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Treg destabilization and reprogramming: implications for cancer immunotherapy

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Abstract

Regulatory T cells (Tregs) are an important contributor to the immunosuppressive tumor microenvironment. To date, however, they have been difficult to target for therapy. One emerging new aspect of Treg biology is their apparent functional instability in the face of certain acute pro-inflammatory signals such as IL-6 and IFN γ . Under the right conditions, these signals can cause a rapid loss of suppressor activity, and re-programming of the Tregs into a pro-inflammatory phenotype. In this review, we propose the hypothesis that this phenotypic modulation does not reflect infidelity to the Treg lineage, but rather represents a natural, physiologic response of Tregs during beneficial inflammation. In tumors, however, this inflammation-induced Treg destabilization is actively opposed by dominant stabilizing factors such as indoleamine 2,3-dioxygenase and the PTEN phosphatase pathway in Tregs. Under such conditions, tumor-associated Tregs remain highly suppressive, and inhibit cross-presentation of tumor antigens released by dying tumor cells. Interrupting these Treg stabilizing pathways can render tumor-associated Tregs sensitive to rapid destabilization during immunotherapy, or during the wave of cell death following chemotherapy or radiation, thus enhancing anti-tumor immune responses. Understanding the emerging pathways of Treg stabilization and destabilization may reveal new molecular targets for therapy.

Keywords

Regulatory T cells; Treg plasticity; Chemotherapy; Indoleamine 2,3-dioxygenase; IDO; PTEN

Introduction

Regulatory T cells (Tregs) inhibit effector T cells and suppress immune-mediated inflammation. In tumors, Tregs are numerous and highly activated (1–3), and are thought to represent a major mechanism of tumor-induced immune suppression (4,5). Not only do Tregs inhibit anti-tumor effector T cells, but they appear to fundamentally alter the entire

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immune milieu associated with the tumor. One crucial aspect of this is that Tregs inhibit the immunogenic antigen-presenting cells (APCs) in the tumor and its draining lymph nodes, that are required for cross-presentation of tumor antigens. In this review, we will propose that by suppressing effective antigen presentation, Tregs interrupt one of the most critical aspects of cancer immunotherapy: the ability of the host immune system to generate spontaneous responses against endogenous antigens released from dying tumor cells (6). For all of these reasons, Tregs represent a potentially important target for cancer immunotherapy. To date, however, the suppressive effect of these cells has been a difficult barrier to overcome (7).

Tregs in the normal immune system

In part, Tregs are difficult to target because they are also an essential component of normal self-tolerance. Thus, if mice are made globally deficient in Tregs – e.g., by germline deletion of the Treg-lineage transcription factor Foxp3 – they spontaneously develop autoimmune disorders throughout life, and die early of chronic, uncontrolled inflammation. There are several varieties of Tregs, and some of these do not express Foxp3; however, much of the research to date has focused on the Foxp3+ lineage, and this population is the best understood. Even in normal, healthy adult mice, if the existing Foxp3+ Treg population is suddenly ablated, then the animals rapidly develop multiple autoimmune disorders, starting within days of depletion (8). Thus, Tregs exert an on-going, “minute-to-minute” dominant control over effector T cells, which is necessary in order to prevent autoimmunity.

Within tumors, however, this dominant suppression becomes exaggerated and pathological, and appears to protect the tumor from immune surveillance and rejection. Consistent with this, ablation of Tregs in mice with established tumors triggers a rapid, spontaneous immune response against the tumor (9,10) – albeit at the cost of collateral autoimmunity. Similarly, mice with defects in Treg stability or activation pathways will spontaneously attack transplantable syngeneic tumor cell lines, which would otherwise grow robustly in a mouse with intact Tregs (11,12). Thus, Tregs represent a potentially attractive target for cancer immunotherapy, but removing them requires a more selective approach than merely global depletion.

Treg destabilization

This review focuses on a recently-identified set of signaling pathways that naturally “destabilize” Tregs – under specific conditions, and in a strictly localized fashion. Under normal circumstances Tregs would be dominant over effector T cells, but under certain conditions a robust effector response is needed – for example, at the site of a viral infection. Thus, the immune system needs some mechanism to suspend Treg-mediated suppression when necessary. Emerging evidence, which will be the subject of this review, suggests that Tregs may accomplish this by sensing specific local pro-inflammatory signals – in particular IL-6 and IFN γ , and perhaps others. The effect of these inflammatory signals is to destabilize the Tregs, causing them to lose their suppressive phenotype.

