Peripheral T-Cell Tolerance Associated with Prostate Cancer Is Independent from CD4+CD25+ Regulatory T Cells

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Abstract

CD4+CD25+Foxp3+ regulatory T cells (Treg) are thought to suppress the natural and vaccine-induced immune response against tumor-associated antigens (TAA). Here, we show that Treg accumulate in tumors and tumor-draining lymph nodes of aging transgenic adenocarcinoma of the mouse prostate (TRAMP) male mice, which spontaneously develop prostate cancer. TAA overexpression and disease progression associate also with induction of TAA-specific tolerance. TAA-specific T cells were found in the lymphoid organs of tumor-bearing mice. However, they had lost the ability to release IFN-γ and kill relevant targets. Neither in vivo depletion of Treg by PC61 monoclonal antibody followed by repeated vaccinations with antigen-pulsed dendritic cells nor the combined treatment with 1-methyl-L-tryptophan inhibitor of the enzyme indoleamine 2,3-dioxygenase, PC61 antibody, and dendritic cell vaccination restored the TAA-specific immune response. Treg did not seem to control the early phases of tolerance induction, as well. Indeed, depletion of Treg, starting at week 6, the age at which TRAMP mice are not yet tolerant, and prolonged up to week 12, did not avoid tolerance induction. A similar accumulation of Treg was found in the lymph nodes draining the site of dendritic cell vaccination both in TRAMP and wild-type animals. Hence, we conclude that Treg accrual is a phenomenon common to the sites of an ongoing immune response, and in TRAMP mice in particular, Treg are dispensable for induction of tumor-specific tolerance. [Cancer Res 2008;68(1):292–300]

Introduction

With a population aging rapidly, prostate cancer is one of the leading causes of death in adult men (1). Indeed, prostatectomy and radiotherapy are potentially curative for organ-confined diseases, and treatment of locally advanced and/or metastatic cancer remains palliative.

Because of the expression of unique tumor-associated antigens (TAA), prostate cancer is an ideal candidate for immunotherapy. Vaccination strategies were tested in phase I trials (2), and prostate cancer was the object of the first randomized phase III vaccine trial (3). Clinical results were encouraging but far from the prediction based on animal models. Indeed, many of the preclinical models were based on transplantable tumors (4), wherein in vitro passages may cause substantial genetic/epigenetic alterations of the tumor cells, and cell engrafment does not mimic the natural dynamic interactions among neoplastic cells, stroma, and the immune response (5). Also, those models may not correctly recapitulate the mechanisms the tumor develops to escape natural and vaccine-induced T-cell responses (6).

Prostate cancer cells may lose expression of relevant antigens, acquire defects in antigen presentation, release immunosuppressive substances, block T-cell function favoring apoptosis, and, finally, promote development and recruitment of regulatory T cells (Treg) inside tumors and draining lymph nodes (7). The latter mechanism, especially the role of CD4+CD25+ Treg in cancer, has been the focus of intense investigation in recent years.

Treg (5–10% of the peripheral CD4+ T cells) are mostly generated in the thymus and represent an essential mechanism of peripheral tolerance to self antigens (8). Absence of Treg, which is caused either genetically or by deletion, favors autoimmunity (9). Treg suppress not only CD4+ T and natural killer cells but also CTL (10). Treg can be identified by several cell surface markers, among which is the interleukin 2 (IL-2) receptor α-chain CD25. In addition, Treg selectively express Foxp3, a forkhead/winged helix transcription factor that controls master genes in Treg development/function (8).

Treg can also be induced in the periphery from naive CD4+CD25− T cells (11), although it is not yet known whether they substantially contribute to peripheral tolerance. Conversion of CD4+CD25− T cells into Treg seems rather common in tumor-draining lymph nodes (TDLN; refs. 12–14).

Prostate cancer (15) and other neoplasms associate with Treg accumulation in the blood and/or in tumors, and this may inversely correlate with patients’ survival (16), therefore suggesting that Treg promote tumor-immune privilege. It remains to be elucidated whether their gathering directly affects the tumor-specific immune response. Data in animal models suggest a role for Treg in tolerance induction against TAA. Indeed, depletion of Treg before tumor challenge favors induction of tumor-specific immune responses and tumor eradication (17). Depletion of Treg also increases the therapeutic index of several cancer treatments (18, 19). Those models, however, were based on transplantable tumors.

Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice express the SV40 large T antigen (Tag) selectively on the prostate epithelium under the control of the rat probasin regulatory element, whose expression is influenced by sexual hormones (20). Hence, male mice remain healthy until puberty. In the following weeks, TRAMP mice progressively overexpress Tag and invariably develop spontaneous mouse prostate intraepithelial neoplasia, adenocarcinoma, and seminal vesicles, lymph node, and visceral metastases, therefore resembling human prostate cancer (21). Also, the immune response against Tag, a surrogate tissue-specific TAA...
antigen in TRAMP mice, has been well characterized in other models and is dominated by CTL specific for the sequence 404 to 411 (Tag-IV; ref. 22). In TRAMP mice, the immune response against Tag is characterized by thymic deletion of high-avidity CTL (23), which allows induction of low-avidity Tag-specific CTL in young healthy mice, upon vaccination with Tag-IV-pulsed dendritic cells. Prostate cancer development and progression parallel induction of a profound state of peripheral tolerance, which cannot be rescued by dendritic cell vaccination (24).

We investigated whether Treg accumulate in the tumor and TDLN of TRAMP mice during disease development and whether they have a role in Tag tolerance.

Materials and Methods

Mice, tumor cell lines, and reagents. Heterozygous C57/BL6 TRAMP mice and wild-type (WT) mice were housed and bred in a specific pathogen-free animal facility and treated in accordance with the European Union guidelines and the approval of the Institutional Ethical Committee. Animals were typed for Tag expression by PCR-based screening assay.

Isolation of mouse tail genomic DNA was performed as described (25). RMA is a H-2d Rauscher virus–induced thymoma (26). B6/K-0 is a kidney cell line expressing Tag (27). TRAMP-C1 is a prostate cancer, originated from a TRAMP mouse (28). C26-GM (H-2k) is a colon carcinoma genetically modified to produce granulocyte/macrophage colony-stimulating factor. Unless specified, all chemical reagents were from Sigma-Aldrich, and monoclonal antibodies (mAb) were from BD PharMingen.

Dendritic cell preparation and immunization protocols. Dendritic cells were prepared and characterized as previously described (29). Dendritic cells were pulsed with 2 μmol/L Tag-IV (Research Genetics) peptide for 1 h at 37°C, washed, and suspended at 1 × 10^6/ml in PBS. Dendritic cells (5 × 10^5) were injected i.d. into mice.

In vitro cytotoxicity assay. Splenocytes were restimulated in vitro in the presence of 1 μmol/L Tag-IV peptide. Day 5 blasts were tested for cytolytic activity in a standard 4 h ^31Cr release assay (29). ^31Cr release of target cells alone was always <25% of maximal ^31Cr release (target cells in 0.25 mol/L SDS).

Flow cytometry analyses. PE-labeled K^b/Tag-IV pentamers (ProImmuno) were generated using a C411L-substituted synthetic peptide to enhance binding to H2-K^b (30). K^b/ova (SIINFEKL) pentamers were used as negative control. Cells were incubated with the pentamer complex for 30 min at +4°C and then, without washing, PerCP-Cy-5.5–conjugated anti-CD8 and the FITC-conjugated anti-CD44 mAbs were added for additional 15 min at +4°C. Dead cells were excluded by physical variables and/or by the addition of ToPro-5 (Molecular Probes) immediately before fluorescence-activated cell sorting analysis. For intracellular cytokine measurement, day 5 blasts were stimulated in vitro with B6/K-0 or RMA cells (1:1 ratio) or phorbol 12-myristate 13-acetate/ionomycin and stained with FITC-labeled anti-CD44, PerCP-Cy-5.5-labeled anti-CD8, and antigen-presenting cells (APC)–labeled anti–IFN-γ antibody as previously described (29). For enumeration of CD^4+CD25^+Foxp3^+ cells, TDLN and collagenase D–digested prostates were processed on a cell strainer, stained with FITC-labeled anti–IFN-γ, PerCP-Cy-5.5-labeled anti-CD8, and APC-labeled anti-CD25 (clone PC61), permeabilized, and finally stained with PE-labeled anti-Foxp3 (eBioscience) according to the manufacturer’s instructions. In all experiments, cells were analyzed on a BD FacsCalibur.

Isolation of CD^4+CD25^+ and CD^4+CD25^- cells and in vitro proliferation assay. CD^4+CD25^-T cells were isolated using a Treg isolation kit (Miltenyi Biotec) following the manufacturer’s instructions. The three cell populations (i.e., unfractionated CD^4+ T cells, CD^4+CD25^- cells, and CD^4+CD25^+ cells) were cultured for 72 h in plates previously coated with 5 μg/ml anti-CD3 mAb. The incorporation of [3H]thymidine by proliferating T cells (triplicate cultures) during the last 16 h of culture was measured. Proliferation index was calculated assigning value of 1 to cpm obtained from unstimulated cultures.

