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PTEN Knockout Prostate Cancer as a Model for Experimental Immunotherapy

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Purpose: Testing immunotherapeutic strategies for prostate cancer has been impeded by the lack of relevant tumor models in immunocompetent animals. This opportunity is now provided by the recent development of prostate specific PTEN knockout mice, which show spontaneous development of true adenocarcinoma arising from prostate epithelium and more faithfully recapitulate the human disease than any previous model. We investigated the feasibility of using tumor cells derived from this model to test tumor vaccination and adoptive immunotherapeutic strategies for prostate cancer.

Materials and Methods: PTEN-CaP8 adenocarcinoma cells derived from the biallelic PTEN knockout prostate cancer model were used to vaccinate nontumor bearing litter mates. Tumor specific effector cells were generated from splenocytes of vaccinated mice by mixed lymphocyte-tumor reactions, and antiproliferative effects and cytokine generation were examined in vitro. The effect of vaccination or adoptive immunotherapy on luciferase marked PTEN-CaP8 subcutaneous tumors was monitored by tumor volumetric measurements and noninvasive bioluminescence imaging.

Results: Vaccination of litter mate mice with irradiated PTEN-CaP8 cells showed a significant prophylactic effect against the subsequent tumor challenge. Effector cells harvested from vaccinated litter mates showed significant interferon-γ secretion upon co-incubation with PTEN-CaP8 target cells and they were capable of efficient target cell growth inhibition in vitro. Intratumor adoptive transfer of effector cells resulted in significant growth inhibition of preestablished prostate tumors in vivo.

Conclusions: The PTEN knockout model serves as a highly useful model in which to investigate tumor cell vaccination and adoptive immunotherapeutic strategies in the context of true adenocarcinoma of the prostate. This model should accelerate efforts to develop effective immunotherapies for human prostate cancer.

Abbreviations and Acronyms

ADI-Ca = androgen depletion independent cancer
CTL = cytotoxic T lymphocyte
ELISA = enzyme-linked immunosorbent assay
E/T = effector-to-target
GFP = green fluorescent protein
IFN-γ = interferon-γ
MLTR = mixed lymphocyte-tumor reaction
MTS = (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PBS = phosphate buffered saline
PTEN = phosphatase and tensin homologue deleted on chromosome 10
ROI = region of interest
RL = Renilla luciferase

Key Words: prostate; prostatic neoplasms; PLIP protein, mouse; cancer vaccines; immunotherapy, adoptive

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PROSTATE cancer is currently the most commonly diagnosed cancer and the second leading cause of cancer death in men in the United States.[1] Generally patients with metastatic prostate cancer are initially responsive to androgen ablation therapy but most patients subsequently progress to ADI-Ca, for which treatment options are limited. The prognosis in patients with ADI-Ca is poor despite aggressive multimodal therapy and there is currently no standard of care. Hence, there is a need to pursue new and potentially more effective treatment strategies.

To improve the long-term outcome it is desirable to develop new therapeutic modalities capable of eliminating metastatic foci of cancer cells that have become resistant to previous therapies. Immunotherapeutic strategies show promise in this regard. The activation of humoral and cellular responses can mobilize antibodies and effector cells, which can circulate systemically and cause cytotoxicity to tumor cells, and immunological memory may be engendered to prevent recurrence. In fact, currently a number of immuno-activating agents are in advanced stages of clinical testing for prostate cancer, including an allogeneic prostate cancer cell vaccine product engineered to express granulocyte-macrophage colony-stimulating factor (GVAX®) and an autologous dendritic cell vaccine produced by ex vivo pulsing with the prostatic acid phosphatase/granulocyte-macrophage colony-stimulating factor fusion peptide sipuleucel-T.[2] However, there have been few studies of prostate cancer exploring the potential of adoptive immunotherapy with activated CTLs, which is a strategy that has shown promise for other malignancies, such as melanoma and glioma.[3]

A number of groups have developed strategies for activating CTLs against prostate cancer cells or for their genetic modification with artificial T-cell receptors.[4–8] Many of these investigators have used adoptive transfer of human CTLs in the setting of human prostate cancer xenografts in immunodeficient rodents, a milieu in which interaction with the endogenous immune system and the immunosuppressive tumor environment cannot be adequately assessed.[4–6] Other groups have pursued studies of adoptive immunotherapy using syngeneic cancer cells and immunocompetent hosts derived from the TRAMP (transgenic adenocarcinoma of mouse prostate) model of prostate cancer.[7–9] However, this transgenic model was generated by prostate specific expression of the SV40 T antigen, which itself represents a foreign target antigen, and it is now known that the cancer arising in this model manifests extensive neuroendocrine differentiation, unlike human adenocarcinoma of the prostate.[10]

