

Altered Immunity Accompanies Disease Progression in a Mouse Model of Prostate Dysplasia

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Abstract

Increasing evidence suggests that altered immune function accompanies, and indeed may facilitate, cancer progression. In this study, we sought to determine the nature of, and cellular mechanisms underlying, changes in immune status during disease progression in a transgenic mouse model of prostate dysplasia. Immune cells in the tumor micro-environment, as well as in the secondary lymphoid tissues, displayed altered phenotypes. Although evidence of anti-tumor immunity was detected, there was a paradoxical decrease in the ability of T cells to proliferate *in vitro* at later stages of disease progression. Detailed analysis of the draining lumbar lymph nodes revealed an increased frequency and number of CD4⁺CD25⁺ T cells and an enhanced production of inhibitory cytokines, which correlated with impaired T-cell function. Functional studies confirmed a role for CD4⁺CD25⁺ regulatory T cells in suppressing T-cell proliferation as well as regulating the growth of transplanted prostate tumor cells. In addition, our studies show for the first time that anti-CD25 antibody treatment reduces, but does not prevent, tumor growth in a transgenic mouse model of prostate dysplasia. Taken together, this work provides compelling evidence that prostate tumor progression is accompanied by altered immune function and, moreover, that regulatory T cells play an important role in this process. These studies thus provide the impetus for development of specific and effective strategies to deplete regulatory T cells, or suppress their function, as an alternative or adjunct strategy for reducing tumor growth. (Cancer Res 2005; 65(7): 2947-55)

Introduction

Prostate cancer is a major cause of morbidity and the second leading cause of cancer mortality among North American men (1). Despite the effectiveness of surgery or radiation therapy for the treatment of early-stage disease, there is currently no effective strategy for late-stage disease. Manipulation of the immune system to induce antitumor immunity has been extensively tested as an alternative approach to specifically and effectively reduce the growth of late-stage tumors. Indeed, immune-based therapies, such as cytokine and vaccine approaches, have been used in clinical trials to treat various types of cancer including melanoma, renal cell carcinoma, and prostate cancer (2–6). One strategy that holds great promise is the use of dendritic cells as a vector for presenting

tumor antigens and thus initiating antigen-specific antitumor immune responses (7). The specificity of this approach ensures that toxic side effects are minimal, a vast improvement over currently existing treatments. However, the majority of cancer patients enrolled in such trials showed only partial response without complete eradication of tumors (3). One possible explanation is that tumor progression has been linked to increasing immune suppression in prostate cancer patients (1, 8). A clearer understanding of how immune function is modulated by tumor cells is required to devise strategies to enhance the efficacy of such therapeutic interventions.

There is evidence that tumor infiltration by dendritic cells, the most efficient antigen-presenting cells in the immune system, is decreased in patients with advanced prostate cancer, suggesting one mechanism by which the tumor suppresses immunity (9). In addition, recent studies have shown that human or murine prostate tumor cells, or the soluble factors they secrete, alter the maturation of bone marrow-derived or monocyte-derived dendritic cells, inhibit their allostimulatory capacity, and induce apoptosis (10–12). Owing to dendritic cells having essential roles in the generation of T-cell-mediated immune responses and in the maintenance of central and peripheral tolerance (13, 14), it is more likely that altered immunity during tumor progression reflects changes in the phenotype and/or function of dendritic cells. Recently, *in vitro* studies have shown that repetitive stimulation of naïve T cells with immature dendritic cells results in the generation of regulatory T-cell activity (15). Additional studies suggest that dendritic cell-mediated peripheral tolerance may involve the induction and/or expansion of regulatory T cells (16). It has been observed that the frequency of CD4⁺CD25⁺ regulatory T cells is elevated in tumor sites and/or the peripheral blood of patients with advanced tumors (17–20). Further evidence to suggest a functional role for these cells in tumor progression and immune suppression is the observation that transient elimination of this suppressive cell population with anti-CD25 monoclonal antibody (mAb) treatment results in effective rejection of transplanted tumors derived from leukemia, myeloma, sarcoma, and B16 melanoma (21–23).

These observations lead to the hypothesis that prostate tumor cells progressively alter the phenotype and/or function of dendritic cells, thus facilitating the accumulation of regulatory T cells. These regulatory T cells play a critical role in suppressing immune responsiveness and thus allow tumor growth to continue. In this study, we tested these hypotheses by assessing immunologic status as a function of disease progression in the 12T-7s LPB-Tag transgenic mouse model of prostate dysplasia. Although adenocarcinoma and metastasis are rarely observed in this model, these transgenic mice develop reproducible prostate-specific tumors that can progress to mouse prostatic intraepithelial neoplasia similar to human prostatic intraepithelial neoplasia (24, 25). Our results show that prostate tumor cells alter the phenotype and frequency of dendritic cells and CD4⁺CD25⁺

Note: A.H. Tien and L. Xu contributed equally to this work.

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regulatory T cells in the tumor microenvironment. Dendritic cells and CD4⁺CD25⁺ regulatory T-cell populations also expand in the lymph nodes draining the prostate. *In vitro* analyses revealed that the proliferative capacity of T cells from the lymph nodes was significantly reduced by the CD4⁺CD25⁺ regulatory T-cell population. As shown previously with other tumors, anti-CD25 mAb treatment reduced the growth of transplanted prostate tumor cells. More significantly, we provide the first experimental evidence that such a therapeutic approach is also capable of reducing tumor growth in the 12T-7s transgenic prostate tumor mouse model. These studies suggest that understanding the mechanisms by which regulatory T cells arise during tumor progression and developing effective strategies to block their generation or function may have important implications for overcoming the limitations of existing immunotherapeutic approaches to prostate cancer treatment.

Materials and Methods

Mouse. The 12T-7s LPB-Tag transgenic mouse model of prostate dysplasia has been described previously (24, 25) and was kindly provided by Dr. Susan Kasper and Dr. Robert J. Matusik. In these mice a large promoter fragment of the probasin gene (*LPB*) is linked to the SV40 large T antigen (Tag) deletion mutant to deliver consistently high levels of transgene expression to the mouse prostate tissue. Tumor formation is observed in 100% of the male transgenic offspring and the characteristics of tumor progression are similar to that observed in humans. Transgenic mouse strains were maintained by mating wild-type (WT) CD1 males with transgenic females and the progeny were screened by PCR analysis of tail DNA. C57BL/6J recipients of the transplanted prostate cancer cell line were purchased from the in-house breeding program. All mice were bred and maintained at the British Columbia Cancer Agency Animal Resource Centre with sterilized food, water, and bedding. All protocols were conducted according to guidelines set forth by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of British Columbia.