In vivo depletion of CD25^+ cells and/or blocking of indoleamine 2,3-dioxygenase enzyme. Five hundred micrograms of monoclonal anti-CD25 antibody (clone PC61; American Type Culture Collection) were injected i.p. into mice at day −4. In selected experiments, treatment was repeated at day +3 or every 2 weeks. Mice received 1-methyl-tryptophan dissolved in drinking water at 5 mg/ml as previously reported (31).

Histology and immunohistochemistry. Organs were fixed in 4% formalin for 6 h, then embedded, and included in paraffin wax. H&E and Tag staining of 5-μm-thick sections were performed as previously described (24). For Foxp3 staining, deparaffinized and rehydrated sections were immersed in EDTA (pH 9.0) and followed the procedures described in

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2 As described in www.jax.org.
ref. (24). Slides were incubated with streptavidin 1:1,000 for 30 min, followed by incubation with 3,3'-diaminobenzidine tetrahydrochloride for 5 min, and the counter stain was done with Mayer hematoxylin. Macroscopic and microscopic specimens were evaluated by a pathologist in a blind fashion. Histology sections were scored as previously described (24) with partial modifications: the score of 5 was given to well-differentiated adenocarcinoma and 6 was given to metastases and/or neuroendocrine tumors.

Statistical analysis. Statistical analyses were performed using Student’s *t* test and log-rank test. Comparison of survival curves was considered statistically significant for *P* < 0.05.

Results

**CD4**<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg accumulate in prostates and TDLN of aged TRAMP mice.** First, we investigated whether Foxp3<sup>+</sup> cells accumulate in prostates of TRAMP mice. The analysis was initially conducted in 25-week-old to 27-week-old mice. At this age, TRAMP mice present a significant enlargement of the urogenital apparatus (2.5 ± 0.9 g; *n* = 6) when compared with age-matched and sex-matched WT littermates (0.9 ± 0.2; *n* = 6; *P* < 0.008) and a well-differentiated adenocarcinoma (Fig. 1A) with penetration of Tag<sup>+</sup> cells through the basement membrane of involved acini (Fig. 1B, black arrows). We also found several Foxp3<sup>+</sup> cells infiltrating the transformed gland and its stroma (Fig. 1C, red arrows), which were absent in WT prostates (not shown).

Prostate tissues were also processed to single cells and analyzed by flow cytometry. After gating on viable cells, a small population of CD8<sup>+</sup> and CD4<sup>+</sup> T cells was found in prostates of both TRAMP and WT mice (Fig. 2). However, the difference in the percentage of infiltrating CD8<sup>+</sup> T cells between TRAMP and WT mice was statistically significant (*P* < 0.001) and reflected the relatively higher number of CD8<sup>+</sup> T cells present in tumor-bearing mice (130.6 ± 92 and 7.9 ± 2.8 × 10<sup>5</sup> cells, respectively; *P* < 0.022). More than 95% of the prostate-infiltrating CD8<sup>+</sup> T cells were antigen-experienced CD44<sup>+</sup> (not shown). Also CD4<sup>+</sup> T cells accumulated in TRAMP prostates (53 ± 18 and 9.9 ± 6.2 × 10<sup>5</sup> cells, respectively; *P* < 0.001). However, the CD4/CD8 ratio was inverted in TRAMP mice when compared with WT littermates (0.5 ± 0.3 and 1.2 ± 0.5, respectively; *P* < 0.017), therefore suggesting a preferential recruitment of antigen-experienced CD8<sup>+</sup> T cells to the tumor site.

Gating on CD4<sup>+</sup> and analyzing for the expression of CD25 and Foxp3, we found that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells were enriched in the prostates of tumor-bearing TRAMP mice (Fig. 2C and D). More strikingly, the absolute number of triple positive cells was 6-fold higher in TRAMP than in WT prostates (Fig. 2E) and correlated well with the increase in prostate cell number (16 ± 9.2 and 2.5 ± 0.6 × 10<sup>6</sup>, respectively; *P* < 0.015). Activated effector CD4<sup>+</sup> T cells (i.e., CD25<sup>−</sup>Foxp3<sup>−</sup>) accumulated as well in TRAMP prostates (7.5 ± 3.7% and 3.3 ± 2%, respectively; *P* < 0.04). Hence, prostate cancer development associated with in situ accumulation of antigen-experienced CD8<sup>+</sup> but also CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells.