The Dunning model is also a syngeneic adenocarcinoma model of prostate cancer that has served as a highly useful model for the development of immunotherapy.[11] Nonetheless, based on the expression of nonprostatic proteins there has also been some controversy as to the true origin of the spontaneous Dunning tumor from which the R3227 subline system was derived.[12]

Thus, to date more comprehensive investigation of immunotherapeutic strategies for prostate cancer has been hampered by the relative dearth of syngeneic cell lines and immunocompetent animal models that represent genetically well-defined examples of true adenocarcinoma and faithfully recapitulate important biological and clinical aspects of the human disease. In this regard the recent development of a unique murine model of spontaneously occurring prostate adenocarcinoma, as generated by biallelic knockout of the PTEN tumor suppressor gene, shows considerable promise as a tool for developing and evaluating novel immunotherapies for this disease. This tumor suppressor gene encodes a phosphatase that antagonizes phosphatidylinositol-3-kinase/protein kinase B (Akt) signaling[13] and is frequently disrupted in various tumors, including prostate cancer.[14] Loss of PTEN leads to the upregulation of pro-survival pathways and contributes to chemoresistance, which correlates with high grade and advanced stage disease, especially in ADI-Ca.[15]

In this recently developed transgenic knockout model biallelic deletion of loxP flanked sequences in the PTEN gene is achieved by prostate specific expression of Cre recombinase. The resultant animals show spontaneous development of hyperplasia and metaplasia of the prostatic epithelium, followed by PIN lesions, and progression to invasive adenocarcinoma and subsequent micrometastasis.[16]

Thus, the prostate specific PTEN knockout model is the first transgenic murine model of true prostate adenocarcinoma that arises reproducibly and spontaneously in vivo, is genetically well-defined and causally linked to a genetic deficiency commonly observed in human disease and faithfully mimics the course of human prostate cancer. Therefore, this model seems highly suitable as a model in which to test immunotherapeutic strategies. Accordingly we have used syngeneic PTEN deleted, androgen receptor positive prostate adenocarcinoma cells derived from this unique model[16,17] to test their potential usefulness for the development and evaluation of tumor vaccination and adoptive immunotherapeutic strategies.

MATERIALS AND METHODS

Cells and Vectors

The murine prostate cancer cell lines PTEN-P8 and PTEN-CaP8 were derived from the PTEN knockout model of prostate cancer, as reported previously,[16,17] and main-

tained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, California), 25 μg/ml bovine pituitary extract (Invitrogen™), 5 μg/ml bovine insulin (Sigma-Aldrich), 6 ng/ml recombinant human epidermal growth factor (Sigma-Aldrich®) and 1% penicillin in a humidified incubator at 37C in 5% CO_2.

To generate PTEN-CaP8 cells that stably expressed RL (PTEN-CaP8/RL) PTEN-CaP8 cells were transduced with lentiviral vector CCL-m4/hrl-IRES-GFP[18] at a ratio of 1 μg p24/1 $\times$ 10^6 cells. After expansion in culture cells were analyzed for GFP expression by fluorescence activated cell sorting analysis using an EPICS® XL™ flow cytometer.

MLTR to Generate Effector Cells

After irradiation with 20,000 Ci to prevent further cell division 5 $\times$ 10^6 PTEN-CaP8 cells were injected subcutaneously into 6 to 8-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, Massachusetts) every 2 weeks for a total of 3 inoculations. Two weeks following the last vaccination splenocytes were harvested and washed after red blood cell removal with lysis buffer (Sigma-Aldrich).

Stimulator PTEN-CaP8 cell monolayers were treated with mitomycin C (Sigma-Aldrich) for 1 hour at 37C, washed and subsequently incubated with responder mouse splenocytes at a responder-to-stimulator ratio of 10:1. Cells were placed into RPMI-1640 medium containing 10% fetal bovine serum and 100 IU/ml recombinant mouse interleukin-2 (Sigma-Aldrich), and incubated at 37C in humidified air with 5% CO_2 for 6 to 7 days. Subsequently effector cells were characterized in vitro or used in adoptive transfer experiments.

Assessment of Antiproliferative Effects in Vitro

The viability of PTEN-P8 cells seeded into 96-well plates at 1 $\times$ 10^3 cells per well with or without MLTR derived effector cells was determined by MTS assay using the soluble tetrazolium salt MTS and a CellTiter 96® nonradioactive cell proliferation assay. Washed effector cells were mixed with PTEN-P8 target cells at a ratio of 1:1 and incubated for 2 days. The medium was changed to remove the nonadherent effector cells and any detached PTEN-P8 target cells, and then adherent cell viability was analyzed by measuring optical density absorbance using an ELISA plate reader at 490 nm to detect formazan produced in the MTS assay after 2 hours of reaction at 37C. The surviving cell fraction was calculated as the ratio of the average absorbance achieved in effector cell treated test cultures to that of conditioned medium from untreated control samples. Results in pentaplicate wells are expressed as the mean percent of metabolically active cells compared to those in untreated wells.