Phenotype analysis by flow cytometry. Spleen and various lymph nodes were collected from WT and transgenic mice of the indicated ages. Single cell suspensions were prepared by incubating the tissues in PBS with 2% fetal bovine serum (StemCell Technologies, Inc., Vancouver, BC, Canada), 5 mmol/L EDTA, and 100 µg/mL hyaluronidase (Sigma, St. Louis, MO) for 10 minutes at room temperature or by incubating tissue fragments with collagenase (StemCell Technologies) for 30 minutes at room temperature with the addition of 10 mmol/L EDTA for the final 5 minutes. Prostate tissues collected from WT and transgenic mice were minced into small pieces and treated with collagenase (StemCell Technologies) for 30 minutes at 37°C with the addition of 10 mmol/L EDTA for the final 5 minutes. Cells were then passed through 70 µm cell strainers. Single cell suspensions were washed twice with PBS/2% fetal bovine serum and then incubated on ice for 20 to 30 minutes with FITC-, phycoerythrin-, or allophycocyanin-labelled mAb. The antibodies used for phenotype analysis included: CD11c (HL3), CD4 (L3T4, RM4-5), CD8α (Ly-2, 53-6.7), CD25 (7D4, PC61), CD69 (H1, 2F3), CD80 (16-10A1), CD86 (GL1), CD40 (HM40-3), and Class II MHC IA/IE (2G9) (Becton Dickinson PharMingen, San Diego, CA). Cells were then washed thrice with the addition of 1 µg/mL propidium iodide (Sigma) in the final wash. Cells were analyzed by flow cytometry using a FACScalibur flow cytometer and CELLQuest software (Becton Dickinson).

Cell proliferation assay. Single cell suspensions of lumbar lymph nodes or spleen were prepared as outlined above. Cells were cultured in triplicate in round-bottomed 96-well plates at 1×10^5 cells per well (0.2 mL) in the presence or absence of various concentrations (0.1-10 µg/mL) of purified anti-CD3 mAb (clone 145-2C11; Becton Dickinson). The culture medium consisted of Iscove's modified Dulbecco's medium (StemCell Technologies) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine, and 115×10^{-6} mol/L

monothioglycerol (Sigma). Proliferation was measured by scintillation counting of each sample following addition of 1 µCi of [³H]thymidine (Perkin-Elmer Life Science, Inc., Boston, MA) per well during the final 16 to 18 hours of a 72-hour culture period at 37°C in 5% CO₂. Data were plotted as either raw counts per minute (cpm) values or cpm ratio in which antibody-induced cpm was divided by the unstimulated cpm levels to control for differences in responder T-cell numbers arising from the altered T-cell frequency in the starting populations.

Purification and functional assessment of dendritic cells and CD4⁺CD25⁺ T cells. Splenic CD11c⁺ dendritic cells were purified from WT or transgenic mice (>20 weeks of age) by positive selection (anti-phycoerythrin-CD11c mAb) using EasySep (StemCell Technologies) and activated with bacterial lipopolysaccharide (Sigma) for 18 hours. Various numbers of activated dendritic cells were then cocultured with 1×10^5 T cells isolated (using SpinSep; StemCell Technologies) from the spleen of C57BL/6J mice. Incorporation of [³H]thymidine was assessed as indicated above.

For analysis of the CD4⁺CD25⁺ T-cell population, CD4⁺ T cells were first isolated from lymph node cell suspensions of WT and transgenic mice (≥20 weeks of age) by negative selection using the mouse CD4⁺ T-cell isolation kit (MACS system; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Following isolation of CD4⁺ T cells, cells stained with phycoerythrin-CD25 mAb were purified using anti-phycoerythrin-coupled magnetic beads (Miltenyi Biotec). CD4⁺CD25⁺ T cells were obtained with purity greater than 90%. Alternatively, CD4⁺CD25⁺ T-cell depletion was achieved from lumbar lymph node cell suspensions of WT and transgenic mice (≥20 weeks of age) by fluorescence-activated cell sorting (FACSVantage, Becton Dickinson). Purity of the resultant cell population was >99%. In one series of experiments, spleen cells (1×10^5 cells per well) were stimulated with soluble anti-CD3 mAb (1 µg/mL) in the presence of various numbers of CD4⁺CD25⁺ T cells purified from either WT or transgenic lumbar lymph nodes. In the second series of experiments, proliferation of the lymph node cell population depleted of CD4⁺CD25⁺ T cells was assessed as described above. [³H]thymidine incorporation was measured at 72 hours and results were analyzed as described above.

Determination of cytokine release. For analysis of cytokine production by CD4⁺ T-cell subpopulations, CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were purified from ≥20-week-old transgenic mice using the MACS system. Purified cells were cultured at 1×10^6 to 1.5×10^6 cells/mL with plate-bound anti-CD3 mAb (2 µg/mL) plus anti-CD28 mAb (1 µg/mL) and cross-linking immunoglobulin G (IgG) mAb (1 µg/mL). Supernatant samples were collected at 24 hours [for interleukin (IL)-2] and 72 hours [IL-10 and interferon (IFN)-γ]. Cytokine levels were analyzed by cytometric bead array (Becton Dickinson PharMingen). Results are the mean ± SE for duplicate wells for four to six samples (each representing cells pooled from three to four mice).

Cell lines and culture. The hybridoma secreting the anti-CD25 mAb (clone PC61, rat IgG1) was purchased from the American Type Culture Collection. The antibody was precipitated from the culture supernatant using two rounds of 40% ammonium sulfate. IgG used for the control injection was precipitated from the serum of normal rats. The murine prostate cancer cell line transgenic adenocarcinoma of mouse prostate (TRAMP)-C2 cell line (26), kindly provided by Dr. N.M. Greenberg, was maintained in high-glucose DMEM (StemCell Technologies) with 5% Nu-Serum IV (Becton Dickinson), 5% fetal bovine serum, 5 µg/mL insulin (Sigma), 10^{-8} mol/L dihydrotestosterone (Sigma), 25 units/mL penicillin, 25 µg/mL streptomycin, and 2 mmol/L L-glutamine. Cultured TRAMP-C2 cells were washed thrice with PBS and resuspended to the appropriate dilution (5×10^6 cells in 0.2 mL PBS) for injection.

