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REVIEW

CD4 regulatory T cells in human cancer pathogenesis

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Abstract Over the past decade, there has been an accelerated understanding of immune regulatory mechanisms. Peripheral immune regulation is linked to a collection of specialized regulatory cells of the $CD4^+$ T cell lineage (i.e., $CD4^+$ Tregs). This collection consists of Tregs that are either thymically derived (i.e., natural) or peripherally induced. Tregs are important for controlling potentially autoreactive immune effectors and immunity to foreign organisms and molecules. Their importance in maintaining immune homeostasis and the overall health of an organism is clear. However, Tregs may also be involved in the pathogenesis of malignancies as now compelling evidence shows that tumors induce or recruit $CD4^+$ Tregs to block immune priming and antitumor effectors. Efforts are underway to develop approaches that specifically inhibit the function of tumor-associated Tregs which could lead to an increased capability of the body's immune system to respond to tumors but without off-target immune-related pathologies (i.e., autoimmune disease). In this review, the biology of human $CD4^+$ Tregs is discussed along with their involvement in malignancies and emerging strategies to block their function.

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Introduction

The complex immune system is responsible for maintaining overall health of humans by eliminating nonconforming tissues, cells, and molecules derived from a number of different sources including malignancy, viral infection, etc. Furthermore, the immune system is extraordinarily powerful but simultaneously very specific relying on keen decision-making capabilities regarding when and how to tolerate cells or antigens derived from the host organism or other organisms when the need for tolerance is deemed important. The decision-making capability of the immune system is mediated by two broad mechanisms, central and peripheral tolerance. Central tolerance shapes the T cell repertoire in the thymus using several mechanisms to delete or preserve effector T cells with the ultimate outcome being the selection of a repertoire of T cells that is capable of recognizing self-antigens, with mild to moderate affinity, bound to major histocompatability complex (MHC) class I or class II molecules [1]. T cells that fail to bind to MHC bound peptide epitope die by a process called "death by neglect" and T cells that have high affinity recognition of self-antigen bound to MHC molecule are deleted by clonal deletion or "negative selection" to avoid pathologic attack on self-tissues. Those T cells which recognize self-antigen bound to MHC molecule with low to moderate affinity are preserved (i.e., positive selection) and constitute the bulk of T cells that can react to either foreign- or self-antigens in the periphery. These

potentially autoreactive T cells that migrate into the periphery are controlled by a number of peripheral tolerization mechanisms [2, 3]. T cells circulating in the periphery must acquire at least two signals to become activated. The first is mediated by the peptide:MHC complex and the second by co-stimulatory molecules (e.g., B7, GITR, and CD40). Without co-stimulation or in the presence of chronic antigen presentation by tolerigenic DC, T cells may undergo either anergy induction or peripheral deletion depending on the strength of the antigenic signal [3]. An alternative mechanism of maintaining tolerance in the periphery is mediated by the regulatory T cells (Treg cells), which comprise a group of either thymically derived or peripherally induced, suppressor $CD4^+$ T cells that control peripheral activation and function of both self- and foreign-antigen reactive T cells [2, 4–9, 10]. While Tregs, in general benefit the host, recently it has become clear that they can be involved in pathogenesis by providing tumors with a mechanism to evade immune detection and destruction. In this review $CD4^+$ Treg cells and their relationship to cancer pathogenesis are discussed along with emerging ideas on how to manipulate them to improve either endogenous or vaccine-induced anti-tumor immune responses.

Human $CD4^+$ regulatory T cells: subtypes, development and mechanisms of immune suppression and motility

Are there subtypes of human CD4 Tregs?

$CD4^+$ Treg cells refers to a collection of different phenotypes of $CD4^+$ T lineage cells whose primary function is regulating the activity of the immune system, in the periphery, against self- and foreign-antigens. In 2005 several hundred research articles and greater than 30 reviews dedicated to Treg cells appeared in the literature making them one of the most well-studied cells in biology in recent years (http://www.pubmed.gov). This is not to say however that Tregs are a newly discovered subset of T cells. In fact, the Tregs may be just a new name for the suppressor T cells that were studied extensively in the 1970s and 1980s. The study of suppressor T cells diminished in popularity in the late 1980s following the repeated failure to identify markers that could be used to isolate and study these cells. Notably, the failure to find the suppressor T cell-specific I-J gene in the place where it had been mapped led to the demise of suppressor T cells until 1995 when Sakaguchi and colleagues detected that T cell suppressor activity was confined to a minor subset of T cells that constitutively expressed the alpha subunit (i.e., CD25) of the high affinity IL-2 receptor [11, 12]. Reemergence led to adopting the new designation of Tregs rather than suppressor T cells.

