Introduction

Optimism has been renewed that many cancers can be cured or forestalled by immune-based therapies, used either alone or as part of multimodal programs. This optimism originates from an improved understanding of tumor immune interactions and the availability of gene, cell, and ligand-based technologies that promote effector antitumor responses. Most tumors develop in the face of normal immune function, and antitumor responses of varying strength result. A strong immune response against the primary tumor is associated with clearance and induced dormancy of metastatic cancer cells, with a resulting enhanced prognosis. Conversely, global immune deficiencies secondary to disease or therapy are associated with an increased frequency, earlier recurrence, more rapid progression of tumors, and poorer prognosis. Responses to chemotherapy and oncolytic virotherapy may, in part, be immune determined, and evidence is persuasive that an intact immune system, specifically determined by CD4+ T cells, is required for sustained tumor regression following oncogene inactivation therapies (1).

Adaptive antitumor immune responses are durable, tumor antigen specific, and acquired through the integrated cellular responses of the innate and adaptive immune systems (Fig. 1, center; ref. 2). Tumor-infiltrating T cells, especially CD8+ CTLs and IFN-γ-secreting CD4+ (Th1) cells, are central to effective immune containment. Adaptive immune responses are initiated when cells of the innate immune system [natural killer T (NKT), γδ T, NK, and macrophages] are recruited to the tumor microenvironment; the continued process of tumor remodeling results in the shedding of cancer cells and debris with a consequent induction of inflammatory signals. The production of IFN-γ (initially from NK and NKT cells) seems critical because it creates a positive feedback loop by inducing some tumor cell death, the further activation of NK cells and macrophages, and the production of chemokines and cytokines, which are also tumoricidal and antiangiogenic. Immature dendritic cells (DC) are activated following uptake of tumor debris and/or antigens and migrate to the regional lymph nodes where they present the tumor antigens to naïve T cells, which can differentiate into Th1, Th2, Th17, or regulatory T cells (Tregs), depending on the cytokine environment. Th1 cells can license DCs to induce tumor-specific CTLs by cross-presentation of antigen on MHC class I. Antigen-specific CD8+ T cells traffic to the tumor, where cell-mediated killing of tumor cells is augmented by Th1- and Th17-derived cytokines. However, these effector responses can be inhibited by Tregs, induced by or recruited to the growing tumor (3).

Immune evasion is a hallmark of cancer, resulting from both passive and active tolerizing conditions that subvert antitumor immune responses (4). Passive tolerization may result from downregulation of MHC class I expression on the tumor cells and/or low antigenicity secondary to immune editing and selective cell growth. Other tolerizing mechanisms involve inhibition of immune cells in the tumor domain by depletion of tryptophan by the enzyme indoleamine 2,3-dioxygenase (IDO). Active tolerization involves suppression of antitumor cell-mediated responses by tumor-infiltrating Tregs and myeloid-derived suppressor cells (5).

Overall, Tregs are considered the most powerful inhibitors of antitumor immunity and the greatest barrier to successful immunotherapy (6). In the early stages of cancer, Tregs are concentrated in the tumor mass, resulting in concomitant immunity, whereby the primary tumor can progress because of local inhibition of effector immune responses, but metastatic cells are eliminated by uninhibited systemic

Abstract

Infiltration of tumors by regulatory T cells confers growth and metastatic advantages by inhibiting antitumor immunity and by production of receptor activator of NF-κB (RANK) ligand, which may directly stimulate metastatic propagation of RANK-expressing cancer cells. Modulation of regulatory T cells can enhance the efficacy of cancer immunotherapy. Strategies include depletion, interference with function, inhibition of tumoral migration, and exploitation of T-cell plasticity. Problems with these strategies include a lack of specificity, resulting in depletion of antitumor effector T cells or global interruption of regulatory T cells, which may predispose to autoimmune diseases. Emerging technologies, such as RNA interference and tetramer-based targeting, may have the potential to improve selectivity and efficacy. Cancer Res; 71(22): 6915–20. ©2011 AACR.
antitumor immune responses. In advanced-stage disease or for poorly immunogenic cancers, the number of Tregs is increased systemically, and there is an absence of concomitant antitumor immunity (7). Although a correlation between increased Treg number and survival, either negative or positive, remains equivocal, the ratio of Treg to effector T cells (Teffector) in the tumor mass seems to have greater prognostic significance (8).

