

try and 10% of whom were of other ethnic backgrounds. The stop codon mutation was screened in 70 Finnish EPM1 carrier parents. All 70 of these individuals contained the common ancestral haplotype around the EPM1 locus on one of their chromosomes. To distinguish mutations from polymorphisms, we considered only the nonancestral haplotype chromosome of these 70 individuals. DNA from these individuals was amplified by PCR, and the products were directly sequenced with the Ampli-Cycle sequencing kit (Perkin-Elmer).

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Enhancement of Antitumor Immunity by CTLA-4 Blockade

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One reason for the poor immunogenicity of many tumors may be that they cannot provide signals for CD28-mediated costimulation necessary to fully activate T cells. It has recently become apparent that CTLA-4, a second counterreceptor for the B7 family of costimulatory molecules, is a negative regulator of T cell activation. Here, *in vivo* administration of antibodies to CTLA-4 resulted in the rejection of tumors, including preestablished tumors. Furthermore, this rejection resulted in immunity to a secondary exposure to tumor cells. These results suggest that blockade of the inhibitory effects of CTLA-4 can allow for, and potentiate, effective immune responses against tumor cells.

Despite expressing antigens recognizable by a host's immune system, tumors are very poor in initiating effective immune responses. One reason for this poor immunogenicity may be that the presentation of antigen alone is insufficient to activate T cells. In addition to T cell receptor engagement of an antigenic peptide bound to major histocompatibility complex (MHC) molecules, additional costimulatory signals are necessary for T cell activation (1). The most important of these costimulatory signals appears to be provided by the interaction of CD28 on T cells with its primary ligands B7-1 (CD80) and B7-2 (CD86) on the surface of specialized antigen-presenting cells (APCs) (2-4). Expression of B7 costimulatory molecules is limited to specialized APCs. Therefore, even though most tissue-derived tumors may present antigen in the context of MHC molecules, they may fail to elicit effective immunity because of a lack of costimulatory ability. Several studies support this notion. In a variety of model systems, transfected tumor cells expressing costimulatory B7 molecules induced potent responses against both modified and unmodified tumor cells (5-8). It appears that

tumor cells transfected with B7 are able to behave as APCs, presumably allowing direct activation of tumor-specific T cells.

Recent evidence suggests that costimulation is more complex than originally thought and involves competing stimulatory and inhibitory signaling events (3, 9-12). CTLA-4, a homolog of CD28, binds both B7-1 and B7-2 with affinities much greater than does CD28 (13-16). *In vitro*, antibody cross-linking of CTLA-4 has been shown to inhibit T cell proliferation and interleukin-2 production induced by antibody to CD3 (anti-CD3), whereas blockade of CTLA-4 with soluble intact or Fab fragments of antibody enhances proliferative responses (17, 18). Similarly, soluble intact or Fab fragments of anti-CTLA-4 greatly augment T cell responses to nominal peptide antigen or the superantigen *Staphylococcus enterotoxin B* *in vivo* (19, 20). It has also been suggested that CTLA-4 engagement can induce apoptosis in activated T cells (21). Finally, mice deficient in CTLA-4 exhibit severe T cell proliferative disorders (22). These results demonstrate that CTLA-4 is a negative regulator of T cell responses and raise the possibility that blockade of inhibitory signals delivered by CTLA-4-B7 interactions might augment T cell responses to tumor cells and enhance antitumor immunity.

