B7 Blockade Alters the Balance between Regulatory T Cells and Tumor-reactive T Cells for Immunotherapy of Cancer

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Abstract

Purpose: In prostate cancer-bearing host, regulatory T (Treg) cells restrain activity of tumor antigen-specific T cells. Because B7:CD28 interactions are needed for both function of CD4+CD25+ Treg cells and CD8+ effective T cells, targeting this pathway may help to overcome the immunotherapy barriers.

Experimental Design: The anti-B7-1/B7-2 monoclonal antibodies were administered to a transgenic mouse model of prostate cancer (TRAMP) ectopically expressing SV40 large T antigen in different tumor development stages for prevention and therapy of prostate cancer. The treatment was also tested in treating transplanted MC38 colon adenocarcinoma in mice.

Results: Here, we showed that short-term administration of anti-B7-1/B7-2 monoclonal antibodies in TRAMP mice leads to significant inhibited primary tumor growth and the size of metastatic lesions. The treatment is effective to inhibit MC38 colon cancer growth. Correspondingly, this treatment results in a transient reduction of Treg in both thymus and the periphery. In vivo cytotoxicity assay revealed T antigen-specific CTL effectors in anti-B7-treated but not control IgG-treated TRAMP mice.

Conclusions: Transient blockade of B7-1/B7-2 alters the balance between Treg and cancer-reactive T cells to enhance cancer immunotherapy.

Many of tumor antigens identified thus far are self-antigens (1–4) and may therefore trigger immune tolerance. Logically, mechanisms that mediate self-tolerance may contribute to inadequacy of tumor immunity. The best characterized mechanism of self-tolerance is clonal deletion (5, 6). In this context, we have shown that tumor antigen controlled by tissue-specific promoter is also expressed in the thymus to trigger clonal deletion (7).

In addition to clonal deletion, CD4+CD25+ regulatory T (Treg) cells play a pivotal role in the maintenance of peripheral self-tolerance (8–12). Accumulating evidence also support a role for Treg in restrained cancer immunity. Thus, cancer patients have elevated numbers of Treg cells in the blood of malignant effusions (13–15). Treg cells are also recruited and accumulated at tumor sites in animal models and in cancer patients (16–18). Correlation between the number of CD4+CD25+ Treg cells and clinical outcomes in some, although not all, cancer patients supported the hypothesis that Treg may suppress the effector function of tumor antigen-specific T cells, allowing tumor growth in the presence of tumor antigen-specific T cells (19, 20). Consistent with this concept, the removal of CD4+CD25+ Treg cells by an anti-CD25 antibody promoted rejection of transplanted tumor cells (21). However, this approach has shown little efficacy in animals with spontaneous tumors, which better reflect the challenge of cancer immunotherapy. In a recent study using a transgenic model of prostate dysplasia, anti-CD25 monoclonal antibody (mAb) treatment at age 12 weeks caused only 25% reduction in the prostate mass at 20 weeks, although extended observation has not been carried out to document long-term effect (22).

Alternatively, it is worth considering conditions that are selectively required for the generation and maintenance of Treg. CD28+ and B7-1/B7-2+ mice have markedly decreased numbers of CD4+CD25+ Treg cells in the thymus as well as in the periphery (23–25). Meanwhile, we and others have reported a significant role for B7-CD28 interaction in clonal deletion of some, although not necessarily all, self-antigens (26, 27). As such, transient blockade of B7-1/B7-2 may reduce Treg while increase the frequency of cancer-reactive T cells, thus overcoming the two major barriers to effective cancer immunity.

Transgenic mouse model of prostate cancer (TRAMP) is a well-established mouse model for prostate cancer with clearly defined progression of prostate cancer that resembles the human disease (28). Metastasis to periaortic lymph nodes and lungs can be detected frequently (29). By the time the mice are 24 to 30 weeks old, the prostate cancer becomes palpable in

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Translational Relevance

Despite the conceptual advances in cancer immunotherapy, clinical development has been slow. Immunotherapy has thus far failed to show clear-cut effect once cancers are established in advanced stage. In this article, we showed that temporary blockade of B7-1 and B7-2 reduced the number of regulatory T cells and conveyed considerable therapeutic effects in transgenic mouse model of prostate cancer mice with spontaneous prostate cancer. To our knowledge, this is the first time that the prostate cancer in the transgenic mouse model of prostate cancer mice can be effectively treated when the large tumors can be shown. Mechanistically, we showed that transient blockade of B7-1/B7-2 resets the balance of regulatory Tcells and cancer-reactive T cells to confer prevention and therapy of prostate cancer. A second major advantage is that the data can be easily translated into human use as the drug that blocks B7-1 and B7-2 (Food and Drug Administration-approved CTLA4 Ig) has already been approved for the treatment of autoimmune diseases. It is possible to dramatically shorten the path of clinical development for the novel immunotherapy.

