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CTLA-4 blockade in tumor models: an overview of preclinical and translational research

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Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a key negative regulator of T cell activation. A complex integration of positive and negative co-stimulatory signals in the well-defined B7:CD28/CTLA-4 pathway modulates the generation and maintenance of immune responses. Inhibiting negative regulation through binding of CTLA-4 has been shown to promote stimulation of adaptive immunity and potentiation of T cell activation. CTLA-4-blocking antibodies have demonstrated efficacy in various murine malignancy models when administered as monotherapy; additionally, they have shown synergistic anti-tumor activity when utilized with other agents, such as vaccines, chemotherapy, and radiation. Preclinical studies have supported the rationale for current clinical development of anti-CTLA-4 antibodies, including ipilimumab and tremelimumab, as novel therapeutic strategies to augment anti-tumor immunity in cancer. Both ipilimumab and tremelimumab have been evaluated extensively in melanoma; notably, ipilimumab was recently approved as monotherapy for the treatment of advanced melanoma. Tremelimumab is currently undergoing evaluation in phase II trials as monotherapy in melanoma and malignant mesothelioma, while ipilimumab is under clinical investigation in phase II and III trials in various tumor types, including in melanoma, prostate, and lung cancers as monotherapy and with other therapeutic modalities, such as chemotherapy and radiation. In this review, we will provide a detailed overview of preclinical advances that have delineated many features of CTLA-4 and have helped define its role in T cell response. We will also highlight clinical application of anti-CTLA-4 therapy in cancer and describe knowledge gaps that future studies may address.

Keywords: CTLA-4, antibody, T cell, preclinical, co-stimulation

Introduction

Cancer is a complex amalgam of host and tumor cells that have acquired multiple traits, including sustained proliferative potential, resistance to apoptosis, induction of angiogenesis, and evasion of the host immune system (1). The role of the immune system in recognizing and suppressing malignant cell growth has been realized for well over a century, and was proven using elegant modern techniques and murine tumor models (2, 3). Indeed, preclinical research has vastly increased our knowledge of the mechanisms that regulate the immune response during tumorigenesis, and this research has led to the development of immunotherapeutic strategies that aim to enhance the inherent anti-tumor capabilities of the immune system. In particular, cellular and murine malignancy models demonstrate that blockade of cytotoxic T lymphocyte antigen-4 (CTLA-4), a negative regulator of T cell responses, augments endogenous responses to tumor cells, thus leading to tumor cell death when utilized on its own or with other therapeutic interventions. Preclinical findings have translated into clinical development of a fully human, IgG1 monoclonal antibody (mAb), ipilimumab (formerly MDX-010 or BMS-734016; Yervoy™, Bristol-Myers Squibb, Princeton, NJ), and a fully human, IgG2 mAb, tremelimumab (formerly ticilimumab; CP-675,206, Pfizer, New York, NY), both of which bind CTLA-4. Of note, ipilimumab was recently approved at a dose of 3 mg/kg in several countries for the treatment of advanced or metastatic melanoma (4-6).

CTLA-4 mechanism of action and cellular expression

The activation of T cells requires not only stimulation of the T cell receptor (TCR) by peptide-major histocompatibility complexes (MHCs) on antigen-presenting cells (APCs), but also an orchestrated balance of co-stimulatory and inhibitory signals that modulate the magnitude and effectiveness of the immune response (7). The activation of effector CD4+ and CD8+ T lymphocytes (T_{effs}) is tightly regulated by multiple mechanisms, including cell surface proteins which expand or downregulate T cell responses (3). Negative regulatory proteins on T cells, such as CTLA-4, programmed death-1 (PD-1), B7 family member B7-H4, T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), and lymphocyte activation gene-3 (LAG-3), interact with their cognate ligands on various cells types, including APCs, regulatory T cells (T_{regs}), and non-hematopoietic cells, resulting in reduced T cell proliferation and functional activity (3, 8). Strict expression control of these ligands is necessary in order to generate productive immune responses and rein in activated immune cells after antigen clearance. Chronic antigen exposure, as is common in cancer and in certain viral infections, drives sustained expression of CTLA-4, PD-1, and LAG-3 on antigen-specific lymphocytes, which culminates in peripheral cell tolerance to the antigens (9, 10). Indeed, one of the primary goals of antibody-mediated negative regulatory blockade is the functional reversal, or reactivation, of tolerized T cells in settings of chronic antigen exposure.

One of the best characterized T cell co-stimulatory signals is mediated by the constitutively expressed Ig-family protein, CD28. CD28 binding to ligands B7-1 and B7-2 on APCs leads to T cell proliferation by inducing production of interleukin-2 (IL-2) and anti-apoptotic factors (11). CTLA-4, an activation-induced T cell surface molecule with homology to CD28 (12), may be detected on activated CD4+ and CD8+ T cells after TCR signaling. No detectable levels of CTLA-4 are found on the surface of naïve T cells, except on T_{regs} (13). Following MHC-peptide/TCR signaling, CTLA-4 is recruited to the immune synapse, an event controlled by the strength of TCR signals (i.e., stronger TCR signals result in greater recruitment of CTLA-4) (14). Like CD28, CTLA-4 binds B7-1 and B7-2, but with greater avidity and affinity (particularly for B7-1) (15).

Table 1
Mechanisms of action for the B7:CD28/CTLA-4 pathway (17-37).

