Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer

(CTLA-4/B7.1/CD28/tumor rejection/cellular immunity)

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ABSTRACT The identification of potentially useful immune-based treatments for prostate cancer has been severely constrained by the scarcity of relevant animal research models for this disease. Moreover, some of the most critical mechanisms involved in complete and proper antitumoral T cell activation have only recently been identified for experimental manipulation, namely, components involved in the costimulatory pathway for T cell activation. Thus, we have established a novel syngeneic murine prostate cancer model that permits us to examine two distinct manipulations intended to elicit an antiprostataste cancer response through enhanced T cell costimulation: (i) provision of direct costimulation by prostate cancer cells transduced to express the B7.1 ligand and (ii) in vivo antibody-mediated blockade of the T cell CTLA-4, which prevents T cell down-regulation. In the present study we found that a tumorigenic prostate cancer cell line, TRAMP1 (pTCl), derived from transgenic mice, is rejected by syngeneic C57BL/6 mice, but not athymic mice, after this cell line is transduced to express the costimulatory ligand B7.1. Also, we demonstrated that in vivo antibody-mediated blockade of CTLA-4 enhances antiprostataste cancer immune responses. The response raised by anti-CTLA-4 administration ranges from marked reductions in wild-type pTCl growth to complete rejection of these cells. Collectively, these experiments suggest that appropriate manipulation of T cell costimulatory and inhibitory signals may provide a fundamental and highly adaptable basis for prostate cancer immunotherapy. Additionally, the syngeneic murine model that we introduce provides a comprehensive system for further testing of immune-based treatments for prostate cancer.

In both its prevalence and incidence, prostate cancer ranks as one of the most common cancers among males in the United States. Rates of death attributable to prostate cancer are steadily rising, estimated at more than 40,000 in 1997 alone, vying with colorectal cancer as the second most common cause of cancer-related death in males (1, 2). Although the mainstay of therapy for patients with the advanced form of this disease is androgen withdrawal or suppression, this treatment is sel-

The most essential mechanisms involved in regulation of T cell activation and inhibition have only recently been recognized. It is now evident that complete T cell activation requires two signals. The first is provided by antigen-specific signals arising from interactions between the T cell receptor (TCR) and antigen/major histocompatibility complex (MHC). The second signal arises from antigen-independent interactions between the CD28 molecule on the T cell surface with the costimulatory B7 family of ligands (CD80 and CD86), which are commonly expressed on professional antigen-presenting cells (APC). We and others have demonstrated that provision of these costimulatory B7 ligands to cancer cells from diverse tissue origins bypasses the requirement for exogenous APC help, culminating in potent and tumor-specific CD8+ T cell activation (8–10).

The CD28 homolog, CTLA-4, also binds to B7, but with an approximately 20-fold higher affinity than CD28 (11). In contrast to the B7/CD28 interaction, however, the B7/ CTLA-4 interaction delivers an inhibitory signal to T cells (12–14). Therefore, the outcome of TCR signaling is depend-

Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell; B7, B7.1; TRAMP, transgenic adenocarcinoma mouse prostate; SV40, simian virus 40; Tag, SV40 large T tumor antigen.

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rectal carcinoma (51BLim10) or murine fibrosarcoma (Sa1N) raised in their syngeneic murine hosts (15).

In the present study, we examine whether these newly elucidated mechanisms regulating T cell costimulation can be manipulated to elicit an antiprostate cancer response. To perform these studies, we established a novel syngeneic model in which an early-passage prostate cancer cell line derived from TRAMP mice (6, 7), or genetically modified derivatives of this cell line, is introduced into normal inbred C57BL/6 mice. The current experiments provide the basis for future trials of immunological treatment of localized and metastatic prostate cancers that develop in TRAMP mice and that closely parallel the biology and progression of human prostate cancer.

MATERIALS AND METHODS

Cell Lines. TRAMPC1 (pTC1) is an early-passage murine prostate cancer cell line derived from TRAMP mice that spontaneously develop prostate cancer due to prostate-specific simian virus 40 (SV40) large T tumor antigen (Tag) expression (6, 7). The medium for growth of this line in culture is Dulbecco’s Modified Eagle Medium (DMEM; Gibco/BRL) supplemented with 5% fetal calf serum (FCS; HyClone), 5% characterized FCS (NuSerum, Collaborative Biomedical Products, Bedford, MA), 5 μg/ml insulin (Sigma), 0.01 mM dithiothreitole (Sigma), and penicillin/streptomycin (Bio-Whittaker). This line, as well as modified derivatives, were maintained at 37°C in 5% CO2 and were passaged weekly in 10-cm dishes. Cells in these studies typically were used between passages 9 and 20. Cells to be used in animal injections were washed with 10 ml of DMEM × 3 per dish. Animal injections were performed using the specified number of viable cells (determined by trypan blue exclusion) suspended in a final volume of 0.1 ml of either PBS or DMEM injected through a 19-gauge needle.

