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What are regulatory T cells (Treg) regulating in cancer and why?

Theresa L. Whiteside, Ph.D.
University of Pittsburgh Cancer Institute Hillman Cancer Center 5117 Centre Avenue, Suite 1.32 Pittsburgh, PA 15213, USA Ph: 412-624-0096 Fax: 412-624-0264 whitesidetl@upmc.edu

Abstract

The role regulatory T cells (Treg) play in cancer development and progression is not clear. Earlier evidence suggested that $CD4^+FOXP3^+CD25^{high}$ Treg accumulate in tumors and the peripheral blood of patients with cancer and through suppression of anti-tumor immune responses promote tumor growth. However, more recent data indicate that in certain cancers, such as colorectal carcinoma (CRC), Treg suppress bacteria-driven inflammation which promotes carcinogenesis and thus benefit the host. Treg appear to play a dual role in cancer. This might explain why the frequency and functions of Treg are associated with a poor prognosis in some cancers but with favorable outcome in others.

The clinical and prognostic significance of Treg in cancer depends on environmental factors, including infectious agents, tumor-derived products and locally-produced cytokines, which shape the nature of immune responses, including Treg generation, recruitment and survival. Adaptive or inducible (i) Treg or Tr1 are the major subset(s) of Treg present in cancer. These iTreg are a distinct subset of regulatory cells that phenotypically and functionally differ from $FOXP3^+$ natural (n) Treg responsible for peripheral tolerance. They mediate powerful suppression of effector T cells via diverse mechanisms, produce immunosuppressive cytokines, notably TGF-β as well as prostaglandin E2 and adenosine, and are resistant to apoptosis or oncological therapies. Strategies for silencing of Tr1 in patients with cancer will require novel approaches that can selectively deplete these cells or block molecular pathways they utilize.

Keywords

Regulatory T cells (Treg); cancer; inducible (i) Treg; inflammation; FOXP3; prognosis

Introduction

It has been well documented that various cancer immunotherapies, including anti-tumor vaccines, have a limited therapeutic efficacy when delivered to patients with advanced disease, and that their impact on disease-free or overall patient survival has been minimal to date [1, 2]. With a few notable exceptions, anti-tumor immune responses generated by these immunotherapies are often weak, short-lived and biased toward Th2- rather than Th1-type responses [3]. The reasons for these unexceptional clinical effects of anti-tumor immunotherapies have been long ascribed to tumor-induced immune suppression, which allows human tumors to disarm the host immune system and thus to escape from immune surveillance and immune destruction [4, 5]. Indeed, human tumors are known to produce a

broad variety of immunosuppressive factors, including adenosine [6, 7], prostaglandin E2 (PGE_2) [8-10], inhibitory cytokines such as TGF-β [11], gangliosides [12] and many others [13, 14]. Immune cells, including cytotoxic T lymphocytes (CTL), are expected to recognize and eliminate tumor cells, but in the tumor microenvironment, these cells are rendered dysfunctional [15]. It is of note that tumor-induced immune cell dysfunction is restricted to tumor-directed responses, as anti-viral and anti-bacterial immune responses remain unimpaired in most patients with cancer while anti-tumor immunity is selectively compromised. Further, tumor-induced dysfunction extends to tumor antigen-specific T cells as well as innate immunity components such as natural killer (NK) cells and macrophages [16]. Tumor-induced dysfunction of adaptive and innate immunity can be local or systemic, can be concomitantly present in T cells, NK cells, dendritic cells (DC), B cells and monocytes [16] and can selectively interfere with signal transduction, activation, cytokine production, proliferation, cytotoxicity or cell migration [16-19]. The profile and severity of immune dysfunction in immune cells of different patients with cancer can vary and appears to be dependent on the ability of each tumor to create a unique immunoinhibitory environment and to engineer an escape from immune control [20].

Human tumors are often infiltrated by immune cells, predominantly T lymphocytes and myeloid cells, which are recruited to the site by chemokines and cytokines secreted by the various cells in the tumor milieu [21]. Although tumor-infiltrating lymphocytes (TIL) can to some degree retain anti-tumor activity, and many express activation markers such as CD25 or CD69, tumors that are most aggressive also appear to be most immunosuppressive for TIL [22-24]. Among T cells recruited to human solid tumors are $CD4^+FOXP3^+CD25^{high}$ regulatory T cells (Treg). These T cells accumulate in tumors and the peripheral blood of patients with cancer [25-30], and the increased Treg frequency has been generally considered as a marker of poor prognosis in cancer presumably due to Treg-mediated suppression of anti-tumor immunity, which benefits the tumor [31-33]. More recently, however, several lines of evidence suggest that Treg might play a dual role in carcinogenesis. On the one hand, Treg can down-regulate inflammation, which in some cancers has been linked to tumor progression [34] and thus Treg benefit the host. On the other hand, Treg accumulations in cancer predict poor outcome [31-33]. It appears that Treg can benefit the host by controlling inflammation but can also be harmful by suppressing anti-tumor immunity and promoting cancer progression. In this review, we will consider the evidence in support of this paradigm. The objective is to document Treg involvement in promoting or inhibiting tumor progression and to provide insights into the role tumor-associated inflammation plays in modulating Treg initiation, differentiation and suppressor functions.

