The Mechanism of Anti-CTLA-4 Activity and the Negative Regulation of T-Cell Activation

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ABSTRACT
The survival rate of patients diagnosed with late-stage melanoma is poor—only 5%–10%. Enlisting the immune system in the fight against cancers such as melanoma could help improve the prognosis of these patients. Data have shown that melanocyte proteins make good targets for immune system–based therapy in this disease. However, self-tolerance, which develops to inhibit autoimmune attack, makes this strategy difficult. Two proteins on the surface of T cells—CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4)—play important roles in the regulation of immune activation and tolerance. CD28 provides positive modulatory signals in the early stages of an immune response, while CTLA-4 signaling inhibits T-cell activation, particularly during strong T-cell responses. CTLA-4 blockade using anti-CTLA-4 monoclonal antibody therapy has great appeal because suppression of inhibitory signals results in the generation of an antitumor T-cell response. Both clinical and preclinical data indicate that CTLA-4 blockade results in direct activation of CD4+ and CD8+ effector cells, and anti-CTLA-4 monoclonal antibody therapy has shown promise in a number of cancers, particularly melanoma. Interestingly, the occurrence of adverse events among patients treated with CTLA-4 blockade helps shed light on the mechanism of action of anti-CTLA-4 monoclonal antibodies. Most adverse events involve immune-related toxicity to the skin and gastrointestinal tract. Major gastrointestinal toxicity develops in up to 21% of treated patients, and while an objective response occurs in approximately 36% of melanoma patients who develop enterocolitis with treatment, an objective response is found in only 11% of patients who do not experience this adverse reaction. The Oncologist 2008;13(suppl 4):2–9

INTRODUCTION
Harnessing the power of the body’s immune system to fight cancers such as melanoma has great appeal. An immune system primed to properly identify and destroy tumor cells would eliminate errant cells in nearby lymph nodes and distant metastases, thus solving one of the most difficult problems in cancer therapy—the treatment of patients with late-stage disease (stage III or IV). Whereas the 5-year survival rate among patients with stage I melanoma is >80%, for those diagnosed with stage IV disease, the 5-year survival rate is only 5%–10% [1].

The immune system is characterized by a complex system of checks and balances to protect the host from exogenous pathogens by distinguishing “self” from “nonself.” This system involves both stimulatory and inhibitory components, and multiple mechanisms of peripheral tolerance.

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Complex Interactions Determine the Functioning of the Immune System

The normal functioning of the immune system involves a series of highly complex interactions. Although tumors express antigens that are recognizable by the immune system, antigen presentation alone is not enough to initiate an effective immune response to cancer or to any other pathological entity [4]. T-cell activation is modulated by stimulatory and inhibitory signals that work together to coordinate the immune system’s response to a threat [5, 6]. The cell surface molecules CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) provide positive (CD28) and negative (CTLA-4) modulatory signals in the early stages of an immune response [5]. CD28 facilitates and maintains a T-cell response, at least partly through increased cytokine expression [4, 7–11] mediated by interaction with its primary ligands B7-1 (CD80) and B7-2 (CD86) on the surface of the antigen-presenting cell (APC). In contrast, by triggering an inhibitory signal, CTLA-4 essentially halts T-cell activation [5, 12, 13]. Inhibition of CTLA-4 can shift the immune system balance toward T-cell activation, resulting in rejection of tumors by the host. In clinical trials, anti–CTLA-4 monoclonal antibody therapy has been associated with a 10%–21% response rate in patients with advanced metastatic melanoma [14–16], and further studies are currently being conducted to evaluate this treatment regimen in other cancers [17–20].

The interaction between antitumor T cells and APCs is central to the development of antitumor T-cell immunity and is modulated through the competing influences of stimulatory and inhibitory molecules. Whereas the first signal in T-cell activation is provided by binding of the T-cell receptor (TCR) to cognate antigen, a second “stimulatory” signal is necessary to initiate T-cell proliferation [21]. This second signal is provided by CD28. CTLA-4 is homologous to CD28, and both molecules are located on the surface of T cells, where they compete to bind to B7 costimulatory molecules on APCs [22]. Compared with CD28, CTLA-4 binds with much greater affinity to B7-1 and B7-2, at rates reported to be 500- to 2,500-fold higher than those of CD28, thus giving CTLA-4 a competitive advantage over CD28 [2].

