Improving Antitumor Immune Responses by Circumventing Immunoregulatory Cells and Mechanisms

Gregory Lizée, Laszlo G. Radvanyi, Willem W. Overwijk, and Patrick Hwu

Abstract Although numerous immunotherapeutic strategies have been studied in patients with cancer, consistent induction of clinical responses remains a formidable challenge. Cancer vaccines are often successful at generating elevated numbers of tumor-specific T lymphocytes in peripheral blood, however, despite this, tumors usually continue to grow unabated. Recent evidence suggests that endogenous regulatory cells, known to play a major role in the induction of immune tolerance to self and prevention of autoimmunity, as well as suppressive myeloid cells invoked in the tumor-bearing state, may be largely responsible for preventing effective antitumor immune responses. This review will focus on the major regulatory cell subtypes, including CD4+CD25+ T-regulatory cells, type 1 regulatory T cells, natural killer T cells, and immature myeloid cells. Studies in humans and in animal models have shown a role for all of these cells in tumor progression, although the mechanisms by which they act to suppress immunity remain largely undefined. Elucidation of the dominant molecular mechanisms mediating immune suppression in vivo will allow more precise targeting of the relevant regulatory cell populations, as well as the development of novel strategies and clinical reagents that will directly block molecules that induce the suppression of antitumor immunity.

The initial wave of enthusiasm experienced by many cancer immunologists with the identification of tumor-associated antigens recognized by immune cells has since given way to a more tempered optimism, based largely on the low response rates using a number of different immunotherapeutic approaches. Several formulations of cancer vaccines designed to induce tumor-specific T cell responses have now been extensively tested in patients with cancer at multiple centers, including immunization with tumor antigen-encoding naked DNA, viral vectors, antigenic peptides, peptide or tumor lysate-pulsed autologous dendritic cells (DC), gene-modified autologous tumor cell vaccines, and tumor-derived heat shock protein preparations (1–7). Although many of these vaccination approaches elicit significant numbers of tumor antigen-specific T cells in the blood of cancer patients, objective clinical regressions remain rare (8–10).

This paradoxical observation of tumor growth in the face of large numbers of activated, circulating tumor-reactive T cells suggests that their antitumor efficacy is actively attenuated in vivo. Evidence for this idea has been accumulating rapidly during the past decade, fueled by the discovery of a number of different immunoregulatory cell types, soluble factors, and mechanisms that limit the strength and duration of immune responses. Such multiple, redundant regulatory mechanisms have probably evolved to induce tolerance to self and seem to be essential for the normal control of autoimmunity. Several of these have now also been strongly implicated as significant barriers to the generation of effective antitumor immune responses.

In light of this recent evidence, it would now seem that the most promising and synergistic approaches for cancer immunotherapy will be ones designed to augment specific antitumor immunity while simultaneously reducing the effect of immunoregulatory mechanisms in vivo. Tumor immunologists have made considerable clinical progress in recent years with the first part of this equation. Following the identification of numerous tumor-associated antigens expressed by different tumor cell types, the induction of tumor antigen–reactive T cells with specific vaccination approaches and expansion of tumor-specific T cells to large numbers for adoptive transfer into patients have now become feasible. Nonetheless, we are just now beginning to clinically consider the latter challenge of preexisting regulatory cells and mechanisms in vivo that present an impediment to the effectiveness of our existing immunotherapies.

In this review, we discuss four major subtypes of immunoregulatory cells (Table 1) and briefly outline the evidence describing their role in impairing antitumor immune responses. One current strategy for eliminating these cells in vivo is through chemotherapeutic lymphoablation, and we review the existing evidence that lymphodepletion of patients with cancer before adoptive T cell transfer substantially increases the antitumor efficacy of the transferred cells. However, this approach can induce a significant degree of collateral damage to the patients' beneficial immune cells...
through the elimination of endogenous antiviral and memory cells. With this in mind, we describe newer strategies designed to more selectively target specific immunoregulatory cell types or specifically block immunosuppressive molecules expressed by regulatory, effector, tumor, or tumor stromal cells in vivo.