Destabilizing local Tregs allows effector T cells to activate, but such a powerful pathway needs to be tightly controlled to prevent autoimmunity. Thus, there also exists a reciprocal

set of pathways that actively prevent this Treg destabilization, and instead intensify local suppression. These stabilizing pathways are elicited, for example, when inflammation is triggered by dying self cells, to which the immune system should remain tolerant. Unfortunately, these Treg-stabilizing signals appear to be highly expressed or rapidly inducible in tumors. Thus, in preclinical models, the success of anti-tumor immunotherapy appears to be critically dependent on the balance between these opposing stabilizing and destabilizing signals for Tregs.

Destabilization is a natural function of the Treg lineage

In the normal immune system, many types of CD4⁺ T cells show some degree of natural plasticity (13). Helper CD4⁺ T cells may change their phenotype, or they may become biased toward a Foxp3⁺ Treg phenotype by factors in the tumor microenvironment. TH17 cells appear particularly susceptible to this latter transformation (14). Thus, plasticity itself is not an abnormal finding in CD4⁺ T cells. In the Treg lineage, many Tregs establish their identity early in the thymus (termed thymic or “natural” Tregs), while a minority differentiate in the periphery (peripheral or inducible Tregs) (15). It is possible that the peripherally-derived Tregs might be more plastic (i.e., more readily destabilized) than thymic Tregs, but this is not known. In tumors, the relative contribution of thymic Tregs versus peripherally-derived Tregs is currently unclear, and both probably contribute (4). Therefore, in discussing Treg destabilization in tumors, we will treat thymic and peripheral Tregs together as a single pool – with the acknowledgement that there may be differences not yet discovered.

In this review, it is important to emphasize that the term “destabilization” signifies only a down-regulation of functional suppressor activity in Tregs – it does not imply a loss of fidelity to the Foxp3⁺ Treg lineage itself. Rather, we propose that Tregs naturally undergo planned, programmed destabilization (suspension of their immunosuppressive phenotype) under specific conditions, in order to permit beneficial effector responses to viral infection or other forms of T cell immunity. Once triggered by the right signals, the change in phenotype can occur rapidly, within 24 hrs (16,17). The Treg cells may continue to express Foxp3 throughout, with the change in phenotype being driven instead by loss of co-transcription factors such as Eos/Ikzf4 (17,18), or alterations in other signaling pathways or molecular complexes with Foxp3 (19). After the phenotypic change, the Tregs lose their functional suppressive activity and may no longer express inhibitory molecules such as CTLA-4. Instead, they may up-regulate pro-inflammatory markers such as IL-17, IL-2 and CD40L, and display functional “helper-like” activity (17,18,20,21). In some models, this new pro-inflammatory activity of the reprogrammed Tregs may be required for the beneficial effect of Treg destabilization on anti-tumor immunity (16,17,21). Although different experimental models show difference in details, a similar inflammation-induced change in Treg phenotype appears to be emerging across multiple models. This phenomenon has been termed “Treg fragility” by Vignali and colleagues (20), and “Treg re-programming” by our group and others (16,17,21,22). By whatever name, we propose that this is not pathologic lineage-infidelity, but rather the natural physiologic response of Tregs to beneficial inflammation.

The long-term fate of such destabilized Tregs is currently unknown. They might remain in a pro-inflammatory phenotype, or they might revert back to suppressive Tregs. It has been somewhat controversial whether true, committed Tregs ever lose their lineage fidelity and stop expressing Foxp3. Under homeostatic conditions this probably does not occur (23), although in pathological settings such as chronic autoimmune disease such “ex-Tregs” may arise (24–26). However, for purposes of anti-tumor immunotherapy, the long-term fate of destabilized Tregs is of less interest than the immediate ability of inflammation to “turn off” Treg suppressor function.

We propose that one major impediment to successful tumor immunotherapy is the excessive resistance of tumor-associated Tregs to undergoing this natural, physiologic de-activation step. As we will discuss in the next section, this excessive resistance to destabilization can be mediated by several known factors in the tumor microenvironment. Thus, to enhance the efficacy of immunotherapy, one goal should be to therapeutically restore the natural pathway of Treg destabilization during inflammation.

