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Immunology

In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance

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ABSTRACT **The efficacy of therapeutic vaccination for the treatment of cancer is limited by peripheral tolerance to tumor antigens. *In vivo* blockade of CTLA-4, a negative regulator of T cell function, can induce the regression of established tumors and can augment the tumor rejection achieved through therapeutic vaccination. These outcomes may reflect enhanced tumor-specific T cell priming and/or interference with the development of tolerance to tumor antigens. We examined the effect of CTLA-4 blockade on the fate and function of T cells specific for a model tumor antigen in the tumor-bearing host. We found that while CTLA-4 blockade enhanced the priming of responsive T cells, it did not prevent the induction of tolerance to tumor antigens. These results demonstrate that there is a critical window in which the combination of CTLA-4 blockade and vaccination achieves an optimal response, and they point to mechanisms other than CTLA-4 engagement in mediating peripheral T cell tolerance to tumor antigens.**

A fundamental difference between prophylactic vaccines against infectious pathogens and therapeutic cancer vaccines is that in the latter case, an attempt is made to prime an immune response against antigens that have been expressed in host tissues long before vaccination occurs. In fact, the majority of human tumor antigens identified to date are not uniquely expressed by cancer cells, but rather are tissue-specific differentiation antigens that are also expressed by cells of the normal tissue from which the cancer originated (1). But even truly tumor-specific neo-antigens that arise as a consequence of mutation may be expressed by cancer cells for years before the malignancy becomes clinically detectable. Consequently, the functional T cell repertoire capable of responding to tumor antigens is likely to be shaped by the same mechanisms that limit autoimmune recognition of antigens expressed by normal tissues (2).

Using a T cell receptor (TCR) transgenic model, we previously demonstrated that $CD4^+$ T cells specific for a model tumor antigen are rendered tolerant early in the course of tumor progression (3). This tolerance was antigen-specific, and it significantly preceded the development of global immunosuppression that is sometimes associated with advanced tumor burdens (4).

One mechanism that may account for the development of tumor antigen-specific T cell tolerance is the delivery of inhibitory signals to T cells through the engagement of CTLA-4. CTLA-4 is a cell-surface receptor expressed by activated T cells that has homology to the T cell costimulatory molecule CD28 (5). Although CD28 and CTLA-4 are both ligands for B7-1 (CD80) and B7-2 (CD86) expressed on antigen-presenting cells, these molecules serve opposing roles in regulating T cell activation (6). CD28 engagement provides costimulatory signals required for T cell activation, whereas CTLA-4 engagement down-modulates T cell responses by raising the activation threshold required for T cell priming (7).

The treatment of tumor-bearing mice with anti-CTLA-4 antibody has been shown to induce T-cell-mediated tumor rejection when given early after a tumor is established (8) and to enhance the efficacy of vaccination with irradiated tumor cells engineered to produce granulocyte–macrophage colony-stimulating factor (GM-CSF) (9). However, the exact mechanism(s) involved in the antitumor responses elicited by CTLA-4 blockade remains to be elucidated. If tumor-specific T cell tolerance develops as a consequence of signaling through CTLA-4, blocking this pathway in the setting of an established tumor would leave a greater number of tumor-specific T cells capable of being primed. Alternatively, CTLA-4 engagement may not play a direct role in the development of T cell tolerance per se. Instead, tumor antigen-specific T cells that have escaped tolerance induction may be more effectively primed in conjunction with CTLA-4 blockade, either by lowering the threshold required for T cell activation and/or by undergoing a more sustained proliferation and expansion that lead to enhanced tumor rejection (8).

We have explored whether the *in vivo* blockade of CTLA-4 engagement prevents the development of tumor antigen-specific tolerance of $CD4^+$ T cells by using the model system described above. These studies demonstrate that anti-CTLA-4-treated tumor-bearing mice vaccinated early after the transfer of antigen-specific T cells—at a time when control antibody-treated mice had impaired responses, but were not yet fully tolerant—responded comparably to tumor-free mice treated in the same fashion. However, vaccination at later time-points demonstrated that tumor antigen-specific T cells from CTLA-4-treated mice followed the same fate as transgenic T cells from untreated tumor-bearing mice—i.e., they were fully unresponsive by all parameters examined. Taken together, these results indicate that CTLA-4 blockade does not prevent the induction of tolerance to tumor antigens, but it significantly enhances the response of those T cells not yet rendered tolerant. Importantly, a critical window exists in which the combination of CTLA-4 blockade and vaccination enhances the response of antigen-specific T cells.

MATERIALS AND METHODS

Mice. Six- to 8-week-old male BALB/c mice were obtained from the National Institutes of Health (Frederick, MD). Transgenic mice expressing an $\alpha\beta$ TCR specific for amino acids 110–120 from influenza virus hemagglutinin (HA) restricted by $I\text{-}E^d$ were a generous gift of Harald von Boehmer (10). These mice were crossed to mice of a BALB/c background for more

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Abbreviations: TCR, T cell receptor; HA peptide, hemagglutinin peptide, residues 110–120; vacc-HA, recombinant vaccinia virus encoding influenza virus hemagglutinin.

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than 10 generations. Transgenic mice used in these experiments were heterozygous for the transgene. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University.