Extrinsic control of immune activation by CTLA-4		
Mechanism	**Outcome**	**Reference(s)**
Reverse signaling through CD80 and CD86 on APCs	Activation of the tryptophan-degrading enzyme IDO leads to suppression of T cell responses by reducing tryptophan levels and/or promoting conversion of naïve T cells to T_{regs}	Grohmann U, *et al.* (17) Fallarino F, *et al.* (18) Munn DH, *et al.*(19) Mellor AL, *et al.* (20) Orabona C, *et al.* (21)
CTLA-4 signaling stimulates the production of regulatory cytokines, such as TGF-β	TGF-β secretion results in inhibition of antigen presentation by APCs and T cell function	Chen W, *et al.* (22)
CTLA-4 binding to CD80 or CD86 reduces the availability of these ligands to engage CD28	Reduced ability of APCs to stimulate functional T cell responses leads to greater threshold of activation	Linsley PS, *et al.* (23) Walunas TL, *et al.* (24) Oaks MK, Hallet KM (25)
CTLA-4 binding to CD80 or CD86 can cause transendocytosis of these ligands	Degradation of co-stimulatory ligands results in decreased APC function	Qureshi OS, *et al.* (26)
Intrinsic control of immune activation by CTLA-4		
Recruitment of inhibitory proteins to T cell synapse	Recruitment of PP2A and PTPN11 to T cell synapse interferes with proximal signaling by either the TCR or CD28, resulting in inhibition of TCR and CD28 proximal signals	Chuang E, *et al.* (27) Parry RV, *et al.* (28) Marengere LE, *et al.* (29)
Ligand competition prevents CD28 signaling	CTLA-4 may prevent CD28 from generating positive signals by acting as a high-affinity competitor for ligands	Thompson CB, Allison JP (30)
Non-ligand binding CTLA-4 splice variant	A splice variant of CTLA-4 that cannot bind to ligands may inhibit T cell activation through an inhibitory signaling pathway, reminiscent to that of the full-length molecule	Vijayakrishnan L, *et al.* (31) Araki M, *et al.* (32) Chikuma S, *et al.* (33) Choi JM, *et al.* (34) Choi JM, *et al.* (35)
Inhibition of T cell stop signal	Increased T cell motility and inhibition of TCR-induced stop signal is required for stable conjugate formation between T cells and APCs. This event leads to reduced contact periods between T cells and APCs that in turn decrease cytokine production and proliferation	Schneider H, *et al.* (36) Schneider H, *et al.* (37)

Thus, small amounts of CTLA-4 can efficiently out-compete CD28 ligand binding and attenuate T cell response (16). Additionally, CTLA-4 has both intrinsic and extrinsic mechanisms of action (Table 1) (17-37), including delocalization of protein kinase C-theta and CARMA1 from the immune synapse (38), limiting the dwell time of T cells (37), transendocytosis of B7 (26), and enhancing T_{reg} function (39). Overall, these mechanisms inhibit cell cycle progression, IL-2 production, and survival pathways, leading to termination of the immune response (40).

The importance of CTLA-4 as a negative regulator is dramatically revealed through the phenotype of CTLA-4 knockout mice. CTLA-4-deficient mice undergo a massive, CD28-dependent expansion of autoreactive T cells in lymph nodes, spleen, and several peripheral organs. These mice die in less than 4 weeks post-birth due to diffuse lymphoproliferative disease (41, 42) but can be rescued from lethality by exogenous expression of the CTLA-4 extracellular domain (43). CTLA-4/B7-1/B7-2 triple knockouts do not present lymphoproliferative disease, indicating a non-redundant role for CTLA-4 in this pathway (44, 45).

In vivo and *in vitro* experiments have shown that when CD4+ and CD8+ T cells do not express CTLA-4, they exhibit an activated phenotype and increased proliferation potential as evidenced by a higher proportion of T cells in the cell cycle (46-49). CTLA-4 null mice exhibit a shift in balance favoring CD4+ T cells over CD8+ T cells, as the population of CD4+ T cells undergoes proliferation. Analyses of CTLA-4-null mice indicate multiorgan infiltration of CD4+ T cells (45, 46), illustrating a critical role for CTLA-4 in the induction of CD4+ T cell tolerance and anergy (50, 51).

In addition to its expression on activated T_{effs}, CTLA-4 is constitutively expressed on the surface of T_{regs} (commonly defined as CD4+ CD25+ FOXP3+ cells) (52, 53). The predominant expression of CTLA-4 on T_{regs} has led to speculation that CTLA-4 may be required for contact-mediated suppression and is associated with T_{reg} production of immunosuppressive cytokines transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10). In fact, conditional knockout mice lacking CTLA-4 in the CD4+ FOXP3+ T-regulatory cellular compartment are characterized by systemic lymphoproliferation, suggesting that CTLA-4 deficiency itself within FOXP3+ T cells can shift immune homeostasis (39). Whereas *in vivo* data are indicative of a role for CTLA-4 in T_{reg} homeostasis, *in vitro* analyses by several groups have provided conflicting results in deciphering such a role for this inhibitory molecule on T_{regs} (40).

While CTLA-4 is known as a central inhibitor of T cell responses, its impact on memory formation of adaptive immune responses is not well understood and is currently an area of ongoing research. One recent study by Rudolph and colleagues (54) found that CTLA-4 blockade modulates the quality of the memory pool by reducing the number of specialized "multifunctional" memory CD4+ T cells, those that coproduce interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and IL-2 in response to antigen. A second study found that *in vivo* administration of anti-CTLA-4 antibody increases memory CD8+ T cell expansion, because treatment resulted in an accumulation of memory cells that were capable of generating cytokines IFN-γ and TNF-α (55).

Table 2
Murine malignancy models: blockade with CTLA-4 (56-85). *All days (d) are relative to implantation of tumor cells.

