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## Basic principles of tumor-associated regulatory T cell biology

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### Abstract

Due to the critical role of Foxp3<sup>+</sup> regulatory T cells (Tregs) in the regulation of immunity and the enrichment of Tregs within many human tumors, a number of emerging therapeutic strategies for the treatment of cancer involve the depletion or modulation of Tregs, with the aim of eliciting enhanced anti-tumor immune responses. Here, we review recent advances in understanding of the fundamental biology of Tregs, and discuss the implications of these findings for current models of tumor-associated Treg biology. In particular, we discuss the context-dependent functional diversity of Tregs, the developmental origins of these cells, and the nature of the antigens that they recognize within the tumor environment. In addition, we highlight critical areas of focus for future research.

### Regulatory T cells and the immunotherapy of cancer

Recent trials demonstrating the efficacy of cancer immunotherapies, including antibody blockade of inhibitory molecules [1], adoptive T cell transfer [2], and autologous cell-based vaccines [3], have provided strong evidence that the immune system can be manipulated for clinical benefit in human cancer patients. In this regard, a number of emerging therapies for the treatment of cancer involve the ablation or modulation of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) concomitant with the administration of cancer vaccines or adoptive cell therapy, in an effort to induce robust anti-tumor immune responses capable of mediating cancer regression [4,5]. Tregs, characterized by expression of the transcription factor Foxp3, are critical for the prevention of autoimmunity, the maintenance of immune homeostasis, and the regulation of immune responses to foreign and self antigens in mice and man [6,7]. Mice with a loss-of-function mutation in *Foxp3* lack functional Tregs, and die of organ-specific autoimmunity and lymphoproliferative disease at an early age [8–11]. Likewise, human patients harboring hypomorphic mutations in *FOXP3* succumb to immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [12,13]. Tregs are often found at elevated frequencies in the peripheral blood and tumors of human patients, and for many cancers, a high density of Tregs correlates with poor disease outcome [14]. Therefore, despite their essential role in maintaining the integrity of the host, Tregs may also play an insidious role in the promotion of cancer development and progression in some types of malignancies. An improved understanding of the fundamentals and complexities of regulatory T cell biology may enable the selective modulation of Tregs for the treatment of cancer.

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In this review, we highlight recent progress in the field of regulatory T cell biology, and discuss the potential implications of these findings on our understanding of tumor-associated Tregs. Strategies for the therapeutic targeting of Tregs have been reviewed extensively elsewhere [15–17], and will not be discussed in depth here. In addition, while many T cell subsets exhibiting regulatory activity have been described [18], we will focus exclusively on CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs, due to their prominent role in immune regulation.

## Tregs in human cancers: correlative or causal?

Many studies indicate that a high density of tumor-infiltrating Tregs correlates with disease outcome in a variety of human cancers (reviewed recently by [14]). For example, multiple reports have demonstrated that in human hepatocellular cancer, high Treg density is correlated with poor prognosis, while in human colorectal carcinoma, high Treg density is associated with improved outcome. Given these associations, it is important to determine whether Tregs play a direct, causal role in either promoting or suppressing disease progression in different types of cancer, as well as the biology underlying these distinct outcomes. For example, Tregs are commonly hypothesized to play a causal role in promoting cancer development via the suppression of anti-tumor immune responses. However, depending on the context, it is also possible that Tregs suppress tumor development by dampening inflammation, which is thought to promote tumor progression in many cancers [19]. Here, we consider three possible scenarios to explain an association between Treg density and disease outcome. First, Tregs may play a causal role in modulating tumorigenesis. Second, Treg density may correlate with that of another cell type or factor that plays a causal role in tumor development. Third, it is possible that high Treg density is associated with cancers of a particular stage and grade, but Tregs play no major functional role in modulating cancer development. In this case, Tregs would have prognostic significance but little functional significance. Addressing the key issue of correlation vs. causality will require loss-of-function *in vivo* evidence demonstrating that the selective depletion or modulation of Tregs causes significant changes in tumorigenesis or tumor progression in genetically engineered mice that are predisposed to the development of indigenous cancer. Such evidence has been elusive due to the fact that sustained Treg depletion in mice induces severe autoimmunity and other secondary adverse effects [20,21]. Thus, it is difficult to induce complete, sustained Treg ablation over the prolonged time periods required for tumor development in many mouse models of primary cancer, thereby limiting the use of this experimental approach. The most striking available data come from multiple studies demonstrating that Treg depletion can induce cancer regression in mouse sarcomas induced by treatment with the chemical carcinogen methylcholanthrene [22–24]. However, data demonstrating a role for Tregs in the modulation of cancer development in genetically-driven mouse models of primary carcinomas are necessary to demonstrate the generality of this concept in different types of cancer (Box 2). Furthermore, in order to elucidate the impact of Tregs on tumorigenesis, it is important to consider recent advances in our understanding of the complexities and specialized roles of Tregs within various inflammatory contexts.

