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CTLA-4-MEDIATED INHIBITION IN REGULATION OF T CELL RESPONSES: Mechanisms and Manipulation in Tumor Immunotherapy

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■ **Abstract** The T cell compartment of adaptive immunity provides vertebrates with the potential to survey for and respond specifically to an incredible diversity of antigens. The T cell repertoire must be carefully regulated to prevent unwanted responses to self. In the periphery, one important level of regulation is the action of costimulatory signals in concert with T cell antigen-receptor (TCR) signals to promote full T cell activation. The past few years have revealed that costimulation is quite complex, involving an integration of activating signals and inhibitory signals from CD28 and CTLA-4 molecules, respectively, with TCR signals to determine the outcome of a T cell's encounter with antigen. Newly emerging data suggest that inhibitory signals mediated by CTLA-4 not only can determine whether T cells become activated, but also can play a role in regulating the clonal representation in a polyclonal response. This review primarily focuses on the cellular and molecular mechanisms of regulation by CTLA-4 and its manipulation as a strategy for tumor immunotherapy.

INTRODUCTION

The first definitive experimental demonstration that T cell antigen receptor (TCR) engagement was insufficient for T cell activation came from the work of Jenkins & Schwartz in the late 1980s (1). They clearly demonstrated that T cell clones that received only a TCR signal did not become activated but were induced into a state of antigen-specific unresponsiveness, or anergy. They showed that a second signal was required and that this costimulatory signal was provided

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exclusively by a cell surface ligand restricted to cells which, for want of a better term, are called professional antigen-presenting cells (APC). Cells with potent costimulatory activity included dendritic cells, activated macrophages, and activated B cells. It was subsequently shown that naïve T cells had a similar requirement for these costimulatory signals in order to produce IL-2 and progress through the cell cycle. This requirement of T cells for costimulation, together with the sharp restriction of the costimulatory ligands to professional APC, was proposed to be a mechanism for maintenance of peripheral T cell tolerance. These observations and ideas naturally provoked considerable interest in the identity of the costimulatory molecules that could suffice, in addition to antigen receptor signals, to allow full activation of naïve T cells and prevent induction of anergy in T cell clones.

CD28 AND CTLA-4: Positive and Negative Costimulators

A considerable literature has developed demonstrating that interactions between the cell surface molecule CD28 and its counter receptor B7 on antigen presenting cells are the major, if not the only, source of costimulatory signals in the sense of the original definition (2). While the term costimulatory has in recent years come to be used as a general term to describe molecules whose engagement enhances T cell responses, it appears that many of these have their effect at later stages in the immune response by influencing cell survival, cytokine production, or other aspects important in elaboration, but not necessarily the initiation, of T cell responses. However, these do not fit the strict definition of signals that, in addition to TCR signals, influence IL-2 production and proliferation. This review focuses on the roles of the classical costimulatory molecules, one positive (CD28) and one inhibitory (CTLA-4; CD152), with a particular emphasis on the regulation of the early stages of the immune response by inhibitory costimulation. Further, it gives some examples of manipulation of these signals in the development of novel strategies for tumor immunotherapy.

The suggestion that CD28 may play an important role in costimulation came from in vitro experiments showing that its engagement could enhance IL-2 (3, 4). Subsequently it was shown that intact anti-CD28 antibodies could prevent the induction of anergy in T cell clones stimulated by chemically fixed APC, whereas Fab fragments of anti-CD28 resulted in induction of hyporesponsiveness in T cell clones stimulated by intact APC (5). In addition, anti-CD28 antibodies are sufficient to provide costimulation to naïve $CD4^+$ T cells stimulated by planar membranes containing purified MHC molecules (6). Engagement of the CD28 ligands B7-1 (CD80) and B7-2 (CD86) is critical for costimulation (7, 8). Antibodies to B7-1 or B7-2 diminished, and a combination of anti-B7-1 plus B7-2 or a CTLA-4-Ig fusion protein (see below) efficiently blocked T cell responses in a variety of in vitro and in vivo settings (9). Finally, evidence from knockout mice also demonstrated the importance of CD28/B7 interactions in T cell responses. While $CD28^{-/-}$ mice retained the ability to reject some viruses,

most T cell responses were severely impaired (10). Subsequent work showed that the determining factor in responses that occur in the absence of CD28 may be the persistence of TCR signaling: repeated administration of antigenic peptides over a 48-h period supported responses in the absence of CD28, but in the absence of this sustained stimulation, T cells from $CD28^{-/-}$ mice failed to respond productively (11). The phenotype of B7-1/B7-2 double knockout mice was quite clear—T cell responses were essentially absent (12).

Very early in the story it became apparent that CD28 was not the only player. A homologue of CD28 named cytotoxic T lymphocyte antigen-4 (CTLA-4) had been previously identified in a subtractive approach directed toward identifying gene products important in the function of cytotoxic T lymphocytes (13). However, even after recognition of the important role of CD28 in costimulation, the role of CTLA-4 remained obscure. It was apparent that the molecules were not only homologous and genetically linked, but shared several intriguing properties, including genomic organization and the presence of a motif, MYPPY, that was implicated as important in ligand binding by CD28 (14). It was also shown that a soluble version of the CTLA-4 ectodomain fused to an immunoglobulin tail (CTLA-4-Ig), like CD28, bound to both B7-1 and B7-2 (15). In fact, binding of both B7-1 and B7-2 by CTLA-4 was of considerably higher affinity than that of CD28, by a factor of 50–2000-fold, depending on the method of analysis (14, 16). Unlike CD28, CTLA-4 mRNA was not readily detectable in naïve resting T cells but was induced by activation (13). Given that CD28 is constitutively expressed by all T cells and seemed to be sufficient and necessary for costimulation, an obvious and intriguing question was the biological role of a second receptor for B7 that was inducible and had an even higher affinity (17).

The role of CTLA-4 in the regulation of T cell responses began to become apparent with the generation of specific monoclonal antibodies. The early reports showed that CTLA-4 antibodies (providing cross-linking was not possible) did not costimulate T cells stimulated via the T cell receptor only, but could enhance proliferation and IL-2 production by cells stimulated by anti-CD3 along with anti-CD28 (18, 19). These findings, together with the observation that anti-B7 antibodies also had the effect of enhancing responses of T cells activated by anti-CD3 along with anti-CD28, suggested that the enhancement was a result of removal of inhibitory signals rather than provision of auxiliary costimulatory signals. This conclusion was also supported by the fact that co-cross-linking of CTLA-4 with CD28 and TCR antibodies resulted in an inhibition of T cell activation in vitro. Finally, the fact that anti-CD28 antibodies inhibited, whereas anti-CTLA-4 antibodies enhanced, T cell responses in vivo suggested that these reagents were blocking costimulatory and inhibitory signals, respectively (20, 21).

CTLA-$4^{-/-}$ Mice: The Importance of Brakes

The most dramatic evidence of the inhibitory function of CTLA-4 came from the knockout mice (22–24). CTLA-4-deficient mice develop a fatal lymphoproliferative disorder. T cell activation is detectable within 5–6 days after birth, and the

mice die at 18–28 days of age due to lymphocytic infiltration into nonlymphoid tissues. This phenotype is one of the most aggressive lymphoproliferative disorders reported in gene-targeted mice. CTLA-$4^{-/-}$ mice die much earlier than those with defects in apoptotic pathways (*lpr* or *gld* mice) (25), cytokine signaling such as in mice with a T cell–specific defect in TGFβ signal transduction (26, 27), or targeted deletion of the inhibitory molecule PD-1 (28). The absence of CTLA-4 results in virtually all of the peripheral T cells displaying an activated phenotype ($CD69^+$, $CD25^+$, $CD44^{hi}$, $CD45RB^{lo}$, $CD62L^{lo}$) (22, 23) and an approximately fourfold increase in the proportion of T cells in cell cycle, as detected by BrdU incorporation, compared to age-matched littermate controls (29). T cells proliferate spontaneously for several days in vitro and secrete a variety of cytokines including IL-2, -4, -6, -3, and GM-CSF (22, 23). The absence of CTLA-4 appears to affect the $CD4^+$ and $CD8^+$ T cell subsets differentially, with $CD4^+$ T cells being preferentially activated at the onset of the lymphoproliferation (29). Although it has not been formally demonstrated, indirect evidence indicates that TCR/MHC engagement is necessary for the polyclonal T cell activation, including the downregulation of the TCR expression levels on the T cells (22, 23), upregulation of CD69, and increased levels of phosphorylation of CD3ζ (30, 31). CD28/B7 interactions are also required for CTLA-$4^{-/-}$ T cell activation, since CTLA-4-Ig treatment from birth or the genetic absence of B7 ligands prevent T cell activation (29, 32–34). This is reversible, since cessation of CTLA-4Ig treatment results in the T cells rapidly becoming activated (32). The phenotype of the T cells and the failure to detect any defect in apoptosis suggest that there is continuous activation of the CTLA-4-deficient T cells in vivo.