Tumor cell transplantation and anti-CD25 monoclonal antibody treatment *in vivo*. C57BL/6J mice (~8 weeks of age) were inoculated s.c. in the flank with 5×10^6 TRAMP-C2 tumor cells (0.2 mL in PBS), Rat IgG or anti-CD25 mAb (PC61), prepared as outlined above, was injected i.v. at a concentration of 1 mg per mouse in 0.25 mL PBS on day -1 and day +3 with respect to the day of tumor transplant. Tumor development was assessed twice to thrice weekly by palpation. 12T-7s mice were treated with two doses of either control rat IgG or anti-CD25 mAb (0.65 or 1 mg/mouse; i.v. injection) at 8 or 12 weeks of age.

TRAMP-C2 recipients were sacrificed for tumor weight analysis at 6 weeks posttransplant whereas 12T-7s mice were sacrificed for analysis at 20 weeks of age. There were at least six mice per group in two to three independent experiments.

Results

Altered phenotype of dendritic cells and regulatory T cells in the prostate tumor microenvironment. To facilitate development of effective immune-based therapies for prostate cancer, a comprehensive understanding of immune function during tumor progression must be achieved. The 12T-7s prostate tumor mouse model mimics the earliest stages of human prostate cancer progression with tumors progressing to mouse prostatic intraepithelial neoplasia. Thus, this model is suitable for examining immune alterations caused by tumors that escape immune surveillance during the earliest stages of tumor development. Recent studies have shown that the tumor microenvironment alters dendritic cell phenotype and function and contains significant numbers of regulatory T cells (9, 20). We thus investigated the immune cellular composition of the prostate tumor microenvironment in the 12T-7s mice at a stage when the tumor has progressed to high-grade dysplasia (24). The frequency of CD11c⁺ dendritic cells (WT: $0.75 \pm 0.32\%$ versus transgenic: $0.99 \pm 0.25\%$) and CD4⁺ T cells (WT: $0.33 \pm 0.13\%$ versus transgenic: $0.49 \pm 0.17\%$) was increased slightly in the tumor sites, whereas the frequency of CD8⁺ T cells was decreased (WT: $1.11 \pm 1.29\%$ versus transgenic: $0.67 \pm 0.46\%$).

We next evaluated the phenotype of the prostate-infiltrating immune cells. The proportion of tumor-infiltrating CD11c⁺ cells expressing the costimulatory molecule CD86 or MHC class II was reduced significantly (29.1% and 32.2%, respectively) as indicated in Fig. 1A. Expression of MHC class II was also significantly reduced on a "per cell" basis (assessed by the mean fluorescence intensity), whereas CD80 levels were significantly higher (Fig. 1B). In addition to the phenotypic alterations observed on the tumor-infiltrating dendritic cells, differences in the T-cell populations were also detected. Intriguingly, the proportion of CD8⁺ or CD4⁺ T cells expressing the activation marker CD69 was elevated in the transgenic prostate tissue. Of further note was the observation that the proportion of CD4⁺CD69⁻ T cells expressing CD25 was also significantly increased in the tumor microenvironment (Fig. 1C). In all cases, there was a corresponding increase in the mean fluorescence intensity (Fig. 1D). These results suggest that the tumor microenvironment alters the phenotype of the tumor-infiltrating immune cells. Further study is clearly required to determine the functional capacity of these tumor-infiltrating lymphocytes.

Immune cell numbers expand in secondary lymphoid tissues during prostate tumor progression. To determine if alterations in immune cell phenotype extend beyond the tumor microenvironment, we analyzed the phenotype of immune cells in the secondary lymphoid tissues (i.e., spleen and lymph nodes) of WT and transgenic mice. Tissues were isolated at various stages of tumor progression, including 11 to 12 weeks of age when the entire epithelium is in the process of becoming hyperplastic, 16 to 17 weeks of age when reactive stromal proliferation is observed, and 20 to 25 weeks of age when the tumor has progressed to high-grade dysplasia (24). At the early stages of tumor development (11-12 weeks) no significant differences were observed in the cellularity of the spleen or the various lymph nodes (i.e., submandibular,

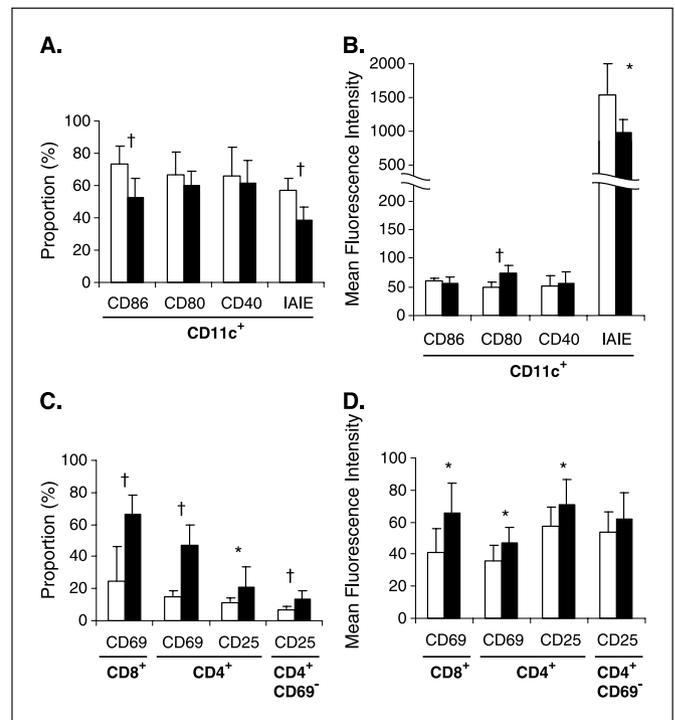


Figure 1. Immunophenotype of tumor-infiltrating dendritic cells and T cells is altered at the late stage (>20 weeks of age) of tumor progression. *A*, proportion of CD11c⁺ cells expressing the indicated cell surface molecules. *B*, mean fluorescence intensity of various cell surface molecules on CD11c⁺ cells. *C*, proportion of CD8⁺ or CD4⁺ T cells expressing CD69 or CD25 and the proportion of CD25⁺ cells within the CD4⁺CD69⁻ cell population. *D*, mean fluorescence intensity of various cell surface molecules on CD8⁺, CD4⁺, and CD4⁺CD69⁻ cells. Columns, mean ($n > 5$); bars, SD. Statistical analysis was carried out using the Student's *t* test. *, $P < 0.05$. †, $P < 0.01$. Open columns, WT; closed columns, transgenic.