## Context-dependent Treg function

Tregs regulate a multitude of inflammatory processes [7], but the mechanisms by which these cells exhibit such diverse functions are not well understood. In recent years, a number of studies have helped to illuminate basic principles underlying the diversity of Treg function (also reviewed by [25]). In one such study, the authors demonstrated that Treg expression of T-bet, a transcription factor that specifies the differentiation of T helper type 1 (Th1) T cells, was required for the regulation of Th1 inflammation [26]. In other studies, it was shown that Treg expression of IRF4 and STAT3, transcription factors that are essential

for the differentiation of Th2 and Th17 T cells, respectively, were critical for the suppression of the respective classes of inflammatory responses [27,28]. More recently, these concepts have been extended by the findings of a study of Tregs associated with the visceral adipose tissue (VAT) [29]. The authors demonstrated that PPAR- $\gamma$ , a nuclear receptor essential for adipocyte differentiation, was highly expressed by VAT-associated Tregs and was required for optimal Treg maintenance and/or infiltration within adipose tissue.

Taken together, these and other studies demonstrate that in order to regulate a particular inflammatory state or anatomical site, Tregs make context-dependent adaptations, adopting transcriptional programs that are central to key cell types operating within the inflammatory milieu. Thus, Tregs can exhibit chameleon-like properties, changing “color” to persist and function within the “foliage” of a given environment.

This concept of Treg “adaptation” could help to explain the potentially paradoxical role of Tregs in human colorectal carcinoma (CRC). Contrary to many other solid tumors, a high density of Tregs in CRCs is generally associated with either a neutral or favorable prognosis [14,30]. Additionally, it was recently reported that high frequencies of IL-17-producing cells were predictive of poor prognosis in CRC [31]. Moreover, complicating the matter, multiple groups have reported that a substantial fraction of Foxp3<sup>+</sup> Tregs isolated from CRC samples produce IL-17 [32–34]. How can these findings be explained? A recent review suggested a model in which Tregs dampen tumor development by suppressing pro-inflammatory Th17 responses, which are thought to promote CRC [30]. However, if Tregs are dampening inflammation, why are they producing “pro-inflammatory” cytokines such as IL-17? In this light, it is possible that Tregs are adapting to contextual cues within a Th17-skewed inflammatory environment, and adopting a Th17-like phenotype in order to suppress inflammation.

The principles of Treg diversity and adaptation have significant implications for our understanding of tumor-associated Tregs. In future work, it will be important to determine the gene expression profiles of tumor-infiltrating Tregs relative to Tregs isolated from lymphoid organs or blood (Box 2), in an effort to identify specific transcriptional programs that may provide insight into the specialized *in situ* function(s) of Tregs infiltrating tumor lesions. In addition, the diversity of Treg function in varying inflammatory contexts suggests the possibility of therapeutic modulation of properties that are unique to tumor-associated Tregs. With this strategy in mind, it was demonstrated that administration of Pio, a synthetic agonist of PPAR- $\gamma$ , induced the specific accumulation of Tregs in the adipose tissue of mice on a high-fat diet, leaving splenic Treg numbers unchanged [29]. Thus, if transcriptional programs that are specifically expressed by tumor-associated Tregs can be identified, it may be possible to selectively modulate these cells, leaving Tregs located elsewhere in the body unaffected. Finally, the versatility of Treg function raises the possibility that Tregs may perform multiple functions within the tumor environment, independently of a role in the suppression of inflammation or adaptive immune responses.

## Newly defined roles for tumor-associated Tregs?

Given the principles of context-dependent diversity of Treg function discussed above, it is important to consider the following questions. What are the environmental cues sensed by tumor-associated Tregs, and what functional responses are elicited under these circumstances? In this regard, emerging evidence suggests that Tregs may exhibit specialized functions that impact both angiogenesis and metastasis within the tumor environment.