Currently, it is envisioned that there are at least three groups of putative Tregs in humans which include [1] $CD4^+CD25^+$($Foxp3^+$) Tregs [2], Tr1 Tregs, and [3] Th3 Tregs, the first of which may actually represent two groups potentially suggesting four subsets (i.e., thymically derived and peripherally induced) (Table 1). One the major issues that makes it difficult to study Tregs in humans is the lack of an exclusive marker or set of markers. Much of what we understand about Tregs is extrapolation from mouse, which has not always held up to scrutiny. In fact, as will be alluded to throughout this section the concept of Treg subsets may be somewhat artificial and as technologic improvements continue to accrue our classification systems for Tregs will likely change. In mice, $CD4^+CD25^+Foxp3^+$ T cells are called "natural" Tregs because they are exclusively derived as a T cell lineage in the thymus [9, 13]. Data demonstrating the existence of a similar population in humans is not yet compelling but, as discussed below, the $CD4^+CD25^+Foxp3^+$ phenotype may represent $CD4^+$ Tregs derived from the thymus and induced in the periphery [14]. Before the discovery of Foxp3, $CD4^+CD25^+Foxp3^+$ Tregs were initially characterized as $CD4^+$ T cells that constitutively co-expressed CD25. As in mouse studies, CD25 (the high affinity IL-2 receptor alpha subunit) marks a peripheral population of T cells that possess immunosuppressive properties in vitro. Indeed, CD25 has been very useful for isolating and studying Tregs function. Initial studies, examining all $CD4^+CD25^+$ T cells (and assuming them all to be Tregs), suggested that Tregs may represent a sizable fraction (e.g., 6–17%) of $CD4^+$ T cells [15–2021]. However, this figure has been substantially revised based on the studies by Baecher-Allan et al. whose work suggested that human $CD4^+$ Tregs could be distinguished from other non-Treg $CD4^+$ T cells based on the intensity of expression of CD25 [22]. In that study, those $CD4^+$ T cells with intermediate to low levels of CD25 expression failed to block T cell proliferation in vitro and thus were excluded from the estimates (~1–2%) of circulating $CD4^+$ Tregs. Newer investigations using the criteria of $CD25^{high}$ confirm that the levels of $CD4^+$ Tregs in normal healthy individuals ranges between 1 and 2% in peripheral blood rather than the 6–17% as previously estimated [22–24, 25]. The high expression of CD25 may be due to a requirement for IL-2 signaling but this remains unclear. It is known that in mouse models, IL-2 signaling is required for Treg homeostasis but it is

Table 1 Putative subtypes of $CD4^+$ Tregs

	Thymically derived Tregs	Inducible Tregs		
		Tr1	*Th3*	*$CD4^+CD25^+Foxp3^+$*
Induction	Lineage commitment in thymus	Peripheral	Peripheral	Peripheral
Identity	$Foxp3^+/CD25^+$, suppressive function	$IL\text{-}10^+/CD25^-$ suppressive function	$Foxp3^+/TGF\text{-}\beta^+CD25?^+$ suppressive function	$Foxp3^+/CD25^+$, suppressive function
Distribution	Systemic	Localized?	Localized?	Localized?
Mechanism of suppression	Contact (CTLA-4, membrane bound TGF-β)	Soluble mediators (IL-10 and possibly TGF-β)	Contact (CTLA-4?) and soluble mediators (TGF-β), both required	?
Role in cancer	Yes	Yes	?	Yes
Diverse TCRs	Yes[a]	?	?	Yes[a]
Potential depleting/ blocking drugs and antibodies	Cyclophosphamide, GITR mAb, CD25 mAb, CTLA-4 mAb, Denileukin Diftitox	IL-10 mAb, TGF-β mAb, cyclophosphamide(?)	TGF-β mAb, CTLA-4 mAb(?), cyclophosphamide(?)	Cyclophosphamide(?), CD25 mAb, CTLA-4 mAb(?), Denileukin Diftitox

[a] Thymically derived Tregs and inducible $CD4^+CD25^+Foxp3^+$ Tregs may be indistinguishable

only ambiguously connected to suppression of T cell proliferation [26, 27]. Despite the improvement that high CD25 expression marks human regulatory T cells, the use of CD25 as a specific Treg marker is limited because it is also upregulated on activated effector T cells. Thus, there have been continued investigations in recent years into understanding other markers for improved detection and isolation, of which Foxp3 is the most notable [28, 29]. Foxp3 expression is detected, nearly exclusively, in the $CD25^{high}$ subset of human $CD4^+$ T cells [30].

Although its cellular function remains obscure, Foxp3, also called Scurfin, belongs to the Forkhead family of winged-helix transcription factors [28] and its gene, FOXP3, is located on the X chromosome [31]. The importance of Foxp3 in regulatory T cell function is demonstrated in certain disease settings in both humans and mice. In humans, loss of Foxp3 function results in an X-linked disease called Immunodysregulation, Polyendocrinopathy Enteropathy, X-linked (IPEX) syndrome [32]. IPEX is a fatal disease, first described in 1982 as X-linked, that presents as multiple autoimmune disorders such as hypothyroidism, enteropathy, Type I diabetes, and psoriasis [33]. Individuals that are destined to develop IPEX usually present during infancy and typically die within 2 years [13]. Subsequent studies traced the disease to the X chromosome and eventually to mutations in Foxp3 [32, 34]. The disease is not traced to a single mutation but rather to many mutations across the gene, some of which are missence mutations [13, 35–39]. The loss of Foxp3 in mice results in a similar X-linked syndrome, called Scurfy, which is a lethal syndrome that is also characterized by immune-mediated destruction of multiple organs [40]. The study of the biology of Foxp3 has revealed some important differences in Treg biology between humans and mouse models (Table 2). In humans, but not mice, there are two isoforms of Foxp3 (Foxp3 and Foxp3Δ2) of which the relevance is unknown [41]. Foxp3Δ2 is a splicing variant that lacks exon 2. Also in contrast to mice, studies from Allan et al. revealed that the ectopic expression of either Foxp3 or Foxp3Δ2 failed to convert naïve T cells to Tregs but rather imparted an anergic phenotype to the T cell (e.g., hyporesponsiveness and suppression of IL-2 production) [41]. Thus, it was concluded from this study that Foxp3 expression is not sufficient to confer a Treg phenotype suggesting that other cellular molecules that are coordinately expressed, work together to convey the regulatory phenotype. Another distinction between mouse and human is that Foxp3 can be naturally induced in human T cells following activation and its induction is associated with the acquisition of Treg activity [42]. In mice, however, there have been no conclusive reports that Foxp3 can be induced in $Foxp3^-$ T cells. The differences between Foxp3 expression and function between human and mouse, as well as its intracellular localization has made it difficult to exploit it as a marker to study the function of human Tregs.

