Modulation of CTLA-4 and GITR for Cancer Immunotherapy

Francesca Avogadri, Jianda Yuan, Arvin Yang, David Schaer, and
Jedd D. Wolchok

Contents

1	Introduction	212
2	CTLA-4 Preclinical Data	213
2.1	CTLA-4: A “Brake” on T Cell Activation	214
2.2	Preclinical Studies Using CTLA-4 Blocking Antibodies	217
3	GITR Preclinical	219
3.1	GITR: “Accelerator” of Effector Function	220
3.2	Preclinical Studies Using GITR Agonist Antibody	220
4	Clinical Experiences with CTLA-4 Blockade	222
4.1	CTLA-4 Monotherapy	223
4.2	Postsurgical Adjuvant Therapy	225
4.3	Combinations	226
4.4	Tremelimumab	226
4.5	Immune Related Adverse Effects	227
4.6	Novel Criteria for Antitumor Response to Ipilimumab and Increased Duration of Response	227
4.7	Other Malignancies	228
5	From Clinical Trials, Back to the Bench	229
5.1	Monitoring Antigen-Specific CD4+ and CD8+ T Cell Activity and Polyfunctionality	229

F. Avogadri and D. Schaer

Immunology Program, Sloan-Kettering Institute, New York, NY 10065, USA

J. Yuan

Immunology Program, Sloan-Kettering Institute, New York, NY 10065, USA

Ludwig Center for Cancer Immunotherapy, Immunology Program, Sloan-Kettering Institute, New York, NY 10065, USA

A. Yang and J.D. Wolchok (✉)

Immunology Program, Sloan-Kettering Institute, New York, NY 10065, USA

Melanoma/Sarcoma Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

Ludwig Center for Cancer Immunotherapy, Immunology Program, Sloan-Kettering Institute, New York, NY 10065, USA

e-mail: wolchokj@mskcc.org

5.2	Cellular Phenotype Analysis: ICOS, Foxp3, HLA-DR and IDO	230
5.3	ICOS	231
5.4	Foxp3	231
5.5	HLA-DR	231
5.6	IDO	232
5.7	Antibody Responses	232
5.8	The “Immunogram”: A Tool to Synthesize Immune Monitoring	233
6	Future Directions	233
	References	236

Abstract The rational manipulation of antigen-specific T cells to reignite a tumor-specific immune response in cancer patients is a challenge for cancer immunotherapy. Targeting coinhibitory and costimulatory T cell receptors with specific antibodies in cancer patients is an emerging approach to T cell manipulation, namely “immune modulation.” Cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor family receptor (GITR) are potential targets for immune modulation through anti-CTLA-4 blocking antibodies and anti-GITR agonistic antibodies, respectively. In this review, we first discuss preclinical findings key to the understanding of the mechanisms of action of these immunomodulatory antibodies and the preclinical evidence of antitumor activity which preceded translation into the clinic. We next describe the outcomes and immune related adverse effects associated with anti-CTLA-4 based clinical trials with particular emphasis on specific biomarkers used to elucidate the mechanisms of tumor immunity in patients. The experience with anti-CTLA-4 therapy and the durable clinical benefit observed provide proof of principle to effective antitumor immune modulation and the promise of future clinical immune modulatory antibodies.

1 Introduction

In the late nineteenth century, the antitumor effects of Coley’s toxin provided the first suggestive evidence that the immune system could be harnessed to combat cancer. Over 100 years later, we now possess a better understanding of mechanisms of T cell activation and the technology to manipulate these findings in the clinical setting. Current clinical, immunotherapeutic treatments, although not exclusively effective in one disease, have been most successful in melanoma. FDA approved treatments for melanoma include the adjuvant use of high-dose interferon alpha and high-dose IL-2 in the metastatic setting. The complete list of approved therapies for melanoma only requires the addition of dacarbazine (DTIC), the only FDA approved chemotherapeutic agent for melanoma. The modest response rates for both IL-2 and DTIC coupled with recent epidemiological data demonstrating an increasing incidence of melanoma provide incentive for alternative strategies. Recent advances in immunology have led to a more profound insight regarding

the function of costimulatory and coinhibitory receptors expressed by different T cell subsets, providing a novel approach to optimize immunotherapies through immune modulation.

During the primary activation of naïve T lymphocytes, the immune system utilizes various checks and balances to maintain tolerance to self while assuring appropriate activation against foreign and self antigens. Although primary antigen recognition occurs through the interactions of the T cell receptor (TCR) and peptide-MHC complexes, without costimulation through CD28 binding to either B7-1 (CD80) or B7-2 (CD86), cognate antigen recognition will result only in T cell anergy induction (Linsley and Ledbetter 1993). This first check is followed by additional signals mediated by coinhibitory/costimulatory receptors, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor family receptor (GITR), which further shape the resulting effector function and dictate its efficacy and duration. Since their discovery, much effort has been put into understanding the immunomodulatory properties of CTLA-4 and GITR in mice. The development of antibodies specifically targeting these receptors and modulating their functions has provided a new perspective for immunotherapeutic approaches.

Under physiological stimulus, TCR binding causes activation of a complex signaling cascade culminating in downstream activation of the NF- κ B, NFAT pathways and target gene transcription (Chan et al. 1995; Zhang et al. 1998; van Leeuwen and Samelson 1999; Tybulewicz et al. 2003). However, naïve T cells require CD28 costimulation to maintain this cascade. When CD28 is activated, it potentiates the cascade through activation of PI3K and Sos resulting in stabilization of mRNA for NF- κ B, NFAT (Pages et al. 1994). The importance of costimulation in T cell physiology is highlighted by CD28 $^{-/-}$ mice which have dramatic reduction in the ability to maintain T cell activation (Lucas et al. 1995). Thus, CD28 provides the first checkpoint in T cell activation, sensing the expression of CD80/CD86 on an activated antigen presenting cell (APC). If the APC has not experienced the proper “danger signals” (e.g., cytokines, TLR, Fc Receptor stimulus), it will not optimally upregulate the expression of CD80/CD86. This need for CD28 costimulation is thought to reduce inadvertent activation of possibly self-reactive T cell clones in the periphery, which have escaped thymic deletion. Reinforcing this checkpoint is CTLA-4, which acts as a coinhibitory molecule. Antagonizing CD28 T cell costimulation, CTLA-4 binds with much greater affinity to CD80/CD86 and effectively shuts off TCR signaling (van der Merwe et al. 1997).

2 CTLA-4 Preclinical Data

CTLA-4 is a member of the CD28:B7 immunoglobulin superfamily. In contrast to CD28, CTLA-4 is normally expressed at low levels on the surface of naïve effector T cells (Teff) and mainly exists in prepackaged vesicles inside the cytosol (Alegre et al. 1996). When the TCR stimulus to the naïve T cell is too strong or lasts too long, CTLA-4 is recruited to the cell surface in a polar manner with release at the

site of the immunological synapse (IS) (Linsley et al. 1996). Once at the IS, CTLA-4 can now compete with CD28 for CD80/CD86 (Chuang et al. 1999; Carreno et al. 2000; Cinek et al. 2000), effectively shutting off TCR signaling (van der Merwe et al. 1997). While CTLA-4 translocation to the cell membrane has been shown to depend on many events downstream of TCR signaling (reviewed in (Rudd et al. 2009)), it is not yet entirely clear how CTLA-4 mediates TCR signaling shut down upon binding to CD80/CD86. CTLA-4 does not have traditional immune tyrosine inhibitory motif (ITIM) domains, which are known to recruit regulatory proteins through binding of SH2 domains to phosphorylated tyrosine residues in the (I/V/L/S)xYxx(L/V) motif (Blank et al. 2009). However, CTLA-4 does have other tyrosine motifs which have been shown to recruit phosphatases SHP-2, and PP2A, yet the functional significance of these interactions remain unclear. For instance, SHP-2 also associates with CD28 and has been shown to potentiate the TCR cascade (Gadina et al. 1998). Although PP2A may act as a negative regulator of the TCR cascade, whether or not it does this through its interactions with CTLA-4 is also not fully established (Chuang et al. 2000). Besides directing downstream signaling events, it is thought that CTLA-4 may function simply to sequester CD80/CD86 away from CD28 because of its higher affinity for these molecules. In fact, mutant CTLA-4 molecules with levels of surface expression that lack tyrosines or prolines in their cytoplasmic tail are still able to suppress CD80-mediated CD28 activation signals (Carreno et al. 2000; Cinek et al. 2000).

Once it has been triggered, CTLA-4 may destabilize components of the super molecular activation complex (SMAC) in the IS. In fact, CTLA-4 was shown to alter lipid raft formation, resulting in reduced phosphorylation of linker for the activation of T cells (LAT) after TCR and CD28 stimulation (Martin et al. 2001). This block in IS formation applies also to ZAP-70 microclusters which are needed to maintain calcium flux and downstream events in TCR signaling (Bunnell et al. 2002; Schneider et al. 2008). Thus, CTLA-4 requires initial TCR signaling for its membrane targeting, and subsequently shuts off these early activation events.

2.1 CTLA-4: A “Brake” on T Cell Activation

The importance of CTLA-4 control of T cell activation was originally demonstrated by studies showing that blockade of CTLA-4:B7 interactions enhanced T cell responses in vitro (Walunas et al. 1994). In vivo adoptive transfer experiments confirmed that anti-CTLA-4 antibodies or Fab fragments greatly enhanced the accumulation of OVA-specific CD4+ T cells following peptide immunization (Kearney et al. 1995). Further proof of the major role for CTLA-4 in inhibiting T cell expansion came from the phenotype of CTLA-4^{-/-} mice, which die within 3–4 weeks of birth because of lymphoproliferation and fatal tissue destruction in multiple organs (Waterhouse et al. 1995; Tivol et al. 1995). Administration of recombinant CTLA-4-Ig protected CTLA-4^{-/-} mice from lethal lymphoproliferation (Tivol et al. 1997), corroborating the concept that CTLA-4 is a negative

regulator of CD28-dependent T cell responses *in vivo*, which is further supported by evidence that CTLA-4/B7-1/B7-2 triple KO mice lack lymphoproliferative disease (Mandelbrot et al. 1999).

2.1.1 Cell Intrinsic Suppression

Both CD4⁺ and CD8⁺ T cells show higher proliferative potential and an activated phenotype when lacking CTLA-4 *in vitro* and *in vivo* (Chambers et al. 1997, 1998, 1999; Greenwald et al. 2001, 2002; McCoy et al. 1999). Interestingly, lack of CTLA-4 had a more dramatic effect on the proliferation of CD4⁺ T cells *in vivo*, resulting in a skewing of the CD4/CD8 ratio toward CD4⁺ T cells. In addition, CD4⁺ T cells were necessary and sufficient for the massive infiltration of peripheral organs, and CD8⁺ T cell activation was entirely CD4⁺ T cell dependent (Chambers et al. 1997). The function of CTLA-4 in CD4⁺ T cells was then more closely investigated using TCR transgenic mice. Lack of CTLA-4 enhanced both primary and secondary peptide-specific responses of CD4⁺ and TCR tg and DO11 TCR tg CD4⁺ T cells (Chambers et al. 1999; Greenwald et al. 2001). CTLA-4^{-/-} DO11 TCR tg T cells were also resistant to tolerance induction. Additionally, experiments with anti-CTLA-4 blocking antibodies consistently showed that CTLA-4 engagement induced peripheral CD4⁺ T cell tolerance (Perez et al. 1997) and regulated CD4⁺ T cell activation in a cell intrinsic manner by modulating cell cycle progression (Greenwald et al. 2001, 2002). To investigate the role of CTLA-4 in CD8⁺ T cells, adoptive transfer experiments were performed with pmel-1 TCR tg mice, specific for the self antigen gp100, which showed that lack of CTLA-4 did not have evident cell intrinsic effects on CD8⁺ T cells. In fact, pmel-1 CTLA-4^{-/-} mice developed autoimmune hypopigmentation in a CD4⁺ T cell dependent manner (Gattinoni et al. 2006). However, experiments with 2CT TCR tg T cells indicated that secondary CD8⁺ responses significantly increased in the absence of CTLA-4 (Chambers et al. 1998). Similarly, in response to a dendritic cell based vaccine, 318 TCR tg T cells proliferated more in the presence of anti-CTLA-4 neutralizing antibodies in a CD4-independent fashion (McCoy et al. 1999). Consistent with CTLA-4^{-/-} mice, TCR transgenic mice lacking CTLA-4 developed lymphoproliferative disease, although with a delayed onset, which was abrogated in a RAG^{-/-} background. This indicated that T cells specific for an endogenous self-antigen are likely required to trigger tissue destruction in the absence of CTLA-4.

