Review

Cancer Research

T-Regulatory Cells: Key Players in Tumor Immune Escape and Angiogenesis

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Abstract

T-regulatory cells (Tregs) are found infiltrating tumors in a vast array of tumor types, and tumor-infiltrating Tregs are often associated with a poor clinical outcome. Tregs are potent immunosuppressive cells of the immune system that promote progression of cancer through their ability to limit antitumor immunity and promote angiogenesis. Here, we discuss the ways in which Tregs suppress the antitumor immune response and elaborate on our recent discovery that Tregs make significant direct contributions to tumor angiogenesis. Further, we highlight several current therapies aimed at eliminating Tregs in cancer patients. Given the multifaceted role of Tregs in cancer, a greater understanding of their functions will ultimately strengthen future therapies. *Cancer Res; 72(9); 2162–71. ©2012 AACR.*

Introduction

The tumor microenvironment is characterized by a multitude of mechanisms that support angiogenesis and immune suppression (1). Many of the immune suppressive regulatory circuits that operate in tumors are part of the physiologic regulatory mechanisms used by the immune system to maintain homeostasis to prevent autoimmunity and temper inflammation after infection or injury (1). T-regulatory cells (Tregs) are considered to be pivotal mediators of peripheral tolerance and immune suppression. Tregs are comprised of natural Tregs, which are thymically derived cells of FoxP3 lineage, and inducible Tregs, which upregulate FoxP3 expression and are derived in the periphery from naïve $CD4^+$ T-cell precursors under tolerogenic conditions (2). Tregs are highly enriched in the tumor microenvironment and are well known for their roles in tumor progression. They are considered to be important for limiting antitumor immune responses and promoting immunologic ignorance (peripheral tolerance) of cancer cells. Recently, we expanded on the roles of Tregs beyond immune suppression in tumors and showed that Tregs are directly involved in promoting angiogenic reprogramming of the tumor microenvironment (3), highlighting a multifaceted role for Tregs in promoting cancer through tumor immune escape and angiogenesis. Thus, we assert that future cancer therapy strategies must take into consideration either the elimination or the functional suppression of Tregs because these cells play an important role in the establishment of aggressive tumor phenotypes.

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Tregs Are Increased in Tumors and Are Correlated with a Poor Prognosis

Woo and colleagues (4) were the first to report an increased number of Tregs in cancer patients. They showed that regulatory $CD4^+CD25^+$ T cells were increased in tumor sites in non–small cell lung and ovarian cancers, and that these cells (now appreciated as Tregs) secreted large amounts of TGF-β that inhibited $CD8^+$ effector T-cell functions *in vitro* (4). An increased number of Tregs has been shown in a multitude of cancers, including melanoma and ovarian, breast, colorectal, lung, and pancreatic cancers [see Zou (5) and references therein]. In a study of ovarian cancer patients, Tregs that were isolated from the tumor site, ascites, or peripheral blood were equally able to suppress tumor-antigen–specific immune responses, suggesting that Tregs contribute to the promotion of ovarian cancer, likely due to their enhanced recruitment or local expansion rather than an enhanced suppressive capacity acquired in the tumor microenvironment (6).

Increased numbers of Tregs in tumors have been associated with poor survival in many solid tumors, including breast cancer (7), gastric cancer (8), and ovarian cancer (6, 9). In ovarian cancer, a low abundance of tumor-infiltrating Tregs can translate into years of added survival, highlighting the importance of these cells for tumor progression (6). However, some groups have identified Treg infiltration to be a biomarker of good clinical outcome [e.g., in colon (10) and ovarian (11) carcinomas], highlighting the complexity of Tregs as a biomarker. We have observed that Treg infiltration increases in proportion to effector T cells in cancer. Thus, Tregs could be associated with improved outcome, if considered as an isolated parameter, possibly reflecting the overall T-cell infiltration, which also predicts improved outcome in colon cancer (12, 13) and ovarian cancer (14). Therefore, of particular importance is the ratio of Tregs to $CD8^+$ effector cells, with a high CD8:Treg ratio representing the best indicator of prolonged survival (9). Mouse models further support the role of Tregs in tumor progression. Depletion of Tregs was shown to facilitate tumor

rejection and induction of antitumor immunity (15, 16), which is associated with a fundamental shift in the tumor microenvironment cytokine milieu (17). Of importance, whereas the transfer of tumor-reactive $CD8^+$ T cells is known to result in tumor elimination experimentally, the cotransfer of Tregs with $CD8^+$ cells abrogates their efficacy in both ovarian cancer and melanoma models (6, 18). Furthermore, Treg depletion *in vitro* allowed for the expansion of NYESO-1-reactive Th1 cells derived from cancer patients (19). Thus, Tregs suppress tumor-specific immunity and significantly affect the course of tumor progression across multiple tumor types.