Murine Tumor	Tumor type / Mouse strain	Anti-CTLA-4 Ab / Tx regimen	Result with CTLA-4 blockade
Brain	SMA-560 Glioma/Vm/Dk (56)	9H10; d7* (100 μg), d10 (50 μg), d13 (50 μg) post-implant	Anti-CTLA-4 monotherapy = 80% survival; increased CD4+CD25- T cells in lymph nodes/spleen
	GL-261 Glioma/C57BL/6 (57)	9H10; d0 (100 μg), d3 (50 μg), d6 (50 μg),	Anti-CTLA-4 monotherapy = 50% survival Anti-CTLA-4 + anti-CD25 = 100% survival; massive amount of IFN-γ secreting CD4 and CD8 TIL
Ovarian	OV-HM/C57BL/6 x C3H/He (58)	UC10-4F10-11;1 mg/mouse	Anti-CTLA-4 monotherapy = 3/5 tumors rejected (d0, d7, d14) 1/5 rejected (d3, d10, d17); 0/5 rejected (d7, d14, d21), (d14, d21, d28)
Bladder	MB49/C57BL/6 (59)	9D9; d7, d10, d13 (200 μg each)	Anti-CTLA-4 monotherapy = tumors rejected Anti-CTLA-4 + CpG-ODN = improved long-term survival; increased levels of tumor-reactive T cells and reduced numbers of T_{regs} at the tumor site
Sarcoma	Meth-A/BALB/c (60)	9H10; d6 (100 μg), d9 (50 μg), d12 (50 μg)	Anti-CTLA-4 monotherapy = 20% survival Anti-CTLA-4 + rMVAp53 = 80% survival
	MC38, 11A1 BALB/c, C57BL/6 (61)	9H10; d14 (100 μg), d17 (50 μg), d20 (50 μg)	Anti-CTLA-4 + MVAp53 + CpG-ODN = 75% survival in both models
Breast	TSA/BALB/c (62)	9H10; d12, d14, d16 (200 μg each)	Anti-CTLA-4 monotherapy = ineffective Anti-CTLA-4 + IR = 80-90% reduction of tumor volume
	4T1 BALB/c (63)	9H10; d14, d18, d21 (200 μg each)	Anti-CTLA-4 monotherapy = 1/9 mice tumor-free Anti-CTLA-4 + IR = 6/9 mice tumor-free
	4T1 BALB/c (64)	9H10; d14, d18, d21 (200 μg each)	Anti-CTLA-4 monotherapy = ineffective Anti-CTLA-4 + IR = tumor rejection/lung metastases inhibition
	4T1 BALB/c (65)	UC10-4F10-11; d7, d11, d15, d19 (100 μg each)	Anti-CTLA-4 monotherapy = ineffective Anti-CTLA-4 + trimAb = 19/26 mice rejected tumors
	SM1/BALB/c (66)	9H10; d4, d7, d10 (100 μg each)	Anti-CTLA-4 monotherapy = ineffective Anti-CTLA-4 + SM1/GM-CSF vaccine = 80% of mice rejected tumors
	EMT6/BALB/c (67)	UC10-4F10-11; d4, d8, d12 (400 μg each) Ixa: d3, d7, d11	Anti-CTLA-4 monotherapy = 40% of mice showed complete tumor regression Ixabepilone = 20% of mice showed complete tumor regression Anti-CTLA-4 + ixabepilone = 100% of mice showed complete tumor regression
Colon	MC38/C57BL/6 (68)	UC10-4F10-11; d7, d11, d16 (100 μg each)	Anti-CTLA-4 monotherapy = ineffective Anti-CTLA-4 + anti-CD25 + DC vaccine = 90% tumor-free survival vs. 53% in DC vaccine only; increased CTL in spleen observed with combination
	MC38 (69)	K4G4, L1B11, L3D10	Created human CTLA-4 knock-in mouse – all 3 clones provided increased survival times with differing autoimmune side effect profiles
	CT26 BALB/c (70)	9H10; d10 (100 μg), d13 (50 μg), d15 (50 μg)	Anti-CTLA-4 monotherapy = 45% survival Anti-CTLA-4 + anti-VEGFR-2 + DC vaccine = 80% survival Monotherapy tx at d0, d3, d5 = 90% tumor rejection
	CT26 BALB/c (67)	UC10-4F10-11; d5, d9, d13 (400 μg each) Ixa: d4, d8, d12	Anti-CTLA-4 monotherapy = 20% of mice showed complete tumor regression Ixabepilone = ineffective Anti-CTLA-4 + ixabepilone = 70% of mice showed complete tumor regression
	MC38/C57BL/6 (71)	UC10-4F10-11; d14, d21, d28 (800 μg each)	Anti-CTLA-4 monotherapy = ineffective Anti-CTLA-4 + anti-4-1BB = >85% of mice rejected tumors Similar results with tx at d2, d9, d16
Lymphoma	BW5147.3/AKR (72)	UC10-4F10-11; d-1 (250 μg), d0 (250 μg), d4 (100 μg), d8 (100 μg), d12 (100 μg)	Hematopoietic stem cell transplantation + anti-CTLA-4 = anti-leukemic effects
	EL4/C57BL/6 (73)	9H10; d3, d5 (100 μg each)	Anti-CTLA-4 monotherapy = ineffective Anti-CTLA-4 + DC vaccine = >60% rejected tumors
Fibrosarcoma	SA1N/A/J (74)	9H10; every 4 days (200 μg each)	Anti-CTLA-4 monotherapy = significant reduction in tumor burden compared to control IFN-γ neutralization abrogated anti-CTLA-4 therapy
	SA1N (67)	UC10-4F10-11; d12, d16, d20 (400 μg each) Ixa: d11, d15, d19	Anti-CTLA-4 monotherapy = 25% of mice showed complete regression of tumors Ixabepilone = ineffective Anti-CTLA-4 + ixabepilone = 71.4% of mice showed complete regression of tumors
Prostate	TRAMP C1[pTC1]/C57BL/6 (75)	9H10; d7, d10, d13 (100 μg each)	Early passage cells = tumor delay observed Late passage cells = tumor rejection observed in 5/5 mice
	TRAMP C2/C57BL/6 (76)	9H10; d4, d7, d10 (100 μg each)	Adjunctive monotherapy CTLA-4 therapy post-resection = 60% metastasis-free compared to 0% with control Ab
	TRAMP/C57BL/6 (77)	9H10; 14-16 week old mice d7, d10, d16 post-IR tx (100 μg each)	(Primary adenocarcinoma and metastases develop in transgenic model at 15-20 weeks) Anti-CTLA-4 + TRAMP C1/C2/GM-CSF vaccine = reduced tumor incidence (15% vs. 75% in control-treated animals)
	TRAMP C2/C57BL/6 (78)	9H10; d29, d33, d40, d50 (100 μg each) d29=1d post-cryoablation	Anti-CTLA-4 monotherapy = ineffective Cryoablation = ineffective Anti-CTLA-4 + cryoablation = 45% tumor-free survival; enhanced intratumoral-specific functional CD8+ T cells
Melanoma	B16/C57BL/6 (79, 80)	9H10; d0, d3, d6 (200 μg each)	Enhanced survival with DC vaccine + anti-CTLA-4; greater survival with inclusion of anti-CD25-depleting antibody
	B16/C57BL/6 (81)	9H10; d6 (100 μg), d8 (50 μg), d10 (50 μg)	Anti-CTLA-4 + Trp-2 peptide vaccine + CpG-ODN = delayed tumor growth by 50 days
	B16/C57BL/6 (82)	9D9; d3, d6, d9	Anti-CTLA-4 + Fvax = rejection of 10% of melanomas Anti-CTLA-4 + Fvax + anti-PD-1 = rejection of 50% of melanomas; increased T_{eff} infiltration
	B16/C57BL/6 (83)	9H10; d3, d6, d9 (100 μg each)	Anti-CTLA-4 + Gvax = 73% long-term survivors; CTLA-4 blockade on both T_{effs} and T_{regs} is synergistic (40% long-term survival if only T_{effs} blocked)
	B16.F10/C57BL/6 (84)	9H10; d5 (100 μg), d7 (50 μg), d9 (50 μg)	Anti-CTLA-4 monotherapy = ineffective Anti-CD40 monotherapy = ineffective Ad-li-GP vaccine = slight survival enhancement Anti-CTLA-4 + anti-CD40 + ad-li-GP vaccine = 30-40% long-term survival
Lung	M109/BALB/c (67)	UC10-4F10-11; d4, d8, d12 (400 μg each) Ixa: d3, d7, d11	Anti-CTLA-4 monotherapy = ineffective Ixabepilone = 50% of mice tumor-free following tumor cell implantation; 20% of mice tumor-free following tumor rechallenge Anti-CTLA-4 + ixabepilone = 80% of mice tumor-free following tumor cell implantation; 75% of mice tumor-free following tumor rechallenge Paclitaxel = 20% of mice tumor-free following tumor cell implantation Anti-CTLA-4 + paclitaxel = 20% of mice tumor-free following tumor cell implantation tumor cell implantation
Plasmacytoma	MOPC-315/BALB/c ANnCrlBr (85)	UC10-4F10-11; 20 mm tumors tx daily for 10 days (100 μg each)	Anti-CTLA-4 monotherapy = ineffective Melphalan = 7/39 mice survived Anti-CTLA-4 + melphalan = 27/37 mice survived