Tregs can be classified into a number of subtypes (8), including natural CD4<sup>+</sup> Tregs (nTreg), which originate in the thymus, express CD25, FOXP3, CTLA-4, LAG3, and GITR, and suppress innate and adaptive immune cells. Induced CD4<sup>+</sup> Tregs (iTreg) control immune responses to tissue antigens, including tumor antigens, and include CD4<sup>+</sup> nTreg-like, Tr1, and Th5 cells that suppress through production of interleukin (IL)–10 and TGF-β. Their cell-surface markers are often indistinguishable from those of nTreg<sup>+</sup> and they differ principally in their mechanism of suppression. Although less well characterized, there are also populations of natural and induced CD8<sup>+</sup> Tregs.

Although the field is still in its infancy, evidence is emerging that inhibition of Treg<sup>+</sup> may help in tumor containment, especially when combined with appropriate immunotherapies that activate effector T cells. Systemic Treg depletion in patients induced regression of melanoma metastases (9), and in mice, when combined with immunogene stimulation of intratumoral immune effector cells, resulted in cure of 90% of animals who had large and weakly immunogenic sarcomas (10). The clinical objective will be to provide sustained reduction of Treg<sup>+</sup> function, particularly in the tumor environment, allowing enhancement of antitumor effector functions and with minimal risk of developing systemic autoimmune diseases.

Figure 1. Targeting Tregs in cancer. The main events involved in mounting an immune response to a tumor. Cells of both the innate and adaptive systems contribute (further details provided in the text). Tregs offer substantial resistance to this immune assault and, thus, 4 different approaches for reducing their immunosuppressive contribution are proposed. A, depletion; B, inhibition of function; C, blockade of trafficking; and D, modulation of T-cell plasticity. Within each approach, numerous existing and novel options for therapeutic manipulation are proposed. Ab, antibody; MDSC, myeloid-derived suppressor cell.
Current Approaches to $T_{\text{reg}}$ Modulation

Regulatory T-cell depletion

Depletion strategies are not specific to the T-cell subset, but they have a selective advantage when the $T_{\text{reg}}$ accumulation provides functional dominance in the tumor environment (Fig. 1A). $T_{\text{reg}}$-depletion strategies have focused on monoclonal antibodies or ligand-directed toxins targeted to cell-surface receptors, such as CD25. Daclizumab and basiliximab are anti-CD25 antibodies that induce cell death by cytokine deprivation (IL-2) and by triggering antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity. Results from an ongoing clinical trial have shown that daclizumab reduces $T_{\text{reg}}$ and, thereby, enhances CTL responses to tumor antigen induced by vaccination (11).

Denileukin diftitox is a fusion protein of human IL-2 and the enzymatically active and membrane-translocating domains of diphtheria toxin. After binding to CD25 and internalization, release of the toxin is cytotoxic. Clinical data on the use of denileukin diftitox for alternative indications have led to its application for CD25 targeting of $T_{\text{reg}}$ and the emergence of similar CD25-targeted immunotoxins LMB-2 and RFT5-SMP7-dgA. With one exception, denileukin diftitox depleted $T_{\text{reg}}$ numbers, albeit transiently, with $T_{\text{reg}}$ nadirs persisting for less than 3 weeks (11). The $T_{\text{reg}}$ elimination was mirrored by a concomitant increase in the prevalence of IFN-γ+CD3+ T cells in the blood and de novo appearance of melanoma antigen-specific CD8+ T cells (9). However, the clinical benefits were modest. Regression of melanoma metastases in 5 out of 16 patients represents the most promising outcome (9). Consistency of response is an issue, as 2 patients who developed antigen-specific T cells failed to show any tumor regression, and another study in patients with melanoma failed to yield a single objective clinical response (11).

Denileukin diftitox is the subject of numerous clinical trials, but to date, it fails to realize its clinical promise. Because CD25 is expressed on activated T-effector cells, denileukin diftitox may also restrain protective antitumor immune responses. Denileukin diftitox transiently depleted various T subsets, including tumor antigen-specific CD8+ T cells (9). Its indiscriminate effects on CD4+CD25− cells are difficult to rationalize.

Low-dose oral metronomic cyclophosphamide induced a profound, selective reduction in $T_{\text{reg}}$ and restored T- and NK-cell function in patients with advanced cancer (12). This restoration of T- and NK-cell function invoked temporary disease stabilization in a number of patients without clinical improvement. The mechanism underpinning its selective toxicity toward $T_{\text{reg}}$ is unexplained. Metronomic cyclophosphamide also has antiangiogenic and direct cytotoxic effects that contribute to tumor stabilization or shrinkage.