Materials and Methods

Experimental animals. C57BL/6 mice and TRAMP mice expressing the SV40 T antigen (Tag) controlled by rat probasin regulatory elements in the C57BL/6 background were purchased from The Jackson Laboratory. The mice were bred at the animal facilities of the Ohio State University and the University of Michigan. All animal experimental procedures were reviewed and approved by The Ohio State University and University of Michigan Institutional Animal Care and Use Committees. Mice were typed for SV40 T antigen by isolation of mouse tumor DNA and PCR-based screening assay was described previously (7). Transgenic mice expressing T-cell receptor (TCR) specific for SV40 large T antigen (Tag) have been described (30). Generation of TRAMP mice expressing TGB TCR (TGB-TRAMP) was also described (7).

Antibody treatment of the TRAMP mice. TRAMP mice were treated with anti-B7-1 and anti-B7-2 antibodies at two different stages. In the first regiment, 4- to 6-week-old TRAMP male mice were injected intraperitoneally with five injections of anti-B7-1 (rat anti-mouse CD80, clone 3A12; ref. 31) and anti-B7-2 (hamster anti-mouse CD86, clone GL-1; American Type Culture Collection; ref. 32) antibodies or control hamster/rat IgG (Sigma) at 100 μg/antibody/injection every other day. Long-term prostate cancer incidence was recorded by physical examination. In the second regiment, 25-week-old TRAMP male mice without palpable prostate cancer were treated intraperitoneally with the anti-B7 or control IgG at 100 μg/antibody/injection for five injections every other day. The magnetic resonance imaging (MRI) examination was carried out before treatment and 8 weeks later at age 33 weeks. In a separate experiment, 25-week-old TRAMP male mice were treated with one intraperitoneal injection of 1 mg anti-CD25 (PC61) or control rat IgG (33). The efficiency of anti-CD25 depletion was examined by flow cytometry with staining PBL using conjugated anti-CD4, anti-CD25 (clone 7D4; American Type Culture Collection), and anti-Foxp3. The MRI examination was carried out before treatment and 5 weeks later at age 38 weeks. For long-term prostate cancer incidence study, anti-B7 and control IgG-treated mice were examined at least weekly for palpable tumor at lower abdomen and were euthanatized when they either become moribund or with tumor size exceeding 5% of body weight.

Six to 8-week-old TRAMP or TRAMP/TGB mice were sublethally irradiated (500 rad) on day 0 and the treatment started on day 1 with either anti-B7-1/B7-2 mAbs (100 μg each) or control rat/hamster IgG (100 μg each) intraperitoneally. The mice were treated six times every other day. One week after the last treatment, the mice were sacrificed and the total thymocytes and splenocytes were harvested and stained with fluorochrome-conjugated antibodies anti-CD4 (RM4.5), anti-CD8 (53-6.7), and anti-V(3)i.8+1.82 (MR5-2; BD).

For transplantable tumor model, MC38 murine colon carcinoma cells were grown in RPMI with 5% fetal bovine serum and subcutaneously injected to male C57BL/6 mice (5 × 105 per mouse). Ten days after injection, mice were divided equally into two groups based on the tumor sizes and administered intraperitoneally with either anti-B7 or control IgG three times every other day. Peripheral blood was collected at 0 and 6 days (0 day is the day before the administration of antibodies) and the splenocytes were collected at 14 days and stained with anti-CD4, CD8, CD25, and Foxp3 antibodies (BD).

Proliferation of T cells to antigenic peptides. Total spleen cells (3 × 105 per well) from control immunoglobulin or anti-B7-treated TRAMP × TGB (H-2b/k) F1 mice were cultured with the given concentrations of SV40 Tag HSV60-568 peptide or control HSV gB peptide in Click's Eagle's-Hank's amino acid medium for 72 h. The proliferation of T cells was determined by incorporation of[^H]thymidine pulse (1 μCi/well) during the last 6 h of culture. The data presented are means of triplicates with variation from the means ± SEM.

Peptide synthesis. All peptides used were synthesized by Research Genetics. The peptides were dissolved in DMSO at a concentration of 10 mg/mL and diluted in PBS or culture medium before use. Peptides used were SV40 Tag S60-568 SEFLEKRI (7) and HSV gB peptide gB498-505 SIEFARL (34).