IFN-γ Assay

MLTR derived effector cell cultures from vaccinated animals were co-cultured for 2 days with or without an equal number of PTEN-P8 cells. The levels of murine IFN-γ present in clarified supernatants of conditioned medium from these co-cultures was measured by ELISA using the Quantikine™ murine IFN-γ immunoassay. Recombinant mouse IFN-γ served as the positive control. Results were normalized as the rate of cytokine production, ie pg/10^6 cells per 24 hours, and comparisons are expressed as the fold difference between effector cells incubated with and without relevant tumor target cells.

Animal Experiments

All animal experiments were performed according to institutional guidelines under approved protocols at UCLA and University of Southern California. Principles of the Helsinki Declaration were followed.

For immunization and prophylactic vaccination experiments 6 to 8-week-old male C57BL/6 mice were vaccinated by subcutaneous injection with irradiated PTEN-CaP8 cells (5 per vaccination group and 5 $\times$ 10^6 cells per mouse with irradiation with 20,000 Ci delivered to 2 $\times$ 10^7 cells per 3 ml culture) into the left flank for a total of 3 vaccinations every 2 weeks, as described. Five each of vaccinated mice and a paired number of control mice were subsequently challenged by subcutaneous injection into the right flank with 1 $\times$ 10^6 naïve PTEN-CaP8 cells. Observations to assess tumor growth were performed for 2 weeks.

For adoptive transfer experiments tumors were established in 6 to 8-week-old male athymic nude mice (Charles River Laboratories) by subcutaneous injection of 1 $\times$ 10^6 PTEN-CaP8/RL cells. Tumor dimensions were measured on days 14, 18, 21, 28, 35, 42 and 60. Tumor volume was calculated using the formula, (length) $\times$ $(\text{width})^2/2$.[19] Two weeks after tumor establishment each mouse was assigned randomly to a treatment arm of 12 per group, followed by a single intratumor injection of 1 $\times$ 10^6 effector cells harvested from PTEN-CaP8 vaccinated mice, as described, or PBS vehicle control.

In Vitro and In Vivo Optical Imaging Analysis

For in vitro imaging studies RL expression in PTEN-CaP8/RL cells was confirmed by bioluminescence optical imaging using a cooled charge coupled device system (Xenogen®) 2 minutes after the addition of its imidazolopyrazine substrate, coelenterazine with maximum emission read at 480 nm upon oxidation by RL.[20]

For in vivo imaging studies mice bearing PTEN-CaP8/RL tumors were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). After the administration of coelenterazine (1 mg/kg) intravenously the anesthetized mice were imaged using the Xenogen system with a 1 minute acquisition time at different time points after tumor implantation according to previously described techniques.[18] Grayscale background photographic images of the tissues were overlaid with color images of bioluminescent signals using Living Image® 2.2 and IGOR Pro image analysis software (WaveMetrics, Lake Oswego, Oregon). The bioluminescence in vivo signal was calculated using the signal in the ROI as photons per second per cm^2/sr.[21]

Statistical Analysis

All statistical analyses were done using GraphPad® Prism®, version 4 for Windows®. Results between different groups were compared using the Mann-Whitney or unpaired t test with $p < 0.01$ considered significant.