The antagonistic roles played by CD28 and CTLA-4 are not only essential for the proper functioning of the immune system but also determine the fate of T cells—activation or anergy [21]. Preclinical studies have shown that, in CTLA-4 knockout mice, the lack of a counterbalance to CD28 T-cell activation results in rampant lymphoproliferative disease and death by age 3–4 weeks. These findings support the hypothesis that CTLA-4 is essential to the downregulation of autoreactive and potentially destructive peripheral T-cell responses. Furthermore, other preclinical studies have demonstrated that, whereas blockade of CD28 inhibits antitumor immunity, blockade of CTLA-4 stimulates antitumor immunity [13, 23]. Additionally, whereas CD28 stimulates the production of cytokines such as interleukin-2 (IL-2) and upregulates antiapoptotic genes such as Bcl-X, the binding of CTLA-4 to B7 molecules results in the inhibition of IL-2 [13, 24, 25].

The Respective Roles of CD28 and CTLA-4

The introduction of the first monoclonal antibodies to CTLA-4 allowed the first direct tests of its function and provided a better understanding of its role [26]. Two models of CTLA-4 regulation of T-cell expansion have been proposed—the threshold model, in which CTLA-4 sets a threshold of activation above the background “noise” of TCR signals, and the attenuation model, in which CTLA-4 limits the capacity of a T cell to divide after initiation of activation [26, 27]. These models are not mutually exclusive, and both mechanisms may be at work, depending on the strength of the TCR signal. It has been proposed that CTLA-4 not only limits the body’s response to autoantigens but also helps diversify the T-cell population so that, during an immune response, T cells specific to only one epitope would not necessarily dominate, and pathogens could thus be more easily targeted and destroyed [26]. CTLA-4 is also important in limiting the severity of autoimmunity, and preclinical studies indicate that animals with a deficiency in B7-2 (the CD28 principal ligand) are protected from some autoimmune diseases, whereas those deficient in B7-1 (the principal CTLA-4 ligand) are more susceptible [28–32] (Fig. 1 and Fig. 2).

The question has been raised as to how the immune system can function and T cells can proliferate at all, when the affinity of CTLA-4 for the B7 family of ligands is so much greater than that of CD28 [26], and additionally, CTLA-4 can form extensive lattice-like protein networks that can effectively exclude CD28 from interacting with the B7 ligands [2, 31, 33]. Part of the answer
The mechanism of anti-CTLA-4 activity involves several key points. CD28, CTLA-4, B7-1, and B7-2 are centrally important for the initial activation of naïve T cells of the clonal composition of the responding repertoire following migration of activated dendritic cells to lymphoid activation organs. As activated effectors traffic back into peripheral tissues, they come under the influence of PD-1–PDL-1 and PD-1–PDL-2–mediated signaling, as a result of interactions with both tissue macrophages and ligands expressed on malignant cells. B7-H3 and B7x might act as the final arbiters of the fate of T-cell effector interactions with nonlymphoid target tissues, and might protect tumor cells that express them from cytotoxic T-cell–mediated killing. The potential for crosstalk between T-cell populations via many of these pathways is complex, particularly because activated cells can upregulate receptors and/or ligands that can potentially signal bidirectionally. Blockade of BTLA might remove inhibitory restraints imposed by HVEM-expressing cells, but effects on T-cell–T-cell interactions mediated by blockade of CTLA-4, PD-1 or PDL-1, or B7-H3 are also possible. Regulatory T cells provide an additional therapeutic target.

**Figure 1.** Inhibitory checkpoints of immune regulation providing potential targets for therapeutic intervention. All members of the immunoglobulin superfamily that act as inhibitory checkpoints are potential targets for manipulation in immunotherapies. CD28, CTLA-4, B7-1, and B7-2 are centrally important for the initial activation of naïve T cells of the clonal composition of the responding repertoire following migration of activated dendritic cells to lymphoid activation organs. As activated effectors traffic back into peripheral tissues, they come under the influence of PD-1–PDL-1 and PD-1–PDL-2–mediated signaling, as a result of interactions with both tissue macrophages and ligands expressed on malignant cells. B7-H3 and B7x might act as the final arbiters of the fate of T-cell effector interactions with nonlymphoid target tissues, and might protect tumor cells that express them from cytotoxic T-cell–mediated killing. The potential for crosstalk between T-cell populations via many of these pathways is complex, particularly because activated cells can upregulate receptors and/or ligands that can potentially signal bidirectionally. Blockade of BTLA might remove inhibitory restraints imposed by HVEM-expressing cells, but effects on T-cell–T-cell interactions mediated by blockade of CTLA-4, PD-1 or PDL-1, or B7-H3 are also possible. Regulatory T cells provide an additional therapeutic target.