### CD4+CD25+ T-Regulatory Cells

The best characterized of the immunoregulatory cells are CD4+CD25+ natural T-regulatory (Treg) cells. They represent a functionally distinct lineage of T cells crucial for the maintenance of peripheral tolerance in vivo. A major distinction from other regulatory cell types is that Treg cells differentiate under normal conditions in the thymus into a mature regulatory cell type. Treg cells play an important role in the suppression of immune responses against self-antigens and pathogens and the induction of tolerance to environmental allergens.

Recent studies of Treg cells in human cancer have shown elevated Treg levels in the peripheral blood and/or tumor microenvironment of patients with melanoma, Hodgkin lymphoma, and non-small cell lung, ovarian, gastrointestinal, pancreatic, and breast cancer (26–31). In a large-scale study of 104 patients with ovarian carcinoma, CD4+CD25+Foxp3+ cells were shown to accumulate in tumors, in which they suppressed tumor-specific T cell immunity (32). Most notably, the number of Treg cells present within tumor biopsy specimens of different patients was highly inversely correlated with patient survival (32).

The mechanism by which Treg cells suppress the activation, proliferation, and cytokine production of antigen-specific helper CD4+ and killer CD8+ T cells is poorly understood, but it is known to require the activation of Treg cells through their T cell receptor and direct contact of the Treg cell with the responding T cell (Fig. 1A; ref. 33). Antibody blocking studies have shown no clear role for the candidate surface molecules GITR or CTLA-4 in directly mediating suppression (34–36). Although CD4+CD25+ Treg cells can produce the immunosuppressive cytokines interleukin 10 (IL-10) and transforming growth factor β (TGF-β) on activation, they do not seem to be important for inducing target cell suppression (37–39). The lack of cell type–specific markers with which to identify and isolate Treg cells has hampered efforts to better understand their mechanism of action and allow for the design of reagents that could specifically target these cells in vivo.

#### Type 1 Regulatory T Cells

Type 1 regulatory T (Tr1) cells comprise a subset of CD4+ T cells that are induced by antigen-specific activation in the presence of IL-10 (40). Such Tr1 cells can induce T cell anergy and suppression of immune responses, primarily via the production of high levels of cytokines IL-10 and TGF-β (41). Human Tr1 cells have been shown to mediate tolerance to kidney or liver allografts, and their presence was also correlated with long-term acceptance of hematopoietic stem cell grafts (42, 43). In general, their physiologic role seems to be the down-regulation of immune responses against self-antigens and pathogens and the induction of tolerance to environmental allergens (44–50).

Although studies on Tr1 cells and cancer have only just begun, Tr1 cells have been reported to be associated with cancer in both humans and in animal models (27, 51). A wide variety of tumor cell types are known to secrete IL-10 directly or induce its secretion by other cells, and increased serum levels of IL-10 have been reported in cancer patients (52–61). Long-term T cell receptor stimulation of tumor antigen–specific CD4+ T cells in the presence of IL-10 could therefore provide conditions that would favor the induction of Tr1 cells in the tumor microenvironment. Interestingly, Tr1-like CD4+ T cells, isolated either from the blood of melanoma patients or induced by repeated in vitro stimulation with tumor cells, have been shown to secrete IL-10 in response to the recognition of MHC class II restricted tumor antigens expressed on melanoma cells (62, 63). The two major effector cytokines produced by Tr1 cells, IL-10 and TGF-β, are potent immunoregulatory cytokines with complex effects on multiple cell types (Fig. 1B). IL-10 can suppress T cell responses indirectly through the inhibition of MHC and costimulatory molecule expression and cytokine production by antigen-presenting cells, including DCs, Langerhans
cells, and macrophages (55, 64–67). IL-10 can also directly regulate T cells by inhibiting their ability to proliferate and produce cytokines, including IL-2 and tumor necrosis factor-α (TNF-α), and has also been implicated in activation-induced cell death of T cells (55, 68–72). TGF-β inhibits T cell proliferation, cytokine production, and cytotoxicity, and can act at all stages of T cell differentiation (73, 74). Under some conditions, TGF-β can also inhibit antigen-presenting cell function by suppressing maturation and IFN-γ production and inducing MHC class II down-regulation (75–77). TGF-β-secreting CD4+ regulatory cells are alternatively referred to as T_{H3} cells. The broadly immunosuppressive role of cytokines produced by Tr1 cells suggests that these cells could play a significant role in shaping the tumor microenvironment to favor the growth of tumor cells.