inguinal, mesenteric, and lumbar) from WT and transgenic mice (data not shown). WT and transgenic mice also had similar frequencies of CD11c⁺ dendritic cells, CD4⁺ T cells, and CD8⁺ T cells in these tissues. In contrast, a modest elevation in total nucleated cell numbers was observed in the spleen (10-50%; data not shown) and various draining lymph nodes (Table 1) isolated from the oldest transgenic mice (≥ 20 weeks of age). This increase reached significance in the lumbar lymph node which is adjacent to the prostate gland. As indicated in Table 1, total nucleated cell numbers in each lumbar lymph node increased as a function of tumor progression (6- to 8-fold in transgenic mice ≥ 16 weeks of age). These observations suggest that tumor progression in this prostate tumor mouse model induces an *in vivo* expansion of immune cells.

Next the cellular compositions of the lumbar lymph nodes isolated from WT and transgenic mice (≥ 20 weeks of age) were compared to determine if there was a preferential expansion of selected cell populations or if all immune cells expanded similarly. Although the frequencies of CD11c⁺, CD4⁺, and CD8⁺ cells in the various lymph nodes and spleen did not differ significantly between WT and transgenic at the late stages of disease progression (data not shown), absolute numbers of all cell types were significantly increased compared with WT in the lumbar lymph nodes of transgenic mice (Table 1). CD11c⁺ cell numbers increased 10-fold versus WT, whereas CD4⁺ and CD8⁺ T-cell numbers increased ~3- to 5-fold versus WT. These data indicate a preferential expansion of CD11c⁺ dendritic cells,

whereas the numbers of CD4⁺ and CD8⁺ T cells increased proportionally with the increased transgenic lumbar lymph node cellularity.

Phenotypic analysis of these expanded cell populations revealed that the proportion of CD11c⁺ cells expressing costimulatory molecules (CD80, CD86, and CD40) or MHC class II was similar in the lumbar lymph nodes, as well as in the spleen, of WT and transgenic mice (data not shown). In addition, no significant differences were detected between WT and transgenic CD11c⁺ dendritic cells in the expression level per cell (i.e., mean fluorescence intensity) of any of these surface molecules. In contrast to the apparently normal phenotype of the CD11c⁺ dendritic cells in the secondary lymphoid tissues of tumor-bearing mice, analysis of the T-cell phenotype indicated that the proportion of CD4⁺ or CD8⁺ T cells expressing the early activation marker CD69 was modestly elevated in the lumbar lymph nodes of ≥20-week-old transgenic mice (Fig. 2A). Additional evidence suggesting T-cell activation was the observation that both the proportion of CD4⁺ T cells expressing CD25 (Fig. 2A) and the absolute numbers of CD4⁺CD25⁺ T cells (Fig. 2B) were significantly elevated in the lumbar lymph nodes during tumor development. Of interest, this increased frequency and number of CD4⁺CD25⁺ T cells was not seen in other lymph nodes (i.e., submandibular, mesenteric, and inguinal lymph nodes; Fig. 2C and data not shown), suggesting that expansion of this population occurs in response to the nearby tumor.

T cells are altered during prostate tumor development.

Expansion of phenotypically defined cell populations within the secondary lymphoid tissues does not necessarily reflect enhanced, or even normal, function. Owing to the increased number of activated T cells suggesting that an immune response is initiated in the secondary lymphoid tissues during tumor progression, we examined the function of selected immune cells in the spleen or lumbar lymph nodes of tumor-bearing mice. WT and transgenic lipopolysaccharide-activated splenic CD11c⁺ dendritic cells were equally capable of stimulating allogeneic

T-cell proliferation regardless of the stage of tumor progression (data not shown). In contrast, as shown in Fig. 3A to C, there was a progressive age-related decrease in the ability of transgenic lumbar lymph node T cells to proliferate in response to anti-CD3 mAb stimulation. These lumbar lymph node T cells also exhibited a significantly decreased ability to proliferate when stimulated with allogeneic C57BL/6 bone marrow-derived mature dendritic cells (data not shown). This reduced proliferation was not observed in transgenic T-cell populations isolated from other lymph nodes regardless of the age of the mice (data not shown).

To explore the possibility that the expanded CD4⁺CD25⁺ T-cell population present in the spleen and lumbar lymph nodes might be responsible for the reduced T-cell proliferation, the function of this population was assessed. CD4⁺CD25⁺ T cells were purified from the lumbar lymph nodes of WT or transgenic mice (≥20 weeks of age) and their ability to inhibit T-cell proliferation was analyzed. As illustrated in Fig. 3D, both the WT and transgenic CD4⁺CD25⁺ T-cell populations inhibited the proliferative response of naïve T cells. Under these conditions, neither the WT nor the transgenic CD4⁺CD25⁺ T-cell population was able to proliferate in response to anti-CD3 mAb stimulation (data not shown). To further confirm the suppressive capacity of the CD4⁺CD25⁺ T cells in the WT or transgenic lumbar lymph nodes, the T-cell population from the lumbar lymph nodes was depleted of CD4⁺CD25⁺ T cells by fluorescence-activated cell sorting and cell proliferation in response to antigenic stimulation was determined. Following removal of the CD4⁺CD25⁺ T-cell population, transgenic T-cell proliferation was restored to WT levels (Fig. 3E), indicating that the elevated number of CD4⁺CD25⁺ T cells could play a significant role in suppressing immune responsiveness in the prostate tumor environment. T cells isolated from transgenic spleens behaved similarly to those from transgenic lumbar lymph nodes (data not shown).