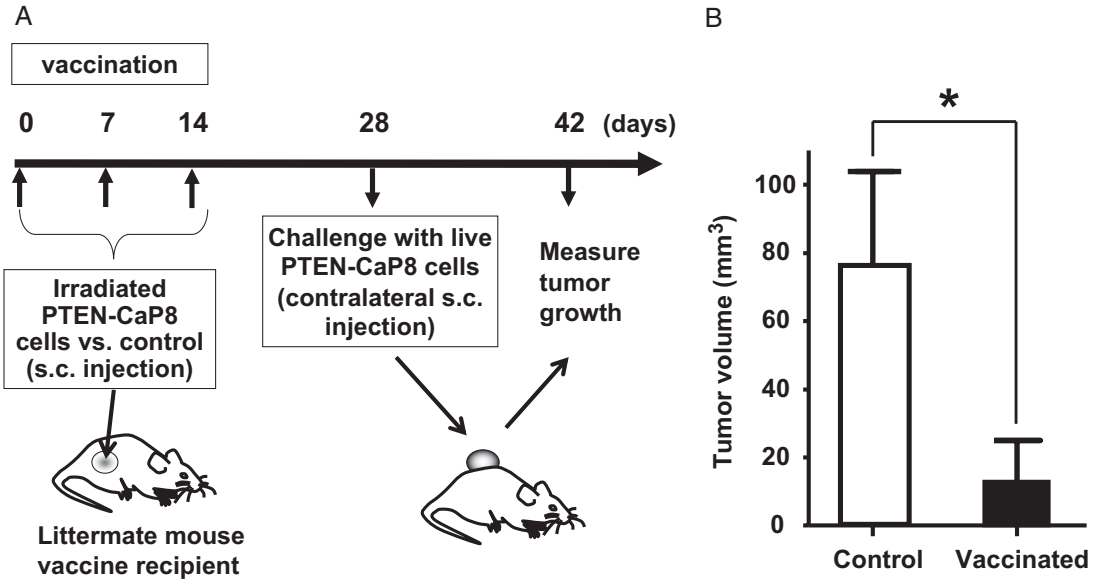


Figure 1. *A,* tumor vaccination protocol schedule. Nontumor bearing litter mates of PTEN knockout prostate cancer mice were vaccinated by subcutaneous (*s.c.*) injection of 1×10^6 irradiated PTEN-CaP8 cells total of 3 times in 2 weeks, followed 2 weeks later by challenge with 1×10^6 live PTEN-CaP8 cells into contralateral flank. *B,* tumor vaccine effect in subcutaneous models. Contralateral flank PTEN-CaP8 tumors were measured on day 14 after challenge in unvaccinated control and vaccinated animals. Error bars indicate SD. Asterisk indicates statistically significant ($p < 0.01$).

RESULTS

Immunization and Tumor Vaccine Prophylaxis

We examined whether the highly tumorigenic murine prostate adenocarcinoma cell line PTEN-CaP8, which was derived from the PTEN knockout model of prostate cancer, would be capable of eliciting a robust antitumor immune response in syngeneic hosts and, thereby, serve as a tumor vaccine. Adult male C57BL/6 background litter mate mice were vaccinated subcutaneously with injections of irradiated PTEN-CaP8 cells and 2 weeks later the animals were challenged with fresh nonirradiated PTEN-CaP8 cells injected subcutaneously into the contralateral flank (fig. 1, *A*). Control mice were also challenged in the same manner but they received injections of saline vehicle instead of irradiated tumor cells. Tumor formation was observed to be significantly inhibited in the vaccinated group compared to the control group 2 weeks after the tumor challenge ($p < 0.01$, fig. 1, *B*). In fact, no tumor establishment was observed in 2 of the 5 vaccinated mice, while all control mice showed approximately 5-fold increased tumor growth compared to the average tumor size in the vaccinated group (fig. 1, *B*). Thus, PTEN-CaP8 could serve as an effective cell vaccine to prevent and/or retard tumor formation in immunocompetent syngeneic hosts.

In Vitro Analysis of Cellular Immune Response

To determine whether this prophylactic vaccination effect was mediated by a cellular immune response and confirm that such responses could be elicited by endogenous tumor antigens litter mate mice were again immunized by subcutaneous injection of irradiated PTEN-CaP8 cells, as described, but this time their splenocytes were harvested for explant culture in mixed lymphocyte tumor cell reactions with PTEN-P8 cells. Parental PTEN-P8 cells, which are the precursor to PTEN-CaP8 cells, were used as target cells in this case because, although they are less tumorigenic, the parental cells lack detectable Cre recombinase expression.[16,17] Hence, this foreign enzyme protein would not serve as a target antigen. Cellular immune responses seen against PTEN-P8 cells in vitro likely represented reactivity to true endogenous tumor antigens.

Accordingly mixed lymphocyte tumor cell reactions were performed by co-culture of splenocytes from PTEN-CaP8 vaccinated mice in the absence and presence of PTEN-P8 target cells and 24-hour conditioned medium was analyzed by ELISA to measure the levels of secreted murine IFN-γ. Effector cells co-cultured with relevant PTEN-P8 target cells produced significantly increased levels of IFN-γ compared to those of effector cells cultured alone (fig. 2, *A*). This suggests that the activated effector cells were capable of a robust T-helper type 1-type cytokine response upon the recognition of endogenous tumor antigens.

We then examined whether effector cells isolated from vaccinated animals could exert antiproliferative effects on PTEN-P8 cells in a dose dependent manner.