We first sought to determine whether

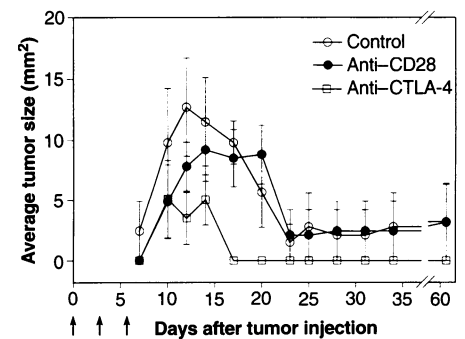


Fig. 1. Treatment with anti-CTLA-4 accelerates rejection of a B7-1-positive colon carcinoma (23). A volume of 100 μ l of cell suspension (4×10^6 cells) was injected subcutaneously into the left flanks of groups of five female BALB/c mice. Two of the groups received three intraperitoneal injections of either anti-CTLA-4 or anti-CD28 (18). Injections of 100, 50, and 50 μ g of antibody were given on days 0, 3, and 6, respectively, as indicated by the arrows. Control animals received no injections. Data points represent the average of the products of bisecting tumor diameters. Error bars represent standard error of the mean.

CTLA-4 blockade with nonstimulatory, bivalent antibody (18, 20) would accelerate rejection of B7-positive tumor cells. Previously, we showed that B7-1 expression was partially successful at inducing rejection of the transplantable murine colon carcinoma 51BLim10 (23). We reasoned that CTLA-4 blockade would remove inhibitory signals in the costimulatory pathway, resulting in enhanced rejection of the tumor cells. We injected groups of BALB/c mice with B7-1-transfected 51BLim10 tumor cells (B7-51BLim10) (23). Two groups were treated with a series of intraperitoneal injections of either anti-CTLA-4 or anti-CD28 (18, 24). Treatment with anti-CTLA-4 inhibited B7-51BLim10 tumor growth as compared with the anti-CD28-treated mice or the untreated controls (Fig. 1). All mice in the untreated and anti-CD28-treated groups developed small tumors that grew progressively for 5 to 10 days and then ultimately regressed in 8 of the 10 mice by about day 23 after injection. The two small tumors that did not regress remained static for more than 90 days. In contrast, three of five mice treated with anti-CTLA-4 developed very small tumors, all of which regressed completely by day 17. Although these results were encouraging and were consistent with our hypothesis, they were not very dramatic because B7-1 expression resulted in fairly rapid rejection of transfected 51BLim10 cells even in the absence of CTLA-4 blockade; however, these results confirmed that anti-CTLA-4 did not inhibit tumor rejection.

We next examined the effects of CTLA-4 blockade on the growth of V51BLim10, a vector control tumor cell line that does not express B7 (23). All mice either injected with 4×10^6 V51BLim10