Depleting $T_{\text{reg}}$ may have further consequences aside from an unintended treatment-mediated elimination of activated T-effector cells (13). Their depletion leads to an increase in tumor-mediated T-effector to $T_{\text{reg}}$ conversion, with a diminution in antitumor immune responses, which does not seem to occur with the other $T_{\text{reg}}$ modulation approaches.

Suppression of $T_{\text{reg}}$ function

Similar to CD25, CTLA-4 is not exclusively expressed on $T_{\text{reg}}$; it is also found on activated CD4+ and CD8+ T cells (Fig. 1B; ref. 8). CTLA-4 inhibits antigen priming of T-effector, by competing with CD28 for the costimulation of CD80/CD86 on antigen-presenting cells (APC). Furthermore, it induces IDO in DCs (14). The consequent depletion of tryptophan and production of tryptophan metabolites, such as kynurenines and picolinic acid, inhibit T-effector proliferation and function. The anti–CTLA-4 antibodies ipilimumab (MDX-010) and tremelimumab (CP-675206) are currently undergoing clinical evaluation. Ipilimumab, as monotherapy or in combination with peptide vaccination, improved survival in patients with previously treated metastatic melanoma (15). Tremelimumab promotes antitumor responses, but recently these have been shown to result from T-effector activation rather than $T_{\text{reg}}$ modulation (16). This finding may also be true of ipilimumab, because its clinical mode of action has yet to be fully defined and could be ascribed to direct effects on $T_{\text{reg}}$, or T-effector, or both.

The glucocorticoid-induced TNF receptor (GITR) is constitutively expressed on $T_{\text{reg}}$ and at lower levels on activated T-effector cells. Intratumoral injection of an agonistic antibody to GITR (DTA-1) invoked potent antitumor immunity and eradicated established tumors in mice (17). The exact mechanism by which this approach achieves its effects is controversial. One study showed that the benefit of DTA-1 was $T_{\text{reg}}$ mediated, facilitated by their selective modulation (18). However, a more recent study suggested T-effector costimulation as the predominant outcome (19). Regardless of mechanism of action, GITR approaches have yet to recapitulate these promising findings in humans. Receptor activator of NF-κB (RANK) ligand (RANKL) expression on $T_{\text{reg}}$ engages the RANK receptor on cancer cells and promotes metastases (20). Inhibitors of RANK signaling, such as the anti-RANKL antibody denisumab, already used against osteoclastic-mediated bone resorption, may block direct $T_{\text{reg}}$-induced metastases of certain cancers.

Targeting FOXP3, the essential transcription factor of $T_{\text{reg}}$, by RNA interference (RNAi) could also modulate their function. Lentiviral-mediated delivery of miR-31 (a negative regulator of FOXP3) to $T_{\text{reg}}$ abolished their suppressor capability (21). Translation to clinical application is challenging, as miR-31 would need to be delivered specifically to $T_{\text{reg}}$, because FOXP3 is also transiently expressed on activated human T-effector. FOXP3 is also expressed (both mRNA and protein) in numerous cancer cell lines (22), but the effects of its downregulation are unknown and could even be counterproductive.

Further options for disrupting $T_{\text{reg}}$ function include Toll-like receptor (TLR) modulation, OX40 stimulation, or interference with the adenosinergic pathway. Exposure of $T_{\text{reg}}$ to the TLR8 ligand poli-G10 abolished their suppressive influence on CD8+ T cells, leading to improved antitumor immunity (23). More recently, a synthetic TLR1/TLR2 agonist, an analogue of bacterial lipoprotein, mediated a dose-dependent tumor regression and a long-lasting protective response against tumor rechallenge through a reciprocal downregulation of $T_{\text{reg}}$ and upregulation of CTL function (24). These findings suggest that
TLR signaling is a worthwhile pursuit, but caution is advised because TLR agonists can promote regulatory as well as effector responses (25). Stimulation of OX40 (a costimulatory member of the TNF receptor family) inhibits the suppressive function of Treg, in vitro (by downregulation of FOXP3) and abolishes protection against graft-versus-host disease in mice (13). The paradoxical stimulatory effects on T effector make it an enticing target for cancer immunotherapy. Another potential target on Treg is ectonucleotidase activity, which facilitates local generation of adenosine, which has immunosuppressive capability. Ectoenzyme inhibitors, such as ARS67156, and other modulators of the adenosinergic pathway, such as inhibitors of the A2A adenosine receptor, have been shown to block Treg-induced immunosuppression (26).