Mechanisms of Immune Suppression by Tregs

Much of what is known about Tregs in tumor progression is related to their ability to limit antitumor immune responses, resulting in immunologic tolerance and ignorance of the tumor. The 4 best-known mechanisms of immune regulation by Tregs are (i) secretion of soluble or membrane-tethered immunosuppressive molecules, (ii) direct cytolytic activity, (iii) metabolic disruption, and (iv) suppression of dendritic cells (DC) [for an extensive review, see Vignali and colleagues (20)].

Suppressive cytokines and secreted molecules

Chief among the mechanisms of T-cell suppression is the secretion of soluble or membrane-tethered mediators that inhibit effector T-cell functions through cell-contact–dependent and –independent mechanisms. The primary established Treg-derived cytokines that are responsible for this are interleukin (IL)-10, TGF-β, and IL-35, which function by inhibiting the activities of effector T cells (20). Of importance for tumor development, both TGF-β and IL-10 derived from Tregs have been shown to be key mediators that contribute to tumor progression by limiting antitumor immunity (21, 22). These cytokines prevent the expansion, cytokine elaboration (e.g., IFN-γ and TNF-α), and effector functions (cytolysis) of effector cells that are critically important for the control of tumor growth but also polarize DCs toward tolerogenic phenotypes. Our recent discovery that Tregs secrete VEGF (3), a known immunosuppressive molecule, adds to the panel of paracrine mechanisms through which Treg can exert suppression and affect the differentiation and function of DCs.

Cytolysis

An additional mechanism of regulation is the killing of effector T cells or possibly tumor antigen-presenting DCs. Tregs have been shown to exert cytolytic functions by using a variety of mediators, such as granzyme B (23, 24), the TRAIL pathway (25), and galectin-1 (26). The activation of these pathways by Tregs induces apoptosis on target effector cells. Of importance, Cao and colleagues (27) were able to show that Treg-derived granzyme-B and perforin are responsible for the suppression of natural killer (NK) cells and the ability of cytotoxic $CD8^+$ cells to eliminate tumors in multiple models.

Metabolic disruption

Investigators have proposed several mechanisms by which Tregs may be able to inhibit the functions of effector T cells metabolically. Although the idea is controversial, Pandiyan and colleagues (28) suggested that Tregs can essentially "starve" effector cells by depleting local resources of IL-2, leading to effector cell apoptosis. Additionally, it was shown that Tregs catalyze ATP to adenosine through expression of CD39 and CD73, and in turn adenosine suppresses effector T-cell functions (29). Finally, Tregs were suggested to inhibit effector T-cell function by the physical transfer of cAMP through membrane gap junctions (30). The contribution of these mechanisms to tumor immune escape is unknown.

Dendritic cell interactions interactions

Some evidence suggests that Tregs may mediate immune suppression through secondary cell types, with the largest body of evidence supporting deleterious interactions with DCs. Tregs induce DCs through cell–cell–mediated reverse signaling by cytotoxic T-lymphocyte antigen 4 (CTLA-4), expressed on Tregs, and CD80 and/or CD86, expressed on DCs, to upregulate indoleamine 2,3-dioxygenase (IDO) in DCs (31). IDO expression is responsible for the catabolism of tryptophan, which suppresses effector T-cell function by simultaneously depleting essential tryptophan and generating immunosuppressive tryptophan metabolites. Further, Tregs have been shown to reduce the capacity for DCs to activate effector T cells through inhibition of costimulatory molecules, suppression of DC maturation via IL-10/TGF-β signaling, or Treg–DC interactions mediated by lymphocyte-activation gene 3 (32, 33).