Tumor Cells. A20 cells were obtained from the American Type Culture Collection (Manassas, VA). A20HA was generated by electroporation-mediated plasmid transfection, and transfected cells were selected and grown as previously reported (11).

Adoptive Transfer. Single-cell suspensions were made from peripheral lymph nodes and spleen harvested from transgenic donors. The percentage of lymphocytes double positive for CD4 and the clonotypic TCR was determined by flow cytometry as described below. Cells were washed in sterile Hanks' balanced salt solution (HBSS), and 2.5×10^6 $CD4^+$ anti-HA TCR^+ T cells were injected into the tail vein of male BALB/c recipients. A20HA cells used for *in vivo* tumor challenge were washed in sterile HBSS and injected via tail vein in a total volume of 0.2 ml, 1×10^6 tumor cells per mouse.

***In Vivo* Treatment with Anti-CTLA-4.** Systemic A20HA lymphoma was established in BALB/c mice as above. After either 18 days (large tumor burden) or 9 days (small tumor burden), transgenic T cells were transferred to these recipients or into tumor-free mice (day 0). On days −1, 0, and +1, a subgroup of these mice received intraperitoneal (i.p.) injections of 100 μg of anti-CTLA-4 antibody per day (mAb 9H10) or 100 μg of isotype control hamster IgG antibody per day (Jackson ImmunoResearch). A third subgroup of mice received no treatment. Half of the mice in each group received subcutaneous (s.c.) immunization with 1×10^7 plaque-forming units of a recombinant vaccinia virus encoding influenza virus hemagglutinin (vacc-HA) on the day indicated. Mice were sacrificed 6 days after immunization and T cells were purified and analyzed.

In Fig. 5, mice received 100 μg of purified mAb 9H10 per injection or isotype-control hamster IgG every 3 days, starting 1 day before the transfer of transgenic T cells (day −1) through day +14. Half of the mice in each group received vacc-HA (1×10^7 plaque-forming units) on day +9. All the animals were sacrificed on day +15, and clonotypic T cells were isolated and analyzed as described below.

Isolation of Clonotypic T Cells after *in Vivo* Transfer. Transgenic T cells previously injected into tumor-free mice or mice with established A20HA tumors were reisolated from the spleen on the days indicated. T cells were enriched by a combination of passage over nylon wool followed by complement lysis with the mAb J11.d.2, which is specific for heat-stable antigen (HSA) expressed by A20 tumor cells.

Flow Cytometric Analysis. T cells were stained with FITC-conjugated goat anti-mouse CD4 (Caltag) and biotinylated rat anti-clonotypic TCR antibody mAb 6.5 followed by phycoerythrin (PE)-conjugated streptavidin (Caltag). Fifty thousand gated events were collected on a FACScan (Becton Dickinson) and analyzed with CELLQUEST software (Becton Dickinson). Values represent the mean + SE of the percentage of cells expressing the clonotypic TCR. Background staining of splenocytes from naive BALB/c mice is usually less than 0.10%. Expression of CD45RB on clonotype$^+$ cells was determined by three-color flow cytometric analysis. Enriched T cells were stained with Cy-Chrome-labeled anti-mouse CD4 (PharMingen), biotinylated anti-TCR clonotype mAb 6.5 followed by PE-labeled streptavidin and FITC-conjugated anti-mouse CD45RB (PharMingen). Live gating on $CD4^+$ T cells was used to collect a total of 100,000 events.

Antigen-Specific Proliferation. T cells (4×10^4 per well) from the different experimental groups were mixed with fresh BALB/c splenocytes (8×10^4 per well) to which 12.5 μg/ml of HA peptide (amino acids 110–120, SFERFEIFPKE) was or was not added. The cells were pulsed with [^{3}H]thymidine (1 mCi per well, Amersham; 1 mCi = 37 kBq) after 3 days in culture. Cells were harvested 18 hr later with a Packard Micromate cell harvester. Thymidine incorporation into DNA was measured as cpm on a Packard Matrix 96 direct β counter. Data are displayed as cpm from which values for cells cultured in medium alone were subtracted. Background [^{3}H]thymidine incorporation for non-stimulated cells from tumor-free and tumor-bearing mice was similar under all the experimental conditions, averaging 2500–3000 cpm.

Cytokine Release. T cells purified and plated as above were cultured with medium alone or HA peptide (12.5 μg/ml) plus fresh BALB/c splenocytes. Forty-eight hours later, supernatants were collected and stored at −70°C until assayed for IL-2 and IFN-γ by ELISA (R&D Systems). Values are the mean + SE of triplicate cultures from three mice in each group. Values for T cells cultured in medium alone were less than 10% of the values for stimulated T cells.

Proliferative Response to Vaccinia Antigens. Normal BALB/c splenocytes were infected with wild-type vaccinia virus (3 plaque-forming units per cell) for 4 hr. Infected cells were washed three times and then cultured with purified T cells from the different experimental groups at a stimulator/responder ratio of 2:1. [^{3}H]Thymidine incorporation was determined after 3 days in culture. Values represent mean + SE of triplicate cultures.