Immunohistochemistry. Mouse organs were fixed with 10% buffered formalin. Tissue sections were stained with H&E and examined under a microscope. Frozen-sections were prepared and stained with 2 μg/mL antibodies specific for CD3 (2C11, hamster IgG), CD4^+ flies were counted using ×20 microscope visual fields.

In vivo cytotoxicity assay. Spleen cells from C57BL/6 mice were pulsed with 10 μg/mL of either SV40 Tag S60-568 SEFLEKRI or a control peptide HSV gB498-505 SIEFARL in the presence of either 0.5 or 5 mmol/L CFSE, respectively. After mixing at a 1:1 ratio, the labeled cells were injected intravenously into recipients and spleen cells were harvested 20 h later and analyzed by flow cytometry for the relative abundance of CFSElow (SV40 Tag peptide) and CFSEhigh (HSV peptide) populations.

Detection of anti-double-stranded DNA. Anti-DNA antibodies were measured by ELISA according to the published procedure (35).

MRI of prostate. The progression of prostate cancer in the TRAMP model was measured by MRI as described (36). Briefly, MRI experiments were done on a Varian system equipped with a 7.0 Tesla, 18.3 cm horizontal bore magnet (300 MHz proton frequency). For MRI examination, the mice will be anesthetized with sodium pentobarbital (70 mg/kg intraperitoneally) and maintained at 37°C inside the magnet using a heated circulation water blanket, with pelvis motion (due to respiration) minimized by a small plastic support placed before
insertion into a 3 cm diameter quadrature birdcage coil (USA Instruments). Multislice images were acquired using a T1-weighted spin echo sequence (TR/TE = 880/13, field of view = 30 x 30 mm using a 128 x 128 matrix, slice thickness = 1.5 mm, and slice separation = 1.0-1.6 mm). Each set contained 9 to 25 slices and enough sets were obtained to provide contiguous image data of the prostate tumor. Prostate volume will be measured using the formula: \( V = \frac{4}{3}(D_1 + D_2) / 4\pi \), where \( D_1 \) and \( D_2 \) correspond to the longest and shortest (transverse and sagittal) diameters measured from the MRI image. The accuracy of this measurement was confirmed by comparing preneoplastic MRI volumes with postnecropsy actual prostate volumes in select cases.

Results

**Anti-B7-1/B7-2 antibody treatment of young TRAMP mice reduced Treg cells in both the thymus and the periphery and delayed development of prostate cancer.** We and others have reported that targeted mutation of CD28 and B7-1/B7-2 abrogated the generation of Treg cells (23). To test whether this pathway can be targeted for transient reduction of Treg, we treated C57BL/6 mice with either anti-B7-1/B7-2 mAbs or control IgG five times every other day. Thymi and spleens were harvested 8 days after the last injection. Cells were stained for flow cytometry analysis. This treatment did not affect either the total cellularity or the numbers of CD4 and CD8 T cells (Fig. 1A). However, the numbers of CD4+FoxP3+CD25+ cells were reduced by 50% in thymus and by 4-fold in the spleen (Fig. 1B). When gated on lymphocyte gate, all CD4+ T cells are CD3+ (Supplementary Fig. S1). Therefore, all FoxP3+CD25+ cells analyzed in this study are Treg. These data indicate that Treg cells can be significantly reduced in both the thymus and the spleen by anti-B7-1/B7-2 antibodies.

To investigate whether anti-B7-1/B7-2 antibody treatment delay the development of prostate cancer, 4-week-old male TRAMP mice were treated with either control IgG or anti-B7-1/B7-2 antibodies and the incidence of cancer development was followed by physical examination. Using 50% of mice with palpable prostate cancer as a reference point, we observed that anti-B7 delayed the tumor development by >14 weeks (Fig. 1C). Therefore, anti-B7 treatment may be valuable for prevention of prostate cancer development.

**Enhanced tumor specific cytotoxicity after anti-B7-1/B7-2 antibody treatment.** To test tumor antigen-specific immunity following anti-B7-1/B7-2 treatment, we further investigated the tumor-specific cytotoxicity by an in vivo killing assay. Six-week-old male TRAMP mice were injected intraperitoneally with anti-B7-1/B7-2 mAbs or control IgG five times every other day. Two weeks after the first injection, they received an intravenous injection of a 1:1 mixture of SV40 TAg peptide-pulsed (CFSElo) and control HSV gB peptide-pulsed (CFSEhi) spleen cells. The spleens were harvested 20 h later and analyzed by flow cytometry. As shown in Fig. 2A, in mice treated with anti-B7 antibodies, the SV40 TAg-pulsed targets were preferentially eliminated, whereas the CFSEbi and CFSElo cells remained at the 1:1 ratio in control immunoglobulin-treated mice. These data showed that anti-B7 treatment enhanced CTL response against the SV40 large TAg without intentional immunization.

