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14. ABSTRACT
Immature myeloid cells lacking NF-kB p50 (p50-IMC) slows growth of prostate cancer (PrCa), given after a dose of 5FU. 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, increasing efficacy. EGFR is also expressed on a subset of aggressive PrCa tumors. We find that wild-type (WT) or p50(-/-) macrophages minimally phagocytose Myc-CaP PrCa cells expressing human PSMA or EGFR, but that PSMA or EGFR Ab and PSMA CAR markedly increase phagocytosis in both IFNg (M1) or IL-4 (M2) culture conditions. We also find that PSMA Ab increased p50-IMC localization to Myc-CaP/hPSMA tumors in NSG mice, with preceding 5FU increasing localization. As immune-competent FVB/N mice reject Myc-CaP cells expressing hPSMA or hEGFR we developed transgenic mice expressing hPMSA in the prostate; however; while RNA was abundant, protein was absent. We also developed transgenic mice expressing truncated hEGFR; we detect protein expression and have germline transmission, which should enable efficacy studies using p50-IMC and EGFR Ab or CAR. We constructed a mutant hPSMA lacking its cytoplasmic domain and with mutation in its enzymatic active site, and confirmed its PSMA Ab binding, with plans to obtain transgenic mice expressing this variant for efficacy studies using p50-IMC with PSMA Ab or CAR.

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

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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. As we found during Yr01 that immune-competent mice do not tolerate human PSMA (hPSMA), in addition to describing our ongoing efforts to target p50-IMC to PSMA on PrCa *in vitro* and in immune-deficient mice and to developing immune-competent mice tolerant to hPSMA to enable anti-tumor efficacy studies, with permission of our previous Science Officer we also include progress on our efforts to direct p50-IMC to aggressive PrCa using EGFR or GD2 Abs or CARs in this report (per our previous Science Officer, this did not require modification to our SOW as we are still doing studies targeting PSMA).

2. KEYWORDS

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

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); Completed.
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). 50% Completed.

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); Completed.
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). 50% Completed.

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 - PrCa growth with 5FU/p50KO-IMC+PMSA Ab, CD47 Ab or *SIRP*α KO, or both (mos 16-36).
Subtask 5 - Assess human tumor myeloid numbers and differentiation state with PSMA Ab (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 - PrCa growth with 5FU/p50KO-IMC vs 5FU/p50KO-IMC/PMSA.CAR, +/- 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 or *SIRP*α KO (mos 18-36).
Subtask 4 - Assess human tumor myeloid numbers and differentiation state with PSMA.CAR (mos 12-30).
Completed.

Accomplishments under these goals
(Yr02 results are shown in the Figures; Yr01 results are summarized in the text except as noted).
Task 1/Subtask 2 - Generate anti-PSMA Ab

We obtained the hybridoma cell lines PSMA3.9 and PSMA10.3 from ATCC. Ab3.9 is fully murine and best for use in immune-competent mice. Ab 10.3 is fully humanized and so most appropriate for use with human p50-IMC. We provided the PSMA3.9 cell line to a commercial vendor (Bio-X-Cell) during Yr01 and they produced 108 mg of purified monoclonal Ab. We used flow cytometry to confirm that PSMA3.9 Ab binds hPSMA on the surface of Myc-CaP (MC) and TRAMP-C1 (TC1) PrCa cells that we engineered to express hPSMA.

We found that purified Ab3.9 and Ab10.3 (impure hybridoma supernatant) do not detect murine PSMA (mPSMA) on MC cells, precluding use of MC/mPSMA cells in immune-competent syngeneic FVB/N mice for proposed efficacy studies with p50-IMC combined with PSMA Ab or PSMA.CAR (our PSMA.CAR derives from Ab10.3, as detailed below). 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. During Yr02, we sent the PSMA10.3 line to Bio-X-Cell in hopes of obtain purified protein useful for studies with human p50-IMC; unfortunately, they found that Ab was produced at only a very low level by this hybridoma.

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

We found that that the large majority of MC/hPSMA cells inoculated into syngeneic FVB/N mice do not form tumors and that the few that form tumors do so slowly and express very little hPSMA; in contrast MC/hPSMA cells grow readily in immune-deficient NSG mice and retain high-levels of surface hPSMA. In addition, MC/mPSMA cells form tumors readily in FVB/N mice and retain surface mPSMA. These data indicate that hPSMA is not tolerated by the FVB/N immune system and not that PSMA prevents tumor growth. Similarly, we found that in contrast to parental TC1 cells, TC1/hPSMA cells do not form tumors in syngeneic C57BL/6 (B6) mice. PSMA has enzymatic activity that removes glutamate from folate-polyglutamate and other polypeptides. Lysine (K) linked to glutatmate (E) via a urea linkage (KEU) is a non-cleavable substrate analog that binds the PSMA active site. We were able to use a KEU-dendrimer-PE/Cy5 reagent developed by the Pomper laboratory (co-Inv.) to detect surfacer mPSMA by flow cytometry.

The key objective of proposed experiments is to determine whether an Ab or CAR directing p50-IMC to PrCa increases anti-tumor efficacy. Human LNCaP prostate cancer cells, PSMA Ab3.9 and our PSMA.CAR construct can be utilized to pursue this objective in immune-deficient NSG mice. However, our finding that murine PrCa lines expressing hPSMA are not tolerated by FVB/N or B6 mice led us to attempt to develop transgenic mice expressing hPSMA throughout development and so tolerant to prostate cancer cell lines expressing hPSMA, to evaluate directing p50-IMC to PSMA using the KEU PSMA ligand that binds mPSMA, and to pursue other PrCa

surface antigens besides PSMA expressed on aggressive prostate cancers (EGFR and GD2) as p50-IMC targets. I will now review our progress related to each of these three approaches.

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. 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 and provided this DNA (**Fig. 1A**), 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 during Yr01 (**Fig. 1B**). Of 17 male offspring obtained from Founder 7, none contained the transgene, whereas 3 of 11 female offspring did, and none of 15 offspring from Founder 28 contained the transgene. In contrast, Founders 20 and 23 transmitted the AR_2Pbn-hPSMA to both male and female offspring. The prostates of males from lines 20 and 23 express hPSMA mRNA, with ~10-fold higher levels in line 20 (**Fig. 1C**). PSMA protein was not detected in the ventral or anterior prostate lobes of male line 20 or 23 offspring (**Fig. 1D** and not shown), with mPSMA evident in the prostate of a six-month-old Hi-Myc mouse. The normal mouse prostate lacks PSMA, in contrast to the normal human prostate, and after initiating this effort I learned that another investigator also encountered difficulty expressing hPSMA as a transgene in mice, using the HoxB13 promoter (C. Bieberich, pers. commun.). These data indicate that hPSMA is toxic when expressed in the prostate as a murine transgene.

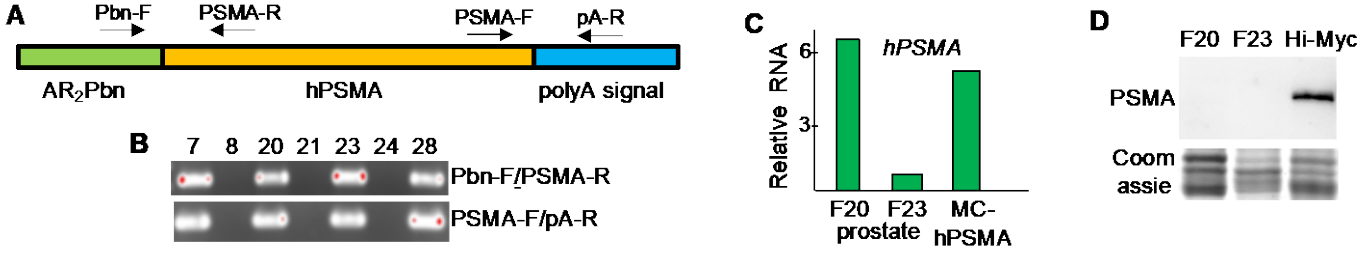
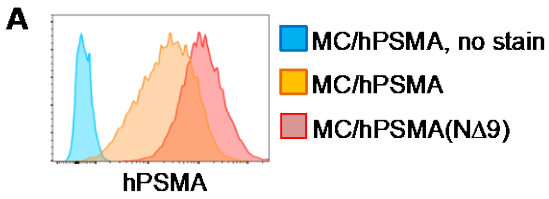


Figure 1 A) Diagram of the AR_2Pbn-hPMSA transgene and PCR primer pairs. AR - androgen receptor; Pbn - probasin promoter. polyA - polyadenylation. **B)** Detection of four transgenic FVB/N founders by tail clip DNA PCR. Data for seven of 34 mice screened are shown. **C)** Total RNA from Founder 20 and 23 (F20, F23) prostates and from MC/hPSMA cells were analyzed by qRT-PCR for *hPSMA*, relative to ribosomal protein S16 mRNA. **D)** Western blot for PSMA in the ventral lobes of prostates from F20 and F23 transgenic mice and from a Hi-Myc mouse, with Coomassie stained samples as loading control. Proteins were prepared by homogenization of snap frozen prostate lobes after addition of RIPA buffer/protease inhibitors.