To further evaluate the properties of the altered transgenic T-cell populations, we determined the levels of various cytokines released following stimulation. This analysis revealed that the transgenic CD4⁺CD25⁺ T-cell population secreted dramatically elevated levels of IL-10 compared with the CD4⁺CD25⁻ T cells (CD4⁺CD25⁺: 8,805 ± 3,212 pg/5 × 10⁵ cells versus CD4⁺CD25⁻: 157 ± 59 pg/5 × 10⁵ cells; *P* < 0.05). In contrast, they produced significantly lower levels of IL-2 (CD4⁺CD25⁺: 288 ± 92 pg/5 × 10⁵ cells versus CD4⁺CD25⁻: 857 ± 193 pg/5 × 10⁵ cells; *P* < 0.05) and IFN-γ (CD4⁺CD25⁺: 4,628 ± 738 pg/5 × 10⁵ cells versus CD4⁺CD25⁻: 37,198 ± 8,536 pg/5 × 10⁵ cells; *P* < 0.005). Surprisingly, WT and transgenic CD4⁺CD25⁺ T cells produced equivalent levels of IL-10 (data not shown) consistent with the similar levels of suppression induced by these two populations. Taken together, these results suggest that regulatory T cells present in the expanded CD4⁺CD25⁺ T-cell population may play a critical role in immune suppression during prostate tumor progression.

Anti-CD25 monoclonal antibody treatment suggests the involvement of CD4⁺CD25⁺ regulatory T cells in prostate tumor progression. Tumor transplantation studies have implicated CD4⁺CD25⁺ regulatory T cells in suppression of antitumor immunity against a wide variety of tumors (21–23). However, no study to date has examined if they also contribute to the immune suppression in prostate cancer. To determine whether CD4⁺CD25⁺ regulatory T cells influence the growth of prostate cancer cells *in vivo*, administration of anti-CD25 mAb was carried

Table 1. Cellularity and cellular composition of various draining lymph nodes during tumor progression

		WT (×10 ⁶)	Transgenic (×10 ⁶)
Lymph nodes*	Submandibular	7.3 ± 3.5	8.7 ± 3.6
	Inguinal	5.4 ± 2.3	10.0 ± 2.3
	Mesenteric	8.4 ± 4.7	13.7 ± 4.4
	Lumbar	1.8 ± 0.8	10.5 ± 4.0 [†]
Lumbar lymph nodes	11-12 wk	1.9 ± 0.4	3.2 ± 0.6
	16-17 wk	1.7 ± 0.6	12.8 ± 1.7 [†]
	20-25 wk	1.8 ± 0.8	10.5 ± 4.0 [†]
Lumbar lymph nodes [‡]	CD11c z	0.0234 ± 0.0067	0.234 ± 0.085 [†]
	CD4	0.926 ± 0.368	4.56 ± 0.71 [†]
	(20-25 wk) CD8	0.825 ± 0.170	2.61 ± 0.26

*Cell number is per lymph node isolated from mice ≥20 weeks of age.

[†]Statistically different (*P* ≤ 0.01) versus WT as determined using the Student's *t* test.

[‡]Absolute numbers of cells expressing CD11c, CD4, and CD8 in each lumbar lymph node were calculated by multiplying total lumbar lymph node cellularity with the percentage of viable nucleated cells expressing the indicated markers as determined by flow cytometry. Each value represents the mean ± SD (*n* ≥ 3 individual experiments).

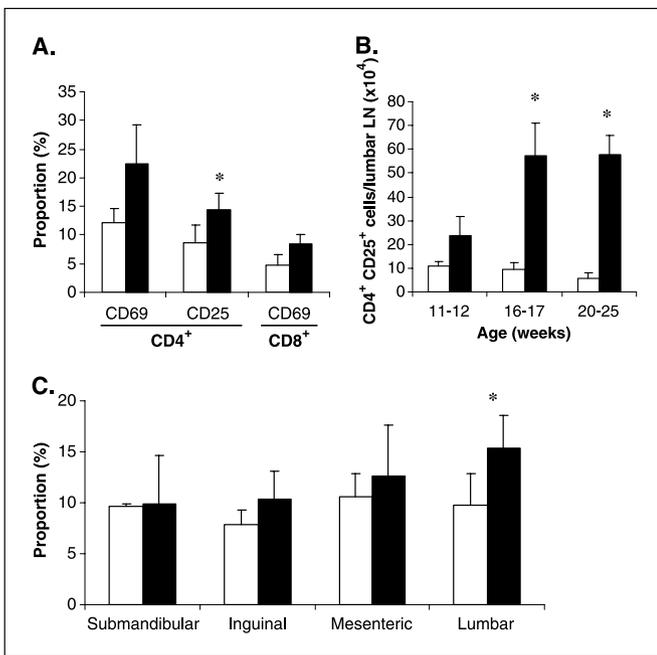


Figure 2. The frequency and number of CD4⁺CD25⁺ T cells increases in the lumbar lymph nodes of transgenic mice during tumor progression. *A*, lumbar lymph nodes were isolated from WT (*open columns*) and transgenic (*closed columns*) mice (≥ 20 weeks of age) and the frequency of CD4⁺ and CD8⁺ cells was determined by flow cytometry. The proportion of CD4⁺ or CD8⁺ T cells expressing CD25 or CD69 was calculated by dividing the percentage of the population positive for the indicated marker by the percentage of the total CD4⁺ or CD8⁺ T-cell population. *B*, absolute numbers of CD4⁺CD25⁺ T cells in the lumbar lymph nodes at the indicated stages of tumor development were calculated by multiplying nucleated lymph node cell numbers with the frequency of double positive cells in the nucleated and viable cell gate. *C*, proportion of CD4⁺ T cells expressing CD25 in various lymph nodes from WT and transgenic mice (≥ 20 weeks of age). *Columns*, mean ($n \geq 3$); *bars*, SD. Statistical analysis was carried out using the Student's *t* test. *, $P \leq 0.01$.

out in mice inoculated with TRAMP-C2 prostate cancer cells. Both the control (rat IgG) and antibody-treated groups developed palpable tumors at ~ 3 weeks posttransplant, which grew progressively until the mice were sacrificed at 6 weeks posttransplant. No metastases were observed in the recipient mice, and thus tumor weight was used as the primary assessment of tumor growth. In this model, anti-CD25 antibody treatment reduced tumor weight by $\sim 28.5\%$ (0.85 ± 0.53 g in the control IgG-treated group versus 0.61 ± 0.43 g in the anti-CD25 mAb-treated group; $P = 0.094$; Fig. 4A, *left pair*). Interestingly, anti-CD25 antibody treatment was significantly more effective on tumors weighing less than 1 g than on those weighing more than 1 g (Fig. 4A, *right and middle pairs*, respectively). To determine if the reduction in tumor growth was accompanied by an altered infiltration of immune cells into the tumor, one to two sections were sliced at random from each tumor and the number of immune cells within a designated field was estimated microscopically. As shown in Fig. 4B and C, there is an increase in the frequency of tumor-infiltrating immune cells in the anti-CD25 antibody-treated group.