**Anti-B7 antibodies rescued SV40 large T-specific T cells from clonal deletion in the TRAMP mice.** Our previous studies have shown that SV40 large TAg is expressed in the thymic peripheral antigen-expressing cells in the TRAMP mice and that such expression caused nearly complete deletion of transgenic T cells expressing a TCR specific for a SV40 large TAg peptide presented by H-2Kk (7). Moreover, we reported that perinatal blockade of B7-1 and B7-2 reduced clonal deletion of autoreactive T cells (26). To test whether the anti-B7 treatment rescues SV40 TAg-specific T cells from clonal deletion in the TRAMP mice, we produced TRAMP mice expressing the SV40 TAg-specific TGB TCR and divided the double transgenic mice with either anti-B7 mAbs or control IgG treatment groups.

As the mice recovered from irradiation, a new wave of bone marrow-derived cells will differentiate into mature T cells in the thymus. This de novo process increases sensitivity of blocking studies (37). To study the effect of anti-B7 treatment on newly formed T cells undergone thymic development and clonal deletion, we gave sublethal irradiation (500 rad) to TGB single transgenic and TRAMP/TGB double transgenic mice. At 1 week after six treatments, the thymic cellularity and mature CD8 T cells were measured by flow cytometry. As shown in Fig. 2B, due to clonal deletion, the numbers of reconstituted thymocytes were extremely low in the double transgenic TGB-TRAMP mice compared with single transgenic TGB. Importantly, anti-B7 treatment increased thymic cellularity by ~10-fold (Fig. 2B). A corresponding increase in the CD8 T cells expressing high levels of Vj88 transgenic TCR was observed in both spleen and thymus (Fig. 2C, left). When the spleen cells were analyzed for CD4/CD8 T cell ratios, it was clear that, perhaps due to clonal deletion, T cells in the control immunoglobulin-treated mice have lost the predominance of CD8 subset due to expression of MHC class I-restricted TCR. This is corrected to a large extent by anti-B7 treatment (Fig. 2C, right). Thus, anti-B7 treatment greatly reduced efficiency of clonal deletion. However, the numbers of transgenic T cells in the anti-B7-treated TGB-TRAMP mice were still much reduced in comparison with TGB mice, which showed that the rescue is only partial.

To test whether the T cells rescued by anti-B7 treatment were responsive to tumor antigen, we stimulated spleen cells from control immunoglobulin or anti-B7-treated mice with different concentration of the SV40 TAg peptide or control peptide from HSV peptide. As shown in Fig. 2D, anti-B7-treated spleen cells underwent a significant proliferation to SV40 TAg peptide. Based on the dose response, the anti-B7-treated spleen cells were at least 100-fold more responsive than the control immunoglobulin-treated spleen cells, which corresponded to increased number of antigen-specific T cells. Therefore, the anti-B7 rescued T cells are functional. However, after in vitro stimulation, the rescued T cells showed poor cytotoxicity (data not shown), which suggests that the rescued T cells may be functionally impaired to some extent.

**Anti-B7-1/B7-2 antibody treatment cause significant albeit transient reduction of Treg in mice with established prostate cancer.** One of the most difficult challenges in cancer immunotherapy is the treatment of established solid tumors. It has been shown that microscopic lesion of prostate cancer can be observed in the TRAMP mice between ages 18 and 24 weeks (29). To confirm the development of tumor in the 25-week-old TRAMP mice in our colony, we used the MRI to compare the size of the prostate at 25 weeks. As shown in Fig. 3A, all of the 12 TRAMP mice tested had considerably larger...
prostate organ sizes compared with non-TRAMP littermate. Thus, essentially all of the 25-week-old TRAMP mice developed cancer in the prostate.

To determine the effect of anti-B7 antibodies for B7-1 and B7-2, we injected either control or anti-B7 mAbs every other day for five times. The blood samples were collected at 0, 1, 2, or 6 weeks after antibody treatment and stained for either anti-CD25 or anti-Foxp3 in conjunction with anti-CD4. As shown in Fig. 3B, in comparison with control immunoglobulin-treated mice, significant reduction of Treg can be observed in the peripheral blood at 1 and 2 weeks after completion of the treatment. Interestingly, the number of Treg is restored to normal levels at 6 weeks after completion of the treatments. Thus, in mice bearing established prostate cancer, anti-B7-1 and anti-B7-2 antibodies caused a significant albeit transient reduction of Treg in tumor-bearing mice.