A recent study presented evidence suggesting a direct role for Tregs in the induction of angiogenesis under hypoxic conditions [35]. In experiments utilizing a transplantable ovarian cancer cell line, it was demonstrated that overexpression of the chemokine CCL28 resulted in increased angiogenesis and VEGFA production, which was diminished following Treg depletion. In other experiments, human Tregs cultured in hypoxic conditions produced VEGFA and promoted the *in vitro* differentiation of endothelial cells. Thus, Tregs may play a functional role in the promotion of angiogenesis through the production of VEGFA. In future work, it will be important to determine whether Treg-derived VEGFA is critical for angiogenesis *in vivo* in this tumor model and in other mouse models of genetically-driven cancer.

Another study reported evidence suggesting a role for RANK ligand (RANKL) expressed by regulatory T cells in the promotion of breast cancer metastasis [36]. In experiments in which a mammary carcinoma cell line was implanted in the mammary gland, and metastasis to the lung was monitored, metastasis did not occur in mice lacking T and B cells. Transfer of CD4<sup>+</sup>CD25<sup>+</sup> Tregs restored metastasis, and this effect was diminished by blockade of RANKL. The authors concluded that Tregs promote cancer metastasis in a RANKL-dependent manner. In a different study, analysis of bone marrow cells from prostate cancer patients demonstrated that RANK-expressing dendritic cells induced Treg expansion *in vitro*, suggesting a role for RANK-RANKL signaling in the proliferation or maintenance of Treg populations [37]. Building from these observations, it will be interesting to determine whether conditional ablation of RANKL expression on Tregs modulates tumor progression or metastasis in murine cancer models, and to elucidate the cellular and molecular mechanisms driving metastasis downstream of RANKL.

Together, these studies present intriguing evidence suggesting that Tregs respond to diverse contextual cues within the tumor environment, and develop functional responses accordingly (Figure 1). These functions appear to be independent of the well-established role for Tregs in the suppression of adaptive immune responses and the regulation of inflammation, and therefore extend our understanding of Treg functional diversity. Thus, heterogeneity of Treg function in different contexts may help to explain the differing prognostic significance of Tregs infiltrating various types of cancer [14].

## Treg lineage stability

The stability of Foxp3<sup>+</sup> Tregs has recently become a topic of substantial debate, and could represent yet another source of Treg functional heterogeneity. Several early studies demonstrated that under certain conditions, Foxp3<sup>+</sup> Tregs can differentiate into Foxp3<sup>neg</sup> cells exhibiting effector function [38–41], suggesting that Tregs may undergo “reprogramming” in response to particular inflammatory stimuli. Potential instability of the Treg lineage raises the possibility of therapeutic intervention to “disarm” tumor-associated Tregs by driving their differentiation into Foxp3<sup>neg</sup> cells lacking suppressor function. On the other hand, several recent reports have challenged this idea by providing evidence that Foxp3<sup>+</sup> Tregs constitute a stable lineage. First, using inducible labeling to track the stability of Foxp3 expression in adult mice, it was found that Foxp3<sup>+</sup> Tregs exhibited a high degree of stability under steady-state and inflammatory conditions [42]. In addition, two studies demonstrated the presence of a minor population of uncommitted nonregulatory T cells that exhibit transient, promiscuous Foxp3 expression [40,43], suggesting that the instability of Foxp3<sup>+</sup> Tregs observed by other groups may in fact reflect the presence of this nonregulatory Foxp3-expressing population. Given the conflicting findings in the field, the stability and plasticity of Treg populations remain active areas of investigation. Further work will be needed to define the factors regulating promiscuous expression of Foxp3 by

nonregulatory T cells, and to determine whether the concepts of Treg instability and reprogramming are relevant in the context of cancer.

