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T_{REG} 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 comprised of many suppressive cell populations that inhibit antitumor immune responses, including regulatory T cells (T_{regs}). The TME is nutrient poor, acidic, and hypoxic, creating a challenging microenvironment for immune cells to function and survive. T_{regs} suppress a wide variety of cell populations through multiple mechanisms and are tasked with limiting tissue damage. T_{regs} are now considered to be a barrier to effective antitumor immunity. Systemic T_{reg} depletion is not favored because of their critical role in maintaining immune homeostasis and preventing autoimmunity. Reducing T_{reg} 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 T_{reg} instability, characterized by loss of critical T_{reg} transcription factors such as Foxp3, could result in conversion into cells that cause immune pathology, tissue damage, and subsequent autoimmune side effects. Interferon- γ (IFN γ) can cause intratumoral T_{reg} ‘fragility’, which results in loss of suppressive activity and increased IFN γ production without loss of Foxp3 expression and gross T_{reg} ‘identity’. We reviewed the impact T_{regs} have on the TME and vice versa, and their implications for responsiveness to cancer immunotherapy. We propose that the extent to which intratumoral T_{regs} develop a ‘fragile’ phenotype following immunotherapy will predict and dictate responsiveness.

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

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

D.A.A.V. declares competing financial interests. He has submitted patents that are pending or granted that cover NRP1, LAG3 and IL35, and is entitled to a share in net income generated from licensing of these patent rights for commercial development.

AUTHORS' CONTRIBUTIONS

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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 (T_{regs}) (4), which remain dominant despite the mechanistic benefits of inhibitory receptor blockade.

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

In the absence of T_{regs} 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 T_{reg} cell transfer within 48 hours of birth (11). Although T_{regs} are critical for preventing autoimmunity, they also suppress the antitumor immune response and promote tumor outgrowth (4). T_{reg} depletion in tumor models has been studied in *Foxp3*^{DTR-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, T_{regs} rely on oxidative phosphorylation and are thought to have a proliferative and functional advantage within hypoxic, acidic environments (15). Increased T_{regs} 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 T_{reg} percentages and poor prognosis in patients. T_{reg} percentages in the tumor mass can increase with severity of stage (18), and higher T_{reg} percentages correlate with poorer disease-free survival in several cancers (19). As a result, T_{regs} 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 T_{regs} (20,21).

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

An anti-CCR4 (mogamulizumab; defucosylated to enhance ADCC) is in clinical trials and targets T_{regs} 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 T_{reg} function, potentially through destabilization or by driving T_{reg} fragility specifically within the TME.

T_{reg} stability is defined as sustained Foxp3 expression, hypomethylation at the CNS2 locus, and maintained suppressive function. However, the prevalence and impact of T_{reg} instability remains controversial (27–31). In contrast to T_{reg} instability, T_{reg} fragility is defined as the retention of Foxp3 expression with loss of suppressive function (32). Fragile T_{regs} produce IFN γ and upregulate the IFN γ 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) (32). In this review, we address the following: (i) How is T_{reg} stability maintained? (ii) How is T_{reg} fragility induced? (iii) Is responsiveness to immunotherapy dependent on T_{reg} fragility?

BUILDING UP: HOW IS T_{REG} STABILITY MAINTAINED?

T_{reg} stability has been discussed across many disease types. T_{reg} 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 T_{regs} 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 T_{reg} stability, but occurs independently of Foxp3 upregulation (34). It was shown that TGF β -induced T_{regs} (iT $_{regs}$) display a somewhat hypermethylated *Foxp3* locus (34), and tumor-derived T_{regs} exhibit a primarily demethylated locus (35). However, variations within CNS2 methylation patterns (~10–60%) suggest that the intratumoral T_{reg} pool may be heterogeneous. Previous studies have identified two subsets of T_{regs} (Foxp3^{Hi} and Foxp3^{Lo}) in the tumor and periphery of colorectal patients that display differences in suppressive capacity and CNS2 methylation status (36). Another