Fig. 1. Anti-B7-1/B7-2 antibody treatment reduced Treg in both the thymus and the periphery of normal mice and delayed the development of palpable tumors in TRAMP mice. A and B, C57BL/6 mice were injected intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μg 3A12 and 100 μg GL-1) or control IgG (1:1 mixture of 100 μg hamster and 100 μg rat IgG) at 6 wk old five times every other day. The mice were sacrificed 8 d after the last injection. Thymocytes and splenocytes were harvested and analyzed by flow cytometry. A, anti-B7-1/B7-2 antibody treatment did not alter the number of thymocytes, spleen cells, and CD4 and CD8 subsets. B, anti-B7-1/B7-2 antibody treatment reduced CD25+Foxp3+ cells in both thymus and spleen. Plots are from CD4+ cells among the lymphocyte gates. *, P < 0.05; **, P < 0.01, Student’s t test.

C, Kaplan-Meier analysis of tumor incidence. The experimental endpoint is 2 cm in tumor diameter as determined by palpation. Data have been repeated three times.
Anti-B7 antibodies delayed growth of established prostate cancer without autoimmune side effects. To determine whether anti-B7 antibodies can confer therapeutic effect in mice with established prostate cancer, we randomly divided 25-week-old TRAMP mice into two groups and measured their tumor size before the treatment with either control immunoglobulin or anti-B7 antibodies, starting at 25 weeks. After five injections, the mice were followed for the tumor progression by either palpation or MRI. As shown in Fig. 3C, at age 33 weeks (8 weeks after first treatment), in the control IgG-treated group, the volume of prostate expanded by 2.5- to 9-fold with an average of >4.5-fold. In contrast, all but one anti-B7-treated mice show <2-fold expansion of the prostate volume. Mann-Whitney test indicate that the difference was statistically significant ($P = 0.04$). Because the tumors are not palpable at the beginning of the treatment, we also used the time when the mice developed palpable tumors as a second endpoint with larger sample size (12 mice for each group). As shown in Fig. 3D, even treated as late as age 25 weeks, the anti-B7 antibodies delayed tumor development by ~7 weeks.

In the TRAMP model, lymph node metastasis has occurred at 25 weeks (29); we therefore tested the effect of anti-B7 treatment on metastatic lesions in other organs, including lung, kidney, and liver. As shown in Fig. 4A, 3 of 6 mice in the control immunoglobulin-treated group have substantially higher number of metastatic lesions in lung. In addition, massive metastatic lesions were found in kidney (1 of 6) and liver (2 of 6) (data not shown). Only one case of metastasis was observed in the anti-B7 treated group, and the metastasis is limited to the lung. In addition, the metastatic lesions in the anti-B7-treated group were substantially smaller than those found in the control immunoglobulin-treated group (Fig. 4A).

Corresponding to reduced tumor growth, we have observed increased T-cell infiltrating into tumors. Immunohistochemistry