In effort to overcome the toxicity of intact hPSMA as a transgene, we have now assembled a hPSMA variant defective in two key functional domains. Deletion of nine cytoplasmic amino acids that include an MXXXL motif from hPSMA prevents cell internalization.[1] We find that hPSMA(NΔ9) expresses at higher levels than hPSMA on the surface MC cells (**Fig. 2A**). Introduction of a H553G mutation into the hPSMA active site eliminates enzymatic activity without affecting protein stability.[2] We introduced these two mutations into hPSMA and used retroviral transduction to obtain MC cells expressing hPSMA(NΔ9/H553G). This double mutant achieves levels identical to hPSMA(NΔ9) on the surface of MC cells and retains binding to two anti-hPSMA Abs and only slightly reduced affinity to KEU PSMA ligand (**Fig. 2B**). We have constructed an AR_2Pbn-hPSMA(NΔ9/H553G) transgene, confirmed its integrity by DNA sequencing, and will now isolate and provide this DNA free of vector



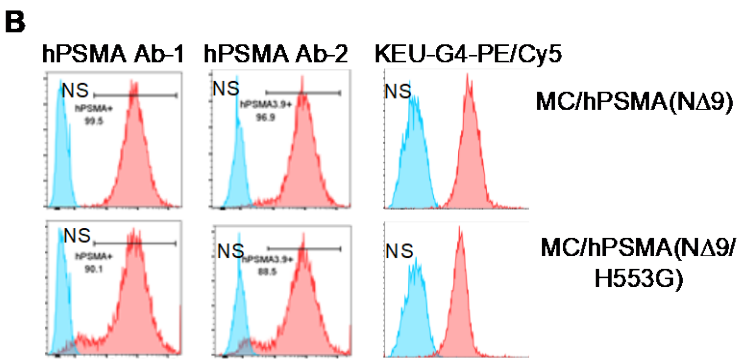


Figure 2 A) Flow cytometry analysis of surface hPSMA on indicated MC lines using hPSMA-APC Ab. **B)** Flow cytometry analysis of hPSMA on indicated MC lines using two hPSMA Abs or KEU-G4(dendrimer)-PE/Cy5. NS – no stain.

DNA to our Transgenic Core as an additional effort to obtain mice tolerant to hPSMA, to allow proposed efficacy studies combining p50-IMC with PSMA Ab or PSMA.CAR.

Streptavidin has high affinity for biotin. Streptavidin is normally dimeric, but 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.[3] We assembled a mSA(RWT) CAR with an internal FLAG peptide to allow detection using FLAG Ab. We introduced mSA(RWT).CAR into Jurkat T cells where its surface expression was detected by flow cytometry using FLAG Ab, and its ability to bind biotin was confirmed using biotin-FITC. However, our inability to detect binding of a biotin-dendrimer-PE/Cy5-KEU molecule to these cells led as to no longer pursue expression of mSA(RWT).CAR in p50-IMC to direct them to MC/mPSMA tumors.

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,[4] 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 (see below). We developed MC/hEGFR and TC1/hEGFR cell lines by constructing a MIPuro-hEGFR retroviral vector, followed by viral packaging, cell transduction, and puromycin selection. Unfortunately, as with MC or TC1 PrCa cells expressing hPSMA, these lines did not grow in FVB/N or B6 mice, respectively. In addition, Lewis Lung Cancer cells expressing hEGFR grew tumors that retain hEGFR in NSG mice but lack hEGFR in syngeneic B6 mice, further indicating that as with hPSMA, immune-competent mice do not tolerate hEGFR.

Full-length hEGFR consists of 1210 amino acids. hEGFRt (**Fig. 3A**), lacking 309 N-terminal extra-cellular and 563 intra-cellular residues, retains the Cetuximab binding-site.[5] We developed LLC cells expressing hEGFRt (**Fig. 3B**); however, tumor derived from these cells in B6 mice lack hEGFRt (**Fig. 3C**). We set out to develop B6 mice expressing an hEGFRt transgene, to induce immune tolerance to TC1 PrCa cells expressing hEGFRt and potentially hEGFR. Plasmid pCAG contains a CMV enhancer, chicken β-actin promoter, and β-globin intron and polyA signal. We inserted a cDNA expressing hEGFRt, preceded by a GM-CSF Receptor-derived leader peptide to direct plasma membrane insertion, into pCAG, with flanking *lox*P sites (**Fig. 3D).** *lox*P sites allow deletion in tolerant adults by 4HT-regulated Cre-ER(T), if needed to minimize off-target effects of EGFR-directed immunotherapies. Expression of hEGFRt from pCAG was confirmed in 293T cells (**Fig. 3E**). We provided CAG-hEGFRt DNA to our Transgenic Core for generation of B6 transgenic mice, and two founders were identified (**Fig. 3F**). Flow analysis of blood of male founder #25 compared with two WT littermates, obtained by facial vein

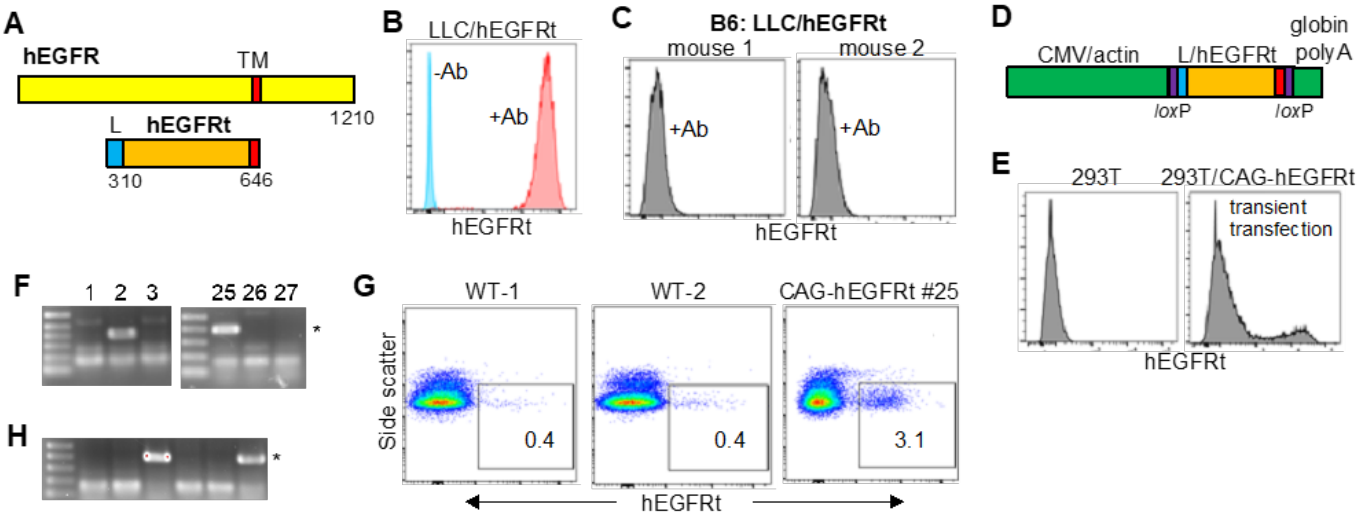


Fig. 3 A) Diagram of hEGFR and hEGFRt; L - leader peptide; TM – trans-membrane domain. **B)** Expression of hEGFRt in LLC cells. **C)** Tumors derived from LLC/hEGFRt cells do not retain hEGFRt. **D)** Diagram of CAG-hEGFRt. **E)** hEGFRt was evaluated in 293T cells (left) or 293T cells transfected two days earlier with pCAG-hEGFRt, without selection (right). **F** Screening 27 B6 pups by tail clip DNA PCR identified two transgenic founders (number 2 and 25) with the expected 400 bp band (*). **G)** Blood obtained of two wild-type B6 mice and CAG-hEGFRt transgenic founder #25 was analyzed for hEGFRt by flow cytometry with anti-EGFR-APC Ab. **H)** Genotyping of F1 litter from founder #2 (two positive pups).