Phenotypic and functional characteristics of Treg in cancer

Treg are a small subset of $CD4^+$ T lymphocytes (about 5%) which suppress functions of autologous conventional T cells (Tconv) [27, 28]. They comprise several subsets of phenotypically similar cells able to suppress Tconv through distinct and often unexpected mechanisms [35-38]. At least two Treg subsets have been recognized in man: (a) natural (n)Treg, which originate in the thymus, mediate suppression via cell contact-dependent mechanisms involving, e.g., the granzyme B/perforin or Fas/FasL pathways and constitute a major regulatory T-cell subset for maintaining peripheral tolerance [39, 40] and (b) inducible or adaptive Treg (iTreg), also referred to as type 1 regulatory T cells (Tr1), which are induced in the periphery in response to environmental signals, including antigens plus IL-2, TGF-β and IL-10 [29, 41, 42]. Tr1 mediate suppression by contact-independent mechanisms through the production of TGF-β, IL-10 and other immunosuppressive factors [29, 43].

In contrast to murine Treg, the phenotype of human Treg is not yet firmly defined. All murine, and most functional human nTreg are characterized by intracellular expression of FOXP3, a transcription factor forkhead box p3, belonging to the forkhead/winged-helix family [44, 45]. Most nTreg are $FOXP3^+$, while Tr1 cells might not be, thus suggesting that these two subsets of Treg represent distinct $CD4^+$ T cell lineages responding to different signals and presenting different phenotypic as well as functional profiles. In addition to intracytoplasmic FOXP3, surface expression of IL-2 receptor alpha chain (CD25) has been the most commonly used marker for Treg definition and also for their isolation [25]. Similar to FOXP3, CD25 is not a marker specific for Treg, as it is expressed in activated $CD4^+$ or $CD8^+$ T cells with no suppressor activity [46]. It has become a common practice in flow cytometry to distinguish between Treg and Tconv based on the level of CD25 expression, with $CD4^+CD25^{high}$ T cells considered as Treg [25, 47]. This has often led to arbitrary decisions regarding the Treg selection, and a functional definition based on suppression of Tconv proliferation had to be used to confirm the presence of Treg in cell fractions separated by CD25 expression levels [25]. An urgent need exists for additional cell markers which could reliably identify Treg and allow for their isolation from tissues and peripheral blood. The cytotoxic T lymphocyte antigen-4 (CTLA-4, CD152), the glucocorticoid-induced tumor necrosis factor receptor (GITR) and ICOS as well as programmed-death-1 (PD-1) were found to be expressed on human Treg, but neither was specific for Treg [48-50]. Similarly, the chemokine receptors CCR4 (CD194) [26, 27], CCR6 (CD196) [28] and CCR7 (CD197) [51, 52], which are expressed by human Treg are also found on other T cells. While these various markers cannot be used to distinguish or isolate Treg, they endow Treg with special functions. For example, the chemokine receptors are critical for Treg migration, while signaling via GITR down-regulates Treg functions [53, 54], and it may serve as a co-stimulatory factor for activated T cells [55]. A relatively new marker, HELIOS, an Ikaros family transcription factor, is said to be present on nTreg but not on iTreg [56]. Treg are also characterized by the absence of certain surface markers that are expressed on Tconv. These include IL-7-receptor, CD127 [57] and an integrin alpha subunit, CD49d [58]. Negative selection of Treg based on the absence of these markers, followed by confirmatory expression of FOXP3, has become a method of choice for Treg isolation [59]. But, in common with CD25, these two surface markers do not provide a distinct cut-off in expression levels between Treg and Tconv in flow cytometry, so that gate setting for these markers is also an arbitrary decision.