To verify whether accumulation of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells correlated with age and disease progression, a similar analysis was conducted on prostate tissue from 6-week-old to 8-week-old mice, age at which TRAMP mice develop scattered foci of mouse prostate intraepithelial neoplasia but are not yet tolerant against Tag (24). Indeed, the macroscopic aspect of TRAMP and WT prostates (24) and their total cell number did not differ (2.4 ± 0.3 and 2.1 ± 0.4 × 10<sup>6</sup>, respectively). In young TRAMP animals, the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells was similar to the one found in prostates of WT mice (Fig. 2E).

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells were enumerated also in para-aortic TDLN. Whereas TDLN of 6-week-old TRAMP and WT mice were macroscopically indistinguishable and contained a similar number of cells (0.4 ± 0.1 and 0.5 ± 0.2 × 10<sup>6</sup>, respectively), TDLN of TRAMP mice ages 25 to 27 weeks were enlarged and contained an...
increased number of cells when compared with age-matched WT controls (1.5 ± 0.7 and 0.6 ± 0.4 × 10⁶, respectively; P < 0.036). Moreover, CD4⁺CD25⁺Foxp3⁺ cells were significantly increased only in TDLN of TRAMP mice ages 25 to 27 weeks (Fig. 2F).

To determine whether the CD4⁺CD25⁺Foxp3⁺ cells accumulating in aged TRAMP mice were functional Treg, we conducted a proliferation assay with CD4⁺ T cells purified from TDLN of 19-week-old TRAMP mice and sorted for CD25 expression. As measured by [³H]thymidine incorporation, proliferation of CD4⁺CD25⁺ cells increased dramatically when CD4⁺CD25⁺ cells were not present in the culture wells (Fig. 3). As expected (9), CD4⁺CD25⁺ cells did not proliferate. A similar proliferation pattern was obtained in the cultures of CD4⁺ cells purified from WT mice, therefore confirming that CD4⁺CD25⁺ Treg from TRAMP mice have phenotypic and functional characteristic undistinguishable from WT Treg.

To further investigate whether CD4⁺CD25⁺Foxp3⁺ cell accumulation paralleled tolerance induction, groups of young and aged TRAMP and WT controls were vaccinated with Tag-IV–pulsed dendritic cells (24) and sacrificed 1 week later. To visualize Tag-specific CD8⁺ T cells in the spleens of vaccinated mice ex vivo, we took advantage of K⁺/Tag-IV pentamers (Supplementary Fig. S1). Upon magnetic bead sorting for CD8, a population of CD8⁺CD44⁺K⁺/Tag-IV⁺ cells (2 ± 0.5%) was found in the spleens of 7-week-old vaccinated TRAMP. In vaccinated WT animals, such population reached 10 ± 3.1% and bound K⁺/Tag-IV pentamers with higher avidity (Supplementary Fig. S2). Upon in vitro restimulation, blasts from both cultures killed an irrelevant target (RMA) pulsed with Tag-IV, as well as B6-K0 that express Tag (27), and not or marginally unpulsed RMA (Fig. 4A and B), therefore demonstrating that those CTL were able to recognize the SV40 epitope as endogenously processed and presented. The lytic activity correlated well with the percentage of CD8⁺CD44⁺IFN-γ⁺ cells as depicted by intracellular staining (Fig. 4E and F). Indeed, blasts of age-matched WT mice were much more frequent and, especially, produced IFN-γ at much higher intensity, therefore confirming that, in TRAMP mice, central tolerance caused loss of high-avidity Tag-specific CTL. As expected from the ex vivo results (Supplementary Fig. S2), the percentage of K⁺/Tag-IV⁺ cells was much higher in vaccinated WT mice than in TRAMP mice (Fig. 4I and J).

When blasts from 16-week-old TRAMP mice were analyzed, neither specific lytic activity nor IFN-γ production could be detected (Fig. 4C and G). Conversely, the lytic activity (Fig. 4B and D) and cytokine release from 6-week-old and 16-week-old vaccinated WT mice were comparable (Fig. 4F and H), therefore confirming that TRAMP mice at 16 weeks of age are fully tolerant to Tag (24). The consistent finding in tolerant TRAMP mice, both ex vivo (Supplementary Fig. S2) and upon in vitro restimulation (Fig. 4K) of a population of unresponsive (gray columns, CD4⁺ total cells; black columns, CD4⁺CD25⁺ cells; white columns, CD4⁺CD25⁺ cells) were cultured for 72 h in 96-well plates previously coated with anti-CD3 mAb. The incorporation of [³H]thymidine by proliferating T cells (triplicate cultures) during the last 16 h of culture was measured. Proliferation index was calculated assigning value of 1 to cpm obtained from unstimulated cultures. Data are representative of at least two independent experiments.

