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14. ABSTRACT

Adoptive transfer of immature myeloid cells lacking NF-kB p50 (p50-IMC) slows growth of prostate cancer (PrCa) tumors, and the large majority of human PrCa expresses PSMA. We hypothesize that co-administering PSMA antibody (Ab) or expressing a PSMA-targeting chimeric antigen receptor (CAR) on p50-IMC will direct p50-IMC to PrCa and enable phagocytosis and Ag presentation to T cells, increasing efficacy. We obtained anti-human PSMA Ab from a hybridoma, constructed a PSMA.CAR, and demonstrated their binding to human PSMA (hPSMA), but not mouse PSMA. We expressed hPSMA in two murine PrCa lines (Myc-CaP and TRAMP-C1); these lines form tumors in immune-deficient NSG mice that retain hPSMA but lose hPSMA in syngeneic mice, indicating immune-rejection. PSMA.CAR increased p50-IMC Myc-CaP/hPSMA phagocytosis. EGFR and GD2 are expressed on a subset of aggressive PrCa tumors. We also constructed a CAR recognizing human EGFR (hEGFR) and developed Myc-CaP and TRAMP-C1 lines expressing hEGFR. These lines retain hEGFR in NSG but again lose hEGFR in syngeneic mice. TRAMP-C1 cells express GD2 retain this ganglioside in syngeneic mice and are slowed by p50-IMC. We are assessing whether hPSMA or GD2 Abs or CARs increase murine or human p50-IMC PrCa tumor localization and efficacy in syngeneic or NSG mice, and are developing transgenic mice expressing hPSMA that will tolerate Myc-CaP/hPSMA cells.

15. SUBJECT TERMS
Prostate cancer, immunotherapy, myeloid cells, NF-kB p50, PSMA

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1. INTRODUCTION

Adoptive transfer of immature myeloid cells lacking NF-κB p50 (p50-IMC) slows growth of prostate cancer (PrCa), although ultimately tumors progress. The large majority of human PrCa expresses PSMA, and PSMA levels increase with tumor aggressiveness. We hypothesize that co-administering PSMA antibody (Ab) or expressing a PSMA-targeting chimeric antigen receptor (CAR) on p50-IMC will direct p50-IMC to PrCa and facilitate tumor cell phagocytosis and thereby antigen presentation to T cells, increasing efficacy. We further hypothesis that addition of T cell checkpoint inhibition, e.g. using PD-1 Ab, or enhancing phagocytosis with SIRPα knockout (KO) or CD47 Ab will further increase efficacy. We intend to pursue these hypotheses using both murine p50-IMC in syngeneic murine models and human p50-IMC as a step towards clinical translation.

2. KEYWORDS

Prostate cancer, immunotherapy, myeloid cells, NF-κB p50, PSMA

3. ACCOMPLISHMENTS

Major goals of the project

Task 1: Assess whether PMSA Ab increases efficacy of p50-IMC or p50KO-IMC against murine PrCa
Subtask 1 - Obtain ACURO approval (mos 1-3), completed prior to 9/01/2022 start date
Subtask 2 - Generate anti-PSMA Ab (mos 1-6
Subtask 3 - Comparison of PrCa tumor growth with 5FU/p50-IMC, PMSA Ab, 5FU/WT-IMC, 5FU/p50-IMC + Ab, or 5FU/WT-IMC + Ab; p50-IMC generated from p50-/- mice (mos 7-15).
Subtask 4 - Comparison of PrCa tumor growth with 5FU/p50-IMC, PMSA Ab, 5FU/WT-IMC, 5FU/p50-IMC + Ab, or 5FU/WT-IMC + Ab; p50-IMC generated by gene-editing (mos 10-24)

Task 2: Assess whether PMSA Ab increases p50-IMC murine PrCa tumor localization and T cell activation
Subtask 1 - Obtain ACURO approval (mos 2-5), completed prior to 9/01/2022 start date
Subtask 2 - Assess tumor myeloid numbers and differentiation state, and assess tumor T cell numbers and activation, after these therapies (mos 9-18).

Task 3: Assess whether PD-1 or CD47 Ab (or IMC SIRPα KO) increase efficacy of PMSA Ab combined with p50KO-IMC against murine prostate cancer
Subtask 1 - Obtain ACURO approval (mos 2-5), completed prior to 9/01/2022 start date
Subtask 2 - Comparison of PrCa tumor growth with 5FU/p50KO-IMC+PMSA Ab, PD-1 Ab, or 5FU/p50-IMC + both PMSA Ab and PD-1 Ab (mos 18-36).
Subtask 3 - Comparison of PrCa tumor growth with 5FU/p50KO-IMC+PMSA Ab, CD47 Ab, or 5FU/p50-IMC +both PMSA Ab and CD47Ab, or *SIRP*α KO (mos 18-36).

Task 4: Assess whether PMSA CAR increases efficacy of p50-IMC or p50KO-IMC against murine PrCa, alone or with PD-1 or CD47 Ab
Subtask 1 - Obtain ACURO approval (mos 2-5), completed prior to 9/01/2022 start date
Subtask 2 - Construct PMSA CAR viral vector (mos 1-5).
Subtask 3 - Comparison of PrCa tumor growth with p50-IMC vs p50-IMC/PMSA.CAR, alone, with PD-1 Ab, or with CD47 Ab or *SIRP*α KO (mos 6-36).
Subtask 4 - Comparison of PrCa tumor growth with p50-IMC vs p50KO-IMC/PMSA.CAR, alone, with PD-1 Ab, or with CD47 Ab or *SIRP*α KO (mos 6-36).
Subtask 5 - Assess tumor myeloid numbers and differentiation state, and assess tumor T cell numbers and activation for 5FU/p50-IMC versus 5FU/p50-IMC/PMSA.CAR (mos 9-30).

Task 5: Assess whether PMSA Ab increases efficacy of p50KO-IMC against human prostate cancer
Subtask 1 - Obtain ACURO approval (mos 2-5), completed prior to 9/01/2022 start date
Subtask 2 - Comparison of human PrCa cell-line-derived tumor growth with 5FU/p50KO-IMC, PMSA Ab, 5FU/NT-IMC, 5FU/p50KO-IMC + Ab, or 5FU/NT-IMC + Ab (mos 7-15).
Subtask 3 - Comparison of human PrCa PDX tumor growth with 5FU/p50KO-IMC, PMSA Ab, 5FU/NT-IMC, 5FU/p50KO-IMC + Ab, or 5FU/NT-IMC + Ab (mos 16-30).