2.1.2 Cell Extrinsic Suppression

Reconstitution of RAG1/2^{-/-} animals with wild type (WT) and/or CTLA-4^{-/-} bone marrow cells or T cells demonstrated that tissue damage induced by transferred CTLA-4^{-/-} cells could be inhibited in a dose-dependent manner by concomitant transfer of WT cells (Bachmann et al. 1999; Tivol and Gorski 2002; Friedline et al. 2009). These experiments suggested that CTLA-4 dependent inhibition is not only

cell autonomous. In fact, CD25+CD4+ T cells and not CD8+ T cells or NKT cells were required to mediate the CTLA-4-dependent extrinsic immune-suppression, in an IL-10 independent fashion (Friedline et al. 2009). This *trans*-regulation depended on the persistent presence of WT suppressor cells and was reversible. Tolerized CTLA-4^{-/-} Teff cells in WT:KO chimeras became pathogenic when transferred into a new Rag1^{-/-} recipient without WT cells. Overall, these experiments showed that regulatory T cells (Tregs) expressing CTLA-4 maintain self tolerance and immune homeostasis in a dominant fashion. This was confirmed by the phenotype of conditional knockout mice lacking CTLA-4 in the CD4+Foxp3+ Treg cell compartment (CKO) (Wing et al. 2008), which developed systemic lymphoproliferation, indicating that CTLA-4 deficiency in Foxp3+ T cells is sufficient to destabilize immune homeostasis. Of note, the onset of disease in CKO mice is delayed as compared to CTLA-4^{-/-}, indicating that CTLA-4 expression on effector cells contributes to control disease progression. These findings indicate that the extrinsic suppressive mechanism of CTLA-4 has a more pronounced role in immune homeostasis and self tolerance as compared to the intrinsic suppression.

The relative contribution of CTLA-4 blockade on Tregs versus Teffs can be different in different disease models. For instance, blocking CTLA-4 on Tregs alone is not sufficient to promote T cell mediated tumor rejection in a mouse melanoma model that is responsive to anti-CTLA-4 treatment in combination with vaccine (Peggs et al. 2009; Quezada et al. 2006). In this case, blocking CTLA-4 on Teffs is necessary to induce tumor immunity, although additional CTLA-4 blockade on Tregs has added effects that lead to maximal tumor immunity. By contrast, in a T cell-mediated colitis model (Read et al. 2000), anti-CTLA-4 mAb induced colitis by primarily affecting Tregs and not colitogenic Teffs (Read et al. 2006). The nature of the target antigen and the inflammatory stimulus that trigger the response may explain the different outcomes. In addition, a CTLA-4 splice variant (liCTLA-4) has recently been identified which is expressed at high levels on resting T cells (Vijayakrishnan et al. 2004). As liCTLA-4 lacks the B7 binding domain, its suppressive function is absent in CTLA-4^{-/-} mice, but is not abrogated by anti-CTLA-4 blocking antibodies, perhaps explaining some of the discrepancies observed. Similarly, the fact that CTLA-4 deficient Tregs express more IL-10 and TGF- β as compared to WT Tregs (Tang et al. 2004) might also account for some of the differences.

The mechanism(s) underlying the extrinsic inhibitory effect of CTLA-4 is/are still largely unknown. It is possible that CTLA-4 expressing Tregs directly inhibit Teffs through the release of immunosuppressive cytokines. Another possible scenario is that CTLA-4 expressing Tregs “condition” other cells, which in turn inhibit Teffs. It has been shown, for example, that CTLA-4 positive TCR tg T cells failed to form long-term interactions with APCs in the presence of antigen *in vitro* and *in vivo* (Schneider et al. 2006), suggesting that dendritic cells may be important to induce/maintain CTLA-4-dependent Treg-mediated immune suppression. Indeed, CD4 + CD25+ cells can induce dendritic cells to express enzyme indoleamine 2,3-dioxygenase (IDO) *in vitro* (Fallarino et al. 2003), possibly via a CTLA-4 dependent mechanism. Similarly, CTLA-4 expression on Foxp3+ Tregs mediates downregulation of CD80 and CD86 on splenic dendritic cells *in vitro* (Wing et al. 2008).

The CTLA-4 dependent suppressive activity of T regs in vivo may therefore be mediated, at least in part, by APCs such as dendritic cells.

2.2 Preclinical Studies Using CTLA-4 Blocking Antibodies

As an immunosuppressive role of CTLA-4 was becoming apparent, the possibility that inhibiting CTLA-4:B7 interaction could have beneficial antitumor effects, by enhancing tumor-specific T cell responses, was hypothesized.

2.2.1 Monotherapy

The initial evidence that blocking CTLA-4 with anti-CTLA-4 antibody could treat established tumors came from studies done in transplantable tumor models of colon carcinoma (51BLim10), fibrosarcoma (Sa1N and CSA1M), ovarian carcinoma (OV-HM), and prostate cancer (TRAMPC1) (Leach et al. 1996; Kwon et al. 1997; Yang et al. 1997). Not only did these models demonstrate that blocking CTLA-4 caused tumor regression as a single agent, but they also showed that the immunity generated provided protection to subsequent challenge with the same tumor. While the use of anti-CTLA-4 as a single agent to treat established tumors showed very promising results in immunogenic cell lines, the beneficial effect was only marginal in the case of established poorly immunogenic tumors (immunogenicity being defined in general as the ability of an irradiated cell line to induce rejection of a subsequent challenge with the same cell line).

2.2.2 CTLA-4 and Active/Passive Immunization

Combination of anti-CTLA-4 with GM-CSF-expressing tumor vaccines resulted in the regression of the poorly immunogenic SM1 mammary tumor (Hurwitz et al. 1998) and B16 melanoma (van Elsas et al. 1999). The same approach showed promising effects in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model, decreasing tumor incidence and grade (Hurwitz et al. 2000). In these preclinical models, treatment with anti-CTLA-4 or the GM-CSF expressing vaccine alone was not sufficient to mediate tumor regression, but the combination of the two treatments had a synergistic effect. The nature of the immune response required for tumor rejection varied depending on the tumor model. In fact, both CD4+ and CD8+ T cell responses could mediate rejection of the SM1 mammary tumor; by contrast, rejection of B16 was mediated by CD8+ T cells and natural killer (NK) cells as well as required perforin and Fas/FasL interactions, but not CD4+ T cells and TNF α (van Elsas et al. 2001).

CTLA-4 blockade has also shown synergistic effects with other vaccination strategies, such as TRP-2 peptide in CpG-ODN adjuvant (Davila et al. 2003) or TRP-2, gp100, and PSMA xenogeneic DNA vaccines (Gregor et al. 2004) for

melanoma and prostate cancer. Interestingly, CTLA-4 blockade did not provide additional benefit when combined with an idiotypic vaccine that induced dormancy in mice with BCL1 lymphoma, suggesting that anti-CTLA-4 might not enhance antigen-specific antitumor effects that are B cell mediated (Pop et al. 2005).

The possibility that CTLA-4 blockade could enhance the antitumor potency of adoptive T cell transfer has also been investigated in mice. Although the transfer of OVA-specific CD8⁺ T cells along with anti-CTLA-4 showed synergistic CD4-dependent effects against the OVA-expressing thymoma E.G7 (Shrikant et al. 1999), lack of CTLA-4 expression did not enhance antitumor efficacy of adoptively transferred gp100-specific CD8⁺ T cells in conjunction with rFPhgp100 vaccine and rIL2 (Gattinoni et al. 2006). This could possibly be due to differences in the antigen and/or the adjuvant used to activate the transferred T cells. Further experiments would be important to address these discrepancies.

2.2.3 CTLA-4 and Chemotherapy/Radiotherapy/Tumor Ablation

Synergy between ionizing radiation therapy and anti-CTLA-4 has been reported in the 4T1 and TSA mammary tumor and MCA38 colon carcinoma models (Demaria et al. 2005). Combination of the two treatments showed a systemic antitumor effect that inhibited the growth of a distant tumor outside the radiation field, suggesting that the combination of CTLA-4 blockade and local radiation enhanced a tumor-specific immune response. It is possible that while radiation provided a source of antigen or increased MHC I expression/peptide processing on tumor cells and stroma (Reits et al. 2006; Zhang et al. 2007), anti-CTLA-4 treatment synergistically empowered the effects of cross-presentation. Anti-CTLA-4 also enhanced antitumor immunity when combined with in situ tumor destructive cryoablation or radiofrequency ablation in a transplantable melanoma model expressing the nonself model antigen OVA (den Brok et al. 2006). It will be interesting to see whether such synergy occurs in nonmanipulated tumors and if tolerance against self antigens can be averted by this means.

Furthermore, CTLA-4 blockade has shown significant therapeutic benefits in mice bearing the MOPC-315 transplantable plasmacytoma tumor, when combined with a subtherapeutic dose of the chemotherapeutic agent melphalan, perhaps reflecting a synergy provided by targeting multiple immunoregulatory pathways (Mokyr et al. 1998a, b). The combination between chemotherapy and CTLA-4 blockade in animal models warrants further investigation. It will be interesting to dissect whether possible synergies require an effect of the chemotherapeutic agents on immune cells or tumors.

2.2.4 CTLA-4 and Other Immunostimulatory Strategies

CTLA-4 blocking antibody dramatically enhanced the antitumor efficacy of a recombinant oncolytic virus developed to preferentially target breast cancer cells

(Gao et al. 2009). Targeting tumor cells with viruses may therefore represent a valuable approach to enhance inflammation at the tumor site and to increase tumor immunogenicity locally, thus providing a window of opportunity for treatment with anti-CTLA-4.

Another way to increase the efficacy of anti-CTLA-4 blockade could be to combine it with other monoclonal antibodies that target immune receptors. It has been recently shown that combining anti-CTLA-4 with the agonistic anti-OX40 mAb (OX86) potentiates the systemic effects of intratumoral CpG treatments in a mouse lymphoma model (Houot and Levy 2009). Several other immunomodulatory mAbs have shown potent anticancer effects in mouse models including the anti-GITR agonistic mAb DTA-1, described later in this chapter, and might be future candidates to combine with anti-CTLA-4.

3 GITR Preclinical

GITR was originally described by Nocentini et al. after being cloned from dexamethasone-treated murine T cell hybridomas (Nocentini et al. 1997). Unlike CD28 and CTLA-4, GITR has a very low basal expression on naive CD4+ and CD8+ T cells (Ronchetti et al. 2004). This is in contrast to Treg cells which express both CTLA-4 and GITR at high levels constitutively. After activation, both naive and Treg cells upregulate expression of GITR which lasts several days and is highest in tumor infiltrating lymphocytes (Tone et al. 2003; Cohen unpublished results).

GITR ligand (GITR-L) is expressed at low levels by APCs such as macrophages, dendritic cells, and B cells. Like CD80/CD86, GITR-L is upregulated upon activation with stimuli such as TLR ligands (Tone et al. 2003; Suvas et al. 2005). Similar to other members of the TNF superfamily, GITR contains TNF receptor associate factor (TRAF) binding domains in its cytoplasmic tail; however, it does not contain so called death domains which directly mediate apoptosis found on Fas and Trail (reviewed in Nocentini and Riccardi (2005)). Through yeast two hybrid screening, GITR was shown to interact specifically with TRAF 1, 2, and 3 which mediate downstream signaling from the receptor. Stimulation of GITR can cause activation of NF- κ B (Nocentini et al. 1997; Ronchetti et al. 2004). In addition, GITR has been shown to activate members of the MAPK pathway, including p38, JNK, and ERK (Esparza and Arch 2005). By activating these pathways and their downstream events, GITR ligation is in turn believed to enhance T cell survival by upregulating IL-2R α , IL-2, and IFN γ , along with rescuing T cells from anti-CD3 mediated apoptosis (Ronchetti et al. 2004). While GITR does not have canonical death domains in its cytoplasmic tail, GITR has been shown to bind to the death domain containing protein, Siva (Spinicelli et al. 2002). As a result of its interaction with Siva, Spinicelli et al. demonstrated that GITR ligation can induce apoptosis in Cos7 cells. However, how this interaction modulates GITR function in primary T cells has yet to be established.

3.1 *GITR: “Accelerator” of Effector Function*

Triggering or blocking GITR signaling on immune cells has provided most of the evidence that GITR may be a T cell costimulatory receptor. GITR ligation by GITR-L enhances both CD4+ and CD8+ T cell proliferation and effector functions, particularly in the setting of suboptimal TCR stimulation (Tone et al. 2003; Kanamaru et al. 2004; Kohm et al. 2004; Ronchetti et al. 2004). In contrast, blocking GITR-GITR-L signaling with anti-GITR-L antibodies inhibited lymphocyte proliferation (Stephens et al. 2004). Consistent with this idea, injection of anti-GITR agonistic antibody in neonatal mice induced overt autoimmune disease (Shimizu et al. 2002).