Tolerance cannot be reverted in TRAMP mice by depletion of CD25⁺ T cells. To verify whether in vivo depletion of Treg would rescue Tag-specific tolerance, PC61 or control rat IgG were injected into TRAMP mice. Four days later, splenocytes were harvested and costained with anti-CD4 and anti-CD25 mAb. At that time, the CD4⁺CD25⁺ cell population in PC61-treated animals dropped to <0.5% as depicted by two anti-CD25 mAb, binding different epitopes on the CD25 molecule (Supplementary Fig. S3). A similar depletion of CD4⁺CD25⁺ cells was found in the blood, and at both sites, CD4⁺CD25⁺ cells remained mostly undetectable for at least 10 days (data not shown). Depletion of Treg was also checked by Foxp3 staining and flow cytometry in prostates and TDLN of TRAMP mice treated with PC61 or rat IgG. As shown in Supplementary Fig. S4, the percentage of CD4⁺CD25⁺Foxp3⁺ cells dropped, as well, in the organs collected from PC61-treated animals.

PC61-treated 16-week-old male TRAMP and WT age-matched littermates were repeatedly vaccinated with Tag-IV–pulsed dendritic cells and killed 1 week after the last boost (week 24). The urogenital apparatuses of TRAMP mice were enlarged (1.4 ± 0.2 g; n = 6) when compared with the ones from WT littermates (0.7 ± 0.1 g; n = 4; P < 0.0001). However, there was no difference with rat IgG–treated and rat IgG–vaccinated TRAMP mice (1.9 ± 0.5; n = 6). Also, we found no difference in the disease score between the two groups of treated TRAMP mice (4.3 ± 0.5 and 4.4 ± 0.5, respectively). Finally, treated TRAMP mice were not able to respond to Tag, as measured both by cytotoxicity (Fig. 5) and IFN-γ release assays (data not shown). Interestingly, in both PC61 and rat IgG–treated and rat IgG–vaccinated TRAMP mice, a population of K⁺/Tag-IV⁺CD8⁺CD44⁺ T cells could be detected, which accounted for 4.9 ± 1.3% and 5.9 ± 2.4% of the restimulated splenocytes, respectively.

In spleens of WT treated mice, we found a strong cytolytic activity against both B6-K0 and Tag-IV pulsed RMA cells and not against unpulsed RMA (Fig. 5). Apparently CD25⁺ cell depletion did not also affect the antigen-specific immune response of WT mice. Indeed, no difference in Tag-specific cytolytic activity, antigen-specific IFN-γ release (not shown), and frequency of K⁺/Tag-IV⁺CD8⁺CD44⁺ T cells
could be detected in PC61-vaccinated mice when compared with rat IgG–vaccinated ones (Fig. 5).

We previously reported that full tolerance against Tag could be found in TRAMP mice as early as by weeks 10 to 11 (24). Although we found evidence of Treg accumulation only at later time points, we hypothesized that failure of PC61 treatment in reverting tolerance against Tag could be due to a deferred schedule. Hence, PC61 antibodies were injected in TRAMP mice starting at week 6, time at which TRAMP mice are not tolerant (Fig. 2). To prolong CD25+ T-cell depletion, PC61 was injected every 2 weeks until week 12, when all mice were vaccinated with Tag-IV–pulsed dendritic cells and killed 1 week later. Ex vivo analysis of lymphoid organs confirmed a 10-fold reduction of CD4+CD25+Foxp3+ cells in PC61-injected animals when compared with their controls (e.g., TDLN, 9.8 ± 0.8% and 0.8 ± 0.8%, respectively). However, such treatment did not break Tag-specific tolerance in TRAMP mice (data not shown).

Those unexpected findings prompted us to verify the antitumor effects of PC61 mAb in a subcutaneous model of cancer. Hence, PC61 or control rat IgG were injected i.p. at days −1 and +3 in WT C57BL/6 mice, and at day 0, animals were challenged subcutaneously with TRAMP-C1 cells, a prostate adenocarcinoma cell line (28). As shown in Supplementary Fig. S5, PC61-treated mice showed a significant delay (P < 0.026) in tumor growth, therefore confirming that PC61 treatment is effective against subcutaneous prostate cancer (32).