***In Vivo* Priming with vacc-HA.** vacc-HA was prepared as previously described (3). Mice were primed by s.c. inoculation of 1×10^7 plaque-forming units of recombinant virus.

RESULTS

CTLA-4 Blockade Enhances the Magnitude of the Antigen-Specific T Cell Response to Immunization in Non-Tumor-Bearing Mice. We examined three determinants of T cell priming in response to immunization: clonal expansion of antigen-specific T cells, enhanced proliferation in response to the nominal peptide antigen *in vitro*, and acquisition of the capacity to produce IFN-γ upon stimulation with antigen *in vitro*. Analysis of non-tumor-bearing mice immunized with vacc-HA reveals clear evidence of T cell priming by each of these parameters (Fig. 1). As has been reported for other systems, treatment with antibody to CTLA-4 increased the magnitude of the response to vaccination. This effect is demonstrated by the increased clonal expansion of HA-specific $CD4^+$ T cells in response to vacc-HA plus CTLA-4 blockade (mean = 3.5%) as compared with mice that received vacc-HA alone (1.6%). No difference in the percentage of clonotype$^+$ T cells was observed in unimmunized mice treated with anti-CTLA-4 compared with those that were untreated (0.18% versus 0.28% respectively) (Fig. 1*A*). CTLA-4 blockade also resulted in enhanced proliferation (Fig. 1*B*) and IFN-γ production (Fig. 1*C*) of HA-peptide-pulsed splenocytes obtained from vacc-HA-immunized mice as compared with mice immunized with vacc-HA alone. Again, there was no effect of CTLA-4 blockade on the *in vitro* response of HA-specific T cells in the absence of immunization. Furthermore, no differences were observed among clonotypic T cells from animals that received vacc-HA alone or vacc-HA plus control antibody (data not shown).

In addition to priming HA-specific TCR transgenic T cells, immunization with vacc-HA primes T cells from the endogenous repertoire that are specific for vaccinia antigens. CTLA-4 blockade also enhanced this vaccinia-specific response to immunization with vacc-HA (Fig. 1*D*, mean cpm: 50,444 versus 30,060 in response to vacc-HA alone). Therefore, *in vivo* administration of anti-CTLA-4 antibodies to non-tumor-bearing mice enhanced the response of both clonotypic and nonclonotypic T cells to immunization with vacc-HA, whereas no effect was observed when anti-CTLA-4 was given alone.

Effect of CTLA-4 Blockade in Tumor-Bearing Mice. Next we sought to determine whether CTLA-4 blockade altered the fate and function of tumor-specific T cells in the tumor-bearing host. Anti-HA transgenic $CD4^+$ T cells were transferred into mice with established A20HA, as well as into tumor-free mice (day 0). Anti-CTLA-4 antibody was given to half these mice on days −1,

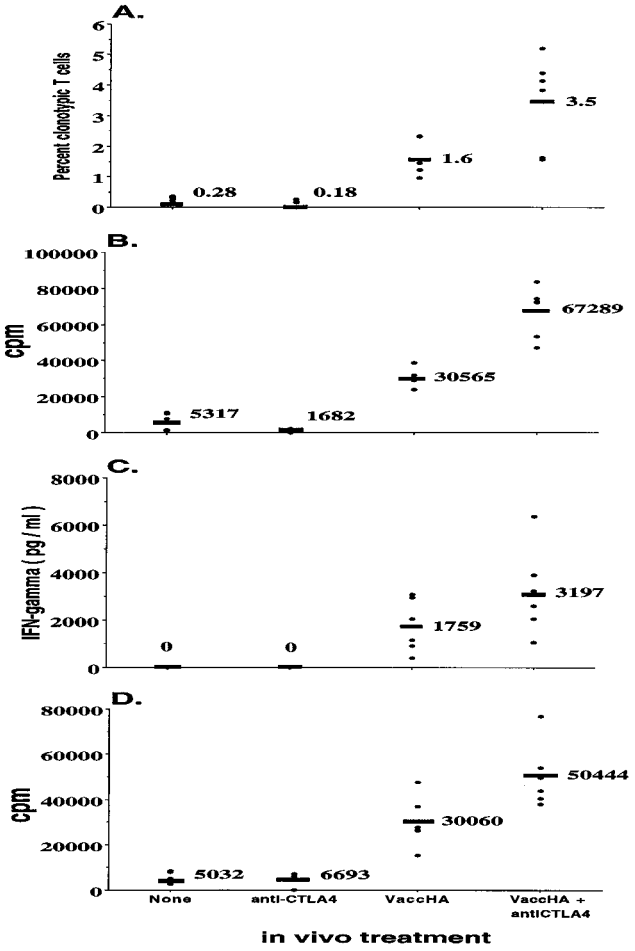

FIG. 1. Effect of CTLA-4 blockade on the response to immunization. BALB/c mice received anti-HA TCR transgenic T cells on day 0 and were treated with the anti-CTLA-4 antibody 9H10 (100 μg/day, i.p.) on days -1, 0, and $+1$ or received no treatment. On day $+2$ after T cell transfer half the mice in each group were immunized with vacc-HA, and 6 days later, T cells were isolated as described in *Materials and Methods*. Data represent the combined results of two independent experiments (three mice per group per experiment). Each point represents the value for an individual animal. Horizontal lines and the numbers indicate the average for six mice in each group. (*A*) Percentage of $CD4^+$ anti-HA TCR^+ cells as determined by FACScan analysis. (*B*) Proliferative response to stimulation with HA peptide *in vitro*. Data are shown as cpm from which values for medium alone were subtracted. (*C*) Production of IFN-γ in response to stimulation with HA peptide. Values for T cells cultured in medium alone were zero. (*D*) Proliferative response to stimulation with splenocytes infected with wild-type vaccinia virus. [^{3}H]Thymidine incorporation was determined after 3 days in culture. Data are shown as cpm from which values for medium alone were subtracted.