Similar to the differential roles that CTLA-4 plays on various T cell subsets, GITR stimulation may affect Tregs differently from Teffs. Treatment of Tregs with anti-GITR polyclonal sera (McHugh et al. 2002) or with the rat monoclonal anti-GITR antibody DTA-1 (Shimizu et al. 2002) inhibited the suppression induced in vitro by CD4+ CD25+ T cells when cocultured with CD4+ CD25– or CD8+ T cells with anti-CD3 stimulation and irradiated APCs. Addition of recombinant GITRL or cells transfected with GITRL to Treg/Teff cocultures had similar outcomes (Tone et al. 2003).

These experiments raised the question regarding the relative contribution of GITR signaling to Tregs and Teffs. Experiments done in vitro have shown that GITR stimulation induced direct proliferation of Tregs (McHugh et al. 2002; Ronchetti et al. 2002) and of effector cells (Ronchetti et al. 2002; Tone et al. 2003; Kanamaru et al. 2004). In addition to the direct proliferative effects, GITR ligation on Teffs may allow these cells to overcome Treg-mediated suppression (Stephens et al. 2004; Zhou et al. 2007). Other data showed that GITR ligation on Tregs could also indirectly contribute to immunostimulation by inhibiting their suppressive activity (Shimizu et al. 2002; Ronchetti et al. 2004).

Interestingly, studies done with GITR KO mice showed that GITR-deficient lymphocytes proliferate more and are more sensitive to activation induced cell death (AICD) as compared to lymphocytes expressing GITR (Ronchetti et al. 2002), supporting a role for this pathway in lymphocyte proliferation/survival. CD4+CD25+ T cells lacking GITR, instead, were equally able to suppress when compared to WT CD4+CD25+ T cells, suggesting that GITR signaling may be more relevant on Teffs rather than Tregs.

3.2 *Preclinical Studies Using GITR Agonist Antibody*

The observation that GITR stimulation has immunostimulatory effects in vitro and induced autoimmunity in vivo prompted the investigation of the antitumor potency of triggering this pathway.

3.2.1 Monotherapy

Injection of the anti-GITR agonistic antibody DTA-1 eradicated established Meth A fibrosarcoma and CT26 carcinoma in an IFN- γ dependent manner and induced tumor specific CD4+ and CD8+ T cell responses (Ko et al. 2005). This immunomodulatory antibody was most effective eight days after tumor inoculation, possibly implying a requirement for tumor-dependent T cell priming and/or the establishment of a structured tumor microenvironment. DTA-1 treatment could also induce concomitant tumor immunity that resulted in rejection of a distal poorly immunogenic B16 melanoma (Turk et al. 2004). The growth of the primary B16 melanoma was transiently slowed by a single DTA-1 dose and the tumor was rejected by multiple DTA-1 doses (at day 1, 4, and 9 after tumor inoculation) in a T cell (both CD4+ and CD8+) and NK cell dependent fashion. In this model, DTA-1's mechanism of action required IFN- γ and FasL, but not perforin while inducing tumor immunity through endogenous T cell response against B16 (Ramirez-Montagut et al. 2006). Furthermore, as observed *in vitro*, DTA-1 treatment also expanded Foxp3+ Tregs *in vivo*. Interestingly, the expanded Tregs retained their suppressive capabilities, as assessed by *ex vivo* suppression assays, but their expansion did not impact negatively on tumor growth. While Tregs are still suppressive in the periphery, we have recently demonstrated, using the B16 melanoma model, that GITR modulation has a profound effect on intra-tumor Tregs. Treatment with DTA-1 causes a significant impairment of intra-tumor Treg accumulation and loss of FoxP3 expression (Cohen et al. 2010). This results in a much more favorable intra-tumor Teff:Treg ratio and enhanced tumor-specific CD8+ T-cell activity. Impairment of Treg infiltration was lost if Tregs were GITR $^{-/-}$, and the protective effect of DTA-1 was reduced in reconstituted RAG1 $^{-/-}$ mice if either the Treg or Teff subset were GITR-negative and absent if both were negative. Our recent data further corroborates the concept that DTA-1 administration has effects on both Teffs and Tregs *in vivo*, both of which may be important to the generation of tumor immunity.

3.2.2 GITR and Active Immunization

Consistent with the hypothesis that DTA-1 has a direct role on Teff cell expansion and activation, administration of DTA-1 enhanced effector and memory CD8+ T cell responses to self antigens improved tumor protection when combined with a xenogenic DNA vaccine for gp100 or TRP-2 (Cohen et al. 2006). Importantly, DTA-1 was most effective when boosting vaccine-induced CD8+ T cells before the second immunization. By contrast, DTA-1 administration before the first immunization failed to enhance tumor rejection, possibly by enhancing AICD rather than T cell expansion. This indicated that triggering GITR signaling with DTA-1 *in vivo* has different outcomes on T cell activation depending on the timing with respect to the antigenic stimulation. The exact reason for this is unknown, but it may reflect differences in the signaling pathways activated at different stages of priming.

Fig. 1 Differential outcomes of CTLA-4 and GITR monoclonal antibody therapy on different T cell lineages. Although CTLA-4 and GITR are present on both Teffs and Tregs, it has become apparent that each lineage incorporates signals from these receptors differently. In Teffs, CTLA-4 provides a strong negative signal which blocks positive stimuli from the TCR and CD28 during priming. GITR on the other hand, provides additional positive stimuli that may overcome CTLA-4's negative effect. Unlike Teffs, Tregs express CTLA-4 and GITR constitutively, an initial clue to a variation in their roles. In fact, Tregs have been shown to be nonfunctional without CTLA-4 expression. This suggests that Tregs may see signals from CTLA-4 as a "positive stimulus" as part of their "suppressive effector functions." Likewise, evidence has shown that under certain conditions, GITR ligation on Tregs may negatively modulate their suppressive ability. The divergent incorporation of signals from these receptors is why the effects of antibody therapy to these receptors may have different effects on each lineage. Blocking CTLA-4 antibodies prevents negative signals to Teffs while at the same time stopping Tregs from receiving positive stimuli. Agonist GITR antibodies work in reverse, providing costimulatory signals to Teffs whereas Tregs may see this stimulus in a negative manner. Thus, while these antibodies enhance desired Teff antitumor activity, they may have the added benefit of negating the suppressive functions of Tregs

This consideration will be extremely important when designing combination cancer immunotherapies based on the use of DTA-1.

Overall, immune modulation of CTLA-4 and GITR with blocking and agonistic monoclonal antibodies, respectively, has proven effective in a variety of pre-clinical tumor models. Although the mechanism of action differs for the two antibodies, they share the complexity of affecting distinct T cell populations with opposing outcomes, as illustrated in Fig. 1.

4 Clinical Experiences with CTLA-4 Blockade

Clinical application of immunomodulatory antibodies has presented a new approach to systemic anticancer therapy. These agents are designed to activate the patient's immune system to eliminate cancer as opposed to administration of cytotoxic agents directed at the tumor itself (treating the patient versus treating the cancer).

Given these fundamental differences from cytotoxic chemotherapy, it was not unexpected to observe differences in the kinetics of the antitumor response, duration of response, and immune related adverse effects. In fact, the efficacy of ipilimumab, an anti-CTLA-4 antibody, appears to be independent of currently accepted negative clinical prognostic factors (Wolchok et al. 2009a).

Currently, there are two available CTLA-4 blocking monoclonal antibodies, ipilimumab (MDX-010) and tremelimumab (CP-675,206) (Phan et al. 2003; Ribas et al. 2005, 2008; Weber 2008). Both antibodies were developed in mice transgenic for human immunoglobulin genes, thereby producing fully human antibodies against CTLA-4. These agents have been most extensively studied in melanoma; however, there have also been durable responses noted in prostate, ovarian, breast, and renal cell cancer (Table 1). For metastatic melanoma in particular, anti-CTLA-4 blockade opens up a new avenue of treatment for patients that have very few options and have only a historical 6–9 month median survival without therapy.

4.1 CTLA-4 Monotherapy

In an initial trial of 17 patients with unresectable melanoma treated with ipilimumab, there were two partial responses (PRs) at the starting dose of 3 mg/kg administered as a single dose. These responses were durable and treatment was well tolerated but for a mild rash (Tchekmedyian et al. 2002). A second pilot trial was conducted with patients that had been prior participants in tumor vaccine studies for metastatic disease. Nine patients who were previously treated with a GM-CSF secreting tumor cell vaccine, including seven with metastatic melanoma and two with ovarian carcinoma, were treated with a single dose of ipilimumab at 3 mg/kg. Here, there was some evidence of efficacy with tumor biopsies of five melanoma patients revealing necrosis, and in the ovarian cancer patients there was a decrease or stabilization of CA-125, an ovarian cancer tumor marker (Hodi et al. 2003). Ipilimumab was then combined with a gp100 peptide vaccine in a phase I clinical trial, where 14 stage IV melanoma patients were treated with 3 mg/kg ipilimumab followed by a gp100 peptide vaccine. While three objective responses were noted including two complete responses (CRs), accrual was discontinued when >3 individuals developed grade III and IV immune related adverse events (irAEs) including dermatitis, enterocolitis, hypophysitis, and hepatitis (Phan et al. 2003). Following this, 56 patients with stage IV melanoma previously vaccinated with gp100 peptide received ipilimumab with an overall response rate (ORR) of 13% (7/56) with two CRs and five PRs. Interestingly, 36% of patients with irAEs experienced tumor regression compared with 5% of patients without irAEs (Attia et al. 2005).

Several dose escalation trials followed; in one trial, 88 patients with either unresectable stage III or IV melanoma received ipilimumab with a dose escalation from 2.8 mg/kg up to 20 mg/kg. Four patients obtained objective responses with one CR and three PRs, and an additional 14 patients had prolonged stable disease (SD)

Table 1 Select anti-CTLA-4 antibody clinical trials

Reference	# Pts	Drug – schedule mg/kg	Response rate	Immune related adverse effects	Median OS
Wolchok et al. (2009c)	217	Ipilimumab	10 mg/kg	70% (50/71)	11.4 months
		Melanoma	BORR 11.1%	Grade 3 or 4 – (18/71)	1 year survival 48.6%
		Dose escalation: 0.3 mg/kg versus 3 mg/kg versus 10 mg/kg q3weeks ×4 cycles, then q3months	Disease control rate 29.2% 2 CR, 6 PR, 13 SD 3 mg/kg BORR 4.2% Disease control rate 26.4% 0 CR, 3 PR, 16 SD	65% (46/71) Grade 3 or 4 – 7% (5/71)	8.7 months 1 year survival 39.3%
			0.3 mg/kg BORR 9% Disease control rate 13.7% 0 CR, 0 PR, 10 SD	26% (19/72) Grade 3 or 4 – 0% (0/72)	8.6 months 1 year survival 39.6%
Weber et al. (2009)	115	Ipilimumab with or without budesonide	Budesonide + Ipilimumab BORR 12.1%	32.7%	17.7 months
		Melanoma	Disease control rate 31%		1 year survival 62.4%
		10 mg/kg q3weeks ×4 cycles followed by q3months	1 CR, 6 PR, 11 SD Ipilimumab BORR 15.8% Disease control rate 35% 0 CR, 9 PR, 11 SD	35%	19.3 months 1 year survival 62.4%
			10 mg/kg (41 pts) BORR 21% 1 CR, 3 PR, 12 SD 15 mg/kg (43 pts) BORR 42% 1 CR, 3 PR, 14 SD	84% (37/44) Grade 3 or 4 – 27% (7/44) 73% (33/45) Grade 3 or 4 – 13% (6/45)	10.0 months 1 year survival 32% 11.5 months 1 year survival 46%
Camacho et al. (2009)	117	Tremelimumab Melanoma 3, 6, 10 mg/kg (28 pts) Phase II 10 mg/kg versus 15 mg/kg (89)			

with a median time of 194 days. The objective responders also maintained a long duration of response of greater than 638 days (Weber et al. 2008). Another phase II trial of 139 patients treated at doses from 3–9 mg/kg of ipilimumab with or without peptide vaccine resulted in a 17% ORR with 3 CRs and 20 PRs. Sixty two percent of the patients had some form of irAEs which in this study was statistically associated with a higher likelihood of antitumor response (Downey et al. 2007).