lancing, demonstrates hEGFRt transgene expression in a subset of cells (**Fig. 3G**) - we have not subjected female founder #2 to this procedure so as to not disturb her ability to breed and care for her pups. hEGFRt expression in only a subset of blood cells may reflect weak CMV promoter activity in the majority of these cells or transgene chimerism; nevertheless, these data indicate that hEGFRt is likely also expressed in other tissues in the adult mice of both lines and, more importantly, is expressed sufficiently during development to render the mice tolerant to hEGFRt, and potentially to full-length hEGFR. Tail vein PCR analysis of an F1 litter from founder #2 identified two positive offspring, confirming germ line transmission (**Fig. 3H**), versus 0/12 for male #25 F1 pups. We will now expand line #2 and determine if males tolerate hEGFR or hEGFRt expression in TC1 tumors, to enable efficacy studies in immune-competent mice with p50-IMC and PrCa-directed Ab or CAR.

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.[6,7] TC1 PrCa cells have a neuro-endocrine phenotype, and during Yr01 we confirmed that they express GD2, as well as its precursor GD3 (**Fig. 4B**). In contrast to PSMA and EGFR, GD2 is conserved between mice and humans. Dinutuximab is an FDA-approved Ab that recognizes GD2, and we recently received a 100 mg supply from the manufacturer; and M. Brenner kindly provided a GD2.CAR used in CAR-T cell clinical trials.[8] Only 9% of TC1 cells express GD2, whereas 85% express its GD3 precursor. GD2 Synthase (GD2S) converts GD3 to GD2. We introduced GD2S into TC1 cells via retroviral transduction; 60% of TC1/GD2S cells express GD2 (**Fig. 4C**). Given low GD2 expression in parental TC1 cells, we had prioritized effort to develop MC/hEGFRt cells for therapeutic studies during Yr02, but will now give equal effort to use of TC1/GD2S and MC/hEGFRt or MC/hEGFR cells (the latter lines in our hEGFRt transgenic mice) for p50-IMC efficacy studies with tumor-targeting Abs or CARs in (immune-competent mice that have T cells available for activation by the myeloid progeny of p50-IMC).

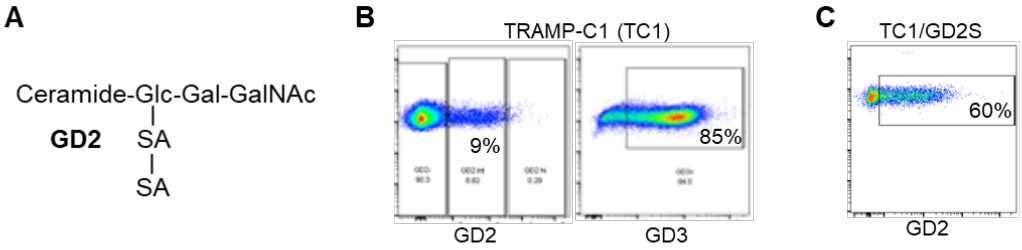


Figure 4 A) Structure of GD2. Glc - glucose, Gal - galactose, SA - sialic acid. **B)** Expression of GD2 and its precursor GD3 on TRAMP-C1 (TC1) cells. **C)** GD2 expression in TC1 cells expressing GD2 Synthase (GD2S).

Task2/Subtask 2 – Effects of PSMA Ab on PrCa phagocytosis, T cell activation, and p50-IMC tumor localization

We hypothesize that combining p50-IMC with an Ab that recognizes a protein on the surface of PrCa cells will increase phagocytosis by tumor macrophages that develop from p50-IMC, with consequent anti-tumor T cell activation via MHC antigen presentation. p50-IMC, derived from the marrow of $p50^{-/-}$ mice, or WT-IMC, derived from wild-type (WT), syngeneic B6 mice, were plated in M-CSF for six days to obtain bone marrow-derived macrophages (BMDM). These were then culture for 24 hours in either IFNγ or IL-4 to promote M1 or M2 gene expression, respectively. Of note, the PrCa tumor microenvironment favors the M2 macrophage phenotype. The macrophages were then released from the culture dishes and combined with either PSMA Ab3.9 or IgG_1 isotype control Ab, each of which can bind to macrophages by interaction of the Ab Fc segment with the macrophage Fc Receptor (FcR, **Fig. 5A**). Ab- or IgG1-bound macrophages were then incubated with CFSE-dye labeled MC/PSMA(NΔ9) cells for 3 hours, followed by flow cytometry for CD11b (which detects the macrophages) and CFSE (which detect the PrCa cells), as diagrammed (**Fig. 5B**). Representative flow cytometry images are shown (**Fig. 5C**), and the results of three independent experiments are provided (**Fig. 5D**). Cells that are positive for both CD11b and CFSE represent macrophages that have phagocytosed the PrCa cells. To confirm that the macrophages have actually internalized the cancer cells, we labelled the macrophages with the CFSE dye (green) and the MC/PSMA(NΔ9) cells with pHRodo Red dye, which is colorless at pH 7.5 (as in the culture media) but becomes red in the acidic environment of the phagocytic lysosome. Red cancer cells were only seen within green macrophages, confirming that phagocytosis has occurred (**Fig. 5E**). Cancer cells that have not been phagocytosed are colorless. This work is included in our accepted SITC abstract.[8]

Several conclusions can be drawn from these data:

i. WT and p50$^{-/-}$ macrophages have similar, low-level phagocytic activity in the absence of PSMA Ab.

ii. PSMA Ab increases phagocytosis by both M1-polarized and M2-polarized WT or p50$^{-/-}$ macrophages

iii. PSMA Ab-mediated phagocytosis is stronger with M1-polarized compared to M2-polarized macrophages.