Preclinical application of CTLA-4 blockade in murine tumor models

CTLA-4 blockade has been explored in preclinical models of various solid and hematological tumors, including malignancies of the bladder, blood, brain, breast, colon, fibrosarcoma, lung, ovary, prostate, skin, lymphomas, and plasmacytomas. In order to examine CTLA-4 blockade in murine malignancy models, CTLA-4 mAbs have been employed, either as monotherapy or in combination with other therapeutic modalities, such as vaccines, chemotherapy, and radiation (Table 2) (56-85).

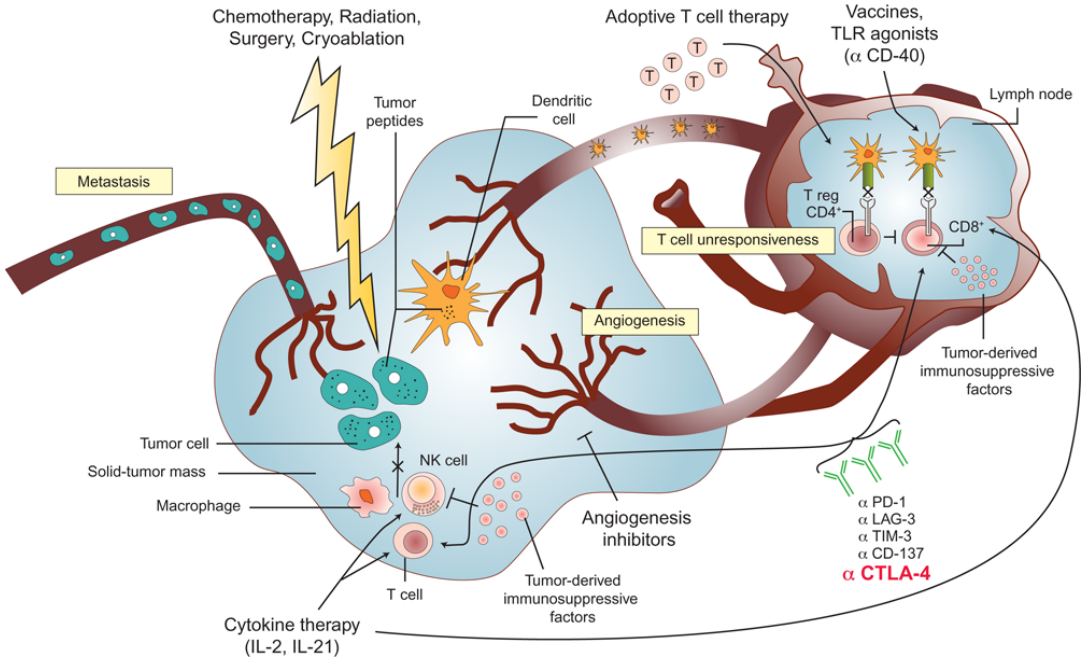
Anti-CTLA-4 monotherapy

Utilization of anti-CTLA-4 monotherapy in murine tumor models has demonstrated a range of cell-line–specific efficacies, as shown in Table 2. Interestingly, few correlations arise between the cell line origin and efficacy; however, several noteworthy trends have been observed. Complete regression or delayed tumor growth is observed in several types of established transplantable murine tumor models, such as cancers of the ovary, bladder, brain, and fibrosarcoma after anti-CTLA-4 immunotherapy (56-59, 67, 74, 86). As expected, mice that rejected the primary tumor developed a memory immune response that prevented tumor growth after a secondary tumor challenge (67, 74, 86). Tumor cell genetics and host backgrounds of these responsive models differ greatly, and predictive markers that determine efficacy have not been defined. Certain variables, such as pretreatment immune cell infiltrates and endogenous tumor-specific T cell clones, have not been fully defined in these models. As discussed below, characterization of these factors in murine models may allow for greater understanding of which human tumor types will respond to antibody therapy.

In contrast to the examples given above, CTLA-4 blockade was ineffective in B16 melanoma, SM1 mammary carcinoma, EL4 lymphoma, M109 lung cancer, and MOPC-315 plasmacytoma models (66, 67, 73, 80, 85). Among transplantable breast cancer models, three cell lines, TSA (62), 4T1 (63-65), and SM1 (66), failed to respond to anti-CTLA-4 blockade, while the EMT6 line was partially sensitive to monotherapy (67). Two models of colon cancer, MC38 and CT26, were also found to be differentially sensitive to anti-CTLA-4 monotherapy: MC38 was insensitive (68, 71), while CT26 displayed a partial response (67, 70).

Based on the numerous studies using anti-CTLA-4 antibody as a monotherapy, two general statements can be made on its efficacy. First, tumor regression mediated by anti-CTLA-4 monotherapy occurs more frequently in tumors that are historically thought to be intrinsically immunogenic. For example, SA1N fibrosarcomas respond well to immunotherapy (67) and have a spontaneous regression rate of approximately 5-10%, highlighting their immunogenicity (Maria Jure-Kunkel, unpublished observations). As discussed below, this trend is also being observed in human tumors, where immune activation signals prior to immunotherapy are predictive of response (87). As ongoing research advances our understanding of tumor immunology, however, the definition of the factors contributing to immunogenicity is evolving. Evidence of ongoing anti-tumor immune responses have been observed in a wide variety of solid tumors beyond those historically considered immunogenic, revealing a potential for anti-tumor activity through CTLA-4 blockade. Secondly, a lower tumor burden upon administration of immunotherapy appears to increase the response rate, as observed in the ovarian cancer model (58). An increase in tumor burden is generally accompanied by enhanced production of immunosuppressive factors such as TGF-β, IL-10,

Figure 1



Complementary approaches to overcome tumor-induced tolerance. Blockade of CTLA-4 on potential effector cells in secondary lymphoid organs is combined with different strategies, such as chemotherapy, radiation, surgery, cryoablation, vaccination, adoptive T cell therapy, cytokine therapy, and antibody blockade of additional negative (i.e., PD-1, LAG-3, Tim-3) or positive (i.e., CD137) regulatory receptors to achieve synergistic anti-tumor effects. [Adapted from Bartlett JB *et al.* (93)].

and soluble MHC class I-related chain A (sMICA), which often leads to a disruption in immune activation signals and adversely affects the anti-tumor immune response.

