Systemic Antitumor Immunity by PD-1/PD-L1 Inhibition Is Potentiated by Vascular-Targeted Photodynamic Therapy of Primary Tumors

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Abstract

Purpose: PD-1/PD-L1 pathway inhibition is effective against advanced renal cell carcinoma, although results are variable and may depend on host factors, including the tumor micro-environment. Vascular-targeted photodynamic (VTP) therapy with the photosensitizer WST11 induces a defined local immune response, and we sought to determine whether this could potentiate the local and systemic antitumor response to PD-1 pathway inhibition.

Experimental Design: Using an orthotopic Renca murine model of renal cell carcinoma that develops lung metastases, we treated primary renal tumors with either VTP alone, PD-1/PD-L1 antagonistic antibodies alone, or a combination of VTP and antibodies and then examined treatment responses, including immune infiltration in primary and metastatic sites. Modulation of PD-L1 expression by VTP in human xenograft tumors was also assessed.

Results: Treatment of renal tumors with VTP in combination with systemic PD-1/PD-L1 pathway inhibition, but neither treatment alone, resulted in regression of primary tumors, prevented growth of lung metastases, and prolonged survival in a preclinical mouse model. Analysis of tumor-infiltrating lymphocytes revealed that treatment effect was associated with increased CD8+:regulatory T cell (Treg) and CD4+FoxP3:Treg ratios in primary renal tumors and increased T-cell infiltration in sites of lung metastasis. Furthermore, PD-L1 expression is induced following VTP treatment of human renal cell carcinoma xenografts.

Conclusions: Our results demonstrate a role for local immune modulation with VTP in combination with PD-1/PD-L1 pathway inhibition for generation of potent local and systemic antitumor responses. This combined modality strategy may be an effective therapy in cancers resistant to PD-1/PD-L1 pathway inhibition alone. Clin Cancer Res; 1–8. ©2017 AACR.

Introduction

Patients with high-risk localized renal cell carcinoma (RCC) are treated with surgery alone as the standard of care despite significant risk of relapse. Among patients with localized or locally advanced RCC treated with partial or radical nephrectomy, up to 30% will experience relapse (1). The risk of recurrence increases with stage: the 5-year progression-free probability is only 31% in patients with pT4 tumors and 27% in N1 patients (2). Currently, there is no standard adjuvant therapy for these patients, and treatment is deferred until recurrence or metastasis is detected. Although VEGF receptor tyrosine kinase inhibitors are active in the metastatic setting, they are not effective in the adjuvant setting for patients with high-risk localized disease (3), although this is an area of ongoing investigation. Subclinical metastases are already present in these patients and there is a need to develop treatments with activity against micrometastases to prevent recurrence and improve survival after surgery.

Immune checkpoint inhibitors targeting the programmed death 1 (PD-1) tumor escape pathway are active against advanced RCC, with response rates around 25% even in previously treated patients (4). Expanding the use of PD-1 pathway inhibitors to earlier in the disease course, when there is a lower burden of systemic disease may provide significant oncologic benefit to patients at high risk of relapse. When PD-1 ligand 1 (PD-L1) is expressed by tumors, it is often in association with tumor-infiltrating lymphocytes (5), which indicates an ongoing antitumor response with the potential to be modulated by anti-PD-1 pathway agents. Although absence of PD-L1 expression on pre-treatment biopsies does not preclude response to PD-1/PD-L1 pathway inhibition (4), the expression of PD-L1 in tumors is
Correlated with higher response rate in some studies (6, 7). Thus, in tumor microenvironments where PD-L1 is constitutively expressed or inducible, strategies that inhibit the PD-1 pathway may be more successful at generating antitumor effect.

Local treatment can enhance the immunogenicity of tumors by increasing inflammation in the tumor microenvironment and, in so doing, increase susceptibility to immune modulation (8). It has been previously shown that vascular-targeted photodynamic (VTP) treatment of primary tumors using the novel photosensitizer WST11 (TOOKAD Soluble) induces a detrimental inflammatory response, including IFNγ production and infiltration of tumors by T cells and neutrophils (9, 10). WST11 is a water-soluble near-infrared–activated compound that sequesters within the blood supply by rapid occlusion, which leads to titratable levels of tumor necrosis and eradication within 48 hours (13, 14). The goal of the current study was to determine whether modulation of the tumor microenvironment with VTP therapy would increase susceptibility to PD-1/PD-L1 pathway inhibition in an orthotopic model of RCC.