Table 2 Notable differences in Foxp3 and Treg biology between human and mouse

Humans have two Foxp3 isoforms while mice appear to have only one
Foxp3 expression is confined only to $CD25^{high}$ cells in humans whereas expression of Foxp3 is distributed throughout the CD25 staining population in mice
Foxp3 is inducible in human $CD4^+CD25^-$ T cells but not in mice
Foxp3 transfection into mouse T cells confers a Treg phenotype while in humans it does not

In fact, it could be suggested that Foxp3 plays at least two roles in human T cell biology, anergy induction, and T cell regulation. Furthermore, it is likely that the T cells that are marked by Foxp3 in humans are a collection of thymically derived Tregs, peripherally induced Tregs, and possibly anergic T cells.

Lymphocyte activation gene-3 (LAG-3) is another candidate Treg marker that has emerged in recent years that may have the potential to further identify Tregs in association with CD4 and CD25. LAG-3 is a cell surface bound MHC class II ligand also referred to as CD223 that was discovered by Triebel et al. in 1990 prior to the reemergence of Tregs in 1995 [43]. As the name suggests, LAG-3 was originally found in activated T cells but not resting T cells [43]. Early studies revealed that rather than augmenting the activation of T cells, LAG-3 competes with CD4 in MHC class II binding acting as a T cell activation suppressor [44]. A recent study in mice by Huang et al. suggested that LAG-3 might in fact be a specific marker of Tregs [45]. That study showed that LAG-3 was nearly exclusively expressed on Foxp3$^+$ thymically derived Tregs but not on effector T cells harvested from recently immunized mice. In human, studies have shown that polyclonal activation of peripheral lymphocytes leads to upregulation of LAG-3 on nearly every cell, and more recent studies showing expression on mouse B cells strongly suggests that LAG-3 may not be useful for definitive Treg identification [46].

Several other markers have been examined in recent years as potentially selective markers for Tregs but to no avail. A key example is the glucocorticoid-induced tumor necrosis factor receptor (GITR), which is constitutively expressed on Tregs, but like CD25 and CTLA-4, it is upregulated on activated T cells [47]. GITR ligation, with agonistic (i.e., non-depleting) antibody, directly blocks the immunosuppressive activity of Tregs by as of yet unknown mechanisms. GITR, like CD25, also acts as a costimulatory molecule for effector T cells so that the net effects of GITR ligation are the simultaneous blockade of CD4$^+$CD25$^+$ Tregs and activation of effector T cells. The identification of specific molecular markers of Tregs remains an important goal in human immunology and will not only aid in the elucidation of Tregs but also provide for a novel target for immunotherapy as described below.

Adding to the complexity of human CD4$^+$CD25$^+$ (Foxp3$^+$) Tregs is the emerging data that multiple minor subsets exist and that a simple panel of cell surface markers may fail to identify all Tregs. For example, Valmori et al. demonstrated that there may be both naïve and antigen-experienced subsets [48]. These studies demonstrated that younger adults carry a naïve population that is characterized as CD45RA$^+$ CD45RO$^-$, a subset that declines with age. In another study, Stassen et al. reported that integrins $\alpha_4\beta_7$ and $\alpha_4\beta_1$ may also distinguish CD4$^+$ Tregs [49]. While the significance of these subsets are unclear, the intriguing finding from this study was that the integrins described unique Tregs that could each give rise to a unique population of induced Tregs (e.g., Tr1 and Th3), supporting an emerging concept of infectious tolerance described by Jonuleit et al. [50].

The inducible Tr1 and Th3 cells represent other Treg cells subsets that are much less understood. Tr1 Tregs are characterized by constitutive expression of the immunosuppressive cytokine, IL-10 [2, 8, 10], and are induced in the periphery by complement and regulatory cytokines. For example, complement C3b dimers can bind to CD46 on T cells and lead to differentiation of naïve CD4 T cells into Tr1 cells [51]. Tr1 Tregs can also be induced by tolerigenic DC and other innate effectors by the action of IL-10 and possibly IL-4 [10]. Although this subset is thought to be devoid of CD25 expression, a recent study suggests that there may be inducible expression of this molecule [52]. Emerging evidence suggests that Tr1 Tregs play a crucial role in maintaining tolerance to normal gut flora and protecting against autoimmune responses in the skin [51, 53, 54]. While it is thought that Tr1 cells do not typically express Foxp3, recent studies show that desmoglein-specific Tr1 cells not only express the transcription factor but that it also plays a role in conferring some immunosuppressive activity and raising the question as to whether peripherally induced CD4$^+$CD25$^+$(Foxp3$^+$) T cells are distinct from peripherally induced Tr1 cells [55]. Th3 Tregs, on the other hand, are characterized by expression of TGF-β, but only low to variable levels of CD25. In mice, Th3 Tregs seem to be important for the induction and maintenance of oral tolerance but little is known of their role or activities in humans [56]. While the designation of distinct subsets may be useful for continued research, one caveat is that it should be kept in mind that our classification strategy is tentative and likely to be dynamic in the coming years. Elements that remain to be sorted out included whether the distinctions that have been identified are in fact permanent or transient, potentially reflective of the in vivo or in vitro environment in which they are measured.

Mechanisms of immune regulation

In vitro studies of human CD4$^+$CD25$^+$(Foxp3$^+$) Tregs show that they block a variety of both adaptive and innate immune effectors mainly by cell contact

mechanisms, including dendritic cells, natural killer cells, and activated T cells [9]. The T cell suppressive activity of the thymically derived $CD4^+CD25^+$ Tregs uses a combination of cell surface bound TGF-β1 and CTLA-4 as shown by Annunziato et al. [14]. Mechanistically, it appeared that the interactions of TGF-β1 and CTLA-4 with effector T cell TGF-β receptors and B7, respectively, led to blockade of upregulation of the high affinity IL-2 receptor on the effector T cells and therefore the ability to respond to the autocrine/paracrine of IL-2. The role of TGF-β1 was corroborated by a recent study from Nakamura et al. who showed that the recombinant latency-associated peptide of TGF-β1 (rLAP), known to block the interactions of TGF-β1 with its receptor, can block the suppressive activity of peripheral $CD4^+CD25^{high}$ Tregs [57]. Furthermore, $CD4^+CD25^-$ T cells that are exposed to $CD4^+CD25^{high}$ Tregs show evidence of activation of the TGF-β signaling pathway as well as upregulation of TGF-β-inducible genes [57]. In addition to signaling through either B7 or TGFβR, Tregs may also induce apoptotic or necrotic T cell death with either granzyme B, perforin, or both [58].