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**Fig. 2.** Anti-B7-1/B7-2 antibody treatment enhanced TAg-specific CTL and partially rescued clonal deleted TAg-specific T cells in the TRAMP mice. A, 6-week-old male TRAMP mice were treated intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μg 3A12 and 100 μg GL-1) or control IgG (1:1 mixture of 100 μg hamster and 100 μg rat IgG) five times every other day. Two weeks after the first injection, mice received an intravenous injection of a 1:1 mixture of TAg peptide-pulsed (CFSE<sup>−</sup>) spleen cells (5 x 10<sup>6</sup> each). Twenty hours later, the spleens were harvested and analyzed by flow cytometry. Left, representative profiles; right, summary of two experiments involving a total of 6 mice per group. CFSE<sup>−</sup> and CFSE<sup>+</sup> cells are gated as indicated. The number shown in the gates are the percentage of gated cells. B to D, anti-B7 treatment rescued tumor-reactive T cells from clonal deletion. TRAMP/TGB double transgenic or TGB single transgenic mice that received sublethal irradiation (500 rad) were treated with either control immunoglobulin or anti-B7 mAbs five times every other days. The thymocytes and splenocytes were harvested on day 7 after final treatment and analyzed by flow cytometry. B, thymocyte and splenocyte cellularity in TGB single transgenic and TRAMP/TGB double transgenic mice treated with control immunoglobulin or anti-B7 antibodies. C, increase of CD8<sup>+</sup> V<sub>α8</sub><sup>high</sup> T cells in thymus and spleen. Left, summary of CD8<sup>+</sup> V<sub>α8</sub><sup>high</sup> cell number change in thymus and spleen from TRAMP/TGB double transgenic mice; right, representative profiles of CD4 and CD8 T cells in the spleens of control immunoglobulin or anti-B7-treated mice. The left flow cytometry shows those for total spleen cells, whereas the right flow cytometry shows those for gated V<sub>α8</sub><sup>high</sup> cells. D, antigen reactivity of T cells rescued by anti-B7. The splenocytes from TRAMP/TGB double transgenic mice were stimulated with either SV40 TAg peptide or control HSV peptide for 72 h and pulsed with [<sup>3</sup>H]thymidine to determine the rate of T cell proliferation. B to D, mean ± SE (n = 5). The conclusions have been confirmed with another independent experiment.
Fig. 3. Anti-B7-1/B7-2 mAb treatments of mice with established prostate cancer inhibited cancer progression. A, MRI measurement of prostate volumes of 25-week-old normal and TRAMP mice. Left, representative local images of male B6 and TRAMP mice. The prostate were identified with thick white outlines. Right, prostates sizes of 3 B6 and 12 TRAMP mice, all at age 25 wk. B and C, anti-B7 treatment initiated at 25-week-old TRAMP mice transiently depleted Treg. Male TRAMP mice were administered intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μg 3A12 and 100 μg GL-1) or control IgG (1:1 mixture of 100 μg hamster and 100 μg rat IgG) five times every other day. Peripheral blood was taken at 0, 1, 2, and 6 wk; 0 wk is the day before injection. Cells were stained for flow cytometry. Plots are gated on CD4+ cells. B, CD25 FoxP3+ cell number started to reduce following the first week of treatment and almost recovered to normal levels 1 mo after the treatment was stopped. Data have been repeated two times, involving a total of 12 mice per group. D, MRI image of TRAMP mice at 25 and 33 wk (8 wk after starting treatments with either control immunoglobulin or anti-B7 mAbs). Summary data are ratio of prostate volumes at 33 versus 25 wk when the treatments started. D, Kaplan-Meier analysis for incidence of palpable tumors in TRAMP mice treated with either control immunoglobulin or anti-B7 antibodies at age 25 wk.
Fig. 4. Anti-B7 blockade in tumor-bearing mice reduces the number and size of metastatic lesions in the TRAMP mice and increases infiltration of T cells into tumors but does not cause autoimmunity. A, internal organs of mice from Fig. 3C were analyzed for metastatic lesions. Three sections of liver, lung, kidney, intestine, and heart, 30 μm apart, were examined double-blind by a pathologist. A representative field of lung sections of control immunoglobulin-treated mice (3 of 6 mice analyzed have metastasis) and the only metastatic lesion in anti-B7 treated group are shown. Yellow arrows, metastatic lesions. In the control immunoglobulin-treated group, massive metastases were also observed in the liver (2 of 6) and kidney (1 of 6). B to D, mice from Fig. 3C were analyzed for infiltrating lymphocytes and autoimmune reactions. B, representative tumor sections stained with anti-CD3 mAb. C, fluorescence-activated cell sorting profiles showing representation of CD4 and CD8 T cells and the CD4+CD25+Foxp3+ T cells. Top left, profiles of mononuclear cells isolated from the tumor; bottom left, profiles from the gated CD4 T cells. Data are from pooled cells from 6 mice per group. Top right, frequencies of CD4 and CD8 T cells among mononuclear cells isolated from the prostate cancer; bottom right, ratio of FoxP3 over CD4 or CD8 T cells. Mean ± SE (n = 6). D, serum anti-double-stranded DNA antibodies. Data are Δ490 from an ELISA using 1:50 dilution of sera. Mean ± SE (n = 6).
staining revealed an increased numbers of T-cell infiltration (Fig. 4B). Quantitative analysis by flow cytometry indicated that the frequency of T cells among the mononuclear cells from the collagenase-treated prostate cancer tissue increased by 4-5 fold, with the majority of the T cells are of CD8 subsets (Fig. 4C). In both groups, higher percentage of CD4+ T cells expressed Foxp3 than what was found in the lymphoid organ (Fig. 4C, bottom left), similar to observations made by others (33). Nevertheless, the percentage of Treg is significantly lower in the anti-B7-treated group. Moreover, the ratio of Treg over effector CD4 and CD8 T cells significantly decreased in anti-B7-treated group (Fig. 4C, bottom right). Therefore, anti-B7 treatment alters the ratio of Treg over effector T cells in the tumor, presumably in favor of local immune response.