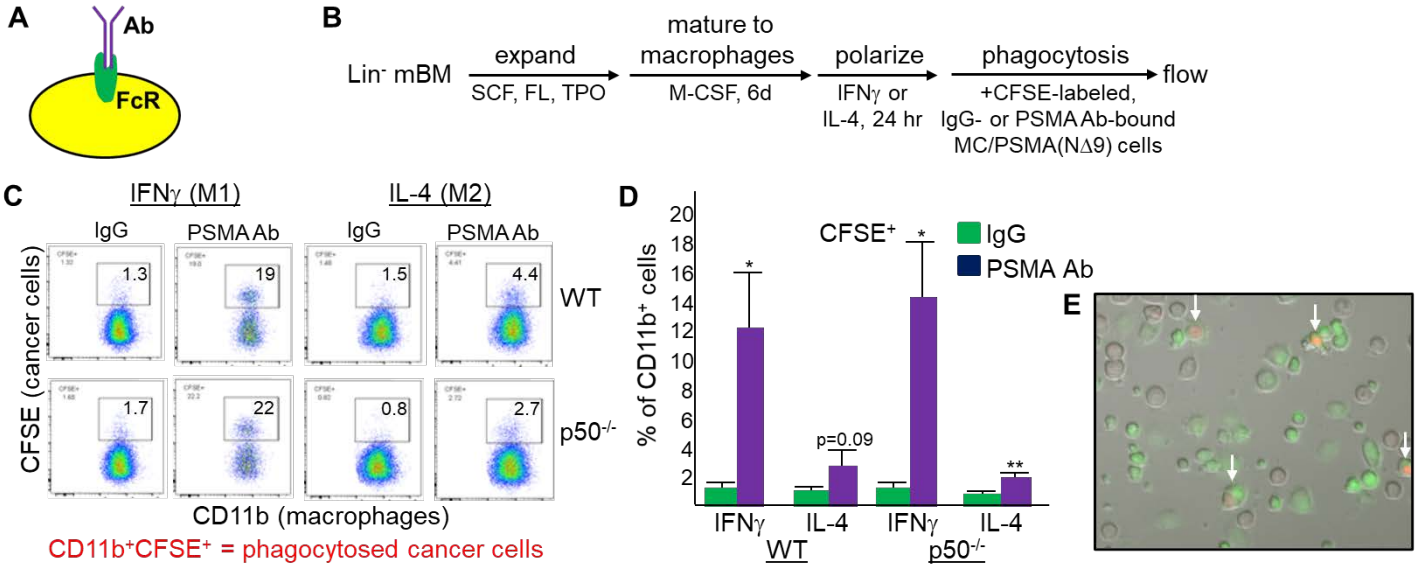


Figure 5. A) Fc domain of an Ab bound to a macrophage via the FcR. **B)** Lineage-negative WT or p50$^{-/-}$ murine bone marrow (mBM) cells were expanded, differentiated to macrophages, M1 or M2 polarized, incubated with PSMA antibody or isotype IgG control, and mixed with CFSE-labeled MC/PSMA(NΔ9), as diagrammed. **C)** Representative flow cytometry data. **D)** Results of three experiments evaluating CFSE$^+$ cells as a percentage of CD11b$^+$ macrophages.*p<.05; **p<.01 **E)** CFSE-labeled macrophages with PSMA Ab (green) were combined with pHRodo-Red-labeled MC/PSMA((NΔ9) cells, followed by microscopy (bright field with the red and green channels). Phagocytosed cancer cells are indicated by white arrows.

We also conducted this experiment with a clinically available EGFR Ab (Cetuximab) and MC/hEGFR cells (**Fig. 6A**). As with PSMA Ab, EGFR Ab significantly increased phagocytosis by p50$^{-/-}$ macrophages in both IFNγ and IL-4 and of WT macrophages in IFNγ. Phagocytosis of MC/hEGFR cells by WT cells in the absence of Ab was higher than that of p50$^{-/-}$ macrophages, in contrast to findings with MC/PSMA(NΔ9) cells. In addition, we conducted this experiment using a clinically available GD2 Ab (Dinutuximab) and TC1/GD2S cells and as a positive control with 9464D/GD2 neuroblastoma (Nb) cells that express high levels of GD2 (**Fig. 6B**).

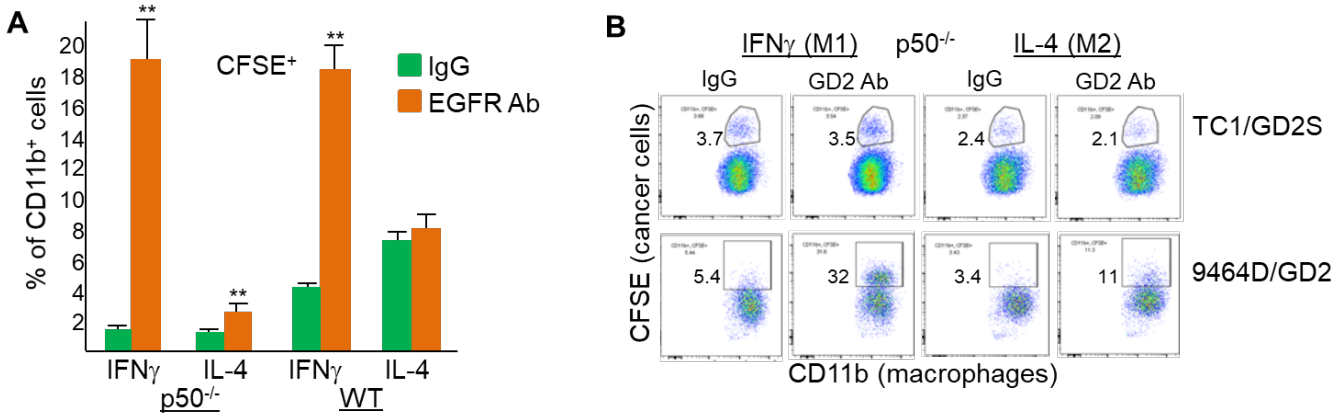


Figure 6. A) Lineage-negative WT or p50$^{-/-}$ murine bone marrow cells were expanded, differentiated to macrophages, M1 or M2 polarized, incubated with EGFR Ab or isotype IgG control, and mixed with CFSE-labeled MC/hEGFR cells for three hours, followed by flow cytometry for CD11b and CFSE. The proportion of CD11b$^+$ cells that are CFSE$^+$ is shown for three experiments (mean, SE). ** - p<0.01 **B)** TC1/GD2S PrCa cells or 9464D/GD2 neuroblastoma cells were assessed for phagocytosis by M1- or M2-polarized p50$^{-/-}$ macrophages in the presence of GD2 Ab or IgG control. Data with TC1/GD2S cells is representative of three experiments. 9464D/GD2 cells serve as a positive control.

While GD2 Ab markedly increased phagocytosis of the Nb cells by p50$^{-/-}$ macrophages, GD2 Ab did not increase baseline low-level phagocytosis of the TC1/GD2S PrCa cells. Of note, TC1 and TC1/GD2S cells are much larger than MC or 9464D/GD2 cells, which may impact their ability to be phagocytosed.

We expect that increased macrophage phagocytosis of PrCa cells mediated by PSMA Ab will lead to increased neoantigen presentation by MHC to activate T cells. To determine whether PSMA Ab-mediated phagocytosis of MC/PSMA(NΔ9) cells increases T cell activation *in vitro*, we will take advantage of OT-I B6 mice (Jackson Laboratory #003831). These mice harbor T cell receptor (TCR) α and TCRβ transgenes that form a TCRαβ complex on CD8$^+$ T cells that recognizes an eight-residue peptide (SIINFEKL) derived from chicken ovalbumin (cOVA) when presented by H-2K^b MHC class I molecules present on antigen presenting cells (macrophages or DCs) obtained from syngeneic B6 mice. We established a colony of homozygous OT-I mice and confirmed that their circulating CD8$^+$ T cells uniformly express the TCRα-V2 and TCRβ-V5 transgenes (**Fig. 7A**). We ligated the cOVA cDNA released from pCI-neo-OVA (Addgene #25097) by restriction enzyme digestion into the pBabeNeo retroviral vector to obtain pBabeNeo-cOVA and used this vector to express cOVA in MycCaP/PSMA(NΔ9) cells, as confirmed by Western blotting (**Fig. 7B**) and qRT-PCR (not shown). We successfully isolated of CD8$^+$ T cells from the spleens of OT-1 mice, labeled these T cells with CFSE dye, and demonstrated that they proliferate extensively when combined with marrow-derived macrophages from p50$^{-/-}$ mice that have been pulsed with the SIINFEKL cOVA peptide (**Fig. 7C**). Of note, T cell proliferation leads to CFSE dye dilution and thus a leftward shift in the CFSE flow cytometry peak. With these reagents in hand, during the coming year we will determine whether PSMA Ab (or CAR) mediated PrCa phagocytosis leads to T cell activation, as assessed by evaluating T cell proliferation and T cell expression of IFNγ.

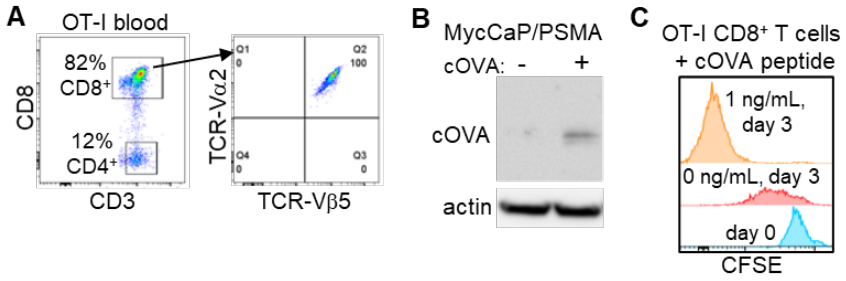


Fig. 7 **A)** Blood from an OT-I mouse was analyzed for TCR-Vα2 and TCR-Vβ5 on CD3$^+$CD8$^+$ T cells. **B)** MycCaP cells non-transduced (-) or transduced (+) with cOVA were subjected to Western blotting. **C)** M1-polarized p50$^{-/-}$ macrophages were incubated overnight with 0 or 1 ng/mL cOVA peptide, followed by culture with CFSE-labeled CD8$^+$ T cells from OT-I mice. CFSE flow was conducted on day 0 and day 3.

