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Immune-Checkpoint Blockade Enhances ^{225}Ac-PSMA617 Efficacy in a Mouse Model of Prostate Cancer

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Running title: PSMA-RNT is Enhanced by PD-1 Blockade

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ABSTRACT

Purpose: Prostate-specific membrane antigen (PSMA)-targeted radionuclide therapy (RNT) may increase tumor immunogenicity. We aimed at exploiting this effect by combining RNT with immunotherapy in a mouse model of prostate cancer (PC).

Experimental Design: C57BL/6-mice bearing syngeneic RM1-PGLS tumors were treated with ^{225}Ac-PSMA617, an anti-PD-1 antibody, or both. Therapeutic efficacy was assessed by tumor volume measurements (computed tomography), time to progression (TTP) and survival.

Results: PSMA-RNT or anti-PD-1 alone tended to prolong TTP (isotype-control 25d; anti-PD-1 33.5d, p=0.0153; RNT 30d, p=0.1038) and survival (control 28d; anti-PD-1 37d, p=0.0098; RNT 32d, p=0.1018). Combining PSMA-RNT and anti-PD-1 significantly improved disease control compared to either monotherapy. TTP was extended to 47.5d (p<0.0199 vs. monotherapies), and survival to 51.5d (p<0.0251 vs. monotherapies).

Conclusion: PSMA-RNT and PD-1 blockade synergistically improve therapeutic outcomes in our PC model, supporting the evaluation of RNT/immunotherapy combinations for PC patients.

INTRODUCTION

Prostate-specific membrane antigen (PSMA)-targeted radionuclide therapy (PSMA-RNT) is effective but not curative in ~50% of metastatic castration-resistant prostate cancer (mCRPC) patients. However, PSMA-RNT may increase PC immunogenicity; this could be exploited by combining RNT with immunotherapy to improve both, RNT and immunotherapy outcomes.

Immunotherapies have been used successfull against several cancer types (*1,2*). Two immunotherapies were approved by the FDA for PC. The cancer vaccine Sipuleucel-T (PROVENGE) improves overall survival by 4 months in patients with non- or minimally-symptomatic mCRPC, but neither lowers prostate-specific antigen levels nor improves radiographic- or progression-free survival (*3*). PD-1 immune-checkpoint blockade (Pembrolizumab) was approved for the ~5% mCRPC patients presenting with mismatch repair gene defects and/or microsatellite instability (*4*). In the KEYNOTE-199 trial (NCT02787005), modest objective response rates to Pembrolizumab (3-5%) were observed in genetically unselected PC patients (*5*). Overall, the efficacy of immunotherapies in PC, especially when applied as monotherapy, has been limited. This lack of efficacy has been attributed to the low immunogenicity of PC that might result from a low mutational burden, impaired T cell activation, and an immunosuppressive tumor microenvironment that limits cytotoxic- but increases regulatory- T cell infiltration and activation (*6-10*).

The cytotoxicity of ionizing radiation may enhance tumor immunogenicity by inducing immunogenic cell death; this, in turn, can lead to the release of tumor-associated antigens, and to an inflammatory phenotype (*1,11-13*). Indeed, several clinical trials explore the

combination of radio- with immunotherapy in PC (*11*). However, little is known about the immunogenicity induced by RNT.

Here we demonstrate synergy between PSMA-RNT and PD-1 blockade in a syngeneic PC mouse model.

MATERIALS AND METHODS

Cell Culture

RM1-PGLS cells were a gift from M. Sadelein (Memorial Sloan Kettering Cancer Center). Parental RM1 cells were derived by transduction of mouse prostate cells with *ras* and *myc* oncogenes (*14*); transduction of parental RM1 cells with human PSMA and SFG-Egfp/Luc yielded RM1-PGLS (*15*). This cell line represents CRPC. Cells were maintained in Rosewell Park Memorial Institute-1640 with 5% fetal bovine serum (Omega Scientific) at 37ºC, 5%CO_2. Mycoplasma contamination was excluded using the Venor™GeM Mycoplasma Detection Kit (Sigma-Aldrich).