Recruitment of Tregs to the Tumor Microenvironment

The increased number of Tregs in tumor sites is probably due to a number of factors. Tumor environments such as ovarian cancer (6) and Hodgkin lymphoma (34) contain large amounts of CC-chemokine ligand 22 (CCL22), which is likely derived from both tumor cells and tumor macrophages. CCL22 can recruit Tregs through CCR4, and Treg migration can be abrogated through CCR4 blockade *in vitro*. Recently, we identified a novel immunosuppressive and angiogenic circuit that establishes a direct role for tumor hypoxia in the recruitment of Tregs in ovarian cancer (3). Hypoxia is a key promoter of tumor angiogenesis and has been previously linked to the infiltration of Tregs in breast cancer (35). We have recently shown that hypoxia upregulates CCL28 in ovarian cancer cells, and CCL28 expression was responsible for the recruitment of $CD4^+CD25^+Foxp3^+$ Treg cells through ligation of the cognate receptor CCR10 expressed on Tregs (3). In ovarian cancer patients, CCL28 expression was correlated with the expression of hypoxia-inducible factor 1α (HIF-1α), which is a poor-prognosis biomarker, and high CCL28 expression in patient tumors was also shown to be an indicator of poor survival. Artificial overexpression of CCL28 in mouse ovarian cancer cells led to enhanced growth of intraperitoneal tumors, as characterized by increased Treg infiltration and increased IL-10 production in the peritoneal ascites (3). Of importance, in the CCL28-overexpressing mouse tumor model, depletion of $CD25^+$ or $CCR10^+$ cells eliminated Tregs from the tumor and

abrogated the tumor growth advantage conferred by CCL28 overexpression. It is possible that numerous additional chemokines regulate Treg recruitment in cancer [see Campbell and Koch (36) for a list of potentially important chemokine receptors], and they may have nonredundant roles in recruiting as-yet-unidentified Treg subsets. In the case of CCL28–CCR10 interactions, recruitment of Tregs to the specific hypoxic environment may serve to enhance their immunosuppressive capacity as part of a biologic program (1, 37), because it has been shown that hypoxia increases the potency of Tregs, and hypoxia-exposed Tregs are more effective at suppressing the proliferation of effector cells (1, 37).

Expansion of Tregs in the Tumor Microenvironment

For the most part, Treg cells can be divided into natural Tregs (nTreg), which are derived from the thymus and maintained peripherally by TGF-β, or inducible Tregs (iTreg), which are induced from naïve $CD4^+$ T-cell precursors and exert suppressive characteristics similar to those observed for nTregs. Both of these Treg subtypes express FoxP3 [a more detailed discussion of this concept can be found in Curotto de Lafaille and Lafaille (2)]. Beyond recruitment of nTregs through chemokines, the tumor microenvironment promotes the continued expansion of nTregs (38) and the generation of iTregs (39) due to a tumor microenvironment that is rich in cytokines such as IL-10 (40), TGF-β (41), and adenosine (42) derived from either the tumor cells or tumor-resident immunosuppressive DCs (43) and $TIE\text{-}2^+$ monocytes (39, 44). These circuits are a reflection of physiologic homeostatic mechanisms that tumors co-opt in tissue-specific and anatomic-compartment–restricted ways. For example, naïve $CD4^+$ cells are converted into iTregs by $CD103^+$ DCs in the mesenteric lymph nodes, a mechanism that helps to maintain gut homeostasis in a Toll-like receptor agonist–rich environment (45).

Tregs in Tumor Angiogenesis

Tumor angiogenesis

Angiogenesis is defined as the sprouting of new blood vessels from preexisting ones. Under physiologic conditions, such as development, angiogenesis occurs in a stepwise manner involving vessel destabilization, endothelial cell migration and proliferation, sprouting, and resolution with vessel stabilization (46). Tumor angiogenesis differs in that the resolution phase generally fails and the vessel network is highly disordered. However, blood vessel development is critical for tumor growth because it provides essential nutrients and growth factors while also providing a conduit for waste, and sustained angiogenesis has long been considered a hallmark of cancer (47).