A general concern for immunotherapy of cancer is autoimmune side effect. To determine whether autoantibodies were induced in tumor-bearing TRAMP mice, the sera were collected at 1, 2, and 6 weeks after the start of anti-B7-1/B7-2 antibody treatments. The anti-double-stranded DNA antibodies were detected by ELISA. As shown in Fig. 4D, although an increase in anti-DNA antibodies was detected at 6 weeks after control immunoglobulin treatment, presumably due to tumor growth, such increase was not observed in the anti-B7-treated mice. Histologic analysis showed no inflammation of internal organs in either group (data not shown). Therefore, anti-B7 antibodies can induce significant protection against established tumor without eliciting autoimmune side effect.

**Anti-B7 antibodies inhibit MC38 colon carcinoma cell growth.** To confirm the general antitumor effect of anti-B7 treatment, we tested it with MC38 colon carcinoma tumor model. Male C57BL/6 mice were injected 5 × 10^6 MC38 tumor cells subcutaneously. Ten days after injection, mice developed palpable tumors and were divided evenly into two groups based on the tumor sizes. MC38 tumor-bearing mice were administered intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μg 3A12 and 100 μg GL-1) or control IgG three times every other day. Peripheral blood samples were taken at 0 and 6 days, and spleens were collected at 14 days after completion of antibody treatments. As shown in Fig. 5A, at 6 and 14 days after anti-B7 antibody treatment, the CD4+CD25+ Foxp3+ Treg cells were significantly reduced. Correspondingly, anti-B7 treatment conferred a significant reduction in the growth rate of MC38 colon carcinoma (P = 0.035; Fig. 5B).

**Transient depletion of Treg by anti-CD25 antibody delays established prostate cancer growth in TRAMP mice.** To test whether transient depletion of Treg alone inhibits tumor growth, we treated 25-week-old TRAMP mice with anti-CD25 antibody to deplete CD4+CD25+ cells. Male 25-week-old TRAMP mice were examined by MRI to measure prostate size (6 mice per group). The TRAMP mice were divided into two groups based on the tumor sizes. The mice were administered intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μg 3A12 and 100 μg GL-1) or control IgG three times every other day. Peripheral blood was taken at 0 and 6 d, and spleen was collected at 14 day; 0 day is the day before the administration of antibodies. Cells were stained for flow cytometry. The average after/before treatment prostate size ratio in anti-CD25 group was substantially less effective than transient B7 blockade (the average after/before treatment prostate size ratio in anti-B7 treated group; however, the Foxp3+CD25+ cells were fully recovered to normal levels at 5 weeks after the treatment. Five weeks after anti-CD25 treatment when mice reached age 30 weeks, two groups of TRAMP mice were reexamined by MRI. As shown in Fig. 6B, the prostate sizes were enlarged by 3- to 5-fold during the 5-week period due to the aggressive prostate cancer growth. Compared with the control group, the prostate sizes were increased by 2- to 3-fold in anti-CD25-treated group (Fig. 6C). The significant difference revealed an effect of Treg depletion on tumor growth. However, this treatment is substantially less effective than transient B7 blockade (the average after/before treatment prostate size ratio in anti-CD25...
treatment group is 2.55 after 5 weeks compared with anti-B7 treatment average ratio is 1.72 after 8 weeks; Fig. 3).

Discussion

Traditionally, blockade of costimulatory molecules B7-1 and B7-2 has been explored for treatment of autoimmune diseases and transplant rejection (38). Recent studies that reveal a critical role for B7-1/B7-2 in the production and maintenance of Treg (23–25) and in clonal deletion of self-reactive (26) as well as cancer-reactive T cells (7) suggest that this pathway may be targeted for overcoming the barrier of immune tolerance in cancer setting. The data described herein showed unexpected efficacy of this new approach.

We have chosen the TRAMP mice, which developed malignant transformation of prostate epithelial cells as early...
as 12 weeks to test this notion. Our data showed that a short-term anti-B7 blockade before the development of pathologic lesions delays the development of palpable tumor for ~14 weeks. These data show that a short-term anti-B7 treatment may prevent the development of prostate cancer among individuals with predisposition of prostate cancer.