Subtask 4 - Comparison of PrCa tumor growth with 5FU/p50KO-IMC+PMSA Ab, CD47 Ab or *SIRP*α KO, or their combination (mos 16-36).
Subtask 5 - Assess human tumor myeloid numbers and differentiation state (mos 10-30).

Task 6: Assess whether PMSA CAR increases efficacy of p50KO-IMC against human prostate cancer
Subtask 1 - Obtain ACURO approval (mos 2-5), completed prior to 9/01/2022 start date
Subtask 2 - Comparison of PrCa tumor growth with 5FU/p50KO-IMC versus 5FU/p50KO-IMC/PMSA.CAR, alone or with CD47 Ab (mos 12-30).
Subtask 3 - Comparison of PrCa PDX growth with 5FU/p50KO-IMC versus 5FU/p50KO-IMC PMSA.CAR, alone or with CD47 Ab (mos 18-36).
Subtask 4 - Assess human tumor myeloid numbers and differentiation state (mos 12-30).

Accomplishments under these goals

Task 1/Subtask 2 - Generate anti-PSMA Ab

We obtained the hybridoma cell lines PSMA3.9 and PSMA10.3 from ATCC. Both produce anti-human PSMA monoclonal Ab. Ab3.9 is fully murine and best for use in immune-competent mice. Ab 10.3 is humanized and so most appropriate for clinical translation. We provided the PSMA3.9 cell line to a commercial vendor (Bio X Cell) and, for a fee, they produced 108 mg of purified monoclonal Ab, sufficient for our proposed *in vivo* studies.

To verify that PSMA3.9 Ab binds PSMA on the surface of cells we utilized LNCaP cells, a human PrCa cell line, Myc-CaP (MC) cells, a murine PrCa line derived from FVB/N mice, and TRAMP-C1 PrCa cells, a murine PrCa line derived from C57BL/6 (B6) mice. We constructed an MIPuro retroviral vector expressing human PSMA (hPSMA) and puromycin-resistance and transduced MC cells, followed by puromycin selection. These were then flow-sorted to obtain a population that expressed a high level of hPSMA. We similarly introduced hPSMA into TRAMP-C1 cells, and a colleague provided MC cells expressing murine PSMA (mPSMA). Western blot analysis confirms hPSMA expression in LNCaP, MC/hPSMA, and MC/mPSMA cells (**Fig. 1A**). Flow cytometry with a commercially available anti-hPSMA-APC Ab similarly confirms expression in LNCaP, MC/hPSMA, and TRAMP-C1/hPSMA cells (**Fig. 1B, top row**). PSMA3.9 Ab also effectively binds and detects surface hPSMA in these three cell lines, but does not bind mPSMA (**Fig. 1B, bottom row**). PSMA Ab10.3 also bound hPSMA but not mPSMA, as assessed by flow cytometry (not shown). Since Ab9.3 and Ab10.3 are mouse monoclonals, it is not surprising that they do not bind mPSMA, as mice are tolerant to mPSMA and therefore may lack B cells that react with this protein.

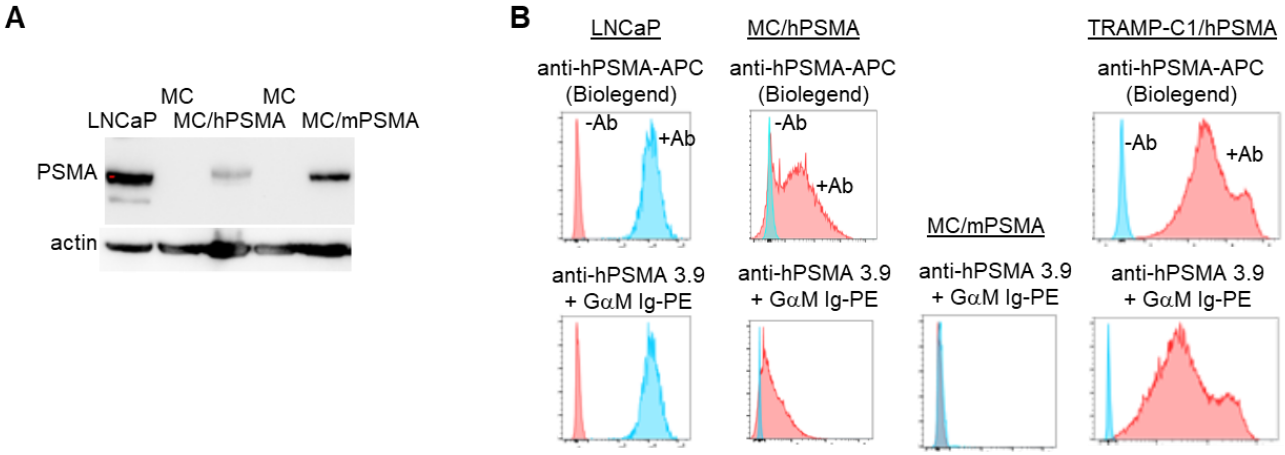

Figure 1. A) Western blot for PSMA and actin in LNCaP (human PrCa), Myc-CaP (MC, murine FVB/N-derived PrCa), and MC cells expressing either human or murine PSMA (hPSMA, mPSMA). **B)** Flow cytometry analysis of surface PSMA on indicated cell lines using either anti-hPSMA-APC (Biolegend) or anti-hPSMA3.9 Ab and GαM Ig-PE secondary Ab.

Task 1/Subtask 3 and Subtask 4 - Murine PrCa tumor growth combining PSMA Ab and p50-IMC or p50KO-IMC

The B6CaP cells we used in our initial study to demonstrate efficacy of p50-IMC against murine PrCa only grows as an allograft, requiring serial subcutaneous (sq) passage, and not as an adherent cell line. Given the difficulty we encountered introducing hPSMA into these cells, we have instead pursued our alternative strategy of using murine cell lines and successfully introduced hPSMA into both MC and TRAMP-C1 cells (Fig. 1).

Parental MC cells form small sq tumors by days 30 post-inoculation into syngeneic FVB/N mice, whether inoculated in Hank's buffered saline solution (HBSS) or Matrigel; in contrast, few tumors formed when MC/hPSMA cells were inoculated, and the few that grew arose far more slowly (**Fig. 2A, left and center**). On the other hand, MC/hPSMA cells readily formed tumors in immune-deficient NSGS mice (**Fig. 2A, right**).