Mice

Animal studies were approved by the UCLA Animal Research Committee (#2005-090). Male, 6-8 weeks old C57Bl/6 mice (Department of Radiation Oncology, UCLA) were housed under pathogen-free conditions (12h-12h light-dark cycle; food, water ad libitum). Animal welfare was ensured daily by vivarium staff and investigators.

^{225}Ac-PSMA617

Actinium-225 was supplied by the U.S. Department of Energy Isotope Program, Office of Science for Nuclear Physics. ^{225}Ac-PSMA617 was synthesized as previously described (*16*) with >92% purity by radio thin-layer chromatography at 130 MBq/μmol (UCLA Biomedical Cyclotron Facility).

Computed Tomography (CT)

Tumor volumes were monitored by CT. Scans were analyzed using OsiriXv.10.0.2 (Pixmeo SARL) (*16*).

Flow Cytometry

PSMA expression was verified using an anti-hPSMA-APC antibody (5µl/test; REA408; Miltenyi; **Figure 1A**) (*16*). Interferon-gamma (mIFN-γ; 10ng/mL) or radiation (x-ray, 10Gy) induced PD-L1 expression was determined 24h after treatment using an anti-mPD-L1-PE antibody (1:20; 10F.9G2; Biolegend). Samples were measured on a LSRII flow cytometer (BD) and analyzed using FlowJo (Three Star).

Therapy Study

RM1-PGLS ($0.1x10^6$ cells in 50µl PBS+50µl Matrigel) were subcutaneously inoculated into the shoulder region of mice. When tumors reached 97 ± 34 mm^3 (day 11), mice were randomized based on tumor volumes into the following groups (n=8-9 mice/group): 1. rat-IgG2a isotype-control (clone 2A3; #BE0089, bxcell); 2. anti-PD-1 (clone RMP1-14; #BE0146, bxcell); 3. ^{225}Ac-PSMA617; 4. ^{225}Ac-PSMA617 + anti-PD-1. Mice were treated with 30kBq ^{225}Ac-PSMA617 (intravenous) on day 12 and with anti-PD-1 or isotype-control (10mg/kg in PBS, intraperitoneal) on days 13, 16, 20, and 23. Tumor volumes and body weights were measured twice weekly until tumors reached ≥3cm^3 or mice reached a humane endpoint.

Statistics

Data were analyzed by investigators blinded for interventions. Data are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey correction and set to $p<0.05$. Time to progression (TTP) to 1.5 cm^3

tumor volume and survival were analyzed using the log-rank test. GraphPad Prism (version 8, GraphPad Software) was used for all statistical calculations.

RESULTS

IFN-γ induces PD-L1 Expression on RM1-PGLS Cells

As a prerequisite for investigating the efficacy of anti-PD-1 *in vivo*, we tested the responsiveness of RM1-PGLS cells to IFN-γ. IFN-γ is released by activated T cells and can induce a reciprocal upregulation of PD-L1 expression on tumors cells to facilitate immune-evasion (*17*). Treatment of RM1-PGLS with IFN-γ resulted in a 5.4±2.3 fold increase in PD-L1 expression (p=0.0056; **Figure 1B**). This finding indicates that tumor control *in vivo* might be enhanced by PD-1 blockade.

Similarly, radiation-induced PD-L1 expression may contribute to immune-suppression and radioresistance (*18*). In our model, PD-L1 expression did not significantly increase in response to irradiation (1.6±0.02 fold; p=0.8010) (**Figure 1C**).

Efficacy of ^{225}Ac-PSMA617 and PD-1 Blockade

We investigated whether PD-1 blockade is effective in our model, and if combining ^{225}Ac-PSMA617 and PD-1 blockade improves therapy responses. Both, anti-PD-1 and RNT monotherapy tended to enhance tumor control. Median TTP increased from 25d (control) to 33.5d (anti-PD-1; p=0.0153) and 30d (RNT; p=0.1038), respectively. Survival tended to improve from 28d (control) to 37d (anti-PD-1; p=0.0098) and 32d (RNT; p=0.1018), respectively (**Figure 2A-B, D-E**).