The observation that tumor lines derived from the same organ exhibit differential responses to anti-CTLA-4 monotherapy is intriguing and may present an opportunity to identify possible predictive biomarkers for treatment of human disease. An understanding of tumor cell growth and cell death characteristics, how individual tumors shape their microenvironment, and the evaluation of their immunomodulatory surface molecules and secreted factors will better enable us to predict cellular resistance and sensitivity to immunotherapy.

The complex and time-consuming process of evaluating and characterizing human tumors is under way, and experiments have demonstrated that nearly all human cancers may be classified into subtypes that may display differential phenotypes, prognoses, and responses to treatment. A tremendous effort has been placed in characterizing these subtypes of human cancers. For instance, gene expression profiling studies have described six subtypes of triple-negative breast cancer, including an immunomodulatory subtype, each with unique sensitivity to chemotherapeutic agents (88). Understanding not only genetic but also phenotypic differences between CTLA-4 responsive versus nonresponsive tumors (i.e., epigenomic profile, inflammatory cytokine/chemokine production, and surface expression of immunomodulatory proteins such as MHC and PD-L1) may all be necessary to understand the observed efficacy patterns of therapeutic CTLA-4 blockade.

In addition to the tumor, the lineage and status of T cells that CTLA-4 blockade alters is of great interest and is likely to affect the anti-tumor immune response. For instance, anti-CTLA-4 monotherapy may not alter the status of CD8+ T cells that are in a tolerized state at the tumor site; therefore, further manipulation, such as decreasing tumor burden or anti-PD-1 therapy (which inhibits T cell activation through a mechanism distinct from that of CTLA-4), may be necessary for tumor rejection. Many combination therapies, as described below, appear to synergize with CTLA-4 blockade and dramatically enhance anti-tumor immunity.

Combination therapy with anti-CTLA-4 immunotherapy

The versatility of CTLA-4 blockade, in combination with multiple therapeutic interventions, has been illustrated in a variety of mouse tumor models. Synergistic effects or augmented anti-tumor activity have been demonstrated in combination with vaccines (60, 61, 66, 68, 70, 73, 77, 79-82), chemotherapy (67, 85), radiation (62-64), cytosine-phosphate-guanine oligodeoxynucleotides (CpG-ODN) adjuvants (59, 61, 81), antibodies (57, 65, 68, 70, 71), cryoablation (78), and surgery (72, 76).

The rationale behind using these interventions is based upon our understanding of how the immune system becomes activated, sustains a functional response, and reverses a preexisting tolerogenic state. The major concepts underlying these treatments are to prime a functional tumor-specific T cell response, release tumor-associated antigens, reduce tumor burden by immunogenic cell death, decrease pro-tumor/anti-immune factors, increase immune cell access to tumor, and restore/enhance anti-tumor immune cell function.

The combination of anti-CTLA-4 immunotherapy with agents that prime immune responses have been successfully employed in multiple tumor models and highlight the importance of immune priming for successful anti-CTLA-4 immunotherapy. For example, in the EL4 lymphoma mouse model, administration of a dendritic cell vaccine, in addition to CTLA-4 blockade, was effective in rejecting more than 60% of tumors, while the vaccine and anti-CTLA-4 antibody were ineffective as monotherapies (73). Likewise, the combination of anti-CTLA-4 antibody and vaccination with B16 or SM1 cells, which have been genetically modified to express granulocyte macrophage-colony stimulating factor (GM-CSF), was administered in the B16 melanoma model and SM1 mammary carcinoma model, respectively, after they did not respond well to CTLA-4 blockade monotherapy (66, 80). Importantly, anti-CTLA-4 antibody in combination with a GM-CSF–expressing tumor cell vaccine demonstrated enhanced efficacy and tumor regression in the B16 melanoma model, along with the presence of certain toxicities, such as skin depigmentation. Taken together, these data suggest that CTLA-4 blockade in combination with antigen-specific immunotherapy could break tolerance to antigens aberrantly expressed in tumors, resulting in tumor clearance and long-term host immunity upon tumor rechallenge (80).

There is preclinical and clinical evidence to suggest that chemotherapy- or radiotherapy-mediated tumor cell death may be immunogenic, in that the dying tumor cells may release tumor antigens for presentation and enhance priming of the immune system (89-91). Addition of CTLA-4 blockade to the cytotoxic epothilone B analog ixabepilone led to synergistic anti-tumor effects in multiple models, including EMT6 (breast cancer), CT26 (colon cancer), and SA1N (fibrosarcoma) (67). In the 4T1 breast cancer model, pairing radiation and CTLA-4 blockade resulted in > 50% increase in tumor-free mice over CTLA-4 blockade monotherapy (63, 64). While chemotherapy and radiotherapy can both be effective at decreasing tumor burden and releasing tumor antigens, radiotherapy may support successful immunotherapy due to its ability to eliminate resident immunosuppressive cells, such as CD4+ T_{regs} and myeloid-derived suppressor cells.

When given with modified vaccinia Ankara-expressing murine *p53*, the combination of CpG-ODN adjuvant and anti-CTLA-4 worked synergistically to reject palpable 11A1 and MC38 tumors (61). Moreover, it was recently demonstrated that coupling a Flt3-ligand vaccine with dual antibody-mediated blockade of CTLA-4 and PD-1 resulted in expansion of infiltrating T_{effs} and reduction of T_{regs}, culminating in a favorable T_{eff}-to-T_{reg} T cell ratio within B16 melanoma tumors (82).

Studies of CTLA-4 blockade in murine models have been primarily conducted in healthy mice bearing transplantable tumors, due to the difficulty of evaluating this type of therapy in mouse models where tumors arise spontaneously. However, data demonstrating the role of CTLA-4 in T cell tolerance supports the hypothesis that blocking CTLA-4 might rescue anergic T cells, which often develop as a result of chronic inflammation. In fact, adding a CTLA-4 mAb to vaccination with GM-CSF in a transgenic mouse model that spontaneously develops prostate adenocarcinoma (TRAMP) effectively reduced tumor incidence (77).