**Materials and Methods**

**Cell culture and orthotopic tumor model**

Renca murine renal adenocarcinoma cells were obtained from ATCC and grown in RPMI medium supplemented with 10% FCS (Life Technologies/Thermo Fisher Scientific), nonessential amino acids 0.1 mmol/L (Life Technologies/Thermo Fisher Scientific), sodium pyruvate 1 mmol/L (Life Technologies), and l-glutamine 2 mmol/L (Life Technologies/Thermo Fisher Scientific). Murine IFNγ (PeproTech) was added to some cultures as indicated at 10 μg/mL. Seven- to 8-week-old male BALB/cAnNCr mice were purchased from the NCI (Frederick National Laboratory for Cancer Research, Frederick, MD) and housed and treated according to approved Institutional Animal Care and Use protocols. Renca tumor cells [5 × 10^5 in 10 μL injection volume in 1:1 PBS: Matrigel (BD Biosciences)] were implanted in the upper pole of the right kidney of each mouse, as described previously (15). Prior to tumor implantation, mice were anesthetized with inhaled isoflurane, meloxicam (2 mg/kg), and buprenorphine (0.5 mg/kg), and the hair and skin overlying the right flank was sterilized using a povidone-iodine and ethyl alcohol solution. Mice were injected with 250 μg anti–PD-1 (clone RMP1-14, Bio X Cell) plus 100 μg anti–PD-L1 (clone 10F.9G2, Bio X Cell) or rat IgG2a isotype control (clone 2A3, Bio X Cell) plus rat IgG2b isotype control (clone LTF-2, Bio X Cell) in 0.2 mL PBS intraperitoneally twice weekly for 3 weeks starting 6 days after tumor implantation.

**VTP administration**

Ten days after tumor implantation, mice bearing Renca tumors were anesthetized with 150 mg/kg ketamine and 10 mg/kg xylazine injected intraperitoneally plus inhaled isoflurane administered via a right-fitting nose cone. A bolus injection of WST11 (9 mg/kg) was administered intravenously via tail vein and the right kidney was exposed via a flank incision. Tumors were illuminated with 753 nmol/L light delivered via diode laser with a 600 μm end-fire fiber (biolitec) for 10 minutes at 150 mW/cm². After illumination, the peritoneum was closed with 5-0 Vicryl sutures and the skin closed with 9-mm metal clips. Xenograft flank tumors were illuminated directly in a similar fashion. VTP was administered at 5 weeks (A-498) or 2 weeks (786-O) after tumor implantation when tumor size was approximately 1 cm².

**Assessment of renal and pulmonary tumor growth and survival**

At the end of the study, mice were euthanized by CO₂ asphyxiation. Kidneys were dissected, weighed, and bisected longitudinally, and the maximal tumor dimensions were recorded. Kidneys were then fixed in formalin for 48 to 72 hours, then processed routinely in ethanol and xylene, paraffin embedded, sectioned at 4-μm thickness, and stained with hematoxylin and eosin (H&E). After euthanasia, lungs and trachea were harvested en bloc, instilled with India ink, and counterstained in Fekete’s solution for 24 hours (16). Surface lung tumors were manually enumerated under a dissecting microscope. To histologically confirm tumor deposits, H&E-stained sections were prepared as described previously and evaluated. The presence of a large ulcerated tumor, loss of ability to ambulate, or labored respiration was used as the endpoint in the survival experiment.

**IHC**

Formalin-fixed paraffin-embedded sections were stained on a Leica Bond RX automated staining platform (Leica Biosystems). Following heat-induced epitope retrieval in a pH 9.0 buffer, the anti-CD3 mAb (Vector Laboratories, catalog # VP-RM01) was applied at a concentration of 1:100 and was followed by application of a polymer detection system (Bond Polymer Refine Detection, Leica Biosystems, catalog # DS9800).

The IHC detection of PD-L1 was performed at the Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center (New York, NY) using Discovery XT processor (Ventana Medical Systems). The tissue sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), antigen retrieval was performed with CC1 buffer (Ventana Medical Systems), and sections...
were blocked for 30 minutes with Background Buster solution (Innovex), followed by avidin–biotin blocking (Ventana Medical Systems) for 8 minutes. Sections were incubated with anti–PD-L1 (Cell Signaling Technology, catalog # 13684, 5 µg/mL) antibodies for 5 hours, followed by 60-minute incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories, catalog # PK6101) at 1:200 dilution. The detection was performed with DAB Detection Kit (Ventana Medical Systems) according to the manufacturer’s instructions. Slides were counterstained with hematoxylin and coverslipped with Permount (Thermo Fisher Scientific).