Molecular mechanisms of Treg destabilization

Role of IL-6 and IFN γ

In order for Tregs to maintain their usual suppressive phenotype, a number of constitutive internal signals are required. Thus, for example, Tregs will become unstable if the transcription factors Eos or Helios are disrupted; or if signaling via PTEN, Ezh2 or TSC1 (inhibitor of the TORC1 complex) is blocked; or if the neuropilin-1 receptor is ablated (11,12,17,18,26–29). However, all of these represent artificial experimental disruptions designed to test the pathway; they do not reveal which physiologic signals are actually responsible for Treg destabilization during inflammation in vivo.

Early in vitro studies found that when Foxp3⁺ Tregs were activated by T cell-receptor (TCR) cross-linking in the presence of IL-6, they down-regulated Foxp3 and lost their suppressive activity (30). The biology of IL-6 is complex, but, in the immune system, data from IL-6-deficient mice and from patients treated with the anti-IL-6 antibody tocilizumab show that IL-6 is an important driver of acute inflammation (31). In vivo, using a vaccine model of inflammatory Treg destabilization, IL-6 was found to be a key signal for Treg reprogramming during inflammation (17). In a tumor model, mice with a targeted deletion of the IL-6-receptor α -chain (IL6Ra) on Tregs lost the ability to destabilize and reprogram Tregs during anti-tumor immunotherapy (12). In these mice, this inability to reprogram the Tregs resulted in essentially complete loss of effector T cell activation in the tumor, and abrogation of anti-tumor efficacy by otherwise potent immunotherapy (12).

In a second model, tumors were implanted in host mice bearing a genetic deletion of the stabilizing receptor neuropilin-1 (Nrp1) on Tregs (20). This caused tumor-infiltrating Tregs to become spontaneously unstable (“fragile”) and produce pro-inflammatory IFN γ . Expression of the receptor for IFN γ (IFN γ R1) on the Tregs was found to be required for their destabilization, thus suggesting an autocrine or paracrine loop driven by IFN γ . Importantly, if the Tregs could not respond to this destabilizing IFN γ (i.e., were IFN γ

receptor-deficient) then the anti-tumor effect of conventional PD-1 checkpoint-blockade immunotherapy was lost (20).

Thus, two key pro-inflammatory cytokines – IL-6 and IFN γ – appear to deliver important signals that destabilize tumor-associated Tregs (Figure 1A); and, in the various models tested, this destabilizing effect on Tregs was required for successful immunotherapy. It may be that IL-6 and IFN γ act independently and redundantly, depending on the model system. However, it seems more likely that they function together as part of a connected pro-inflammatory network. Further work is needed to elucidate this important relationship.

Role of TCR engagement

In the preceding models, it is likely that Treg destabilization also required activating signals via the TCR, in addition to the signals from inflammatory cytokines. It is known that TCR engagement is required for Tregs to trigger their functional suppressive activity (32). However, it is less well understood how signals from the TCR complex might participate in the opposite process of Treg destabilization. Signaling via the TCR in Tregs appears to differ in several important respects from TCR signaling in effector T cells (15). The TCR α and β chains, which confer antigen specificity, are not themselves intrinsically different between Tregs and effector T cells. However, the signals from the TCR are generated by a complex array of proteins that are recruited around the $\alpha\beta$ heterodimer, and these signals show significant differences in Tregs (15,33). One major difference is that TCR signaling in Tregs does not result in high levels of phosphorylation and activation of the downstream Akt kinase, as would normally occur in effector T cells. Indeed, if Akt becomes highly activated in Tregs, then their suppressor function is lost (34). Another difference is that protein kinase C theta (PKC- θ), which is normally a key component of the TCR complex in effector T cells, is actively excluded from the TCR complex in Tregs (35). If PKC- θ is not excluded, then the Tregs lose their suppressive function and become destabilized during inflammation. Thus, the TCR appears to contribute to destabilization if it is allowed to generate “effector-like” signals (Akt phosphorylation and PKC-theta activity) in the Tregs. However, exactly how the TCR generates these different patterns of signals in Tregs is still a subject of active investigation (15).

The PI3K→Akt→mTOR axis

The IL-6-receptor, IFN γ -receptor and TCR complex are all upstream signals. It is also important to understand the downstream signals within Tregs that actually abrogate the suppressive phenotype. One important such destabilizing signal appears to be activation of the pathway comprising PI3K, Akt and mTOR kinases (Figure 1A). In the case of non-Treg (effector) T cells, this PI3K→Akt→mTOR axis is important for coordinating proliferation, metabolic activation and gain of effector function following activation (36). However, in Tregs this same PI3K→Akt→mTOR pathway can have a profound destabilizing effect on suppressor activity. Thus, the process of Treg activation appears to be a balancing act: signals from the TCR are needed in order to trigger suppression (32), but the usual downstream activation of the PI3K→Akt→mTOR pathway must be kept at a low level or the Tregs will become unstable.