One possible origin of the phenotype observed in the CTLA-$4^{-/-}$ mice is a defect in central tolerance (22, 23). Failure to negatively select thymocytes expressing TCRs with a high affinity for self-MHC and/or self-peptide would permit the emigration of highly autoreactive T cells to the periphery. However, thymocyte development appears normal in CTLA-$4^{-/-}$ mice expressing an unmanipulated repertoire (24), as well as in CTLA-$4^{-/-}$ mice bearing MHC class I- or class II-restricted transgenic TCRs (35–38). These results suggest that there is no defect in thymocyte development but rather that CTLA-4 is necessary for the regulation of peripheral T cell tolerance and homeostasis.

The lymphadenopathy that occurs in CTLA-$4^{-/-}$ mice appears to be initiated by $CD4^+$ T cells that are preferentially activated in young CTLA-$4^{-/-}$ mice. Depletion of $CD4^+$ T cells from birth prevented the onset of lymphoproliferation (29). Restriction of the TCR repertoire by the introduction of MHC class II–restricted TCR transgenes delays, but does not prevent, the development of lymphoproliferative disease (37, 38). A predominant role for $CD4^+$ T cells in the lymphadenopathy was also supported by introduction of MHC class I–restricted TCR transgenes (35, 36). CTLA-$4^{-/-}$ mice bearing the HY, 2C, or an LCMV-specific TCR developed lymphoproliferative disorder that was a result of activation and expansion not of the predominant $CD8^+$ population, but rather of $CD4^+$ cells that presumably expressed endogenous TCR. Further restriction of the TCR repertoire in the $CD4^+$ T cells by introduction of the *rag-1* null mutation into the AND TCR Tg^+

CTLA-4$^{-/-}$ mice resulted in a considerable delay in the onset of, but did not prevent CD4$^+$ T cell activation (39).

TCR transgenic T cells from CTLA-4-deficient mice have been used to examine the role of CTLA-4 in the regulation of peptide-specific T cell responses. Naïve CD4$^+$ T cells from AND Tg$^+$Rag$^{-/-}$CTLA-4$^{-/-}$ mice had a moderately enhanced proliferative response upon primary stimulation as compared to comparable cells from wild-type mice (37). Upon restimulation of resting, previously activated T cells, however, there was a much more dramatic increase in the magnitude of the response of the CTLA-4-deficient T cells. This enhanced response was evident at the level of proliferation, bulk cytokine secretion, and frequency of cytokine secreting T cells (37). In both cases, the differences were more pronounced at the high end of the dose response. An increased secondary response in the absence of CTLA-4 was also observed in the proliferative and cytokine responses of 4-day blasts of CD4$^+$ T cells expressing the DO.11.10 TCR (38).

A role for CTLA-4 in regulation of CD8$^+$ T cell responses has been controversial. The observations that naïve CD8$^+$ T cells from CTLA-4$^{-/-}$ mice bearing a transgene specific for an MHC class I–restricted epitope of LCMV have no alterations in response to primary peptide stimulation in vitro or in the ability to resolve LCMV infection in vivo led to the proposal that CTLA-4 does not regulate CD8$^+$ T cell responses (40). However, the results in this system may not reflect all situations. For example, the response of wild-type and CTLA-4-deficient T cells bearing the 2C TCR are essentially identical upon primary stimulation in vitro. However, upon secondary stimulation the CTLA-4-deficient cells exhibit a marked increase in the magnitude of both the proliferative and cytokine responses, an effect that was most pronounced at the high end of the dose response curve (36). As is discussed below, experiments using CTLA-4 blockade in vivo also support a direct role in the regulation of CD8$^+$ T cell responses.

These results suggest two features of CTLA-4 regulation of T cell responses. First, consistent with the fact that it is expressed by both CD4$^+$ and CD8$^+$ T cells, CTLA-4 can play a role in attenuating the response of both. Second, the observation that the effects of the absence of CTLA-4 are most evident in secondary responses suggests that the activational history of the T cell will influence the role of CTLA-4. It is unclear if this simply occurs due to the fact that previously activated T cells express higher basal levels of CTLA-4 compared to naïve T cells (41, 42) and/or if previously activated T cells are more responsive to CTLA-4 signals. Differential sensitivity of T cells to CTLA-4-mediated costimulation would have obvious implications for the generation and maintenance of memory T cells, and the induction of memory responses.

CTLA-4 Regulates Cell Cycle Progression, Not Cell Death

Taken together, these data provide a strong case for CTLA-4 as a crucial negative regulator of T cell responses in the periphery. The process by which this regulation occurs is clearly distinct from activation-induced cell death. An early experiment cross-linking CTLA-4 on human T cell clones suggested that CTLA-4 inhibits

T cell responses by inducing apoptosis (43). Similarly, cross-linking CTLA-4 on mouse ConA T cell blasts was reported to induce apoptosis (44). Conversely, there is no evidence that CTLA-4 ligation in conjunction with TCR and CD28 cross-linking on resting murine T cells induces apoptosis (45–48). Further, CTLA-4 ligation does not alter the CD28-mediated upregulation of survival factor bcl-x (49), arguing against apoptosis induction being the major mechanism of CTLA-4 inhibition. In addition, no defect in Fas/FasL-mediated apoptosis has been observed in T cells deficient in CTLA-4 (22, 50). It seems likely that the induction of apoptosis in experiments utilizing human T cell blasts and clones may have been an indirect result of cytokine deprivation as a result of CTLA-4-mediated inhibition of IL-2 secretion, rather than direct induction of apoptosis.

The fact that CTLA-4 deficiency is lethal relatively early in life and that the Fas pathway is intact indicates that CTLA-4 can play a critical role in limiting T cell expansion. CTLA-4 has effects on at least two aspects of activation that have critical relevance to proliferation. The first is on IL-2 production. CD28 costimulation enhances IL-2 production both at the level of transcription and mRNA stabilization (51, 52). Extensive ligation of CTLA-4 under suboptimal conditions of stimulation by TCR plus CD28 can result in inhibition of IL-2 production, probably at the level of transcription (45, 46, 53). CTLA-4 ligation does not prevent IL-2 from causing degradation of the cell cycle inhibitor $p27^{kip}$, but does inhibit TCR-induced production of cdk4, cdk6, and cyclin D3, all of which are required for G0/G1 progression (53). Thus CTLA-4 can limit expansion not only by reducing production of an important growth factor, but also by inhibiting TCR-mediated induction and assembly of essential components of the cell cycle machinery.

THE CELL BIOLOGY OF COSTIMULATION

Protein trafficking and localization during the process of T cell activation is thought to play a major role in the ability of a protein to regulate the T cell response. The finding that CTLA-4 and CD28 deliver opposing signals to the T cell yet bind the same ligands on APCs suggests that a balance may exist between the signals generated by these two molecules. Shifting this balance in one direction or another could influence to what extent an individual T cell becomes activated. It is likely that one mechanism of controlling the function of CTLA-4 and CD28 involves their differential trafficking to the T cell–APC interface, where these molecules interact with their B7 ligands.

While CTLA-4 and CD28 are homologous molecules, they have very different lifestyles (for brief reviews, see 17). CD28 is constitutively expressed on the surface of T cells, with levels increasing slightly upon activation. CTLA-4 is not readily detectable in naïve T cells but is rapidly upregulated upon T cell activation. CTLA-4 mRNA can be readily detected within 1 h of TCR engagement and peaks at about 24–36 h (54). CTLA-4 is not readily detectable at the cell surface until 24–48 h after activation (18). However, an accurate assessment of the kinetics

of CTLA-4 protein expression is complicated by the fact that it is not primarily expressed at the cell surface.