The accumulation of Tregs at tumor sites has been correlated with biomarkers of accelerated angiogenesis such as VEGF overexpression and increased microvessel density in endometrial (48) and breast cancers (49), providing clinical cues for an association between Tregs and angiogenesis. Tregs can contribute to tumor angiogenesis through both indirect and direct mechanisms. Tregs promote angiogenesis indirectly by suppressing the activities of Th1 effector T cells that release angiostatic cytokines like TNF-α and IFN-γ, as well as interferon-induced chemokines such as CXCL9, 10, and 11. Indeed, Tregs have been shown to promote tumor angiogenesis by specifically inhibiting tumor-reactive T cells (52). However, we have also shown that Tregs can make significant contributions to the direct promotion of tumor angiogenesis (ref. 3, Fig. 1). We showed that tumor hypoxia in ovarian cancer leads to the recruitment of Tregs via CCL28 upregulation (3). The forced expression of CCL28 in mouse ovarian carcinoma resulted in striking increase of *in vivo* tumor growth. CCL28 expression also resulted in robust Treg accumulation, increased VEGF levels, and significantly increased blood vessel development. Of importance, depletion of $CD25^+$ or $CCR10^+$ cells eliminated Treg cells from the tumor microenvironment and significantly suppressed VEGF expression and angiogenesis at these sites (3). We showed that $CD4^+CD25^+$ Treg cells secreted higher amounts of VEGF in the steady state as well as under hypoxic conditions when compared with $CD4^+CD25^-$ T cells, and media conditioned by Tregs in hypoxia promoted capillary tube formation *in vitro*, an effect that was dependent on VEGF signaling. Further, using an entirely cell-free Matrigel implant, we showed that supernatants of hypoxic Tregs were able to significantly promote angiogenesis *in vivo* (3). Our results are supported by early observations that T cells exposed to hypoxia express VEGF, and T cells within tumors express VEGF (53). Thus, we established a new mechanism whereby tumor hypoxia recruits Tregs to tumor sites, leading to substantial, direct contributions to the proangiogenic tumor microenvironment.

Tregs as Targets for Cancer Immunotherapy

On the basis of the information provided above, it is apparent that Tregs make important contributions to tumor immune escape and have newly described functions in angiogenesis. Therefore, the elimination of Tregs in cancer patients, and particularly within the tumor microenvironment, should be considered to be an essential component of any successful cancer therapy. Several available therapeutics can either reduce the number of Tregs or disrupt their functions. Of interest, several chemotherapeutic drugs that interfere with Tregs, such as methotrexate and cyclophosphamide, have well-described immunostimulatory and antiangiogenic effects in cancer patients.

Nonspecific targeting of Tregs

A number of commonly used chemotherapeutics have been shown to reduce either the number or the immunosuppressive capacity of Tregs. These drugs include antimitotics such as cyclophosphamide, gemcitabine, mitoxantrone, and fludarabine, as well as thalidomide analogues and cyclooxygenase 2 (COX-2) inhibitors. Thus, it is intriguing to speculate that these drugs have some off-target antitumor effects mediated through the modulation of Tregs.

Cyclophosphamide has been shown to preferentially deplete $CD4^+CD25^+$ Tregs in rats, and in one study (54), only a single injection prior to tumor challenge with a rat colon cancer line was sufficient to delay tumor growth. Cyclophosphamide