Stable Transduction of pTC1. pTC1 was stably transduced to express the costimulatory murine B7.1 (B7-TC1) using an ecotropic retrovirus (16) containing the murine B7.1 (B7) gene. For controls, pTC1 was also transduced with the empty vector lacking the murine B7 gene (pTC1). The retroviral vector also contains a hygromycin resistance gene for positive selection in vitro, as well as the herpes simplex virus thymidine kinase gene (HSVTK) to permit negative selection in vivo following ganciclovir administration (16). After about 1 month in hygromycin selection, B7 is expressed by greater than 95% of the cells.

Flow Cytometry. Murine B7.1 expression was evaluated by staining with the B7-binding CTLA-4-immunglobulin fusion protein, followed by FITC-conjugated goat antihuman immunglobulin (Caltag, South San Francisco, CA). MHC class I and II expression was evaluated by flow cytometry after staining the cells with an anti-pan MHC class I (M1/42.3.9.8.HLK), anti-pan class II MHC (N22), anti-D b (28–11-S), or anti-K b (Y-3; American Type Culture Collection), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster immunglobulin or goat anti-rat immunglobulin (Caltag) second-step reagents. Flow cytometry was performed using Coulter Epics Elite ESP flow cytometer.

Isolation and Analysis of SV40 Large Tumor T Antigen (Tag) mRNA. Analyses for Tag mRNA expression in pTC1 and its derivatives, from tumors raised in BALB/C mice or cells grown in culture, were performed as previously described (6).

Anti-CTLA-4 Production, Purification, and Titering. Anti-CTLA-4 antibody used in these studies was protein G-purified from nine supernatants from the 9H10 hybridoma line raised in either a Cell Pharm System 2000 (UniSyn, Hopkinton, MA) or Cellco Bioreactor (Cellco, Kensington, MD). Antibody concentrations were quantified by ultraviolet spectrophotometry (15).

Animal Studies. Animal experiments were conducted in the Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute (National Institutes of Health), and the Cancer Research Laboratory at the University of California at Berkeley according to National Institutes of Health animal care and use guidelines. Six- to 8-week-old male C57BL/6, C57BL/6 nu/nu, and BALB/C nu/nu mice, were obtained from The Jackson Laboratory, Charles River Breeding Laboratories, or Taconic Farms. Mice receiving subcutaneous injections of tumor cells (at the doses specified) received Metophane (Mallinckrodt) inhalational anesthesia at the time of tumor cell injection. Tumor growth was monitored by measuring bisecting diameters of the tumor base with vernier calipers.

RESULTS

MHC I Expression by pTC1. Cytofluorimetric analysis of pTC1 grown in vitro reveals that approximately 50–70% of early passage (passage ≤10) pTC1 cells express low but detectable MHC class I molecules (Fig. 1). Further passaging of these cells, however, is associated with a decline in MHC I expression, and by passage 15, ≤5% of pTC1 cells express MHC I (Fig. 1). Among those pTC1 cells positive for MHC I, both K b and D b are expressed at equivalent levels (data not shown). No MHC II is detectable as determined by cytofluorimetric analysis (data not shown).

Tag Is Not Expressed by pTC1 or Its Modified Derivatives in Vitro or in Vivo. To eliminate the possibility that Tag could serve as a potential antigen in our model, pTC1 and its derivatives were analyzed for Tag mRNA by reverse transcription–polymerase chain reaction followed by Southern blot analysis. No Tag mRNA is apparent in pTC1 and derivative cells maintained in vitro (Fig. 2A) or obtained from tumors raised in BALB/C nu/nu mice (Fig. 2B).

Murine B7 Expression by Murine Prostate Cancer Cells Is Sufficient to Cause Their Rejection in Vivo. To test whether B7 expression by prostate cancer cells can provide sufficient direct costimulation to elicit an immune response that results in the rejection of these cells, passage 9 pTC1 was modified to express the murine costimulatory ligand B7 (B7-TC1) or the empty vector –polymerase chain reaction followed by Southern blot analysis. No Tag mRNA is apparent in pTC1 and derivative cells maintained in vitro (Fig. 2A) or obtained from tumors raised in BALB/C nu/nu mice (Fig. 2B).