Several models have demonstrated that activity associated with CTLA-4 blockade was dependent on CD8+ T cell expansion and, in some models, CD4+ T cells and IFN-γ (64, 86). Additionally, blocking CTLA-4 interactions reversed tolerance of CD8+ T cells by a mechanism dependent on CD4+ T cells and IL-2, providing evidence for the reactivation of tolerized cytotoxic T lymphocyte (92). Overall, the data suggest that anti-CTLA-4 monotherapy is efficacious in a select popula-

Table 3
CTLA-4 blockade: Ongoing and recruiting phase II trials (98). *Denotes phase I/II trial.

Clinical Trials.gov Identifier	Treatment Arm(s)	Patient Population	Primary Outcome Measure	Status	Sponsor
NCT00162123	Ipilimumab (3 or 10 mg/kg) vs. Ipilimumab (0.3, 3, or 10 kg/mg) vs. No Intervention	Extended-treatment monotherapy or follow-up for melanoma patients previously enrolled in MDX-010	Safety	Ongoing	Bristol-Myers Squibb
NCT00378482	CP-675,206 (Tremelimumab)	Colorectal neoplasms, melanoma, prostatic neoplasms, renal cell carcinoma, neoplasms, patients who have/have had melanoma and other tumors	To allow access to CP-675,206 for subjects who received CP-675,206 in other trials, Measure safety and efficacy	Ongoing	AstraZeneca
NCT00471887	Ticlimumab (CP-675,206)	Stage IIIC or Stage IV melanoma	Change in percent tumor infiltration by CD8 positive CTL	Ongoing	Jonsson Comprehensive Cancer Center
NCT00610857	High-dose Interferon Alfa-2b/Anti-CTLA-4 mAb	Recurrent inoperable Stage III or Stage IV melanoma	Improved response rate with combination	Ongoing	University of Pittsburgh
NCT00623766	Ipilimumab vs. Ipilimumab/Corticosteroids	Melanoma with brain metastases	Tumor assessment	Ongoing	Bristol-Myers Squibb
NCT00871481	Ipilimumab/ Cyclophosphamide/Biopsy/ Aldesleukin/IHC/PCR/ Immunoenzyme technique/ Therapeutic allogeneic CTL	Metastatic melanoma	Numeric frequency and functional persistence of transferred CTL, Toxicity assessment of study treatment by CTCAE v3.0	Recruiting	Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium
NCT01034787	CP-675,206	Unresectable or metastatic uveal melanoma	6-month PFS	Ongoing	Alberta Health Services
NCT01119508	Ipilimumab/Temozolomide	Metastatic melanoma	6-month PFS	Ongoing	MD Anderson Cancer Center
NCT01134614	Ipilimumab vs. Ipilimumab/Sargramostim	Stage III or Stage IV melanoma that cannot be removed by surgery	OS	Ongoing	Eastern Cooperative Oncology Group
NCT01216696	Ipilimumab	Advanced melanoma and spontaneous preexisting immune response to NY-ESO-1	Disease control rate (irRC)	Recruiting	National Center for Tumor Diseases, Heidelberg
NCT01302496	Ipilimumab/TriMix-DC	Previously treated, unresectable Stage III or Stage IV melanoma	Disease control rate (irRC)	Ongoing	Bart Neyns
NCT01323517	Ipilimumab/Melphalan/ Dactinomycin	Advanced unresectable melanoma of the extremity	1-year PFS	Recruiting	Memorial Sloan-Kettering Cancer Center
NCT01355120	Ipilimumab	Ocular melanoma	OS	Recruiting	Prof. Dr. med. Dirk Schadendorf
NCT01363206	Ipilimumab/GM-CSF	Unresectable, metastatic malignant melanoma	Disease control rate at 24 weeks (irRC)	Recruiting	Lynn E. Spitler, M.D.
NCT01400451*	Ipilimumab/Vemurafenib	Metastatic melanoma and expression an activated mutant form of the BRAF oncogene (V600E)	OS	Recruiting	Bristol-Myers Squibb
NCT01497808*	Ipilimumab/Stereotactic body radiation therapy	Metastatic melanoma	Dose-limiting toxicity	Recruiting	Abramson Cancer Center of the University of Pennsylvania
NCT01565837	Ipilimumab/Stereotactic ablative radiosurgery	Oligometastatic but unresectable malignant melanoma	Safety and tolerability	Recruiting	Wolfram Samlowski
NCT01604889*	Ipilimumab/INCB024360 vs. Ipilimumab/Placebo	Unresectable or metastatic melanoma	OS	Recruiting	Incyte Corporation
NCT01654692	Ipilimumab/Fotemustine	Unresectable locally advanced or metastatic malignant melanoma	irDCR	Ongoing	Italian Network for Tumor Biotherapy
NCT00323882*	MDX-010	Metastatic hormone-refractory PC	Safety	Ongoing	Bristol-Myers Squibb
NCT01194271	Ipilimumab/ Leuprolide acetate/ Radical prostatectomy	PC	Longitudinal peripheral blood values	Recruiting	MD Anderson Cancer Center
NCT01377389	Ipilimumab/Androgen deprivation therapy	Castrate-sensitive PC	Achieving a PSA ≤ 0.2 ng/mL at 7 months	Ongoing	MD Anderson Cancer Center
NCT01498978	Ipilimumab/Androgen suppression therapy	Metastatic hormone-resistant PC	Fraction of patients who achieve an undetectable PSA	Recruiting	OHSU Knight Cancer Institute
NCT01471197	Ipilimumab vs. Pemetrexed	Non-squamous NSCLC	OS	Recruiting	Bristol-Myers Squibb
NCT01331525	Ipilimumab/Carboplatin/Etoposide	Extensive-stage SCLC	1-year PFS	Recruiting	University Hospital Southampton NHS Foundation Trust
NCT01524991	Ipilimumab/Gemcitabine/Cisplatin	Metastatic urothelial carcinoma	1-year OS	Recruiting	Hoosier Oncology Group
NCT01585987	Ipilimumab vs. BSC	Unresectable locally advanced/ metastatic gastric or gastro-esophageal junction cancer	irPFS	Recruiting	Bristol-Myers Squibb
NCT01611558	Ipilimumab	Recurrent, platinum-sensitive ovarian cancer	Adverse events of grade ≥ 3 during induction	Recruiting	Bristol-Myers Squibb
NCT01649024	Tremelimumab	Malignant mesothelioma	Objective tumor response by modified RECIST	Ongoing	Azienda Ospedaliera Universitaria Senese
NCT01655888	Tremelimumab	Malignant mesothelioma	Objective response	Recruiting	Azienda Ospedaliera Universitaria Senese