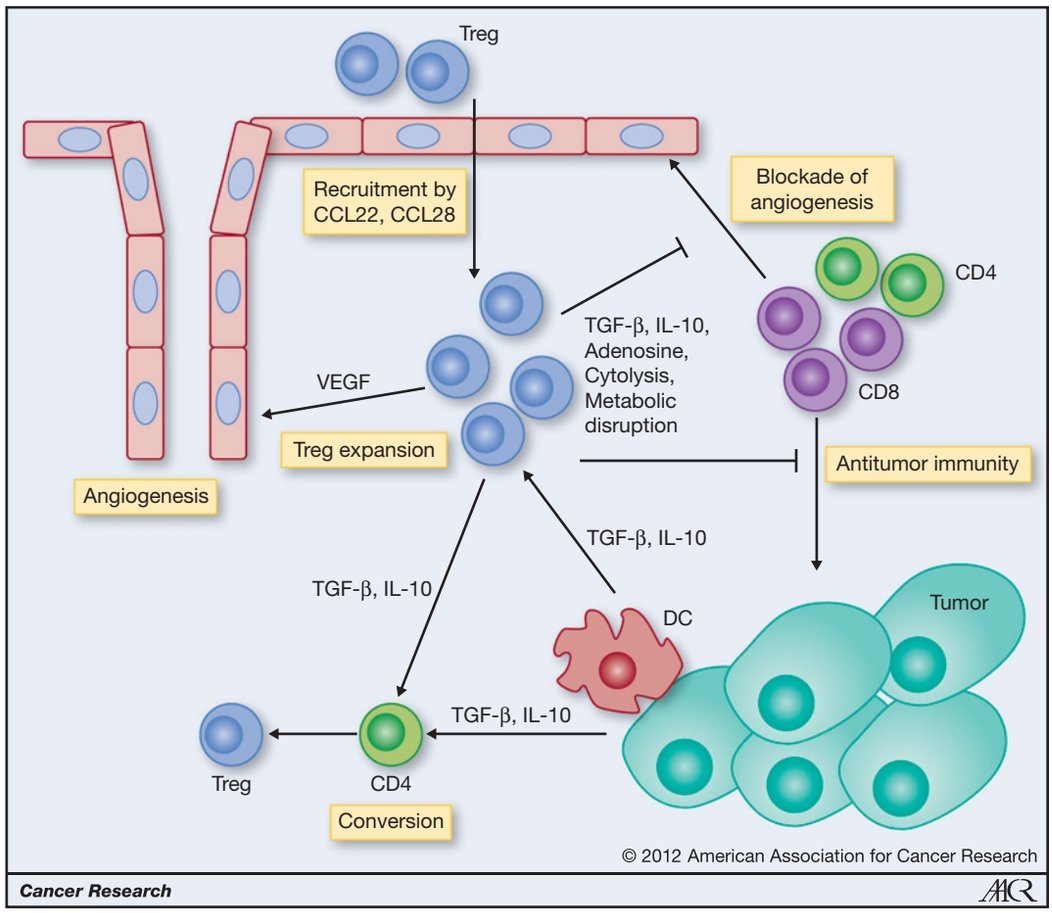

Figure 1. Role of Tregs in tumor progression. Tregs are recruited to tumors from the periphery by tumor-derived, hypoxia-induced CCL28, but also by DC and tumor-derived CCL22. Within the tumor microenvironment, Tregs can be expanded by TGF-β and possibly IL-10, which can also convert $CD4^+$-naïve precursors into induced Tregs. Tregs promote tumor progression by direct inhibition of antitumor effector $CD4^+$ and $CD8^+$ T cells through inhibitory cytokines, cytolysis, and metabolic disruption. Further, Tregs that have been recruited to hypoxic areas directly stimulate angiogenesis through production of VEGF. Tregs also indirectly encourage angiogenesis by blocking effector-cell–derived angiostatic cytokines such as IFN-γ and CXCL-10.

alkylates DNA, resulting in crosslinks between (interstrand) and within (intrastrand) DNA strands, which leads to cell death, and it has been suggested that Tregs are more sensitive to cyclophosphamide-induced apoptosis (55). For example, Ercolini and colleagues (56) showed that low-dose cyclophosphamide given to HER-2/neu transgenic mice with HER-2/neu-expressing mammary tumors selectively depleted Tregs that were progressing through the cell cycle. Of interest, in untreated tumor-bearing mice, Tregs were shown to be the predominant cycling T-cell population, although fewer than half of the $Foxp3^+CD4^+CD25^+$ Treg cells were cycling and thus susceptible to depletion by cyclophosphamide. Redmond and colleagues (57) reported that effector cells that are in the process of being tolerized or deleted are also cycling and proliferating, indicating that depletion of cycling cells with cyclophosphamide may be beneficial for clearing out tolerizing cells. Further, low-dose cyclophosphamide also disrupts the homeostatic proliferation of Tregs and decreases their immunosuppressive functionality by decreasing FoxP3 and glucocorticoid-induced TNF receptor (GITR; ref. 55). Recent evidence also suggests that inhibition of Treg function by low-dose cyclophosphamide may be the result of selective depletion of intracellular stores of ATP caused by increased surface expression of CD39 (an ATP-to-adenosine conversion enzyme; ref. 58). However, in one study (59), cyclophosphamide was shown to deplete effector T cells in addition to Tregs. Therefore, cyclophosphamide disrupts Tregs via a multitude of mechanisms, but it may also have unintended effects on tumor-reactive effector T cells.