possibility that could contribute to the heterogeneity of T_{regs} within the tumor is the conversion of conventional T (T_{conv}) cells to T_{regs}. However, TCR repertoires between these two populations appear to be distinct (37,38). Whether Foxp3⁺ T_{regs} with a partially methylated *Foxp3* locus are a functionally unstable population or a heterogeneous population comprised of both stable and unstable T_{regs} 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 T_{regs}. 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, T_{reg} development is reduced (Fig. 1) (40), while Foxo1 and Foxo3a translocate to the nucleus of T_{regs} and prevent effector functions (41). Induction of Foxp3 and subsequent T_{reg} 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. 'ExT_{regs}', that no longer express Foxp3, upregulated IL7R, secreted IFN γ and IL17, took on a pathogenic role, and worsened disease (29). Other groups report that Foxp3 expression is stable and that previously identified exT_{regs} were likely T cells that transiently upregulated Foxp3 during differentiation or activation (31,44). Very few exT_{regs} 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 T_{regs} secreted IFN γ in the TME (45,46). However, whether unstable T_{regs} exist in patients is unknown and remains difficult to assess without cell lineage tracing capabilities or definitive markers.

BREAKING DOWN: HOW IS T_{REG} FRAGILITY INDUCED?

Multiple factors have been reported previously to be important for preventing T_{reg} fragility, including Nr1p, Foxo1, and Eos (14,32,46–49). Although Nr1p can be expressed on a number of cell types, it is upregulated on T_{regs} and supports T_{reg} function through binding of semaphorin-4a (Sema4a). In the absence of Nr1p signaling, either through Nr1p blockade or genetic deletion (using *Nr1p^{fl/L}Foxp3^{Cre}-YFP* mice), intratumoral T_{regs} show reduced cell survival, reduced expression of suppressive 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 T_{regs}, but they displayed no signs of autoimmunity. Nr1p⁺ T_{regs} are also increased in metastatic melanoma and head and neck squamous cell carcinoma patients compared to healthy donors (32). Patients with a larger population of Nr1p⁺ T_{regs} correlated with reduced disease-free survival. It was later shown that Nr1p⁻ T_{regs} produce IFN γ and were less suppressive than wildtype (WT) T_{regs} but maintained *Foxp3* expression. Secretion of IFN γ only occurred within the TME, likely as a result of sustained or increased Hif1 α 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 T_{reg} stability, as they are more adept at tolerating this ‘harsh environment’ compared with effector T cells. Distinct metabolic differences between T_{regs} and other T cell subsets have been found (52,53), which may also underlie the preferential restriction of a fragility T_{reg} phenotype to the TME following Nr1 deletion/blockade or immunotherapy.

Previous reports have shown that although Foxo1 is key for Foxp3 upregulation during T_{reg} development, the loss or mutation of Foxo1 from mature T_{regs} leads to a fragile phenotype rather than an unstable one. Specifically, Foxo1-deficient T_{regs} are less suppressive and secrete IFN γ , but Foxp3 expression is maintained and the percentage of T_{regs} increases *in vivo*. Foxo1 deletion in T_{regs} leads to an IFN γ -dependent lethal inflammatory phenotype (54). However, unlike the phenotype observed in the absence of Nr1, 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 T_{regs}, and when removed, T_{reg} suppression is reduced, IFN γ and IL2 are upregulated, but Foxp3 expression remains unchanged (47). It has been reported that when LAG3 is deleted on T_{regs}, Eos is increased and leads to better suppressive function in an autoimmune diabetes setting (55). It is possible that LAG3 limits Eos expression in T_{regs}, thereby, promoting fragility, but further studies are required.

IS RESPONSE TO IMMUNOTHERAPY DEPENDENT ON T_{REG} FRAGILITY?

Intratumoral T_{regs} display a distinct profile, suggesting specific markers could be targeted within the TME that might lead to T_{reg} instability or T_{reg} fragility. However, whether this is observed in cancer patient intratumoral T_{regs} and predicts patient responsiveness remains unknown. We hypothesize that T_{regs} within the TME upregulate stabilizing molecules, such as NR1, and that patients who respond to immunotherapy exhibit a more fragile intratumoral T_{reg} phenotype.