Slides were digitally scanned with Panoramic Flash 250 (3DHistech) using Zeiss 20 ×/0.8NA objective. Relevant tissue regions were denoted and exported into tiff images using Panoramic Viewer (3DHistech). For CD3 staining, the areas of pulmonary tumors were manually identified with a controlled area, and the number of CD3-positive cells was manually enumerated. For PD-L1 analysis, the xenograft images were quantified using ImageJ/Fiji (NIH, Bethesda, MD), and appropriate threshold values were set for PD-L1 and DAPI signals separately, and the area of positive staining was measured and normalized to the area of total tissue determined by DAPI signaling.

Flow cytometry
Right kidneys, lungs, and spleens were harvested and manually disrupted to create single-cell suspensions by pipetting through a 70-µm nylon filter (kidneys and lungs) or a 40-µm nylon filter (spleen). Cells were stained with combinations of surface and intracellular antibodies according to the manufacturers’ protocols. Antibodies were used to detect the following surface proteins: CD45-APC/Cy7, CD8-PerCP, CD4-V450, CD25-PE. Staining for intracellular FoxP3-APC and Ki67-FITC was done according to the manufacturers’ protocols. Dead cells were excluded using Fixable Viability Dye eFluor 506 stain (eBioscience). Fc block (anti-mouse CD16/32 antibody, BioLegend) was used on all specimens. Antibodies were obtained from eBioscience or BD Biosciences. Anti–PD-L1 (MIH5; eBioscience) was used to stain in vitro cultured Renca cells after coculture with IFNγ. Cells were analyzed using a BD LSR multi-channel flow cytometer (BD Biosciences) and FlowJo data analysis software.

Statistical analyses
GraphPad Prism was used for all statistical analyses. The Kruskal–Wallis test was used to assess differences between all groups, and t test was used for pairwise comparison of means between groups. P ≤ 0.05 was defined as significant.

Results
VTP and PD-1/PD-L1 blockade synergize to mediate rejection of orthotopic renal tumors and prolong survival
To ensure that it would be feasible to target the PD-1 pathway in this model, we confirmed that PD-L1 expression was induced in the Renca cell line following in vitro culture with IFNγ as assessed by flow cytometry (Fig. 1). We then developed an orthotopic model system to test the capacity of VTP and anti–PD-1/PD-L1 combination therapy to reject primary renal tumors (Fig. 2A). Renca tumors were implanted in the right kidney of male BALB/c mice and treated with VTP 10 days after implantation. Mice received either anti–PD-1/anti–PD-L1 Abs only, VTP only, or the combination therapy (VTP + Abs). Growth of primary renal tumors was assessed on day 21 after implantation. When assessed by kidney weight and tumor area, treatment of the primary tumor with VTP + Abs (n = 15) resulted in regression of primary tumors, whereas treatment with VTP alone (n = 16) or Abs alone (n = 21) did not (Fig. 2B and C). Histologic analysis of the kidneys from mice treated with the combination therapy confirmed the absence of viable tumor and presence of necrosis (Fig. 2D). Treatment with the combination therapy also resulted in prolonged survival with median survival time of 27 days after VTP treatment for the VTP + Abs group (n = 20, P = 0.006; Fig. 2E) compared with 15.5 days in the control group (n = 20).

Combination therapy with VTP and PD-1 pathway inhibition increased the ratio of effector T cells to regulatory T cells in primary renal tumors
To identify whether there was change in T-cell infiltration that correlated with tumor rejection in the group receiving combination treatment, we isolated renal tumors after treatment and assessed tumor-infiltrating lymphocytes by flow cytometry. The VTP + Abs group showed an increase in the ratio of both CD8+ regulatory T cells [Treg (CD4+ FoxP3+)] and conventional T cells [Tconv (CD4+ FoxP3-)] : Tregs by 1 day after VTP, suggesting a role for both CD8+ and Tconv cells in the response to combination therapy. The ratios of CD8+Tregs and Tconv:Tregs returned to baseline by 3 days after treatment (Fig. 3B and C).

VTP treatment of primary renal tumor plus systemic anti–PD-1/anti–PD-L1 blockade prevents growth of distant lung tumors and is associated with increased T-cell infiltration in lungs
After establishing the effect of combined VTP therapy plus PD-1 pathway blockade in the treatment of primary renal tumors, we next investigated whether there was evidence of effect on distant sites of disease. Twenty-one days after tumor implantation, lungs were harvested and assessed for the presence of lung tumors. When assessed by enumeration (Fig. 4A), mice from the control group had 70 ± 17 (mean ± SEM) lung lesions per animal (Fig. 4D), which was confirmed histologically (Fig. 4B). Significant numbers of lung tumors were also seen in animals treated with either VTP alone (81 ± 23), or Abs.
alone (107 ± 23), whereas mice treated with combination therapy (VTP + Abs) had only 18 ± 11 lung tumors per animal (P = 0.025; Fig. 4D). A study of 9 mice with control Abs and 9 with anti–PD-1/PD-L1 Abs (no VTP administered) showed that the mean number of lung nodules at this treatment day was 81 and 69, respectively (Fig. 4E; P = 0.8).

