Cancer Immunology Research

Treg Fragility: A Prerequisite for Effective Antitumor Immunity?

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Abstract

Inhibitory checkpoint blockade has significantly improved patient response rate across numerous tumor types. However, most patients remain unresponsive to immunotherapy, suggesting that unappreciated mechanisms of resistance exist. The tumor microenvironment (TME) is unique and composed of many suppressive cell populations that inhibit antitumor immune responses, including regulatory T cells (Tregs). The TME is nutrient poor, acidic, and hypoxic, creating a challenging microenvironment for immune cells to function and survive. Tregs suppress a wide variety of cell populations through multiple mechanisms and are tasked with limiting tissue damage. Tregs are now considered to be a barrier to effective antitumor immunity. Systemic Treg depletion is not favored because of their critical role in maintaining immune homeostasis and preventing autoimmunity. Reducing Treg function specifically within the TME may provide a more effective, targeted approach to limit the immunosuppressive environment within the tumor without inducing systemic adverse consequences. Targeting molecules that cause Treg instability, characterized by loss of critical Treg transcription factors such as Foxp3, could result in conversion into cells that cause immune pathology, tissue damage, and subsequent autoimmune side effects. Interferon-γ (IFNγ) can cause intratumoral Treg "fragility," which results in loss of suppressive activity and increased IFNγ production without loss of Foxp3 expression and gross Treg "identity." We reviewed the impact Tregs have on the TME and vice versa, and their implications for responsiveness to cancer immunotherapy. We propose that the extent to which intratumoral Tregs develop a "fragile" phenotype following immunotherapy will predict and dictate responsiveness. *Cancer Immunol Res; 6(8); 882–7. ©2018 AACR.*

Introduction

Immunotherapy became a major pillar of cancer treatment around 2010, when an antibody targeting the inhibitory receptor, CTLA-4 (Ipilimumab), showed a ∼20% increase in overall survival in metastatic melanoma patients, followed by FDA approval (1, 2). Interest in immunotherapy grew rapidly in 2014, when another antibody targeting PD-1 (Nivolumab) was approved with better than anticipated patient responses, showing a 40% objective response rate in melanoma (3). Unfortunately, many patients remained unresponsive. The underlying mechanisms of immunotherapy resistance remain obscure. However, one potential roadblock is the presence of suppressive cell populations, such as regulatory T cells (Tregs; ref. 4), which remain dominant despite the mechanistic benefits of inhibitory receptor blockade.

Tregs function as the master regulators of the immune system, maintaining homeostasis and preventing autoimmunity. Initially identified as "suppressive cells" (5), Tregs are characterized by the transcription factor Foxp3, which is required for their development and function in mice (6, 7). Treg-suppressive function is exerted through a variety of mechanisms, such as cytokine secretion (including IL10, IL35, and TGFβ), metabolic disruption through CD39:CD73 adenosine production or IL2 deprivation, direct cytolysis through granzyme B, and modulation of DC development and function via LAG3 and CTLA-4 (8). Tregs also play a key role in limiting tissue damage, but the mechanisms utilized remain to be fully elucidated (9). Tregs can develop in the thymus (tTregs), arise in the periphery (pTregs), or be generated *in vitro* with the addition of TGFβ (iTregs; ref. 10). We primarily focus on tTregs (herein denoted as "Tregs"). However, the role of pTregs in tumors, their stabilizing factors, and whether they become fragile in tumors remains unclear and warrants further investigation.

In the absence of Tregs or when the *Foxp3* locus is disrupted, rampant systemic autoimmunity ensues. This presents as IPEX (immune dysregulation polyendocrinopathy, enteropathy, x-linked) in patients and is lethal without a bone marrow transplant. In mice that lack expression of *Foxp3* through genetic deletion or the "*Scurfy* mutation" (6), autoimmune symptoms can be substantively delayed by Treg cell transfer within 48 hours of birth (11). Although Tregs are critical for preventing autoimmunity, they also suppress the antitumor immune response and promote tumor outgrowth (4). Treg depletion in tumor models has been studied in *Foxp3*$^{DTR\text{-}GFP}$ mice (12), where the majority of mice clear the tumor but subsequently succumb to systemic autoimmunity (13, 14).