We and others have recently described the presence of an ectonucleotidase, CD39, as a new functional surface marker for Treg [60-62]. CD39 hydrolyzes exogenous ATP to ADP and 5′AMP, which is further hydrolyzed to adenosine by another ectonucleotidase, CD73 [63]. Murine Treg express CD73 on the cell surface, while in human Treg it is found in the cytoplasm but only after vigorous cell permeabilization (T. Whiteside, unpublished data). Therefore, its presence on the surface of human Treg is difficult to demonstrate by conventional methods [64, 65]. Our data suggest that CD73 surface expression may be restricted to a subset of iTreg [66]. It has been shown that CD39 expression defines a subset of $CD4^+$ T cells which mediate suppression *in vitro* [67] and also *in vivo*, as shown in murine studies [68]. Therefore, this marker is considered suitable for positive selection of Treg from $CD4^+$ T cells [62]. More recently, it has become clear that human Treg selected by the surface expression of CD39 consist of two closely interacting cell subsets, a subset of $CD25^+FOXP3^+$ cells, which mediate suppression, and a subset of $CD25^{neg}FOXP3^{neg}$ cells, which are not able to suppress T cell proliferation but always accompany $FOXP3^+$ T cells, perhaps serving as precursor cells [69, 70]. Because cells in both subsets are $CD39^+$ and can hydrolyze ATP to AMP and eventually to adenosine, they are operationally considered to be suppressor cells. Adenosine is known to modulate functions of a variety cell types via adenosine receptors and 3′5′-cAMP up-regulation [71]. Thus, Treg able to produce adenosine could be involved in multiple cellular functions from immune suppression to

regulation of the vascular proliferation and other cellular responses. Most likely, an excess of extracellular ATP, such as exists in tumors infiltrated by inflammatory cells, serves as a recruiting signal for iTreg which express purinergic receptors, P2X7 [72]. This may be, in part, responsible for Treg accumulations at tumor sites. Once recruited, iTreg perform a variety of tasks, including the hydrolysis of endogenous ATP, which at elevated levels may be toxic to surrounding cells [72].

Functional attributes of Treg are clearly the best marker for their identification. The most widely used suppressor cell assays measure inhibition of responder T cell proliferation or cytokine production. Unfortunately, T-cell proliferation assays, which have to be run at several different T suppressor/T responder cell ratios, require substantial numbers of preferably freshly-harvested cells and are not practical. On the other hand, flow cytometry-based cytokine assays, which can be performed with relatively small numbers of cryopreserved and thawed lymphocytes, lend themselves well to measurements of inhibition of cytokine expression levels in responder cells. Intracytoplasmic cytokine assays could serve as surrogate functional markers for Treg, especially iTreg which use TGF-β or IL-10 for suppression and express TGF-β-associated membrane-tethered GARP (garpin) and latency-associated peptide (LAP). Further, iTreg generated in the presence of COX-2^+ tumors have been shown to be COX-2^+ and produce PGE_2 as well as express CD73 on the cell surface, which might allow for discrimination of Tr1 and their partial functional characterization by flow cytometry [66]. More recently, Krammer's group reported that human Treg can suppress T cell receptor (TCR)-induced Ca^{2+}, NF-κB and NFAT signaling in Tconv, providing a broader repertoire of suppression assays that can be used with human cells [73]. Another alternative is to measure increases in cAMP levels as an indicator of suppression in responder T cells co-incubated with Treg, using classical biochemical methods. However, cell numbers might again be a limiting factor in these assays. It is always advisable to measure suppressor function of isolated T cell subsets in addition to phenotyping Treg, so that the presence of $FOXP3^+$ $CD25^{high}$Treg can be supported by evidence of suppression. It is necessary to remember that several different molecular pathways responsible for Treg-mediated suppression exist [35-37, 74] and may or may not be utilized by $FOXP3^+CD25^{high}$ Treg. Another confounding aspect of Treg phenotyping is the finding that iTreg generated *in vitro* and those present in tumor tissues may not be $CD25^{high}$, but instead tend to express CD123 (IL-2Rβ) and CD132 (IL-2Rγ) and are variably positive for FOXP3 [29]. This has to be taken into account when studies of the Treg frequency in cancer patients are conducted. Also, while immunostaining of sections cut from paraffin-embedded tumor for $FOXP3^+$ cells is reliable and allows for their enumeration, it is necessary to remember that activated Tconv or tumor cells can also express FOXP3 [46, 75, 76]. Further, Abs specific for markers other than FOXP3 expressed on Treg might not be reliable for immunohistochemistry (IHC). Counting of Treg in tumor sections stained by IHC is a demanding task that requires the use of image analysis and the systems biology methods, as so elegantly demonstrated by Galon and collaborators [77]. Visual examination and manual cell counts in tissue are clearly less reliable. Thus, tissue studies of $FOXP3^+$ Treg must be interpreted with caution.