An alternative explanation to our findings is that antibody treatment also depleted activated/effecter CD25+ T cells. Using a similar depletion protocol, concomitant depletion of effectors and consequent reduction of the antigen-specific immune response has not been reported thus far (e.g., refs. 17–19). Also, in WT animals, we found that depletion of CD25+ cells by PC61 treatment did not impair the immune response induced by Tag-IV–pulsed dendritic cells (Fig. 5), therefore suggesting that CD25+ effectors were not altered by PC61 pretreatment. To further address this issue, we attempted to impair Treg function by cyclophosphamide, which, at low doses, decreases Treg number and functionality (33) and...
increases the antitumor effect of dendritic cell vaccines (34). Hence, 100 mg/kg cyclophosphamide were injected i.p. in 16-week-old TRAMP mice and WT controls 4 days before Tag-IV–pulsed dendritic cell vaccination. One week after dendritic cell vaccination, the percentage of CD4+CD25+Foxp3+ cells dropped significantly in the spleens of both WT and TRAMP mice. Furthermore, cyclophosphamide treatment increased the immune response induced by Tag-IV–pulsed dendritic cells in WT mice (Supplementary Fig. S6), therefore confirming that cyclophosphamide augments the antitumor effect of dendritic cell vaccines (34). Conversely, no Tag-specific immune response could be detected in vaccinated TRAMP, either untreated or injected with cyclophosphamide (Supplementary Fig. S6).

The combined treatment with 1-MT, PC61 mAb, and Tag-IV–pulsed dendritic cells cannot revert tolerance in TRAMP mice.

Indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the oxidative breakdown of the essential amino acid tryptophan and, therefore, blocks T-cell function, accumulates in prostate cancer tissues (31) and may favor induction of Treg (35). We reasoned that accumulation of Treg in aged TRAMP mice could be due also to the release of IDO. Hence, we tested whether treatment with 1-MT, an IDO inhibitor (31), could synergize with PC61 mAb and dendritic cell vaccination in restoring the TAA-specific immune response. In preliminary experiments, we verified that administration of 1-MT was effective in causing tumor growth delay. BALB/c mice challenged subcutaneously with C26-GM colon carcinoma cells (36) and fed thereafter with 1-MT in the drinking water showed a significant delay in tumor growth when compared with control untreated animals \( (P < 0.0006); \) Supplementary Fig. S7).

Hence, PC61 or rat IgG mAb were injected in 6-week-old TRAMP mice, and animals were fed thereafter with 1-MT. Mice were repeatedly treated with PC61 mAb at weeks 8, 10, and 12 and vaccinated once with Tag-IV–pulsed dendritic cells 4 days after the last injection of PC61 mAb. Animals were killed at week 13, and their splenocytes were restimulated \textit{in vitro}. Neither Tag-specific cytolytic activity (Fig. 6) nor IFN-\( \gamma \) production \( (\text{data not shown}) \) could be detected in TRAMP mice, irrespective of the treatment. In WT mice, PC61 treatment in combination with 1-MT did not increase the immunogenic potential of the vaccine (Fig. 6).

Taken together, our data suggest that Treg, although clearly accumulating at the tumor site and in the TDLN, do not seem to have a dominant role in tolerance induction against this TAA.

\( \text{CD4}^+\text{CD25}^+ \) Treg accumulate at the site of an active immune response. Neither enlargement of lymph node not draining (NDLN) the prostate gland nor accumulation of CD4+CD25+Foxp3+ cells in those lymph node could be found in TRAMP mice at all weeks tested \( (\text{from week 6 to week 34}; \) data not shown), therefore suggesting that accumulation of CD4+CD25+Foxp3+ cells was directly related to tumor progression and local activation of an immune response. A tumor-dependent activation of the immune response in enlarged TDLN was

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Tolerance cannot be reverted by \textit{in vivo} depletion of CD25+ cells. Five hundred micrograms of monoclonal anti-CD25 antibody PC61 \( (A, E, B, \) and \( F) \) or control rat IgG \( (C, G, D, \) and \( H) \) were injected i.p. at day \(-4\) into 16-wk-old male TRAMP \( (n = 6; A, E, C, \) and \( G) \) and WT mice \( (n = 5; B, F, D, \) and \( H) \). At day 0, mice were vaccinated with Tag-IV–pulsed dendritic cells. Animals were boosted at days 7, 21, 35, and 49. Mice were sacrificed at 24 wk of age \( (\text{day 56}) \), and splenocytes were stimulated in vitro with irradiated B6/K-0 cells. Cytotoxicity assay \( (A–D) \) and K\textsuperscript{b}/Tag-IV pentamer staining \( (E–H) \) were performed as described in Fig. 3.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Tolerance cannot be reverted in \textit{vivo} by the association of PC61 with inhibition of IDO enzyme. TRAMP \( (A \) and \( C) \) and WT \( (B \) and \( D) \) mice were injected i.p. with 500 \( \mu \text{g} \) of monoclonal anti-CD25 antibody PC61 \( (A \) and \( B) \) at the age of 6 wk or left untreated \( (C \) and \( D) \). The group of mice that received the antibody was also fed with 1-MT \( (5 \text{mg/mL in the drinking water, ad libitum until killing of mice}) \). Administration of PC61 was repeated at weeks 8, 10, and 12. All mice were vaccinated with Tag-IV–pulsed dendritic cells at week 12 and killed 1 wk later. Splenocytes were restimulated \textit{in vitro} and tested 5 d later in \( ^{51} \text{Cr} \) release assays against specific targets, as described in Fig. 3. Each panel is representative of at least two independent experiments.