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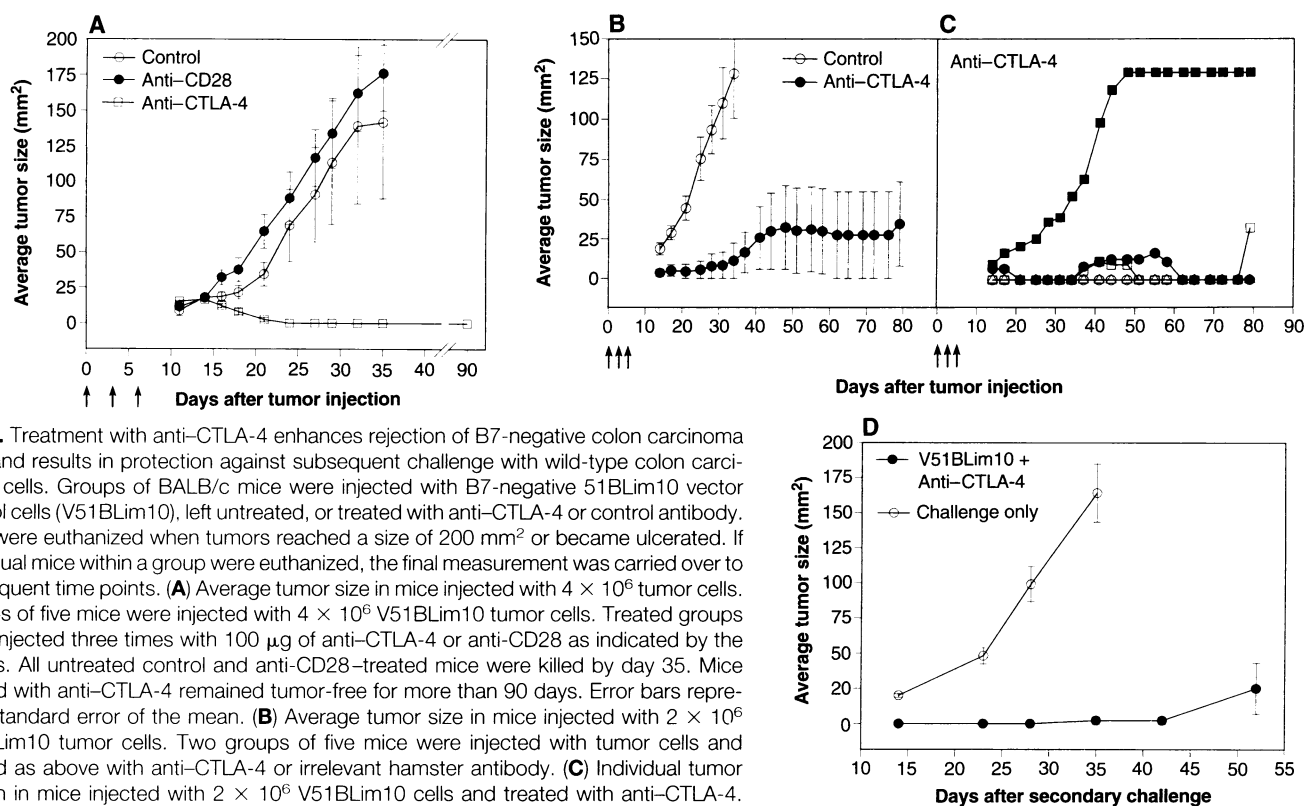


Fig. 2. Treatment with anti-CTLA-4 enhances rejection of B7-negative colon carcinoma cells and results in protection against subsequent challenge with wild-type colon carcinoma cells. Groups of BALB/c mice were injected with B7-negative 51BLim10 vector control cells (V51BLim10), left untreated, or treated with anti-CTLA-4 or control antibody. Mice were euthanized when tumors reached a size of 200 mm² or became ulcerated. If individual mice within a group were euthanized, the final measurement was carried over to subsequent time points. **(A)** Average tumor size in mice injected with 4×10^6 tumor cells. Groups of five mice were injected with 4×10^6 V51BLim10 tumor cells. Treated groups were injected three times with 100 μ g of anti-CTLA-4 or anti-CD28 as indicated by the arrows. All untreated control and anti-CD28-treated mice were killed by day 35. Mice treated with anti-CTLA-4 remained tumor-free for more than 90 days. Error bars represent standard error of the mean. **(B)** Average tumor size in mice injected with 2×10^6 V51BLim10 tumor cells. Two groups of five mice were injected with tumor cells and treated as above with anti-CTLA-4 or irrelevant hamster antibody. **(C)** Individual tumor growth in mice injected with 2×10^6 V51BLim10 cells and treated with anti-CTLA-4. Three of the mice remained tumor-free beyond 80 days. **(D)** Challenge tumor growth in anti-CTLA-4-treated mice. Five anti-CTLA-4-treated mice that had completely rejected V51BLim10 tumor cells were rechallenged 70 days later with 4×10^6 wild-type tumor cells injected subcutaneously in the opposite flank. Five naïve mice were also injected as controls. All control mice developed progressively growing tumors and were euthanized on day 35 after inoculation. Three of five previously immunized mice remained tumor-free 70 days after rechallenge.