**Invariant Natural Killer T Cells**

A third subset of immunoregulatory T cells express the NK cell marker, NK1.1, and recognize glycolipid antigens presented by the MHC class Ibmolecule CD1d through a highly restricted T cell receptor repertoire (78, 79). Invariant NKT (iNKT) cells from both humans (expressing the Vα24 T cell receptor chain) and mice (expressing the Vα14 chain) are strongly activated by the marine sponge–derived glycolipid, α-galactosylceramide (α-GalCer), leading to the rapid secretion of both T helper 1 and 2 (T_{H1} and T_{H2}) cytokines (80–83). Paradoxically, iNKT cells have been implicated in both the augmentation and suppression of antitumor responses (79, 84).

IFN-γ, which is rapidly produced by iNKT cells on activation with α-GalCer, triggers prolonged IFN-γ production by NK cells, which can subsequently inhibit tumor growth by blocking angiogenesis and inducing NK tumor cell killing via a tumor necrosis factor–related apoptosis inducing ligand (TRAIL)–dependent mechanism (85–87). iNKT activation also influences adaptive antitumor immune responses by increasing DC maturation, cross-presentation, and T cell priming against tumor antigens (88–90). Early clinical trials studying direct α-GalCer injection or infusion of α-GalCer-loaded DCs into patients with solid tumors reported no dose-related toxic effects, and although significant and sustained increases in iNKT cell numbers have been observed, no patients showed objective responses to this treatment (91–93). The recent identification of endogenous mammalian glycolipid ligands

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**Fig. 1.** The four major subtypes of immunoregulatory cells and their proposed mechanisms of action. (A) CD4+CD25+ Treg cells, (B) Type 1 regulatory T cells, (C) invariant natural killer T cells, and (D) immature myeloid cells. Please refer to the text for details.
In contrast to their documented antitumor effects, iNKT cells have also been reported in a number of mouse models to suppress antitumor immune responses (97–99). This suppression seems to be mediated at least in part by secretion of Th1 cytokines IL-4 and IL-13 by iNKT cells in response to tumor-derived factors (Fig. 1C; ref. 98). There is evidence that the IL-13 produced by iNKT cells induces myeloid dendritic cells to produce TGF-β which, in turn, directly suppresses CD8+ CTLs (100).

Patients with cancer frequently show reduced levels of peripheral blood iNKT cells compared with healthy donors and, in addition to a diminished ability to proliferate in response to α-GalCer, they produce little IFN-γ while still maintaining functional IL-4 production (91, 101–105). The cytokine polarization by iNKT cells, in which Th1 cytokines are deficient and immune responses are skewed toward the production of Th2 cytokines, seems to be consistent in both mouse models and cancer patients (83). This defect seems to be reversible in vitro by stimulation with α-GalCer-loaded DCs, suggesting a possible therapeutic means to relieve iNKT-induced immune suppression in patients with cancer (105).

Immature Myeloid Cells and Immature Dendritic Cells

There is accumulating evidence from mouse models and cancer patients that progressive tumor growth is associated with an increased frequency of immature myeloid cells (iMC) and immature DCs (iDC) that can inhibit tumor-specific T lymphocytes (106–108). iMCs and iDCs can be induced by tumor-derived soluble factors, including vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor, IL-6, granulocyte-macrophage colony-stimulating factor, IL-10, and gangliosides (108). These factors inhibit the differentiation of myeloid cells in vivo, leading to decreased levels of functionally competent, mature antigen-presenting cells, accumulation of iDCs that are unable to up-regulate MHC class II and costimulatory molecules or produce appropriate cytokines, and increased production of iMCs (109–112). The proangiogenic cytokine VEGF seems to be a major mediator of this disrupted DC differentiation, and tumors can secrete copious amounts of VEGF detectable in the serum of patients with cancer (113–115). In vitro studies have shown that these VEGF levels block DC maturation, leading to the appearance of iMCs, whereas blocking VEGF augments normal DC differentiation and function (116).