The tumor microenvironment (TME) is unique in that it is nutrient poor, hypoxic, and acidic, making it a taxing environment for many immune subsets, such as effector T cells (T_{effs}), that are primarily glycolytic. In contrast to this, Tregs rely on oxidative phosphorylation and are thought to have a proliferative and functional advantage within hypoxic, acidic environments

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(15). Increased Tregs have been observed in a variety of cancer patient peripheral blood and tumors (16, 17), and many cancer types show a positive correlation between higher Treg percentages and poor prognosis in patients. Treg percentages in the tumor mass can increase with severity of stage (18), and higher Treg percentages correlate with poorer disease-free survival in several cancers (19). As a result, Tregs have been targeted in the clinic, albeit with limited success. Depletion strategies targeting the IL2 pathway, through use of antibodies or other small molecules, led to off-target effects such as depletion of T_{effs} or loss of DC-mediated T-cell activation, in addition to incomplete depletion of Tregs (20, 21).

CTLA-4 blockade has been identified as a potential Treg target due to high surface expression. Previous studies have shown that CTLA-4 antibodies with FcγR ADCC activity reduced Tregs in the TME and that a positive correlation exists between reduced Tregs and CTLA-4 blockade response in bladder cancer patients (22). CTLA-4 blockade was initially thought to work on Tregs through either depletion or reduction of suppression (23). However, effects on both T_{eff} and Treg compartments are required for full antitumor function (24). Blockade led to increased peripheral Tregs and reduced intratumoral Tregs due to higher CTLA-4 expression on intratumoral Tregs and the presence of FcγR-expressing macrophages. Subsequent studies suggested that CTLA-4 blockade also led to increased T_{effs} in both the tumor and periphery, highlighting a role for both Tregs and T_{eff} in patient response (25).

An anti-CCR4 (mogamulizumab; defucosylated to enhance ADCC) is in clinical trials and targets Tregs through the CCL22:CCR4-mediated recruitment to the tumor, which has shown some clinical efficacy (26). Although encouraging, these strategies still show limited efficacy, thereby further highlighting the need to identify new avenues to target Treg function, potentially through destabilization or by driving Treg fragility specifically within the TME.

Treg stability is defined as sustained Foxp3 expression, hypomethylation at the CNS2 locus, and maintained suppressive function. However, the prevalence and impact of Treg instability remains controversial (27–31). In contrast to Treg instability, Treg fragility is defined as the retention of Foxp3 expression with loss of suppressive function (32). Fragile Tregs produce IFNγ and upregulate the IFNγ receptor, as well the transcription factor Tbet. They have reduced expression of suppressive molecules, such as CD73 and IL10, and are functionally less suppressive in the TME (Fig. 1; ref. 32). In this review, we address the following: (i) How is Treg stability maintained? (ii) How is Treg fragility induced? (iii) Is responsiveness to immunotherapy dependent on Treg fragility?

Building Up: How Is Treg Stability Maintained?