## Treg trafficking to tumors

In recent years, there has been considerable interest in understanding the inflammatory signals that drive Treg recruitment into tumors, in an effort to develop strategies for the selective blockade of Treg trafficking. An early study implicated the chemokine CCL22 and its receptor CCR4 as important factors driving Treg trafficking to human ovarian cancers [44]. In this study, it was shown that tumor ascites contained high amounts of CCL22, and that CCL22 induced Treg migration *in vitro*. Furthermore, in a xenotransplant tumor model, administration of anti-CCL22 antibody reduced the trafficking of adoptively transferred human Tregs to the transplanted tumor. Following this initial report, a growing number of chemokine ligand-receptor pairs, often referred to as chemokine-receptor “axes”, have been implicated in the trafficking of Tregs to different types of cancer (reviewed recently by [45]).

The importance of chemokine-driven Treg trafficking to tumors lesions raises the possibility of therapeutic intervention based on the blockade of this process. In this regard, a number of considerations may influence the feasibility and efficacy of such approaches. First, while many studies have demonstrated that a particular chemokine is sufficient to induce Treg trafficking, due to the redundancy and promiscuity of many chemokine ligand-receptor pairs [46], it will be important to determine whether the chemokine-receptor axis in question is essential for Treg trafficking (Box 2). If Tregs are able to use alternate chemokine-receptor axes, blockade of a specific axis is unlikely to be effective. In this regard, a recent study demonstrated that treatment of mice bearing established pancreatic tumors with a CCR5 antagonist reduced Treg infiltration within the tumor and slowed tumor growth [47], demonstrating a non-redundant role for CCR5 signaling that could be exploited to slow tumor growth in this model. Second, as discussed above, the ability of Tregs to adopt transcriptional programs similar to the target cells under regulation suggests that a given chemokine receptor may not be expressed only by tumor-infiltrating Tregs, but may also be expressed by other immune cell types within the tumor. Thus, disruption of a given axis may inhibit the trafficking of both Tregs and immune effector cells, potentially reducing therapeutic efficacy. Third, in treating established tumors, it will be critical to determine whether blockade of a chemokine-receptor axis will impact tumor-infiltrating Treg populations that are already present within the tumor. Future work will be required to address these concepts for chemokine-receptor axes that have been implicated in Treg trafficking to different types of cancer.

## Treg localization within the tumor

Our heightened appreciation of the diversity of Treg function suggests that following recruitment into the tumor environment, Tregs may play multiple roles within a single tumor. For example, it is possible that Tregs localized in different regions within a cancer tissue may have distinct functions. Consistent with this idea, a survey of human mammary carcinomas [48] revealed that a high density of Foxp3<sup>+</sup> cells within lymphoid-enriched areas of the tumor was associated with poor survival, while a high density of Foxp3<sup>+</sup> cells distributed elsewhere in the tumor exhibited no association with disease outcome. As an additional example, a survey of colorectal carcinoma revealed that elevated Treg density within normal mucosa was associated with poor prognosis, while a high Treg density within tumor tissue correlated with improved outcome [49]. These and other studies highlight the importance of quantifying Foxp3<sup>+</sup> cells in different areas of a tissue sample (Box 2), including the tumor bed, the stroma, clusters or aggregates of lymphoid cells, preinvasive

lesions, the zone of tumor invasion, and normal tissue adjacent to a malignant region. In-depth analyses of the relationship between disease outcome and the density of Tregs within these different regions may provide important clues as to the function and impact of tumor-infiltrating Tregs, and may serve as useful diagnostic markers.

## Treg development and antigen specificity

If Treg heterogeneity exists within a given tumor, what are the drivers of functional diversity? The antigen specificity of a T cell, conferred by expression of the T cell antigen receptor (TCR), is an important determinant of many aspects of T cell biology. For tumor-infiltrating Tregs, antigen recognition is likely to drive development into the *Foxp3*<sup>+</sup> lineage, activation and differentiation within the tumor environment, and co-localization with cellular interaction partners (antigen presenting cells) within the tumor. Thus, an understanding of the nature of the antigens recognized by tumor-associated Tregs would provide important insights into the biology of these cells.