Parental TRAMP-C1 cells form small sq tumors by days 49 post-inoculation in syngeneic B6 mice and by day 34 in NSGS mice (**Fig. 2B**); in contrast, no TRAMP-C1/hPSMA tumors grew, even by 90 days post-inoculation (not shown). Flow cytometry of dissociated tumor cells indicates that MC/hPSMA cells retain hPSMA in NSGS but not in FVB/N mice (**Fig. 2C, left and center**). Finally, MC/mPSMA cells readily formed tumors in FVB/N mice, and these retain surface mPSMA (**Fig. 2C, right**) - to detect mPSMA by flow cytometry we utilized a dendrimer nanoparticle linked to KEU (a small molecule PSMA ligand) and Cy5.5 - developed by M. Pomper (co-Inv). These data with MC/hPSMA and TRAMP-C1/hPSMA cells demonstrate that cells expressing hPSMA are subject to immune rejection in immune-competent mice.

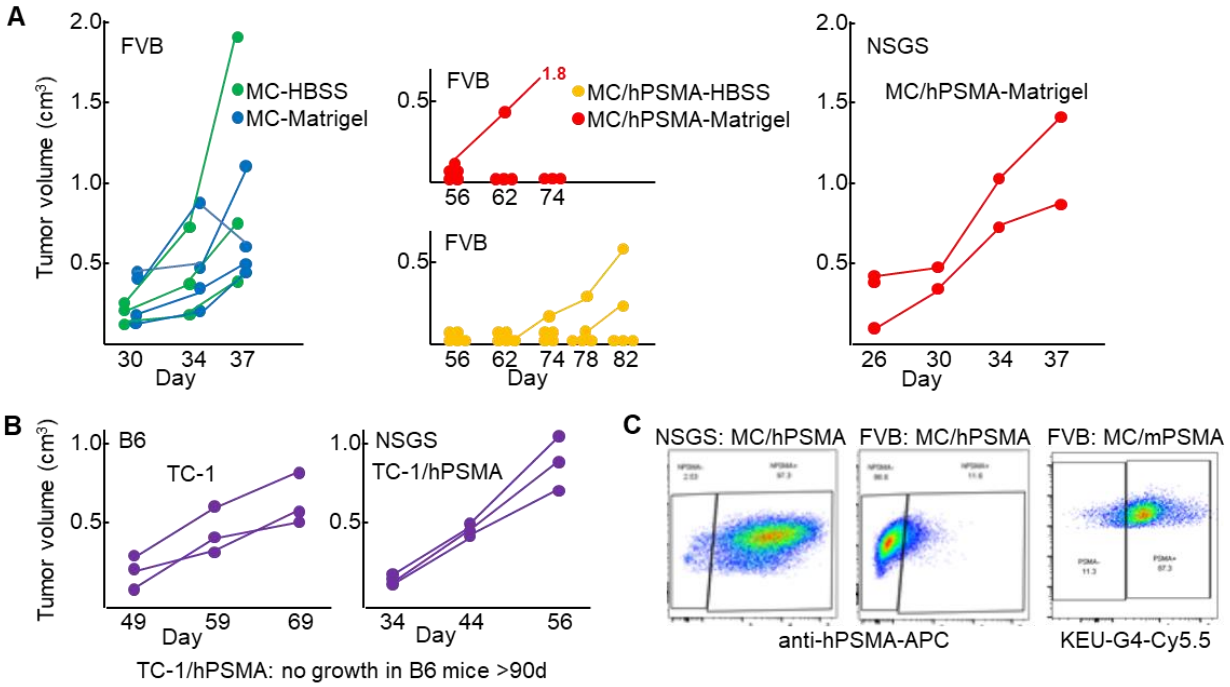

Figure 2 A) MC or MC/hPSMA sq tumor growth in FVB/N (FVB) mice in HBSS or Matrigel and in NSGS mice in Matrigel. B) TRAMP-C1 (TC-1) and TC-1/hPSMA tumor growth in B6 or NSGS mice. **C)** Surface PSMA retention by MC/hPSMA tumors in NSGS or FVB/N mice and by MC/mPSMA tumors in FVB/N mice.

The key objective of proposed experiments is to determine whether an Ab or CAR directing p50-IMC to prostate cancer increases anti-tumor efficacy. Human LNCaP prostate cancer cells, PSMA Ab3.9, and the PSMA.CAR construct we have developed (described below) can be utilized to pursue this objective in immune-deficient mice (Task 5 and Task 6). However, our finding that murine PrCa lines expressing hPSMA are not tolerated by FVB/N or B6 mice led us to evaluate directing p50-IMC to PSMA using a PSMA small molecule ligand that binds mPSMA, to develop transgenic mice expressing hPSMA throughout development and so tolerant to prostate cancer cell lines expressing hPSMA. and to pursue other PrCa surface antigens as p50-IMC targets.

Epidermal Growth Factor Receptor (EGFR) is expressed on 41% of newly diagnosed PrCa cases, 76% of castration-resistant prostate cancers, and 100% of metastatic prostate cancers,[1] making it a highly relevant target. In addition, we have available Cetuximab, a high-affinity, FDA-approved anti-human EGFR Ab, and also the

DNA sequence of Cetuximab that allowed us to assemble a hEGFR.CAR. We developed MC/hEGFR and TRAMP-C1/hEGFR cell lines by constructing a MIPuro-hEGFR retroviral vector, followed by viral packaging, cell transduction, and puromycin selection (**Fig. 3A**). Unfortunately, as with MC or TRAMP-C1 cells expressing hPSMA, these lines did not grow in FVB/N or B6 mice, respectively (not shown). In addition, Lewis Lung Cancer (LLC) cells expressing hEGFR grew tumors that retain hEGFR in NSG mice but lack hEGFR in syngeneic B6 mice (**Fig. 3B**), further indicating that as with hPSMA, immune-competent mice do not tolerate hEGFR.

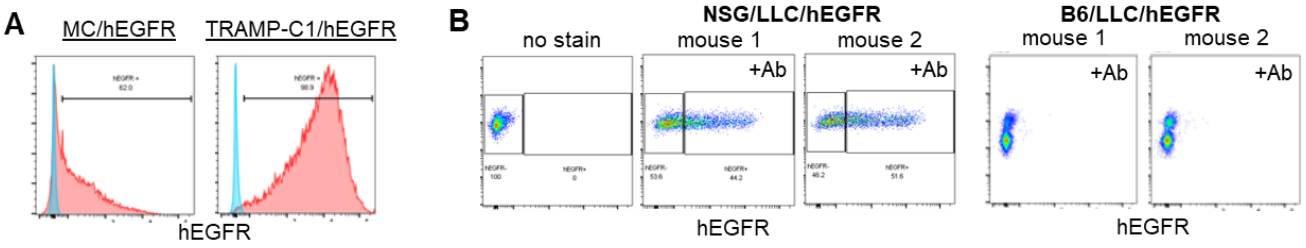

Figure 3 A) Detection of hEGFR in (MC/hEGFR or TRAMP-C1/hEGFR cells. **B)** LLC/hEGFR cells express hEGFR when grown in NSG mice but not in B6 mice.