Human $CD4^+CD25^+(Foxp3^+)$ Tregs regulate not only T cells but also other immune effectors as well. Houot et al. recently showed that maturation and cytokine production of human myeloid dendritic cells with toll-like receptor ligands were inhibited by Tregs [59]. Inhibition required both cell-to-cell contact as well as IL-10 suggesting that multiple mechanisms are used to ensure complete suppression. Ghiringhelli et al. recently showed that natural killer cells can also be inhibited by $CD4^+CD25^{high}$ Tregs. The rationale for their investigation was the preliminary finding that NK cell activity in tumor bearing patients was inversely correlated with the levels of Tregs. Subsequent in vitro studies showed that the direct application of Tregs to NK cells could block NK-mediated cytolysis and IFN-γ production as well as downregulation of the NK-activating receptor NKG2D [60].

While most of these mechanistic studies demonstrate killing or suppression of effector cells in vitro, it remains unclear which of these mechanisms are operative and dominant in vivo [6]. Importantly, it remains unknown if cell-to-cell contact is required in vivo or if inhibition of T cell responses is mediated by soluble factors or a combination of soluble and contact-dependent mechanisms.

In general, less is known about the immunosuppressive mechanisms of peripherally induced Tregs [2]. Tr1 Treg-mediated immune suppression appears to require both IL-10 and TGF-β but unlike thymically derived Tregs, does not require cell-to-cell contact indicating that both IL-10 and TGF-β are released as soluble mediators [61]. Unlike the Tr1 Tregs, the Th3 Treg subset does not typically produce IL-10 but rather confers immune suppression through the elaboration of soluble TGF-β and by an undefined cell-contact mechanism that may involve CTLA-4 [10, 62].

Human Treg migratory mechanisms

The findings in the past decade that tumors can preferentially recruit Tregs into the tumor microenvironment underscores the importance of identifying mechanisms by which Tregs migrate from the periphery into the tissues. Iellem et al. found in early studies that human Tregs express both CCR4 and CCR8. Furthermore, they showed that Tregs are attracted to mature dendritic cells by secretion of CCL17 and CCL22 [63]. As will be discussed below, several tumor types attract Tregs by releasing CCR4 binding ligands [64, 65]. In addition to the CCR4/CCR8 axes, Tregs use other mechanisms for migration which are just now being elucidated but as of yet have no established role in cancer pathogenesis. For example, Lim et al. found that tonsillar-localized Tregs switch their trafficking pattern from the CCR7/CCL19 axis to the CXCR5/CXCL13 axis upon activation providing them with the capability of migrating from the T-cell rich areas of the tonsils to the B-cell rich germinal centers [66]. Thymically derived Tregs can also migrate toward CCL1, perhaps using CCR8, as a means to localize to specific regions within the thymus [14]. Thus, several migratory mechanisms have been identified and the evidence suggests migration or acquisition of migratory capabilities is a dynamic process that may reflect the microenvironment in which Tregs may find themselves. Tumors may use these dynamic migratory mechanisms to selectively recruit in Tregs to block normal immunosurveillance and immune destruction.

The field of Tregs is an area that is being aggressively pursued by many laboratories. Based on the striking new findings that continue to emerge regularly, it is conceivable that we have only the most rudimentary understanding of Tregs. Studies continue to be published that identify new Treg subsets (other than $CD4^+$ Tregs), including one by Cosmi et al. who recently showed the existence of $CD8^+$ Tregs that share many of the same properties of the $CD4^+$ Treg [67]. How these various subsets will relate to each other and contribute to immune tolerance will also become clearer with the continued discovery of cell surface markers that uniquely identify Treg subsets such as CCR8 and Neuropilin-1 [52, 68]. For example, in studies by Freeman et al. it was found that the chemokine

receptor, CCR8 may be a cell surface marker for IL-10 producing $CD4^+CD25^+$Foxp3-Tregs [52]. Such markers will ultimately prove useful for purifying Tregs for ex vivo expansion and ex vivo analysis of phenotype and functions [69].

Tregs in human cancer pathogenesis

Studies over the last several years now suggest that a natural function of the immune system is to prevent tumor growth by a process called immunosurveillance and immunoediting [70]. Although controversial, several lines of evidence from mouse modeling studies strongly support a role for immunosurveillance in controlling tumors. For example, mice that lack IFN-γ and lymphocytes have higher incidences of spontaneous and chemically induced tumors [71]. However, in other studies of sporadic tumorigenesis, tumors avoid immune destruction by simply inducing tolerance [72]. The reasons for the discordance in results are not known, largely because it is unclear how the immune system, thought to be trained to avoid self-tissues, can target tumors of non-viral origin. One possibility is that the immune system relies on its peripheral mechanisms of suppressing autoimmunity greater than previously thought. In mouse models of tolerance, rather than being deleted, high affinity tumor antigen-specific T cells remain latent but can be recovered in vitro or in vivo [73, 74]. The fact that we can find functionally active human tumor antigen-specific T cells in patients with cancer suggests that there are similar tolerance mechanisms in the human [75–79, 80]. Other circumstantial lines of evidence also support natural immunosurveillance in humans. Human tumors, for example, are often infiltrated with T cells, some of which are known to be self-antigen-specific and correlate with disease outcome [81, 82, 83]. Assuming then that immunosurveillance is important, how do tumors evade immunity? The possibility that Tregs interfere with antitumor immunity was suggested in the years immediately following the re-emergence of $CD4^+$ Tregs, which may have at least two roles in blocking tumor-specific immunity; first by blocking the generation of immunity to tumor antigens in the periphery (i.e., lymph nodes) and by neutralizing tumor infiltrating effector T cells.