To determine whether there was evidence of immune infiltration in the distant lung tumors, we assessed CD3+ T-cell infiltration by IHC (Fig. 4C). There was a trend toward increased tumor-infiltrating CD3+ cells in lung tumor nodules in the VTP + Abs group but not with either treatment alone, suggesting that infiltrating T cells are important for distant tumor eradication or growth prevention (Fig. 4F).

In a separate group of animals, we harvested lungs 3 days after VTP treatment (day 13 after tumor implantation), created single-cell suspensions, and assessed for the presence of infiltrating T cells by flow cytometry. In the combination group, there was a trend toward increased absolute number of CD45+ Treg cells and Tconv:Treg cells when compared with control

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**Figure 2.** VTP and PD-1/PD-L1 blockade synergize to reject orthotopic renal tumors and prolong survival. **A,** Treatment scheme. VTP, vascular-targeted photodynamic therapy; IP, intraperitoneal. Renal tumor growth was assessed on day 21 by kidney weight (B) or maximal cross-sectional tumor area (C); mean ± SEM is shown; data represent results from two pooled experiments, n = 15–21 per group, P < 0.001 (Kruskal–Wallis). Abs, anti–PD-1 + anti–PD-L1 antibodies. **D,** Kidneys were harvested on day 21 and stained with H&E and analyzed by light microscopy at 2× and 20×. * Represents necrosis, and ** represents viable tumor, with delineation marked by dotted line. Scale bar, 200 mm. **E,** In a separate experiment, mice were treated per the schema in A and sacrificed upon development of a large necrotic tumor, loss of ambulation, or labored respiration (n = 20 per group), and median survival time (MST) after VTP treatment was compared (Kaplan–Meier).
VTP and PD-1 Blockade Synergize for Systemic Antitumor Immunity

Figure 3.
Combination therapy with VTP and PD-1/PD-L1 blockade decreases the proportion of regulatory T cells in treated tumors. Animals were treated with combination VTP and PD-1/PD-L1 blockade per the schema in Fig. 2A. Kidneys were harvested 1, 3, or 5 days after VTP treatment and analyzed by flow cytometry for infiltrating immune cells. Day 0 indicates untreated Renca tumors. A, Representative flow cytometry plots of tumor-infiltrating Tconv (CD4+ FoxP3−) and CD8+ and Treg (CD4+ FoxP3+) cells from a gated CD45+ cell population. B and C, Ratios of CD8+:Treg (B, P = 0.15) and Tconv:Treg cells (C, P = 0.07) were calculated. Three to 5 animals per group were analyzed per time point. Mean ± SEM is shown, and means were compared using Kruskal–Wallis test.

Discussion
We have shown that VTP treatment of a primary renal tumor in combination with PD-1 and PD-L1 antagonistic antibodies produces a potent antitumor effect that is active in both the primary tumor and metastases. The orthotopic model system involved photoablation of an established renal tumor combined with systemic PD-1 pathway blockade and allowed the opportunity to assess response in an established primary renal tumor and spontaneous lung metastases. The orthotopic Renca model has been previously shown to develop lung metastases by 7 days after renal tumor implantation (17). We confirmed that lung metastases were present in our model at the time of VTP treatment (day 10). Thus, micrometastases are present during immune activation and treatment of the primary tumor. In this model, only the combination of photoablation of the primary tumor plus systemic PD-1/PD-L1 inhibition, but neither treatment alone, induced regression of the primary renal tumor and lung metastases and prolonged survival.

We assessed the cellular responses associated with tumor rejection in our model by looking at effector T cell and Treg responses. We found that after combination therapy, both CD8+:Treg and Tconv:Treg ratios increased in the primary tumors, providing evidence of a T cell–mediated immune response. Increased intratumoral ratios of effector T cells to Tregs are associated with increased tumor rejection with costimulatory blockade (18). In particular, the PD-1 pathway is known to be associated with increased intratumoral ratios of effector T cells to Tregs and decreased proliferation of Tregs at the metastatic site, providing evidence of a systemic response with the potential to eliminate distant disease.