First, to review the necessary conceptual framework, we will discuss recent advances in our understanding of the developmental origins of Tregs, and the nature of the antigens recognized by these cells, two concepts that are closely interrelated (Figure 1). *Foxp3*<sup>+</sup> Tregs are thought to develop by two pathways. “Natural” Tregs (nTregs), also referred to as thymic Tregs (tTregs), originate in the thymus, the major site of T cell development and maturation [50]. nTregs are thought to recognize self antigens [51–53], although little is known regarding the identity or nature of these antigens. “Induced” Tregs (iTregs), also called adaptive Tregs (aTregs) or peripherally-induced Tregs (pTregs), develop from conventional T cell precursors at extrathymic sites throughout the body, in a process that is augmented by exposure to TGF- $\beta$  and retinoic acid [54]. Whether iTregs recognize a similar array of self antigens as nTregs, or whether they recognize a distinct set of antigens has been unclear. In addition, it has been unclear whether nTregs and iTregs serve redundant functions, or whether there is a “division of labor” between these two subsets [54,55].

Recent work has provided considerable insight into these issues [56,57]. These studies demonstrated that CNS1, a conserved non-coding regulatory element in the *Foxp3* locus, is dispensable for nTreg development in the thymus, but is crucial for iTreg generation in the periphery [56,57]. Importantly, mice deficient in iTregs due to a targeted mutation in CNS1 exhibited spontaneous pathology exclusively at mucosal sites such as the lungs and gastrointestinal tract [56]. Extending these findings, a recent study utilizing CNS1-mutant mice demonstrated a role for iTregs in the maintenance of fetal-maternal tolerance during pregnancy, in a process involving the recognition of paternal antigens [58]. Together, these findings provide strong support for a model in which iTregs serve a crucial non-redundant function in the maintenance of immune tolerance and homeostasis, by functioning primarily at surfaces that interface with the external environment, with iTreg differentiation driven by recognition of foreign antigens that are not presented in the thymus.

This model of iTreg distribution and function is consistent with recent findings showing that a substantial fraction of colonic Tregs recognize antigens that derive from commensal microorganisms [59]. Expression of colonic Treg-derived TCRs did not facilitate Treg development in the thymus, but instead drove the accumulation of Tregs in the colon, consistent with an extrathymic origin of these Tregs.

Integrating these new findings with existing evidence, we can envision a simplified model of Treg development and specificity. This model predicts that nTregs recognize self antigens and function to maintain organ-specific immune tolerance to self tissues, while iTregs recognize foreign antigens (such as those derived from commensal bacteria, food, or allogeneic tissue), and function to maintain immune and tissue homeostasis at surfaces that

interface with the external environment. Thus, iTregs extend the Treg repertoire to include Tregs specific for antigens that are not presented for recognition in the thymus during T cell development [60]. In addition, iTreg development enables new Treg specificities to be generated throughout the lifetime of an organism in response to changes in the environment.

What are the implications of these concepts on our understanding of the developmental pathways and antigen specificities of tumor-associated Tregs? We consider two potential scenarios (Figure 1), which are not mutually exclusive. First, a developing tumor may recruit pre-existing thymic-derived nTregs reactive to self antigens (Figure 1, Pathway 1). Second, tumors may induce the *de novo* generation of Tregs by driving the differentiation of CD4<sup>+</sup> effector T cells reactive to tumor-associated antigens into the Foxp3<sup>+</sup> iTreg lineage (Figure 1, Pathway 2). This second process is a potential mechanism by which tumors evade immune destruction, due to the simultaneous diversion of effector T cells into a different fate and the generation of new Tregs with suppressive activity. In order to determine the relative contributions of these two pathways in the context of cancer, it is important to elucidate not only the developmental origins of Treg populations, but also the nature of the antigens recognized by these cells.

It has been difficult to assess the developmental origins of endogenous tumor-associated Treg populations for two reasons. First, it is not possible to recapitulate thymic and peripheral development of human Tregs using *in vitro* approaches. Second, the lack of definitive markers for the delineation of nTregs and iTregs (Box 2) precludes direct determination of the developmental origin of these cells. In the absence of such information regarding Treg development, there is considerable interest in determining the nature of the antigens recognized by Tregs. The rationale is that the antigens recognized by nTregs must be presented in the thymus for recognition during T cell development. If Tregs are found to recognize tumor-associated or tumor-specific antigens that are aberrantly or uniquely expressed by tumor cells following cancer development, these antigens are unlikely to be presented in the thymus to drive nTreg development. Therefore, Tregs recognizing tumor-associated or tumor-specific antigens are likely to be iTregs (Figure 1).