It is generally agreed that immunotherapy is very inefficient for treatment of established tumors (39). This can be more challenging in transgenic tumor models where malignant tumor cells continue to arise due to transgenic expression of oncogenes. Our data showed that, even when administered at a time when the TRAMP mice show >3-fold enlargement of prostate size, transient blockade of B7-1 and B7-2 dramatically reduced the rate of tumor growth. Thus, at 8 weeks after initiation of the treatment, the prostate of the control immunoglobulin-treated expanded by 5-fold in volume. In contrast, those from anti-B7-treated mice expanded by <2-fold during the same period. When the palpable tumors were used as endpoint, the anti-B7 treatment at 25 weeks reduced tumor development by 7 weeks. Nevertheless, perhaps because of the continuous production of new cancer cells from the germ-line insertion of SV40 large Tag and waning of antibodies, short-term treatment did not completely eradicated the tumors. Because the majority of tumors that developed in human have clonal origin, the malignant transformation is likely less frequent that what is observed in transgenic model of spontaneous tumors. Therefore, the relatively simple treatment may show greater efficacy. Given the broad function of B7-1 and B7-2 in host immune system, including T-cell costimulation at both priming and effector phases, Treg generation and maintenance, and clonal deletion, it is unlikely that a single mechanism is responsible for the therapeutic efficacy reported herein.

First, we have shown significant albeit transient reduction of Treg in both thymus and the peripheral blood. Because the treatment with anti-CD25 antibody also showed some efficacy in slowing prostate tumor growth in TRAMP mice, Treg depletion alone is sufficient to convey significant, although less marked, protection. It is worth noting that anti-CD25 depletion alone is sufficient to convey significant, although less marked, protection. It is worth noting that anti-CD25 antibody depletes almost 95% of CD4+CD25+ cells in 6 days; however, 60% of CD4+Foxp3+ cells still remained in peripheral blood at the same time. Because the treated mice had more CD25 Foxp3+ cells than the untreated cells, anti-CD25 ablated part of CD25+Foxp3+ cells and down-regulation of CD25 on others. On the other hand, anti-B7 treatment caused similar extent of reduction in the CD4+Foxp3+ cells regardless of their CD25 phenotype. It is unclear whether the different depletion profile contributed to different efficacy.

Interestingly, the number of Treg returns to normal levels at 6 weeks after reconstitution. It is therefore of interest why the antitumor effect appears to have lasted long after the frequency of Treg is restored. In this regard, it should be emphasized that in vitro Treg reconstitution is almost universal for all methods of Treg depletion, including antibody elimination and treatment of toxin targeting Treg that express the specific receptor for the toxin (40–42). In all cases, however, restoration of Treg did not prevent the immune response against antigen or pathogen. These studies suggested that numerical restoration of Treg is usually not accompanied by immune suppression of ongoing immune response and therefore made it plausible that temporary reduction of Treg can promote cancer immunity.

Second, in line of the function of B7 in clonal deletion of autoreactive T cells, including some tumor-reactive T cells, it is possible that anti-B7 treatment also rescues some tumor-reactive T cells that are otherwise deleted. In this regard, we showed that transient blockade of B7-1 and B7-2 reduced the clonal deletion of SV40 T-reactive CTL. Therefore, it is likely that anti-B7 blockade may also increase the frequency of tumor-reactive T cells. Taken together, by reducing the burden of Treg and increasing the frequency of cancer-reactive T cells, B7 blockade resets the balance between regulatory burden and effector function. These two factors provide plausible explanation for the prevention described herein. Because the TGB mice do not survive long enough for us to study clonal deletion at 25 weeks, due to insertional mutation by TCR transgene (43), the effect of rescue of tumor-reactive T cells in the therapy setting remains to be shown.

It is possible to argue that because the majority of cancer patients developed cancer late in their life when the thymic function has deteriorated, the rescue of TCR repertoire may be less relevant for cancer immunotherapy in humans. Nevertheless, we would like to point out that continuous production of T cells has been shown throughout the lifespan (44). Moreover, it is worth pointing out that hormone ablation is part of the standard therapy for prostate cancer. An unexpected benefit of this therapy is reinvigoration of thymic function (45). Therefore, it may be valuable to combine anti-B7 blockade with hormone ablation in human prostate cancer treatment.

Finally, it is worth pointing out that blockade of B7-1 and B7-2 with their soluble receptor CTLA4Ig has been approved for therapy of autoimmune disease with little side effect (38). In this study, we showed that, despite the modulation of Treg and rescue of potentially self-reactive T cells, anti-B7 blockade does not trigger autoimmune side effect. The availability of a safe drug makes blockade of B7-1 and B7-2 an attractive approach for the cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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