**Abbreviations:** BTLA, B- and T-lymphocyte attenuator; CTLA-4, cytotoxic T-lymphocyte antigen 4; HVEM, herpes virus entry mediator; IDO, indoleamine 2,3-dioxygenase; IL-2, interleukin-2; LIGHT, lymphotoxins, inducible, competes with herpes simplex virus glycoproteins D for HVEM, expressed by T cells; PD, programmed death; PDL, programmed death ligand; TGF-β, transforming growth factor β.

more stable protein than CTLA-4, which has a half-life of approximately 2 hours, thus linking its expression tightly to gene transcription and translation [26]. B7-2 appears to be the principal ligand involved in CD28 concentration at the immunological synapse, whereas B7-1 is the primary ligand involved in CTLA-4 concentration [28]. Although the presence of B7-1 and B7-2 increases CD28 localization, their absence does not preclude CD28 localization. However, the presence of these ligands on APCs and subsequent binding are essential to the recruitment of CTLA-4 to the synapse.

The ability of CTLA-4 to inhibit the activation of any particular T cell is dependent upon a number of factors, including the strength of the TCR signal and the activation state of the APC [26]. CTLA-4 signals via an immunoreceptor tyrosine-based inhibitory motif to inhibit both CD4+ and CD8+ T-cell responses [24, 26]. Studies suggest that CTLA-4 localization to the immunological synapse is favored under conditions of stronger TCR signaling, with the accumulation of CTLA-4 at the immunological synapse being proportional to the strength of the TCR stimulus [24]. Thus, CTLA-4 is more likely to inhibit strong T-cell responses, which has considerable implications for the respective roles of CD28 and CTLA-4 in regulating the T-cell response to antigens. The preferential restriction of cells bearing higher affinity TCRs for any given antigen may allow for greater representation of cells bearing lower affinity TCRs and thus diversify the T-cell response to a threat. This diversified population of T cells may have greater crossreactivity to similar antigenic epitopes and may be important in the development of a protective T-cell response.

**PROPOSED CELLULAR MECHANISMS OF ANTI–CTLA-4 ANTITUMOR ACTIVITY**

The identification of key players in the stimulatory and inhibitory mechanisms of the immune system is very promising for the treatment of cancers. Because melanocytic differentiation proteins have been shown to be targets for cytotoxic T lymphocytes, CD4+ T cells, and antibodies in both patients with melanoma and healthy individuals, these self-proteins are ideal targets for immune system–based therapy.
in melanoma [34]. These pigmentation-related proteins are expressed by most melanomas at all stages. Furthermore, immunological tolerance to melanocyte antigens appears to be incomplete.

Evidence from preclinical trials supports enhanced activation of effector CD8+ T cells as the mechanism involved in tumor rejection and the associated autoimmune effects after combination therapy with a vaccine and an anti–CTLA-4 monoclonal antibody [34]. Although CD8+ T cells were required for rejection of tumors in mice, B cells and CD4+ T cells were not. In murine models, tumor rejection was followed by autoimmune depigmentation, suggesting that a major target of the immune system response was a pigmentation-related antigen shared by the tumor, vaccine, and normal melanocytes. However, there was no evidence of a specific B-cell response observed in the depigmented skins of the mice. The addition of an anti–CTLA-4 monoclonal antibody obviates the need for CD4+ T-cell help, which is necessary when a tumor-cell vaccine is used as monotherapy. This finding is supported by other evidence [35] that CTLA-4 blockade enhances the CD8+ T-cell response independently of CD4+ T cells, although the mechanism involved is not yet understood [34].

Both clinical and preclinical data suggest that CTLA-4 blockade with anti–CTLA-4 monoclonal antibody therapy results in direct activation of CD4+ and CD8+ effector cells [36]. Studies in preclinical melanoma models suggest that anti–CTLA-4 therapy does not significantly affect the suppressive capacity of regulatory T cells, discrediting the competing theory that CTLA-4 blockade results in inhibition of CD4+ CD25+ cells, with the consequent enhancement of effector T-cell activity secondary to reduction in regulation [17]. Human leukocyte antigen (HLA)-DR expression is a marker of T-cell activation, and in clinical trials evaluating patients with melanoma treated with anti–CTLA-4 monoclonal antibody therapy, HLA-DR expression increased in both CD4+ and CD8+ cells. In contrast, neither the expression nor function of CD4+ CD25+ cells was affected in those patients. However, anti–CTLA-4 monoclonal antibody therapy, most likely through a direct stimulation of effector T cells, does result in an altered ratio of effector cells to regulatory cells within the tumor itself, with a marked increase in CD4+ effector cells and an even more marked increase in CD8+ effector cells, as demonstrated in murine models [17]. Other evidence from a murine model indicates that stimulation of T cells against tumor antigens is the dominant mechanism of action involved in CTLA-4 blockade [36]. In that study, after mice were treated with an anti–CTLA-4 antibody, CD8+ T cells that were reactive against the melanoma antigen tyrosinase-related protein 2 were isolated. In summary, the preponderance of preclinical studies in mice suggests that the dominant activity of anti–CTLA-4 is to stimulate effector T cells in the tumor, while further immune monitoring of patients is necessary to confirm whether the same is true in humans.