Two drugs that inhibit DNA synthesis, fludarabine and gemcitabine, have also been shown to disrupt Tregs. Fludarabine administration as a standard 5-day course induces lymphopenia, which has been shown to be favorable for patients with chronic lymphocytic leukemia and other hematologic malignancies. In a clinical setting, fludarabine treatment of chronic lymphocytic leukemia resulted in surprising increases of Treg apoptosis and decreases of Treg inhibitory functions (60). Further, fludarabine blocked the expansion of IL-10–producing $CD4^+$ Tregs *in vitro*, which was associated with higher numbers of antigen-specific CTLs (61). In a phase I study in non–small cell lung cancer, gemcitabine

administration induced lymphopenia with a decrease in effector T-cell populations (62). However, another phase I study of patients with colon cancer showed that gemcitabine caused an increase in CTLs with a concomitant decrease in $CD25^+CD4^+$ T cells in clinical responders (63). Although both studies showed positive results, the direct effects of this drug on lymphocyte populations is unknown. Gemcitabine affects a variety of immunosuppressive cells, including myeloid-derived suppressor cells (MDSC). When given at a clinically equivalent dose, gemcitabine resulted in a dramatically reduced number of MDSCs in animal spleens, accompanied by an increase in the antitumor activity of $CD8^+$ T cells and activated NK cells (64). In light of the observation that MDSCs are capable of converting naïve CD4 cells to Tregs, it is entirely plausible that gemcitabine limits Tregs through its effects on MDSCs (65). Although both fludarabine and gemcitabine show little to no direct specificity for Tregs, it is possible that a particular dosing regimen could provide optimal disruption of Tregs while concomitantly inhibiting tumor growth.

Thalidomide and thalidomide derivatives have been used in the treatment of various nonmalignant diseases, including cutaneous and systemic inflammatory disorders (66–68). Lenalidomide is a U.S. Food and Drug Administration (FDA)–approved oral thalidomide analogue that is used to treat multiple myeloma and low- to intermediate-risk myelodysplastic syndrome caused by deletion of chromosome 5q (5q syndrome). Lenalidomide induces myeloma cell apoptosis directly and indirectly by inhibition of bone marrow stromal cell support, by antiangiogenic and antiosteoclastogenic effects, and through immunomodulatory activity. Lenalidomide has a broad range of immunomodulatory properties that can be exploited to treat many hematologic and solid cancers. Lenalidomide inhibits human Treg cell proliferation in response to IL-2 and downregulates FoxP3 expression (69). It was also shown to significantly reduce Treg cells in mouse lymph nodes (69). In a recent clinical trial in chronic leukocyte leukemia, the administration of lenalidomide resulted in a decrease of Tregs and an increase of Th17 cells in peripheral blood (70), supporting a potential role for thalidomide analogues in the elimination of Tregs in patients (69). Of importance, lenalidomide has also been shown to exert costimulatory effects on T cells and enhance T-cell proliferation, effector function (71–73), and Th1 reprogramming (74), and in combination therapy it has augmented tumor lysate vaccines (75).

COX enzymes, and particularly COX-2, are known to contribute to many facets of tumor progression. Patients who take a nonsteroidal anti-inflammatory drug (NSAID), such as aspirin, are significantly less likely to develop colorectal cancer (76, 77), and several investigators have shown an adjuvant property of COX-2 inhibitors in combination with cancer vaccines (78–80). Experimentally, COX-2 inhibition was shown to reduce Treg cell frequency and suppressive activity, attenuate FoxP3 expression in tumor-infiltrating lymphocytes, and decrease tumor burden *in vivo* (81). In patients with colon cancer, treatment with an oral NSAID significantly increased $CD8^+$ tumor-infiltrating T cells and decreased expression of FoxP3 and IL-10 (82). Patients with colorectal cancer have increased concentrations of prostaglandin E_2 (PGE_2) in the peripheral blood, and although tumors express large amounts of PGE_2, it has been shown that Treg cells also express COX-2 and produce PGE_2 in a manner that suppresses effector T cells. A role for Treg-derived PGE_2 in immune suppression is supported by the observation that indomethacin (a COX-2 inhibitor) reverses Treg-mediated antitumor suppression *in vitro* (83).

Specific targeting of Tregs

In light of the important role played by Tregs in different kinds of tumors and other pathologies, investigators have developed several compounds (often depletion antibodies) that directly target Tregs through recognition of Treg markers such as CD25, CTLA-4, and GITR.