\textbf{CD4+CD25+Foxp3+ Treg accumulate at the site of an active immune response.} Neither enlargement of lymph node not draining (NDLN) the prostate gland nor accumulation of CD4+CD25+Foxp3+ cells in those lymph node could be found in TRAMP mice at all weeks tested \( (\text{from week 6 to week 34}; \) data not shown), therefore suggesting that accumulation of CD4+CD25+Foxp3+ cells was directly related to tumor progression and local activation of an immune response. A tumor-dependent activation of the immune response in enlarged TDLN was
suggested by the increased population of activated CD4⁺CD25⁺ Foxp3⁺ cells only in TDLN from aged and tumor-bearing TRAMP mice (12.3 ± 7 × 10³; n = 6) and not in age-matched WT animals (3 ± 1.3 × 10³; n = 6; P < 0.021) or young TRAMP mice (3.9 ± 3.3 × 10³; n = 5).

Hence, we verified whether CD4⁺CD25⁺Foxp3⁺ cells accumulated also in lymph node draining the site of vaccination (VDLN). As found in TDLN, the absolute number of cells in VDLN, from WT mice vaccinated 1 week before with Tag-IV–pulsed dendritic cells, increased dramatically from an average of 0.95 ± 0.4 × 10⁶ for a NDLN to 12.9 ± 3.6 × 10⁶ cells in VDLN (n = 10; P < 0.000002). The absolute number of CD4⁺CD25⁺Foxp3⁺ cells increased as well, being only 30 ± 16 × 10³ in NDLN and reaching the 299 ± 78 × 10³ units in VDLN (P < 0.000006). Curiously enough, also in VDLN, we found a significant increase in the absolute number and not in the percentage of Treg, therefore confirming that, during an active immune response, increase in Treg number in VDLN parallels increase of other lymph node cell populations, such as total CD4 (3.4 ± 1 and 0.3 ± 0.1×10⁶ cells in VDLN and NDLN, respectively), CD8 (2.5 ± 0.5 and 0.2 ± 0.1 × 10⁶), and especially Kbt/Tag-IV⁺ cells (9.5 ± 2.5%). Similar results were obtained in vaccinated TRAMP mice (data not shown).

All together, our data suggest that Treg accumulation at the site of an active immune response is physiologic and, at least in the TRAMP model, is not essential for tolerance induction to a TAA.

Discussion

The mechanism by which peripheral tolerance is induced in tumor-bearing subjects is ill defined (4), although findings in animal models suggest that TAA drained or carried to the lymph nodes and presented by APC in a noninflammatory context drive clonal deletion or anergy (37). Tolerance in 10-week-old to 11-week-old TRAMP mice (24) is likely induced in the absence of local inflammation because the prostate gland at that time lacks an inflammatory infiltrate and TDLN are not enlarged (data not shown). Although several previous findings were consistent with peripheral deletion of TAA-specific T cells in tumor-bearing TRAMP mice (24, 38), in those mice, we consistently found a population of Kbt/Tag-IV⁺CD8⁺CD4⁺ T cells, which neither expanded in culture nor killed or produced IFN-γ upon specific stimulation, therefore implying that a sizable population of Tag-specific T cells survive peripheral deletion and undergo anergy in tumor-bearing TRAMP mice.

As it occurs in human prostate cancer patients (15), we found evidence of a progressive accumulation of functional Treg both at the tumor site and in TDLN of TRAMP mice developing spontaneous prostate cancer. However, Treg depletion by PC61 mAb followed by repeated vaccinations did not rescue T cells from anergy. Also, repetitive PC61 injections followed by dendritic cell vaccination did not break tolerance. Most importantly, the combined treatments did not modify both the early and advanced phases of disease progression. We excluded malfunction of the mAb, because mice that received a single dose of PC61 antibody showed approximately a 10-fold reduction in the CD4⁺CD25⁺ T-cell population in lymphoid organs. Furthermore, PC61 treatment was effective against TRAMP-C1 tumors.