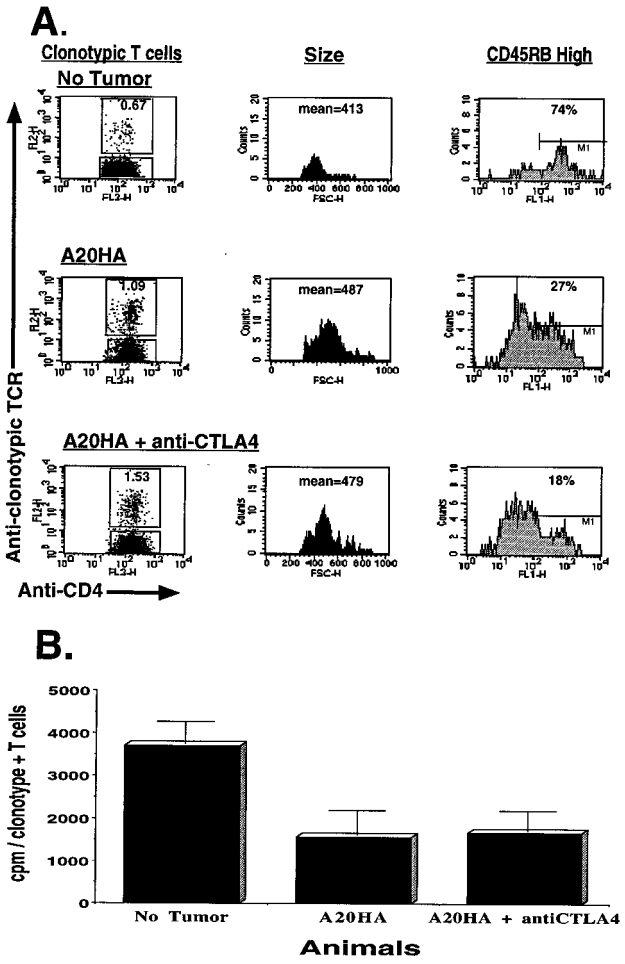

FIG. 2. Effect of CTLA-4 blockade in tumor-bearing mice. Tumor-bearing mice or tumor-free mice received anti-HA TCR^+ transgenic T cells and were treated with anti-CTLA-4 antibody (100 μg/day, i.p.) on days -1, 0, and $+1$ or received no treatment. On day $+8$ after T cell transfer, splenocytes were isolated as described in the text. (*A*) Phenotypic changes associated with antigen recognition in untreated and CTLA-4 treated tumor-bearing mice. Purified T cells were stained with Cy-Chrome-labeled anti-mouse CD4, biotinylated anti-TCR clonotype mAb 6.5 followed by phycoerythrin-labeled streptavidin and FITC-conjugated anti-mouse CD45RB. Live gating on $CD4^+$ T cells was set, and 100,000 events were collected per sample. Forward light scatter (FSC) and the level of CD45RB expression were determined on $clonotype^+$ T cells. The results are representative of two experiments with three mice per group. (*B*) Proliferative response to stimulation with HA peptide was determined as in Fig. 1. Data represent the mean + SE cpm per 100 $clonotype^+$ T cells per well from three mice in each group.

0, and $+1$, so as to prevent CTLA-4 engagement during T cell encounter with cells presenting the nominal antigen. Eight days after T cell transfer, the percentage and phenotypic characteristics of the $clonotype^+$ T cells was determined. As we have previously observed, there was an expansion of $clonotype^+$ T cells in A20HA-bearing mice relative to tumor-free mice at this early time point (3). This expansion was slightly greater in A20HA-bearing mice treated with anti-CTLA-4 antibody (Fig. 2*A Left*). $Clonotype^+$ T cells from both untreated and anti-CTLA-4-treated A20HA-bearing mice increased in size (FSC) (Fig. 2*A Center*) and decreased the level of expression of CD45RB compared with T cells from tumor-free mice (Fig. 2*A Right*), consistent with their having encountered antigen *in vivo*.

Functional analysis at this early time point revealed that transgenic T cells from both untreated and anti-CTLA-4-treated tumor-bearing mice had a diminished proliferative response to HA peptide *in vitro* as compared with those isolated from non-tumor-bearing mice, although they were not yet fully unresponsive (Fig. 2*B*). Therefore, in spite of the increased expansion of HA-specific $CD4^+$T cells observed in anti-CTLA-4-treated A20HA-bearing mice, these T cells display phenotypic and functional characteristics similar to those of T cells isolated from untreated A20HA-bearing mice.