A large number of Treg-targeting strategies rely on specific recognition of CD25. In several mouse tumor models, $CD4^+CD25^+$ Treg depletion via antibodies targeting CD25 produced significant antitumor activity, although it was often associated with an increased incidence of autoimmunity. Combinatorial approaches using monoclonal antibodies and vaccines have been investigated in murine models, and the positive results of these preclinical studies clearly highlight the potential of the Treg-depletion approach in cancer immunotherapy (84). In an early phase I clinical trial in patients with metastatic breast cancer, the anti-CD25 antibody daclizumab significantly depleted Treg cells and enhanced the immunogenicity of a cancer vaccine. Five of the 10 patients who received the vaccine had stable disease for several months (85, 86). However, in another study using daclizumab in combination with a DC vaccine, investigators noted a detrimental role of daclizumab treatment, which may have been due to the timing of administration (87).

Denileukin diftitox (Ontak; Esai, Inc.) is a fusion protein of human IL-2 and diphtheria toxin. The IL-2 portion of the fusion protein binds preferentially to cells expressing intermediate- to high-affinity IL-2 receptors (IL-2R) comprised of IL-2Rα(CD25)/β(CD122)/γ(CD132) subunits or IL-2Rβ/γ subunits, and results in cell death by interfering with protein synthesis following endocytosis. Denileukin diftitox was shown to be efficacious in advanced chronic T-cell lymphoma with high CD25 expression, where high CD25 expression is associated with clinical response to denileukin diftitox (88). In patients with melanoma, the application of recombinant Ontak significantly but transiently reduced the frequency of Tregs in peripheral blood. However, another study that evaluated the treatment of melanoma patients with denileukin diftitox failed to show any Treg depletion or clinical benefit (89).

LMB-2 is a fusion protein that is obtained by fusing a single-chain variable fragment antibody (scFv) against CD25 to *Pseudomonas* exotoxin A. *In vitro*, treatment of human peripheral blood mononuclear cells (PBMC) with LMB-2 resulted in specific $CD4^+CD25^+$ Treg depletion (90). In a phase I clinical trial, treatment of $CD25^+$ T-cell malignancies with a dose of >60 μg/kg of LMB-2 showed encouraging results, with 8 objective responses in a cohort of 20 patients, indicating that LMB-2 is efficacious in patients (91). In patients with melanoma, LMB-2 administration in combination with peptide vaccination

showed a significant decrease of $FoxP3^+CD4^+CD25^+$ Tregs in peripheral blood; however, the effect was transient and the quantity of Tregs returned to pretreatment levels within days. As might be expected, there was no objective clinical response (92). Thus, the utility of LMB-2 is not yet clear.

When CTLA-4 was first cloned in 1987, it was not clear whether CTLA-4 was involved in stimulatory or inhibitory pathways in T cells. However, the generation of CTLA-4 knockout mice allowed investigators to solve this riddle, because the knockout mice developed a progressive and uncontrolled accumulation of activated T cells and died of lymphoproliferative disease (93). The seminal study by Leach and colleagues (94) showed that CTLA-4 blockade could attenuate the growth of several implanted murine tumors, and the mechanism of inhibition was immune mediated. CTLA-4 is expressed on the surface of Tregs, but blockade could actually expand functionally suppressive Tregs (95). Although various CTLA-4 blockade therapies reduce tumor-infiltrating Tregs (84), this effect may be due entirely to the ability of CTLA-4 blockade to promote the generation of memory and promote effector T-cell functions (96).