Tumors from patients with cancer often contain iDCs with reduced allostimulatory capacity (117, 118). Furthermore, such tumor-derived iDCs often express low levels of the costimulatory molecules CD80 and CD86 and often fail to up-regulate them even in the presence of the DC maturation factors TNF-α or CD40 ligand (119, 120). Increased circulating levels of such iDCs have also been observed in the peripheral blood of patients with lung, breast, head and neck, and esophageal cancer (108, 110, 111). The failure of iDCs to provide adequate costimulation during T cell priming, together with their inability to secrete IL-12, can lead to T cell anergy, and in some cases, activation-induced cell death (108, 119, 121, 122).

Importantly, patients with cancer often show a significant reduction in circulating levels of mature DCs that can be reversed on surgical removal of tumors, an observation that has also been reported in several mouse models (109, 111, 112, 123–125). Thus, abnormal differentiation and maturation of DCs in vivo, mediated by tumor-derived soluble factors, likely plays a substantial role in preventing the effective priming of a productive, T cell–mediated antitumor immune response.

iMCs are a heterogeneous population that include immature cells of the granulocyte and monocyte/macrophage lineage (108). These cells have been shown to accumulate in the peripheral blood of patients with breast, lung, or head and neck cancer, and this accumulation is often associated with a decrease in DC numbers (112). Similar to iDCs, circulating levels of iMCs have been well correlated with stage of disease and poorer prognosis, and surgical resection of tumors has been shown to decrease the number of peripheral blood iMCs in both human and animal models (126–128).

iMCs mediate their immunosuppressive activity through the inhibition of IFN-γ production by CD8+ T cells in response to MHC class I–associated peptide epitopes presented on the iMC surface (129). This effect requires direct cell-to-cell contact and is mediated by reactive oxygen and nitrogen species, such as hydrogen peroxide (H2O2) and nitric oxide (NO), secreted by the iMCs in close proximity to the T cell (Fig. 1D; refs. 130, 131). Although the precise mechanism of action on T cells has yet to be fully elucidated, there is some indication that iMCs act, in part, through down-regulation of the CD3ζ chain on responding CD8+ T cells (130, 132). Recently, a population of iMCs has been described in the peripheral blood of cancer patients having high arginase-1 activity capable of depleting local arginine levels and down-modulating CD3ζ levels on T cells. Depletion of this iMC subset in vitro restored CD3ζ expression and normal T cell responses (133, 134). Although freshly isolated iMCs do not seem to be capable of suppressing CD4+ T cells, cultured iMCs have been shown to induce CD4+ T cell apoptosis through the production of arginase and NO (135).

In summary, there is much evidence to suggest that the balance of immature and mature myeloid cells in vivo can have a significant effect on both naturally occurring and vaccine-induced antitumor T cell responses. It is becoming increasingly apparent that effective cancer immunotherapy may require the correction of aberrant myeloid cell differentiation frequently observed in tumor-bearing hosts.

Elimination of Regulatory Cells by Lymphodepletion

The development of clinical reagents designed to eliminate relevant subsets of immunoregulatory cells in vivo remains an important goal for cancer immunotherapy. Currently, the best strategy for eliminating regulatory cells in patients with cancer involves lymphoablative conditioning, which has been best studied in the context of adoptive T cell transfer studies in patients with melanoma. Evidence from both animal studies and human clinical trials suggests that the host immune environment can significantly affect the efficacy of
adoptive T cell transfer therapy (136). Mouse tumor models have shown that sublethal, lymphodepleting doses of irradiation before adoptive transfer of tumor antigen–specific lymphocytes substantially increases the persistence and antitumor activity of the transferred cells (137, 138). In addition, preconditioning mice with low doses of chemotherapeutic agents, such as cyclophosphamide and 5-fluorouracil, prior to antitumor vaccination has been found to markedly augment antigen-specific T cell responses and tumor control (139–143).