CTLA-4 Blockade Enhances the Response to Early Immunization in Tumor-Bearing Mice. The most stringent determinant of T cell tolerance is the loss of responsiveness to a potent immunogen *in vivo*. We therefore, evaluated the response of A20HA bearing mice to immunization with vacc-HA with or without CTLA-4 blockade. In mice with a small tumor burden (established on day -9), immunization with vacc-HA 2 days after T cell transfer still resulted in the expansion of $clonotype^+$ T cells, although their *in vitro* proliferation and IFN-γ production were

diminished relative to non-tumor-bearing mice (Fig. 3 *A–C*). Nevertheless, at this early time point, the response to immunization with vacc-HA demonstrated that not all these cells were fully tolerant. Similar results were obtained with mice harboring a large tumor burden (tumor given on day −18), the only difference being the more pronounced impairment in IFN-γ production in response to immunization (Fig. 3*C*).

In contrast, regardless of the tumor burden, T cells from tumor-bearing mice treated with anti-CTLA-4 antibodies responded to vacc-HA priming comparably to tumor-free mice (Fig. 3, hatched bars). Indeed, in most instances, tumor-bearing mice treated with anti-CTLA-4 generated a similar or even greater response to vacc-HA than did non-tumor-bearing mice immunized with vacc-HA alone. One interpretation of these results is that CTLA-4 blockade may interfere with the development of T cell unresponsiveness in the tumor-bearing host. However, because of the potent ability of CTLA-4 blockade to enhance the T cell response to priming, analysis at this early time point (when HA-specific T cells from A20HA-bearing mice are still responsive) cannot distinguish between the prevention of tolerance and augmentation of the response of T cells that have yet to be tolerized.

CTLA-4 Blockade Does Not Prevent the Induction of Tolerance to Tumor Antigen. Given the above results, a similar analysis was performed at a time when HA-specific T cells from control treated A20HA-bearing mice were fully unresponsive. Our previous studies demonstrated that the extent of T cell tolerance increases rapidly as a function of how long the T cells have resided in the tumor-antigen-bearing host (3). Therefore, we delayed vacc-HA immunization by 4 days (vaccination: day +6) and analyzed the T cell response 6 days later. As we have previously reported, HA-specific clonotype$^+$ T cells from A20HA-bearing mice were fully tolerant as ascertained by their failure to expand (Fig. 4*A*) as well as their blunted proliferative response (Fig. 4*B*) and lack of IFN-γ production (Fig. 4*C*). In contrast to the results obtained with early vaccination, CTLA-4 blockade failed to preserve the response to vacc-HA given 6 days after T cell transfer in A20HA-bearing mice (Fig. 4 *A–C*, hatched bars). Importantly, this unresponsiveness was antigen-specific as demonstrated by the equivalent response of these same splenocytes from A20HA-bearing and non-tumor-bearing mice to vaccinia antigens (Fig. 4*D*).

In the experiments presented above, anti-CTLA-4 antibody administration bracketed the period of transfer of HA-specific T cells (days −1, 0, and +1). It is possible that insufficient CTLA-4 blockade at later time points may have allowed the engagement of CTLA-4 on clonotype$^+$ T cells in A20HA-bearing mice, resulting in the loss of responsiveness to vacc-HA. To evaluate this possibility, we treated A20HA-bearing mice with anti-CTLA-4 antibody every 3 days, beginning 1 day prior to T cell transfer until day +14 after T cell transfer. vacc-HA was given on day +9, and responses were evaluated 6 days after immunization (day +15). As seen in Fig. 5, CTLA-4 blockade maintained throughout the entire course of T cell exposure to antigen did not prevent the development of T cell unresponsiveness, suggesting that the induction of tumor antigen-specific tolerance involves mechanisms other than CTLA-4 engagement.

DISCUSSION

Monoclonal antibodies that block CTLA-4 engagement *in vivo* have been used to examine the role of CTLA-4 in normal and