So far, 2 humanized anti-human CTLA-4 neutralizing antibodies, MDX-010 (ipilimumab) and CP-675206 (tremelimumab), have been tested in phase I–III trials. The first phase I clinical trial with anti-CTLA-4 blocking antibody was carried out in 2002 at the University of California, Los Angeles and The University of Texas MD Anderson Cancer Center. The majority of enrolled patients had measurable metastatic melanoma. This trial tested doses ranging from 0.01 to 15 mg/kg in 7 cohorts. Objective tumor responses were noted in a subset of patients starting at a dose of 3 mg/kg and becoming more frequent at 15 mg/kg (97). Of interest, supporting the immune-modulatory effects of CTLA-4, treatment of metastatic melanoma patients with ipilimumab resulted in tumor regression in 36% of the patients and was associated with autoimmune toxicity, but patients without autoimmune toxicity were less likely to experience tumor regression (98). A further trial combining high-dose IL-2 and varied doses of ipilimumab showed synergy compared with earlier studies that evaluated IL-2 alone in metastatic melanoma (99). Further analysis of PBMCs in patients undergoing anti-CTLA-4 treatment for stage IV metastatic melanoma and renal cell carcinoma revealed, by *in vitro* coculture, no inhibition of the suppressive activity of $CD4^+CD25^+$ T cells per se, but a probable enhancement of effector T-cell function. The results of a phase III clinical trial that included 502 untreated patients with metastatic melanoma were recently reported (100). Ipilimumab (10 mg/kg) in combination with dacarbazine, as compared with dacarbazine plus placebo, improved overall survival in the patients, leading to its FDA approval for the treatment of metastatic melanoma (100). Tremelimumab has been shown not only to suppress Treg activity but also to induce expansion of effector and memory $CD4^+$ and $CD8^+$ T cells, with antitumor efficacy (101). Thus, it has been suggested that depletion of Tregs may be of secondary importance for modulating the ratio of $CD8^+$ effector cells to Tregs, which may be mediated through direct interactions of anti-CTLA-4 antibody with effector cells.

GITR is a TNF receptor family member that is expressed at low levels on resting $CD8^+$ and $CD4^+Foxp3^-$ T cells but is constitutively expressed at high levels on $CD4^+CD25^+Foxp3^+$ Tregs (102). Treg cells express even higher levels of GITR in tumors than elsewhere (103). Although it may not affect systemic Treg, GITR ligation specifically depletes Tregs in tumors, increasing tumor Teff/Treg ratios (104). DTA-1, a GITR agonistic Ab, may disable Treg, depletes intratumoral Treg, and enhances T-cell immunity against tumors (105–108). Of importance, it also costimulates $CD4^+$ and $CD8^+$ T-cell proliferation and effector functions, renders Teff cells resistant to Tregs, and enhances a variety of T-cell responses (109, 110). However, some studies have shown that DTA-1 does not affect the number or functions of Tregs. In one study (104), administration of an agonistic antibody prevented the infiltration of Tregs into the tumor microenvironment, promoting a high CD8:Treg ratio and resulting in the control of tumor growth in mice. Thus, although targeting Tregs through GITR is an interesting approach, it may require additional therapeutics to promote systemic antitumor immune responses.

We believe our recent studies have added a new possible target to this list: CCR10. Tumor hypoxia induced CCL28 expression, leading to the recruitment of $CCR10^+$ Tregs, whereas the depletion of $CCR10^+$ cells by an anti-CCR10 immunotoxin resulted in complete Treg depletion and loss of the tumor growth advantage conferred by CCL28 overexpression (3). Further, it appears that CCR10 expression on Tregs is associated with a peripheral homing phenotype, and is a marker of highly suppressive Treg cells (111). Although CCR10 expression is not entirely restricted to Tregs, $CCR10^+$ cell depletion has been shown to be beneficial in a mouse model of ovarian cancer. Thus, CCR10 is an attractive new target for disrupting Tregs in cancer.

Conclusions

On the basis of the information presented above, it should be apparent that Tregs are instrumental in establishing tumor immune tolerance and are important cellular mediators of tumor progression in patients. In our recent work we expanded this view and showed that, in addition to immune suppression, Tregs can make significant contributions to the direct promotion of tumor angiogenesis. Thus, we believe that Tregs are key orchestrators of tumor development, linking immune suppression and angiogenesis in one biologic program. This highlights the need to specifically target these cells to promote antitumor immunity and tumor regression. Indeed, reducing the functions and/or numbers of Tregs in patients with cancer should allow more effective immune-based therapies, alone or in combination with traditional chemotherapeutics. Here, we have presented numerous preclinical and clinical data that support the notion that the elimination of Tregs should be considered crucial for many cancer therapies. However, a major therapeutic challenge remains because of the paucity of tools available to target Tregs effectively in the clinic. The effort to unravel the complexity of Tregs is only just beginning, and a further understanding of their biology and

characterization of targets will undoubtedly enhance future therapeutic opportunities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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