Surface expression of CTLA-4 is tightly regulated as a result of the presence of a tyrosine-based intracellular localization motif in its cytoplasmic tail (55). This motif results in both the rapid endocytosis of CTLA-4 from the cell surface to endosomal compartments, as well as the targeting of at least some CTLA-4 to the lysosomes for degradation (56, 57). It is unclear whether endocytosed CTLA-4 can recycle back to the surface or if this represents a terminal pathway for the protein. Through the use of a yeast-two-hybrid screen to search for proteins that bound to the unphosphorylated tail of CTLA-4, several groups demonstrated an association with the medium subunit of the clathrin-coated pit adaptor complex, AP2, thus providing a mechanism for CTLA-4 cellular localization (58–61). CTLA-4 expression on the T cell surface is stabilized and increased by tyrosine phosphorylation of the endocytosis motif, which inhibits AP2 association. It is noteworthy that the intracellular portion of CTLA-4 is 100% conserved among many different species of animals, suggesting that control of intracellular trafficking may be extremely important for its function. Indeed, a recent report proposed that the T cell lymphoproliferative disorder in the human disease Chediak-Higashi syndrome was a result of defective CTLA-4 trafficking and localization (62).

It has been reported that upon antibody cross-linking CD28 may also associate with PI3K and be shuttled into intracellular compartments to be degraded and/or recycled (63). However, since CD28 cell surface expression does not vary markedly following T cell activation, the functional importance of this internalization is unclear (42, 64).

Upon focal engagement of the TCR by antibody-coated beads, slides, or allogeneic cells, the bulk of intracellular CTLA-4 within an activated cell is reorganized toward sites facing TCR engagement (65). This finding is not unexpected given experiments showing reorganization of the microtubule organizing center, and all of its associated membrane structures, toward sites facing T cell interactions with APC (66). However, whether the localization of intracellular CTLA-4 beneath the T cell-APC contact site is accompanied by surface expression and phosphorylation-induced surface stabilization of CTLA-4 remains unclear. In addition, the source of CTLA-4 that localizes to the T cell–APC interface is unknown. Long-lasting intracellular stores of CTLA-4 may be rapidly mobilized to the T cell–APC interface upon antigen encounter, as well as newly translated CTLA-4 leaving the *trans*-Golgi Network.

MOLECULAR MECHANISMS OF COSTIMULATION

Despite considerable interest and effort, the mechanisms by which CD28 and CTLA-4 exert their effects remain poorly understood. Several models for CD28 signal transduction have been proposed. An extensive and detailed discussion of the literature is beyond the scope of this review, but the proposed pathways for CD28 costimulation can be grouped into three broad categories. Based on

the identification of unique CD28-responsive elements in the IL-2 promoter, it has been proposed that CD28 utilizes a signaling pathway distinct from that of the TCR and induces a unique transcription factor (67). Alternatively, it has been proposed that CD28-mediated signals converge distally with the TCR signaling pathways, possibly at the level of JUN-kinase (68) and synergize in the induction of multiple transcription factors. Based on visualization of T cell activation by a number of laboratories (69–72), there is a new and growing appreciation for the physical changes that occur spatially during T cell activation. The plasma membrane reorganizes to form cholesterol-rich rafts of proteins involved in T cell signaling (73), and CD28 may play a critical role in recruitment of these rafts to the T cell/APC interface (72). Collectively these observations have led to a new model of T cell activation. In this model, the CD28 and TCR-mediated signals intersect proximally and act synergistically to enhance the TCR signaling pathway.

Examination of the consequences of CTLA-4-ligation in conjunction with TCR and CD28-mediated signals have provided data consistent with this model of the integration of TCR and costimulatory signals. A number of proteins known to play a role in various signaling pathways including TCR signals have been reported to bind to CD28 and CTLA-4 cytoplasmic tails (74). However, controversy over the proteins and the potential substrates involved in the biochemical signal transduction pathways for costimulatory molecules remains. The details of the mechanism or mechanisms by which CTLA-4 inhibition occurs are even more unclear. Several possibilities have been proposed including direct effects on phosphorylation levels and/or indirect effects due to competition with CD28 for ligand, sequestration of signaling molecules, or disruption of signaling complexes.

The observation that many proteins known to be involved in T cell signaling were hyperphosphorylated in CTLA-$4^{-/-}$ T cells led to the notion that CTLA-4 might decrease CD3/CD28-mediated phosphorylation by the recruitment of phosphatases (31). One candidate was the protein tyrosine phosphatase SHP-1, which is involved in transducing inhibitory signals initiated by NK receptors (75). SHP-1 binds to a phosphorylated polypeptide of the CTLA-4 cytoplasmic tail (61). However, normal levels of SHP-1 are not required for CTLA-4-mediated inhibition (76). Another protein tyrosine phosphatase, SHP-2, associates with the cytoplasmic tail of CTLA-4 and it has been proposed that catalytically active SHP-2 binds to the SH2-binding domain (YxxM motif) in the CTLA-4 cytoplasmic tail (30, 31) following phosphorylation of the motif by the src family kinase $p56^{lck}$ (60, 77). However, there is conflicting data regarding whether SHP-2 binds directly to CTLA-4 (30, 78) and, if so, whether there is a requirement for phosphorylation of the YxxM tyrosine (30, 31, 61, 77, 78). Further, SHP-2 was reported to bind to phosphorylated peptides corresponding to the cytoplasmic tails of CD28 as well as CTLA-4 in pull-down assays (61). Thus, while the accumulated data suggests a role for SHP-2 in signaling, its role in CTLA-4-mediated inhibition remains to be definitively established.

Several other proteins have been reported to associate with CTLA-4. JAK2 associates with the proline-rich box1-like domain of the cytoplasmic tail of

CTLA-4, and this kinase may phosphorylate the tyrosine in the YxxM motif of the CTLA-4 tail (79). Most recently it has been reported that the serine/threonine phosphatases PP2A and PP6 bind to the phosphorylated YxxM motif of CTLA-4 and to the similar motif in the CD28 cytoplasmic tail (80). What proteins are required for CTLA-4 signal transduction remains unclear. Because the phosphorylation status of the tyrosine in the YxxM motif also regulates CTLA-4 intracellular localization, it has been difficult to separate the regulation of CTLA-4 intracellular trafficking versus signal transduction.

Despite the confusion concerning the specific role of the YxxM motif, and the identity and the role of proteins involved, some evidence implicating a role for tyrosine phosphatase activity has been reported. CTLA-4 cross-linking on recently activated T cell blasts resulted in a decrease in the level of tyrosine phosphorylation of proximal T cell signaling molecules such as CD3ζ (30). This offers a compelling scenario for CTLA-4 mediated inhibitionn of proximal steps in TCR signaling. CTLA-4 cross-linking was also found to inhibit downstream activation events including the phosphorylation of ERK and JUN-N-terminal kinase (30, 81). Identification of potential substrates for the tyrosine and serine/threonine phosphatases, and how these proteins function in the integration of CTLA-4, CD28, and TCR signal transduction, awaits further experiments but offers interesting possibilities.

Evidence that CTLA-4 might function at least in part by competing with CD28 for B7 ligands and thereby acting as an indirect attenuator of costimulatory signals comes from both in vitro and in vivo systems. Experiments utilizing T cell transfectants of CTLA-4 with mutations or truncations suggest that the CTLA-4 cytoplasmic tail is not always necessary for the inhibitory function of CTLA-4 (82–84). Competition may be most effective when B7 levels are low, but direct signaling through the tail seems to be necessary if B7 levels are high (83). Constitutive expression of high levels of a tailless CTLA-4 mutant on the cell surface delayed but did not prevent T cell activation and lymphoproliferation in CTLA-4$^{-/-}$ mice, indicating that competition for B7 ligands is not sufficient for normal CTLA-4 function (85).