Combining RNT and anti-PD-1 synergistically improved therapeutic efficacy. Median TTP and survival were 47.5d (p≤0.0199 vs. monotherapies) and 51.5d (p≤0.0251 vs. monotherapies) in the combination therapy group (**Figure 2A-B,F-G**). In addition, 2/8 mice

in the RNT/anti-PD-1 group had stable disease and remained alive at the end of the observation period; all mice in the monotherapy groups, and 6/8 of mice in the RNT/anti-PD-1 group exhibited tumor growth delay. None of the mice exhibited signs of toxicity (e.g., weight loss) and had to be euthanized prematurely (**Figure 2C**).

DISCUSSION

To our knowledge, this is the first report demonstrating synergistic anti-tumor efficacy between PSMA-targeted RNT and PD-1 blockade in a syngeneic PC mouse model.

The immunological consequences of RNT have not been well-documented. In NCI-H727 neuroendocrine-tumors in NMRI-mice (deficient in T- and mature B-cells) increased numbers of tumor-infiltrating antigen-presenting and natural killer cells following ^{177}Lu-DOTATATE were observed (*19*). Treatment of a syngeneic lymphoma mouse model with ^{90}Y-NM600 (targeting alkylphosphocholine) increased $CD8^+$ T cell infiltration and $CD8^+$ T cell : regulatory T cell (T_{reg}) cell ratios, and generated tumor-specific immunological memory (*20*). Two studies demonstrated improved tumor control with RNT/immune-checkpoint blockade in MC38 colorectal cancer (integrin $\alpha_v\beta_3$-targeted ^{177}Lu-EB-RGD+anti-PD-L1) (*21*), and B16F10 melanoma (very late antigen 4-targeted ^{177}Lu-LLP2A+anti-CTLA-4+anti-PD-1 or anti-PD-L1) (*22*). The present study is in line with these findings and supports the exploration of RNT/immunotherapy combinations for the treatment of advanced PC.

The anti-PC synergy between PSMA-RNT and PD-1 blockade might be explained by mechanisms similar to those observed when external beam radiotherapy was combined with PD-1 blockade. Based on these observations, RNT might cause immunogenic cell death leading to the release of tumor-associated antigens and the enhancement of T cell diversity, priming and activation (*23,24*). Because RNT delivers radiation to all PSMA-positive metastases, it would rely less on abscopal effects for launching successful systemic anti-tumor immune responses than radiotherapy (*23*). Blockade of PD-1/PD-L1 signaling may prevent exhaustion of tumor-infiltrating cytotoxic T cells and their conversion to immunosuppressive T_{reg}. In addition, PD-1 blockade might counteract the

up-regulation of PD-L1 expression that has been observed following radiation-induced activation of Ataxia telangiectasia and Rad3 related, a key-effector kinase in the DNA damage/replication stress response (*18*). In future studies, we will investigate the exact mechanisms underlying PSMA-RNT-induced immunogenicity and the synergy between RNT and PD-1 blockade in PC.

The RNT/immunotherapy regimen was effective but not curative in the current PC mouse model. One explanation might include activation of immunosuppressive mechanisms beyond the reactive PD-L1 upregulation on PC tumors exploited in the current study. These include mechanisms mediated by the release of intracellular adenosine triphosphate from dying cells (*25*). Another reason for the non-curative outcome might be the pre-treatment tumor volume (97 ± 34 mm^3) that might negatively correlate with immune cell infiltrates (*26*). Clinically, the intra- and inter-patient, spatial and temporal heterogeneity of PC might prove to be an additional challenge for generating curative outcomes after RNT/immunotherapy.