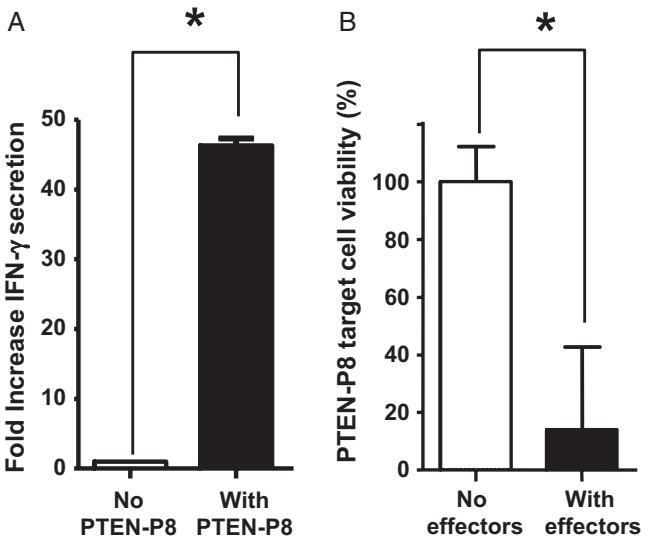


Figure 2. *A*, IFN-γ production by activated effector cells upon co-culture with PTEN-P8 prostate cancer cells. Conditioned medium after overnight culture of CTLs in absence (*No PTEN-P8*) or presence (*With PTEN-P8*) (E/T ratio 1:1) of target cells was assayed by ELISA. Results are expressed as fold increase compared to negative control. *B*, PTEN-P8 target cell viability after culture in absence (*No effectors*) or presence (*With Effectors*) (E/T ratio 1:1) of effector cells isolated from PTEN-CaP8 vaccinated litter mates was evaluated by MTS assay for metabolic activity. Results are expressed as percent of cell viability compared to negative control. Error bars indicate SD. Asterisk indicates statistically significant ($p < 0.01$).

Activated effector cells from PTEN-CaP8 vaccinated mice were co-cultured with PTEN-P8 target cells at different E/T cell ratios. Subsequently nonadherent effector cells as well as any nonviable target cells were removed by washing and metabolic activity was measured by MTS assay as an indicator of the viability of any remaining adherent target cells. At a low E/T ratio of 1:10 there was no significant cytotoxicity to PTEN-P8 target cells (data not shown). However, activated CTLs incubated with PTEN-P8 target cells at higher E/T ratios of 1:1 and 10:1 showed significant antiproliferative effects. As measured by MTS assay, at an E/T ratio of 10:1 metabolic activity of the prostate cancer cells was completely suppressed (data not shown). Even at an E/T ratio of 1:1 activated effector cells achieved greater than 80% suppression of PTEN-P8 target cell growth during the 2 day co-culture period, as measured by metabolic activity (fig. 2, *B*). These results further suggest that vaccination results in the generation of activated effector cells, which can exert antiproliferative effects against PTEN knockout prostate cancer cells.

Generation of PTEN Knockout Prostate Cancer Cells Expressing RL

Bioluminescence optical imaging is a noninvasive method for serially monitoring tumor progression and the response to treatment in living animals with time. The availability of PTEN knockout prostate adenocarcinoma cell lines provides an opportunity to take advantage of this imaging technology because genetically engineering these cells to express a luciferase enzyme enables detection by bioluminescence upon the addition of the appropriate substrate. Accordingly the lentiviral vector CCL-m4/hrl-IRES-GFP, which encodes RL and Aequorea GFP, was used to stably transduce the PTEN-CaP8 cell line. The resultant population of transduced cells, designated PTEN-CaP8/RL, showed high levels of GFP expression on fluorescence microscopy (fig. 3, *A*). Fluorescence activated cell sorting analysis confirmed that highly efficient gene delivery to