**Disrupting tumoral homing of regulatory T cells**

Chemokine–chemokine receptor and integrin–integrin ligand interactions attract Treg, to the tumor, a phenomenon first observed for the CCL22–CCR4 interaction in ovarian cancer (Fig. 1C; ref. 27). Importantly, CCL22 expression was not confined to tumor cells but also included stromal cells, such as tumor-associated macrophages. Further chemokines and/or integrins have been implicated in the selective recruitment and retention of Treg at tumor sites, including CXCR4, CD103, and CCR2 (8). Because chemokatraction is ubiquitous in the immune system, efforts to block Treg recruitment to the tumor mass may be limited by the concurrent effects on T effector. Nevertheless, disruption of CCR5/CCL5 signaling blocks Treg migration to tumors and inhibits pancreatic tumor growth in mice (28). Methyl gallate has also recently been shown to inhibit infiltration of Treg in tumors, resulting in reduced tumor growth and prolonged survival rates (29).

Immunostimulatory therapies may inadvertently promote tumoral homing of Treg. Therapy with IL-2 can enhance CCR4 expression on Treg, which stimulates their migration to the tumor mass and an upregulation of CXCR4, the receptor for CXCL12, a chemokine linked to development of organ-specific metastases (8). These findings endorse a more prudent use of IL-2 or perhaps its use in combination with agents such as AMD-3100, which antagonize the CXCR4–CXCL12 interaction.

**Exploiting T-cell plasticity**

The origins of iTreg within the tumor microenvironment are diverse, because varying degrees of plasticity exist within the helper CD4+ T-cell population (Treg, Th1, Th2, Th17, Thf; Fig. 1D; ref. 30). Predifferentiated Treg may migrate under the influence of chemokines (27), and Treg may arise from de novo generation via differentiation and expansion or may derive from conversion of CD4+CD25- T cells. The plasticity inherent in each of these processes is a potentially exploitable therapeutic niche.

IL-6 is central to T-cell plasticity (30). It helps to convert FOXP3+ Treg into IL-17–secreting T cells (Th17). It potently abolishes conversion of conventional T cells into iTreg and in its absence, no other cytokine can substitute for this inhibition. Thus, IL-6 merits further investigation as a therapeutic for cancer. TGF-β acts at the axis between Treg and Th17 differentiation, enhancing the function of FOXP3 and inhibiting the function of RORγt, their essential transcription factors, respectively. TGF-β–induced FOXP3 expression is inhibited by proinflammatory cytokines (e.g., IL-6 and IL-21) in a STAT3-dependent manner. Thus, STAT3 may also represent a therapeutic option; indeed, forced expression of STAT3 augmented IL-17 production, most likely through increased RORγt expression (30). Redirecting differentiation toward a Th17 phenotype might also be achieved by direct introduction of RORγt, as this has been shown to induce IL-17 expression upon transduction of naive CD4+ T cells (30). Conversely, selective methylation at the FOXP3 locus would likely hinder differentiation along a suppressor pathway. Aside from the epigenetic level, targeting FOXP3 at the mRNA and protein levels would also be worthwhile. Other approaches include antagonists for retinoic acid receptors that facilitate differentiation into Th17 cells over Treg (30). Treg differentiation can be redirected toward lineages other than Th17. Specific inactivation of the transcription factor IFN regulatory factor 4 elevates Th2 cytokine production, whereas IL-4–driven growth factor independent 1 facilitates optimal Th2 differentiation (30).

Blocking Treg proliferation is an obvious goal, which can be achieved by direct inhibition of TGF-β, inhibition of IDO directly with 1-methyl-1-tryptophan, or inhibition of IDO indirectly by CTLA-4 blockade. Aside from directly stimulating Treg expansion, COX-2–derived PGE2 facilitates tolerogenic APC-led Treg recruitment and is itself a functional instrument of Treg, in certain tumors (8). Thus, use of COX-2 inhibitor like celecoxib may be justified. Alternatively, bevacizumab or blockade of PDI-L on Treg with MDX-1106 (phase II) may halt Treg proliferation.