As for our TRAMP-C1 model, most of the reports on successful PC61-mediated depletion of Treg were conducted in mice in which tumor cells from in vitro established tumor cell lines were injected s.c. The engraftment of even a small number of tumor cells does not mimic the natural development of a tumor mass (37) and likely causes inflammation at the site of injection (39). This may dramatically alter the dynamic interactions between tumor and immune cells (5). Hiura et al. recently reported that both Treg and antitumor effector T cells were primed in the same TDLN of a tumor growing subcutaneously. (12). PC61 mAb are usually injected a few days before or at the time of tumor cell implantation. Such treatment schedule may give an unrealistic advantage to effector T cells, likely depleting thymic-derived Treg and preventing the induction of peripherally induced tumor-specific Treg. Those models may more closely mimic the condition of a prophylactic vaccination, wherein PC61 pretreatment increases the immunogenic potential of the vaccine (18), likely recruiting high-avidity T cells (40) and natural killer cells (17).

However, those tumor transplantation models did not address the peculiar condition of tumor-bearing subjects, wherein effector T cells, negatively selected for avidity or exhausted by prolonged TAA presentation in the draining lymph nodes (41), are at competition with both thymus-derived and peripherally induced Treg expanded by conversion of CD4⁺CD25⁺ lymphocytes (13). Indeed, when high-avidity tumor-specific T cells were lacking because of deletion (42), as it occurs in TRAMP mice and most likely in humans, or when PC61 treatment was begun at later time points since subcutaneous tumor challenge (43), depletion of Treg by PC61 treatment did not increase vaccine efficacy even against a subcutaneous tumor.

Tien et al. (32) found increased CD4⁺CD25⁺ T cells in the transgenic model of spontaneous prostate dysplasia 12T-7s LPB-Tag, PC61 treatment in those mice reduced, but did not prevent, tumor growth. The different findings obtained in TRAMP and 12T-7s LPB-Tag models may depend on 12T-7s LPB-Tag mice developing a far less aggressive prostate disease. Indeed, the low amount of antigen released from prostate cells in 12T-7s LPB-Tag mice may likely spare prostate-specific T-cell clones from tolerance induction and favor their activation in conditions of reduced competition (i.e., depletion of CD25⁺ cells).

Our experimental settings addressed the role of Treg in a model of spontaneous prostate cancer development, wherein endogenous low avidity CD8⁺ T cells are tolerized during cancer development and progression. Hence, our data substantially extend, in a model that more closely resemble the human disease, the observation made in a more artificial context (44) by Mihalyo et al. (45). Indeed, PC61 pretreatment did not avoid tolerization also of high-avidity HA-specific transgenic CD4⁺ T cells upon adoptive transfer into TRAMP mice expressing HA in the prostate.

The process of Treg generation and accrual in tumor-bearing subjects may follow a kinetic common to autoantigens: the persistence of the Ag; in this case, the TAA would lead to continuous generation of Treg in the periphery. As suggested (46), removal of Treg might have only limited or no effect in that condition, as Treg may be continuously replenished from the memory pool. In the TRAMP model in particular, tolerance is so profound that even transient depletion of Treg is not sufficient to restore TAA-specific immune response. This might occur also in prostate cancer patients.

Depletion of Treg in vivo has been already attempted in humans with variable results. Dannull et al. (47) reported that in renal cancer patients pretreated with the recombinant IL-2-diphtheria toxin conjugate denileukin, Treg were selectively eliminated and a vaccine-mediated antitumor immunity was enhanced. However, another group reported no reduction in the number of Treg or their
function in melanoma patients receiving denileukin (48). Furthermore, depletion is only transient, and Treg rapidly repopulate the human body (49).

Other strategies may be implemented to break tolerance in prostate cancer patients and in TRAMP mice. Also, inhibition of IDO by 1-MT did not revert tolerance in our transgenic model. On the other hand, high levels of nitrotyrosines in T cells infiltrating IDO by 1-MT did not revert tolerance in our transgenic model. More, depletion is only transient, and Treg rapidly repopulate the function in melanoma patients receiving denileukin (48). Further-}


despite the kinetics of dendritic cells activation on the presentation of both tumor cells and APC, as well as altered expression of B7 family molecules by APC, may likely reduce effective immunosurveillance of prostate cancer (7). Finally, FasL expressed on tumor cells, together with other proapoptotic mechanisms, may favor programmed T-cell death at the tumor site (7). Hence, further studies are warranted to better define tumor immunosuppressive mechanisms and design more effective and vigorous combinatorial strategies for generating productive immune responses.

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Peripheral T-Cell Tolerance Associated with Prostate Cancer Is Independent from CD4 +CD25+ Regulatory T Cells

Elena Degl'Innocenti, Matteo Grioni, Giusy Capuano, et al.


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