CTLA-4 may also function by physically disturbing the assembly or organization of molecules in the synapse. This could occur by sequestration of proteins involved in signal transduction away from the immunological synapse, thereby reducing the resultant signaling. An alternative mechanism is suggested by the recently solved structures of B7-1 and CTLA-4 (86, 87). Both molecules are dimers, and the structures suggest that one CTLA-4 homodimer may bind two B7 molecules and form a very stable multimeric complex. This mode of binding, combined with the higher avidity of CTLA-4 than CD28 for B7 molecules, leads to the suggestion that the formation of stable CTLA-4/B7 lattices in the immunological synapse may disrupt the organized assembly of key components involved in the generation of TCR/CD28 signals (86, 87). The possibility of disruption of the synapse as a method of inhibition is especially intriguing, and could account for many of the functional observations.

A final possibility for an indirect mode of action of CTLA-4 is that CTLA-4 engagement costimulates the secretion of inhibitory cytokines, such as TGFβ (48). This more global and indirect effect is discussed below.

A schematic representation of trafficking and possible signal transduction mechanisms is shown in Figure 1.

DYNAMIC INTEGRATION OF TCR AND COSTIMULATORY SIGNALS

As previously discussed, CD28 is constitutively expressed on T cells, whereas CTLA-4 appears after activation. Because of this, and perhaps as a result of our innate appreciation for symmetry, the idea arose that CD28 engagement allowed initiation, while CTLA-4 provided for termination of immune responses (87g). Surprisingly, the majority of the in vitro data has demonstrated an inhibitory role for CTLA-4 in the early stages of T cell activation. A summary of the effects of CTLA-4 ligation on early events in T cell activation are summarized in Table 1. As can be seen, IL-2 production, expression of early markers such as CD69 and CD25, and a number of other aspects of activation are inhibited upon CTLA-4 cross-linking. These events take place within hours of T cell activation with anti-CD3 and CD28. In fact, the inhibition of the induction of IL-2 transcription was detected 4 h after stimulation (53). This suggests either that there is a physiologically relevant intracellular pool of CTLA-4 present in naïve T cells or that protein expression is induced rapidly upon activation.

Appreciation for the possibility that CTLA-4 can inhibit early stages of T cell activation has led to the development of models that stress that the dynamic interplay of costimulatory and TCR signals depends on the activation state of the T cell as well as the activation state of the antigen-presenting cell (88). Such a model is shown in Figure 2. As in the classical two-signal model, an encounter of a naïve T cell with a cell expressing appropriate MHC/antigen complex but lacking B7 does not result in activation of the T cell owing to lack of costimulation. The cells receiving a TCR signal in the absence of CD28-mediated costimulation may be rendered anergic. However, engagement of the TCR can lead to rapid induction and/or mobilization of small amounts of CTLA-4. Under conditions where there is an incompletely activated APC expressing only low amounts of B7, CTLA-4 could, by virtue of its higher affinity, outcompete CD28 for B7 and/or deliver inhibitory signals. This could effectively raise the threshold of CD28 and/or TCR signals needed for full activation. However, when a T cell encounters an antigen on a fully activated antigen-presenting cell expressing high amounts of B7, the scant amount of CTLA-4 induced after TCR engagement would become limiting. This would leave B7 available to engage CD28, allowing costimulation, IL-2 production, and proliferation of the T cells. One consequence of this activation would be further induction of CTLA-4, which could serve to terminate the response or attenuate it in more subtle ways, e.g. by limiting the burst size of the activated T cell.

For the purposes of a reductionist and admittedly oversimplified discussion, it is useful to consider the impact of CTLA-4 expression and function under two extreme situations: (*a*) Where B7 levels are low and TCR signals are weak, the amount of CTLA-4 induced is low but may be sufficient to minimize costimulation and prevent activation. Under these conditions CTLA-4 may set a threshold for activation. (*b*) Where B7 levels are high and TCR signals strong, the higher levels of CTLA-4 induced after activation may be able to attenuate the response of activated cells at relatively early stages of the response (Figure 2).

Threshold Model

There are two scenarios in which CTLA-4 may play a role in establishing a threshold for CD28 and/or TCR signals needed for activation of naïve T cells. Both presume that low levels of CTLA-4 pre-exist or can be rapidly induced in naïve T cells upon engagement of the TCR and CD28. Due to the exponential expansion of T cells that follows activation, CTLA-4 need only have a very subtle effect on T cell activation threshold to have a large impact on the magnitude of the T cell response in vivo.

The first scenario suggests a role for CTLA-4 in maintaining peripheral tolerance of T cells with specificity for tissue-specific antigens that are not expressed in the thymus and have not been deleted as a consequence of negative selection. This is essentially the same context in which the two-signal model originally proposed that the absence of costimulatory ligands from all but very specialized APC would provide a fail-safe mechanism for self-tolerance (1). CTLA-4 may provide an additional level of regulation to ensure peripheral tolerance by preventing activation when a T cell encounters a normal self-antigen in the context of low B7, as discussed above. This scenario may explain the observation that CTLA-4 blockade or deficiency accelerates the onset and severity of insulitis and diabetes in nonobese diabetic (NOD) mice expressing a transgenic $\alpha\beta$ TCR cloned from an islet-β-cell-specific $CD4^+$ T cell clone isolated from a NOD mouse (89, 90). T cells bearing this TCR are efficiently selected, rather than deleted in the thymus, demonstrating that central tolerance is not effective for T cells with this specificity. The observation that blockade or loss of CTLA-4 dramatically accelerates disease in this model system is a testament to the significance of CTLA-4 in limiting autoreactive T cells bearing TCRs specific to autoantigens. It is possible that CTLA-4 could also similarly regulate T cell activation in response to low levels of foreign antigens in the absence of inflammation-mediated upregulation of B7 on activated APC.

The second scenario deals with the regulation of the response of T cells to tonic signaling by self-peptide/MHC interactions. The idea that T cells are regularly stimulated by such signals was initially suggested by the observation that the TCRζ chain is partially phosphorylated in freshly isolated, unstimulated T cells (91). These continuous TCR interactions with self-peptide/MHC provide important signals to the T cells, although there is some controversy about whether these are necessary for T cell survival, homeostasis, or readiness for activation (92–94).

Some of these tonic interactions, under conditions of low levels of CD28/B7 interaction, might be sufficiently stimulatory to lead to the activation of $CD4^+$ T cells and the induction and/or mobilization of CTLA-4. Based on the analysis of the CTLA-$4^{-/-}$ mice and the kinetic properties of CTLA-4 and CD28 interactions with B7 molecules, we have speculated that CTLA-4 might prevent the signals generated by these interactions from leading to full activation of $CD4^+$ T cells but would enable the T cells to receive partial signals (29). Full T cell activation could be prevented by mechanisms described earlier. In this scenario, due to limiting B7 levels, competition for ligand may be the primary mechanism of action, and CTLA-4 will predominantly affect the CD28-mediated signals. Thus, we propose that CTLA-4 is involved in maintaining naïve $CD4^+$ T cells and previously activated T cells in a resting state.

This model is supported by several observations with the CTLA-$4^{-/-}$ mice. First, the expansion of T cells that occurs in these mice is polyclonal, as assessed by Vβ repertoire analysis and TCR α and β CDR3 spectratyping (22, 24, 95). This suggests that the expansion may not be a result of a failure to terminate responses to a few environmental pathogens. It is also consistent with the observation that the phenotype of the mice is independent of the peripheral peptide repertoire. For example, H-2M$\alpha^{-/-}$ mice display a very restricted peptide repertoire that seems to be entirely of self-origin (96, 97). Despite the restricted peptide repertoire, $CD4^+$CTLA-$4^{-/-}$ T cells become activated in H-2M$\alpha^{-/-}$ mice (CA Chambers, JP Allison, unpublished results). Results of experiments using transgenes to restrict the TCR repertoire are mixed. The results from mice expressing restricted TCR repertoires are consistent with there being an initial preferential activation of $CD4^+$ T cells in vivo (35–37). However, there is a lag in the development of the phenotype and expansion is primarily of $CD4^+$ MHC class II–restricted TCR Tg^+ and T cells expressing endogenous TCR chains (36, 38). In mice that also bear the *rag*-$1^{-/-}$ mutation, T cell activation is delayed longer, and there is no dramatic lymphoproliferation (39). This result is on first glance not consistent with the threshold scenario. However, it is unclear whether this effect is due to defects in the RAG-$1^{-/-}$ mice, such as alterations in the splenic and lymph node architecture. Finally, the threshold model would predict that an alternative source of a dominant inhibitory signal should rescue the phenotype. Consistent with this, Ly49A expression on CTLA-$4^{-/-}$ T cells delays and reduces lymphoproliferation and T cell activation in the presence of the appropriate MHC class I allele (98).