Subsequently, there was a dose–response relationship demonstrated in a double-blind phase II trial of ipilimumab with three dose levels administered as a monotherapy to 217 unresectable stage III/IV patients. These patients received 0.3, 3, or 10 mg/kg of antibody every 3 weeks for four doses, followed by a maintenance dose once every 12 weeks starting at week 24 in the setting of SD or better without grade III/IV toxicity. The 10 mg/kg cohort had the greatest response rate at 11% with a median overall survival of 14 months. Consequently, the 10 mg/kg dosage is considered the optimal dosing to achieve clinical benefit although associated with the greatest rate of irAEs (Wolchok et al. 2009c). It is important to note that most irAEs can be easily medically managed with corticosteroids using simple algorithms. In fact, use of immunosuppressive drugs does not interfere with antitumor effects, for unclear reasons at this time. In an additional phase II trial of ipilimumab in advanced stage melanoma patients progressing on prior therapies, 150 patients were treated with 10 mg/kg of ipilimumab and the best overall response rate (BORR) was shown to be 5.8% (O'Day et al. 2008). A phase III registration trial which began in 2006 is currently ongoing comparing combined DTIC and ipilimumab with DTIC alone as front-line therapy. A separate phase III registration trial with 676 enrolled patients for second-line therapy with ipilimumab has just been completed, assessing the safety and efficacy of ipilimumab in combination with gp100 peptide vaccines, or single agents.

4.2 Postsurgical Adjuvant Therapy

Ipilimumab has also been tested in the adjuvant setting for high risk patients that had been rendered free of disease by surgery. In this trial, anti-CTLA-4 therapy was given at 0.3, 1 or 3 mg/kg every 4 weeks combined with a gp100, MART-1, and tyrosinase peptide vaccine to 19 stage III and IV resected melanoma patients. Eight patients experienced evidence of irAEs with three patients experiencing grade III gastrointestinal toxicity. Interestingly, 3 of 8 patients with irAEs experienced relapse while 9 out of 11 patients without irAEs experienced relapse (Sanderson et al. 2005). Similar results were seen in a subsequent phase II trial of ipilimumab at 3 mg/kg with a multi-peptide vaccine in 25 patients who had resected stage III/IV melanoma with no evidence of disease, which resulted in ten relapses at 22 months (Weber et al. 2006). Finally, the efficacy of ipilimumab in the adjuvant setting will soon be determined by an ongoing phase III double blinded placebo controlled clinical trial for stage III melanoma patients with no evidence of disease, with an estimated enrollment of 950 patients.

4.3 Combinations

Ipilimumab has also been combined with standard therapies. In a phase I/II clinical trial, 36 patients were treated with 0.1–3 mg/kg of ipilimumab every 3 weeks combined with IL-2. However, the ORR of 22% with three CRs and five PRs suggested no synergistic effect in combining IL-2 compared to ipilimumab alone (Maker et al. 2005b). Chemotherapy has also been rationally combined with ipilimumab on the basis of the potential for tumor antigen release, or depletion of Tregs. In a phase II randomized trial, 72 patients were treated with 3 mg/kg of ipilimumab, with or without DTIC. From this trial, it appeared that DTIC augmented the efficacy of ipilimumab with a 21.6% disease control rate in the ipilimumab alone arm and 31.4% in the combination arm (Hersh et al. 2008). In a follow up report, the investigators demonstrated an overall 57% 1 year survival rate with the combined DTIC and ipilimumab arm having a superior survival rate (Hersh et al. 2009). Currently ongoing is another three arm trial with 60 patients, where ipilimumab is being administered alone or in combination with carboplatin and paclitaxel, or with DTIC (NCT00796991).

4.4 Tremelimumab

A dose escalation study with tremelimumab ranging from 0.03 to 15 mg/kg in 34 patients demonstrated durable responses with eight objective responses, including two CRs, two PRs and four patients with SD (Ribas et al. 2005). A follow up phase II study of 20 patients receiving tremelimumab at 10 mg/kg monthly and ten patients receiving 15 mg/kg every 3 months resulted in five total responders with four occurring in individuals who developed irAEs (Reuben et al. 2006). In a third phase II study, 84 patients received tremelimumab at 10 mg/kg or 15 mg/kg with an ORR of 10% and median survival of 10.2 months at the 10 mg/kg dose, and with a 7% ORR and 11.5 month median survival at the 15 mg/kg dose (Camacho et al. 2009). A further phase II trial at 15 mg/kg for stage IV malignant melanoma patients who had progressed on prior chemotherapy was conducted with an endpoint of achieving at least a 15% ORR. Reported at the American Society of Clinical Oncology (ASCO) 2008 Meeting, this trial of 258 patients treated with tremelimumab showed an ORR of 8.3% with a median survival of 10.0 months (Kirkwood et al. 2008). A large randomized phase III trial has been completed with tremelimumab 15 mg/kg compared to either DTIC or temozolomide in 550 patients. Unfortunately this trial was halted in its second interim analysis with a median overall survival of 10.7 months for the chemotherapy arm and 11.7 months for the tremelimumab arm. The hazard ratio was 1.05 with survival curves overlapping and thus it did not meet its primary endpoint (Ribas 2008).

4.5 Immune Related Adverse Effects

Since the initial studies with ipilimumab, there has been evidence of irAEs, most of which included diarrhea, colitis, rash, hypophysitis, and rare incidences of uveitis and adrenal insufficiency (Blansfield et al. 2005). To determine if the incidence of diarrhea could be decreased, 115 stage IV melanoma patients participated in a randomized phase II trial with ipilimumab with or without budesonide, a nonabsorbed oral steroid. These patients were treated with or without budesonide and received ipilimumab at 10 mg/kg every 3 weeks for four doses. Overall, ipilimumab was well tolerated with the best ORR of 12.1% in the budesonide arm and 15.8% in the placebo controlled arm, with no effect of budesonide on the rate of grade III or greater diarrhea (Weber et al. 2009). As noted above, most irAEs are easily managed with corticosteroids and those few patients with steroid-refractory disease respond to TNF-blocking agents or mycophenolate. Intriguingly, despite the rapid resolution of symptoms from irAEs using immunosuppressive medications, such drugs do not seem to temper antitumor effects.

4.6 Novel Criteria for Antitumor Response to Ipilimumab and Increased Duration of Response

Traditional standards for evaluation of antitumor response have been based on the Response Evaluation Criteria in Solid Tumors (RECIST) or WHO criteria, which may not adequately reflect the patterns of response for immunotherapies. A set of novel immune related response criteria (irRC) was recently proposed to more accurately describe the clinical activity of immunotherapies. It has not been uncommon to see late responses, sometimes happening 5–6 months after initiating treatment, even occurring after progression of index and/or new lesions. This may be related to the time needed for specific activation of the immune system to recognize antigens expressed by individual tumors, or a function of the kinetics regarding immune mediated tumor destruction. This should be contrasted with relatively short periods of time expected for observation of direct cytotoxic effects from chemotherapy. In addition, radiological evidence of progression of disease at early time points may reflect a heterogeneous mixture of inflammation, edema, and lymphocytic infiltration as opposed to true increase in tumor volume.

To develop irRC, phase II clinical trial data from the ipilimumab program were utilized to define four patterns of response: (1) decrease in baseline lesions without evidence of new lesions, (2) durable SD with possible slow, steady decline in tumor burden, (3) response of tumor volume after initial increase in total tumor burden, and (4) response in the presence of new lesions. All of these response patterns were associated with increased overall survival (Wolchok et al. 2009b). Duration of response also appears to be increased in comparison to standard therapies. In fact, a recent ASCO abstract provided updated survival data from three phase II clinical trials of pretreated advanced stage melanoma patients. Here, they showed that the

18 month overall survival ranged from 34.5 to 39.4% for the 288 previously treated melanoma patients (O'Day et al. 2009).

4.7 Other Malignancies

While the vast clinical experience of CTLA-4 blockade has been with melanoma, there is increasing evidence of efficacy in other malignancies. In prostate cancer, ipilimumab was studied in a phase II trial of comparing ipilimumab with or without docetaxel in chemotherapy-naïve hormone refractory prostate cancer patients. Six of 43 patients with three in each arm had decreases of PSA >50%. Three patients had durable PSA responses greater than 79, 169, and 280 days. Immune related adverse effects were similar to those noted in melanoma, including adrenal insufficiency and colitis (Small et al. 2006). Other combinatorial treatment strategies include a phase I/II clinical trial in 45 metastatic castrate resistant prostate cancer patients treated with ipilimumab and radiation therapy. Here, ten patients had decreases of PSA greater than 50% while radiation therapy did not appear to impact the effects of ipilimumab (Slovin et al. 2009). A combinatorial approach using a PSA-TRICOM vaccine, a pox based viral vector expressing PSA and three costimulatory molecules including ICAM-I, LFA-3, and B7.1 and GM-CSF, has been tested in combination with ipilimumab in 30 metastatic castrate resistant prostate cancer patients as well. Twenty-four chemotherapy naïve patients had an increased PSA doubling time from 2.5 to 6 months. In addition, 14 of 30 patients had PSA decreases from baseline or peak PSA on study. The therapy was well tolerated with no dose limiting toxicity, although again there was a correlation with increasing dose and incidence of irAE (Mohebtash et al. 2009). While most trials have been performed in solid malignancies, a phase I trial of ipilimumab has been performed in 18 non-Hodgkin's lymphoma patients. The therapy was safely tolerated and two patients had a clinical response, with one CR and one durable PR (Ansell et al. 2009). Ipilimumab has also been tested in relapsed allogeneic transplant patients, based on the hypothesis that CTLA-4 blockade could augment the graft versus tumor effect. Interestingly, in the 29 patients receiving a single infusion of ipilimumab at either 0.1 mg/kg or 3 mg/kg, there was no exacerbation of graft versus host disease, although four patients experienced organ specific immune related adverse effects including grade 3 arthritis, grade 2 hyperthyroidism, and recurrent grade 4 pneumonitis. In that trial, there were three overall responders with two CRs and one PR (Bashey et al. 2009). Tremelimumab has been evaluated in 25 advanced breast cancer patients. A phase I open label trial of tremelimumab combined with exemestane resulted in a best response of eight patients with SD lasting a median of 8 months. The expected dose limiting toxicities included transient grade 3 transaminitis, diarrhea, and thyroiditis (Vonderheide et al. 2009). A phase II trial of tremelimumab versus best supportive care in 87 patients with nonsmall cell lung cancer has also been reported. Two of 44 (4.8%) patients treated with tremelimumab experienced an objective response, compared with zero in the best supportive care group (Zatloukal et al. 2009). Ipilimumab is also being tested in lung cancer in a phase II trial comparing

carboplatin and paclitaxel with or without ipilimumab (NCT00527735). For renal cell carcinoma, anti-CTLA-4 antibodies have been tested alone and with sunitinib. In a phase II trial of ipilimumab, 61 patients were treated in sequential cohorts of increased doses. Twenty-one patients were treated at 3 mg/kg followed by 1 mg/kg q3weeks with one PR. Five of the 40 patients at 3 mg/kg every 3 weeks had PRs (Yang et al. 2007). Finally, tremelimumab has been combined with sunitinib, a multitargeted tyrosine kinase inhibitor, in a phase I clinical trial. The combination with sunitinib was chosen on the basis of evidence of sunitinib inhibiting myeloid-derived suppressor cells. There were five PRs noted in the 21 patients treated. There were multiple adverse effects noted including a ruptured diverticula, grade 3 mucositis, sudden death, and acute renal failure (Gordon et al. 2009).

There are other clinical trials with either ipilimumab or tremelimumab that are still ongoing and have not yet been reported. These include ipilimumab in unresectable pancreatic cancer and advanced hepatocellular carcinoma (NCT00836407) (NCT01008358) along with neoadjuvant ipilimumab being assessed in urothelial carcinoma undergoing surgical resection (NCT00362713). The later trial will provide an ideal trial design to assess the impact of immune modulation on the local tumor microenvironment. Adoptive T cell therapy is also being combined with ipilimumab in treatment of metastatic melanoma patients. NY-ESO-1 specific CD8⁺ T cells are currently being studied in cyclophosphamide treated metastatic melanoma patients with or without CTLA-4 blockade (NCT00871481). If successful, this strategy would support anti-CTLA-4 therapy preventing possible local tumor microenvironment inhibition of T effs. (NCT00871481).

5 From Clinical Trials, Back to the Bench

As described above, anti-CTLA-4 therapy has shown clinical activity as an immune modulator, inducing durable objective responses as a monotherapy, and demonstrating efficacy with combination therapies that direct the immune response to the cancer. However, we have yet to fully investigate the heterogeneity of responses that have been observed with anti-CTLA-4 therapy. Detailed analysis of anti-CTLA-4 treated patients may assist us in discovering mechanisms that are critical to the antitumor response. This section outlines several current directions in the field of immune monitoring i.e., the study of individual responses to anti-CTLA-4 therapy.