tumor cells and left untreated, or treated with anti-CD28, developed progressively growing tumors and required euthanasia by 35 days after inoculation (Fig. 2A). In contrast, all mice treated with anti-CTLA-4 completely rejected their tumors after a short period of limited growth. Similarly, control mice injected with 2×10^6 tumor cells developed rapidly growing tumors and required euthanasia by day 35 (Fig. 2B). Anti-CTLA-4 treatment had a dramatic effect on tumor growth, but one mouse did develop a tumor quickly (accounting for a majority of the growth indicated in Fig. 2B) and another developed a tumor much later (Fig. 2C). Anti-CTLA-4 appeared to be less effective at a tumor dose of 1×10^6 cells, where treatment resulted in significantly reduced tumor growth rates, but four of five mice developed progressively growing tumors (25). Thus, although curative responses were not obtained in all cases, it is clear that CTLA-4 blockade significantly enhanced rejection of B7-negative tumor cells.

We next sought to determine whether tumor rejection as a consequence of CTLA-4 blockade was associated with enhanced immunity to a secondary challenge. Mice that had rejected V51BLim10 tumor cells as a result of treatment with anti-CTLA-4 were challenged with 4×10^6 wild-type 51BLim10 cells 70 days after their ini-

tial tumor injections. These mice showed significant protection against a secondary challenge as compared with naïve controls (Fig. 2D). All control animals had progressively growing tumors by 14 days after injection, and required euthanasia by day 35. Only one of the previously immunized mice had a detectable tumor by day 14, and growth of this tumor was very slow. Ultimately, two more tumors developed in the immunized mice 42 days after challenge. Two mice remained tumor-free throughout the course of the experiment. These results demonstrate that tumor rejection mediated by CTLA-4 blockade results in immunologic memory.

To determine whether anti-CTLA-4 treatment could have an effect on the growth of established tumors, we injected groups of mice with 2×10^6 wild-type 51BLim10 tumor cells and treated them with anti-CTLA-4 beginning on day 0 as before, or beginning 7 days later at which time most mice had palpable tumors. Mice treated with anti-CTLA-4 at either time period had significantly reduced tumor growth compared with untreated controls (Fig. 3). In fact, delaying treatment appeared to be more effective, with two of five mice remaining tumor-free beyond 30 days after inoculation.

The effects of anti-CTLA-4 treatment

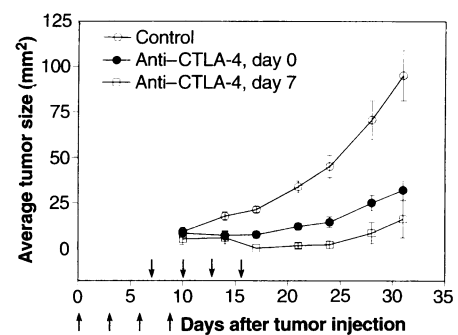


Fig. 3. Treatment with anti-CTLA-4 reduces the growth of established tumor. Groups of mice were injected subcutaneously with 2×10^6 51BLim10 tumor cells. Control animals ($n = 10$) were injected intraperitoneally with 100 μ g of irrelevant hamster antibody on days 0, 3, 6, and 9, as indicated by the upward-pointing arrows. One anti-CTLA-4 treatment group ($n = 10$) received intraperitoneal injections on the same days. The other treated mice ($n = 5$) were given intraperitoneal injections of anti-CTLA-4 beginning on day 7 and subsequently on days 10, 13, and 16 (downward-pointing arrows).

were not limited to variants of the murine colon carcinoma 51BLim10. Similar results were obtained with a rapidly growing fibrosarcoma of A/JCr mice, Sa1N (26) (Fig. 4). All control mice injected subcutaneously with 1×10^6 Sa1N cells developed measur-