Attenuation Model

The scenarios presented above would occur primarily when T cells are weakly stimulated under conditions of limited B7. The dynamic integration model holds that when B7 levels are high, CD28-mediated costimulation overwhelms the inhibitory effects of low levels of CTLA-4, allowing T cell activation to proceed. However, CTLA-4 can have an effect on regulating early T cell responses primed by activated APCs when B7 levels are high.

Regulation of T cell responses primed under inflammatory conditions by CTLA-4 has been demonstrated in in vivo systems using anti-CTLA-4 antibodies or Fab fragments to disrupt CTLA-4/B7 interactions. The first report employed an adoptive transfer system to study a monoclonal $CD4^+$ T cell response primed with the agonist ligand in adjuvant (20). Blockade of CTLA-4 resulted in increased numbers of transferred cells at the peak of the response. The kinetics of expansion and contraction were similar between anti-CTLA-4 and control treated animals, suggesting that CTLA-4 may have affected the expansive phase but did not affect termination of the response. This study did not distinguish between effects on the proliferative capacity or the frequency of responders in the transferred pool, or both. Nevertheless, the data demonstrated a role for CTLA-4 in regulating the magnitude of a T cell response to a strong agonist signal under inflammatory conditions.

CTLA-4 also regulates antigen-specific polyclonal T cell responses. Experimental autoimmune encephalomyelitis (EAE), a murine model system of multiple sclerosis (MS), has been widely used for these studies. EAE is induced in susceptible strains by immunization of animals with myelin sheath-derived proteins or peptide antigens in adjuvant. This primes autoreactive $CD4^+$ T cells that differentiate to a Th1 phenotype and mediate demyelination. Blockade of CTLA-4 enhances disease (99–101) and is associated with an increased frequency of inflammatory lesions in the CNS (101), enhanced secretion of pro-inflammatory cytokines (99, 100), and increased proliferative responses to antigen stimulation in vitro (99). CTLA-4 also plays a critical role in regulating relapsing EAE mediated by T cell responses to myelin epitopes distinct from the disease-inducing epitope (99, 102). This process of relapse by epitope spreading was enhanced if CTLA-4 was blocked during the initial priming or the acute phase of disease. Enhanced relapse was also associated with increased in vitro proliferation to the relapse-associated antigens (102).

Blockade of CTLA-4 in tumor models has provided additional evidence for a role of CTLA-4 in attenuation of antigen-specific polyclonal T cell responses. As is discussed in more detail below, rejection of weakly immunogenic tumors can be accomplished by concomitant administration of anti-CTLA-4 along with tumor vaccines expressing GM-CSF, presumably by enhancing T cell cross-priming by activated host APC (103–105). Interestingly, anti-CTLA-4/tumor vaccine treatment resulted in tissue-specific autoimmunity in the melanoma and prostate systems (104, 105). CTLA-4 blockade also enhances $CD8^+$ T cell responses primed in vivo by GM-CSF activated peptide-pulsed dendritic cells, even in the absence of helper T cells (106).

The mechanisms by which CTLA-4 regulates polyclonal T cell responses in the systems discussed above are likely to be complex. In the context of the integrated three signal model, if the potential for CD28/B7 interactions is not limiting, the remaining critical parameters for the activation of any given antigen-specific T cell are: (*a*) the quantity of TCR signals, which is a function largely of peptide/MHC (pMHC) density on the APC; (*b*) the quality of TCR signals, which is a function of

the affinity and duration of TCR/pMHC interactions; and (*c*) the level of expression and, perhaps more importantly, the cellular localization of CTLA-4.

It has been speculated that the threshold mechanism may also apply to responses primed by activated APC (101, 102, 104). If so, CTLA-4 most likely sets a threshold for the quantity and/or quality of TCR signals necessary for T cell activation and would affect clonal representation in the response by lowering the number of T cells bearing low-affinity TCRs. Thus, the enhanced T cell responses observed in these systems would be as a result of increased recruitment of T cells bearing low-affinity TCRs into the responding pool.

An alternative model is that CTLA-4 preferentially attenuates the expansion of T cells that have been strongly acivated (197). This notion is supported by the observation that proliferation of naïve $CD4^+$ TCR transgenic T cells stimulated by agonist peptide is most tightly regulated by CTLA-4 at high ligand density (37). Additionally it has been suggested that CTLA-4 preferentially regulates T cells bearing TCRs that bind pMHC with high stability/affinity (107). CTLA-4 would prevent this high-affinity population from dominating the primed pool by restricting proliferation early in the response. Engagement of CTLA-4 would then serve to broaden the pool of T cells by limiting clonal representation of the high-affinity population, thus allowing more equal representation of the cells bearing lower affinity TCRs in the early stages of the clonal evolution of the response.

Predictions of the threshold and attenuation models were tested in two in vivo systems under conditions where costimulation by B7 would not be limiting. Adoptive transfer experiments using $CD4^+$ TCR transgenic T cells were performed to examine the effects of CTLA-4-blockade on the proliferative capacity, or average number of daughter cells per responder, for cells primed with agonist or weak agonist peptides in adjuvant. Blockade significantly increased the proliferative capacity of T cells primed with the agonist ligand but had a minimal effect on the cells responding to the weak agonist peptide. In normal, unmanipulated mice, blockade of CTLA-4 increased not only the frequency, but also the overall affinity of a polyclonal population of antigen-specific $CD4^+$ T cells primed with peptide in adjuvant (MS Kuhns, PA Savage, JG Egen, MM Davis, JA Allison, manuscript in preparation). These results suggest that CTLA-4 preferentially regulates the proliferation of T cells receiving strong agonist signals.

A variety of mechanisms could explain the ability of CTLA-4 to preferentially act on the high-affinity population of T cell responders. The most straightforward of these mechanisms would be that CTLA-4 expression is upregulated to highest levels in those cells receiving the strongest TCR signal. While differential expression of CTLA-4 probably does impact a T cell response, there is no evidence that the absolute level of CTLA-4 expression correlates with function (108).

Another, more subtle mechanism that could explain preferential regulation of the high-affinity T cells by CTLA-4 would be a correlation between strength of TCR signal and the extent of CTLA-4 localization to sites of T cell contact with APC. Thus, a certain strength of TCR signal would be needed to achieve localization of critical levels of CTLA-4 at the immunological synapse. As

previously discussed, the majority of intracellular CTLA-4 polarizes toward sites facing T cell contact (65). Recent studies indicate that this polarization can occur under conditions where the T cell receives either agonist or weak agonist signals. This is not an unexpected result, given that any TCR signal sufficient for MTOC reorganization will result in relocalization of associated intracellular membrane compartments (66). However, localization of CTLA-4 to the T cell–APC interface was not directly related to the polarization of intracellular stores of CTLA-4. Significantly, translocation of CTLA-4 to the immunological synapse primarily occurred when previously activated T cells were stimulated by strong agonist peptide, and to a much lesser extent when stimulated by weak agonist peptide. CD28 localization was comparable in response to both stimuli (JG Egen, JA Allison, manuscript in preparation). Thus, it appears that translocation of CTLA-4 into the synapse correlates with the strength of TCR signals.

The dynamic integration model of T cell activation originally proposed that T cell activation upregulated CTLA-4, which would then terminate the response when antigen was encountered again. As this model evolves in light of new data, it appears that increased CTLA-4 expression upon activation modulates T cell responses differentially and might serve to limit the burst size of responding T cells. Overall, these results suggest that the quality of the TCR signal is critical to determining if and/or how dramatically CTLA-4 regulates the proliferative capacity of any antigen-specific clone selected from the T cell repertoire. While the TCR and CD28 might primarily determine the range of T cells responding to antigen, CTLA-4 would limit clonal representation of those T cells with high-affinityTCRs. As discussed below, this may have implications for the type and ultimate outcome of a T cell response primed under inflammatory conditions.