We next sought to determine if PSMA Ab increases p50-IMC localization to MC/PSMA(NΔ9) tumors in NSG mice. p50-IMC were CFSE-labeled, incubated with PSMA Ab3.9 or IgG and injected into NSG mice bearing

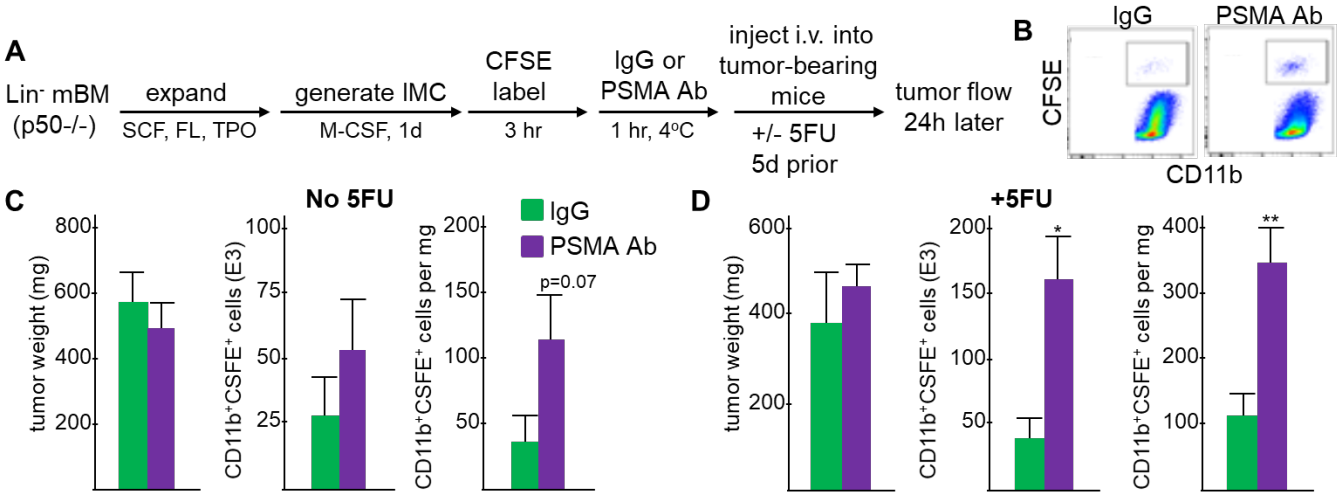


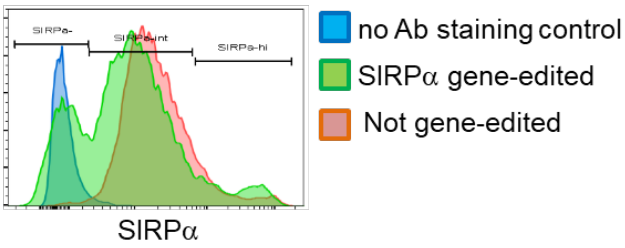
Figure 8. A) Lineage-negative p50-/- murine bone marrow cells were expanded, cultured with M-CSF for one day to obtain p50-IMC, CFSE-labeled, incubated with PSMA antibody or IgG control, and injected into NSG mice bearing subcutaneous tumors derived from MyC/CaP-hPSMA(NΔ9) cells, +/- 5FU five days prior to cell injection, as diagrammed. **C, D)** Tumor weight, total tumor CD11b$^+$CSFE$^+$ cells, and CD11b$^+$CSFE$^+$ cells per mg of tumor (mean, SE; n=4 per group). * p<0.05; ** p<0.01.

tumors resulting from sq injection of PrCa cells 28 days earlier, followed by tumor isolation, dissociation into single cells and flow cytometry 24 hour later, as diagrammed (**Fig. 8A**). Representative tumor CD11b/CSFE flow cytometry shows increased tumor localization of p50-IMC bounds to PSMA Ab (**Fig. 8B**). In an initial experiment, we did not give the mice 5FU five days prior to intravenous p50-IMC injections. Mean tumor volumes, average total number of $CD11b^{+}CSFE^{+}$ cells that reached the tumors, and $CD11b^{+}CSFE^{+}$ cells per mg of tumor weight is shown (**Fig. 8C**). In a second experiment, 5FU was given five days prior to p50-IMC (**Fig. 8D**). In the absence of 5FU there was a trend towards increased tumor localization of labeled p50-IMC when bound to PSMA Ab; in the presence of 5FU (which reduces circulating myeloid cells and tumor myeloid cells) PSMA Ab significantly increased the total number of labeled p50-IMC and the number per mg of tumor weight that reached the PrCa tumors, compared with p50-IMC bound to control IgG. Also, comparing No 5FU and +5FU, total $CD11b^{+}CSFE^{+}$ cells (60 vs 160 E3) and $CD11b^{+}CSFE^{+}$ cells/mg tumor (115 vs 340) are each significantly higher with 5FU, in the presence of PSMA Ab. This work is included in our accepted SITC abstract.[8]

Task 3/Subtask 3– Adding CD47 Ab or SIRPa KO to p50-IMC with PSMA Ab (or CAR, Task 4/Subtask 3)

Commercially available CD47 Abs do not block murine CD47. We evaluated SIRPα gene-editing using a mix of sgRNAs designed by Synthego, finding complete KO in 23% of marrow cells (left-most green peak), as assessed by flow cytometry (**Fig. 9**). We will try to improve on this with sgRNAs that we design or will flow-sort the SIRPα negative population for efficacy studies.

Figure 9. Mouse lineage-negative marrow cells where gene-edited with three sgRNAs that target SIRPα, and SIRPα expression was assessed by flow cytometry.



Task4/Subtask 2 - Construct a PSMA.CAR in a viral vector

During Yr01 we constructed a cDNA encoding a PSMA.CAR using the available PSMA10.3 Ab sequence. 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 Ab10.3 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. 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. Finally, we verified PSMA.CAR expression in transduced p50-IMC.

During Yr02, we constructed a hEGFR.CAR using the scFv from Cetuximab (**Fig. 10A, top**) and confirmed its high-level expression in Jurkat T cells (**Fig. 10B, left**) and its ability to efficiently bind the extra-cellular domain of hEGFR linked to biotin after enrichment for CAR-expressing cells by cell sorting (**Fig. 10B, right**). Our initial hEGFR.CAR construct was made using a murine CD3ζ cytoplasmic domain carrying point mutations that favor T cell proliferation. We then made a version with the hCD3ζ domain known to be capable of mediating phagocytosis, confirmed its expression in Jurkat cells and ability to bind EGFR-biotin, using an unsorted pool of transduced cells (**Fig. 10C**), and demonstrated its high-level expression in transduced murine bone marrow cells, with empty MIPuro retroviral vector-transduced cells serving as an negative control (**Fig. 10D**).

During Yr01, we also confirmed that a clinically validated GD2.CAR[9] is expressed well in Jurkat cells and in transduced murine bone marrow cells (not shown).