Two recent reports present indirect evidence that provide insight into Treg antigen specificity. In these studies, sequence analyses of TCRs expressed by purified T cell subsets isolated from transplantable [61] or chemically-induced murine tumors [62] revealed that the TCR repertoire of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs was distinct from the repertoire expressed by CD4<sup>+</sup>Foxp3<sup>neg</sup> T effector cells. These data suggest that the antigens recognized by Tregs within the tumor are different than those recognized by T effector cells. Furthermore, since TCR sequence overlap was not observed between Foxp3<sup>+</sup> and Foxp3<sup>neg</sup> subsets, these data suggest that the *in situ* differentiation of T effector cells into iTregs is negligible within the tumor. If this is a common process, one would anticipate at least a small, but detectable, overlap between the TCR repertoires of each subset, representing a “snapshot” of effector T cell populations undergoing conversion to the iTreg lineage. An important caveat of these studies is that due to the low number of TCR sequences analyzed in these studies, it is unclear whether the sampling of sequences represents the true breadth of the TCR repertoire. In future work, it will be important to extend these findings to other murine cancer models, including models of oncogene-induced malignancies in other tissues, as well as CD4<sup>+</sup>FOXP3<sup>+</sup> and CD4<sup>+</sup>FOXP3<sup>neg</sup> T cell subsets isolated from primary human tumors (Box 2).

A number of reports provide data suggesting the existence of Tregs reactive to tumor-associated antigens (TAAs) in human cancer patients. In this regard, two studies have utilized *in vitro* culture-based assays to detect TAA-specific Tregs in the peripheral blood of melanoma patients [63,64]. In these experiments, antigen-specific T cells are first expanded

by extended *in vitro* culture with peptides derived from TAAs, and the presence of antigen-specific Tregs is then determined by assessing proliferation [63] or CD3 downregulation [64]. However, in these assays, it is unclear whether the antigen-specific T cells detected are derived from *bona fide* Tregs, or originate from naïve T cell precursors that expand and develop regulatory characteristics following *in vitro* culture. In another approach, a short-term *in vitro* culture assay was developed to examine the antigen specificities of Tregs isolated from the blood of human colorectal carcinoma (CRC) patients [65]. Using this assay, the authors demonstrated that culture of CD4<sup>+</sup>CD25<sup>+</sup> T cells with dendritic cells pulsed with defined TAA peptides conferred the capacity to suppress the proliferation of polyclonally-activated effector T cells. This activity was observed for a percentage of samples from CRC patients, but was not observed in samples from healthy donors. Collectively, data from these and other studies suggest the possibility that Tregs reactive to peptides derived from TAAs are present at detectable levels in the blood of cancer patients.

In order to solidify the concept that human cancer patients harbor Tregs specific for TAAs, two types of studies will be critical in future work. First, the *ex vivo* detection of TAA-specific Tregs using peptide/MHC multimer reagents [66–68] would provide direct evidence that Tregs reactive to tumor antigens are present at detectable frequencies in human cancer patients (Box 2). In one such study [69], *ex vivo* staining of samples from melanoma patients revealed that HLA-DQ multimers bearing a peptide from the Melan-A antigen stained a CD4<sup>+</sup> T cell population comprising <0.1% of CD4<sup>+</sup> T cells in the peripheral blood, a fraction of which expressed FOXP3. Thus, while FOXP3<sup>+</sup>CD4<sup>+</sup> T cells of this specificity are detectable in some patients, they do not appear to contribute substantially to the polyclonal Treg repertoire in these patients. Second, it will be important to characterize individual TCRs expressed by human Tregs reactive to tumor-associated antigens, in order to evaluate the fine specificity of these receptors (Box 2). This could be achieved by the generation of T cell clones expressing Treg-derived TCRs [70,71], or by retroviral or lentiviral transduction of Treg-derived TCRs into immortalized cell lines [72]. These approaches would allow determination of whether TCRs expressed by tumor-infiltrating Tregs are “specific” for tumor-associated antigens, or whether these TCRs are cross-reactive, conferring promiscuous recognition of multiple peptide antigens.

In all, despite the available evidence highlighted above, much remains to be learned about the developmental origins and antigen specificities of tumor-associated Tregs. Substantial advances in these areas will require the identification of definitive markers of Treg origin, and means to directly characterize and enumerate antigen-specific Tregs isolated from primary tumors (Box 2).