Treg stability has been discussed across many disease types. Treg instability was initially defined as the loss of Foxp3 expression in cells and subsequent loss of suppressive function. This is thought to be, in part, due to lack of demethylation or remethylation at certain sites within the *Foxp3* locus (33). Demethylation in this locus was first described in 2007 when a region in the 5′ UTR of the *Foxp3* locus containing a number of conserved demethylated CpG motifs, was identified (known as the TSDR/CNS2). This demethylation pattern is observed in both thymic and mature peripheral Tregs in mice and peripheral blood of humans. In contrast, the CNS2 is methylated in effector $CD4^+$ T cells. Hypomethylation of the *Foxp3* locus is thought to be required for Treg stability, but occurs independently of Foxp3 upregulation (34). It was shown that TGFβ-induced Tregs (iTregs) display a somewhat hypermethylated *Foxp3* locus (34), and tumor-derived Tregs exhibit a primarily demethylated locus (35). However, variations within CNS2 methylation patterns (∼10%–60%) suggest that the intratumoral Treg pool may be heterogeneous. Previous studies have identified two subsets of Tregs ($Foxp3^{Hi}$ and $Foxp3^{Lo}$) in the tumor and periphery of colorectal patients that display differences in suppressive capacity and CNS2 methylation status (36, 39). Another possibility that could contribute to the heterogeneity of Tregs within the tumor is the conversion of conventional T (T_{conv}) cells to Tregs. However, TCR repertoires between these two populations appear to be distinct (37, 38). Whether $Foxp3^+$ Tregs with a partially methylated *Foxp3* locus are a functionally unstable population or a heterogeneous population comprised of both stable and unstable Tregs remains unclear. The possibility that there are other factors that lead to the remethylation of the locus in the TME warrants further investigation.

In addition to epigenetic alterations of the *Foxp3* locus, loss of Foxp3 expression is a hallmark of unstable Tregs. A number of factors involved in maintaining Foxp3 expression have been identified, including IL2/STAT5 and Foxo1/3a. STAT5 binds to the *Foxp3* locus, and in its absence, Treg development is reduced (Fig. 1; ref. 40), while Foxo1 and Foxo3a translocate to the nucleus of Tregs and prevent effector functions (41). Induction of Foxp3 and subsequent Treg development can also be prevented by persistent TCR stimulation, leading to constitutive activation of the PI3K/Akt/mTOR pathway (42), or in the absence of the microRNA processing enzyme Dicer (43).

Loss of Foxp3 expression has also been reported in certain disease settings, such as lymphopenia and autoimmune diabetes (27). Fate-mapping mice were used to trace all cells that currently or previously expressed Foxp3, regardless of subsequent downregulation. "ExTregs," which no longer express Foxp3, upregulated IL7R, secreted IFNγ and IL17, took on a pathogenic role, and worsened disease (29). Other groups report that Foxp3 expression is stable and that previously identified exTregs were likely T cells that transiently upregulated Foxp3 during differentiation or activation (31, 44). Very few exTregs have been observed in mouse models of cancer (32), suggesting Foxp3 is stable in these models. Other studies have highlighted a role for Helios in maintaining Foxp3 expression and showed that in its absence, Foxp3 was reduced, and Helios-deficient Tregs secreted IFNγ in the TME (45, 46). However, whether unstable Tregs exist in patients is unknown and remains difficult to assess without cell lineage tracing capabilities or definitive markers.

Breaking Down: How Is Treg Fragility Induced?

Multiple factors have been reported previously to be important for preventing Treg fragility, including Nrp1, Foxo1, and Eos (14, 32, 46–49). Although Nrp1 can be expressed on a number of cell types, it is upregulated on Tregs and supports Treg function through binding of semaphorin-4a (Sema4a). In the absence of Nrp1 signaling, either through Nrp1 blockade or genetic deletion (using $Nrp1^{L/L}Foxp3^{Cre\text{-}YFP}$ mice), intratumoral Tregs show reduced cell survival, reduced expression of suppressive