GD2 is a cell surface ganglioside (**Fig. 4A**) found on neuro-endocrine cancers such as neuroblastoma and melanoma. Castration-resistant prostate cancers (CRPCs) often acquire a neuro-endocrine phenotype and a subset express GD2.[2,3] TRAMP-C1 prostate cancer cells have a neuro-endocrine phenotype, and we confirmed that they express GD2, as well as its precursor GD3 (**Fig. 4B**). Of note, GD2 is conserved between mice and humans. We have available an FDA-approved Ab, Dinutuximab, that recognizes GD2 and is used for neuroblastoma therapy, and M. Brenner kindly provided a GD2.CAR that had been used in CAR-T cell clinical trials.[4] While only a subset of TRAMP-C1 cells express GD2 at any one time, we expect this to be sufficient to attract p50-IMC bound to GD2 Ab or expressing GD2.CAR to the tumors and to enable tumor cell phagocytosis and antigen presentation to T cells to augment anti-tumor immunity. A similar subset of the neuroblastoma cell line 9464D expresses GD2 and yet we and others find that this line shows markedly reduced tumor growth in response to GD2 Ab. In an initial experiment, we evaluated efficacy of 5FU followed by three doses of p50-IMC against TRAMP-C1 cells, finding that 5FU/p50-IMC slows TRAMP-C1 neuro-endocrine prostate cancer tumor growth ~2.6-fold (**Fig. 4C**). **This is an exciting finding, indicating p50-IMC efficacy against a second prostate cancer, besides B6CaP.**

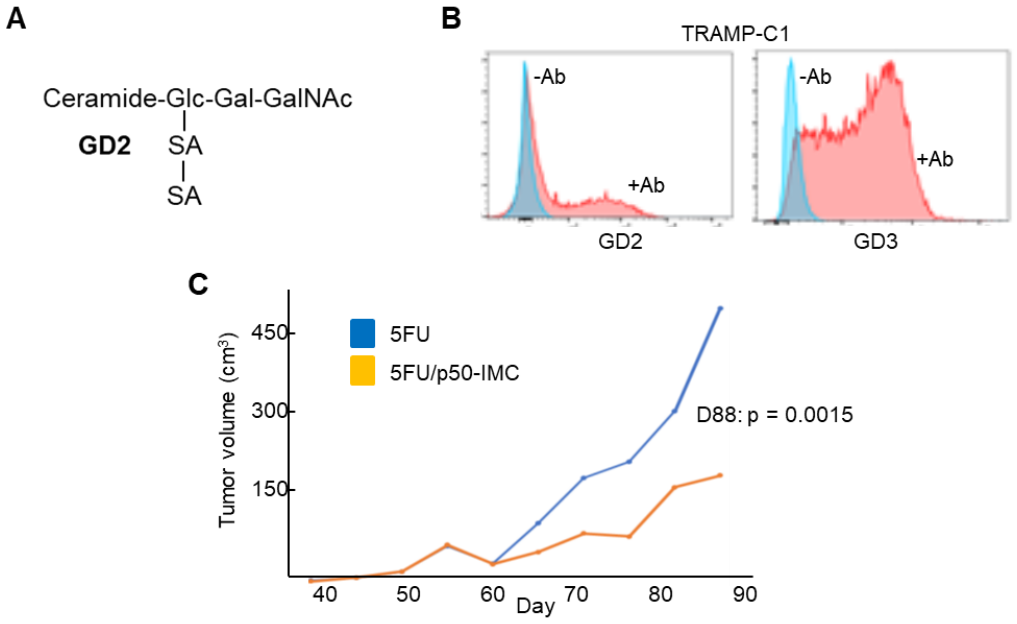

Figure 4 A) Structure of GD2 ganglioside. Glc - glucose, Gal - galatctose, SA - sialic acid. **B)** Expression of GD2 and its precursor GD3 on TRAMP-C1 cells, indicative of their neuro-endocrine phenotype. **C, D)** B6 mice inoculated sq with 3E6 TRAMP-C1 cells received 5FU on day 57, followed by no treatment or p50-IMC (1E7 cells/dose) iv on days 63, 65, and 67 (n=5/group). Data were fit to an exponential model and mean tumor volumes are shown.

PSMA is an enzyme with an active site that releases glutamate from folate-polyglutamate and other molecules. M. Pomper (Co-Inv) has done extensive work developing small molecule, non-cleavable substrate analogs that binds PSMA and can be used for PET imaging of prostate cancer patients. One such ligand, KEU, links lysine (K) to glutamate (E) via a urea (U) rather than a normal peptide bond linkage. Streptavidin has high affinity for biotin. Streptavidin is normally dimeric, but a monomeric streptavidin (mSA) carrying mutations in three amino acids (mSA_RWT) that retains high biotin affinity and can be expressed on the cell surface has been developed.[5] We assembled a mSA(RWT) CAR containing a spacer, TM domain, and CD3ζ cytoplasmic domain capable of mediating phagocytosis, with an internal FLAG peptide to allow detection using FLAG Ab (**Fig. 5A**). We introduced mSA(RWT).CAR into Jurkat T cells where its surface expression at high levels could be detected by flow cytometry using FLAG Ab (**Fig. 5B, top**), and its ability to bind biotin was confirmed using biotin-FITC (**Fig. 5B, bottom**). We intend to express mSA(RWT).CAR in p50-IMC and combine these cells with biotin-KEU to direct them to MC/mPSMA tumors growing in immune-competent syngeneic FVB/N mice.

