheumatoid arthritis. We could also develop Abs to block OX40L from interacting with OX40 on Tregs. Finally, is a combinatorial therapy possible and necessary? Given that there may be a 2-fold defect in Treg number and function in T1D, combinatorial therapy may be most useful. One could combine Treg adoptive transfer with approaches that promote Treg stability prior to and/or following transfer. Of course, these approaches may also be combined with current therapies that are in clinical trials for T1D, such as teplizumab (non-FcR–binding anti-CD3). Indeed, one might argue that as combinatorial approaches are the mainstay of effective cancer therapy, it is likely that combinatorial approaches will be required for the treatment of T1D, with perhaps the inclusion of therapies that promote Treg stability and function.

# Disclosures

D.A.A.V. has submitted patents covering LAG3 and NRP1/SEMA4A that are pending and will be entitled to a share in net income generated from licensing of these patent rights for commercial development. The other author has no financial conflict of interest.

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