Parallel findings in humans have shown that the addition of a lymphodepleting conditioning regimen prior to adoptive T cell transfer therapy for patients with metastatic melanoma significantly improves clinical response rates (144, 145). Stage IV melanoma patients enrolled in a recent clinical trial received a lymphodepleting chemotherapy regimen that consisted of cyclophosphamide and 5-fluorouracil before administration of highly selected, expanded, tumor-reactive tumor-infiltrating lymphocyte cultures, and IL-2 therapy (146). The lymphodepletion regimen resulted in a transient myelosuppression and the elimination of all endogenous circulating lymphocytes for ~1 week, after which time, patients recovered endogenous marrow function and reconstituted their lymphocyte compartments toward normal levels within 3 to 4 weeks (144, 147).

Eighteen (51%) of 35 patients treated in this trial experienced objective clinical responses, including 3 ongoing complete responses and 15 partial responses with a mean duration of 11.5 months. Significant levels of tumor regression were observed in metastatic deposits in the liver, lungs, brain, cutaneous and subcutaneous tissues, and lymph nodes (146, 148). Interestingly, a number of responding patients showed marked evidence of lymphoproliferation of the transferred cells, and clinical responses were highly correlated with their in vivo persistence (146, 149–151). These results underscore the significant potential that lymphodepleting regimens, which eliminate circulating regulatory cells, have to enhance the efficacy of adoptive T cell transfer immunotherapy in patients with cancer. It will be critical to determine the relationship between T cell persistence, lymphoproliferation, and the role of any re-emerging regulatory T cell subsets in long-term tumor control and what the differences are between responding and nonresponding patients in this regard.

**Future Approaches to Circumventing Immunoregulation**

Lymphoablative chemotherapy, although currently beneficial for patients with cancer in adoptive transfer settings, is clearly a “sledgehammer” approach to eliminating immunoregulatory cells that may abrogate the efficacy of immunotherapy. In addition, these regimens are fairly toxic and lead to increased susceptibility to viral and/or bacterial infection. An important future goal for cancer immunologists is to develop more refined methods of eliminating specific immunoregulatory cells that will not result in collateral damage to patients’ beneficial immune cells. Increasing our knowledge of the precise mechanisms of antitumor immunoregulation will inevitably lead to the development of more specific and effective clinical reagents capable of interfering with suppression at the molecular level.

CD4+CD25+ Treg cells are currently the only regulatory cell subtype that has been specifically targeted for elimination in human studies. Two approved compounds, human anti-Tac (anti-CD25) and ONTAK (IL-2 receptor-binding domain of IL-2 fused to diphtheria toxin), have been used in clinical trials for the treatment of cancer with, thus far, limited success (152, 153). Although trials are still proceeding, a major concern is the specificity of this approach. CD25 is also expressed on activated CD4+ helper T cells and activated CD8+ cytotoxic T cells, in which it helps drive the expansion and survival of effector cells (154). Thus, elimination of the CD25+ cell subsets may prove more detrimental than helpful. Preclinical mouse models on which these trials are based have eliminated Treg cells before activation of relevant tumor-reactive T cell subsets (20, 22). By contrast, in patients with cancer, tumor-specific T cells are often activated before therapeutic intervention, suggesting that the two systems may not be comparable.

The systemic depletion of any of the regulatory cell subsets presents at least two important caveats: (a) none of the regulatory cell subsets express currently known unique markers that can be targeted without affecting nonregulatory cells, and (b) removal of an entire population of regulatory cells could potentially result in the induction of autoimmunity. With these considerations in mind, strategies designed to target subsets of regulatory cells present within the local tumor microenvironment or tumor-draining lymph nodes may be warranted. Although the means to accomplish this are not currently available, this type of approach may, in some cases, be enough to tip the immunologic balance to favor antitumor immunity, with minimal adverse effects.

A promising alternative strategy for alleviating antitumor immunoregulation in vivo involves the specific blocking of molecules that can mediate immune suppression. The list of potentially targetable molecules is rapidly growing, and these can be expressed either on the cell surface or secreted by tumor cells, tumor stroma, regulatory cells, or effector cells (Table 2). One of the best-studied blocking reagents targets CTLA-4, a transmembrane protein expressed by both CD4+CD25- Treg cells and effector T cells. CTLA-4 is up-regulated on conventional T cells on antigen stimulation and functions as a coinhibitory receptor to attenuate activation during T cell priming and limiting effector cell expansion (155). The constitutively high CTLA-4 expression on Treg cells has been shown to induce DCs to turn on tryptophan catabolism via indoleamine 2,3-dioxygenase, causing local depletion of this essential amino acid and effectively crippling T cell priming and proliferation (156, 157).