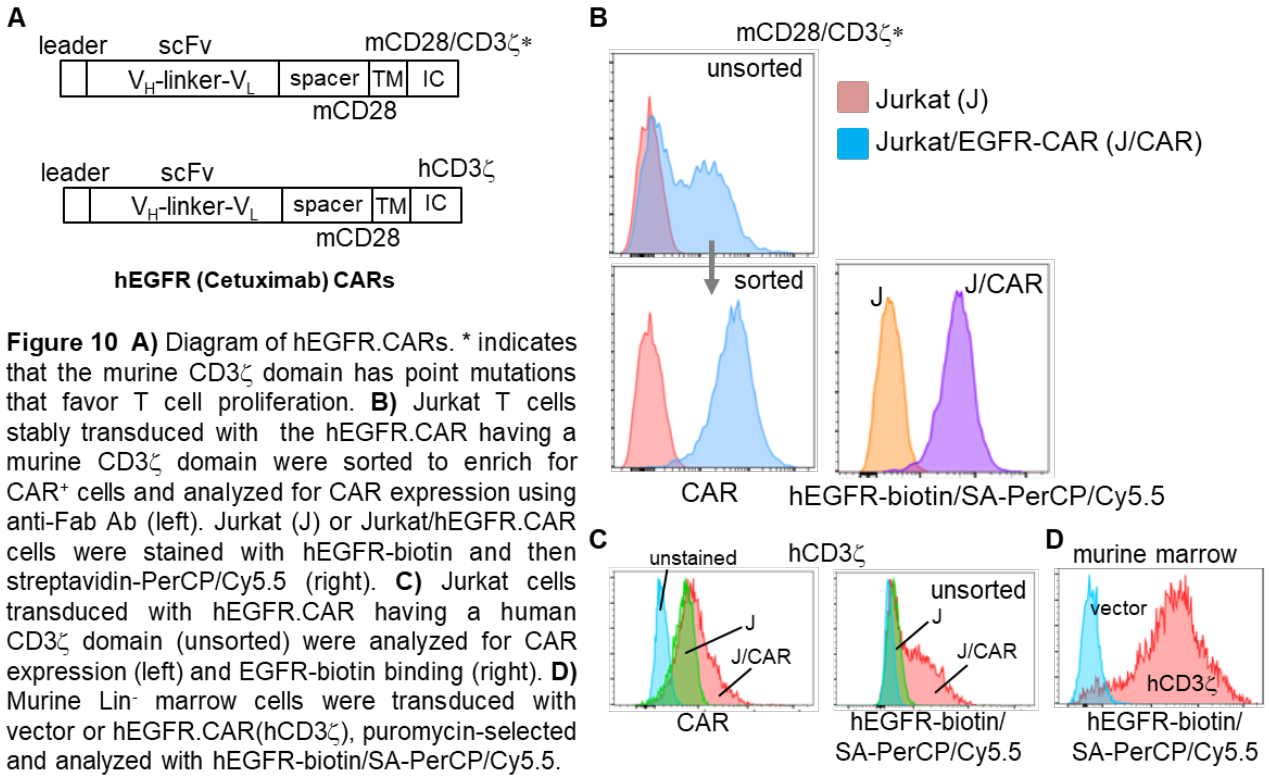


Figure 10 A) Diagram of hEGFR.CARs. * indicates that the murine CD3ζ domain has point mutations that favor T cell proliferation. **B)** Jurkat T cells stably transduced with the hEGFR.CAR having a murine CD3ζ domain were sorted to enrich for CAR+ cells and analyzed for CAR expression using anti-Fab Ab (left). Jurkat (J) or Jurkat/hEGFR.CAR cells were stained with hEGFR-biotin and then streptavidin-PerCP/Cy5.5 (right). **C)** Jurkat cells transduced with hEGFR.CAR having a human CD3ζ domain (unsorted) were analyzed for CAR expression (left) and EGFR-biotin binding (right). **D)** Murine Lin- marrow cells were transduced with vector or hEGFR.CAR(hCD3ζ), puromycin-selected and analyzed with hEGFR-biotin/SA-PerCP/Cy5.5.

Task 4/Subtask 3 and Subtask 4 - Murine PrCa tumor growth combining PSMA CAR and p50-IMC, p50KO-IMC

Data presented above in the section entitled "Task 1/Subtask 3 and Subtask 4," Figures 1-4, are relevant to these subtasks as well. In particular, these data show the following:

- hPSMA is not tolerated by immune-competent B6 or FVB/N mice
- Wild-type hPSMA RNA is expressed in the prostate of AR_2Pbn-hPSMA transgenic mice, but hPSMA protein is not detected, suggesting toxicity.
- We have developed a hPSMA variant mutant in two critical domains (the cytoplasmic signaling domain and the extra-cellular active site that cleaves glutamate from polypeptides), and demonstrated its cell surface expression, with the intent of now generating a mouse expressing this variant to allow tolerance of MC/hPSMA cells for p50-IMC anti-tumor efficacy studies +/- PSMA Ab or +/- PSMA.CAR
- Development of CMV-hEGFRt transgenic B6 mice, with hEGFRt expression and germline transmission, with the intent to verify tolerance of TC1/hEGFR or TC1/hEGFRt cells and then conduct p50-IMC efficacy studies +/- hEGFR Ab or +/- hEGFR.CAR
- Development of TC1/GD2S cells that express GD2 in 60% of the cells (compared with 9% of TC1 parental cells) that we can now use for p50-IMC efficacy studies +/- GD2 Ab or +/- GD2.CAR in B6 mice.

Task4/Subtask 5 – Effects of PSMA.CAR on PrCa phagocytosis, T cell activation, and p50-IMC tumor localization

A diagram of PSMA.CAR expressed in a macrophage is shown (**Fig. 11A**). Macrophages developed from marrow cells transduced with PSMA.CAR or vector were incubated with CFSE-dye labeled MC/PSMA(NΔ9) cells for 3 hours, followed by flow cytometry for CD11b (which detects the macrophages) and CFSE (which detect the PrCa cells), as diagrammed (**Fig. 11B**). CAR expression in WT or $p50^{-/-}$ marrow cells after puromycin selection is shown (**Fig. 11C**). Representative flow cytometry images are shown (**Fig. 11D**), and the results of three independent experiments are provided (**Fig. 11E**). To confirm that the macrophages have internalized the cancer cells, we labelled the macrophages with the CFSE dye (green) and the MC/PSMA(NΔ9) cells with pHRodo Red dye. Red cancer cells were only seen within green macrophages, confirming that phagocytosis has occurred (**Fig. 11F**). This work is included in our accepted SITC abstract.[8] In addition, with OT-I mice and MC/PSMA(NΔ9)/OVA cells in hand (Fig. 7), we can now determine whether PSMA.CAR-mediated PrCa phagocytosis leads to increased T cell activation *in vitro*.

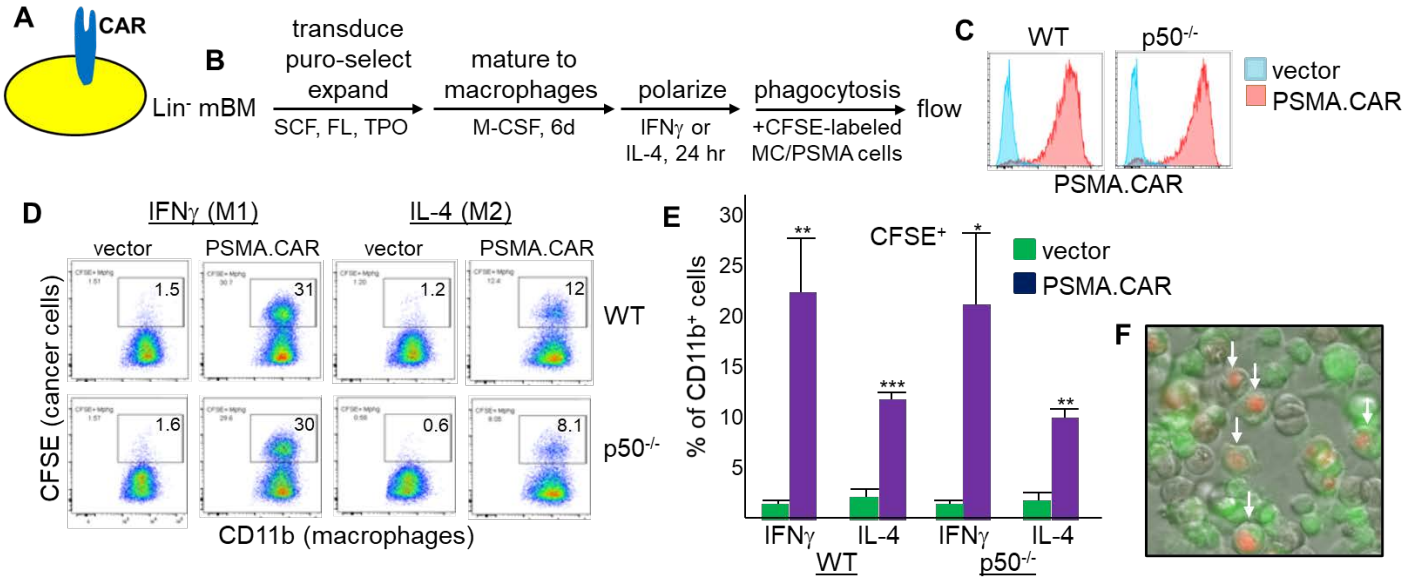


Figure 11. A) CAR expressed in a macrophage. **B)** WT or p50$^{-/-}$ murine bone marrow (mBM) cells were expanded and transduced with vector or PSMA.CAR, differentiated to macrophages, M1 or M2 polarized, and mixed with CFSE-labeled MC/PSMA(NΔ9), as diagrammed. **C)** Flow cytometry for PSMA.CAR after puromycin-selection. **D)** Representative flow cytometry showing phagocytosis by vector versus CAR-expressing macrophages. **E)** Results of three experiments evaluating CFSE$^+$ cells as a percentage of CD11b$^+$ macrophages. * p<.05, ** p<.01, *** p<.001. **F)** CFSE-labeled macrophages expressing PSMA.CAR (green) were combined with pHRodo-Red-labeled MC/PSMA((NΔ9) cells, followed by microscopy (bright field with the red and green channels). Phagocytosed cancer cells are indicated by white arrows.