Genetic models show that excessive activity of Akt kinase prevents Treg differentiation in the thymus (36). One way in which Akt may disrupt Treg function is via inactivation of the transcription factors FoxO1 and FoxO3a (37). These do not physically associate with Foxp3, but they are important for maintaining normal Treg differentiation and function (38). FoxO1 and FoxO3a participate in up-regulation of key genes for Treg biology, including transcription of Foxp3 itself, as well as CTLA-4 and other genes (37). FoxO1 and FoxO3a are both phosphorylated and inactivated by Akt; thus, excessive activity of the PI3K/Akt/mTOR loop results in loss of the FoxO1/FoxO3a contribution to maintaining the Treg phenotype. Eventually, excessive Akt signaling may result in loss of Foxp3 itself (26). However, it is important to emphasize that down-regulation of FoxO1/FoxO3a, and loss of attendant suppressor activity, can occur even when Foxp3 itself remains unchanged (17).

Similarly, uncontrolled activation of the mTOR pathway also disrupts the stability and suppressor function of Tregs (29,39). In large part this may be mediated by the feedback loop between mTOR and Akt activation (Figure 1A). The role of mTOR in Tregs is complex, because some basal level of mTOR signaling appears necessary for homeostatic maintenance of the Treg pool (40); whereas excessive mTOR signaling during the functional (suppressor) phase rapidly destabilizes Tregs (17,29,41). Another possible effect of excessive mTORC1 activity may be to increase Treg glycolytic metabolism, which has been reported to abrogate suppressor activity (39,42).

Down-regulation of Eos

In addition to loss of FoxO1 and FoxO3, destabilized Tregs may decrease their suppressive activity via active down-regulation of the co-transcription factor Eos (Ikzf4). Eos forms a molecular complex with Foxp3. This complex is required for normal Treg function, as shown by the fact that induced ablation of Eos causes loss of suppressor activity (18). In the context of inflammation, destabilization and reprogramming of Tregs has been linked to IL-6-mediated down-regulation of Eos (17). Mechanistically, ligation of the IL-6 receptor has been shown to induce the micro-RNA species miR-17 (43), which inhibits Eos mRNA transcripts and results in loss of suppressor function and up-regulation of pro-inflammatory IFN γ . Importantly, down-regulation of Eos (and hence loss of those functions of Foxp3 that require Eos as a binding partner) can occur while Foxp3 itself is still expressed (17). This is one example of an important layer of regulation in Tregs, namely the incorporation of Foxp3 into different molecular complexes in order to turn on and off certain functions (44). The fact that this IL-6-induced down-regulation of Eos can occur rapidly, while Foxp3 remains expressed, emphasizes the fact that Treg destabilization is not a loss of fidelity to the Foxp3 lineage, but rather a planned, physiologic response of Foxp3-expressing cells to specific external signals.

Currently, the interconnections between the upstream receptors (IL-6R, IFN γ R, TCR), the downstream signaling cascades (PI3K, Akt, mTOR), and modulation of co-transcription factors such as Eos, remain a subject of on-going research. However, all of these appear to play a role in the destabilization and re-programming process during inflammation.

Opposing pathways: active stabilization of Tregs

In the preceding discussion we have proposed that destabilization of Tregs is important to permit beneficial immune responses. However, if Tregs always became unstable every time they encountered inflammation then they could not perform their function, and autoimmunity would result. This suggests that there must also exist a set of counter-acting “stabilization” signals (Figure 1B), which instruct the Tregs when to maintain their dominant suppressive phenotype, even in the face of inflammation. One such recently discovered set of signals is linked to the PTEN phosphatase pathway in Tregs. PTEN is a lipid phosphatase that inhibits the activity of PI3K. PI3K would normally phosphorylate Akt on threonine-308, which is a key permissive event for initiation of the PI3K→Akt→mTOR cascade (which, as discussed above, destabilizes Tregs). Thus, PTEN phosphatase activity functions to maintain Treg stability and prevent destabilization. Mice with a targeted deletion of PTEN in Tregs cannot maintain Treg stability, and progressively develop autoimmune disorders as they age (26,45). When tumors are implanted in mice with PTEN-deficient Tregs, the tumors are unable to create their normal suppressive microenvironment (12). Instead, the Tregs spontaneously reprogram into pro-inflammatory helper-like cells within the tumor, and the tumors are chronically attacked by the immune system and can barely grow. Thus, Treg PTEN appears to be an important control-point in Treg stability, and a key suppressive pathway within the tumor microenvironment.