Based on successful preclinical murine tumor therapy models (158, 159), a number of ongoing and completed clinical trials have used anti-CTLA-4 to treat patients with cancer of different histologic types. The initial reports have been encouraging, with two groups documenting significant increases in antitumor immunity in vaccinated melanoma patients following anti-CTLA-4 treatment, including the induction of some objective tumor regressions (160–162). However, this treatment has also been associated with inducing a wide variety of grade 3 and 4 autoimmune manifestations, potentially limiting its usefulness as a clinical reagent (161, 162).

Another promising molecular target is the immunoinhibitory receptor programmed death 1 (PD-1), which is expressed by both helper CD4+ and cytokotoxic CD8+ T cells. The ligand for PD-1, the B7 family member B7-H1, is expressed on a wide
variety of human cancer cells, including lung, ovarian, colon, and melanoma (163). Ligation of PD-1 on T cells by B7-H1 has been shown to decrease proliferation and cytokine secretion and induce apoptosis of human T cells (164, 165). Furthermore, antibody blockade of PD-1 and/or B7-H1 has been shown to significantly augment immunotherapy in mouse tumor models, suggesting that clinical trials using this approach may be warranted (166–168).

Targeting of soluble immunoregulatory molecules could also prove useful for enhancing the immunotherapy of cancer. Neutralization of immunosuppressive cytokines IL-10, IL-13, TGF-β, and VEGF through the administration of specific monoclonal antibodies or soluble receptors may prove synergistic with other cancer vaccine or adoptive transfer strategies by reducing anti-inflammatory signals that limit antitumor immunity and inhibit DC differentiation. One promising addition to the anticancer armamentarium is bevacizumab, a humanized monoclonal antibody against VEGF that is proving effective in metastatic colon cancer, and more recently in metastatic breast cancer, as an antiangiogenic agent (169–172). Although bevacizumab is proposed to act by blocking tumor angiogenesis (which has not been proven directly in humans yet), it may also inhibit the immune suppressive effects of VEGF on DC differentiation and iMC production. Indoleamine 2,3-dioxygenase, an enzyme produced by macrophages, DCs, and many human tumor cells, inhibits T cell proliferation by catabolizing and depleting local tryptophan, and thus, might represent another attractive molecular target (173, 174). Immune suppression may also be circumvented by targeting two enzymes expressed by myeloid suppressor and some tumor cells, inducible NO synthase 2 and arginase-1 (175, 176). These metabolic enzymes act to generate NO and deplete local arginine, respectively, and can act synergistically in the tumor microenvironment to induce the apoptosis of activated T cells and enhance tumor growth (177–182).

Conclusions
Two decades after the pioneering studies by North and colleagues demonstrating induction of suppressor cells in murine sarcoma models (183–186), there now exists overwhelming evidence from both human and animal studies that regulatory immune cells play an active and significant role in the progression of cancer. Thus, there is a clear rationale for developing clinical strategies to diminish these regulatory influences, with the ultimate goal of augmenting antitumor immunity. It should be mentioned that although this review has focused on four of the most well characterized regulatory cell types, other subsets have also been reported (including CD8+ regulatory cells, CD4+ cells that show features of both natural and adaptive Treg, and γδ T cells), further underscoring the heterogeneous nature of immunoregulation.

Future challenges include the identification of unique regulatory cell markers that can be used to more specifically target these cells in vivo and a more accurate characterization of the molecular nature of immune suppression. The ability to diagnose which regulatory mechanisms are dominant within the tumor microenvironment of individual patients and when they are initiated during cancer progression will also be of paramount importance for implementation of effective immunotherapy. Meeting these challenges should lead to substantially increased specificity of immune interventions, thus allowing for harnessing of the benefits of enhanced antitumor immunity, while avoiding the simultaneous induction of unwanted autoimmunity.

Acknowledgments
The authors would like to thank Linda Duggan for providing graphical assistance on Figure 1.

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