We next sought to determine whether PSMA.CAR increases p50-IMC localization to MC/PSMA(NΔ9) tumors in NSG mice. p50-IMC transduced with vector or PSMA.CAR were CFSE-labeled and injected into NSG mice bearing tumors resulting from sq injection of PrCa cells 28 days earlier, followed by tumor isolation, dissociation into single cells and flow cytometry 24 hour later, as diagrammed (**Fig. 12A**). In an initial experiment, we did not give the mice 5FU five days prior to intravenous p50-IMC injections. Mean tumor volumes, average total number of CD11b$^+$CSFE$^+$ cells that reached the tumors, and CD11b$^+$CSFE$^+$ cells per mg of tumor weight is shown (**Fig. 12B**). PSMA.CAR did not increase p50-IMC tumor localization. As 5FU increased the ability of PSMA Ab to mediate p50-IMC tumor localization, we will include 5FU in our next experiment with PSMA.CAR.

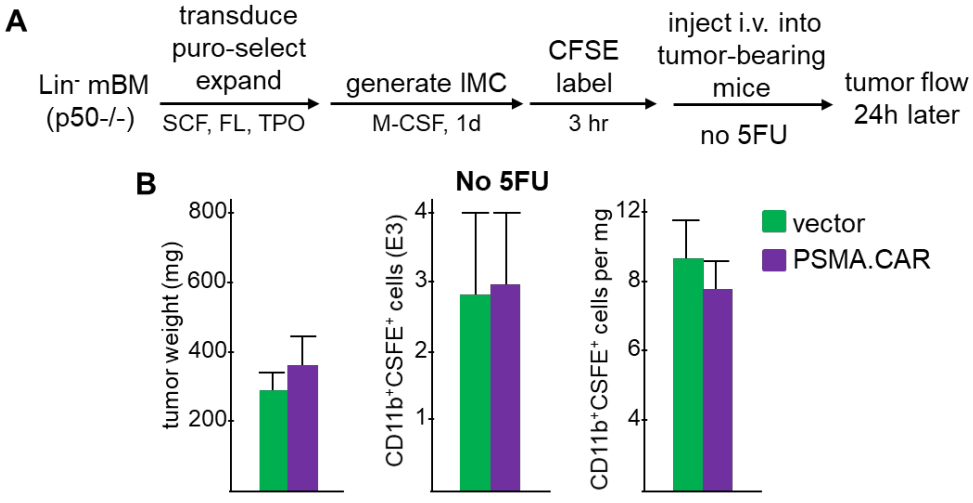


Figure 12. A) Lineage-negative p50-/- murine bone marrow cells were expanded, transduced with vector or PSMA.CAR, cultured with M-CSF to obtain p50-IMC, CFSE-labeled, and injected into NSG mice bearing subcutaneous tumors derived from MyC/CaP-hPSMA(NΔ9) cells, as diagrammed. **B)** Tumor weight, total tumor CD11b$^+$CSFE$^+$ cells, and CD11b$^+$CSFE$^+$ cells per mg of tumor (mean, SE; n=3 per group).

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

We find that human LNCaP cells express high levels of hPSMA and readily form tumors in NSG mice, and as described above we have obtained a large supply of anti-human PSMA3.9 Ab from a hybridoma, which has a murine Fc domain. As the murine Fc Ab domain has some affinity for the human FcR, we can now determine efficacy of combining human p50-IMC with Ab 3.9 against LNCaP tumor in NSG mice. We would have preferred to used humanized PSMA Ab10.3, but as noted above Bio-X-Cell failed to obtain a sufficient amount when we sent them the corresponding hybridoma.

Importantly, during the past year we extensively characterized gene expression by macrophages derived from human p50-IMC obtained by gene-editing, finding markedly increased expression of three proinflammatory M1 cytokines, IL-12β, IL-1β, and TNFα, extending findings we previously obtained for murine tumor macrophages lacking NF-κB and validating the clinical relevance of these experiments.[10] In addition, we found that four days of culture in M-CSF is required to obtain human p50-IMC equivalent to those obtained by the one day of culture in M-CSF we have used to obtain murine p50-IMC with anti-tumor efficacy.[10]

Task 5/Subtask 4 - Adding CD47 Ab or SIRPa KO to human p50-IMC with PSMA Ab (or CAR, Task 6/Subtask 3)

We evaluated SIRPα gene-editing using a mix of sgRNAs designed by Synthego, finding that sgABC or sgAB optimally reduced SIRPα expression (**Fig. 13**). We will now combine this with p50KO for proposed studies.

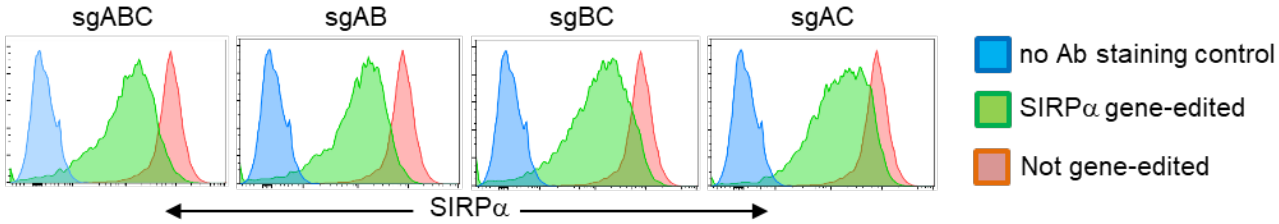
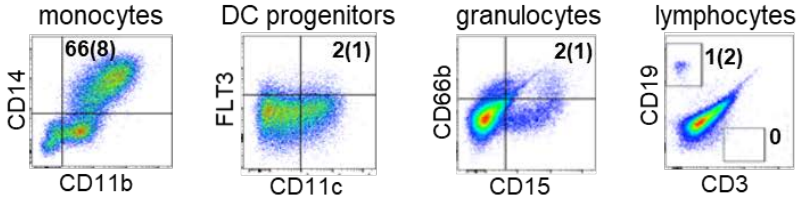


Figure 13. Human marrow CD34⁺ stem/progenitor cells were gene-edited with a combination of three sgRNA or three different combinations of two sgRNAs targeting the SIRPα gene, followed by flow cytometry for SIRPα.

Task 5/Subtask 5 – Fate of human p50-IMC with PSMA Ab (with PSMA.CAR, Task 6/Subtask 5)

During Yr01, we found that human p50-IMC do not mature in NSG mice. During Yr02, we therefore evaluated their *in vitro* maturation in M-CSF, finding that predominantly generate $CD11b^+CD14^+$ macrophages, with few $FLT3^+CD11b^+$ DC precursors, $CD15^+CD66b^+$ granulocytes, $CD19^+$ B cells, or $CD3^+$ T cells (**Fig. 14**).[10]

Figure 14. Human marrow CD34⁺ cells were subjected to p50 gene-editing, expanded, cultured in M-CSF for five days and then evaluated for the presence of monocytes, granulocytes, and B and T lymphocytes by flow cytometry. Data representative of three experiments is shown.



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

During Yr01 we found that retroviral transduction of human marrow cells with PSMA.CAR was inefficient. We generated a lentivirus (LV) expressing PSMA.CAR and compared several transduction procedures using LV-PSMA.CAR, finding that the combination of Polybrene, PGE2, and LentiBoost during spinoculation led to the highest proportion of $CD34^+$ cells expressing PSMA.CAR. We will now optimize viral titer by using more plates of 293T cells for packaging, concentration with Amicon filters, and avoidance of phenol red in our media which can be toxic to the lentivirus (as advised by an expert on LV packaging) to pursue proposed experiments.