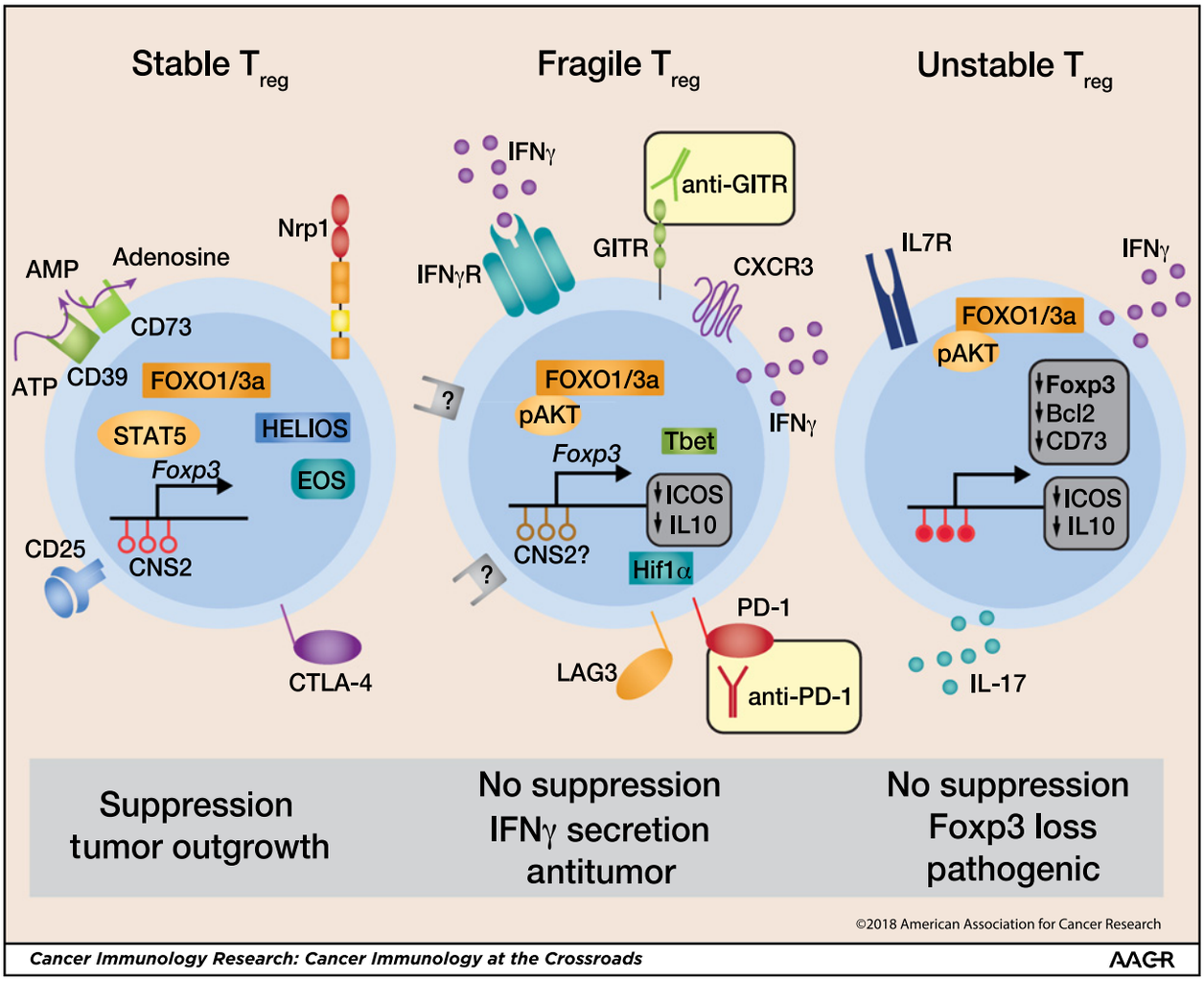

Figure 1.
Contributing factors of stable, fragile, and unstable Tregs. Stable Tregs: hypomethylated at CNS2 and express other stabilizing markers like Nrp1 and Helios. Fragile Tregs: secrete IFNγ while maintaining Foxp3 expression. Unstable Tregs: loss Foxp3 expression and secrete proinflammatory cytokines.