Several upstream stabilizing signals for Tregs appear to converge on PTEN, or otherwise inhibit the PI3K→Akt→mTOR axis. The neuropilin-1 receptor, which has been shown to maintain Treg stability in tumors (11,20), activates PTEN. This blocks PI3K-induced activation of Akt, thereby maintaining FoxO3a expression and preventing Treg destabilization (11). The ligand for neuropilin-1 in this context is probably semaphorin-4a, expressed on tumor-associated dendritic cells (46,47).

Similarly, the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) is known to promote the suppressive function of tumor-associated Tregs (48), and to stabilize Tregs against inflammation-induced reprogramming (16,17,22). Like PTEN, IDO functionally inhibits the PI3K→Akt→mTOR signaling loop in Tregs (12); but IDO appears to target the mTOR component of the loop, preventing mTORC2 from phosphorylating of Akt on serine-473, and thus blocking Akt activation (12). One mechanism by which IDO exerts its effects is by degrading the essential amino acid tryptophan. Lack of tryptophan can activate the amino-acid sensitive kinase GCN2 (49,50), which can then cross-inhibit mTOR (51). mTOR itself is also sensitive to amino-acid insufficiency. Thus, the net effect of IDO is to block the PI3K→Akt→mTOR axis in Tregs, and hence maintain expression of FoxO3a and Treg stability (12).

Importantly, when Akt is inhibited and the FoxO1/FoxO3a axis is active, this appears to drive up-regulation of PTEN itself, and also expression of PD-1 by the Tregs (12). Ligation of PD-1 in turn is known to activate PTEN phosphatase activity (52). Therefore, when Tregs are activated in the presence of IDO, up-regulation of FoxO3a, PTEN and PD-1 establishes a self-sustaining feedback loop (FoxO3a→PD-1→PTEN) that helps maintain Treg stability (12). This self-sustaining loop may be an important contributor to the effects of IDO on

Tregs, because IDO itself is often restricted to just a small number of APCs. Thus, the period when Tregs are directly exposed to IDO would be limited to just the time of antigen-presentation. However, if this initial transient exposure to IDO sets up a self-sustaining PTEN→FoxO3a→PD-1 feedback loop, then the effects of IDO on Tregs could persist long after the Treg leaves the IDO-expressing APC (12).

The preceding discussion focused on a metabolic effect of IDO (tryptophan deficiency) as a way to block mTOR signaling and stabilize Tregs. More speculatively, a number of other metabolic abnormalities often exist in the tumor microenvironment, such as low glucose, hypoxia or acidosis. These can also inhibit mTOR signaling, and thus might have a similar stabilizing effect on Tregs. Unlike effector T cells, Tregs are tolerant of low glucose, and glucose deprivation appears to actively promote Treg suppressive function (53). Hypoxia and acidosis can also inhibit mTOR; however, these conditions create complex signals, and exactly how they affect Treg function during inflammation is still a subject of on-going investigation (54,55).

Inflammatory versus tolerogenic DCs in tumors

Taken together, the pathways in Figure 1 emphasizes the importance of the interaction between Tregs and their cognate APCs (dendritic cells) during inflammation. If the DC is immunogenic and pro-inflammatory, then it becomes a source of Treg-destabilizing IL-6. Inflammatory DCs also activate effector T cells that express IFN γ , which is likewise destabilizing for Tregs. Conversely, if the tumor-associated DCs are tolerogenic, they may express IDO, PD-L1 or semaphorin-4a (the ligand for Nrpl), in which case they may confer resistance to destabilization, and promote suppression. It is not currently known whether such “inflammatory” and “tolerogenic” DCs represent two different cell types, or simply reflect different activation or maturation states of the same cell type. In either case, the tumor-associated DC pool appears pivotal both for destabilizing Tregs, and also for maintaining their suppressive activity.