Despite the successes using anti-CD25 mAb treatment to reduce the growth of transplanted tumors in this and previous reports, prior studies have not yet shown that inhibition of the regulatory T-cell population has efficacy against spontaneously developing tumors. We therefore assessed the ability of anti-CD25 mAb treatment to reduce or prevent tumor growth in the 12T-7s mouse

model. On day 3 following antibody treatment, $\sim 60\%$ to 80% of the CD4⁺CD25⁺ T cells were depleted from the spleen and lymph nodes of anti-CD25 mAb-treated mice, although only 15% depletion was achieved in the tumor mass (data not shown). As indicated in Fig. 5, mice treated at 8 weeks of age had similar tumor weights at 20 weeks regardless of the treatment given (IgG: 8.88 ± 3.48 g versus anti-CD25 mAb: 9.42 ± 3.90 g). The average tumor weights from either group were not significantly different from those of mice without any treatment (8.23 ± 3.29 g). In contrast, analysis of tumors from mice treated at 12 weeks of age and examined 8 weeks postinjection revealed a 23.4% reduction in tumor weight (7.12 ± 2.10 g for IgG versus 5.45 ± 1.41 g for anti-CD25 mAb; $P = 0.025$). Again, the average tumor weights of the control IgG-treated group were similar to those of untreated mice (8.23 ± 3.29 g). These observations suggest that anti-CD25 mAb treatment may reduce tumor growth if administered at the appropriate time during tumor development. Furthermore, these data confirm the involvement of CD4⁺CD25⁺ regulatory T cells in the immune suppression observed during spontaneous prostate tumor progression.

Discussion

Altered immune function has been reported in patients with various types of cancer. However, the exact nature and level of the impairment, as well as its clinical and biological significance, have not been accurately assessed (12). To date, no comprehensive analyses of immune cell phenotype and function have been carried out in prostate cancer patients or in mouse models of this disease. Such information is of critical importance for designing effective immune therapeutic strategies for both early- and late-stage disease. In this study, we thus attempted to determine if immune function is altered with disease progression in a prostate tumor mouse model and, if so, to define the nature of the changes that accompany, and are ultimately responsible for, the development of impaired immunity.

Previous studies have shown that patients with early-stage non-small lung cancer and late-stage ovarian cancer have tumor-associated T cells with an activated phenotype at initial diagnosis (17). This observation suggests that at least during the early stages of tumor development an active antitumor immune response is initiated. Our analyses of the 12T-7s prostate tumor mouse model support this possibility. Phenotypically normal dendritic cells and activated CD4⁺ T cells expanded in the transgenic lumbar lymph nodes. Similarly, activated T cells were also detected in the tumor. Despite these indications of antitumor immunity, there was a progressive decline in immune responsiveness during the course of tumor development (Fig. 3). In addition, although the frequency of tumor-infiltrating dendritic cells was increased slightly in the 12T-7s prostate tumor mouse model, these cells exhibited an immature phenotype as evidenced by reduced expression of CD86 and MHC class II. Previous studies by Troy et al. (9) indicated that minimally activated tumor-infiltrating dendritic cells infiltrate the tumor mass of prostate cancer patients. In contrast, our studies and those of Ciavarrà et al. (27, 28) suggest that the tumor-infiltrating dendritic cells have an immature phenotype. These conflicting observations may be due to analysis at different stages of tumor progression in the prostate cancer patients versus the mouse models. Of interest, it has been shown that the tumor-infiltrating dendritic cells induce a partial state of T-cell tolerance to the tumor cells, suggesting that alterations in dendritic cells

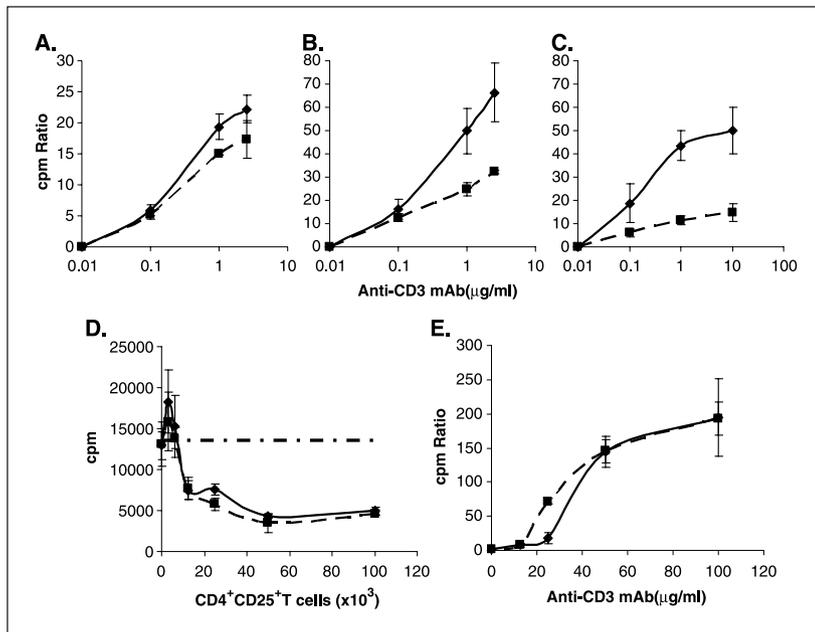


Figure 3. The suppressive activity of CD4⁺CD25⁺ T cells contributes to decreased antigen responsiveness of transgenic T cells during tumor development. Lumbar lymph node cells isolated from WT (solid lines) and transgenic (dashed lines) mice at 12 weeks (A), 16 weeks (B), and over 20 weeks (C) of age were stimulated with the indicated concentrations of anti-CD3 antibody and proliferation was determined as outlined in Materials and Methods. D, syngeneic T-cell proliferation was measured following addition of CD4⁺CD25⁺ T cells isolated from WT (solid line) or transgenic (dashed line) lumbar lymph nodes. Horizontal dashed line, responder T-cell proliferation in the absence of CD4⁺CD25⁺ T cells. E, proliferation of WT (solid line) and transgenic (dashed line) lumbar lymph node cells depleted of CD4⁺CD25⁺ T cells was assessed as outlined in Materials and Methods. Representative experiments (n ≥ 3). Points, mean of triplicate determinations; bars, SD.

survival or maturation prevent them from activating antigen-specific T cells (29). The decreased levels of MHC class II observed in this study may also play a role in tolerance induction.