A sphere of influence

Tumors may block the generation of tumor antigen-specific immunity by releasing diffusible factors (e.g., cytokines or antigens) that increase the numbers of $CD4^+$ Tregs in the lymphactics and the peripheral circulation. Indeed, increased levels of $CD4^+$ Tregs in the peripheral blood of cancer patients, as compared to normal healthy control, have been reported in recent years for many cancers, including head and neck, hepatocellular, gastric, breast, ovarian, lung, melanoma, renal cell, and pancreatic [15–18, 19, 23, 25, 84]. Studies examining all $CD4^+CD25^+$ cells, regardless of the level of expression of CD25, suggested that in cancer patients Tregs represented 13–52% of the total $CD4^+$ T cells [15, 18, 19]. However, when confined to $Foxp3^+$ or $CD25^{high}$ the increases are more moderate ranging from 4 to 10% which is still significantly higher than the 1–2% observed in the normal healthy population [16, 23, 25]. The reason and the biological significance of the increased peripheral levels of Tregs is unclear but perhaps an important question to be asked is whether this represents a non-specific expansion or if the Tregs are responding to tumor antigen (i.e., tumor antigen-specific Tregs)? Indeed, if the latter were true, then peripheral Tregs may aid in the identification of tumor antigens to which the immune system may be responding. Some evidence supports that Tregs associated with tumors are antigen-specific Tregs. For example, Wang et al. recently described their findings that LAGE-1-specific T cells cloned from melanoma TIL demonstrated suppressive activity as well as Foxp3 expression [85]. Given our current understanding of the inducibility of Foxp3 in human T cells, however, it is plausible that the cloning process resulted in the in vitro generation of antigen-specific T cells with acquired immune suppressive activities [13]. The development of HLA class II tetramers may be one way to circumvent the problems associated with ex vivo expansion and enrichment to define the prevalence and specificity of tumor-associated Tregs [86].

Increases of Tregs in the circulation may also be indicative of Treg expansion in the draining lymph nodes and subsequent spillover into the circulation. Studies are emerging showing elevated levels of Tregs in tumor draining lymph nodes, including cervical, endometrial, gastric cancers, and melanoma [87–89, 90]. Tregs in the tumor draining lymph nodes may directly result in tolerance induction manifesting itself as a lack of functional tumor antigen-specific immune effectors. Although the numbers of studies pertaining to Tregs in the lymph nodes is small yet, the information indicates that tumors create a sphere of influence that results in blocking the generation of immune responses where tumor antigens are present (i.e., proximal lymph nodes). For example, a recent study by Kawaida showed that in gastric cancer patients the levels of $CD4^+CD25^+$ Tregs in the lymph nodes are

elevated the closer the proximity to the tumor [87]. In melanoma, the nodal $CD4^+CD25^+$ Tregs at sites proximal to the tumor are activated despite the fact that the nodes are free of tumors whereas more distant sites have normal levels of non-activated Tregs [90]. It seems unlikely that a tumor-derived systemic factor is responsible for activation and expansion of the Tregs as this might be expected to result in increased levels of Tregs in all of the lymph nodes throughout the body. Likely, the tumor relies on establishing a concentration gradient of antigens and other factors (TGF-β, chemokines, etc.) that result in elevated Tregs that are detectable in the peripheral blood because of convergence of the lymphatics and the blood veins. In addition to $CD4^+CD25^+$ Tregs, Viguier and colleagues observed that $CD4^+CD25^-$ T cells from tumor draining nodes released IL-10, TGF-β, or both, suggesting that Tregs are also induced indicating either that tumors can increase activate multiple subtypes of Tregs or that tolerance is infectious (i.e., Tregs promote more Tregs) [90, 91]. Lastly, proof that the tumors directly lead to elevated Tregs was demonstrated by Kono et al. who showed that resection of gastric cancers results in a near complete restoration of elevated peripheral Treg levels [24].

A curtain of protection?

Tumors may also block the activities of tumor infiltrating immune effectors by recruiting or inducing one or more of the Treg subsets into the tumor microenvironment as has been shown in animal models [92–94, 95]. Indeed, many studies in humans have shown intratumoral localization of $CD4^+CD25^+(Foxp3^+)$ Tregs in several human cancers including breast, ovarian, lung, non-Hodgkin lymphoma, liver, and melanoma [15, 64, 65, 84, 90, 96–100] (Table 3). Because recent human studies have shown that Foxp3 can be induced in activated T cells, it is not possible at present to determine if tumor-associated $Foxp3^+CD4^+$ Tregs are induced or natural Tregs [90]. Understanding whether tumors recruit or induce Tregs in the tumor microenvironment is potentially important when designing targeted agents aimed at reducing tumor-associated Tregs as one could envision differences in approaches targeting migration or induction.

Tumors can directly induce Tr1 ($nonCD25^+Foxp3^+$) $CD4^+$ Tregs in the tumor microenvironment. For example, it has been shown that Hodgkin lymphomas have an abundance of Tr1 Tregs within the tumor beds [101]. In vitro, these Tr1 cells can block effector T cell activation mediated by soluble IL-10. Furthermore, studies in ovarian cancer suggest that a specialized $CD8^+$ subset of Tr1 Tregs are induced by infiltrating tolerigenic plasmacytoid dendritic cells [102]. Thus, it appears that tumors possess multiple mechanisms of recruiting and inducing Tregs in the tumor microenvironment, which may block the function of infiltrating potentially tumor destructive immune effectors.