CTLA-4 IN T HELPER SUBSET DIFFERENTIATION

It has been proposed that CD28 directs Th2 differentiation (2), whereas CTLA-4 serves to counter CD28 by preventing Th2 differentiation and thus promoting Th1 differentiation (38). The primary evidence linking CTLA-4 to the prevention of Th2 differentiation is that $CD4^+$ TCR Tg^+ CTLA-$4^{-/-}$ T cells produced IL-4, while CTLA-4 expressing littermate control cells produced IFN-γ upon secondary in vitro stimulation (38). Similarly, IL-4 was produced during secondary stimulation of $CD4^+$ T cells if CTLA-4 had been blocked by anti-CTLA-4 Fab fragments during priming, but production was limited if anti-CTLA-4 antibodies were used to cross-link CTLA-4 (109). In addition, blockade of CTLA-4 during activation by SEB in vivo resulted in enhanced Th2 responses (110). Likewise, Th2 responses to *Nippostrongylus brasiliensis* were enhanced by CTLA-4 blockade (111).

In contrast, blockade of CTLA-4 facilitated resistance to *Leishmania major* in susceptible Balb/c mice by enhancing Th1 responses (112). Th1 responses to the model antigen KLH were also enhanced by CTLA-4 blockade during priming, at least at a population level (113). Finally, as discussed above, CTLA-4 blockade enhanced Th1-mediated disease in EAE models (99, 100). Together, these data

suggest that CTLA-4 engagement limits Th1 differentiation. The opposing results obtained in the different systems suggest that there is no inherent capacity for CTLA-4 to regulate Th1 vs. Th2 differentiation.

There are other ways in which CTLA-4 might influence the T helper responses. It has been suggested that the strength of TCR and costimulatory signals can have an effect on cytokine polarization (2, 114). CTLA-4 might influence polarization by affecting the overall strength of the integrated signals. It is also likely that CTLA-4 can influence the development of Th responses not at the single cell, but at the population level. A primed polyclonal $CD4^+$ T cell population contains mixed subsets of cells with different T helper functions and the relative ratios of these cells may dictate the overall T helper response. An analysis of cytokine production at the single cell level in an EAE system, for example, revealed that both IFN-γ and IL-4-producing cells were present in primed mice (107). The frequency of both subsets increased upon CTLA-4 blockade, but the IFN-γ-producing population was increased to a larger extent. These changes in the size of the primed population and T helper ratios corresponded with exacerbated disease under normal EAE-inducing conditions and amelioration of disease antagonism by an altered peptide ligand. These data argue that the disease differences upon CTLA-4 blockade occurred because of a shift in the relative proportions of T helper subsets in the population. Thus, CTLA-4 may not serve to directly regulate T helper differentiation but may shift the response at the level of the T cell population.

Additionally, CTLA-4 could regulate the response of a specific T helper subset after differentiation. However, both Th1 and Th2 cells express CTLA-4, and CTLA-4 engagement is known to inhibit the responses of both types of clones (108, 115). Clearly, regulation of T helper responses is complex. Altogether the data suggest that the regulation of Th1 or Th2 responses by CTLA-4 is dependent on the context under which T cell responses occur.

CTLA-4 AND PERIPHERAL TOLERANCE

The role of CD28 costimulation in the maintenance and loss of tolerance has been reviewed in (2). Recent data suggests that CTLA-4 /B7 interactions may also be very important in tolerance. This has been most effectively demonstrated in models of autoimmunity, graft rejection, and tumor rejection. One possible mechanism, the raising of the threshold of activation when B7 levels are limiting, has been discussed above. Additional mechanisms have been proposed, including a direct role in regulating T cell anergy, an indirect effect via the costimulation of secretion of inhibitory cytokines, and finally direct mediation of the suppressive activity of immunoregulatory T cells.

Anergy

The original characterization of the phenomenon of costimulation demonstrated that T cell clones activated solely by TCR engagement became anergic. The discovery that there was a second ligand for B7 molecules that could inhibit T cell

activation raised the possibility that CTLA-4/B7 interactions may play a role in the induction of anergy in the presence of low B7. The requirement of CTLA-4/B7 interactions for anergy induction has been examined in a number of experimental models. The first study to examine this used adoptive transfer of $CD4^+$ T cells from OVA-specific DO11.10 TCR transgenic mice (116). Blockade of CTLA-4 prevented the induction of hyporesponsiveness when antigen was administered in tolerigenic (i.e. weak adjuvant) conditions. This was taken as evidence that engagement of the TCR when CTLA-4 could not be ligated was a neutral event and that CTLA-4 engagement was required for the induction of nonresponsiveness. Similar results were obtained when the peptide was administered intravenously, also shown to be a strongly tolerigenic route (117). A role for CTLA-4 ligation in superantigen-induced tolerance in vivo has also been reported (110). Finally, a requirement for CD4 was reported in the induction of T cell nonresponsiveness by CTLA in an in vitro system (41). However, in other systems CTLA-4 was not required for induction of hyporesponsiveness. In a study using the DO11.10 adoptive transfer system that examined the induction of anergy by intranasal administration of soluble peptide, CTLA-4 blockade had no effect (118). It has also been reported that stimulation of CTLA-$4^{-/-}$ T cells with anti-CD3 still resulted in anergy (119).

Overall, there may be a role for CTLA-4 in the induction of T cell anergy, but the data are obviously mixed. The experimental approaches used to examine the issue are very complicated and often suffer from an unclear definition of anergy. The line between hyporesponsiveness and anergy is often a quantitative rather than qualitative one, and results are often difficult to interpret.

CTLA-4 in the Induction of Inhibitory Cytokines

It has been reported that cross-linking of CTLA-4 may enhance production of TGFβ by activated T cells (48, 120). This raises the possibility that CTLA-4 does not directly inhibit T cell activation but does so by the active induction of this inhibitory cytokine. One observation taken as evidence for an indirect role for CTLA-4 in the inhibition of T cell responses was the failure of mixed bone marrow chimeric mice generated with CTLA-$4^{-/-}$ and wild-type bone marrow to develop a phenotype equivalent to the *ctla-4* null mice (121). It was proposed that there is no primary defect in CTLA-$4^{-/-}$ T cells but that the CTLA-$4^{-/-}$ phenotype is due to a failure of T cells to secrete inhibitory cytokines such as TGFβ.

Although the lack of an intrinsic defect is the most simplistic interpretation for the failure of mixed bone marrow chimeric mice to develop a phenotype resembling that of CTLA-$4^{-/-}$ mice, the results were clearly more complicated (121). The fact that mice reconstituted entirely with CTLA-$4^{-/-}$ bone marrow often failed to develop organ infiltration, and none showed signs of lymphoproliferative disease, although all eventually died, indicates that the situation is more complex. An alternative possibility is that there is an intrinsic defect in the cells that can be regulated by extrinsic factors. Still, the basis for the observed differences between

the mixed bone marrow radiation chimeras and CTLA-4$^{-/-}$ mice are intriguing and need to be resolved.

Recent studies showed that CTLA-4 cross-linking resulted in the inhibition of proliferation of T cells from TGFβ $^{-/-}$ mice or of T cells from mice lacking Smad3, a critical downstream signaling molecule in the TGFβ pathway (122; and J Letterio, TJ Sullivan, CA Chambers, A van Elsas, JP Allison, manuscript in preparation). This suggests that neither TGFβ production nor its signaling pathway is required for CTLA-4-mediated inhibition of T cell responses. These studies also failed to show a role for CTLA-4 in regulating TGFβ production, since it was produced by CTLA-4 $^{-/-}$ T cells. Finally, CTLA-4 ligation failed to induce production of TGFβ by normal naïve T cells (122).