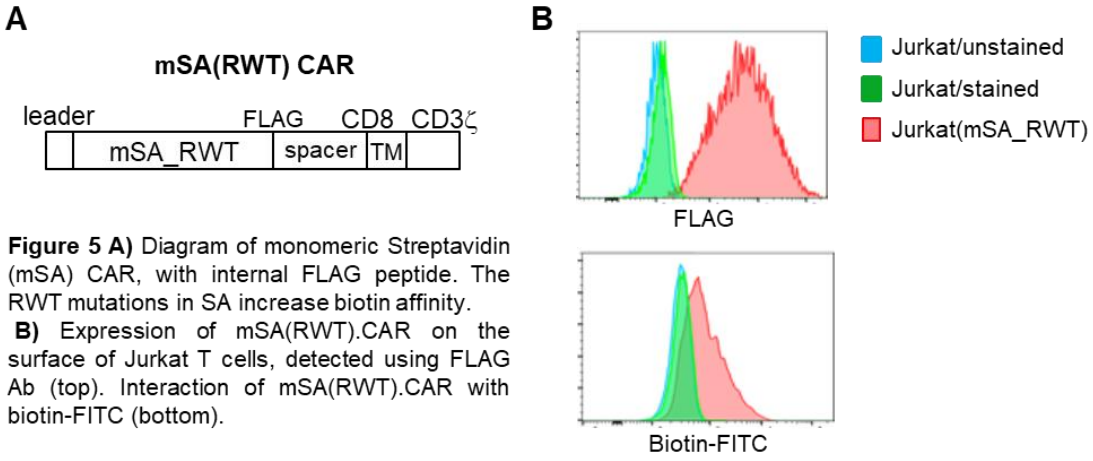

Figure 5 A) Diagram of monomeric Streptavidin (mSA) CAR, with internal FLAG peptide. The RWT mutations in SA increase biotin affinity. **B)** Expression of mSA(RWT).CAR on the surface of Jurkat T cells, detected using FLAG Ab (top). Interaction of mSA(RWT).CAR with biotin-FITC (bottom).

We set out to develop mice expressing transgenic hPSMA, inspired by the finding that mice expressing luciferase and GFP in the pituitary have increased tolerance to cell lines expressing these transgenes.[6] Hi-Myc mice (from which MC cells derive) express c-Myc from the Pbn promoter combined with two additional AR elements (AR_2Pbn). We constructed an AR_2Pbn-hPSMA transgene so that AR_2Pbn-Myc/AR_2Pbn-hPSMA (Myc/hPSMA) mice would express both transgenes in the same cells with the same temporal pattern, facilitating potential cooperative malignant transformation. Hi-Myc mice in the FVB/N background develop prostate cancer at least six months earlier than do B6 Hi-Myc mice, and we have in hand a colony of FVB/N Hi-Myc mice. We therefore provided AR_2Pbn-hPSMA-polyA DNA (**Fig. 6A**), free of plasmid vector DNA, to our Transgenic Core and requested that they conduct micro-injection into FVB/N zygotes. PCR analysis of tail clip DNA using both a 5' and 3' primer pair identified four founders from 34 mice (18 male, 16 female) screened (**Fig. 6B**). We will next obtain male offspring from these founders and determine whether hPSMA is expressed in their prostate glands, whether MC/hPSMA cells form tumors with kinetics similar to parental MC cells in these mice, and whether these tumors retain hPSMA, to enable experiments with p50-IMC combined with PSMA Ab or CAR.

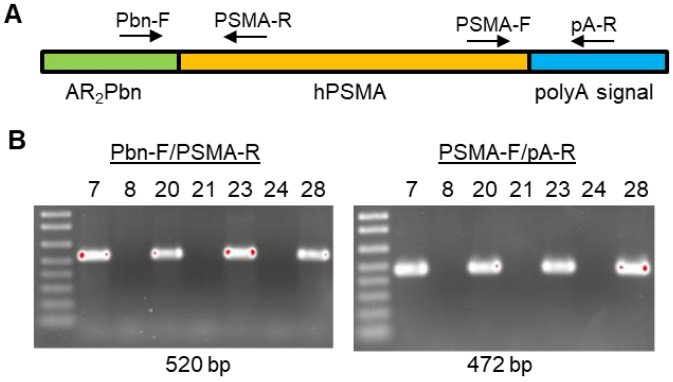

Figure 6 A) Diagram of the AR_2Pbn-hPMSA transgene and PCR primer pairs. AR - androgen receptor; Pbn - probasin promoter. polyA - polyadenylation. **B)** Detection of four transgenic FVB/N founders by tail clip DNA PCR. Data for seven of 34 mice screened are shown.

Task 4/Subtask 2 -Construct a PSMA.CAR in a viral vector

We constructed a cDNA encoding a PSMA.CAR using the available PSMA10.3 Ab sequence, as diagrammed (**Fig. 7A**). In particular, we had a commercial vendor synthesize a DNA sequence encoding a 19 amino acid leader peptide derived from human IgG (for membrane localization) and an scFv domain with the V_H and V_L domains connected by a flexible linker. Of note, this scFv is fully humanized making it optimal for later clinical translation. This DNA was ligated upstream of a spacer and a trans-membrane domain (TM) derived from human CD8α, and an intracellular signaling domain derived from hCD3ζ. The hCD3ζ domain was reported to be sufficient to mediate phagocytosis by both murine and human macrophages. We inserted the cDNA encoding this PSMA.CAR into the MIPuro retroviral vector and used this vector to transduce human Jurkat T cells, followed by puromycin selection. Our PSMA.CAR was expressed at high levels in these cells, as detected by anti-human Fab Ab (**Fig. 7B**). We utilized a commercially available extra-cellular domain of hPSMA linked to biotin, together with streptavidin-PerCP/Cy5.5 to confirm that PSMA.CAR on Jurkat T cells effectively binds hPSMA (**Fig. 7C**).

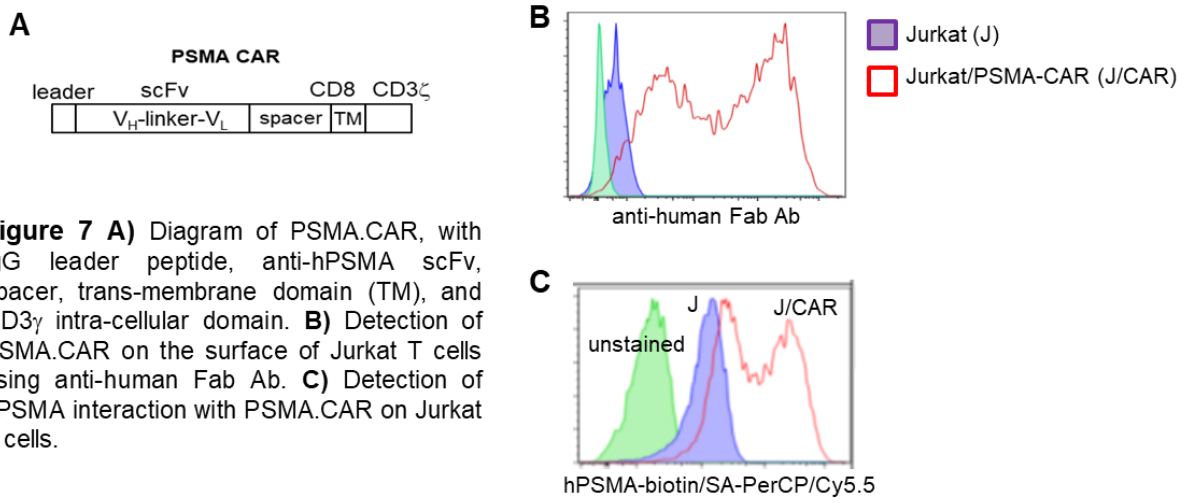

Figure 7 A) Diagram of PSMA.CAR, with IgG leader peptide, anti-hPSMA scFv, spacer, trans-membrane domain (TM), and CD3γ intra-cellular domain. **B)** Detection of PSMA.CAR on the surface of Jurkat T cells using anti-human Fab Ab. **C)** Detection of hPSMA interaction with PSMA.CAR on Jurkat T cells.