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In Vivo Blockade of CTLA-4 by Anti-CTLA-4 (9H10) Antibody in C57BL/6 Mice Slows Wild-Type pTC1 Growth or Causes Its Rejection. To test whether blockade of CTLA-4 augments an antitumoral immune response against wild-type pTC1, the following experiments were performed. Early-passage unmanipulated pTC1 cells (passages 9, 10, or 11) were injected at three different challenge doses (1.25, 2.5, and 5.0 × 10^6 cells) subcutaneously into the backs of male inbred C57BL/6 mice. Subsequently, mice received 100-μg intraperitoneal injections of either an irrelevant hamster antibody or anti-CTLA-4 antibody (Ctrl Ab or αCTLA-4, respectively; see Fig. 4) on days 7, 10, and 13 following tumor cell injection. In the representative experiment depicted in Fig. 4A, administration of anti-CTLA-4 significantly delayed the growth and in one animal induced the complete rejection of tumor cells in mice challenged with 2.5 × 10^6 pTC1 cells. Similar responses to anti-CTLA-4 administration were observed following challenges with either 1.25 or 5 × 10^6 pTC1 cells, with a slightly greater effect being observed at the lower pTC1 challenge dose (data not shown). Interestingly, however, in two separate experiments using 2 × 10^6 pTC1 cells of a higher passage (passage >15), which express far less MHC I in vitro, a more complete response was observed (Fig. 4B). In a total of five experiments (including those shown in Fig. 4A and B), 21 of 50 (42%) anti-CTLA-4-treated mice exhibited complete rejection of their tumors, while the majority of the remaining mice demonstrated delayed growth of pTC1 tumors. In contrast, pTC1 was uniformly tumorigenic in animals treated with the control hamster antibody.

**DISCUSSION**

We describe a new syngeneic model to investigate whether recently identified mechanisms involved in the costimulatory pathway of T cell activation can be appropriately manipulated for immunotherapy of prostate cancer. To perform these experiments, we used a transplantable prostate cancer cell line, pTC1, that does not express Tag and is readily tumorigenic in the nontransgenic, syngeneic C57BL/6 mouse. Our studies demonstrate that when pTC1 is modified to express the murine B7.1 costimulatory ligand, a host immune response is elicited leading to the complete rejection of these cells in the syngeneic C57BL/6 mouse. Given that B7-TC1, but not pTC1 or vTC1, is rejected by syngeneic C57BL/6 mice (Fig. 3A) and that B7-TC1 is as tumorigenic as pTC1 and vTC1 in athymic mice (Fig. 3B), the mechanism for the rejection of B7-TC1 is likely T cell-mediated. It should be noted, however, that in two experiments, B7-TC1 failed to be completely rejected due to poor initial B7 expression by these cells (data not shown). As has been proposed for other MHC I^+ B7-expressing cancer cells, rejection of B7-TC1 likely involves direct, antigen-specific costimulation and activation of CD8^+ T cells (8, 9) by these modified prostate cancer cells.

We also demonstrate that in vitro antibody-mediated blockade of CTLA-4 can “tip the balance,” favoring immune-mediated rejection of unmodified prostate tumor cells presumably by removing inhibitory signals in the costimulatory pathway that down-regulate T cell responses. CTLA-4 blockade is capable of slowing the growth of unmanipulated wild-type pTC1 tumors (Fig. 4A). Because these tumor cells do not express costimulatory ligands, it is likely that the effect is at the level of cross-priming of T cells by host APCs rather than by the tumor cells themselves (15, 17, 18). The enhancement by CTLA-4 blockade may be a result of more efficient recognition of antigenic peptides by T cells whose threshold for activation has been lowered or by more extensive proliferation of activated T cells, or both (19).

The effectiveness of transduction with B7 or of CTLA-4 blockade in inducing regression of those pTC1 cells that express little or no MHC I is perhaps surprising. One possi-
Days post injection

Fig. 3. (A and B) Rejection or growth of B7-TC1, and growth of pTC1 and vTC1 tumors, in male syngeneic C57BL/6 mice (A) or C57BL/6 nu/nu mice (B). (A) Male C57BL/6 mice received subcutaneous injections to the back on day 0 with 5 × 10^6 cells of p-, v-, or B7-TC1. (B) Male C57BL/6 nu/nu mice received similar injections on day 0 with 1 × 10^6 pTC1 cells or its derivatives. Data are presented as the mean tumor base area (mm^2) ± SD from a single representative experiment that was repeated three times. Each group contained 5–7 mice. The fraction of animals in each group that formed tumors is provided in parentheses next to corresponding marker designations. Animals were euthanized when their tumors achieved 250 mm^2.

Fig. 4. (A and B) Effects of intraperitoneal anti-CTLA-4 administration on growth of early-passage pTC1 (A, passages 9, 10, and 11) and late passage pTC1 (B, passages >15), C57BL/6 mice were injected with 2.0 × 10^6 (B) or 2.5 × 10^6 (A) wild-type pTC1 cells. Data are mean tumor areas from a single, representative experiment in which groups of five mice received either 100 mg of anti-CTLA-4 antibody (aCTLA-4) or 100 µg of a control hamster antibody (Ctrl Ab) or 100 mg of anti-CTLA-4 antibody (aCTLA-4) on days 7, 10, and 13 following tumor injection. The fraction of animals in each group that formed tumors is provided in parentheses next to corresponding marker designations. Experiments in A were performed three times, and experiments in B were performed two times.