markers (such as CD73, IL10, and IL35), and significantly impaired suppressive function (Fig. 1). Surprisingly, this did not result in loss of Foxp3 expression. Mice cleared tumors similarly to mice lacking Tregs, but they displayed no signs of autoimmunity. Nrp1$^+$ Tregs are also increased in metastatic melanoma and head and neck squamous cell carcinoma patients compared with healthy donors (32). Patients with a larger population of Nrp1$^+$ Tregs correlated with reduced disease-free survival. It was later shown that Nrp1$^-$ Tregs produce IFNγ and were less suppressive than wild-type (WT) Tregs but maintained *Foxp3* expression. Secretion of IFNγ only occurred within the TME, likely as a result of sustained or increased Hif1α expression due to hypoxia and heightened Akt activity (14, 32, 50, 51). It is possible that the unique metabolic environment within the TME contributes to Treg stability, as they are more adept at tolerating this "harsh environment" compared with effector T cells. Distinct metabolic differences between Tregs and other T-cell subsets have been found (52, 53), which may also underlie the preferential restriction of a fragility Treg phenotype to the TME following Nrp1 deletion/blockade or immunotherapy.

Previous reports have shown that although Foxo1 is key for Foxp3 upregulation during Treg development, the loss or mutation of Foxo1 from mature Tregs leads to a fragile phenotype rather than an unstable one. Specifically, Foxo1-deficient Tregs are less suppressive and secrete IFNγ, but Foxp3 expression is maintained and the percentage of Tregs increases *in vivo*. Foxo1 deletion in Tregs leads to an IFNγ-dependent lethal inflammatory phenotype (54). However, unlike the phenotype observed in the absence of Nrp1, wherein fragility appears to be restricted to the TME (14, 32), the loss of Foxo1 results in a fragile phenotype that is systemic, leading to the inflammatory phenotype. Similarly, Eos is upregulated in Tregs, and when removed, Treg suppression is reduced, IFNγ and IL2 are upregulated, but Foxp3 expression remains unchanged (47). It has been reported that when LAG3 is deleted on Tregs, Eos is increased and leads to better suppressive function in an autoimmune diabetes setting (55). It is possible

that LAG3 limits Eos expression in Tregs, thereby promoting fragility, but further studies are required.

Is Response to Immunotherapy Dependent on Treg Fragility?

Intratumoral Tregs display a distinct profile, suggesting specific markers could be targeted within the TME that might lead to Treg instability or Treg fragility. However, whether this is observed in cancer patient intratumoral Tregs and predicts patient responsiveness remains unknown. We hypothesize that Tregs within the TME upregulate stabilizing molecules, such as NRP1, and that patients who respond to immunotherapy exhibit a more fragile intratumoral Treg phenotype.

Treg fragility appears to be required for response to anti–PD-1 in murine tumor models. In an adenocarcinoma mouse model that is sensitive to PD-1 blockade, treatment of WT mice with anti–PD-1 led to the upregulation of IFNγ$^+$ Tregs, consistent with an increased fragile phenotype. When Tregs were insensitive to IFNγ (through the use of an *Ifngr1*$^{L/L}$*Foxp3*$^{Cre\text{-}YFP}$ mouse), mice were completely resistant to PD-1 blockade in comparison with ∼40% response in WT mice (32), suggesting a role for Treg fragility in responsiveness to immunotherapy. Similarly, reduction of tumor burden through the use of a GITR agonist antibody (DTA-1) was due to an increase in IFNγ$^+$ Tregs and reduction in Helios expression (45, 56). As mentioned, CD8:Treg ratios have been shown to be indicative of patient response to therapy. However, the idea that Treg fragility is the key component to determining response to immunotherapy was previously unappreciated.