Task 4/Subtask 3 and Subtask 4 - Murine PrCa tumor growth combining p50-IMC or p50KO-IMC with PSMA.CAR with or without PD-1 Ab or CD47 Ab

We will undertake these subtasks using TRAMP-C1 cells and GD2.CAR, while pursuing development of mice that tolerate hPSMA. We obtained a validated GD2.CAR from M. Brenner that contains an scFv derived from a murine anti-GD2 Ab and spacer, TM, and intra-cellular domains derived from CD3ζ (**Fig. 8A**). We confirmed expression of GD2.CAR after retroviral transduction and puromycin-selection of Jurkat T cells (**Fig. 8B**).

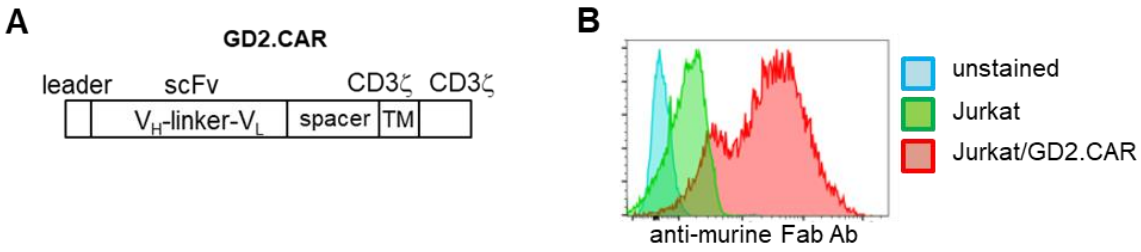

Figure 8 A) Diagram of GD2.CAR, with leader peptide, anti-GD2 scFv, and CD3γ spacer, TM domain, and intra-cellular domains. **B)** Detection of GD2.CAR on the surface of Jurkat T cells using anti-murine Fab Ab

We have also demonstrated that we can efficiently transduce murine immature myeloid cells (IMC) with both GD2.CAR and PSMA.CAR, to obtain cells for *in vivo* experiments. Murine lineage-negative (Lin^-) bone marrow (BM) cells growing in media with SCF, FL, and TPO were transduced with GD2.CAR or PSMA.CAR by spinoculation, followed by puromycin selection to eliminate untransduced cells, expansion, and then transfer to

M-CSF for either one day (to mimic how we make p50-IMC) or seven days (to generate macrophages to mimic the *in vivo* maturation of p50-IMC). Both CARs were detected at high levels in cells growing in SCF/FL/TPO, and were retained even after culture for seven days with M-CSF (**Figs. 9A, 9B**). The anti-human Fab Ab used to detect PSMA.CAR gave high background on empty vector (MIPuro)-transduced cells in M-CSF (Fig. 9B, right). In addition, we optimized a protocol for gene-editing the *Nfkb1* gene encoding NF-κB p50 in wild-type murine marrow cells followed by CAR expression by retroviral transduction and puromycin selection, again obtaining CAR expression in the large majority of cells (not shown).

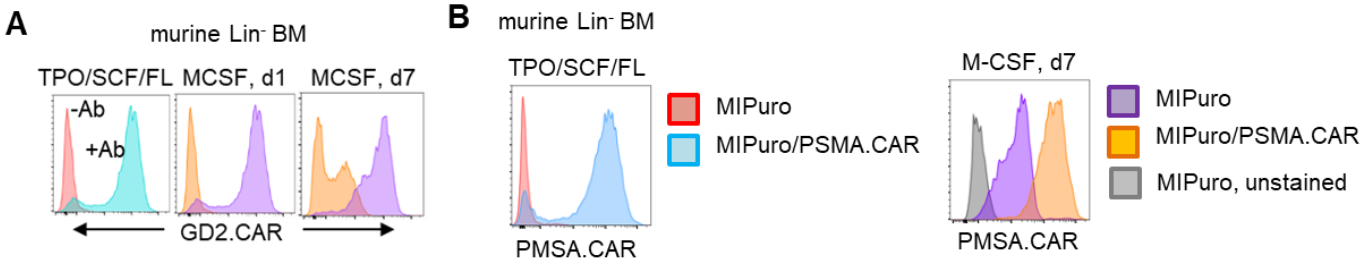

Figure 9 A) Murine lineage-negative bone marrow cells growing in IMDM/FBS with TPO, SCF, and FL were transduced with GD2.CAR by spinoculation with Polybrene, followed by puromycin-selection, expansion, and then transfer to M-CSF for 1 or 7 days. Cells were analyzed for GD2.CAR expression by flow cytometry. **B)** Cells transduced with the empty MIPuro vector or with MIPuro-PSMA.CAR and cultured similarly were analyzed for PSMA.CAR in TPO/SCF/FL or M-CSF (day 7).

To determine whether CAR expression on IMC increases phagocytosis we transduced wild-type murine marrow cells with either MIPuro (empty vector) or MIPuro-PSMA/CAR, followed by culture for seven days to generate macrophages and then for one day with IFNγ to induce an M1 phenotype (to mimic the effect of p50KO). These macrophages were then mixed with CSFE-dye labeled MC/hPSMA cells for 2 hr, followed by flow cytometry for CD11b (a myeloid cell marker) and CSFE (**Fig. 10**). Presence of the PSMA.CAR on the IMC led to a 10-fold increase in the proportion of cells that are $CD11b^{+}CSFE^{+}$, representing macrophages that have phagocytosed MC/hPSMA cells.