5.1 *Monitoring Antigen-Specific CD4⁺ and CD8⁺ T Cell Activity and Polyfunctionality*

Antigen-specific T cell activity can be evaluated using in vitro stimulation assays, or by staining peripheral T cells with antigen-specific MHC tetramers. Such analyses were recently conducted on selected melanoma patients receiving ipilimumab (Yuan et al. 2008). Of eight clinical responders, five demonstrated antibody, CD4⁺, and CD8⁺

responses to NY-ESO-1, a prototypical cancer testis antigen. In contrast, only 1/7 nonresponders mounted a CD4⁺ response, but no CD8⁺ or antibody response (Yuan et al. 2008). These data imply that antigen-specific immunity may be associated with clinical benefit, and suggest a rationale for future trials combining vaccination to NY-ESO-1 or other antigens with anti-CTLA-4 therapy.

Using the same techniques, specificity to other antigens has also been investigated. In a phase I trial of 24 prostate cancer patients treated with ipilimumab +GM-CSF, no antigen-specific T cell responses were detected against PSA or other known prostate differentiation antigens, including PAP, PSMA, EphA2, or survivin (Fong et al. 2009). However, in an analysis of a melanoma patient experiencing CR from treatment with ipilimumab, both Melan-A-specific peripheral T cells and tumor-infiltrating T cells were identified (Klein et al. 2009). These Melan-A/MART1 specific CD8⁺ cells were expanded in culture, and were demonstrated to exhibit potent killing activity in vitro against SK-Mel-14, a Melan-A-expressing cell line. This case report calls for further studies examining Melan-A-specific immunity as a possible marker for positive therapeutic response (Klein et al. 2009). In some cases, peripheral T cell specificity may not capture intratumoral immune events as analysis of patients receiving tremelimumab showed no trend of increased gp100, Melan-A/MART1, or tyrosinase-specific peripheral CD8⁺ cells (Comin-Anduix et al. 2008). Still, analysis of tumor infiltrate in regressing lesions demonstrated marked enrichment of gp100-specific CD8⁺ cells, which suggests that peripheral specificity may not always correlate with intratumoral T cell specificity (Comin-Anduix et al. 2008).

Polyfunctional T cell subsets – T cell subsets that generate multiple cytokines – can be measured by multiparametric flow cytometry, and are markers of robust immune activity. Polyfunctional T cells have been associated with nonprogression of HIV2 infection (Makedonas and Betts 2006; Duvall et al. 2008) and vaccine responses to Hepatitis B virus (De Rosa et al. 2004) and vaccinia (Precopio et al. 2007). Recently, polyfunctional CD8⁺ T cells were also demonstrated in a melanoma patient receiving adjuvant therapy with GM-CSF DNA and gp100/tyrosinase peptide vaccines with polyfunctionality coinciding at the time point of maximal IFN- γ production (Perales et al. 2008). An analysis of polyfunctionality in ipilimumab patients found that patients experiencing clinical benefit demonstrated CD4⁺, CD8⁺, and/or antibody responses to NY-ESO-1. In fact, T cells from clinical benefitters were found to be polyfunctional, producing IFN- γ , MIP-1 β , and/or TNF α in response to NY-ESO-1 peptide stimulation, while none of the nine nonresponders demonstrated a polyfunctional T cell response. These results suggest that antigen-specific polyfunctionality could be a possible marker of anti-CTLA-4 activity (Yuan et al. 2008).

5.2 Cellular Phenotype Analysis: ICOS, Foxp3, HLA-DR and IDO

Another immune monitoring strategy is to utilize flow cytometric analysis and other techniques to examine changes in cellular phenotype, with the hope of identifying biomarkers for clinical response.

5.3 ICOS

ICOS is a T cell specific surface protein structurally related to CD28 and CTLA-4, which becomes expressed on the cell surface following T cell activation (Hutloff et al. 1999; Liakou et al. 2008). T cells expressing intermediate levels of ICOS are associated with synthesis of the T_H2 cytokines IL-4, IL-5, and IL-13, whereas T cells expressing high levels of ICOS (ICOS^{high}) correlate with the production of IL-10, an immunosuppressive cytokine, in some studies (Lohning et al. 2003). Studies showing a relationship between ICOS expression with increased Teff cell survival (Burmeister et al. 2008) serve to highlight the significance of ICOS as a possible marker of anti-CTLA-4 activity. This has been recently investigated in a series of six bladder cancer patients receiving anti-CTLA-4 therapy preoperatively. In these patients, an increase in the number of CD4⁺ICOS^{high} cells was observed in peripheral blood mononuclear cells and in tumor infiltrates. These cells exhibited increased IFN- γ production, and in 3/3 of these patients who had NY-ESO-1 expressing tumors, IFN- γ was produced in response to stimulation with NY-ESO-1 peptides (Liakou et al. 2008). These data imply that ICOS expression could be correlated with clinical activity; however, this must be further investigated.

5.4 Foxp3

Foxp3, a member of the forkhead box family of transcription factors, is necessary for Treg function. The overall frequency of Foxp3⁺ Treg cells in untreated cancer patients tends to be higher than in normal healthy donors, which implies that Foxp3 levels may correlate with tumor immune evasion (Liakou et al. 2008). The role of Foxp3 as a biomarker of anti-CTLA-4 induced Treg modulation is being investigated. In the series of bladder patients above, Foxp3 expression was not consistently altered by CTLA-4 blockade (Liakou et al. 2008). In an analysis of eight patients receiving tremelimumab, no statistical change was seen in predosing and postdosing levels of peripheral Foxp3 mRNA production. However, a recent analysis of tumor biopsies taken at week 0 and week 4 in ipilimumab patients showed a statistical correlation of clinical benefit with changes from pretreatment Foxp3 expression (Hamid et al. 2009).

5.5 HLA-DR

Several markers of T cell activation have been screened as possible biomarkers for anti-CTLA-4 activity. HLA-DR is a cell surface molecule which appears on both CD25⁺ and CD25⁻ T cells late after activation. In a recent analysis of 30 patients treated with ipilimumab, posttreatment increases in the percentage of HLA-DR expression were seen in both CD4⁺CD25⁻ and CD4⁺CD25⁺ cells. (Maker et al.

2005a) A separate analysis demonstrated that HLA-DR expression on CD8⁺ cells after two doses of tremelimumab could differentiate responders from nonresponders (comin-Anduix et al. 2008). Further work must be performed to validate HLA-DR expression as a biomarker.

5.6 IDO

Treg cells from tumor-draining lymph nodes in mouse models have been shown to become highly activated by exposure to the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO), an enzyme expressed in specific subsets of plasmacytoid dendritic cells (Sharma et al. 2009). Additionally, blockade of IDO expression has been shown to convert Tregs into a nonsuppressive, proinflammatory phenotype similar to T_H17 cells. As such, IDO has been implicated as a “switch” for Treg mediated immune regulation, and thus levels of IDO expression could be used as a biomarker. To date, this has yet to be validated. Analysis of pre and postdosing tumor biopsies in four patients receiving tremelimumab failed to show an association of IDO⁺ T cell staining with tumor response (Ribas et al. 2009). Analysis of tumor biopsies in ipilimumab patients showed a statistical correlation of clinical benefit with week 0 IDO expression (Hamid et al. 2009). Further studies are pending.

5.7 Antibody Responses

Differentiation antigens (for example, PSA, Melan-A/MART1, and gp100), cancer testis antigens (for example, NY-ESO-1 and MAGE), and products of DNA damage (for example, Bcr/Abl or mutated p53) have all been associated with antigen-specific humoral and/or T cell responses (Houghton et al. 1982; Chen et al. 1997; Perales et al. 2002; Nakada et al. 2003). CTLA-4 blockade may indirectly activate the humoral response by stimulating CD4-mediated priming of B cells, thereby inducing antibody production against tumor antigens.

In a recent serologic analysis of melanoma patients treated with ipilimumab, 22% of patients (9/41) produced antibodies against NY-ESO-1, an antigen expressed in a significant fraction of melanoma, lung, and bladder cancer specimens (Gnjatic et al. 2008). The majority (66%) of these patients experienced clinical benefit, and of the three nonresponders, one had a transient mixed response and another had a PR prior to requiring steroids for treatment-related colitis (Gnjatic et al. 2008). Anti-CTLA-4 induced NY-ESO-1 antibody responses have also been demonstrated in prostate cancer patients (Fong et al. 2009). Further investigation must be conducted to determine whether such antibodies are protective, or are simply surrogates for another protective immune process.

Antibodies against products secreted from tumors have also been observed with CTLA-4 blockade. Some patients who have responded clinically to anti-CTLA-4

therapy developed high titers of antibodies against MHC class I chain-related protein A (MICA) (Fong and Small 2008). MICA and related proteins including ULBP normally stimulate NK or CD8⁺ T cell mediated cytotoxicity by binding to NK cell receptor NKG2D. However, tumor metastases may evolve to shed soluble MICA or ULBP, thereby suppressing cytolytic activity (Paschen et al. 2009). It is possible that part of the clinical benefit from anti-CTLA-4-induced anti-MICA antibody production may be the reduced soluble MICA enabling better NK-mediated tumor killing (Jinushi et al. 2006; Fong and Small 2008).

5.8 *The “Immunogram”: A Tool to Synthesize Immune Monitoring*

The ever expanding and evolving data from correlative immunologic studies of patients undergoing immune modulation may in fact become overwhelming. To mitigate this, we introduced the concept of the “immunogram,” which synthesizes current immune monitoring data and allows us to distill important lessons from individual patients. The immunogram is simply a modular, chronological illustration of treatment history, dissected radiographic response, blood counts, serologic measurements of immune response, cellular phenotype markers, and measurements of antigen-specific response. With changes in technology and data collection, the immunogram can evolve to include different types of information.

The immunogram of patient IMF-16 (Fig. 2) anecdotally validates a wealth of data regarding the mechanism of anti-CTLA-4 therapy (Yuan et al. 2010). For example, the immunogram illustrates that steroid treatment in this patient did not clearly affect therapy and that early lymphocyte expansion, along with ICOS increases, may predict clinical outcome. In addition, responses seen may also be correlated with NY-ESO-1 specific T cell and B cell activity. This immunogram prompted a follow-up study analyzing the tumor-antigen and tumor-infiltrating lymphocyte profile of the resected “escape” lesion. Here, we learned that NY-ESO-1-specific T cells were engaging the tumor, which was still expressing NY-ESO-1 antigen, but that Tregs were heavily abundant in the tumor. This suggests that, in IMF-16, tumor escape was mediated by Treg suppression of antigen-specific Teff activity. The immunogram – a newly-described graphical synthesis of treatment data and immune correlates in individual patients – may help us to confirm, reject, or formulate hypotheses of the mechanism of anti-CTLA-4 activity.

6 Future Directions

Immunomodulation with monoclonal antibodies targeting immune receptors, such as CTLA-4 and GITR, has shown efficacy as an anticancer therapeutic approach both as a single agent and in combination with other drugs in different pre-clinical tumor models. Experiments done in mice indicate that anti-CTLA-4 is effective on

Fig. 2 Immunogram of patient IMF-16. (a) Patient IMF-16 received ipilimumab therapy as part of protocol CA183-008, and received four induction doses at 10 mg/kg every 3 weeks followed by maintenance doses every 3 months. The patient received intermittent low-dose oral steroids to treat grade 2 rash and pruritis; (b) Response of target lesions is illustrated as percent change of maximal tumor diameter from baseline. The patient underwent resection of progressive iliac lymph nodes (“x”); (c) WBC and ALC are graphed in $\times 10^3/\text{mm}^3$ units; (d) Peripheral CD4⁺ and CD8⁺ lines expanded with therapy, measured by flow cytometry; (e) Peripheral expressions of phenotype markers ICOS and Foxp3 were both transiently expanded with therapy; (f) NY-ESO-1 titers are graphed in log₁₀ as an inverse titer. CD8⁺ NY-ESO-1 specific function was measured by flow cytometry using HLA-A*0201/NY-ESO-1_{157–165} tetramer stain. (Reproduced with permission from Yuan et al. 2010). *Ipi* ipilimumab, *LN* lymph node, *Tet* tetramer, *ALC* absolute lymphocyte count

tumor bearing hosts if a certain threshold of “tumor-immunogenicity” is reached. For example, the tumor itself can be intrinsically immunogenic or rendered immunogenic by other means such as transfection, vaccination, or immunomodulation. Conventional chemotherapeutic agents or radiotherapy have also shown efficacy in combination with anti-CTLA-4. This might depend on effects of the chemo/radiotherapy on “tumor-immunogenicity” either by directly affecting immune cell populations (e.g., Inducing lymphopenia and promoting activation of adaptive immune responses during recovery from lymphopenia) or by favoring antigen release, increasing MHC I expression and/or inducing local inflammation. Further dissecting the effects of conventional therapies on tumor immunogenicity when combined with CTLA-4 blockade will be important to provide more rationale for clinical studies.