The ultimate question is, are the intratumoral Tregs clinically relevant? In some human tumors, intratumoral Tregs are associated with a poorer outcome. Because it is difficult to identify Tregs with certainty in humans, it has not yet been possible to thoroughly evaluate their role in the pathogenesis of cancer conclusively and there are many conflicting reports about whether these intratumoral Tregs are important in blocking infiltrating immune effectors (i.e., DC and T cells). The best evidence for a pathogenic role of Tregs is in ovarian cancer as indicated by two independent studies. In the first study by Curiel et al., it was found that regardless of stage, an increased number of $CD4^+CD25^+$ T cells in the tumor is associated with a poorer disease outcome [64]. In that study, tumor-infiltrating Tregs were analyzed both in vitro (i.e., T cell immunosuppression assays) and in vivo (NOD/SCID), demonstrating both the ability to block tumor-specific immunity as well as tumor growth. In another corroborating study in ovarian cancer, Wolf et al. observed that increased levels of intratumoral Foxp3, assessed

Table 3 Intratumoral Tregs

Cancer	Phenotype	Associated with survival or tumor control	Reference
Breast cancer	$IL\text{-}10^+$, $TGF\text{-}\beta^?$? (Tr1) and $CD4^+CD25^+Foxp3^+$	Unknown (TR1), No ($CD4^+CD25^+Foxp3^+$)	[13, 94]
Ovarian cancer	$CD4^+CD25^+$	Yes, negatively	[62, 99]
Hodgkin's lymphoma	IL-10 (Tr1) and $Foxp3^+CD4^+CD25^+$	Ambiguous	[97]
Non-Hodgkin's lymphoma	$CD4^+CD25^+Foxp3^+CTLA\text{-}4^+$	Unknown	[63]
Lung	$CD4^+CD25^+$	Unknown	[81]
Liver	$CD4^+CD25^+Foxp3^+TGF\text{-}\beta^+$	No	[95, 96]
Pancreas	$IL\text{-}10^+$, $TGF\text{-}\beta^?$?(Tr1)	Unknown	[13]
Head and neck	$CD4^+CD25^+$ and $CD4^+Foxp3^+$	Yes, positively	[101]

by RT-PCR, within the tumor bed was highly associated with decreased survival [103]. In that study, individuals who had low Foxp3 had a mean survival of 77 months (median not reached) while those with high Foxp3 levels had a median survival of 30.2 months. Furthermore, in a previous study, the authors of this study had shown that patients with increased levels of IFN-γ within the tumor had significantly improved survival [104]. This updated study, however, revealed that co-localization of high Foxp3 neutralized this improved survival, strongly suggesting that Tregs can block effector immunity if recruited or induced in the tumor microenvironment. However, one caveat is that it remains unclear what the source of the Foxp3 is (i.e., tumor or T cells).

Despite the evidence in ovarian cancer, studies of Tregs in other cancers have failed to demonstrate a role of intratumoral Tregs in tumor pathogenesis. Badoual et al. examined $Foxp3^+CD4^+$ T cells and $CD69^+CD4^+$ T cells (activated effector T cells) in head and neck cancers, observing that both subsets were associated with improved locoregional control. The $CD69^+$ subset was also positively associated with improved overall survival while the $Foxp3^+$ only demonstrated a trend toward survival [105]. In another study, Alvaro et al. were also unable to demonstrate a significant association of $Foxp3^+$ cells with poorer survival in Hodgkin's Lymphoma [106]. In fact, their study also showed some evidence that Foxp3 expression in the tumor bed was associated with improved survival. The reasons for the discrepancies are unclear, but a number of potential explanations emerge with the first being that each tumor type behaves differently with some benefiting from infiltration of immune effectors, perhaps by benefiting from a rich source of cytokine-associated signals, which may actually enhance tumor growth.

Modulating $CD4^+$ Tregs to augment tumor rejection immunity

As previously discussed, Tregs are potent immunoregulatory cells and play a critical role in maintaining immune homeostasis. In addition to playing a key role in the maintenance of immunologic self-tolerance and prevention of autoimmunity, Tregs are also known to regulate immune responses against infectious agents, tumor antigens and transplantation antigens [107, 108]. While the current literature on Tregs is substantial, the exact mechanisms of how Tregs control or regulate normal and pathological immune responses (i.e., mechanism of activation, specificity for antigen, mode of action, etc.) have not been fully defined. Thus, the impact of modulating this specific T cell population is not trivial and may result in unwanted off-target effects. Additionally, the lack of accessible (i.e., cell surface bound) molecules that can be used to identify Tregs and serve as targets makes this therapeutic strategy increasingly difficult.

However, given that Tregs have been demonstrated to down-regulate immune responses to self-antigens, such as tumor antigens, careful depletion or inactivation of Tregs may result in generation of functional immune effector cells and enhancement of endogenous tumor-specific immunity [109]. The initial studies that suggested that modulation of Tregs could result in the development of an antitumor response came from North and colleagues who showed that elimination of tumor-induced suppressor T cells with cyclophosphamide resulted in immune-mediated regression of an advanced lymphoma [110, 111]. Cyclophosphamide is an alkylating agent that mediates DNA cross-linking and has been used extensively to treat human diseases including various cancers. While high doses of cyclophosphamide which are required for effective tumor cytotoxicity result in immunosuppression, low doses of cyclophosphamide can induce immunostimulatory effects resulting in improved immune responses in various animal tumor models [112]. Preclinical studies in mice have shown that the immunostimulatory effects observed at the lower doses of cyclophosphamide are due to the selective depletion of cycling (i.e., proliferating) Tregs [73, 113]. For example, Lutsiak et al. found that low-dose cyclophosphamide significantly reduced the $CD4^+CD25^+$ T cells but not the total $CD4^+$ and $CD8^+$ T cell populations [113]. Furthermore, depletion of Tregs increases the capability of mice to overcome tolerance to tumor-associated antigen neu [73]. Ercolini et al. showed that $CD4^+CD25^+$ T cells in the periphery are selectively downregulated and latent pools of high avidity tumor antigen-specific T cells are recruited to the antitumor immune response when vaccines are combined with cyclophosphamide, in the neutransgenic mouse [73]. In clinical studies, the use of cyclophosphamide as a pretreatment in adoptive T cell therapy strategies has been effective in causing regression of tumors in patients with advanced metastatic melanoma. In a study by Dudley and colleagues, pretreatment of patients with a non-myeloablative chemotherapy regimen of fludarabine and cyclophosphamide followed by infusion of ex vivo expanded melanoma antigen-specific T cells resulted in persistence of tumor-specific T cells in vivo, evidence of antitumor functional activity (i.e., 50% clinical response rate), and the ability of the infused T cells to traffic to tumor