Treg found in tumors and in the peripheral circulation of cancer patients have distinctive properties. Induced in the tumor microenvironment that is dominated by the tumor, these Treg acquire properties necessary for the control of immune responses taking place locally. Treg present in cancer patients are, by and large, adaptive or inducible Treg (iTreg, Tr1). They differ from thymus-derived natural Treg (nTreg) responsible for the maintenance of peripheral tolerance in healthy donors. The phenotypic differences that exist between these subpopulations of Treg are not as apparent or as well defined as functional differences: iTreg, especially those in TIL isolated from human tumors, mediate stronger suppression and may utilize a broader range of suppressor mechanisms than nTreg [25]. In fact,

subpopulations of iTreg seem to exist that "specialize" in the type of regulatory mechanisms they employ. This functional heterogeneity of iTreg accounts for difficulties in assigning to them a definitive phenotype. Also, the origin of iTreg is still a mystery, although they seem to arise by conversion of Tconv responding to signals generated on site [78]. However, the nature of these signals as well as the elements controlling iTreg differentiation are not entirely clear. Importantly, $CD4^+$ T cells with characteristics similar to those of iTreg present in cancer patients are found in chronic inflammatory lesions and chronic viral infections such as HIV-1 or HPV [79].

Cancer and inflammation

Many human tumors are preceded by or associated with inflammation [80]. In liver cancers caused by viral hepatitis, gastric cancer caused by *H. pylori* infection, head and neck cancers which are HPV^+ or in inflammatory bowel disease preceding the development of colorectal cancer (CRC), inflammation is considered a direct cause of malignancy [81]. On the other hand, while the initial development stages of some human tumors may not be associated with inflammation, once established, these tumors invariably produce factors attracting inflammatory cells and thus create an inflammatory environment, which promotes tumor growth [17]. These two pathways of tumor genesis share in common a tumor-promoting process in inflammation. Further, a vast majority of human cancers are treated with adjuvant, neo-adjuvant or definitive chemoradiotherapy (CRT). CRT causes a long-lasting imbalance of the host immune system, resulting in a state of chronic inflammation [82]. It is suspected, but not proven, that post-therapy chronic inflammation plays a role in the development of secondary cancers or in recurrence of the disease. The frequency of Treg is increased in chronic inflammation, in cancer patients with active disease, and especially, in cancer patients after oncologic treatments [26]. In all these cases, the increased frequency of Treg serves as a signal for enhanced levels of immune regulation to bring inflammation under control and restore the equilibrium.

When persistent inflammation threatens to cause tissue destruction, Treg accumulate and proceed to contain inflammation, thus benefiting the host. As the promotion of tumor growth is dependent on the destruction of normal tissue homeostasis by chronic inflammatory cells, Treg activities in this context can be seen as a limiting factor in the tumor development. On the other hand, in the presence of an established tumor, Treg-mediated suppression eliminates anti-tumor functions of immune effector cells, thus benefiting the tumor. The questions that remain unanswered in this scenario are whether the same or different Treg subsets mediate both these series of events; how the tumor influences Treg activity; and whether Treg use the same or different suppression mechanisms to control tumor-associated inflammation vs. that occurring in the tumor absence. To be able to begin addressing these issues, it is necessary to consider the current information available about Treg associated with human cancers and their role in cancer progression.

The role of Treg in colorectal carcinoma (CRC) progression

There has been intense interest in the role Treg play in human cancer and in chronic viral infections. Increased numbers of $CD4^+FOXP3^+CD25^{high}$ Treg as well as high levels of suppressor function have been observed not only among TIL obtained from various human carcinomas but also in the peripheral circulation of cancer patients [reviewed in 28]. Further, Treg accumulations in cancer have been generally linked to unfavorable disease outcome as reported for many human solid tumors [30-32]. This might be expected, as Treg are able to inhibit anti-tumor immunity and mediate immune tolerance favoring tumor growth. In this context, Treg could be viewed as the major component of tumor escape from the host

immune system and thus might serve as a marker of a poor prognosis and represent a new target for immunotherapy.

At the same time, evidence exists that accumulations of $FOXP3^+$ Treg in tumors, as evaluated by IHC, is not always associated with a poor prognosis. In some cancers, notably in CRC, the presence and density of $FOXP3^+$ Treg have been linked to an improved prognosis. Salama et al recently reported an improved survival (p=0.001) for patients whose tumors had a high density of intratumoral $FOXP3^+$ Treg [83]. This finding was more recently confirmed by Frey et al [84]. In patients with head and neck squamous cell carcinoma (HNSCC), tumor infiltration by $CD4^+FOXP3^+$ Treg was positively associated with a better locoregional control of the tumor [85]. Recently, Ghiringhelli and colleagues examined all published studies referring to $FOXP3^+$ T cell infiltration and prognosis in CRC [86]. A uniformly consistent results of these studies confirmed a significant positive correlation between the density of $FOXP3^+$ T cell infiltrations and an improved prognosis and/or survival in CRC [86]. Looking for an explanation for these unexpected results, it is necessary to consider a possibility that $FOXP3^+$ or $CD25^+$ T cells counted in tissues were not Treg but rather activated $CD4^+$ or $CD8^+$ effector T cells. However, functional studies, while limited, appeared to confirm that $FOXP3^+$ T cells isolated from CRC mediated immune suppression [87], and thus the “mistaken identity” could not explain the paradoxical results.