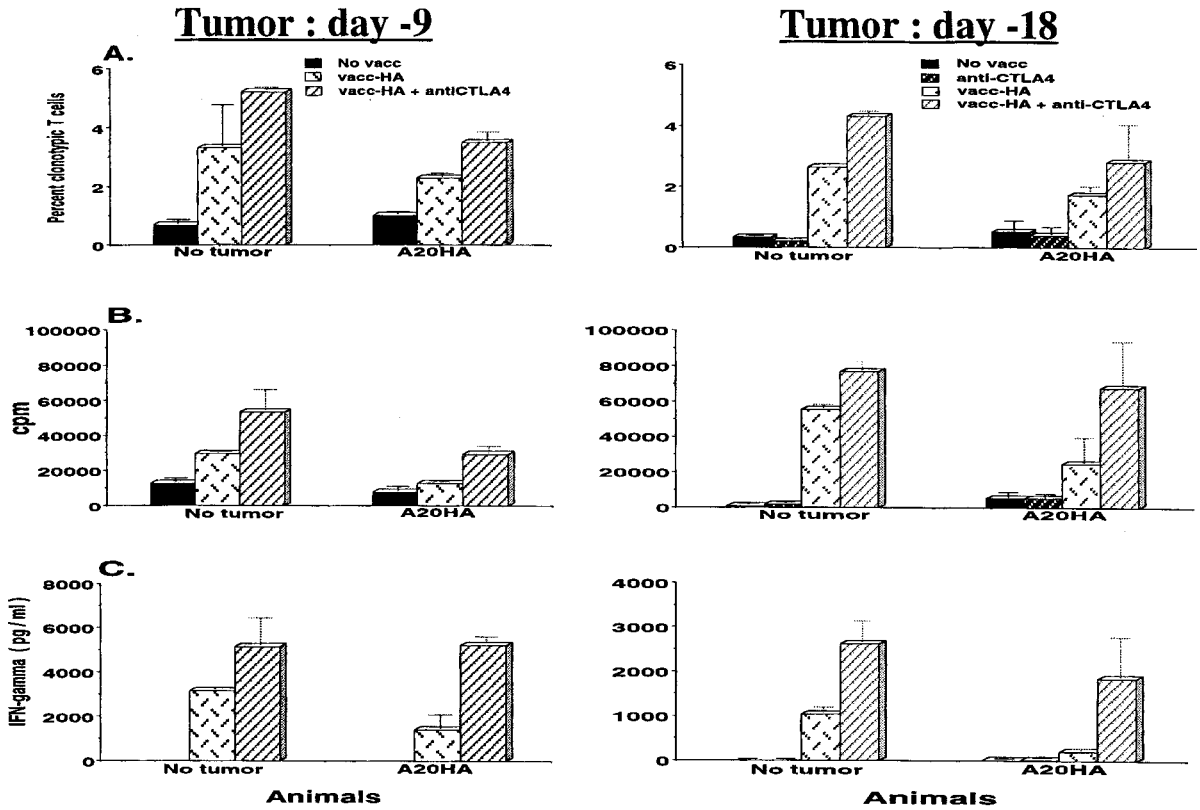

FIG. 3. Effect of CTLA-4 blockade on the response of tumor-bearing mice to early immunization. Mice with or without established tumors received anti-HA TCR$^+$ transgenic T cells on day 0 and were treated with anti-CTLA-4 antibodies or a similar amount of hamster IgG antibody on days −1, 0, +1 or were left untreated. Half the mice in each group were immunized with vacc-HA on day +2, and all mice were analyzed 6 days later. The responses of mice with a small tumor burden (tumor given day −9) are displayed on the *Left* and those with a large tumor burden (day −18) on the *Right*. (*A*) T cells from unimmunized mice (solid bars), vacc-HA-immunized mice (interrupted cross-hatched bars), and mice treated with anti-CTLA-4 antibodies plus vacc-HA (hatched bars) were analyzed by FACScan for CD4 versus anti-HA TCR. Values represent mean + SE of percentage of T cells expressing the clonotypic TCR for three mice per group. Proliferation of T cells in response to HA peptide *in vitro* (*B*) as well as IFN-γ production (*C*) were also measured. Values are the mean + SE of triplicate cultures from three mice in each group.