Table 4
CTLA-4 blockade: Ongoing and recruiting phase III trials (98)

Clinical Trials.gov Identifier	Treatment Arm(s)	Patient Population	Primary Outcome Measure	Status	Sponsor
NCT00324155	Ipilimumab/Dacarbazine vs. Placebo/Dacarbazine	Untreated unresectable Stage III or Stage IV melanoma	OS	Ongoing	Bristol-Myers Squibb
NCT00636168	Ipilimumab vs. Placebo	Completely resected high-risk Stage III melanoma	RFS	Ongoing	Bristol-Myers Squibb
NCT01274338	Ipilimumab at high-dose vs. Ipilimumab at low-dose vs. Recombinant interferon alfa-2b	High-risk Stage III or Stage IV melanoma that has been removed by surgery	RFS, OS	Recruiting	Eastern Cooperative Oncology Group
NCT01515189	Ipilimumab 3 mg/kg vs. Ipilimumab10 mg/kg	Unresectable or metastatic melanoma	OS	Ongoing	Bristol-Myers Squibb
NCT00861614	Ipilimumab vs. Placebo, each following a single dose of RT	Advanced CRPC post-docetaxel	OS	Recruiting	Bristol-Myers Squibb
NCT01057810	Ipilimumab vs. Placebo	Asymptomatic or minimally symptomatic chemotherapy-naïve metastatic CRPC	OS	Recruiting	Bristol-Myers Squibb
NCT01285609	Ipilimumab/Paclitaxel/ Carboplatin vs. Placebo/Paclitaxel/ Carboplatin	Squamous NSCLC	OS	Recruiting	Bristol-Myers Squibb
NCT01450761	Ipilimumab/Etoposide/ Cisplatin or Carboplatin vs. Placebo/Etoposide/ Cisplatin or Carboplatin	Extensive-stage SCLC	OS	Recruiting	Bristol-Myers Squibb

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tion of tumor models. Furthermore, combination modalities that employ another immunotherapy with a divergent mechanism of action (i.e., vaccination or blockade of additional negative regulatory proteins) or immunosupportive anti-cancer therapies (i.e., chemotherapy, radiation, surgery, or others), have the potential to greatly enhance the scope and overall efficacy of CTLA-4 blockade immunotherapy (Figure 1) (93).

Clinical development of CTLA-4 monoclonal antibodies in oncology

Studies conducted with various anti-CTLA-4-specific mAbs have demonstrated that selective blockade of CTLA-4 leads to enhancement of endogenous or induced anti-tumor immune responses, providing support for clinical development of mAbs that target CTLA-4. Two fully human antibodies, ipilimumab and tremelimumab, both bind CTLA-4 and block its interaction with B7 ligands to augment T cell activation and proliferation (94, 95). Ipilimumab and tremelimumab have been under clinical investigation for the past 10 years, and both have undergone the most extensive study in melanoma. Notably, ipilimumab was approved in 2011 at a dose of 3 mg/kg for treatment of unresectable or metastatic melanoma by regulatory agencies in the United States, European Union, and Australia (4-6).

Ipilimumab approval came following a phase III study in which patients with advanced metastatic melanoma demonstrated statistically significant survival improvement when treated with 3 mg/kg ipilimumab, either as monotherapy or in combination with melanoma vaccine glycoprotein 100 (gp100), compared with patients given gp100 monotherapy alone (96). More recently, another phase III trial in chemotherapy-naïve patients with metastatic melanoma demonstrated improved overall survival in cohorts treated with 10 mg/kg ipilimumab in combination with the chemotherapeutic agent dacarbazine, compared with those given dacarbazine and placebo (97). Based on these successful trials, ipilimumab is presently being investigated as monotherapy and in combination with other standard of care agents, such as chemotherapy, radiation, hormonal ablation, and immunotherapy, in phase II and III trials across a number of tumor types, including but not limited to melanoma, small cell lung cancer, non-small cell lung cancer, and prostate cancer (Table 3 and Table 4) (98).

Four patterns of tumor response to ipilimumab have been noted, and while some of these responses resemble those observed with cytotoxic chemotherapeutic agents, others may differ (99). Patients treated with either a chemotherapy regimen or ipilimumab therapy may demonstrate an immediate response in baseline lesions without the presence of new lesions; however, ipilimumab treatment has also yielded durable stable disease, which may be followed by a slow, steady decline in total tumor burden (99). Ipilimumab treatment has also resulted in a response in the presence of new lesions (which may have been present at baseline, but were radiographically undetectable) or in a response following an increase in total tumor burden (99). While ipilimumab has demonstrated substantial clinical benefit, it is also characterized by adverse events that manifest as inflammatory conditions; these adverse events have been managed in clinical studies by protocol-specific treatment guidelines (4, 96, 97).

Early phase I and II trials with single-agent tremelimumab report durable clinical response to the agent in melanoma patients (100-102). Based on this earlier success with tremelimumab in phase I and II trials in melanoma, a subsequent larger phase III study was carried out in patients with previously untreated metastatic melanoma to test the survival benefit of single-agent tremelimumab, relative to standard of care dacarbazine or temozolomide (103). This phase III study, however, was closed early, as an interim analysis reported that tremelimumab failed to demonstrate improvement in overall survival in the experimental group compared with the control (104).

Clinical experience with tremelimumab indicates that this agent is well-tolerated in advanced cancers (95, 104). A retrospective analysis of several trials in which tremelimumab monotherapy was evaluated reveals that most of the patients treated with a median of single dose of tremelimumab experienced at least one treatment-related adverse event, the

majority of which were mild to moderate in nature (104, 105). Common adverse events related to treatment were diarrhea, rash, and fatigue (104, 105), which are likely reflective of the agent's immune-based mechanism of action.

In 2011, AstraZeneca's MedImmune took over global clinical development of tremelimumab (106), and began exploring utility of this agent in a number of tumor types, including malignant mesothelioma, as shown in Table 3. Most recently, results were reported from a small phase II trial in which tremelimumab's clinical activity was evaluated in patients with advanced hepatocellular carcinoma due to chronic hepatitis C infection (107). Of 21 evaluable patients in this trial, tumor burden was reduced in 2 patients, and 11 patients had disease stabilization for more than a year. Importantly, investigators also observed a reduction of hepatitis C virus in the blood of patients, which was complemented by objective enhancements of anti-viral immunity (107). Tremelimumab was well-tolerated in this trial, and most patients experienced mild to moderate adverse events, such as itching and rash (107), consistent with observations in previous trials with this agent (104, 105).