It is important to recall that CRC develop in a special microenvironment enriched in the intestinal microorganisms. Numerous lymphoid and myeloid cells are present in the lamina propria and infiltrate between the epithelial cells arranged in a monolayer forming the intestinal mucosal barrier. Many of the T cells infiltrating intestinal mucosa might be specific for bacterial antigens. These T cells and other inflammatory cells rich in toll-like receptors (TLRs) probably play a key role in initiating and maintaining cellular activation *in situ*. Gastrointestinal bacteria are known to trigger cascades of pro-inflammatory cytokines, which drive pro-angiogenic and tumor-enhancing effects via activation of transcription factors such as STAT3 and NFκB [88, 89]. A chronic inflammatory reaction in the intestine has to be strictly controlled to prevent the onset of inflammatory bowel disease. Thus, Treg are necessary to impose control, and this explains their accumulation in the intestinal mucosa. Mice deficient in T cells were shown to be highly susceptible to inflammatory bowel disease and to the development of carcinomas. Adoptive transfer of Treg ameliorated inflammation and carcinogenesis, but only if Treg were previously exposed to enteric bacteria and secreted IL-10 [90]. These experiments showed that through suppression of inflammation driven by bacteria, $FOXP3^+$ Treg prevented carcinogenesis. In fact, their presence was essential for control of destructive inflammation culminating in CRC. Thus Treg, whose normal job is to control and suppress potentially destructive immune responses, acquire anti-tumorigenic properties when such responses promote carcinogenesis. In CRC, the relationship between the $FOXP3^+$ Treg abundance and favorable prognosis is the result of a physiologically normal process designed to control excessive inflammation. It is important to remember, however, that the role of Treg in early stages of carcinogenesis may be different from that in established tumors. Once established, the tumor has an opportunity to influence Treg functions and re-direct them into exercising not anti- but pro-tumorigenic activities designed to suppress functions of tumor antigen-specific effector T cells. In CRC, Treg appear to attenuate destructive inflammation in early stages of carcinogenesis, while at late stages, they might down-regulate anti-tumor immune responses.

In contrast to CRC, in other human solid tumors, which develop in non-infected tissues, tumor cells have the capability to produce TGF-β, PGE2, adenosine and other suppressive factors which inhibit Th1 responses [6-9, 91]. Tumors also produce chemokines, which promote Treg accumulation. In the presence of TGF-β and tumor antigens conversion of

Tconv to iTreg is encouraged. Accumulations iTreg further inhibit Th1 responses by interfering with anti-tumor functions of effector T cells and ultimately contribute to poor outcome. It appears that septic vs. non-infective nature of the tissue in which carcinogenesis occurs influences the role Treg are destined to play in respect to inhibition vs. promotion of tumor progression.

Treg and effector T cell interactions vs. prognosis

Human tumors are usually infiltrated with T cells other than Treg which could influence prognosis. For example, in a recent IHC study of over 1300 breast cancer specimens, tumor-infiltrating $CD8^+$ T lymphocytes were found to predict clinical outcome: the total count of tumor-infiltrating $CD8^+$ T cells was found to be an independent prognostic factor of better patient survival [92]. Numerous other IHC studies have suggested that infiltration of solid human tumors with CD8+ T cells is associated with improved prognosis [93]. Not only the number of tumor-infiltrating $CD8^+$ effector T cells but also their activation and the functional capability to eliminate tumor targets are considered critical for the control of tumor growth [16]. In this context, Treg and their interactions with $CD8^+$ T effector or $CD4^+$ T helper cells could influence prognosis. In a series of studies in CRC by Fridman's group, it has been shown via immunostaining of hundreds of tumor specimens, that a strong local immune reaction, including $CD3^+$, $CD8^+$ and memory $CD45RO^+$ T cells, correlates with a favorable prognosis regardless of the local extent of the tumor or regional lymph node involvement [94, 95]. Within dense infiltrates of $CD8^+$ effector memory T cells in CRC, the high frequency of $FOXP3^+$ T cells was singled out as the independent and favorable prognostic factor in Salama's report [83]. In aggregate, these studies suggested that in CRC, high-density infiltrations by effector $CD8^+$ Tcells and $FOXP3^+$ Treg are associated with improved outcome. If so, then the $CD8^+$/Treg ratio could serve as a marker of favorable prognosis in CRC and perhaps other solid tumors. Indeed, based on positive correlations between effector T-cell density in CRC and favorable outcome [96], the CD8/Treg ratio has been used in some studies to monitor lymphocyte changes in the course of therapies. However, the lack of consistency in phenotyping of human Treg *in situ* does not yet allow for definitive conclusions about the general utility of the $CD8^+$/Treg ratio as a potential surrogate marker of prognosis.