We studied the efficacy of immune-checkpoint blockade combined with RNT in a single PC mouse model that is based on murine, hPSMA-overexpressing PC cells with basal and IFN-γ induced PD-L1 expression. A large fraction of PC are immune-desert or -excluded, two phenotypes associated with resistance to PD-1/PD-L1 blockade and reduced IFN-γ signalling (*1*). However, the presence of an IFN-γ responsive gene signature has recently been reported in 9 mCRPC patients responding to Ipilimumab, but not in 10 non-responders (*27*). In line with this study, the Keynote-199 trial concluded that anti-PD1 therapy is effective in some mCRPC patients, while highlighting the need to identify predictive biomarkers (*5*). Therefore, RM1-PGLS cells epresent a relevant subset

of patients with mCRPC. Moreover, RNT might change the immune-phenotype of PC to an inflammatory one in which immune-checkpoint blockade is likely effective. The recent registration of two clinical trials testing ^{177}Lu-PSMA617 + Pembrolizumab in genetically unselected patients with mCRPC (NCT03658447, NCT03805594) underscores the high interest in combining RNT with immunotherapy. Therefore, and despite investigating only one mouse model, the current study timely and strongly supports exploration of RNT/immunotherapy combinations for PC patients.

CONCLUSION

Combining PSMA-RNT and PD-1 blockade synergistically reduces tumor burden and improves TTP and survival. While the mechanistic explanation for this synergy has to be elucidated in future studies, RNT might support the conversion of PC from an immunological cold to a hot tumor. Thus, RNT/immunotherapy combinations represent promising therapeutic options even for those PC patients without microsatellite instability and/or mismatch-repair deficiencies, and with heterogenous PSMA expression (*16*).

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KEY POINTS

Question: Does ^{225}Ac-PSMA617 radionuclide therapy synergize with PD-1 blockade in a mouse model of prostate cancer?

Pertinent Findings: In this therapeutic efficacy study, PSMA-RNT or anti-PD-1 alone tended to prolong TTP and survival. Combining PSMA-RNT and anti-PD-1 synergistically improved disease control and survival compared to either monotherapy.

Implications for Patient Care: Our data suggest that RNT can promote PC immunogenicity, and strongly support exploration of RNT/immunotherapy combinations for PC patients.