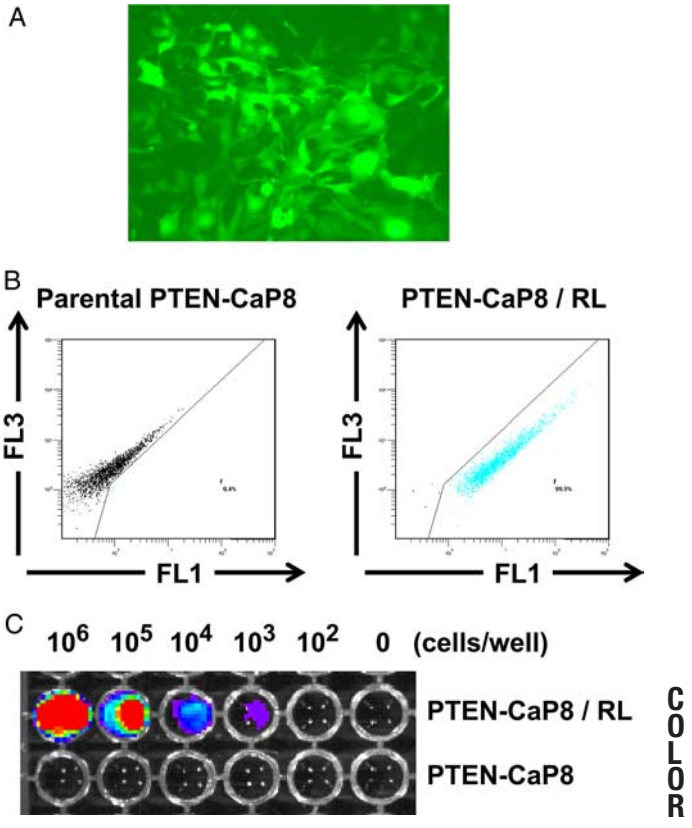


Figure 3. Characterization of PTEN-CaP8/RL cells. Parental PTEN-CaP8 cells were transduced with a recombinant lentiviral vector expressing RL and GFP, as described. *A*, fluorescence microscopy 72 hours after lentiviral transduction shows high GFP expression. *B*, after expansion in culture viral transduction was quantified by flow cytometry. Compared to parental PTEN-CaP8 cells fluorescence profile of PTEN-CaP8/RL cells showed that entire population was completely shifted along FL1 (green) fluorescence axis but not along FL3 (red) fluorescence axis, indicating complete transduction with GFP marker gene. *C*, PTEN-CaP8/RL cells were examined by bioluminescence optical imaging in vitro. Bioluminescence signals from PTEN-CaP8/RL cells increased in correlation with number of cells per well, while no bioluminescence was detected from parental PTEN-CaP8 cells regardless of number of cells plated.

99.5% of the cell population had been achieved (fig. 3, *B*).

We further confirmed RL expression in the PTEN-CaP8/RL cells by the detection of bioluminescence signals using a charge coupled device imaging system upon incubation with the substrate coelenterazine in vitro. Bioluminescence emission was confirmed to be cell dose dependent by measuring the signals obtained from 10-fold serial dilutions of the cells (range 10^2 to 10^6) (fig. 3, *C*).

Adoptive Transfer of Effector Cells Generated by MLTR From the Splenocytes of Vaccinated Animals

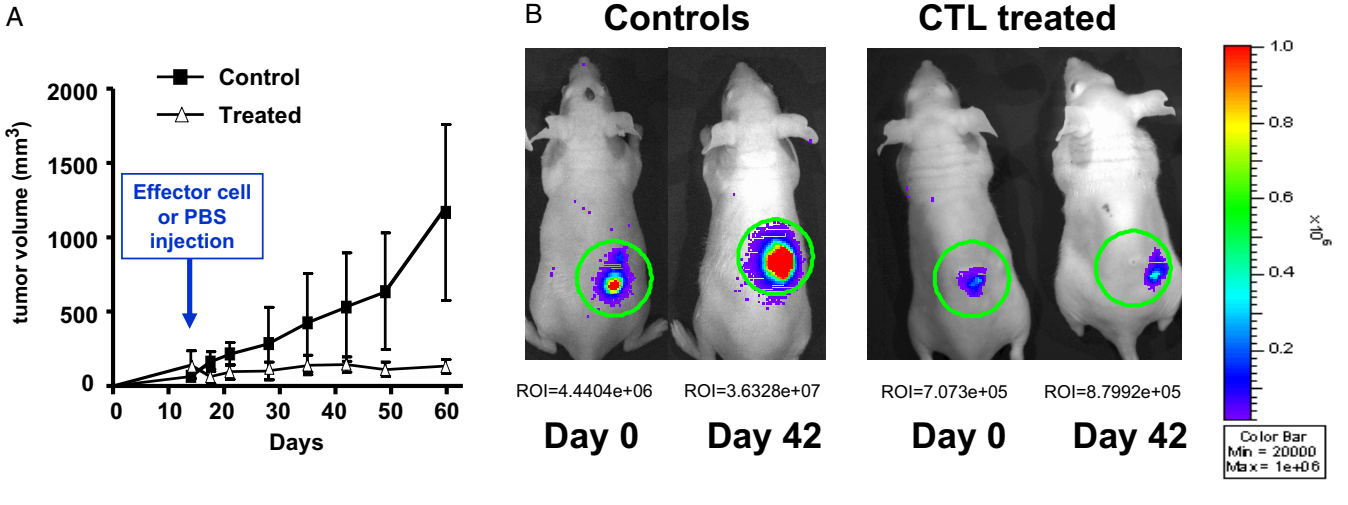
Luciferase marked PTEN-CaP8/RL prostate adenocarcinoma cells were used in experiments to monitor the efficacy of immunotherapy by the adoptive transfer of effector cells in vivo. To assess therapeutic efficacy in the absence of potential additional effects from endogenous immune responses athymic nude mice served as hosts for the establishment of subcutaneous PTEN-CaP8/RL tumors. Two weeks later activated effector cells generated as described by vaccination of nontumor bearing litter mates with irradiated PTEN-CaP8 cells were administered by direct intratumor injection. Tumor growth was then examined by bioluminescence imaging as well as by confirmatory direct measurement of tumor size at serial time points.