Similarly, previous studies have identified IFNγ$^+$ Tregs in human samples in autoimmune diseases, such as multiple sclerosis and type 1 diabetes, where these cells have reduced suppression and altered methylation while maintaining Foxp3 expression, suggesting a fragile phenotype (34, 57, 58). Patients with malignant glioma (GBM) exhibit a higher percentage of circulating PD-1^{Hi} Tregs that are less suppressive and express IFNγ. PD-1^{Hi} Tregs bear a distinct transcriptional profile, are phenotypically exhausted as defined by upregulation of LAG3 and Tim3, and show a slight reduction in CNS2 demethylation. When GBM patients were treated with anti–PD-1 (nivolumab), the exhausted PD-1$^+$IFNγ$^+$ Treg population increased (59). This population has been observed in other tumor types, such as late advanced rectal cancer, where it correlated with poorer patient response (60). These data suggest that PD-1 blockade, as well as other immunotherapies, may act, in part, through inducing a fragile Treg phenotype in patients. Whether this is a direct effect of anti–PD-1 on Tregs or an indirect effect of increased IFNγ in the TME acting on Tregs to drive fragility remains to be determined.

Given that the impact of Treg fragility in immunotherapy has not been fully elucidated in the clinic, assessing the extent to which patient Tregs develop a fragile phenotype following immunotherapy could aid in both prediction of patient susceptibility to anti–PD-1, as well as provide a rationale for patient responsiveness to immunotherapy. Sensitizing Tregs to become fragile may be an effective strategy to utilize alongside PD-1 blockade. Although PD-1 blockade has been shown to upregulate IFNγ in CD8$^+$ T cells, whether this directly affects Tregs remains unclear in the clinic. However, IFNγ-sensitive Tregs have been observed in patient samples and were found to be less suppressive following IFNγ treatment *in vitro* (32). Although PD-1 blockade has been the primary focus thus far, it is possible that Treg fragility plays a key role in responsiveness to other immunotherapies in the clinic or perhaps the efficacy of any immunotherapy. One possibility could be to target a known driver of Treg fragility prevention, such as NRP1, through antibody blockade. Although Tregs become fragile in mice upon loss of Nrp1 through either genetic deletion or antibody blockade, whether this is conserved in human Tregs after Nrp1 depletion remains to be further investigated. Although a higher percentage of Nrp1$^+$ Tregs in patients appears to correlate with reduced disease-free survival, it remains unclear whether this is due to enhanced Treg stability. We would argue that inducing Treg fragility may be a preferred therapeutic strategy compared with Treg depletion or destabilizing Tregs because the effect on Tregs seems to be restricted to the TME, thereby preventing autoimmune side effects.

Identifying ways to target Treg fragility while leaving Treg stability intact may be critical, given the previously identified pathogenic nature of unstable Tregs or exTregs in various diseases (Fig. 1; refs. 27–29). It is possible that local Treg destabilization strategies may be efficacious. However, the potential systemic autoimmune effects of this are unknown, and distinguishing between the two Treg subsets can be challenging. Although some clear markers of fragile Tregs have been identified, including Nrp1, PD-1, and IFNγR1, specific markers do not exist for unstable Tregs that distinguish them from Th-like cells, and tracking the presence of exTregs in patient samples is not yet feasible. There may be more unappreciated markers of Treg fragility that warrant further investigation.

Although targeting molecules that prevent Treg fragility in the clinic may represent the clearest step forward, many interrelated questions remain: (i) What are the markers of unstable or exTregs in patients? (ii) Are there other drivers of Treg fragility? Although IFNγ has been shown to drive Treg fragility, it is possible that other cytokines or soluble factors could lead to a similar phenotype. (iii) Do fragile Tregs display hypermethylation at the *Foxp3 CNS2* locus, and does this lead to reduced suppressive function? (iv) What is the level of Treg fragility and instability in checkpoint blockade responders and nonresponders, and do they correlate? (v) Is Treg fragility a biomarker of patient response to immunotherapy? (vi) Does patient response to immunotherapy depend on Treg fragility? Although loss of Nrp1 and increased IFNγ sensitivity have been identified as drivers of Treg fragility, other molecules that contribute to this phenotype have yet to be defined. We propose that the development of combinatorial immunotherapies that maximize Treg fragility may maximize efficacy and improve patient response to immunotherapy.

Disclosure of Potential Conflicts of Interest

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