Although the amount of preclinical work done with anti-GITR agonist antibody is not yet as thorough as what has been done with anti-CTLA-4, it is very clear that DTA-1 has striking antitumor properties in mouse models both as a monotherapy and in combination with vaccines. The mechanisms of action of DTA-1 are still under investigation and its relative effect on Tregs versus Teffs or perhaps other cells, such as endothelial cells, is not yet entirely clear. Anti-GITR is therefore an interesting new drug to test in patients and to consider for combinations with other immunomodulatory antibodies and vaccines.

Conceptually, optimal tumor vaccination would involve production of increased amounts of tumor antigen in order to drive a tumor specific response. There are currently promising small molecule inhibitors targeting melanoma harboring genetic mutations including BRAF, the presence of which appears to confer sensitivity to BRAF inhibitors. Preliminary phase I and II clinical trials have reported high rates of response, however, with a median duration of response reported to be only 6 months. We hypothesize that the antigenic release with these small molecule inhibitors coupled with immune modulating antibodies to lower the immune threshold for antitumor response may be synergistic in providing long-lasting tumor immunity.

Finally, durable responses induced by anti-CTLA-4 therapy correlate with markers of immune activity such as antigen-specific CD4⁺ or CD8⁺ cytokine release, antitumor antibody formation, or cellular phenotype differentiation. Some patients exhibit atypical responses to anti-CTLA-4 therapy, demonstrating transient/delayed responses or heterogeneity by lesion site. Such atypical responses may offer insight into the mechanism of anti-CTLA-4 therapy. Immune monitoring of individual patients enables us to hypothesize regarding the mechanism of anti-CTLA-4 therapy. When analyzed in a compendium, such analyses might assist us in determining which immune processes are key, which combination therapies to pursue, and which immune parameters can be used to guide treatment fine-tuning of the immune system to achieve an optimal clinical result.

Acknowledgments The authors would like to thank Dr. Stephanie Terzulli and Brian Ginsberg for reviewing the manuscript and Drs. Lloyd Old and David Page for their scientific guidance and assistance in generating the immunogram.

References

- Alegre ML, Noel PJ, Eisfelder BJ, Chuang E, Clark MR, Reiner SL, Thompson CB (1996) Regulation of surface and intracellular expression of CTLA4 on mouse T cells. *J Immunol* 157:4762–4770
- Ansell SM, Hurvitz SA, Koenig PA, LaPlant BR, Kabat BF, Fernando D, Habermann TM, Inwards DJ, Verma M, Yamada R, Erlichman C, Lowy I, Timmerman JM (2009) Phase I study of ipilimumab, an anti-CTLA-4 monoclonal antibody, in patients with relapsed and refractory B-cell non-Hodgkin lymphoma. *Clin Cancer Res* 15:6446–6453
- Attia P, Phan GQ, Maker AV, Robinson MR, Quezado MM, Yang JC, Sherry RM, Topalian SL, Kammula US, Royal RE, Restifo NP, Haworth LR, Levy C, Mavroukakis SA, Nichol G, Yellin MJ, Rosenberg SA (2005) Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. *J Clin Oncol* 23:6043–6053
- Bachmann MF, Kohler G, Ecabert B, Mak TW, Kopf M (1999) Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. *J Immunol* 163:1128–1131
- Bashey A, Medina B, Corringham S, Pasek M, Carrier E, Vrooman L, Lowy I, Solomon SR, Morris LE, Holland HK, Mason JR, Alyea EP, Soiffer RJ, Ball ED (2009) CTLA4 blockade with ipilimumab to treat relapse of malignancy after allogeneic hematopoietic cell transplantation. *Blood* 113:1581–1588
- Blank U, Launay P, Benhamou M, Monteiro RC (2009) Inhibitory ITAMs as novel regulators of immunity. *Immunol Rev* 232:59–71
- Blansfield JA, Beck KE, Tran K, Yang JC, Hughes MS, Kammula US, Royal RE, Topalian SL, Haworth LR, Levy C, Rosenberg SA, Sherry RM (2005) Cytotoxic T-lymphocyte-associated antigen-4 blockade can induce autoimmune hypophysitis in patients with metastatic melanoma and renal cancer. *J Immunother* 28:593–598
- Bunnell SC, Hong DI, Kardon JR, Yamazaki T, McGlade CJ, Barr VA, Samelson LE (2002) T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol* 158:1263–1275
- Burmeister Y, Lischke T, Dahler AC, Mages HW, Lam KP, Coyle AJ, Kroczeck RA, Hutloff A (2008) ICOS controls the pool size of effector-memory and regulatory T cells. *J Immunol* 180:774–782
- Camacho LH, Antonia S, Sosman J, Kirkwood JM, Gajewski TF, Redman B, Pavlov D, Bulanhagui C, Bozon VA, Gomez-Navarro J, Ribas A (2009) Phase I/II trial of tremelimumab in patients with metastatic melanoma. *J Clin Oncol* 27:1075–1081
- Carreno BM, Bennett F, Chau TA, Ling V, Luxenberg D, Jussif J, Baroja ML, Madrenas J (2000) CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. *J Immunol* 165:1352–1356
- Chambers CA, Sullivan TJ, Allison JP (1997) Lymphoproliferation in CTLA-4-deficient mice is mediated by costimulation-dependent activation of CD4+ T cells. *Immunity* 7:885–895
- Chambers CA, Sullivan TJ, Truong T, Allison JP (1998) Secondary but not primary T cell responses are enhanced in CTLA-4-deficient CD8+ T cells. *Eur J Immunol* 28:3137–3143
- Chambers CA, Kuhns MS, Allison JP (1999) Cytotoxic T lymphocyte antigen-4 (CTLA-4) regulates primary and secondary peptide-specific CD4(+) T cell responses. *Proc Natl Acad Sci USA* 96:8603–8608
- Chan AC, Dalton M, Johnson R, Kong GH, Wang T, Thoma R, Kurosaki T (1995) Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. *EMBO J* 14:2499–2508
- Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ (1997) A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 94:1914–1918
- Chuang E, Lee KM, Robbins MD, Duerr JM, Alegre ML, Hambor JE, Neveu MJ, Bluestone JA, Thompson CB (1999) Regulation of cytotoxic T lymphocyte-associated molecule-4 by Src kinases. *J Immunol* 162:1270–1277

- Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, Gardner JP, Hamor JE, Neveu MJ, Thompson CB (2000) The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity* 13:313–322
- Cinek T, Sadra A, Imboden JB (2000) Cutting edge: tyrosine-independent transmission of inhibitory signals by CTLA-4. *J Immunol* 164:5–8
- Cohen AD, Diab A, Perales MA, Wolchok JD, Rizzuto G, Merghoub T, Huggins D, Liu C, Turk MJ, Restifo NP, Sakaguchi S, Houghton AN (2006) Agonist anti-GITR antibody enhances vaccine-induced CD8(+) T-cell responses and tumor immunity. *Cancer Res* 66:4904–4912
- Cohen AD, Schaer DA, Liu C, Li Y, Hirschhorn-Cymerman D, Kim SC, Diab A, Rizzuto G, Duan F, Perales MA, Merghoub T, Houghton AN, Wolchok JD (2010) Agonist anti-GITR monoclonal antibody induces melanoma tumor immunity in mice by altering regulatory T cell stability and intra-tumor accumulation. In Press
- Comin-Anduix B, Lee Y, Jalil J, Algazi A, de la Rocha P, Camacho LH, Bozon VA, Bulanhagui CA, Seja E, Villanueva A, Straatsma BR, Gualberto A, Economou JS, Glaspy JA, Gomez-Navarro J, Ribas A (2008) Detailed analysis of immunologic effects of the cytotoxic T lymphocyte-associated antigen 4-blocking monoclonal antibody tremelimumab in peripheral blood of patients with melanoma. *J Transl Med* 6:22
- Davila E, Kennedy R, Celis E (2003) Generation of antitumor immunity by cytotoxic T lymphocyte epitope peptide vaccination, CpG-oligodeoxynucleotide adjuvant, and CTLA-4 blockade. *Cancer Res* 63:3281–3288
- De Rosa SC, Lu FX, Yu J, Perfetto SP, Falloon J, Moser S, Evans TG, Koup R, Miller CJ, Roederer M (2004) Vaccination in humans generates broad T cell cytokine responses. *J Immunol* 173:5372–5380
- Demaria S, Bhardwaj N, McBride WH, Formenti SC (2005) Combining radiotherapy and immunotherapy: a revived partnership. *Int J Radiat Oncol Biol Phys* 63:655–666
- den Brok MH, Suttmoller RP, Nierkens S, Bennink EJ, Frielink C, Toonen LW, Boerman OC, Figdor CG, Ruers TJ, Adema GJ (2006) Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. *Br J Cancer* 95:896–905
- Downey SG, Klapper JA, Smith FO, Yang JC, Sherry RM, Royal RE, Kammula US, Hughes MS, Allen TE, Levy CL, Yellin M, Nichol G, White DE, Steinberg SM, Rosenberg SA (2007) Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. *Clin Cancer Res* 13:6681–6688
- Duvall MG, Precopio ML, Ambrozak DA, Jaye A, McMichael AJ, Whittle HC, Roederer M, Rowland-Jones SL, Koup RA (2008) Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur J Immunol* 38:350–363
- Esparza EM, Arch RH (2005) Glucocorticoid-induced TNF receptor functions as a costimulatory receptor that promotes survival in early phases of T cell activation. *J Immunol* 174:7869–7874
- Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, Belladonna ML, Fioretti MC, Alegre ML, Puccetti P (2003) Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 4:1206–1212
- Fong L, Small EJ (2008) Anti-cytotoxic T-lymphocyte antigen-4 antibody: the first in an emerging class of immunomodulatory antibodies for cancer treatment. *J Clin Oncol* 26:5275–5283
- Fong L, Kwek SS, O'Brien S, Kavanagh B, McNeel DG, Weinberg V, Lin AM, Rosenberg J, Ryan CJ, Rini BI, Small EJ (2009) Potentiating endogenous antitumor immunity to prostate cancer through combination immunotherapy with CTLA4 blockade and GM-CSF. *Cancer Res* 69:609–615
- Friedline RH, Brown DS, Nguyen H, Kornfeld H, Lee J, Zhang Y, Appleby M, Der SD, Kang J, Chambers CA (2009) CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. *J Exp Med* 206:421–434
- Gadina M, Stancato LM, Bacon CM, Lerner AC, O'Shea JJ (1998) Involvement of SHP-2 in multiple aspects of IL-2 signaling: evidence for a positive regulatory role. *J Immunol* 160:4657–4661