Adoptive transfer of activated effector cells to PTEN-CaP8/RL tumors resulted in significant tumor growth suppression compared to that of untreated control tumors, which continued to show progressive growth. By day 60 there was a significant difference in the average ± SD tumor volume of 117 ± 53 mm^3 in the treated group vs 1,168 ± 627 mm^3 in the control group ($p < 0.01$, fig. 4, *A*). F4

These findings were corroborated by the bioluminescence imaging results, which were obtained by recording sequential images of the PTEN-CaP8/RL tumors in individual animals at serial time points after intravenous injection of coelenterazine. Figure 4, *B* shows representative images of control and treated mice. The bioluminescent signal within the ROI, ie the tumor area, in the control mouse was measured as 2.0×10^6 photons per second per cm^2/sr on day 14, ie just before treatment, which then increased to 3.6×10^7 photons per second per cm^2/sr on day 49. However, the ROI signal in the treated mouse remained at 1.6×10^6 and 7.1×10^5 photons per second per cm^2/sr on days 14 and 49, respectively (fig. 4, *B*). As demonstrated in vitro, bioluminescence signal intensity can be used as a semiquantitative measure that correlates in a cell dose dependent manner with the number of viable tumor cells. Hence, the results indicate that the number of cells in the control tumor had increased approximately 15-fold between days 14 and 49, while the tumor treated with effector cells remained static.

DISCUSSION

We report the feasibility of using syngeneic cell lines, derived from the PTEN knockout model of prostate cancer, as tumor vaccines to elicit prophylactic effects against tumor establishment and pro-



COLOR COLOR

Figure 4. *A*, growth of PTEN-CaP8/RL subcutaneous tumors in nude mice after adoptive immunotherapy by intratumor injection of effector cells harvested from PTEN-CaP8 vaccinated mice (*Treated*) or injection of PBS vehicle (*Control*). Tumor volume was measured as described. Error bars indicate SD. Statistically significant differences in tumor size between 2 groups was noted on day 60 ($p < 0.01$). *B*, representative in vivo bioluminescence images show PTEN-CaP8/RL tumors on day 14 before treatment and on day 49, 5 weeks after injection of saline (*Controls*) or effector cells (*CTL treated*). Values below images indicate bioluminescence signal measured from indicated ROI (circled areas) in photons per second per cm^2/sr.

gression. We further confirmed that vaccination and tumor inhibition were associated with a cellular immune response resulting in the generation of effector cells capable of exerting highly potent antiproliferative effects against these prostate cancer cells. Finally, we also noted that effective growth suppression could be achieved upon the adoptive transfer of these effector cells to preestablished tumors.

The PTEN-P8 cell line was isolated directly from true prostate adenocarcinoma arising spontaneously in this model, as generated by mating mice with biallelic floxed PTEN loci with mice expressing Cre recombinase under the control of a prostate specific promoter.[16] Notably PTEN-P8 cells are deleted in only 1 PTEN allele, they are only weakly tumorigenic in vivo and they no longer express the recombinase.[17] Subsequently PTEN-CaP8 cells were derived from PTEN-P8 by the introduction of a retroviral vector constitutively expressing Cre, resulting in deletions in the 2 PTEN alleles and showing much more robust tumorigenicity in vivo.[17]

Therefore, in these experiments we used the PTEN-CaP8 prostate cancer cell line for immunization and tumor establishment in vivo, and its parental cell line PTEN-P8 for confirmatory in vitro studies to characterize the cellular immune response. Effector cells were generated by MLTR after vaccinating nontumor bearing litter mates of PTEN deleted mice, which were of an identical genetic background except for the lack of prostate specific Cre. Hence, vaccination with PTEN-CaP8 best mimics the situation in the mice in which PTEN deleted prostate cancer developed, whose tumors initially expressed Cre. However, in vitro assays demonstrated that effector cells generated through vaccination and resensitization with PTEN-CaP8 showed robust reactivity against PTEN-P8 cells, which no longer expressed detectable levels of Cre. This indicated that the cellular immune response was not exclusively directed against Cre as a foreign antigen, but rather likely recognized endogenous tumor antigens.