CTLA-4 and Regulatory T cells

Over the last several years, a considerable literature has documented a role for CD25$^+$CD45RB^{low}CD4$^+$ regulatory T cells (Treg) in the maintenance of peripheral tolerance to organ-specific self-antigens (123). The observation that these cells constitutively express CTLA-4 has raised the possibility that CTLA-4 may be directly involved in their function (42, 124–126). One interesting characteristic of Treg cells is a failure to secrete IL-2 or to proliferate in response to ligation of the TCR and CD28, despite a requirement of CD28 for their generation and homeostasis (124). The hyporesponsiveness might be attributed to the inhibitory properties of CTLA-4. However, to date, attempts to release the block on proliferation in response to TCR engagement by CTLA-4 blockade have not been successful (125, 127; B Metzler, JP Allison, unpublished data).

Perhaps a more relevant issue is the role of CTLA-4 in the suppressive function of these cells. Here the results are mixed. Administration of either anti-CTLA-4 antibodies or anti-TGFβ reversed the inhibitory effects of transferred CD25$^+$ Treg cells on the induction of colitis by transferred CD4$^+$ CD25$^-$ cells in SCID mice (126). This was taken as evidence for a blocking of the suppressive effects of Treg cells by preventing CTLA-4-mediated induction of TGFβ production. However, the results are correlative and do not exclude the possibility that the effect of anti-CTLA-4 was a result of enhancement of the effector T cells. In a separate study, depletion of CD25$^+$ Tregs or anti-CTLA-4 treatment alone failed to induced colitis in normal Balb/c mice. However, colitis was induced when CD25$^+$ Tregs were depleted and CTLA-4 was blocked (125). Some of the data in this report may in fact suggest that CTLA-4 and CD25$^+$ Tregs represent independent but complementary mechanisms of maintaining peripheral tolerance. For example, CD25$^+$CD4$^+$ T cells from CTLA-4$^{-/-}$ mice retained inhibitory activity in in vitro inhibition assays. This would suggest that CTLA-4 expression was not required for the function of CD25$^+$CD4$^+$ Treg cells.

A role for CTLA-4 in the function of Treg cells is also not supported by recent findings in a tumor immunotherapy system (128). Depletion of either CD4$^+$ T cells or CD25$^+$ T cells improved the effectiveness of a GM-CSF-producing

tumor cell vaccine in the B16 melanoma system. This finding confirms a role for Treg cells in partially suppressing the T cell response in this system. However, depletion of $CD4^+$ T cells or $CD25^+$ cells also enhances, rather than diminishes, the effectiveness of anti-CTLA-4 in inducing both tumor rejection and the increase in T cells specific for a D^b-restricted peptide derived from the *trp-2* gene product. The effectiveness of CTLA-4 blockade was not reduced, but rather was considerably enhanced, by the removal of $CD25^+$ T cells, strongly suggesting that CTLA-4 is not directly involved in the function of Treg cells.

On balance, the evidence for a direct role of CTLA-4 in the function of Treg cells is indirect and not strongly supported by all results. It seems likely that CTLA-4 and Treg cells are independent mechanisms of regulating responses to self-antigen. This is an area that clearly needs further work.

MANIPULATION OF COSTIMULATORY SIGNALS IN TUMOR IMMUNOTHERAPY

The idea that one of the major roles of the immune system is to provide protection against constantly arising tumors is an old one, proposed by Ehrlich almost a century ago. While the experimental evidence for tumor immunosurveillance is mixed, considerable data support the notion that tumor cells can express antigens potentially capable of eliciting T cell responses. It is also clear that these responses may be largely ineffective. Still, the prospect of manipulating the immune system to obtain tumor rejection and protection against metastasis and recurrence is an attractive therapeutic approach, especially given the alternatives.

One of the reasons for the poor immunogenicity of tumors is a failure to express costimulatory ligands. Thus, despite expression of relevant antigens, a tumor may be invisible to the immune system until T cells are alerted by cross-priming by professional APC. The importance of costimulation to tumor immune responses was shown by the fact that in many cases introduction of B7 expression to tumor cells was sufficient not only to result in a rejection, but also to induce prophylactic immunity. This work demonstrated the relevance of costimulation to tumor immunity and offered a new strategy for immunotherapy. However, this approach was limited to application to inherently immunogenic tumors. And, while capable of inducing prophylactic immunity, $B7^+$ tumor cell vaccines were generally of limited effectiveness in treating established tumors (for review, see 129).

Given the demonstrated ability of CTLA-4 blockade to enhance T cell responses in a variety of settings, it was reasonable to determine whether it could also enhance anti-tumor responses. The importance of costimulation provided by host APC to anti-tumor responses was confirmed in experiments showing that the growth of relatively immunogenic transplanted tumors was accelerated by blockade of costimulation with anti- CD28 antibodies (130). CTLA-4 blockade, on the other hand, has been shown to lead to rejection of a number of immunogenic transplanted tumor cell lines, including colorectal carcinoma, renal carcinoma, lymphoma, and

fibrocarcoma cell lines (131–134). In some experiments, administration of anti-CTLA-4 resulted in rejection even when treatment was delayed until weeks after tumor implantation and when there were sizable tumor masses (130). These results demonstrated that weak responses elicited by tumors could be converted into potent responses sufficient for tumor rejection by removal of the inhibitory effects of CTLA-4. In other tumor systems, CTLA-4 blockade was effective if given only during early stages of tumor growth (131). In some, but not all tumor models, CTLA-4 blockade was capable of reversing tumor-induced T cell hyporesponsiveness (133, 134).

While this work clearly demonstrated the potential of this approach as a strategy for tumor immunotherapy, CTLA-4 blockade by itself was not effective against all experimental tumors—susceptibility appears to correlate with the inherent immunogenicity of the tumor (for review, see 132). This led to an examination of the effectiveness of CTLA-4 blockade in combination with other strategies for therapy of poorly immunogenic tumors. One approach that appeared to be complementary to CTLA-4 blockade was the use of irradiated tumor cell vaccines engineered to express the cytokine GM-CSF (135). GM-CSF tumor cell vaccines were effective in stimulating responses to the poorly immunogenic melanoma B16 by a mechanism that involved enhancement of cross-priming by host APC (136). While effective in prophylaxis, the GM-CSF tumor cell vaccine by itself was not sufficient to obtain rejection of established tumors. When combined with CTLA-4 blockade, however, GM-CSF-producing tumor cell vaccines were effective in inducing rejection of the B16 melanoma and another poorly immunogenic tumor, mammary carcinoma SM-1 (103, 104). Similarly, CTLA-4 blockade in combination with a GM-CSF-vaccine has been effective in delaying and reducing the severity of primary adenocarcinoma in the TRAMP system, a transgenic model of prostate cancer (105). In each of these systems, therapeutic effectiveness is lost if initiated too late, which may be a result of the tumor passing a critical mass or inactivation of potentially reactive T cells. However, the potent synergy observed in these systems clearly demonstrates the potential of CTLA-4 as part of a combinatorial approach to tumor immunotherapy.

Use of CTLA-4 blockade in combinatorial strategies is not limited to conventional immunomodulators. Anti-CTLA-4 can synergize with low doses of chemotherapeutic drugs to achieve tumor rejection (137). CTLA-4 blockade initiated following surgical resection of transplanted prostatic adenocarcinoma at the primary site reduces the incidence of metastases in the draining lymph nodes (138).

Studies of the cellular requirements for the therapeutic effect of CTLA-4 blockade have provided insight into the mechanisms involved. The response elicited by the GM-CSF B16 cell vaccine is absolutely dependent on $CD4^+$ T cells (139). While $CD8^+$ T cells are involved in the response, their contribution is minimal. Tumor protection requires Th2 cytokines, and the effector cells for rejection of the tumor cells, which do not express MHC class II gene products, are probably eosinophils (139). In the combinatorial treatment with anti-CTLA-4, however, $CD4^+$ cells are totally dispensable—only $CD8^+$ T cells and $NK1.1^+$ cells are

required (104). Indeed, the anti-tumor effect is even more vigorous in the absence of $CD4^+$ T cells. This finding has two important implications. The first is that CTLA-4 blockade may lower the threshold for activation of $CD8^+$ T cells by host APC, perhaps by eliminating the requirement for licensing by CD40L or other $CD4^+$ T cell–dependent mechanisms. Second, as discussed in more detail above, the mechanism by which CTLA-4 blockade enhances anti-tumor responses is at the level of the effector cell, and not inhibition of the suppressive effects of Treg cells.