This dependence on DCs is important to remember when considering the location where Treg destabilization may occur. While DCs can enter the tumor and capture antigen, the actual antigen-presenting step may occur after they exit the tumor and migrate to the tumor-draining lymph (or tumor-associated lymphoid structures) (56). Thus, the factors influencing Treg stabilization or destabilization may be more than just the physical microenvironment of the tumor itself, and also include the signals expressed by the migratory DCs – signals such as IDO, PD-1 and semaphorin-4a.

PTEN+ Tregs and IDO help define the host response to dying cells

Signals that act to stabilize Tregs would be appropriate in settings where T cell tolerance must be enforced despite potential signals of inflammation. A classic example of this could be during cell death. While an occasional apoptotic cell might be removed so quickly that it remains “invisible” to the immune system, larger waves of cell death – such as tissue injury or wound healing – often create local inflammation, and clearly release self antigens. The tumor is faced with an analogous wave of antigen-release following chemotherapy or

radiation. In these settings, both the PTEN and the IDO systems appear to play important, closely-linked roles in maintaining dominant Treg suppressor function, and thereby enforcing tolerance to antigens from dying cells.

Mice with a targeted deletion of PTEN in Tregs progressively develop spontaneous lupus-like autoimmunity as they age (12,26,45). Lupus autoimmunity classically results from a breakdown in tolerance to apoptotic cells. The onset of spontaneous autoimmunity was gradual; but if even young and healthy PTEN-deficient mice were challenged with a large bolus of apoptotic cells, then they rapidly lost self-tolerance and developed lethal autoimmunity (12).

Consistent with the link between IDO and PTEN+ Tregs, interruption of the IDO pathway also results in a loss of tolerance to apoptotic cells. When normal mice are challenged with a bolus of apoptotic cells, this induces high levels of IDO expression (12,57). In the spleen, this IDO is highest in marginal-zone macrophages expressing CD169 and the scavenger receptor MARCO (57). These IDO+ macrophages rapidly recruit Tregs, and it is the Tregs which actually create the IDO-dependent tolerance induction (50,58). In the case of apoptotic tumor cells, the identity of the IDO-expressing cells observed in tumor-draining lymph nodes is not yet defined, but experimental models show that this IDO activity is required for induction of PTEN-expressing Tregs (12). Functionally, mice with a deletion of the IDO1 gene, or mice treated with the IDO-pathway inhibitor drug indoximod (1-methyl-D-tryptophan), cannot maintain normal self-tolerance when challenged with a large wave of apoptotic cells, and rapidly develop lupus-like autoimmunity (50,57). Likewise, mice treated with IDO-inhibitor lose their unresponsiveness to apoptotic tumor cells, and become able to respond to tumor-associated neo-antigens (12).

Thus, taken together, the IDO pathway and PTEN+ Tregs appear to be integral participants in one of the basic decisions faced by the immune system: whether to remain tolerant to antigens from dying cells, or to treat them as immunogenic. For apoptotic cells, the default is normally tolerance; but – as shown by interruption of PTEN or IDO – this “default” tolerance must be actively created. If the IDO or PTEN pathways are blocked, then the response is immediately changed to immune activation instead. Even granted the limitations of experimental mouse systems, this is a striking finding: that a decision as fundamental as self-tolerance versus immunity to dying cells could be subject to control by simple, small-molecule drugs. As will be discussed below, this may have significant implications for generating a therapeutic immune response to dying tumor cells.

Effects on the tumor milieu when Tregs are destabilized

Genetically-defined mouse models suggest that destabilizing Tregs in tumors has two effects that may be important for therapy. The first is that tumors cannot create their normal suppressive milieu: instead, the tumors are spontaneously recognized by the immune system and attacked. This is seen, for example, when the stabilizing receptor neuropilin-1 is deleted in Tregs (11,20); or when Tregs lack the PTEN pathway (12,59); or if the EZH2 histone methyltransferase pathway is disrupted (21). One caveat is that these data were obtained with transplantable tumor cell lines, which – although aggressive and uniformly lethal – tend

to be somewhat more immunogenic than autochthonous tumors. Nevertheless, the findings are important, because they show that tumors may be much more inherently immunogenic than they appear, if the dominant Treg-mediated suppression can be removed.

A second effect of Treg destabilization is that the antigen-presenting milieu in the tumor may be profoundly transformed. Suppressive Tregs have been shown to interact with tumor-associated DCs and inhibit their function (10,60,61). When Tregs are destabilized (e.g., by disruption of PTEN) the endogenous tumor associated DCs can become highly activated following chemotherapy or immunotherapy (12). In one such model, the tumor milieu became dominated by immunogenic, Batf3-expressing CD103⁺ DCs that cross-presented tumor antigens and re-activated anergic T cells (59). Since cross-presentation of endogenous tumor antigens is totally dependent on the DC population in the tumor and its draining lymph nodes, removing Treg-mediated suppression of these DCs might enhance response to tumor antigens.