Under normal circumstances, whenever $CD8^+$ effector and $CD4^+$ helper T cells accumulate in response to local signals, so do $FOXP3^+$ nTreg to maintain the homeostatic balance and prevent potential tissue damage. In solid tumors, however, a conversion of Tconv into Tr1 occurs, creating a pool of highly activated and indiscriminately suppressive adaptive Treg, which interfere with functions of immune cells, including anti-tumor effector T cells. Tumor-associated Tr1 are pro-tumorigenic, as they produce immunosuppressive cytokines (IL-10, TGF-β) as well as other immunoinhibitory factors, such as adenosine and PGE_2 [66]. It is of interest to note that PGE_2 was recently identified as the single inflammatory signal that triggers DNA methylation, thereby shutting off tumor suppressor and DNA repair genes in colon cancer models [97]. Further, tumor-associated Tr1 appear to be resistant to chemo-, radio-, and immunotherapies, and thus their increased numbers following oncologic therapies and persistent suppression of anti-tumor immune responses might create conditions favorable to disease recurrence. This aspect of Tr1 induction and persistence in cancer is currently under investigation.

Our understanding of the role $FOXP3^+$ Treg play in cancer has been further complicated by a recent discovery of another subset of $CD4^+$ T cells, Th17 cells, which normally arise in response to intestinal bacteria and reside in gut-associated lymphoid tissue (GALT). Th17, like $FOXP3^+$ Treg, accumulate in human tumors, although the relationship between $FOXP3^+$ Treg and Th17 is not clear [98]. Th17 express the transcription factor RORC,

which is linked to STAT-3, produce IL-17, a pro-angiogenic cytokine, as well as pro-inflammatory IL-6, IL-1, and TNF-α [99]. Normally Th17 mediate protection against extracellular pathogens but in tumors they contribute to tumor growth [99]. Galon et al reported that in CRC, infiltration of the tumor with Th17 predicted a poor prognosis ($p=0.0009$), contrasting with a favorable prognosis for tumors infiltrated by Th1 and $CD8^+$ T cells [100]. In the same study, a favorable prognosis correlated with the high frequency of $FOXP3^+$ Treg. Based on these data, it is possible to speculate that tumor-promoting capabilities of Th17 could be decreased or abolished by $FOXP3^+$ Treg thus, in part, accounting for a favorable role of $FOXP3^+$ Treg in CRC prognosis. On the other hand, in ovarian carcinoma, patients with higher numbers of tumor-infiltrating Th17 cells had significantly better overall survival, irrespective of the tumor stage, and their frequency inversely correlated with that of tumor-infiltrating $FOXP3^+$ Treg [98]. The prognostic impact of Th17 in cancer remains to be determined as is the extent and results of inter-cellular interactions between Th17 and Treg in the tumor microenvironment.

In aggregate, current evidence suggests that in CRC, the density of effector T cells and the frequency of $FOXP3^+$ Treg in the tumor are associated with improved prognosis. The question remains as to whether the same relationship exists in other human solid tumors that do not arise and develop in the inflammatory environment dominated by gastrointestinal microorganisms.

Treg in prognosis of other solid cancers

The favorable impact of $FOXP3^+$ Treg accumulations on prognosis has also been reported for head and neck carcinomas (HNC), which are often characterized by dense infiltrates of activated T cells [101]. Situated in the oral cavity, nasopharynx or larynx, these tumors, like CRC, are permanently exposed to microorganisms, and microbial flora appears to play a role in tumor progression [102]. Like CRC, HNSCC are reported to be heavily infiltrated by Th17 cells [103]. Using flow cytometry and CFSE-based suppressor assays, we evaluated the frequency of $FOXP3^+CD25^{high}$ Treg and their suppressor functions, respectively, in isolated tumor-infiltrating lymphocytes (TIL) obtained from tumors and the peripheral circulation of untreated patients with HNSCC. The frequency and function of Treg in the tumor as well as the patients' blood were significantly increased relative to values seen in the blood of age- and sex-matches normal donors [26]. $CD4^+FOXP3^+CD25^{high}$ Treg were more numerous and mediated significantly higher suppression in HNSCC patients with T3/T4 tumors, nodal involvement and advanced disease than in patients with T1/T2 tumors and early disease [26]. The data suggested that a high frequency of $FOXP3^+CD25^{high}$ Treg and, as shown later of $CD4^+CD39^+$ Treg, in the tumor and peripheral blood, was associated with poor prognostic parameters in HNSCC [104]. Two other studies, both using immunostaining, reported the increased $FOXP3^+$ Treg frequency in HNC specimens. In the study of 84 HNSCC by Badual et al [85], a high density of $CD4^+FOXP3^+$ Treg was associated with a better locoregional control of the tumor at $p=0.026$ but did not influence overall survival ($p=0.07$). In another study of 106 tumor specimens from patients with nasopharygeal carcinoma (about half were positive for Epstein Barr Virus (EBV)), high density of tumor-infiltrating $FOXP3^+$ Treg was negatively associated with tumor stage ($p<0.05$) and positively associated with better overall and disease-free survival ($p<0.01$) [105]. The discrepancy in prognostic significance of Treg between these studies could be explained by the methods used as discussed above creating a bias in favor of activated T cells, whose presence in the tumor clearly favors an improved prognosis. Also, by exclusive consideration of $FOXP3^+$ Treg, the *in situ* studies disregard the presence of iTreg, by far the most active suppressor cells in cancer.