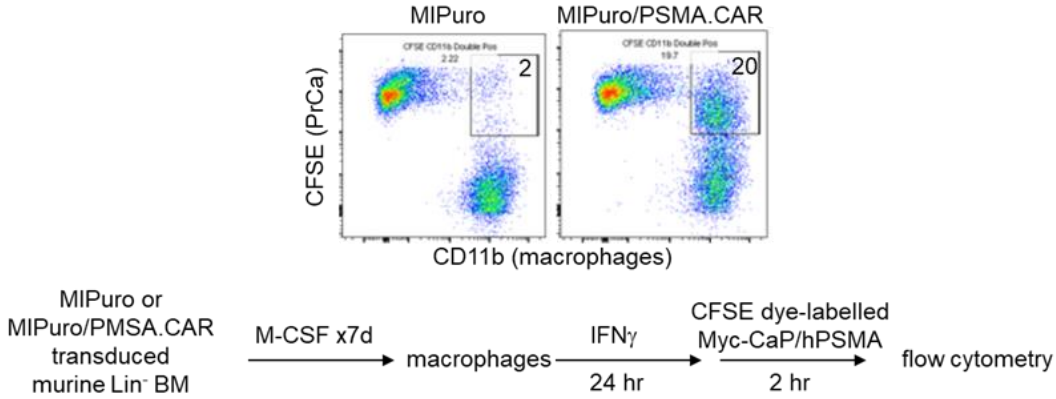

Figure 10 Murine lineage-negative bone marrow cells were transduced with MIPuro or MIPuro/PSMA.CAR and then cultured for 7 days in M-CSF to generate macrophages. These were then treated with IFNγ for 24 hr (to induce M1 phenotype) followed by mixing with CSFE-dye-labelled Myc-CaP(MC)/hPSMA cells for 2 hrs and then flow cytometry. $CD11b^{+}/CSFE^{+}$ cells represent MC/hPSMA cells that have been phagocytosed.

Task 5/Subtask 2 - Assess effect of human p50-IMC and PSMA Ab on human tumor growth

We find that human LNCaP cells express high levels of hPSMA (Fig. 1) and readily form tumors in NSG mice (not shown), and as described above we have obtained a large supply of anti-human PSMA3.9 Ab from a hybridoma. We also have developed MC/hPSMA cells that form tumors in NSG mice and retain hPSMA (Fig. 2C). In an initial experiment, we combined human IMC and PSMA3.9 Ab on ice for 1 hour prior to injection, loaded the cells with CSFE dye, injected NSG mice bearing MC/hPSMA with these cells or with control IMC

cells not exposed to PSMA3.9 Ab. We then assessed IMC tumor localization two days later by flow cytometry. PSMA3.9 Ab did not increase IMC tumor localization (data not shown). We plan to repeat this experiment using LNCaP cells, which express much higher levels of hPSMA than MC/hPSMA cells (Fig. 1A).

Task 6/Subtask 2 – Assess effect of human p50-IMC and PSMA.CAR on human tumor growth

We constructed MIPuro retroviral vectors that express GD2.CAR and PSMA.CAR efficiently in murine marrow, as described above. In addition, we find that we can efficiently transduce human BM $CD34^+$ stem/progenitor cells with our GD2.CAR using a retroviral vector (**Fig. 11A**). To improve human $CD34^+$ transduction we transferred our GD2.CAR and PMSA.CAR constructs into a lentiviral vector that also contains a puromycin-resistance cassette. We then compared several transduction procedures using LV-PSMA.CAR and found that the combination of Polybrene, PGE2, and LentiBoost during spinoculation led to the highest proportion of $CD34^+$ cells expressing PSMA.CAR (**Fig. 11B**).

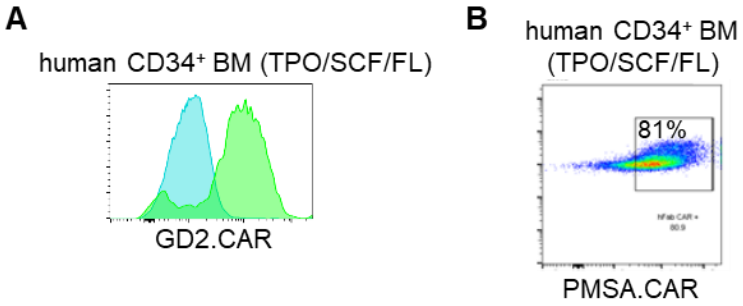

Figure 11 A) GD2.CAR expression in human $CD34^+$ marrow cells after transduction and puromycin-selection. **B)** Human $CD34^+$ BM cells expanding in serum-free media with SCF, TPO, FL, SR-1, and UM171 were spinoculated with an SIN lentiviral vector expressing PSMA.CAR and puromycin-resistance. The transduction cocktail included Polybrene (8 μg/mL), PGE2 (10 μM), and LentiBoost (1 mg/mL). PSMA.CAR expression was analyzed 4 days later.

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Opportunities for training and professional development

During the past year, this proposal facilitated laboratory-based training and professional development in the fields of prostate cancer research and immunotherapy for a post-doctoral fellow, Mohammad Alzubi PhD, and for a medical student, Patrick Beck. In addition to conducting the above experiments, these trainees attended and presented at weekly laboratory meetings held by Dr. Friedman and attended numerous scientific seminars at the Johns Hopkins Comprehensive Cancer Center, including Oncology Grand Rounds, Translational Research Conference, and Journal Club.

Dissemination of research results

Nothing to Report.

Plans during the next reporting period

Task 1 We will determine whether GD2 Ab increases efficacy of p50-IMC or p50KO-IMC against tumor arising in syngeneic B6 mice from TRAMP-C1 cells. In addition, we will continue our effort to generate transgenic FVB/N mice expressing human PSMA from the prostate-specific Probasin promoter (by evaluating offspring from our four AR_2Pbn-hPSMA founders) which we anticipate will be tolerant of hPSMA and allow us to conduct related studies using anti-human PSMA Ab and MC/hPSMA cells.

Task 2 We will determine whether GD2 Ab increases localization of p50-IMC or p50KO-IMC to TRAMP-C1 tumors and will determine the effect of addition of GD2 Ab on changes in tumor myeloid cells and T cells induced by p50-IMC or p50KO-IMC. Related studies will also be conducted with PSMA Ab should we succeed in developing B6 mice tolerant to MC/hPSMA cells.

Task 3 We will determine whether PD-1 Ab increases efficacy of GD2 Ab combined with p50-IMC or p50KO-IMC against murine TRAMP-C1 prostate cancer in immune-competent B6 mice. We will also optimize SIRPα CRISPR/Cas9 gene editing in murine lineage-negative bone marrow cells.

Task 4 We will determine whether GD2.CAR increases localization and efficacy of p50-IMC or p50KO-IMC against tumor arising in syngeneic B6 mice from TRAMP-C1 cells, alone or together with PD-1 Ab. We will also determine whether mSA(RWT).CAR combined with biotin-KEU increases efficacy of p50-IMC or p50KO-IMC against tumor arising in syngeneic FVB/N mice from MC/mPSMA cells, alone or together with PD-1 Ab. In addition, we will continue our effort to generate transgenic FVB/N mice expressing human PSMA, which we anticipate will be tolerant of hPSMA and allow us to conduct studies using PSMA.CAR and MC/hPSMA cells.