It should be noted that cell-death in tumors is abnormal, and is probably not directly analogous to classical apoptosis (62). Tumor cells die in various stressed and aberrant ways, and tumors may release antigens via multiple pathways. The relevant point, however, is that – by whatever pathway – tumor antigens are usually released into a profoundly immunosuppressive milieu, and do not generate an immune response. However, by destabilizing the local Tregs, this milieu may be rendered much more pro-inflammatory and immunogenic. Thus – just as in the case of normal apoptotic cells, when IDO or PTEN are inhibited (12,57) – tumor antigens may become immunogenic when the tolerogenic mechanisms are blocked.

Implications for translational therapy

In this final section we discuss possible clinical/therapeutic strategies for destabilizing Tregs in tumors. In this regard, it is important to emphasize that destabilization does not necessarily require a special pharmacologic intervention: acute inflammation by itself (assuming this can be achieved by immunotherapy) may physiologically destabilize local Tregs. Thus, for example, in preclinical mouse models, conventional checkpoint-blockade or T cell adoptive transfer can cause spontaneous Treg destabilization, as long as it is intense enough to cause the tumor to actively shrink (59). Therefore, in the clinic, if a patient's tumor already has a robust IFN γ -driven inflammatory signature at diagnosis (63), and is treated with checkpoint blockade and responds, then the hypothesis would predict that intratumoral Tregs will have likely been physiologically destabilized. The problem, however, is that most tumors do not achieve this ideal pattern of success. Thus, the question becomes whether Tregs can be forced to destabilize by some therapeutic intervention, and thus remove a major barrier to immunotherapy.

One possible approach would be to block one of the known Treg-stabilizing signals in the tumor, such as IDO, PD-1 or PTEN. Several IDO-inhibitor drugs are currently in clinical trials. No PTEN-inhibitor drugs are yet in clinical trials; but PTEN is a potentially “drug-able” enzymatic target, with a number of preclinical inhibitors (64). PD-1 blocking

antibodies are in the clinic with some notable successes. However, whether PD-1 blockade contributes to destabilization of intratumoral Tregs has not yet been tested.

In all of these approaches, however, two complicating factors arise. The first is redundancy: blocking only one of these pathways may not give optimal effect if the others are still active. In this regard, PTEN may present an attractive target, because it is downstream of multiple signals (PD-1, neuropilin-1, and, indirectly, downstream of IDO via the FoxO3a→PD-1 feedback loop). The second complication is that simply blocking one or more stabilizing signals does not provide the active, inflammatory destabilizing signal that is needed to reprogram the Tregs. Thus, blocking these stabilizing signals will likely need to be combined with active immunotherapy such as T cell transfer, or with a stimulus such as chemotherapy or radiation to elicit tumor killing. In preclinical models, for example, in order to trigger rapid regression of large established tumors, PTEN-inhibitor needed to be combined with at least a modest dose of chemotherapy (12).

This point is also important when considering IDO as a therapeutic target. Blocking the IDO pathway does not, by itself, provide the active inflammation needed to destabilize Tregs and create a “hot” tumor milieu. For this, an inflammatory stimulus is also needed – e.g., combining an IDO-inhibitor with tumor-cell death from chemotherapy or radiation (65–67), or with effector cells such as CAR-T cells (68). This concept may be relevant to the recent failure of attempts to combine the IDO-inhibitor epacadostat (Incyte Corp.) with PD-1 checkpoint blockade (69). PD-1 blockade is effective in the subset of immunogenic tumors that already have a pre-existing, spontaneous T cell response, driven by intrinsic neoantigens in the tumor. In this select group of already “hot” tumors, adding an IDO-inhibitor drug may be redundant (i.e., PD-1 blockade alone is sufficient). In the remaining patients with “cold” tumors where PD-1 blockade is ineffective, blocking IDO by itself is also not sufficient, because it needs to be combined with something that can kill tumor cells and release antigens. To this end, clinical trials are in progress using blockade of the IDO pathway combined with chemotherapy and radiation (70,71). Mechanistically, this may provide a better opportunity for synergy than attempts at combination with checkpoint blockade.