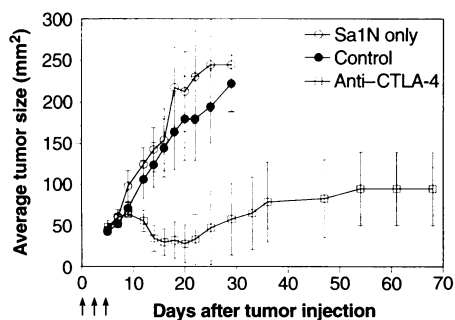


Fig. 4. Treatment with anti-CTLA-4 reduces the growth of the murine fibrosarcoma Sa1N. Groups of five mice were injected subcutaneously in the flank with a suspension of 1×10^6 Sa1N fibrosarcoma cells. Treated groups were injected intraperitoneally with 100 μ g of anti-CTLA-4 or irrelevant hamster control antibody at days 0, 3, and 6 as indicated by the arrows. All control animals were killed by day 30. Two of five animals treated with anti-CTLA-4 remained tumor-free at day 55.

able, rapidly growing tumors within 7 days, whereas only two mice treated with anti-CTLA-4 had tumors by day 30, and one additional mouse developed a tumor around day 40 after injection. The remaining mice were still tumor-free 70 days after injection. In another experiment, control mice injected with 4×10^5 Sa1N tumor cells also developed rapidly growing tumors, whereas 7 of 10 mice treated with anti-CTLA-4 were tumor-free by day 25 after injection (25).

Our results indicate that removing inhibitory signals in the costimulatory pathway can enhance antitumor immunity. Although it has been shown that anti-CTLA-4 interferes with signals that normally down-regulate T cell responses in vivo (17, 18), the exact mechanisms of antitumor immunity elicited by CTLA-4 blockade are not clear. In the case of B7-negative tumors, antigens are most likely transferred to and presented by host APCs (27), where CTLA-4 blockade might effect T cell responses in two nonexclusive ways. First, removal of inhibitory signals may lower the overall threshold of T cell activation and allow normally unreactive T cells to become activated. Alternatively, CTLA-4 blockade might sustain proliferation of activated T cells by removing inhibitory signals that would normally terminate the response, thus allowing for greater expansion of tumor-specific T cells.

Regardless of the mechanism, it is clear that CTLA-4 blockade enhances antitumor responses. Most importantly, we have observed these effects against unmanipulated, wild-type tumors. Current methods of enhancing antitumor immunity generally require the engineering of tumor cells (8). Some of these methods, such as the induction of B7 expression, rely on enhancing the costimulatory activity of the tumor cells

themselves. Others, such as engineering tumor cells to express MHC class II molecules (26, 28, 29) or to produce granulocyte-macrophage colony-stimulating factor (27, 30, 31) or pulsing dendritic cells with tumor antigen ex vivo (32, 33), seek to enhance antigen presentation, antigen transfer, or both. Thus, CTLA-4 blockade, by removing potentially competing inhibitory signals, may be a particularly useful adjunct to other therapeutic approaches involving the costimulatory pathway.

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Light-Induced Degradation of TIMELESS and Entrainment of the *Drosophila* Circadian Clock

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Two genes, *period* (*per*) and *timeless* (*tim*), are required for production of circadian rhythms in *Drosophila*. The proteins encoded by these genes (PER and TIM) physically interact, and the timing of their association and nuclear localization is believed to promote cycles of *per* and *tim* transcription through an autoregulatory feedback loop. Here it is shown that TIM protein may also couple this molecular pacemaker to the environment, because TIM is rapidly degraded after exposure to light. TIM accumulated rhythmically in nuclei of eyes and in pacemaker cells of the brain. The phase of these rhythms was differentially advanced or delayed by light pulses delivered at different times of day, corresponding with phase shifts induced in the behavioral rhythms.

Circadian rhythms, found in most eukaryotes and some prokaryotes (1), are ~24-hour rhythms governed by an internal clock that functions autonomously but can

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be entrained by environmental cycles of light or temperature. Circadian rhythms produced in constant darkness can also be reset by pulses of light. Such light pulses will shift the phase of the clock in different directions (advance or delay) and to a varying extent in a manner that depends on the time of light exposure (2).

In the fruit fly *Drosophila melanogaster*, two genes, *period* (3) and *timeless* (4), are

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