In contrast to CRC or HNC, most human breast tumors, except perhaps ductal breast carcinomas, contain fewer inflammatory cells and develop away from infectious agents. Nevertheless, inflammatory cells, especially $CD8^+$ T cells are often present in and around tumor nests and are prognostically significant [92]. The Treg number is increased in the peripheral blood and breast cancer tissue [106] and is significantly elevated in patients with metastatic disease [107]. However, in other studies evaluating the clinical significance of $FOXP3^+$ circulating or tumor-infiltrating cells, respectively, the absolute numbers of Treg were not found to be prognostically important [108, 109].

The hypothesis that associates high density of $FOXP3^+$ Treg in CRC and, less convincingly in HNSCC, with a favorable prognosis is based on the ability of these cells to suppress tumor-promoting inflammatory reactions initiated by infectious agents. By extending this hypothesis to other human solid tumors, many of which are, in fact, preceded by numerous infectious episodes, an argument for a beneficial role of Treg in regulating carcinogenesis gains strength. On the other hand, if Treg in tumors suppress anti-tumor immunity thereby promoting tumor progression, then their presence and activities would predict a poor outcome. Further, their presence and activities would likely interfere with immunotherapy. This latter view has been dominating the oncology field, and it provided a rationale for considering Treg depletion in order to increase the efficacy of immunotherapy.

Treg and cancer therapy

The role of Treg in cancer therapy, similar to their prognostic value, remains controversial. Although *in vivo* and *in vitro* studies in murine models of cancer and in patients with cancer have shown that Treg compromise the host's anti-tumor immunity, more recent data indicate that their role in cancer therapy is complex and diverse [110] Nevertheless, it is still widely believed that *in vivo* elimination of Treg may enhance tumor anti-tumor immunity. The immunomodulatory properties of low-dose cyclophosphamide regimen are well known, and in various experimental animal cancer models, Treg depletion by cyclophosphamide has been linked to the recovery of T-cell immune responses[111] However, recent studies show that Treg depletion by cyclophosphamide is less effective in humans, and that it does not enhance the potency of cancer immunotherapies [112, 113]. Other Treg-depleting regimens used to improve endogenous anti-tumor immunity or the efficacy of immunotherapies include administration of daclizumab (anti-CD25 Ab), denileukin diftitox also known as ONTAC or tyrosine kinase inhibitors such as Sunitinib [114-116]. These anti-Treg regimens transiently reduce Treg numbers in the blood of some patients. To investigate the impact of the above listed Treg-depleting agents administred in combination with anti-tumor vaccines on the frequency of $FOXP3^+$ Treg, and not on other activated T cells, a recent study used a MS-qPCR method to follow the fate of circulating T cells with demethylated FOXP3 intron 1 [117]. In melanoma patients receiving this immunotherapy, none of the three strategies resulted in a sustained reduction in the frequency of circulating $FOXP3^+$ Treg that exceeded 50%. In most patients, this reduction was much more modest, while the treatment with IL-2 used as a control, increased the frequency of circulating $FOX\text{-}2^+$ Treg at least two fold [117].

Because the role of Treg in cancer therapy remains unclear, it is possible that Treg depletion might serve as a proverbial double edged sword. Schwartzentruber et al recently reported an association between the Treg frequency and clinical responses in a phase III study of melanoma patients treated with high-dose IL-2 alone or in combination with a peptide vaccine [118]. The responding patients enrolled in the IL-2 plus vaccine arm showed a significantly higher increase in Treg frequency than the patients who did not clinically respond [118]. Here, the results point to a beneficial clinical role of expanded Treg. In another study, tumor specimens obtained from colon cancer patients prior to systemic