T_{reg} 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 IFN γ ⁺ T_{regs}, consistent with an increased fragile phenotype. When T_{regs} were insensitive to IFN γ (through the use of an *Ifngr*^{L/L} *Foxp3*^{Cre-YFP} mouse), mice were completely resistant to PD-1 blockade in comparison to ~40% response in WT mice (32), suggesting a role for T_{reg} 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 IFN γ ⁺ T_{regs} and reduction in Helios expression (45,56). As mentioned, CD8:T_{reg} ratios have been shown to be indicative of patient response to therapy. However, the idea that T_{reg} fragility is the key component to determining response to immunotherapy was previously unappreciated.

Similarly, previous studies have identified IFN γ ⁺ T_{regs} 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} T_{regs} that are less suppressive and express IFN γ . PD-1^{Hi}

T_{regs} 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⁺IFN γ ⁺ T_{reg} 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 T_{reg} phenotype in patients. Whether this is a direct effect of anti-PD-1 on T_{regs} or an indirect effect of increased IFN γ in the TME acting on T_{regs} to drive fragility remains to be determined.

Given that the impact of T_{reg} fragility in immunotherapy has not been fully elucidated in clinic, assessing the extent to which patient T_{regs} 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 T_{regs} to become fragile may be an effective strategy to utilize alongside PD-1 blockade. Although PD-1 blockade has been shown to upregulate IFN γ in CD8⁺ T cells, whether this directly affects T_{regs} remains unclear in the clinic. However, IFN γ -sensitive T_{regs} have been observed in patient samples and were found to be less suppressive following IFN γ treatment *in vitro* (32). Although PD-1 blockade has been the primary focus thus far, it is possible that T_{reg} 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 T_{reg} fragility prevention, such as NRP1, through antibody blockade. Although T_{regs} become fragile in mice upon loss of Nrp1 through either genetic deletion or antibody blockade, whether this is conserved in human T_{regs} after Nrp1 depletion remains to be further investigated. Although a higher percentage of Nrp1⁺ T_{regs} in patients appears to correlate with reduced disease-free survival, it remains unclear whether this is due to enhanced T_{reg} stability. We would argue that inducing T_{reg} fragility may be a preferred therapeutic strategy compared to T_{reg} depletion or destabilizing T_{regs} because the effect on T_{regs} seems to be restricted to the TME, thereby, preventing autoimmune side effects.

Identifying ways to target T_{reg} fragility while leaving T_{reg} stability intact may be critical, given the previously identified pathogenic nature of unstable T_{regs} or exT_{regs} in various diseases (Fig. 1) (27–29). It is possible that local T_{reg} destabilization strategies may be efficacious. However, the potential systemic autoimmune effects of this are unknown, and distinguishing between the two T_{reg} subsets can be challenging. Although some clear markers of fragile T_{regs} have been identified, including Nrp1, PD-1, and IFN γ R1, specific markers do not exist for unstable T_{regs} that distinguish them from Th-like cells, and tracking the presence of exT_{regs} in patient samples is not yet feasible. There may be more unappreciated markers of T_{reg} fragility that warrant further investigation.

Although targeting molecules that prevent T_{reg} fragility in the clinic may represent the clearest step forward, many interrelated questions remain: (i) What are the markers of unstable or exT_{regs} in patients? (ii) Are there other drivers of T_{reg} fragility? Although IFN γ has been shown to drive T_{reg} fragility, it is possible that other cytokines or soluble factors could lead to a similar phenotype. (iii) Do fragile T_{regs} display hypermethylation at the *Foxp3* CNS2 locus, and does this lead to reduced suppressive function? (iv) What is the

level of T_{reg} fragility and instability in checkpoint blockade responders and non-responders, and do they correlate? (v) Is T_{reg} fragility a biomarker of patient response to immunotherapy? (vi) Does patient response to immunotherapy depend on T_{reg} fragility? Although loss of Nr1 and increased IFN γ sensitivity have been identified as drivers of T_{reg} fragility, other molecules that contribute to this phenotype have yet to be defined. We propose that the development of combinatorial immunotherapies that maximize T_{reg} fragility may maximize efficacy and improve patient response to immunotherapy.

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Figure 1.

Contributing factors of stable, fragile, and unstable T_{regs}. *Stable T_{regs}*: hypomethylated at CNS2 and express other stabilizing markers like Nrp1 and Helios. *Fragile T_{regs}*: secrete IFN γ while maintaining Foxp3 expression. *Unstable T_{regs}*: loss Foxp3 expression and secrete pro-inflammatory cytokines.