It has been previously shown that patients whose tumors express PD-L1 have increased risk of metastatic progression and decreased survival (20). Although PD-L1 expression has been associated with response to therapy, intratumoral PD-L1 expression can be dynamic and therefore may not be a definitive biomarker for identifying patients that will respond to treatment (5). Instead, identifying strategies that increase PD-L1 expression may help clinicians increase patients’ susceptibility to PD-1 pathway blockade. In this setting, blocking signaling from newly induced PD-L1 in the tumor microenvironment may prevent tumor escape.

groups or animals treated with single-agent therapy (Fig. 4H). Although there was an increase in the number and ratio of Tregs, they exhibited a lower level of proliferation when assessed by Ki-67 staining (e.g., 15% in the VTP+Abs group vs. 26% in the control groups; P = 0.01; Fig. 4I).
Photodynamic therapy alone, including VTP, can lead to antigen-specific immune responses against some tumors (9, 21), and when combined with immunotherapy may also improve rejection of established tumors (22). Here, we show generation of a systemic antitumor response that extends beyond the effects of the local therapy. Renca cells have a low level of PD-L1 expression at baseline; in the absence of PD-L1 expression, there is no target for pathway inhibition, and PD-1/PD-L1 blockade is unlikely to be effective. We show that Renca tumor cells upregulate PD-L1 in response to IFNγ stimulation, which is known to be induced by

**Figure 4.**
VTP of the primary renal tumor plus systemic PD-1/PD-L1 blockade prevents growth of distant lung tumors and increases T-cell infiltration in distant tumors. Animals were treated per the schema in Fig. 2A. Lungs were harvested on day 21 and analyzed for lung nodules by staining with Fekete's solution (A), H&E staining (B), or CD3 IHC (C) and analyzed by light microscopy. D, Absolute number of lung lesions per animal was determined; mean ± SEM is shown. Data represent results from two pooled experiments, n = 15–23 per group, P = 0.025. E, Absolute number of lung lesions from animals not treated with VTP was determined; mean ± SEM is shown, n = 9 per group, P = 0.8. F, Lungs were harvested on day 21, and CD3⁺ cell infiltration was assessed in tumor nodules by IHC as in C. Percent of tumor-infiltrating CD3⁺ cells per field (tumor nodules assessed only); mean ± SEM is shown, n = 5 per group, P = 0.136. (G–I) In a separate group of animals, lungs were harvested 3 days after VTP treatment and analyzed by flow cytometry for infiltrating immune cells (n = 5 per group). G, Absolute numbers of CD45⁺, CD8⁺, Tconv, and Treg cells per gram of lung. H, Ratios of CD8⁺/Treg and Tconv/Treg cells were calculated. I, Ki-67 expression on tumor-infiltrating CD8⁺ and Tconv cells was assessed by flow cytometry. Mean ± SEM is shown.
photoablation with WST11 (9). By increasing intratumoral PD-L1 expression with VTP, the PD-1/PD-L1 pathway becomes an actionable target and increases the likelihood that antagonistic antibodies will be effective.

Alternatively, the upregulation of PD-L1 after VTP photoablation may be a marker of immune activation or infiltrative immune response like that known to be induced by effector T-cell cytokines, especially IFNγ (23). Patients with activated immune responses in the tumor microenvironment may respond better to systemic immune therapies. The idea that local treatment of a primary tumor can prime an immune response when combined with checkpoint inhibition has been proposed, leading to strategies utilizing radiation (8, 24) and cryoablation (25) to create an “inflamed” tumor microenvironment that synergizes with checkpoint inhibition. Whether there are advantages to each of these ablative techniques combined with PD-1 pathway blockade remains to be seen, but this is an area of ongoing investigation.

Importantly, in our study, PD-L1 expression was induced in human RCC xenografts by VTP photoablation, supporting evaluation of this combined approach in patients with RCC, including patients with metastatic disease or patients with high-risk non-metastatic disease for whom adjuvant systemic therapy does not convey a survival benefit (3). We propose that combined neoadjuvant treatment with VIP and PD-1 pathway inhibition will prime the tumor microenvironment to initiate a systemic antitumor response and reduce risk of relapse in patients for whom there is no effective adjuvant therapy. The novel combined modality strategy presented here has the potential to overcome resistance to PD-1 pathway inhibition and should be investigated further in a clinical trial.

Disclosure of Potential Conflicts of Interest

A. Scherz has ownership interest (including patents) as the inventor of WST11. No potential conflicts of interest were disclosed by the other authors.

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