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Enhancement of Antitumor Immunity by CTLA-4 Blockade

Dana R. Leach, Matthew F. Krummel, James P. Allison*

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, transplanted 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 CTLA-4 blockade with nonspecific, 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 51Blml0 (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 51Blml0 tumor cells (B7-1Blml0) (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 B7-1Blml0 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 51Blml0 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 V51Blml0, a vector control tumor cell line that does not express B7 (23). All mice either injected with 4 × 10^6 V51Blml0
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 initial tumor injections. These mice showed significant protection against a secondary challenge as compared with naive controls (Fig. 2D). All control animals had progressively growing tumors by 14 days after injection, developed massive tumors, 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 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-
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<sup>5</sup> 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 affect T cell responses in 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.

**REFERENCES AND NOTES**

17. T. L. Walunas et al., ibid., p. 405.
24. na-corna cells were transfected with a plasmid construct containing the gene for murine B7-1 and (cloned by limit dilution). Fresh cultures of tumor cells were established from early passage frozen stocks and maintained in culture for no more than 30 days before use. Tumor cells were harvested by trypsinization from tissue culture plates, washed three times in ser-
25. um-free medium, and suspended at 4 × 10<sup>6</sup> cells per milliliter. Expression of B7-1 molecules on transfected cells was verified by flow cytometry before injection. V51BLim10 and wild-type 51BLim10 tumor cells do not express detectable amounts of B7-1, B7-2, or CTLA-4 as determined by flow cytometric analyses.
27. D. R. Leach, M. F. Krummel, J. P. Allison, data not shown.
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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 eye 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 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* (2) and *timeless* (4), are...