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FIGURES

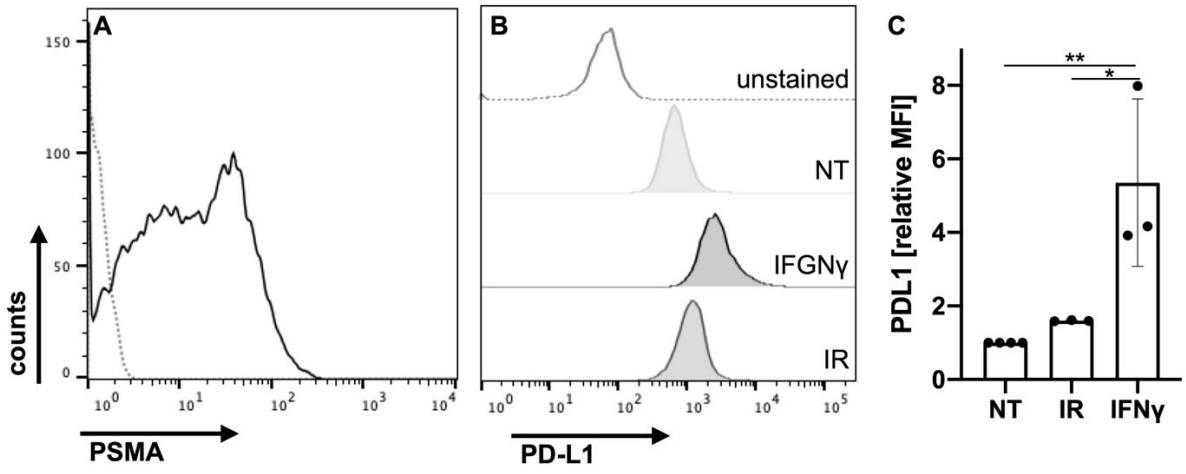

Figure 1. IFN-γ induces PD-L1 expression on RM1-PGLS cells. (**A**) PSMA expression (dotted line: unstained control). (**B**) Basal (NT), IFN-γ and radiation (IR) induced PD-L1 expression. 1/3 representative experiments is shown. (**C**) Quantification of PD-L1 expression shown in B (n=3). Columns represent mean values, bars SD, individual values are shown as dots. Relative MFI – mean fluorescent intensity normalized to NT.

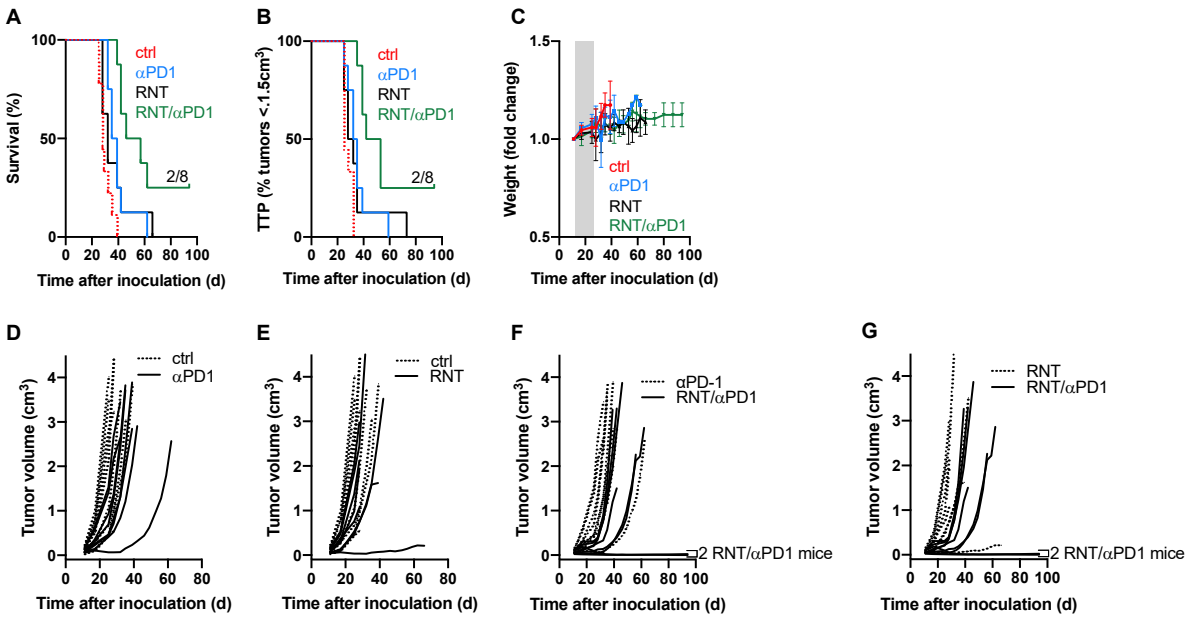

Figure 2. Synergy between ^{225}Ac-PSMA617 and anti-PD-1. (**A**) Survival: control, 28d; anti-PD1, 37d (p=0.0098 vs. control); RNT, 32d p=0.1018 (vs. control); RNT/anti-PD-1, 51.5d (p≤0.0251 vs monotherapies). (**B**) TTP to half-maximal tumor volume: control, 25d; anti-PD1, 33.5d (p=0.0153 vs. control); RNT, 30d (p=0.1038 vs. control); RNT/anti-PD-1, 47.5d (p≤0.0199 vs. monotherapies). (**C**) Bodyweights (mean±SD). The grey-shaded area indicates the treatment duration. (**D-G**) Tumor volumes for individual mice (n=8-9 mice/group). Two mice in the RNT/anti-PD-1 group had stable disease and remained alive at the end of the observation period.