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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 two post-doctoral fellows, Mohammad Alzubi PhD and Rahila Kuhroo, PhD. 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. Dr. Alzubi will attend the upcoming SITC meeting, in November, 2023.

Dissemination of research results

Two posters describing a portion of the above unpublished work have been accepted for presentation at the Annual Meeting of the Society for Immunotherapy of Cancer (SITC), to be held November, 2023 in San Diego:

Alzubi MA, Barberi T, Friedman AD. Impact of PSMA antibody or chimeric antigen receptor on phagocytosis and tumor localization by wild-type and NF-κB p50-deficient macrophages. Abstract #404.

Barberi T, Friedman AD. Human macrophages lacking NF-κB p50 display increased proinflammatory cytokine expression. Abstract #344.

We anticipating expanding on the work presented and submitted two manuscripts in the coming months.

Plans during the next reporting period

Task 1 We will determine whether GD2 Ab increases efficacy of p50-IMC or p50KO-IMC against tumors arising in syngeneic B6 mice from TC1/GD2S PrCa cells that express increased GD2. We will determine whether our transgenic hEGFRt B6 mice tolerate TC1/hEGFR or TC1/hEGFRt cells and if so determine whether EGFR Ab increases efficacy of p50-IMC or p50KO-IMC against these tumors. We will generate transgenic AR_2Pbn-hPSMA(NΔ9/H553G) FVB/N mice and determine whether the doubly-mutated hPSMA is expressed as a protein in the prostate and whether these mice tolerate MC/hPSMA or MC/hPSMA(NΔ9/H553G) cells, to allow us to then determine whether PSMA Ab increases efficacy of p50-IMC or p50KO-IMC against these tumors.

Task 2 We will determine whether PSMA Ab-mediated phagocytosis of MC/hPSMA(NΔ9)/OVA cells leads to stimulation of T cell proliferation and IFNγ production, utilizing splenic $CD8^+$ T cells from OT-I mice. We will repeat our experiment showing that PSMA Ab increases localization of p50-IMC to MC/hPSMA(NΔ9) tumor in NSG mice, when the mice receive 5FU five days prior to IMC injection.

Task 3 We will determine whether PD-1 Ab or SIRPα KO increases efficacy of GD2 Ab combined with p50-IMC or p50KO-IMC against murine TC1/GD2S prostate cancer in immune-competent B6 mice. SIRPα KO cells will be flow sorted prior to injection if gene-editing is inefficient. If mice tolerant to MC/hPSMA or MC/hEGFR cells are obtained, we will conduct related experiments in these models using PSMA Ab or EGFR Ab.

Task 4 We will determine whether GD2.CAR increases efficacy of p50-IMC or p50KO-IMC against tumors arising in syngeneic B6 mice from TC1/GD2S PrCa cells that express increased GD2, alone or with PD-1 Ab or SIRPα KO. We will determine whether our transgenic hEGFRt B6 mice tolerate TC1/hEGFR or TC1/hEGFRt cells and if so determine whether EGFR.CAR increases efficacy of p50-IMC or p50KO-IMC against these tumors. We will generate transgenic AR_2Pbn-hPSMA(NΔ9/H553G) FVB/N mice and determine whether the doubly-mutated hPSMA is expressed as a protein in the prostate and whether these mice tolerate MC/hPSMA or MC/hPSMA(NΔ9/H553G) cells, to allow us to then determine whether PSMA.CAR increases efficacy of p50-IMC or p50KO-IMC against these tumors, alone or with PD-1 Ab or SIRPα KO.

Task 5 We will determine whether PSMA Ab 3.9 increases efficacy of human p50KO-IMC against tumors derived from human LNCaP prostate cancer cells in immune-deficient NSGS mice, alone or with SIPRα or KO or CD47 Ab. We will determine whether PSMA Ab increases p50KO-IMC tumor localization to LNCaP tumors, with IMC injections preceded by 5FU.

Task 6 We will determine whether PSMA.CAR increases efficacy of human p50KO-IMC against tumors derived from human LNCaP prostate cancer cells in immune-deficient NSGS mice, alone or with SIPRα or KO or CD47 Ab. We will determine whether PSMA.CAR increases p50KO-IMC tumor localization to LNCaP tumors, with IMC injections preceded by 5FU.

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 mouse models of aggressive prostate cancer. Prostate cancer cells also express additional molecules, such as EGFR and 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 bound to PSMA antibody or expressing a PSMA-targeting CAR uptake prostate cancer cells much more effectively and localize to prostate cancer tumors in mice more effectively than do control cells. This result, and additional findings we anticipate obtaining, has implications for the treatment of prostate cancer. In particular, 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

Based on our experimental progress and our plan to publicly disclose data in Figures 5, 8, 11, and 12 at the 11/2023 SITC meeting, on 4/28/2023 I made an invention disclosure to the Johns Hopkins Technology Ventures (JHTV) office entitled "PSMA Chimeric Antigen Receptor-Modified p50-IMC." JHU Reference number C17876. After review, on 7/10/2023 JHTV informed me that they will proceed with a patent application for this invention, and I recently met with a lawyer they enlisted for this purpose. JHTV also assured me that they would inform DOD of this invention disclosure through the appropriate mechanism. We intend to strengthen data including in this provisional patent application with additional results obtained during the coming year.

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 by immune-competent mice we are conducting Task 1-4 experiments using GD2 Ab or GD2.CAR and hEGFR Ab or hEGFR.CAR. 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, to be used also for Tasks 1-4, we are developing mice expressing a mutant version of hPSMA in the prostate using the AR_2-Probasin promoter used previously to develop Hi-Myc mice (after finding that expression of wild-type PSMA in this manner leads to expression of hPSMA RNA but not protein in the prostate).

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

Alzubi MA, Barberi T, Friedman AD. Impact of PSMA antibody or chimeric antigen receptor on phagocytosis and tumor localization by wild-type and NF-κB p50-deficient macrophages. Abstract #404, 2023 SITC Annual Meeting (November, 2023).

Barberi T, Friedman AD. Human macrophages lacking NF-κB p50 display increased proinflammatory cytokine expression. Abstract #344, accepted for presentation at the 2023 Society for Immunotherapy of Cancer (SITC) annual meeting.

Websites or other internet sites

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patents, or licenses

On 4/28/2023 I made an invention disclosure to the Johns Hopkins Technology Ventures (JHTV) office entitled "PSMA Chimeric Antigen Receptor-Modified p50-IMC." The Johns Hopkins University reference number is C17876. JHTV has expressed their intent to proceed with patenting this invention.

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:	4
Contribution to Project:	Experimental supervision, CAR construction and retroviral packaging, generated cell lines expressing hPSMA(NΔ9), GD2S, and hEGFR, generated and bred hPSMA and hEGFRt transgenic mice.
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:	Theresa Barberi
Project Role:	Research Associate
ORCID ID:	0000-0002-7816-1969
Person months worked:	12
Contribution to Project:	Ab- and CAR-mediated phagocytosis and tumor localization, SIRPα gene-editing, evaluation of fate of human p50-IMC
Funding Support:	this award

Name: Mohammad Alzubi
Project Role: Post-Doctoral Fellow
ORCID ID: 0000-0002-3953-8436
Person months worked: 4
Contribution to Project: Ab- and CAR-mediated phagocytosis and tumor localization
Funding Support: T32 CA060441 (salary support); supplies provided by 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)
None.

new (grant number/funding agency, PI, dates, % effort)
2023-MSCRFD-6101, Maryland Stem Cell Research Fund, Friedman, 6/30/23 - 6/29/24, 0.9 Cal

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

Name: Martin G. Pomper
Changes: closed (grant number/funding agency)
P41 EB024495, NIH/NIBIB
No number, American Heart Association
R01NS104283, NIH
210PA35310888, American Heart Association
R01 AG066464, NIH
R33 AG054802, NIH
W81XWH-21-1-0920, CDMRP
No number, NexImmune
No number, Precision Molecular, Inc.
R21 CA267374, NIH/NCI

new (grant number/funding agency, PI, dates, % effort)
W81XWH2210130, CDMRP, Maragakis, 10/01/23-9/30/24, 0.24 Cal

Dr. Pomper's 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.