In addition to the altered dendritic cells population, there was an increased frequency of tumor infiltrating CD4⁺CD69⁻CD25⁺ T cells,

in the 12T-7s prostate tumor mass. We hypothesize that these cells are regulatory T cells that contribute to the immune suppression in the tumor environment. Recent studies have shown that the frequency of CD4⁺CD25⁺ regulatory T cells is increased in the peripheral blood and tumor microenvironment of patients with

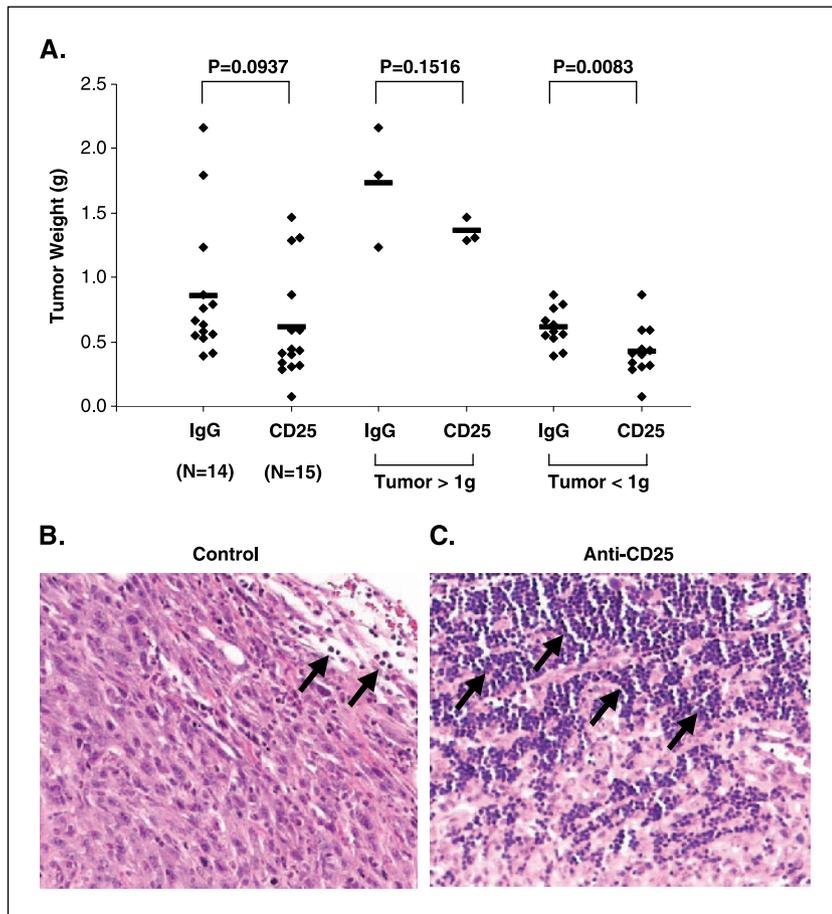


Figure 4. Anti-CD25 mAb treatment reduces the growth of transplanted prostate tumor cells. A, C57BL/6J mice treated with either rat IgG or anti-CD25 mAb were transplanted with TRAMP-C2 tumor cells and tumor weight was determined for individual mice in each group at 6 weeks posttransplant. Dots, tumors isolated from individual mice. Horizontal bars, average tumor weight in each treatment group. Statistical analysis was carried out using the Student's *t* test. B and C, lymphocytic infiltration into the tumor is increased by anti-CD25 mAb treatment. Tumors recovered from mice treated with either rat IgG or anti-CD25 mAb were fixed, sectioned, and stained as indicated in Materials and Methods. Arrows, areas of lymphocytic infiltration. Final magnification, ×200. Experiments included one to two sections per tumor (14-15 mice per group over 3 independent experiments).

pancreas, breast, non-small cell lung, colorectal, and ovarian carcinomas (17–19), contributing to immune tolerance to the tumor. It has been shown that CD80 and CD86 differentially modulate the suppressive activity of human CD4⁺CD25⁺ regulatory T cells (30). Interestingly, we observed that the expression of CD80 was increased significantly on the tumor-infiltrating dendritic cells suggesting that the dendritic cells phenotypically altered within the tumor environment may enhance the suppressive activity of the regulatory T cells found within the tumor.

These observations raise an interesting question regarding the mechanism(s) by which CD4⁺CD25⁺ regulatory T cells increase in the tumor environment, both within the tumor site as well as in the draining (lumbar) lymph nodes. Several studies have shown a role for dendritic cells in the generation and expansion of regulatory T cells. For example, it has been shown that human CD4⁺ T cells stimulated repetitively with immature dendritic cells differentiate into IL-10-producing cells with suppressive activity (15), whereas antigen-loaded dendritic cells can expand CD4⁺CD25⁺ regulatory T cells directly (31). Owing to dendritic cells incubated with prostate cancer cells or tumor culture supernatant exhibiting an immature phenotype (refs. 32–34 and our unpublished data), it is tempting to speculate that in the 12T-7s prostate tumor mouse model the phenotypically immature dendritic cells detected in the tumor mass behave as tolerogenic dendritic cells that induce regulatory T cells and thus increase the frequency of regulatory T cells within the tumor microenvironment. Alternatively, in ovarian carcinoma it has been shown that tumor cells and tumor-infiltrating macrophages secrete CCL22, a chemokine that attracts regulatory T cells to the tumor microenvironment (20). Thus, CCL22 expression by the prostate tumor may recruit and retain regulatory T cells to suppress any ongoing immune response (35). Further investigation is clearly required to determine how the complex interaction among dendritic cells, T cells, and tumor cells results in immune suppression.