- Gao Y, Whitaker-Dowling P, Griffin JA, Barmada MA, Bergman I (2009) Recombinant vesicular stomatitis virus targeted to Her2/neu combined with anti-CTLA4 antibody eliminates implanted mammary tumors. *Cancer Gene Ther* 16:44–52
- Gattinoni L, Ranganathan A, Surman DR, Palmer DC, Antony PA, Theoret MR, Heimann DM, Rosenberg SA, Restifo NP (2006) CTLA-4 dysregulation of self/tumor-reactive CD8+ T-cell function is CD4+ T-cell dependent. *Blood* 108:3818–3823
- Gnjatic S, Yuan J, Ritter E, Jungbluth AA, Gallardo H, Terzulli S, Ritter G, Houghton A, Old LJ, Allison JP, Wolchok JD (2008) Serum antibodies as predictive markers of clinical response to anti-CTLA-4 (Ipilimumab) treatment in advanced melanoma patients. In: ASCO 2008 Annual Meeting
- Gordon MS, Stein M, Shannon P, Eddy S, Tyler A, Catlett L, Hsyu P, Huang B, Healey D, Rini BI (2009) Phase I dose escalation trial of tremelimumab plus sunitinib in patients (pts) with metastatic renal cell carcinoma (mRCC). *J Clin Oncol (Meeting Abstracts)* 27 Abstract no: 5115
- Greenwald RJ, Boussiotis VA, Lorschach RB, Abbas AK, Sharpe AH (2001) CTLA-4 regulates induction of anergy in vivo. *Immunity* 14:145–155
- Greenwald RJ, Oosterwegel MA, van der Woude D, Kubal A, Mandelbrot DA, Boussiotis VA, Sharpe AH (2002) CTLA-4 regulates cell cycle progression during a primary immune response. *Eur J Immunol* 32:366–373
- Gregor PD, Wolchok JD, Ferrone CR, Buchinshky H, Guevara-Patino JA, Perales MA, Mortazavi F, Bacich D, Heston W, Latouche JB, Sadelain M, Allison JP, Scher HI, Houghton AN (2004) CTLA-4 blockade in combination with xenogeneic DNA vaccines enhances T-cell responses, tumor immunity and autoimmunity to self antigens in animal and cellular model systems. *Vaccine* 22:1700–1708
- Hamid O, Tsuchihashi Z, Alaparthi S, Galbraith S, Berman D (2009) Association of baseline and on-study tumor biopsy markers with clinical activity in patients (pts) with advanced melanoma treated with ipilimumab. In: 2009 ASCO annual meeting, Orlando, FL
- Hersh EM, Weber JS, Powderly JD, Khan K, Pavlick AC, Samlowski WE, O'Day SJ, Nichol G, Yellin MJ, Cramner L (2008) Disease control and long-term survival in chemotherapy-naïve patients with advanced melanoma treated with ipilimumab (MDX-010) with or without dacarbazine. *J Clin Oncol (Meeting Abstracts)* 26 Abstract no: 9022
- Hersh E, Weber J, Powderly J, Pavlik A, Nichol G, Yellin M, Cramner L, Urba W, O'Day S (2009) Long-term survival of patients (pts) with advanced melanoma treated with ipilimumab with or without dacarbazine. *J Clin Oncol (Meeting Abstracts)* 27 Abstract no: 9038
- Hodi FS, Mihm MC, Soiffer RJ, Haluska FG, Butler M, Seiden MV, Davis T, Henry-Spires R, MacRae S, Willman A, Padera R, Jaklitsch MT, Shankar S, Chen TC, Korman A, Allison JP, Dranoff G (2003) Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci USA* 100:4712–4717
- Houghton AN, Eisinger M, Albino AP, Cairncross JG, Old LJ (1982) Surface antigens of melanocytes and melanomas. Markers of melanocyte differentiation and melanoma subsets. *J Exp Med* 156:1755–1766
- Houot R, Levy R (2009) T-cell modulation combined with intratumoral CpG cures lymphoma in a mouse model without the need for chemotherapy. *Blood* 113:3546–3552
- Hurwitz AA, Yu TF, Leach DR, Allison JP (1998) CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. *Proc Natl Acad Sci USA* 95:10067–10071
- Hurwitz AA, Foster BA, Kwon ED, Truong T, Choi EM, Greenberg NM, Burg MB, Allison JP (2000) Combination immunotherapy of primary prostate cancer in a transgenic mouse model using CTLA-4 blockade. *Cancer Res* 60:2444–2448
- Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczeck RA (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397:263–266

- Jinushi M, Hodi FS, Dranoff G (2006) Therapy-induced antibodies to MHC class I chain-related protein A antagonize immune suppression and stimulate antitumor cytotoxicity. *Proc Natl Acad Sci USA* 103:9190–9195
- Kanamaru F, Youngnak P, Hashiguchi M, Nishioka T, Takahashi T, Sakaguchi S, Ishikawa I, Azuma M (2004) Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells. *J Immunol* 172:7306–7314
- Kearney ER, Walunas TL, Karr RW, Morton PA, Loh DY, Bluestone JA, Jenkins MK (1995) Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J Immunol* 155:1032–1036
- Kirkwood JM, Lorigan P, Hersey P, Hauschild A, Robert C, McDermott DF, Gomez-Navarro J, Liang JQ, Bulanhagui CA (2008) A phase II trial of tremelimumab (CP-675,206) in patients with advanced refractory or relapsed melanoma. *J Clin Oncol* (Meeting Abstracts) 26 Abstract no: 9023
- Klein O, Ebert LM, Nicholaou T, Browning J, Russell SE, Zuber M, Jackson HM, Dimopoulos N, Tan BS, Hoos A, Luescher IF, Davis ID, Chen W, Cebon J (2009) Melan-A-specific cytotoxic T cells are associated with tumor regression and autoimmunity following treatment with anti-CTLA-4. *Clin Cancer Res* 15:2507–2513
- Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, Shimizu J, Nomura T, Chiba T, Sakaguchi S (2005) Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3 + CD25 + CD4+ regulatory T cells. *J Exp Med* 202:885–891
- Kohm AP, Williams JS, Miller SD (2004) Cutting edge: ligation of the glucocorticoid-induced TNF receptor enhances autoreactive CD4+ T cell activation and experimental autoimmune encephalomyelitis. *J Immunol* 172:4686–4690
- Kwon ED, Hurwitz AA, Foster BA, Madias C, Feldhaus AL, Greenberg NM, Burg MB, Allison JP (1997) Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc Natl Acad Sci USA* 94:8099–8103
- Leach DR, Krummel MF, Allison JP (1996) Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271:1734–1736
- Liakou CI, Kamat A, Tang DN, Chen H, Sun J, Troncso P, Logothetis C, Sharma P (2008) CTLA-4 blockade increases IFN γ -producing CD4 + ICOS $^{+}$ cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci USA* 105:14987–14992
- Linsley PS, Ledbetter JA (1993) The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* 11:191–212
- Linsley PS, Bradshaw J, Greene J, Peach R, Bennett KL, Mittler RS (1996) Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity* 4:535–543
- Lohning M, Hutloff A, Kallinich T, Mages HW, Bonhagen K, Radbruch A, Hamelmann E, Kroczek RA (2003) Expression of ICOS in vivo defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. *J Exp Med* 197:181–193
- Lucas PJ, Negishi I, Nakayama K, Fields LE, Loh DY (1995) Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. *J Immunol* 154:5757–5768
- Makedonas G, Betts MR (2006) Polyfunctional analysis of human T cell responses: importance in vaccine immunogenicity and natural infection. *Springer Semin Immunopathol* 28:209–219
- Maker AV, Attia P, Rosenberg SA (2005a) Analysis of the cellular mechanism of anti-tumor responses and autoimmunity in patients treated with CTLA-4 blockade. *J Immunol* 175:7746–7754
- Maker AV, Phan GQ, Attia P, Yang JC, Sherry RM, Topalian SL, Kammula US, Royal RE, Haworth LR, Levy C, Kleiner D, Mavroukakis SA, Yellin M, Rosenberg SA (2005b) Tumor regression and autoimmunity in patients treated with cytotoxic T lymphocyte-associated antigen 4 blockade and interleukin 2: a phase I/II study. *Ann Surg Oncol* 12:1005–1016
- Mandelbrot DA, McAdam AJ, Sharpe AH (1999) B7-1 or B7-2 is required to produce the lymphoproliferative phenotype in mice lacking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). *J Exp Med* 189:435–440

- Martin M, Schneider H, Azouz A, Rudd CE (2001) Cytotoxic T lymphocyte antigen 4 and CD28 modulate cell surface raft expression in their regulation of T cell function. *J Exp Med* 194: 1675–1681
- McCoy KD, Hermans IF, Fraser JH, Le Gros G, Ronchese F (1999) Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) can regulate dendritic cell-induced activation and cytotoxicity of CD8(+) T cells independently of CD4(+) T cell help. *J Exp Med* 189:1157–1162
- McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC (2002) CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16:311–323
- Mohebtash M, Madan RA, Arlen PM, Rauckhorst M, Tsang KY, Cereda V, Vergati M, Poole DJ, Dahut WL, Schlom J, Gulley JL (2009) Phase I trial of targeted therapy with PSA-TRICOM vaccine (V) and ipilimumab (ipi) in patients (pts) with metastatic castration-resistant prostate cancer (mCRPC). *J Clin Oncol (Meeting Abstracts)* 27 Abstract no: 5144
- Mokyr MB, Kalinichenko T, Gorelik L, Bluestone JA (1998a) Realization of the therapeutic potential of CTLA-4 blockade in low-dose chemotherapy-treated tumor-bearing mice. *Cancer Res* 58:5301–5304
- Mokyr MB, Kalinichenko TV, Gorelik L, Bluestone JA (1998b) Importance of the B7-2 molecule for low dose melphalan-induced acquisition of tumor-eradicating immunity by mice bearing a large MOPC-315 tumor. *J Immunol* 160:1866–1874
- Nakada T, Noguchi Y, Satoh S, Ono T, Saika T, Kurashige T, Gnjjatic S, Ritter G, Chen YT, Stockert E, Nasu Y, Tsushima T, Kumon H, Old LJ, Nakayama E (2003) NY-ESO-1 mRNA expression and immunogenicity in advanced prostate cancer. *Cancer Immun* 3:10
- Nocentini G, Riccardi C (2005) GITR: a multifaceted regulator of immunity belonging to the tumor necrosis factor receptor superfamily. *Eur J Immunol* 35:1016–1022
- Nocentini G, Giunchi L, Ronchetti S, Krausz LT, Bartoli A, Moraca R, Migliorati G, Riccardi C (1997) A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. *Proc Natl Acad Sci USA* 94:6216–6221
- O'Day SJ, Ibrahim R, DePril V, Maio M, Chiarion-Sileni V, Gajewski TF, Pehamberger H, Hoos A, Humphrey R, Wolchock J (2008) Efficacy and safety of ipilimumab induction and maintenance dosing in patients with advanced melanoma who progressed on one or more prior therapies. *J Clin Oncol (Meeting Abstracts)* 26: 9021
- O'Day S, Weber J, Lebbe C, Maio M, Pehamberger H, Harmankaya K, Siegel J, Hoos A, Humphrey R, Wolchok J (2009) Effect of ipilimumab treatment on 18-month survival: update of patients (pts) with advanced melanoma treated with 10 mg/kg ipilimumab in three phase II clinical trials. *J Clin Oncol (Meeting Abstracts)* 27 Abstract no: 9033
- Pages F, Ragueneau M, Rottapel R, Truneh A, Nunes J, Imbert J, Olive D (1994) Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature* 369: 327–329
- Paschen A, Sucker A, Hill B, Moll I, Zapatka M, Nguyen XD, Sim GC, Gutmann I, Hassel J, Becker JC, Steinle A, Schadendorf D, Ugurel S (2009) Differential clinical significance of individual NKG2D ligands in melanoma: soluble ULBP2 as an indicator of poor prognosis superior to S100B. *Clin Cancer Res* 15:5208–5215
- Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP (2009) Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med* 206:1717–1725
- Perales MA, Blachere NE, Engelhorn ME, Ferrone CR, Gold JS, Gregor PD, Noffz G, Wolchok JD, Houghton AN (2002) Strategies to overcome immune ignorance and tolerance. *Semin Cancer Biol* 12:63–71
- Perales MA, Yuan J, Powel S, Gallardo HF, Rasalan TS, Gonzalez C, Manukian G, Wang J, Zhang Y, Chapman PB, Krown SE, Livingston PO, Ejadi S, Panageas KS, Engelhorn ME, Terzulli SL, Houghton AN, Wolchok JD (2008) Phase I/II study of GM-CSF DNA as an adjuvant for a multi-peptide cancer vaccine in patients with advanced melanoma. *Mol Ther* 16:2022–2029

- Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB, Abbas AK (1997) Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* 6:411–417
- Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, Duray PH, Steinberg SM, Allison JP, Davis TA, Rosenberg SA (2003) Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci USA* 100:8372–8377
- Pop LM, Smallshaw JE, Tucker TF, Stevenson FK, Vitetta ES (2005) Failure of vaccination with idiotype protein or DNA, (+/-IL-2), the depletion of regulatory T cells, or the blockade of CTLA-4 to prolong dormancy in mice with BCL1 lymphoma. *J Immunother* 28:525–534
- Precopio ML, Betts MR, Parrino J, Price DA, Gostick E, Ambrozak DR, Asher TE, Douek DC, Harari A, Pantaleo G, Bailer R, Graham BS, Roederer M, Koup RA (2007) Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 204:1405–1416
- Quezada SA, Peggs KS, Curran MA, Allison JP (2006) CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J Clin Invest* 116:1935–1945
- Ramirez-Montagut T, Chow A, Hirschhorn-Cymerman D, Terwey TH, Kochman AA, Lu S, Miles RC, Sakaguchi S, Houghton AN, van den Brink MR (2006) Glucocorticoid-induced TNF receptor family related gene activation overcomes tolerance/ignorance to melanoma differentiation antigens and enhances antitumor immunity. *J Immunol* 176:6434–6442
- Read S, Malmstrom V, Powrie F (2000) Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192:295–302
- Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, Sharpe AH, Powrie F (2006) Blockade of CTLA-4 on CD4 + CD25+ regulatory T cells abrogates their function in vivo. *J Immunol* 177:4376–4383
- Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK, Camphausen K, Luiten RM, de Ru AH, Neijssen J, Griekspoor A, Mesman E, Verreck FA, Spits H, Schlom J, van Veelen P, Neefjes JJ (2006) Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J Exp Med* 203:1259–1271
- Reuben JM, Lee BN, Li C, Gomez-Navarro J, Bozon VA, Parker CA, Hernandez IM, Gutierrez C, Lopez-Berestein G, Camacho LH (2006) Biologic and immunomodulatory events after CTLA-4 blockade with ticilimumab in patients with advanced malignant melanoma. *Cancer* 106:2437–2444
- Ribas A (2008) Overcoming immunologic tolerance to melanoma: targeting CTLA-4 with tremelimumab (CP-675, 206). *Oncologist* 13(Suppl 4):10–15
- Ribas A, Camacho LH, Lopez-Berestein G, Pavlov D, Bulanhagui CA, Millham R, Comin-Anduix B, Reuben JM, Seja E, Parker CA, Sharma A, Glaspy JA, Gomez-Navarro J (2005) Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675, 206. *J Clin Oncol* 23:8968–8977
- Ribas A, Hauschild A, Kefford R, Gomez-Navarro J, Pavlov D, Marshall MA (2008) Phase III, open-label, randomized, comparative study of tremelimumab (CP-675,206) and chemotherapy (temozolomide [TMZ] or dacarbazine [DTIC]) in patients with advanced melanoma. *J Clin Oncol* (Meeting Abstracts) 26 Abstract no: LBA9011
- Ribas A, Comin-Anduix B, Economou JS, Donahue TR, de la Rocha P, Morris LF, Jalil J, Dissette VB, Shintaku IP, Glaspy JA, Gomez-Navarro J, Cochran AJ (2009) Intratumoral immune cell infiltrates, FoxP3, and indoleamine 2, 3-dioxygenase in patients with melanoma undergoing CTLA4 blockade. *Clin Cancer Res* 15:390–399
- Ronchetti S, Nocentini G, Riccardi C, Pandolfi PP (2002) Role of GITR in activation response of T lymphocytes. *Blood* 100:350–352

- Ronchetti S, Zollo O, Bruscoli S, Agostini M, Bianchini R, Nocentini G, Ayroldi E, Riccardi C (2004) GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. *Eur J Immunol* 34:613–622
- Rudd CE, Taylor A, Schneider H (2009) CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev* 229:12–26
- Sanderson K, Scotland R, Lee P, Liu D, Groshen S, Snively J, Sian S, Nichol G, Davis T, Keler T, Yellin M, Weber J (2005) Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and Montanide ISA 51 for patients with resected stages III and IV melanoma. *J Clin Oncol* 23:741–750
- Schneider H, Downey J, Smith A, Zinselmeyer BH, Rush C, Brewer JM, Wei B, Hogg N, Garside P, Rudd CE (2006) Reversal of the TCR stop signal by CTLA-4. *Science* 313:1972–1975
- Schneider H, Smith X, Liu H, Bismuth G, Rudd CE (2008) CTLA-4 disrupts ZAP70 microcluster formation with reduced T cell/APC dwell times and calcium mobilization. *Eur J Immunol* 38:40–47
- Sharma MD, Hou DY, Liu Y, Koni PA, Metz R, Chandler P, Mellor AL, He Y, Munn DH (2009) Indoleamine 2, 3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood* 113:6102–6111
- Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S (2002) Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 3:135–142
- Shrikant P, Khoruts A, Mescher MF (1999) CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity* 11:483–493
- Slovin SF, Beer TM, Higano CS, Tejwani S, Hamid O, Picus J, Harzstark A, Scher HI, Lan Z, Lowy I, Prostate Cancer Clinical Trials Consortium (2009) Initial phase II experience of ipilimumab (IPI) alone and in combination with radiotherapy (XRT) in patients with metastatic castration-resistant prostate cancer (mCRPC). *J Clin Oncol (Meeting Abstracts)* 27 Abstract no: 5138
- Small E, Higano C, Tchekmedyian N, Sartor O, Stein B, Young R, Vestal J, Moseley W, Fischkoff S, Lowy I (2006) Randomized phase II study comparing 4 monthly doses of ipilimumab (MDX-010) as a single agent or in combination with a single dose of docetaxel in patients with hormone-refractory prostate cancer. *J Clin Oncol (Meeting Abstracts)* 24 Abstract no: 4609
- Spinicelli S, Nocentini G, Ronchetti S, Krausz LT, Bianchini R, Riccardi C (2002) GITR interacts with the pro-apoptotic protein Siva and induces apoptosis. *Cell Death Differ* 9:1382–1384
- Stephens GL, McHugh RS, Whitters MJ, Young DA, Luxenberg D, Carreno BM, Collins M, Shevach EM (2004) Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4 + CD25+ T cells. *J Immunol* 173:5008–5020
- Suvas S, Kim B, Sarangi PP, Tone M, Waldmann H, Rouse BT (2005) In vivo kinetics of GITR and GITR ligand expression and their functional significance in regulating viral immunopathology. *J Virol* 79:11935–11942
- Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA (2004) Distinct roles of CTLA-4 and TGF-beta in CD4 + CD25+ regulatory T cell function. *Eur J Immunol* 34:2996–3005
- Tchekmedyian S, Glaspy J, Korman A, Keler T, Deo Y, Davis TA (2002) MDX-010 (human anti-CTLA4): a phase I trial in malignant melanoma. *Proc Am Soc Clin Oncol* 21 Abstract no: 56
- Tivol EA, Gorski J (2002) Re-establishing peripheral tolerance in the absence of CTLA-4: complementation by wild-type T cells points to an indirect role for CTLA-4. *J Immunol* 169:1852–1858
- Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH (1995) Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541–547
- Tivol EA, Boyd SD, McKeon S, Borriello F, Nickerson P, Strom TB, Sharpe AH (1997) CTLA4Ig prevents lymphoproliferation and fatal multiorgan tissue destruction in CTLA-4-deficient mice. *J Immunol* 158:5091–5094

- Tone M, Tone Y, Adams E, Yates SF, Frewin MR, Cobbold SP, Waldmann H (2003) Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. *Proc Natl Acad Sci USA* 100:15059–15064
- Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN (2004) Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J Exp Med* 200:771–782
- Tybulewicz VL, Ardouin L, Prisco A, Reynolds LF (2003) Vav1: a key signal transducer downstream of the TCR. *Immunol Rev* 192:42–52
- van der Merwe PA, Bodian DL, Daenke S, Linsley P, Davis SJ (1997) CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J Exp Med* 185:393–403
- van Elsland A, Hurwitz AA, Allison JP (1999) Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* 190:355–366
- van Elsland A, Suttmüller RP, Hurwitz AA, Ziskin J, Villaseñor J, Medema JP, Overwijk WW, Restifo NP, Melief CJ, Offringa R, Allison JP (2001) Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy. *J Exp Med* 194:481–489
- van Leeuwen JE, Samelson LE (1999) T cell antigen-receptor signal transduction. *Curr Opin Immunol* 11:242–248
- Vijayakrishnan L, Slavik JM, Illes Z, Greenwald RJ, Rainbow D, Greve B, Peterson LB, Hafler DA, Freeman GJ, Sharpe AH, Wicker LS, Kuchroo VK (2004) An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity* 20:563–575
- Vonderheide RH, LoRusso PM, Khalil M, Heath E, Khaira D, Soulieres D, Dorazio P, Mariani GL, Usari T, Domchek SM (2009) Tremelimumab in combination with exemestane as novel immunotherapy for patients with advanced breast cancer. *J Clin Oncol* (Meeting Abstracts) 27 Abstract no: 3034
- Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA (1994) CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1:405–413
- Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Griesser H, Mak TW (1995) Lymphoproliferative disorders with early lethality in mice deficient in Ctlα-4. *Science* 270:985–988
- Weber J (2008) Overcoming immunologic tolerance to melanoma: targeting CTLA-4 with ipilimumab (MDX-010). *Oncologist* 13(Suppl 4):16–25
- Weber JS, Targan S, Scotland R, Snively J, Garcia M, Yellin M, Fischkoff S, Nichol G (2006) Phase II trial of extended dose anti-CTLA-4 antibody ipilimumab (formerly MDX-010) with a multi-peptide vaccine for resected stages IIIC and IV melanoma. *J Clin Oncol* (Meeting Abstracts) 24 Abstract no: 2510
- Weber JS, O'Day S, Urban W, Powderly J, Nichol G, Yellin M, Snively J, Hersh E (2008) Phase I/II study of ipilimumab for patients with metastatic melanoma. *J Clin Oncol* 26:5950–5956
- Weber J, Thompson JA, Hamid O, Minor D, Amin A, Ron I, Ridolfi R, Assi H, Maraveyas A, Berman D, Siegel J, O'Day SJ (2009) A randomized, double-blind, placebo-controlled, phase II study comparing the tolerability and efficacy of ipilimumab administered with or without prophylactic budesonide in patients with unresectable stage III or IV melanoma. *Clin Cancer Res* 15:5591–5598
- Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S (2008) CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322:271–275
- Wolchok JD, de Pril V, Linette G, Waterfield W, Gajewski T, Chiarion-Sileni V, Ibrahim R, Chin K, Hoos A, Hamid O (2009a) Efficacy of ipilimumab 10 mg/kg in advanced melanoma

- patients (pts) with good and poor prognostic factors. *J Clin Oncol* (Meeting Abstracts) 27 Abstract no: 9036
- Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, Maio M, Binder M, Bohnsack O, Nichol G, Humphrey R, Hodi FS (2009b) Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* 15:7412–7420
- Wolchok JD, Neyns B, Linette G, Negrier S, Lutzky J, Thomas L, Waterfield W, Schadendorf D, Smylie M, Guthrie T Jr, Grob JJ, Chesney J, Chin K, Chen K, Hoos A, O'Day SJ, Lebbe C (2009c) Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol* 11:155–164
- Yang YF, Zou JP, Mu J, Wijesuriya R, Ono S, Walunas T, Bluestone J, Fujiwara H, Hamaoka T (1997) Enhanced induction of antitumor T-cell responses by cytotoxic T lymphocyte-associated molecule-4 blockade: the effect is manifested only at the restricted tumor-bearing stages. *Cancer Res* 57:4036–4041
- Yang JC, Hughes M, Kammula U, Royal R, Sherry RM, Topalian SL, Suri KB, Levy C, Allen T, Mavroukakis S, Lowy I, White DE, Rosenberg SA (2007) Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis. *J Immunother* 30:825–830
- Yuan J, Gnjjatic S, Li H, Powel S, Gallardo HF, Ritter E, Ku GY, Jungbluth AA, Segal NH, Rasalan TS, Manukian G, Xu Y, Roman RA, Terzulli SL, Heywood M, Pogoriler E, Ritter G, Old LJ, Allison JP, Wolchok JD (2008) CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. *Proc Natl Acad Sci USA* 105:20410–20415
- Yuan J, Page DB, Ku GY, Li Y, Mu Z, Ariyan C, Gallardo HF, Roman RA, Heine AI, Terzulli SL, Gnjjatic S, Ritter G, Jungbluth AA, Allison JP, Old LJ, Wolchok JD (2010) Correlation of clinical and immunological data in a metastatic melanoma patient with heterogeneous tumor responses to ipilimumab therapy. *Cancer Immun* 10:1
- Zatlouk P, Heo DS, Park K, Kang J, Butts C, Bradford D, Graziano S, Huang B, Healey D (2009) Randomized phase II clinical trial comparing tremelimumab (CP-675,206) with best supportive care (BSC) following first-line platinum-based therapy in patients (pts) with advanced non-small cell lung cancer (NSCLC). *J Clin Oncol* (Meeting Abstracts) 27 Abstract no: 8071
- Zhang W, Sloan-Lancaster J, Kitchen J, Tribble RP, Samelson LE (1998) LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92:83–92
- Zhang B, Bowerman NA, Salama JK, Schmidt H, Spiotto MT, Schietinger A, Yu P, Fu YX, Weichselbaum RR, Rowley DA, Kranz DM, Schreiber H (2007) Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. *J Exp Med* 204:49–55
- Zhou P, L'Italien L, Hodges D, Schebye XM (2007) Pivotal roles of CD4+ effector T cells in mediating agonistic anti-GITR mAb-induced-immune activation and tumor immunity in CT26 tumors. *J Immunol* 179:7365–7375