CTLA-4 blockade has been, and continues to be, evaluated in various model systems, and ongoing basic science and clinical research has led to an improved delineation of the detailed regulation of this and other components involved in the immune response pathway.

Emerging biomarkers for ipilimumab in clinical studies

Monitoring immune cell phenotype and activation states prior to and during the course of anti-CTLA-4 immunotherapy in patients is an intensely active area of study. Data from these studies aim to enhance our understanding of the drug's mechanism of action, identify predictive markers that correlate with clinical response and/or toxicity, and identify pharmacodynamic markers that determine drug activity.

As reviewed by Callahan and colleagues, several promising factors have been described that may potentially serve as biomarkers for response to ipilimumab therapy (108). Biomarkers that correlate with anti-CTLA-4 clinical activity include a rise in absolute lymphocyte counts, sustained inducible T cell co-stimulator (ICOS) expression on T cells (109), and an upregulation of HLA-DR/CD45RO on T cells (110). In ipilimumab-treated patients with melanoma, a strong correlation between clinical benefit and an increase in tumor-infiltrating lymphocytes (TILs) was observed, as assessed by histology (111). In addition, this study also demonstrated benefit in patients who were forkhead box P3 (FOXP3)-positive and indoleamine 2,3-dioxygenase (IDO)-positive within the tumor microenvironment.

In a study conducted at Memorial Sloan-Kettering Cancer Center and Yale University, patients with melanoma who developed antibodies to cancer antigen NY-ESO-1 prior to or during ipilimumab therapy, and displayed NY-ESO-1-specific T cell reactivity were highly responsive to therapy, compared to NY-ESO-1-seronegative patients (112). This intriguing result suggests that patients with ongoing immune responses against their tumors may have greater benefit with immunotherapy, and that combination of anti-CTLA-4 therapy with agents that elicit anti-tumor immune responses (i.e., vaccines, certain types of chemotherapies or radiotherapy) may greatly enhance efficacy.

Gene expression analysis of tumor biopsies before and 3 weeks following ipilimumab monotherapy in patients with melanoma has revealed interesting patterns of gene expression in response to therapy. These include an increase in IFN-γ-related genes and decrease in genes associated with cellular proliferation and melanoma-specific antigens. Notably, responders to ipilimumab therapy had higher levels of immune-related genes at baseline compared to non-responders (87). Taken together, these findings support the concept described in murine models that patients with an ongoing immune response may respond successfully to anti-CTLA-4 immunotherapy.

Discussion

CTLA-4 blockade offers a new paradigm in modulating the immune system and it represents a landmark discovery in cancer therapy. The body of preclinical research on CTLA-4 has expanded our knowledge of the molecular mechanisms involved in immune response to tumor cells and has increased our understanding of clinical responses observed with use of human antibodies to target CTLA-4. Furthermore, preclinical findings on CTLA-4 have laid the groundwork for major advances in targeting other T cell co-inhibitory receptors, such as PD-1, PD-L1, Tim-3, and LAG-3. As combinatorial approaches of CTLA-4 blockade with other therapeutic agents, such as chemotherapy and radiation, have proven to be effective in regression of tumors in various malignancy murine models, such concepts are now under intense clinical investigation in multiple tumor types, including melanoma, small cell lung cancer, non-small cell lung cancer, and prostate cancer.

Preclinical studies, including cellular analyses and mouse models, have added a great deal to our understanding of the B7:CD28/CTLA-4 pathway in support of the above clinical advances. However, there are details in this T cell activation pathway that still require elucidation, such as the specific molecular and spatio/temporal mechanisms by which CTLA-4 regulates TCR/CD28 signaling, how CTLA-4 signaling contributes to the formation of T_{regs}, the regulation of intracellular CTLA-4 trafficking, and the contribution of CTLA-4 variants to tumor-mediated immunosuppression and anti-CTLA-4 immunotherapy.

Our understanding of the immune system components that are necessary for a functional, long-lasting anti-tumor response has greatly evolved, largely due to the use of murine tumor models; however, there is a dearth of suitable pharmacodynamic and predictive biomarkers for CTLA-4 blockade immunotherapy. In general, the presence of TILs is considered a favorable indicator of outcome, yet a clear correlation between TILs and clinical response to immunotherapy has yet to be demonstrated. Recently, a phase II trial in metastatic melanoma showed that an increased number of TILs post-ipilimumab treatment is associated with clinical response (111). Furthermore, patients whose tumors had higher levels of expression of genes involved in immune function at baseline responded better to ipilimumab treatment; in fact, expression of genes associated with T cell responses were increased post-ipilimumab therapy (87). These findings support the concept that ipilimumab may be more efficacious in subjects who have endogenous, albeit ineffective, anti-tumor immune responses, as expected from the preclinical data. Although these findings are encouraging, future trials investigating TILs and other markers are warranted to confirm their clinical utility. It is possible that anti-CTLA-4 antibodies modulate specific subsets of T cell populations. Utilization of polychromatic flow cytometry techniques to characterize the phenotype and activation state of effector, regulatory, naïve, and memory T cells in tumor and peripheral compartments (113) will be vital to

understanding the mechanisms of immunomodulatory therapies and may help elucidate which T cell subsets may be the most beneficial for study. Tumor gene expression and/or proteomic profiling prior to and after CTLA-4 blockade may unveil pharmacodynamic and predictive markers to therapy. A rigorous profiling of anti-CTLA-4 responsive and nonresponsive tumors may reveal distinct gene expression patterns and pathways that help determine therapeutic outcome. In addition, correlating antibody pharmacokinetics and dose levels to efficacy and pharmacodynamic endpoints, particularly during combination therapy treatments, will vastly increase our understanding of drug mechanisms and the anti-tumor responses, and further the rational design and sequencing of combination modalities employing CTLA-4 blockade.

Our increasing knowledge of the immune system and its interplay with tumors will undoubtedly lead to novel therapeutic clinical regimens. A continuous effort to evaluate tumor models will not only enable an efficient development of these drugs, but also assist in the development of personalized medicinal treatments.

Abbreviations

CTLA-4, cytotoxic T lymphocyte antigen-4; T_{reg}, regulatory T cell; T_{eff}, effector T cell

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