Regardless of the mechanisms by which regulatory T cells expand in the tumor environment, it has been shown that depletion of CD4⁺CD25⁺ regulatory T cells using anti-CD25 mAb can abrogate immunologic unresponsiveness to syngeneic implanted leukemia, lymphoma, sarcoma, and B16 melanoma cell lines *in vivo*, resulting in endogenous antitumor immunity (21–23). The response is mediated by tumor antigen-specific CD8⁺ CTL and tumor antigen-nonspecific CD4⁺CD8⁻ cytotoxic cells akin to natural killer cells (36, 37). In the B16 melanoma model there is an enhancement of the IFN α -induced, CD8⁺ T-cell-dependent immune response following elimination of CD4⁺CD25⁺ regulatory T cells *in vivo* (23). It has also been shown that treatment with anti-CD25 mAb facilitates long-term CD4⁺ T-cell-mediated tumor immunity (38). This evidence suggests that regulatory T cells play an important role in immune suppression in at least some tumors.

Our research is the first to show that blockade of CD4⁺CD25⁺ regulatory T cells using anti-CD25 mAb reduces prostate cancer cell growth both in a prostate tumor transplant model (TRAMP-C2) and in a spontaneous prostate tumor mouse model (12T-7s mice). Moreover, we have shown that the timing of anti-CD25 antibody treatment, as well as unidentified factors regulating tumor growth, is likely to be important in the efficacy of this treatment in reducing tumor growth. In the 12-7s mouse model, the expansion of regulatory T cells in the draining lymph nodes or tumor mass likely does not occur until 12 weeks of age or

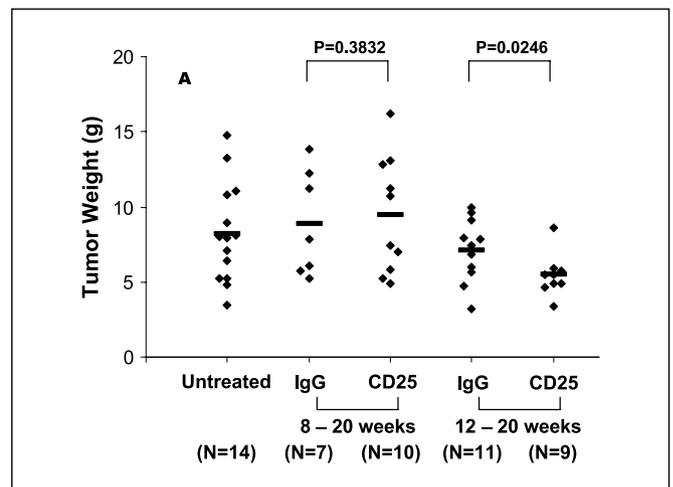


Figure 5. Anti-CD25 mAb treatment reduces the growth of spontaneous prostate tumors. 12T-7s mice were treated with either rat IgG or anti-CD25 mAb at 8 or 12 weeks of age and tumor weight was determined at 20 weeks of age. Similarly, the tumor weight in untreated mice was also determined. Dots, tumors isolated from individual mice. Horizontal bars, average tumor weight in each treatment group. Statistical analysis was carried out using the Student's *t* test.

later. Thus, treating the mice too early (i.e., at 8 weeks of age) did not eliminate CD4⁺CD25⁺ regulatory T cells. Instead, it was most likely that activated T cells were depleted, thus accounting for the slight increase in tumor weight in the anti-CD25 antibody-treated mice. In contrast to untreated control mice, or those receiving rat serum IgG, the reduction in tumor weight induced following anti-CD25 antibody treatment at 12 weeks of age is likely due specifically to CD4⁺CD25⁺ T-cell elimination. Because of only modest depletion of the regulatory T-cell population being achieved within the tumor, it is not surprising that complete tumor elimination was not achieved. Moreover, based on previous work in other tumor models (21, 22, 38), regulatory T-cell frequency in either lymph nodes or peripheral blood returns to pretreatment levels ~1 to 3 weeks following treatment. Obviously improved strategies for complete and sustained removal of the tumor antigen-specific regulatory T cells from the tumor environment, as well as secondary lymphoid tissues, must be developed to improve the efficacy of this treatment strategy.

It has been shown that reduction of tumor mass by anti-CD25 antibody treatment involves CD4⁺, CD8⁺ T cells, or natural killer cells in different types of tumors and the immune response mediated by these cells may be affected by the immunogenicity of various tumors (21–23, 36–38). In our studies, we observed increased tumor-infiltrating immune cells in the TRAMP-C2 recipients treated with anti-CD25 antibody. We have not yet determined whether this immune response is tumor antigen specific or nonspecific (i.e., resulting from enhanced release of inflammatory cytokines induced by the cell death resulting from anti-CD25 antibody treatment). Regardless, in the anti-CD25 antibody-treated 12T-7s mice we have detected a slightly elevated frequency of CD8⁺ T cells infiltrating the tumors (data not shown). It will be important to determine the antigen specificity of these cells and further elucidate their role in reducing tumor growth. Further studies are obviously required to understand the mechanisms by which anti-CD25 antibody

treatment reduces the growth of transplanted and spontaneous prostate tumors.

In the long term, elimination of the regulatory T-cell population using antibody therapy may favor the generation and/or expansion of activated T cells capable of reducing tumor growth. Validation of this hypothesis will require a detailed analysis of immune function following antibody therapy at various times during tumor progression. Although these results are encouraging, our studies and others suggest that simply blocking or even eliminating CD4⁺CD25⁺ regulatory T-cell function *in vivo* using anti-CD25 mAb might not be adequate to overcome immune suppression and eradicate the tumor (refs. 21, 39 and our data). A major concern is that CD25 is also transiently expressed on activated T cells, and thus use of anti-CD25 mAb *in vivo* could potentially eliminate recently activated tumor-specific T cells. A second concern is that this antibody treatment likely results in only temporary blockade of regulatory T-cell expansion or function. Determination of appropriate doses, administration routes, and time schedules may circumvent these concerns. It is also possible that a combination of dendritic cell- or T-cell-mediated immune therapy to initiate an antitumor response and anti-CD25 mAb (or equivalent) treatment to reduce the level of active immune suppression may result in the generation of an effective, long-lasting antitumor immune response.

In summary, these studies show for the first time in a prostate dysplasia mouse model that CD4⁺CD25⁺ regulatory T cells

contribute to the development of a progressively immune suppressed environment. Although antitumor immune responses seem to be initiated, our data suggest that the balance between immune activation and suppression (tolerance) is altered during tumor growth, ultimately allowing the tumor to escape immune destruction. Taken together, our studies have important implications for designing effective antitumor immune therapy strategies that involve manipulation of both dendritic cells and regulatory T cells. It is anticipated that these and other such studies will pave the way to development of more effective therapeutic strategies to induce endogenous antitumor immunity.

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