Inhibiting the peripheral conversion of CD4+CD25+ T cells into CD4+CD25- Treg may be a useful therapeutic approach. The TGF-β–blocking antibody 1D11 abolished this conversion and reduced tumor burden in mice. Subsequently, other TGF-β modulators, including antibodies, soluble TGF-β receptors, and the antisense oligonucleotide AP-12009, have reached phase I and II clinical trials. However, systemic TGF-β blockade may carry the risk of developing autoimmune disorders. Furthermore, under immunosuppressive conditions, T-cell conversion can occur in the absence of TGF-β; IL-10 and IDO have also been shown to promote induction of Treg (8).

**Novel Approaches to Treg Modulation**

The multitude of strategies discussed in this review deliver only marginal efficacy. Although some strategies have lacked potency, the majority flounder on specificity. This dearth of specificity is understandable, given the intersecting differentiation pathways shared by all cells of the T-cell lineage. Selective approaches to Treg modulation are warranted. Simple depletion of Treg may be naive and the benefit short-lived, whereas inhibiting their migration to the tumor ignores the in situ generation of these cells. Thus, strategies focused on negating Treg function or reprogramming their functional phenotype would seem more meritorious.

A unique cell-surface marker that facilitates selective targeting of Treg has yet to be uncovered. Thus, targeting CD25 or CTLA-4 has been encumbered by a concomitant effect on...
Introducing a second layer of specificity, so-called dual specificity, to receptor targeting would likely be synergistic. This strategy is under investigation in our laboratory, whereby a relatively \( T_{reg} \)-specific gene therapy approach is coupled to ligand selectivity.

A global \( T_{reg} \) modulation is undesirable because it may increase susceptibility to autoimmunity. Tumor \( T_{reg} \) could be targeted via their antigen-specific TCRs; antigen-specific \( T_{reg} \) engaged melanoma-expressed LAGE1 and ARTC1 (31), and in patients with colorectal cancer, carinoembryonic antigen, telomerase, HER2/neu, and MUC-1 reactive \( T_{reg} \) were detected in the peripheral blood (32). On a practical level, targeting tumor \( T_{reg} \) may be achieved by harnessing tetramer technology; saporin-coupled MHC class I tetramers specifically ablated islet-specific glucose-6-phosphatase catalytic subunit-related protein–autoactive T cells and delayed diabetes in nonobese diabetic mice (33). Identification of CD4\(^+\) \( T_{reg} \) specific for a given tumor antigen would facilitate their targeting with MHC class II tetramers by similar means.

Although such agents would be specific for a given subset of \( T_{reg} \), they would also target other CD4\(^+\) helper cells expressing the same antigen specificity; CD8\(^+\) cells would be unaffected. To circumvent this issue, the effector component attached to the tetramer could be modified to confer another level of specificity. It could be miR-31, as 100% of target cells internalize the tetrameric complexes (33). Although the consequence of FOXP3 knockdown in non-\( T_{reg} \) is unknown, TCR engagement in these cells may simply lead to activation, further augmenting the immune effector response.

Alternatively, one could target tumor \( T_{reg} \) indirectly by modulating DC activation. This indirect targeting could be achieved by blockade of DC p38 mitogen-activated protein kinase, COX-2, or phosphoinositide 3-kinase, which inhibit innate production of TGF-\( \beta \) and IL-10 and, thereby, suppress induction of \( T_{reg} \). Such strategies enhance the efficacy of TLR agonists or HSPs as immunotherapeutics or adjuvants for DC vaccines and permit an unrestrained development of protective Th1 and Th17 cells (25).

Conclusions

\( T_{reg} \) inhibition in the cancer environment would permit an antitumor immune effector competency with containment or elimination of disease. Such responses would be tumor specific and durable and should be effective against systemic disease, particularly micrometastases. \( T_{reg} \) inhibitory strategies have clinical potential as part of multimodal programs or combined with targeted therapies or local immunogene stimulation of antitumor immune effector cells. The objective should be to selectively modulate \( T_{reg} \) within the tumor microenvironment, rather than their global depletion, to minimize the risk of autoimmune manifestations. Strategies targeting \( T_{reg} \) function or differentiation seem currently to be the best option because they are less susceptible to compensatory mechanisms. Emerging technologies, such as tetramer or RNAi approaches, should improve specificity and efficacy and, thus, favor the preferential inhibition of \( T_{reg} \) within the tumor environment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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