chemotherapy were evaluated for tumor infiltration by $FOXP3^+$ Treg [119]. In patients with tumors characterized by high numbers of tumor-infiltrating Treg, overall survival, progression-free survival and treatment-relative survival were all significantly higher relative to patients whose tumors were poorly infiltrated with $FOXP3^+$ Treg [119]. In view of these clinical trial results, and the observations that therapies inducing anti-tumor immune responses also induce Treg which, in turn, influence clinical responses and prognosis in cancer patients, it becomes highly important to determine whether this paradigm is valid. In other words, is Treg depletion necessary or effective in improving results of immunotherapy in cancer?? Is Treg expansion, e.g., by IL-2 delivery, rather than Treg depletion a better strategy for improving results of immune therapies?? To confirm the validity of Treg depletion for improving clinical efficacy of immune therapies in humans, it will be necessary to design and conduct future clinical trials based on new insights into the Treg biology. In cancer, the emphasis should be on iTreg or Tr1, because these cells appear to have a major role in cancer progression. These Treg are induced in the tumor microenvironment and empowered by the tumor to eliminate or dampen anti-tumor immunity. Their selective depletion may thus be necessary. Fortunately, they have distinct phenotypic and functional attributes [29, 91]. For example, we have recently reported on the utilization of the adenosinergic pathway by human iTreg [66, 67]. In pathologic conditions, such as cancer or chronic infections, it is these iTreg recruited and conditioned by the tumor that mediate high levels of suppression by producing adenosine and up-regulating 3′,5′-cAMP levels in responding immune cells [118]. Our evidence suggests that while the iTreg induction may be driven by tumor-associated antigens in a cytokine-permissive environment, and in that sense might be antigen specific, it appears that suppression these Tr1 exert at the effector cell level is non-specific, targeting adaptive as well as innate immune cells.

Conclusions

Regulatory functions exercised by Treg are critically important for maintaining immune responses in balance. In health, this balance is largely controlled by thymus-derived $CD4^+FOXP3^+$ T lymphocytes utilizing contact-independent suppression mechanisms. In cancer or chronic viral infections, the rules for nTreg-mediated control of immune responses change. A rapid expansion of adaptive T cells and powerful activation of innate immune cells necessary for an effective containment of insulting agents has to be regulated to avoid excessive tissue destruction. Whether in cancer or during infections, Treg are called upon to restore the immune equilibrium and prevent tissue damage. In early carcinogenesis, which in tumors such as CRC is associated with prolonged pro-inflammatory insults driven by gastrointestinal bacteria, Treg are instrumental in limiting the local inflammation that ultimately leads to cancer. The abundant presence of $FOXP3^+$ Treg within inflammatory infiltrates into tumor tissues signifies control of carcinogenesis, ameliorates pro-inflammatory signals and is associated with improved outcome.

In human tumors developing in a non-inflammatory milieu, immune cells are recruited to the tumor site to: (a) provide factors supporting tumor growth and (b) exert anti-tumor functions. This recruitment and activation of immune effector cells to these tumor-associated inflammatory sites always includes Treg. The tumor now proceeds to block functions of accumulating anti-tumor effector cells and instigates a massive conversion of Tconv into iTreg with powerful and varied suppressive capabilities. iTreg are resistant to apoptosis, accumulate and promote tumor escape. Their numbers increase as tumor progresses, and they could serve as surrogate markers of poor outcome.

In the scenario presented in this review, Treg play a double role of protecting the host and protecting the tumor. However, the latter function is conferred on them by the tumor. Thus,

in cancer, iTreg are directed to down-regulate immune responses against the tumor to enable its progression. These iTreg are a distinct subset of regulatory cells that phenotypically and functionally differ from $FOXP3^+$ nTreg normally in charge of peripheral tolerance. These iTreg need to be depleted or disabled in cancer patients, especially in patients to be treated with immunotherapy. In contrast, depletion of $FOXP3^+$ nTreg probably should be avoided. For oncologic therapies, this may be a difficult and complex challenge, and additional knowledge of cellular and molecular mechanisms underlying interactions of iTreg and nTreg within the tumor microenvironment will be necessary for future success.

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Abbreviations

TGF-β	transforming growth factor beta
nTreg	natural regulatory T cells
iTreg	inducible regulatory T cells
PGE_2	prostaglandin E_2
COX2	cyclooxygenase 2
TIL	tumor infiltrating lymphocytes
CTL	cytotoxic T lymphocytes
NK cells	natural killer cells
DC	dendritic cells
FOXP3	forkhead box p3
CTLA-4	cytotoxic T lymphocyte antigen-4
PD-1	programmed cell death-1
GITR	glucocorticoid-induced tumor necrosis factor receptor
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CRT	chemoradiotherapy
CRC	colorectal cancer
HNSCC	head and neck squamous cell carcinoma
EBV	Epstein Barr Virus
CFSE	carboxyfluorescein diacetate succinimidyl ester
IHC	immunohistochemistry

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