Task 5 We will determine whether PSMA Ab increases efficacy of p50KO-IMC against tumors derived from human LNCaP prostate cancer cells in immune-deficient NSG or NSGS mice. We will determine whether PSMA Ab increases p50KO-IMC tumor localization to LNCaP-derived tumors. We will determine the effects of p50KO-IMC alone or with PSMA Ab on tumor myeloid cell phenotypes. We will also optimize SIRPα CRISPR/Cas9 gene editing in human $CD34^+$ bone marrow cells.

Task 6 We will determine whether PSMA.CAR expression increases efficacy of p50KO-IMC against tumors derived from human LNCaP prostate cancer cells in immune-deficient NSG or NSGS mice. We will determine whether PSMA.CAR increases p50KO-IMC tumor localization. We will also determine the effects of p50KO-IMC alone or with PSMA.CAR on tumor myeloid cell phenotypes.

4. IMPACT

Impact on the development of the principal discipline(s) of the project

Prostate cancers include normal white blood cells called macrophages that contribute to tumor growth. These tumor-associated macrophages suppress the immune system's ability to fight prostate cancer. We previously found that white blood cells lacking a protein called p50 slows the growth of prostate cancer in mice. Prostate cancers express prostate-specific membrane antigen or PSMA, and the level of PSMA is highest in the most aggressive human cancers. We plan to direct p50-deficient white blood cells to prostate cancer either by using PSMA antibody or by expressing a PSMA-targeting chimeric antigen receptor (CAR) on the cells. Once the p50-deficient white blood cells reach the tumor, they are expected to "eat" PSMA-expressing cancer cells and thereby stimulate broad anti-tumor immunity. We will determine the effectiveness of these strategies in models of local or metastatic prostate cancer. Prostate cancer cells also express additional molecules, such as a complex sugar termed GD2, that we can similarly use to direct p50-deficient white blood cells to prostate cancer tumors. We have already seen that while blood cells expressing a PSMA-targeting CAR uptake prostate cancer cells much more effectively than do control cells lacking a PSMA CAR. This result, and additional findings we anticipate obtaining, has implications for the treatment of prostate cancer, as we expect to pursue a therapy in which we target p50-deficient white blood cells to prostate cancer to increase their efficacy,

Impact on other disciplines

In addition to prostate cancer, many other cancers contain TAMs that inhibit tumor growth by suppressing the immune system and express molecules analogous to PSMA that can be used to target direct p50-deficient white blood cells to the tumor to stimulate anti-tumor immunity, including brain, pancreatic, and breast cancers. Our findings are therefore also relevant to these and other cancers.

Impact on technology transfer

Nothing to Report.

Impact on society beyond science and technology

Nothing to Report.

5. CHANGES/PROBLEMS

Changes in approach

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

In addition to directing p50-IMC to human prostate cancers using anti-human PSMA Ab or PSMA.CAR (Task 5 and Task 6), given our finding of immune-rejection of murine prostate cancers expressing hPSMA in immune-competent mice we are conducting Task 1-4 experiments using GD2 Ab or GD2.CAR and Task 4 using mSA(RWT).CAR and biotin-KEU to target PSMA. The objective and scope of proposed experiments, to determine whether directing p50-IMC to prostate cancer with Ab or CAR, has not changed. In effort to obtain immune-competent mice tolerant to hPSMA, we are developing mice expressing hPSMA in the prostate using the AR_2-Probasin promoter used previously to develop Hi-Myc mice.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. PRODUCTS

Publications, conference papers, and presentations

Nothing to Report.

Websites or other internet sites

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patents, or licenses

Nothing to Report.

Other products

Nothing to Report.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who worked on the project (effort round up to nearest whole month)

Name:	Alan D. Friedman
Project Role:	Principal Investigator
ORCID ID:	0000-0002-5615-7061
Person months worked:	1
Contribution to Project:	Experimental supervision, constructed CARs and cell lines expressing hPSMA,
Funding Support:	this award

Name:	Martin G. Pomper
Project Role:	Co-investigator
ORCID ID:	0000-0001-6753-3010
Person months worked:	1
Contribution to Project:	Experimental supervision
Funding Support:	this award

Name:	Mohammad Alzubi
Project Role:	Post-doctoral Fellow
ORCID ID:	None
Person months worked:	6
Contribution to Project:	tumor inoculation and growth monitoring, p50-IMC generation and mouse treatments
Funding Support:	this award

Name:	Theresa Barberi
Project Role:	Research Associate
ORCID ID:	None
Person months worked:	6
Contribution to Project:	tumor inoculation and growth monitoring, p50-IMC generation and mouse treatments, cell and tumor flow cytometry, Western blotting
Funding Support:	this award

Change in the active other support of the PI and senior/key personnel

Name: Alan D. Friedman
Changes: closed (grant number/funding agency)
R01 HL130034, NIH
611711, The Andrew McDonough B+ Foundation
T2018-002, V Foundation
2020-MSCRFD-5380, Maryland Technology Development Corporation
PHPA-1104, Maryland Cigarette Restitution Fund
90090815, Johns Hopkins Univ./Univ. of Pennsylvania SPORE of Ovarian Cancer

new (grant number/funding agency, PI, dates, % effort)
00153062, Vita Therapeutics, Inc. (Friedman), 11/01/21-10/31/23, 1.2 Cal

Dr. Friedman's 1.2 Cal annual effort on this DoD award (contract # W81XWH-21-1-0671) will not change as a result of his new Other Support.

Name: Martin G. Pomper
Changes: closed (grant number/funding agency)
None, Sinotau Pharmaceuticals
None, FiveEleven
None, Roche Laboratories
HHHSN271201800716P, NIDA
0073JHU/R44CA17724, Sibtech Inc/NCI
15050162, Cornell

new (grant number/funding agency, PI, dates, % effort)
75N95021P00602, NIDA (Pomper), 9/20/21-9/19/24, 0.12 Cal
None, Precision Molecular (Pomper), 12/21/21-12/20/22, 0.12 Cal
None, Genetech (Pomper), 9/15/21-9/14/24, 0.12 Cal

Dr. Pomper's 0.2 Cal annual effort on this DoD award (contract # W81XWH-21-1-0671) will not change as a result of his new Other Support.

Other organizations involved as partners
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS
Not applicable.

9. APPENDICES
None.