Hence, it is possible that the expression of Cre as a foreign protein may have served an adjuvant function to stimulate an initial immune response against PTEN-CaP8 cells during vaccination, which then further resulted in epitope spreading to natural endogenous tumor antigens. However, notably no conventional immune adjuvants were used in the vaccination protocol. It is certainly possible that initial irradiation of the cells used in vaccination might also have somehow resulted in the exposure of epitopes that facilitated the immune response but the subsequent in vitro MLTR restimulation procedures used PTEN-CaP8 cells that had been treated with mitomycin C, rather than irradiated. Thus, a significant contribution of irradiation as an adjuvant seems less likely. In this context using radiation in situ is likely to have limited efficacy as an immuno-activating strategy due to intrinsic local mechanisms of tumor immunoresistance, including the expression of immunosuppressive cytokines, such as transforming growth factor-β, and the presence of inhibitory dendritic cells expressing anergizing co-regulators, such as B7-H1, as well as immunotolerizing T-regulatory cells in the tumor and draining lymph nodes. In fact, the ex vivo pre-activation of cytolytic T cells away from the immunosuppressive tumor environment and their removal from these inhibitory influences may indeed represent a critical factor in the effectiveness of adoptive transfer strategies for immunotherapy.

In these experiments we used bioluminescence imaging to detect the presence and quantity of luciferase marked PTEN-CaP8/RL cells in vitro and in vivo. Bioluminescence signal intensity showed good correlation with the cell number in vitro and tumor size in vivo, and it confirmed the usefulness of this methodology for monitoring tumor growth and the response to adoptive immunotherapy in living animals with time. In these studies unmarked parental PTEN-CaP8 cells were used for vaccination in nontumor bearing litter mates. Hence, the effector cells that mediated effective tumor growth inhibition upon adoptive transfer were not reacting to luciferase as a foreign antigen. Further studies using this methodology should be greatly facilitated by the recent generation of a new version of the prostate specific PTEN knockout model, which is also transgenic for prostate specific luciferase and, hence, spontaneously arising orthotopic prostate tumors are already marked.[22]

The antiproliferative effects in vitro and tumor growth suppression in vivo achieved by effector cells derived from PTEN-CaP8 vaccinated hosts were found to be highly potent, particularly considering that the responses were not likely to be directed against xenogenic antigens, such as Cre or RL. Based on the MLTR culture conditions it was expected that in the presence of target antigens on the sensitizing tumor cells the effector cell preparation would be enriched for CTLs. However, it is certainly possible that the effector cell culture also may have contained subpopulations of natural killer or lymphokine activated killer cells, which contributed to the potent antiproliferative responses in vitro and in vivo. In this context it should also be noted that the MTS assay measures only the metabolic activity of viable adherent target cells, and so it is difficult to distinguish between decreased target cell proliferation, eg in response to immunocytokine signals released from effector cells, and actual target cell death due to effector cell mediated cytotoxicity.

Therefore, future studies will focus on the further characterization of effector cells generated by vaccination with the syngeneic prostate cancer cell lines derived from the PTEN knockout model as well as the identification of relevant endogenous tumor antigens that are expressed by these cancer cells and may be specifically recognized by CTLs. Additionally, this model is also likely to be highly informative and useful for testing various augmentation strategies, such as lymphodepleting chemotherapy, which has shown promise as a preconditioning regimen to augment adoptive immunotherapy in experimental models dating back 25 years[23–25] as well as in recent clinical trials.[26,27]

In addition to adoptive transfer strategies, the reliable and reproducible nature of spontaneous prostate cancer arising in this model is well suited for assessing vaccination strategies administered before carcinogenesis or at an early (PIN) stage. To date cancer vaccine strategies have been considerably more effective in a prophylactic setting or against minimal residual disease. In fact, they have proved rather ineffective against established bulky disease. As noted, it is increasingly recognized that solid tumors create a highly immunosuppressive environment through a wide variety of mechanisms, and so preventive vaccination may prove to be the most effective approach.

In this context it is also interesting to note a recent report showing that targeted deletion of PTEN in T cells regulated the peripheral homeostasis of Tregs in vivo and allowed their expansion in response to interleukin-2. Because prostate specific expression of Cre recombinase results in biallelic deletion of PTEN only in prostate cells in our current model, we are not currently able to directly observe the effects of PTEN deletion on T-cell function. However, in the human disease setting it is certainly conceivable that PTEN loss may represent a more generalized genetic predisposition in each cell compartment, which may conspire to simultaneously promote carcinogenesis and tolerize the immune system to the developing malignancy. Therefore, in future studies it may be valuable to evaluate the consequences of PTEN loss in prostate epithelium and T cells in a dual compartment knockout model.

CONCLUSIONS

We propose that this unique PTEN knockout model of prostate cancer can serve as a highly useful experimental system in which to assess immunotherapy strategies against true prostate adenocarcinoma in a fully immunocompetent rodent model that mimics the human disease with high fidelity. In particular we propose that this model would be especially useful in future studies for investigating the therapeutic potential of adoptive immunotherapy in ADI-Ca, which develops spontaneously in this model after castration.[16,22]

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