## Concluding remarks

The studies discussed in this review exemplify our emerging appreciation for the multifaceted role that Tregs may play in the context of cancer. Recent progress in the field reveals that Tregs, once thought to be of “single-minded” purpose of dampening innate and adaptive immunity, instead exhibit substantial versatility, responding to varying contextual cues and adopting specialized functions accordingly. This functional diversity may be a critical factor underlying the observed differences in the prognostic significance of Treg density in different types of cancer, and illustrates the importance of studying Tregs contextually. We anticipate that the complexity of Treg biology will provide a fertile area for future research, and may reveal novel means for the selective therapeutic targeting of tumor-associated Tregs.



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## Glossary Box

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| <b>1. “naturally occurring Treg”</b>      | any Foxp3 <sup>+</sup> Treg that exists naturally, in the absence of experimental manipulation (likely a mixture of nTregs and iTregs)          |
| <b>2. “natural Treg (nTreg)”</b>          | a Foxp3 <sup>+</sup> Treg that develops in the thymus   |
| <b>3. “induced Treg (iTreg)”</b>          | a Foxp3 <sup>+</sup> Treg that differentiates into the Foxp3 <sup>+</sup> lineage extrathymically   |
| <b>4. “tumor-specific (neo-) antigen”</b> | a unique antigen that is exclusively expressed by tumor cells, usually generated by mutation or changes in post-translational modification [73] |
| <b>5. “tumor-associated antigen”</b>      | a non-mutated self antigen that is aberrantly expressed or over-expressed by tumor cells, but is also expressed by some normal cells [73]       |

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### Box 1. Markers for the analysis of Tregs

As described below, progress in the field has been slowed by the lack of definitive markers for the identification of Tregs and the delineation of Tregs of thymic (nTreg) or extrathymic (iTreg) origin.

#### Identification of Tregs

##### Foxp3

Foxp3, a transcription factor that is essential for Treg development and lineage specification [8–11], is considered to be the most definitive marker of CD4<sup>+</sup> regulatory T cells. However, in humans, FOXP3 can be expressed by activated T cells lacking regulatory activity [13,74], complicating its use as a Treg marker in human studies. Approaches incorporating the use of additional cell-surface markers have been used to more precisely define the phenotypic subsets of human FOXP3<sup>+</sup> cells that exhibit suppressive properties *in vitro*. For example, in experiments utilizing the cell surface marker CD127 (the IL-7 receptor  $\alpha$ -chain), it was found that suppressive activity was restricted to the CD4<sup>+</sup>Foxp3<sup>+</sup>CD127<sup>low</sup> subset [75]. In another study using the markers CD45RA (an isoform of the CD45 phosphatase) and CD25 (the IL-2 receptor  $\alpha$ -chain), it was found that CD45RA<sup>+</sup> FOXP3<sup>low</sup> and CD45RA<sup>-</sup> FOXP3<sup>high</sup> subsets exhibited suppressive activity, while CD45RA<sup>-</sup> FOXP3<sup>low</sup> cells produced cytokine and were not suppressive [74].

##### CD25

Seminal work by Sakaguchi and colleagues [76] demonstrated that regulatory T cell activity in mice was highly enriched in the CD4<sup>+</sup>CD25<sup>+</sup> T cell subset. Based on these and other studies, CD25 was the most commonly used Treg marker until the development of anti-Foxp3 antibodies [77] and Foxp3 reporter mice [78] in 2005. However, subsequent work has revealed the presence of suppressive FOXP3<sup>+</sup> T cells lacking CD25 expression in both mice [78] and humans [75].