An interesting consequence of the induction of tumor immunity in the B16 and TRAMP models is the development of tissue-specific autoimmunity. Mice that have rejected the B16 melanoma develop a progressive depigmentation as a result of elimination of normal melanocytes (104). This depigmentation has never been observed in mice treated with the GM-CSF producing B16 cell vaccine alone. Similarly, immunization of mice with the GM-CSF-producing TRAMP tumor cell line together with anti-CTLA-4 results in the development of autoimmune prostatitis, but not depigmentation. Together, these results suggest that the anti-tumor response enhanced by CTLA-4 blockade is in large part directed against normal tissue-derived gene products, rather than tumor-specific antigens. In support of this idea, the main target of T cells from mice cured of melanoma is a normal, unmutated peptide derived from the trp-2 gene, which is expressed in normal melanoma (A van Elsas, J Ziskin, N Restif, and JP Allison, unpublished results).

These findings suggest that the anti-tumor effect of CTLA-4 blockade in these models is a consequence of removal of constraints on T cells directed against normal tissue antigens, allowing the elaboration of a response that eliminates tumor cells on the basis of their tissue of origin. This has two important implications. First, this approach to therapy will not require knowledge of expression of individually specific tumor antigens and may be more generally useful in the clinic. However, this same feature might limit the usefulness of the approach to the treatment of tumors of nonessential tissues. Even with this limitation, the list would include many prevalent cancers, such as melanoma, prostate, testicular, ovarian, and mammary tumors.

SUMMARY AND CONCLUSION

Recent advances in our understanding of the mechanisms involved in costimulation have led to an appreciation of the importance of inhibition by CTLA-4 in the regulation of early stages of the T cell response. Although controversial areas require further inquiry for resolution, significant advances have been made in our understanding of CTLA-4 activity at the molecular, single cell, and population levels. Results demonstrating the effectiveness of CTLA-4 blockade in enhancing anti-tumor responses in experimental systems offer the exciting possibility of translating our basic knowledge of costimulatory regulation into new strategies for tumor therapy.

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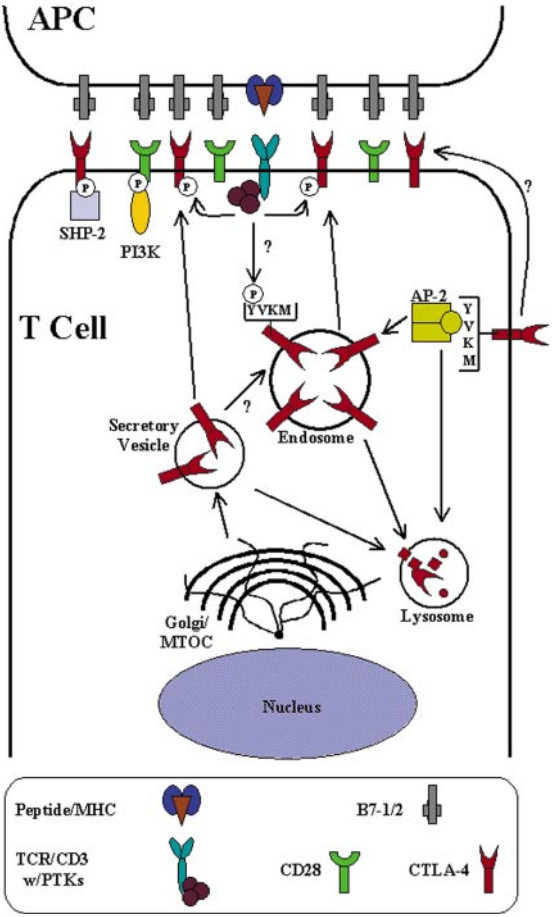

Figure 1 Protein trafficking of CTLA-4 in T cells. Newly translated CTLA-4 emerging from the Golgi apparatus in secretory vesicles may traffic to endosomal compartments or directly to the cell surface. Upon TCR stimulation, the Microtubule Organizing Center (MTOC) relocates to a site facing the APC, thereby polarizing associated compartments such as endosomes and the Golgi apparatus. This allows for directed secretion of CTLA-4 to the T cell-APC interface. Once at the interface the intracellular localization motif of CTLA-4 may be phosphorylated by protein tyrosine kinases (PTKs) associated with the TCR, resulting in surface-stabilization of CTLA-4 by inhibiting association with the clathrin-coated pit adaptor protein AP-2. This phosphorylated CTLA-4 may then interact with SH2 domain containing proteins such as the phosphatase SHP-2 and/or physically disrupt the assembly of molecules at the immunological synapse. Alternatively, unphosphorylated CTLA-4 will associate with AP-2 and enter the endosomal pathway where it may recycle back to the T cell surface or traffic to lysosomes for degradation. Further complexities of CTLA-4 trafficking are shown.

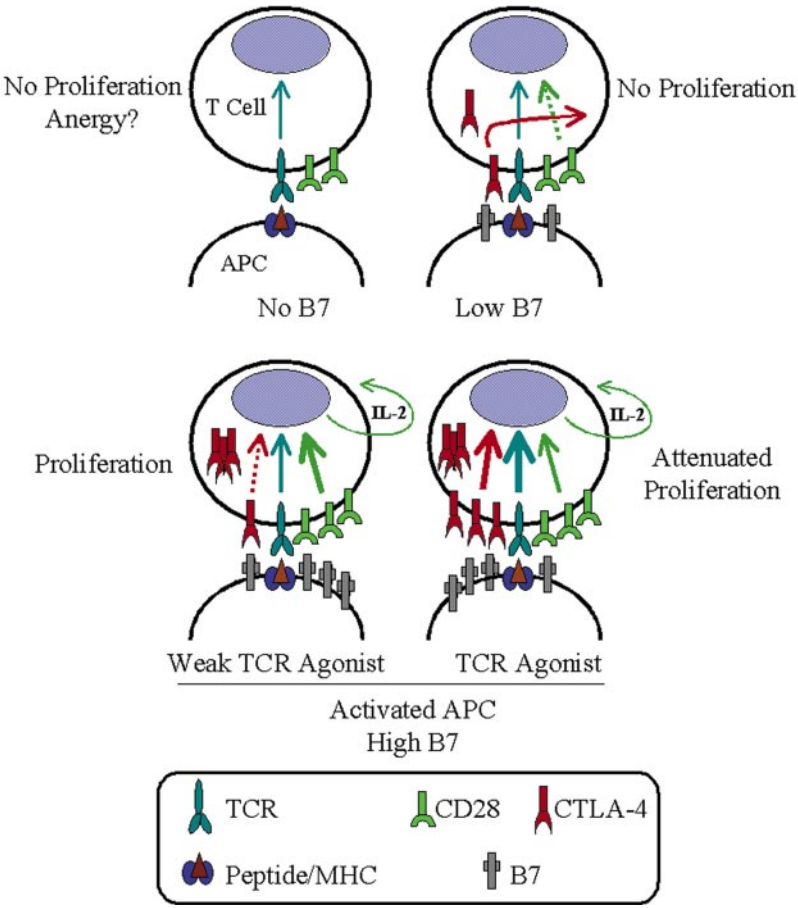

Figure 2 Dynamic integration of TCR and costimulatory signals. The contribution of signals generated by the T cell antigen receptor (TCR), CD28, and CTLA-4 depends on the activation state of the T cell as well as the nature and activation state of the antigen presenting cell (APC). TCR engagement in the absence of B7 expression by an APC, does not result in proliferation and may induce anergy. However, if the T cell encounters an APC expressing low levels of B7, rapidly induced CTLA-4 can compete with CD28 for B7 binding and/or deliver inhibitory signals which prevent T cell activation. Under conditions where the APC is activated and expressing high levels of B7, CD28 costimulation may dominate and activation proceeds. However, this results in induction and/or mobilization of CTLA-4 that may be proportional to the strength of the TCR signal, resulting in differential inhibition. Importantly, the proliferation of T cells receiving agonist signals may still be greater than cells receiving weak agonist signals due to differences in the ability of these ligands to induce T cell activation. Therefore, TCR signals may directly regulate the balance between activating and inhibitory costimulatory signals thereby determining the outcome of T cell interactions with APC.