**CTLA-4 Blockade—The Potential for Use as a Single Therapy and in Combination Immunotherapies**

Anti–CTLA-4 therapy is one of the first treatments to demonstrate definite clinical benefit through direct T-cell activation. A number of strategies relying on enhancing costimulation have been evaluated, including the use of irradiated tumor cells expressing GM-CSF to enhance crosspriming of T cells by APCs, dendritic cells pulsed with peptides or RNA to afford immunization in conjunction with an APC, anti-CD40 antibodies to upregulate expression of APC costimulatory ligands, and IL-2 to eliminate the need for costimulation [23, 28, 37]. Although the outcomes of these studies have not been entirely successful, data from these trials suggest that further refinements in the approach to the modulation of costimulation will yield more benefit for more patients with less toxicity.

When used alone, CTLA-4 blockade causes general, rather than tumor-specific, immunity [38]. Trials of CTLA-4 blockade as monotherapy in advanced-stage melanoma and renal cell carcinoma have shown objective response rates in the range of 12%–19% [2, 39–41]. Other patients have had stable disease in response to anti–CTLA-4 monoclonal antibody therapy. Many of these responses have been durable, with most trials documenting ongoing responses lasting from 18 months to >35 months [2], which is longer than the response times generally seen with conventional cytotoxic chemotherapy in these patients with advanced cancer.

The clinical trials evaluating anti–CTLA-4 monoclonal antibody monotherapy were based on preclinical data that showed that, when used alone, this treatment regimen was associated with the rejection of several kinds of tumors, including colon carcinoma, fibrosarcoma, prostatic carcinoma, lymphoma, and renal carcinoma [2, 4, 42–45]. However, anti–CTLA-4 monoclonal antibody monotherapy failed in less immunogenic tumors, such as B16 melanoma [2].

Preclinical studies also have shown antitumor efficacy for anti–CTLA-4 monoclonal antibody therapy in conjunction with vaccines [3, 46]. Based on preclinical data, it has been hypothesized that CTLA-4 blockade used with vaccines directed against tumor cells may result in a synergistic combination, but the potential role of vaccines in humans is poorly understood at this time. Although GM-CSF–secret-
ing vaccines had demonstrated considerable efficacy as prophylaxis before tumor challenge experiments in mice, they were not effective as treatment against established tumors. However, when a GM-CSF–secreting vaccine was combined with CTLA-4 blockade, synergy was evident in melanoma, mammary carcinoma, and prostate carcinoma models [2]. Blockade of CTLA-4 also has been shown to substantially enhance the efficacy of xenogeneic DNA vaccines in preclinical models. Increased T-cell responses, antitumor immunity, and autoimmunity were evident [47]. The results of trials in animals also indicate that the scheduling of anti–CTLA-4 antibody therapy in combination with vaccines must be carefully evaluated, because timing appears to affect efficacy. Future studies may show that combination immunotherapeutic approaches using anti–CTLA-4 therapy can be successfully translated into the clinical setting.

The occurrence of immune-related adverse events (irAEs) among patients treated with CTLA-4 blockade may reveal some of the mechanisms involved in this therapy. The most common irAEs involve the skin and gastrointestinal tract [2]. Among patients with melanoma and renal cell carcinoma treated with CTLA-4 blockade monotherapy, the major toxicity was most often reported to be in the gastrointestinal tract, and up to 21% of treated patients developed grade 3 or 4 clinical enterocolitis [48]. In addition, there was a significant association between these irAEs and tumor response. This is not to imply that the occurrence of an irAE is required for clinical response. Among patients with melanoma, objective responses were observed in 36% of those who developed enterocolitis, but in only 11% of those who did not; similarly, among patients with renal cell carcinoma, objective responses were reported in 35% of those who developed enterocolitis, but in only 2% of those who did not. In another trial, among patients with melanoma treated with anti–CTLA-4 antibody and concomitant vaccination with two modified HLA-A*0201-restricted peptides (gp100:209-217[210M] and gp100:280-288[288V]), investigators noted a strong correlation between tumor regression and grade 3 or 4 irAEs with CTLA-4 blockade [15]. The fact that the normal organs targeted by the T cells (e.g., colon, liver, eye, and pituitary) do not carry the antigen contained in the peptide vaccine indicates that the toxicities resulted from disrupted self-tolerance induced by CTLA-4 blockade, rather than from the vaccine itself. This is supported by findings from murine models in melanoma. The hypopigmentation that accompanies tumor rejection is uncommon with vaccination alone and is not seen with anti–CTLA-4 monotherapy, suggesting that CTLA-4 blockade is important in breaking peripheral tolerance [2].