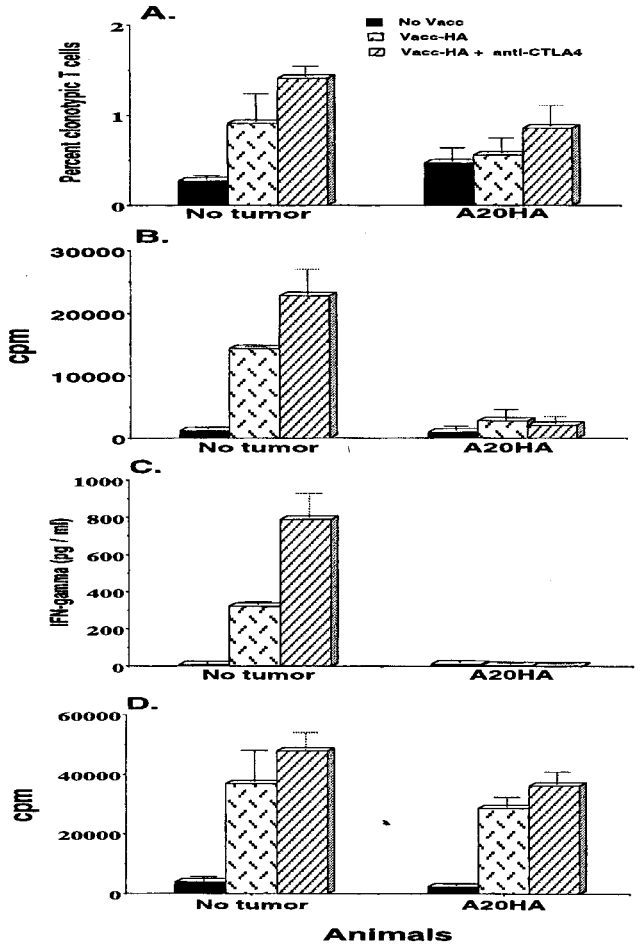

FIG. 4. Effect of CTLA-4 blockade on the response of tumor-bearing mice to delayed immunization. Tumor-bearing mice or tumor-free mice received anti-HA TCR^+ transgenic T cells on day 0 and were treated with anti-CTLA-4 antibodies or hamster IgG antibodies (days -1, 0, $+1$) or were left untreated as in Fig. 3. vacc-HA immunization was delayed until day $+6$ and the analysis was performed 6 days later. (*A*) T cells from unimmunized mice (solid bars), vacc-HA-immunized mice (interrupted cross-hatched bars), and mice treated with anti-CTLA-4 antibodies plus vacc-HA (hatched bars) were analyzed by FACScan. Values represent mean + SE of percentage of T cells expressing the clonotypic TCR for three mice per group. T cells from the mice in *A* were analyzed for their *in vitro* proliferation (*B*) as well as IFN-γ production (*C*) in response to stimulation with HA peptide. Values are the mean + SE of triplicate cultures from three mice in each group. (*D*) Proliferative response to stimulation with splenocytes infected with vaccinia virus was determined as in Fig. 1.

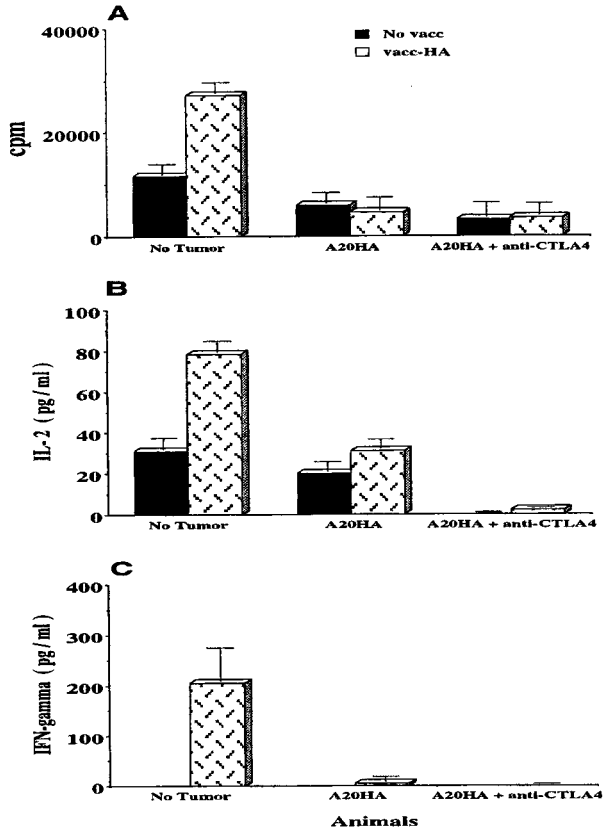

FIG. 5. Effect of sustained CTLA-4 blockade on the response of tumor-bearing mice to immunization. Tumor-bearing or tumor-free mice received anti-HA TCR^+ transgenic T cells on day 0 and were treated with anti-CTLA-4 antibodies every 3 days from day -1 to day $+14$ after the transfer of T cells, or received no treatment. On day $+9$, half the mice in each group were immunized with vacc-HA, and all mice were analyzed 6 days later. (*A*) HA-specific proliferative response of T cells from unimmunized (solid bars) or vacc-HA-immunized (interrupted cross-hatched bars) mice was determined. Three mice per group were analyzed. In a parallel plate T cells from *A* were cultured with medium alone or HA peptide plus fresh BALB/c splenocytes for 48 hr. Then, supernatants were collected and assayed for IL-2 (*B*) and IFN-γ (*C*) by ELISA.

pathologic immune responses. These studies have demonstrated that CTLA-4 blockade enhances T-cell-mediated responses. Specifically, treatment with anti-CTLA-4 antibodies has been shown to amplify the expansion of antigen and superantigen-specific T cells in response to priming (12); to exacerbate experimental autoimmune encephalomyelitis (13); as well as to increase the incidence and accelerate the development of autoimmune diabetes in a transgenic model (14). The ability of CTLA-4 blockade to unmask and/or amplify immune responses to normal self-antigens suggests that CTLA-4 may play a role in the induction and maintenance of peripheral tolerance.

However, the immunologic basis for tolerance to antigens found outside the thymus remains incompletely understood (15). Full activation of resting T cells requires both an antigen-specific signal provided by engagement of the TCR with the appropriate peptide/MHC complexes and a second "costimulatory" signal delivered exclusively by bone-marrow-derived antigen-presenting cells (APCs). A prediction of the "two-signal" model of T cell activation is that antigen-specific T cell unresponsiveness results from TCR engagement with peptide/MHC complexes on cells that are incapable of delivering adequate costimulation. Recent studies, however, have demonstrated that bone-marrow-derived APCs not only are required for T cell priming but also participate in the induction of T cell tolerance to peripheral tissue-specific antigens (16, 17). This observation implies that the tolerizing cell population has the capacity to provide costimulation through the expression of B7-1 and B7-2 as well as other ligands required for T cell priming. Accordingly, the central determinant of T cell priming versus tolerance may well be the regulated expression of these molecules during APC activation (18, 19)