#### Delineation of nTregs and iTregs

##### Helios

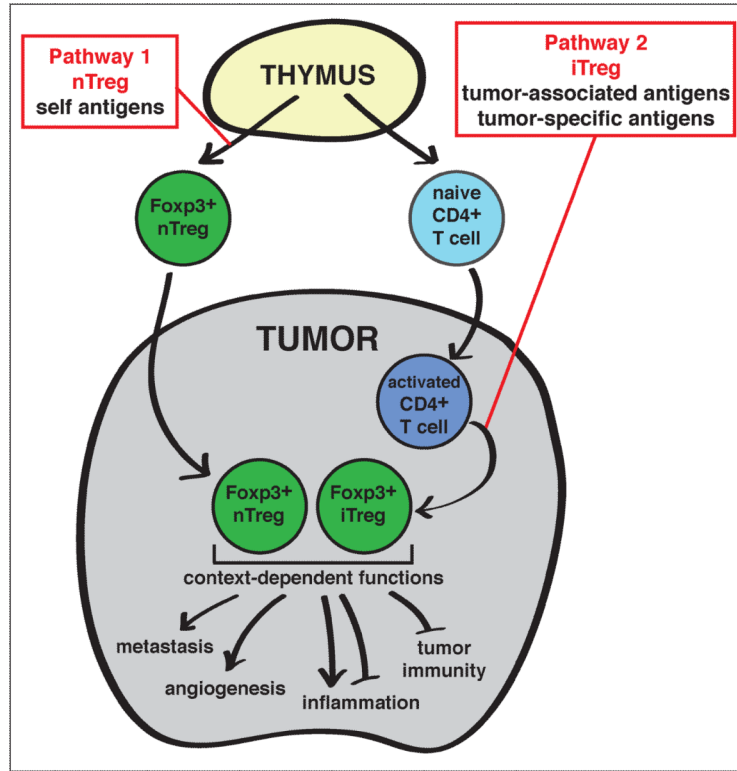
While initial reports suggested that the transcription factor Helios is a marker of thymic-derived nTregs [79], subsequent studies have revealed that Helios can also be expressed by iTregs generated experimentally [80–82]. Therefore, while Helios may serve as a marker of activated Tregs of increased suppressive capacity [82,83], it is not considered a definitive marker of Treg origin.

#### Methylation status of *Foxp3* gene

Recently, several assays have been developed for the epigenetic analysis of the methylation status of CpG motifs within conserved elements of the *Foxp3* locus [84–89]. Complete demethylation of these regions is associated with stable Foxp3 expression by T cells, and is a hallmark of thymic-derived nTregs [85–88]. However, while Foxp3-expressing cells generated by experimental manipulation often exhibit partial demethylation and unstable Foxp3 expression [85–88], it is unclear whether naturally occurring iTregs (existing in the absence of experimental manipulation) exhibit complete or partial demethylation of this region. In addition, available assays rely on the analysis of bulk populations, and do not provide information at the single-cell level.

**Box 2. Important areas of future research**

1. Loss-of-function evidence demonstrating that Tregs modulate tumor progression *in vivo* in genetically-driven murine cancer models
2. Gene expression profiling of tumor-associated Tregs to identify unique transcriptional programs
3. Quantification of Tregs localized in different regions of a cancer, and multivariate analysis of association with disease outcome
4. Identification of definitive markers to delineate and isolate Tregs in human samples
5. Identification of definitive markers to delineate nTregs and iTregs
6. Direct identification and enumeration of antigen-specific Tregs using peptide/MHC multimers
7. Characterization of the fine specificity / cross-reactivity of TCRs expressed by Tregs
8. Analysis of TCR repertoire overlap for CD4<sup>+</sup>FOXP3<sup>+</sup> and CD4<sup>+</sup>FOXP3<sup>neg</sup> T cell subsets isolated from human tumors
9. Characterization of chemokine receptors and homing receptors critical for Treg function and trafficking to the tumor



**Figure 1. Conceptual model describing the biology of tumor-associated Tregs**

Tumor-associated Tregs are thought to follow one of two developmental pathways in order to enter the Fxp3<sup>+</sup> Treg lineage. First, a developing thymocyte may recognize self antigen presented within the thymus during T cell maturation (Pathway 1, referred to as natural Tregs (“nTregs”)). Alternatively, a conventional CD4<sup>+</sup> T cell may encounter a tumor-associated (self) or tumor-specific (“neo”) antigen in the tumor environment, become activated, and under the influence of an immunosuppressive tumor microenvironment, differentiate into a Fxp3<sup>+</sup> Treg (Pathway 2, referred to as induced Tregs “iTregs”). Next, within the tumor environment, Tregs may respond to context-dependent inflammatory signals (e.g. Th1, Th2, or Th17 inflammation), the tissue or organ type (e.g. colon, breast, or prostate) and even the immediate proximal microenvironment (e.g. stroma, tumor bed, or lymphoid cluster). From these environmental cues, Tregs are capable of mediating distinct functions, which may include promotion of angiogenesis or metastasis, regulation of inflammation, and suppression of anti-tumor adaptive immune responses.