Blocking the EZH2 histone methyltransferase is another potentially attractive strategy for destabilizing Tregs (21), and EZH2-inhibitor drugs are in clinical trials for other indications. Finally, CTLA-4 is another checkpoint molecule that is expressed by Tregs, and anti-CTLA-4 antibodies are in clinical use. The effect of CTLA-4 blockade on Tregs is complex, and different antibodies may differ in their mechanisms of action (e.g., depletion of Tregs, versus functional inhibition without depletion) (72,73). Thus, while CTLA-4 is an attractive target for modulating Tregs, it is not yet known whether any of the effects of CTLA-4 blockade involve Treg destabilization.

Conclusion: taking the brakes off the cancer-immunity cycle

This review has been based largely on preclinical research in mouse models. There are intriguing hints that similar Treg reprogramming can also occur in humans (74,75), but whether this will apply to human tumors during immunotherapy remains to be established. One of the challenges in this regard is that Treg destabilization, by definition, occurs only in

the context of active inflammation. Thus, the best setting in which to look for it will be on-treatment biopsies of regressing tumors – which are difficult tissues to obtain. However, the fact that (at least in mice) acute destabilization of Tregs can occur while the cells continue to express Foxp3, may facilitate the identification of these cells in biopsies.

These uncertainties notwithstanding, the concept of Treg destabilization and reprogramming is an important one. If destabilizing the intratumoral Treg population can indeed alter the immune response to dying tumor cells, and thus allow for immunogenic cross-presentation of endogenous tumor antigens, then this could have profound implications for therapy. Most obviously, it could affect the immune response to the antigens released by chemotherapy and radiation – modalities which are just beginning to be demonstrated as powerful partners for immunotherapy (76). More broadly, enhanced antigen cross-presentation could affect the response to multiple forms of immunotherapy. Chen and Mellman (6) elegantly summarized the importance of establishing a self-amplifying loop (the “cancer-immunity” cycle) in response to endogenous tumor antigens released during immunotherapy. Whatever the initial immunotherapy treatment, in order to achieve the maximum response and the best hope of long-term cure, it seems crucial that these endogenous tumor antigens be cross-presented in an immunizing fashion. This allows the host immune system to continually respond and adapt to the tumor’s own antigenic landscape, optimizing responses and suppressing emergence of escape variants. Currently, very few forms of immunotherapy are targeted specifically at changing the antigen-presenting milieu of the tumor, from tolerizing to immunizing. If this key step is being dominantly suppressed by tumor associated Tregs, and if these Tregs can be therapeutically induced to destabilize, then this could be an important new target for therapy.

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Figure 1. Stabilizing and destabilizing signals for Tregs.

(A) Treg destabilizing signals. During immune-mediated inflammation, activated DCs secrete IL-6 and ligate the TCR on Tregs. In the same milieu, activated effector T cells produce IFN γ . Through various intermediate pathways, these upstream signals result in activation of PI3K; phosphorylation of Akt on the activating S473 and T308 sites; and activation of mTOR (mTORC1 and mTORC2 complexes). mTOR activation also feeds back to activate Akt, so the pathway functions as a loop. Activated Akt phosphorylates and inactivates the transcription factors FoxO3a and FoxO1, contributing to down-regulation of a suite of Treg-associated genes, and inhibition of the Treg suppressor phenotype. The IL-6-receptor, via induction of miR-17, also inhibits the transcription factor Eos, which is a binding partner for Foxp3, further destabilizing the suppressive phenotype. **(B) Dominant stabilizing signals.** In the tumor microenvironment, tolerogenic DCs and other APCs may express IDO (e.g., in response to local IFN γ , or induced by apoptotic cells). IDO degrades

local tryptophan, activating the amino-acid sensitive kinase GCN2, which can inhibit mTOR. Low tryptophan and other metabolic deficiency states (e.g., low glucose) in the tumor microenvironment may also depress mTOR signaling. Surface receptors such as neuropilin-1 and PD-1 activate the PTEN phosphatase, which inhibits PI3K activity. In the absence of PI3K and mTORC2 activity, Akt is not phosphorylated and activated. Without Akt activity, FoxO1 and FoxO3a are allowed to remain active, and the Tregs are not destabilized. The FoxO1/FoxO3a axis also appears to feed back and up-regulate PD-1 and PTEN expression, thereby creating a self-reinforcing loop that stabilizes the suppressive Treg phenotype.