sites [5]. Thus, while current data supports the idea that low dose cyclophosphamide can be used clinically to reduce Treg-mediated immunosuppression, the specific effects of cyclophosphamide on Tregs remain unclear. In fact, it remains unclear whether cyclophosphamide depletes Tregs in humans.

Preclinical murine cancer models have also demonstrated in vivo depletion of Tregs with resultant regression of tumors after treatment with anti-CD25 monoclonal antibodies [114] Thus, a logical technique for depleting Tregs based on these animal studies, is the use of anti-CD25 monoclonal antibodies which have already been developed for clinical use in humans for the treatment of acute cellular rejection of allograft transplants, demonstrating both a good safety profile as well as clinical efficacy [115, 116, 117]. As previously mentioned, CD25 is the high affinity IL-2 receptor alpha subunit and while it is constitutively expressed on some subsets of Tregs, it is also expressed, albeit transiently and at high levels, on effector T cells during activation. The upregulation of CD25 imparts responsiveness of the effector T cells to the growth factor effects of IL-2 generated during the immune response, and it is this subset of lymphocytes that are targeted in the setting of allograft rejection, rather than the Tregs. Thus, based on its efficacy at inhibiting $CD25^+$ effector T cells, anti-CD25 may also be effective at depleting $CD25^+$ Tregs to augment antitumor immunity. However, because expression of CD25 expression would occur during the effector phase of an emerging antitumor immune response that would be targeted by the antibody, it is likely that dosing with anti-CD25 antibodies in the setting of cancer therapy will be different from the strategies used in the transplant setting. For example, CD25-expressing Tregs might be amendable to rapid depletion with a high dose of anti-CD25 antibody followed by its rapid elimination (i.e., the antibodies) which may subsequently allow for the natural or pharmacologically induced development of effector antitumor T cell immunity during the Treg deplete period (1–2 months) [30]. The ability of anti-CD25 antibody alone to augment endogenous immunity depends where in the development of an antitumor response the effector T cells were inhibited by the tumor. If the effectors are inhibited following the priming phase within the tumor by the Tregs, then the elimination of Tregs may result in spontaneous tumor rejection by releasing blocks on endogenous effectors. This is suggested by preclinical murine models in murine sarcoma which have revealed that simply eliminating intratumoral $CD4^+CD25^+$ Tregs with an anti-CD4 antibody can directly lead to the unmasking of tumor-rejecting immunity, suggesting that anti-CD25 monoclonal antibody monotherapy may be possible with some tumor types and possibly with intratumoral injections [92]. However, if blockade of the development of a tumor-specific immune response involves a combination of different elements (e.g., tolerance), then other strategies (e.g., vaccines) may be needed to boost the immune response following Treg depletion. In the neu-transgenic preclinical mouse model of breast cancer, for instance, some studies have shown that the combination of depletion with anti-CD25 antibodies and vaccines is a more effective approach than using either depletion or vaccines alone [73]. Treg killing may be enhanced through conjugation of anti-CD25 antibodies to toxins. Attia et al. recently reported that LMB-2, a conjugate of an anti-CD25 single chain antibody and *Pseudomonas* exotoxin, selectively impairs $CD25^+$ Tregs in vitro without harming bystander T lymphocytes [118].

The use of IL-2 or IL-2 conjugates to selectively target CD25-expressing Tregs is currently being explored as an alternative to anti-CD25 monoclonal antibody. Our group has used Denileukin Diftitox (Ontak, DAB_{389}IL-2), an IL-2 diphtheria toxin fusion protein, to directly kill Treg in preclinical models [119]. Denileukin Diftitox down-regulates $CD4^+CD25^+Foxp3^+$ Tregs and when used early in the course of disease can lead to a sustained antitumor response in tumor growth in the neu-transgenic mouse. Coincidental with the development of a sustained antitumor response, Denileukin Diftitox also resulted in the ability of the transgenic animals to generate both T cell and B cell immune responses that were specific for tumor antigens. Furthermore, the immunotoxin also led to reduced Tregs in the tumor bed. Like the anti-CD25 antibody, the central concern when using Denileukin Diftitox is the most appropriate dosing of the immunotoxin so that effector T cells are not inhibited. Recently, Denileukin Diftitox has been evaluated as an adjunct to deplete Tregs and improve the immunogenicity of cancer vaccines. Dannull et al. used the immunotoxin to deplete Tregs from PBMC from renal cancer patients [30]. Patients were treated with a single dose of the immunotoxin followed by vaccination with tumor antigen RNA-transfected dendritic cells. Treatment resulted in the elimination of Tregs, identified as $CD4^+CD25^{high}$ T cells. Interestingly, the administration of Denileukin Diftitox did not affect CD25-intermediate effector/memory T cells. Compared to vaccination alone (i.e., in the absence of Treg depletion), the administration of Denileukin Diftitox seemed to have greatly improved the immune response to the vaccine. These results provide strong evidence that Denileukin Diftitox can modulate the Treg population in humans

and that immunity to self-antigens can be augmented. Continued development and determining its most effective use (e.g., tumor and vaccine settings) seem to be warranted.