CTLA-4 blockade therapy in combination with more conventional therapies, such as chemotherapy or radiotherapy, also may be a viable alternative for treating melanoma. Both chemotherapy and radiotherapy can induce tumor cell death, which may cause the release of antigens that can improve the efficacy of anti–CTLA-4 monoclonal antibody therapy. Consistent with this hypothesis, data from a preclinical study show that treatment with CTLA-4 blockade alone did not provide a therapeutic benefit, but with the addition of melphalan, greater antitumor immunity was observed [49]. In a recent study, a poorly immunogenic metastatic mouse mammary carcinoma was used to test the combination of local radiation and CTLA-4 blockade. Mice injected with mammary carcinoma cells were randomized to receive one of four treatment regimens: IgG (control), radiotherapy plus IgG, anti–CTLA-4 monoclonal antibody therapy, or anti–CTLA-4 monoclonal antibody therapy plus radiotherapy. Because the tumor was poorly immunogenic, CTLA-4 blockade by itself had no effect on primary tumor growth or survival, whereas radiotherapy delayed growth of the tumor but with a survival rate similar to that of the control group. However, among mice treated with both CTLA-4 blockade and radiotherapy, there was a statistically significant survival advantage. As noted earlier, efficacy of CTLA-4 blockade requires CD8+ but not CD4+ T cells [50]. Because the mice were not treated until they had well-established disease—equivalent to advanced disease in humans—complete cure of these tumors was rare. The investigators suggested that large tumors may develop an environment that inhibits T-cell function.

In humans, multiple clinical studies have been undertaken to determine whether CTLA-4 blockade might synergize with conventional chemotherapy and/or radiation. A study published by Hersh and colleagues of 76 patients using ipilimumab with or without dacarbazine yielded a 17% overall response rate in the combination arm compared with a 5% response rate in the ipilimumab arm alone [51]. A phase III trial of dacarbazine with or without ipilimumab recently completed accrual and results are awaited [52]. Because the time course and kinetics of response are complex for anti–CTLA-4 blockade, the determination as to whether responses represent a synergistic or additive effect of both agents may require time and close analysis of the duration of responses. Meanwhile, a trial in prostate cancer of ipilimumab with or without docetaxel was also recently completed [53], and a trial of anti–CTLA-4 therapy in prostate cancer with or without a single dose of focal radiotherapy is currently accruing patients [54]. Data from this last study may help to test the hypothesis that directly cytotoxic therapies may enhance the efficacy of CTLA-4 blockade by increasing antigen release from the tumor.
CONCLUSION

Immunological approaches to cancer therapy have tremendous potential, particularly in late-stage disease such as stage III or IV melanoma, where the prognosis for patients is very poor. The immune system is regulated by a series of stimulatory and inhibitory signals that interact to produce an appropriate response to a pathogenic threat. Identifying key inhibitory checkpoints of immune regulation and developing therapies to target those points is a major goal of cancer research. Evidence from numerous studies indicates that CTLA-4 provides a braking mechanism on T-cell activation and serves a critical role in immune response. CTLA-4 competes for the B7 family of ligands with CD28, a key costimulatory molecule that is essential for the effective activation of T-cell–mediated immunity. CTLA-4 blockade results in enhanced antitumor immunity, most likely through the direct activation of T cells. Anti–CTLA-4 monoclonal antibody therapy, either as a monotherapy or in combination with a vaccine, may potentially allow for a more specific immune response against tumor targets. Data suggest that the use of anti–CTLA-4 antibody therapy results in a break in self-tolerance, as evidenced by the irAEs that occur with the therapy. Chemotherapy and radiotherapy also may enhance crosspresentation of tumor antigens and therefore heighten the effect of anti–CTLA-4 therapy. Clinical trials of anti–CTLA-4 monoclonal antibody therapy, either as monotherapy or in combination with vaccines or other adjuvant therapies, are ongoing in patients with cancer.

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