Notably, some studies have demonstrated a requirement for B7-1/B7-2–ligand interaction in the establishment of tolerance (20, 21). Consistent with this is the observation that the development of anergy *in vivo* phenotypically resembles a "partial activation state," which may require some degree of T cell costimulation to develop. *In vivo* models of T cell tolerance to exogenously administered peptide antigen (22), to tissue-specific self-antigen (16, 17), and to antigen expressed by tumor cells (3), all demonstrate that the induction of T cell tolerance is preceded by cell division, leading to an initial clonal expansion of T cells specific for the nominal antigen, increase in cell size, and loss of their naive phenotype. In the studies demonstrating a role for B7-1/B7-2 in tolerance induction, blocking B7-1/B7-2 from

interacting with their ligand(s) on T cells not only prevented the induction of anergy but also preserved the naive phenotype, indicative of unstimulated T cells. In this regard, the prevention of tolerance by blocking B7-1/B7-2 may be similar in effect to blocking signaling downstream of the TCR such as with cyclosporin A, which also prevents the induction of anergy (23).

An alternate hypothesis for the participation of B7-1/B7-2 in the development of T cell tolerance is that preferential engagement of CTLA-4 over CD28 is directly responsible for induction of the unresponsive state. Using the adoptive transfer of TCR transgenic T cells specific for an MHC class II epitope of ovalbumin (OVA), Perez and colleagues (21) demonstrated that administration of an otherwise tolerizing form of antigen (i.p. injection of OVA peptide) resulted in T cell priming rather than anergy when antibody to CTLA-4 was delivered during the initial response to antigen.

These findings are at odds with the results of the present study examining the role of CTLA-4 in the development of peripheral tolerance to tumor antigen. In this system, we find that clonotype$^+$ T cells from anti-CTLA-4-treated tumor-bearing mice display phenotypic and functional characteristics that are similar to those found in tumor-bearing mice in the absence of CTLA-4 blockade (Fig. 2). Furthermore, administration of anti-CTLA-4 antibodies, either during the initial period of interaction between clonotype$^+$ T cells and the tumor antigen (Fig. 4) or during the whole period that the clonotype$^+$ T cells reside in the tumor-bearing host (Fig. 5), resulted in the same outcome—i.e., induction of antigen-specific T cell tolerance. Importantly, the failure of CTLA-4 blockade to prevent tolerance in tumor-bearing mice occurred in the setting where the T cell response to vaccination is enhanced by CTLA-4 blockade in tumor-free mice used as controls. Notably, FACScan analysis failed to demonstrate any differences in the expression of CTLA-4 by clonotype$^+$ T cells isolated from tumor-free or tumor-bearing mice (data not shown), making it unlikely that the failure to prevent tolerance in the latter group was a result of having a subsaturating concentration of the blocking antibody in tumor-bearing mice.

One possible explanation for these seemingly discordant results relates to the time of analysis of T cell function after exposure to tolerogenic antigen. Early analysis in both systems demonstrated an apparent preservation of T cell responsiveness in mice treated with antibody to CTLA-4 as compared with controls. However, given the demonstrated influence of time on the induction of T cell unresponsiveness *in vivo* (3, 17, 24), it is possible that these observed effects of CTLA-4 blockade reflect augmented responses of T cells that were not yet rendered tolerant, rather than interference with the induction of tolerance per se. Consistent with this interpretation, vaccination at later times demonstrated that when untreated controls were uniformly unresponsive (after 6 or 9 days of exposure to antigen), CTLA-4 blockade failed to preserve the ability to prime tumor specific T cells in the tumor-bearing host (Figs. 4 and 5).

The time of T cell exposure to antigen was also found to affect the outcome of CTLA-4 blockade in a TCR transgenic model of autoimmune diabetes (14). In this system, the injection of anti-CTLA-4 into very young mice (months before they would normally become diabetic) rapidly induced diabetes. However, these effects were observed only if anti-CTLA-4 was injected during a narrow time window, before the initiation of insulitis. Similarly, in a tumor rejection model, Yang *et al.* (25) demonstrated that while *in vivo* administration of anti-CTLA4 antibodies resulted in tumor regression when given at early time points after tumor challenge, no antitumor effect was observed when the delivery of anti-CTLA4 was delayed. Interestingly, in our tumor model, the duration of T cell exposure to antigen appears to have an even greater influence on the development of tolerance than does the size of the systemic tumor burden itself.

Given these results, we favor a model in which the induction of antigen-specific T cell anergy is not fully attributable to CTLA-4 engagement. In this model, the anti-tumor effect of CTLA-4 blockade, as well as the apparent ability to "break" peripheral tolerance to self-antigens, results from the enhanced responsiveness of T cells that have escaped tolerance induction. The normal T cell repertoire contains a spectrum of TCRs having various affinities for tumor antigen, and individual tumor-specific T cells may be more or less susceptible to the induction of tolerance. As demonstrated by Morgan *et al.* (26), in a setting where high-affinity T cells for antigen expressed on both normal tissues and tumor cells were tolerant, T cells having low affinity for the antigen remained responsive to priming and could mediate tumor rejection. It is perhaps this population of T cells on which the combined effects of vaccination and CTLA-4 blockade are likely to have the greatest impact. Just as this population of T cells may be less susceptible to the induction of tolerance, its response to immunization may be modest when conventional vaccine strategies are used, further underscoring the utility of CTLA-4 blockade in its ability to lower the threshold required for T cell priming.

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