High dose IL-2 has been used extensively in patients with metastatic melanoma and renal cell carcinoma and results in approximately a 20% response rate [120]. While in vitro studies support the role of low-dose IL-2 in promoting the survival and differentiation of Tregs, the in vivo effect of high-dose IL-2 on the Treg population and its mechanism of therapeutic activity in responding patients are not known, but a recent study by Cesana et al. suggests that high-dose IL-2 alone (i.e., unconjugated) may down-modulate Treg levels, at least in the periphery [23]. Administration of high-dose IL-2 in melanoma or renal cancer patients resulted in a significant decrease of peripheral Tregs in those patients who achieved an objective clinical response to IL-2 therapy. Intratumoral Tregs were not evaluated so it is unclear if the treatment resulted in decreased microenvironmental suppression or increase in effector T cells or both.

Targeting of suppressive molecules on Tregs such as CTLA-4 is another therapeutic strategy to reverse immunosuppression and enhance endogenous tumor-specific immunity. Tregs constitutively express CTLA-4 and use it to suppress effector T cells, as previously mentioned above. Like CD25, CTLA-4 is not expressed exclusively on Tregs but unlike CD25, CTLA-4 is only associated with inhibiting T cell responses and, therefore, blocking its function at multiple sites may lead to an immune response that may be greater than if it was expressed only on Tregs [121]. Current data suggest that in vivo administration of anti-CTLA-4 antibody blocks CTLA-4-specific signaling in CTLA-4$^+$ Tregs and thereby promotes tumor immunity. Phan et al. showed that treatment with anti-CTLA-4 antibody induced cancer regression in some patients that were vaccinated with HLA-A2 restricted gp100-derived peptides [122]. Importantly, blockade of CTLA-4 led to severe autoimmunity (grade III/IV dermatitis, enterocolitis, hepatitis, and hypophysitis) in 43% of patients. The phase I clinical trial was instrumental in defining the central role of CTLA-4 in blocking autoimmunity. However, it remains unclear if anti-CTLA-4 acted by reducing Tregs in the tumor, augmenting antigen-specific T cells in the periphery or both. In recent studies, the administration of anti-CTLA-4 antibody in patients with renal-cell cancer or metastatic melanoma did not inhibit the suppressive activity of Tregs in vivo or in vitro and there was no decrease in the number of peripheral blood Tregs [123]. Thus, a mechanistic link between the effects of anti-CTLA-4 antibody on Treg function and improved tumor immunity, if any, remains to be defined.

Two preclinical studies have shown that administration of GITR-specific antibody protected mice from tumor challenge and induces tumor regression in mice bearing advanced cancers [112, 124]. In the latter study, Ko et al. showed that treatment of mice with agonistic antibody resulted in the reduced recruitment of Tregs into the tumor microenvironment thereby permitting reduction of the microenvironmental suppression of the effector T cells and better tumor eradication [124]. Another possibility that remains to be tested is whether depletion of Tregs can be accomplished with depleting anti-GITR monoclonal antibodies in a manner analogous to anti-CD25 antibodies.

Other strategies that have emerged in recent years, which may be useful for inhibiting Treg function, include blocking soluble suppressive molecules such as IL-10 and TGF-β. Notably, IL-10 and TGF-β can be produced by multiple cell types in the tumor microenvironment. Although, Tregs might not be the main source of IL-10 and TGF-β it is clear that Tregs can mediate suppression through the actions of IL-10 and TGF-β in vivo [112, 125]. More specifically, the primary mechanism of action of induced Tregs (Tregs generated in the periphery from CD4$^+$CD25$^-$ precursors) is mediated through IL-10 or TGF-β as described above. IL-10 is a pleiotropic cytokine and is suppressive to effector T cells but is an important B cell differentiation and maturation factor [126]. IL-10-specific antibodies have been shown to result in more prolonged antitumor CTL responses in vitro [127]. Additionally, IL-10-specific antibodies can block the severity of Systemic Lupus Erythmatosus (SLE), a disease in which autoantibodies are important in pathogenesis [128]. In that early study, five of six SLE patients were treated into clinical remission with blockade of IL-10. Furthermore, the treatment was safe and well tolerated. The findings that IL-10-secreting Tregs are induced and accumulate within some tumors (e.g., Hodgkin's lymphoma) suggests that blockade of IL-10 with antibody may be worthwhile to block the local immune suppression if the antibody can be specifically targeted to the tumor.

Conclusion

In closing, modulating the action of Tregs may be important in enhancing endogenous tumor immunity for some cancers, as recent evidence suggests that they may be involved in pathogenesis. Potential therapeutic strategies in targeting Tregs include depletion, blocking trafficking into tumors, or reducing their

differentiation and suppressive mechanisms. Again, one of the major concerns with targeting Tregs for the purposes of treating tumors is the potential for off-target effects. As previously mentioned above, agents like anti-CTLA-4, have significant toxicity profiles. Besides blocking intratumoral immune effectors or preventing the induction of antitumor immunity, Tregs are also dominant in controlling generalized autoimmunity. Thus, treatment strategies that can specifically target these agents, such as to the intratumoral microenvironment would be of considerable interest so as to prevent off-target effects. Indeed, recombinant DNA techniques, agents like anti-IL-10 or anti-CTLA-4 could be conjugated with other agents that localize them to the tumor microenvironment. For example, anti-CTLA-4 antibodies could be linked to nanoparticles that have been pre-coated with a tumor-localizing antibody (e.g., HER-2/neu) [129, 130]. Such strategies may ultimately reduce the amount of anti-CTLA-4 antibody thereby reducing the possibility of off-target effects. Lastly, although our understanding of peripheral regulatory mechanisms has greatly improved in the last decade, the continued discoveries of new Tregs and new Treg properties drives one to the conclusion that, in reality, our understanding is still only minimal.

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