Modulation Of Anti-Tumor T Cell Responses By CD3 Redirection Therapies Targeting Prostate Cancer

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MODULATION OF ANTI-TUMOR T CELL RESPONSES

BY CD3 REDIRECTION THERAPIES TARGETING PROSTATE CANCER

Abstract

By

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PSMA is overexpressed on prostate cancer with increasing levels corresponding to severity of disease making it an ideal target for CD3 redirection bispecific antibody therapy targeting metastatic castrate resistant prostate cancer. PSMAxCD3 antibodies are currently being evaluated clinically and despite promising preclinical antitumor responses, minimal clinical response has yet been observed. As evidenced by lack of response to T cell checkpoint blockade therapy in the clinic, solid tumors with suppressive tumor microenvironment including low immune cell infiltration, mutational burden, and PD-L1 levels, such as prostate cancer will likely prove challenging to treat with CD3 redirector therapies. The work outlined here utilizes two mouse preclinical models that may be more clinically translatable with possible immune suppressive TME in the context of a complete immune system to evaluate the effects of PSMAxCD3 therapy: human xenografts in human CD34+ engrafted immune incompetent mice and immune “hot” CT26 and “cold” TRAMP.C2 tumors in immune competent transgenic mice expressing human CD3e. PSMAxCD3 treatment resulted in antitumor efficacy correlating with infiltration of T cells demonstrating an activated, effector memory CD8+ phenotype in both model systems. Our results demonstrated that overexpression of PD-

L1 on tumor cells conferred resistance to PSMAxCD3 therapy with minimal T cell infiltration that was overcome by combination with PD-1 blockade therapy. Combination treatment resulted in enhanced infiltration of T cells, with complete responses in some tumors whereby T cell responses protected against disease rechallenge. Additionally, we demonstrated that PSMAxCD3 treatment elicited antitumor responses against an immunologically "hot" tumor model, while minimal responses were observed against an immunologically "cold" tumor. PSMAxCD3 treatment resulted in minimal intratumor T cell infiltration in TRAMP.C2 tumors and combination with CBI therapy did not significantly enhance tumor growth control, despite expansion of CD8+ T cell effector memory cells. These results suggest solid tumors with suppressive TME may require further combination strategies that improve T cell trafficking to tumors. Taken together, the findings here demonstrate that PSMAxCD3 therapy elicits activated, effector memory T cell responses but combination strategies will most likely be required for improved T cell trafficking and durable T cell responses in the clinic.

WORKING MODEL

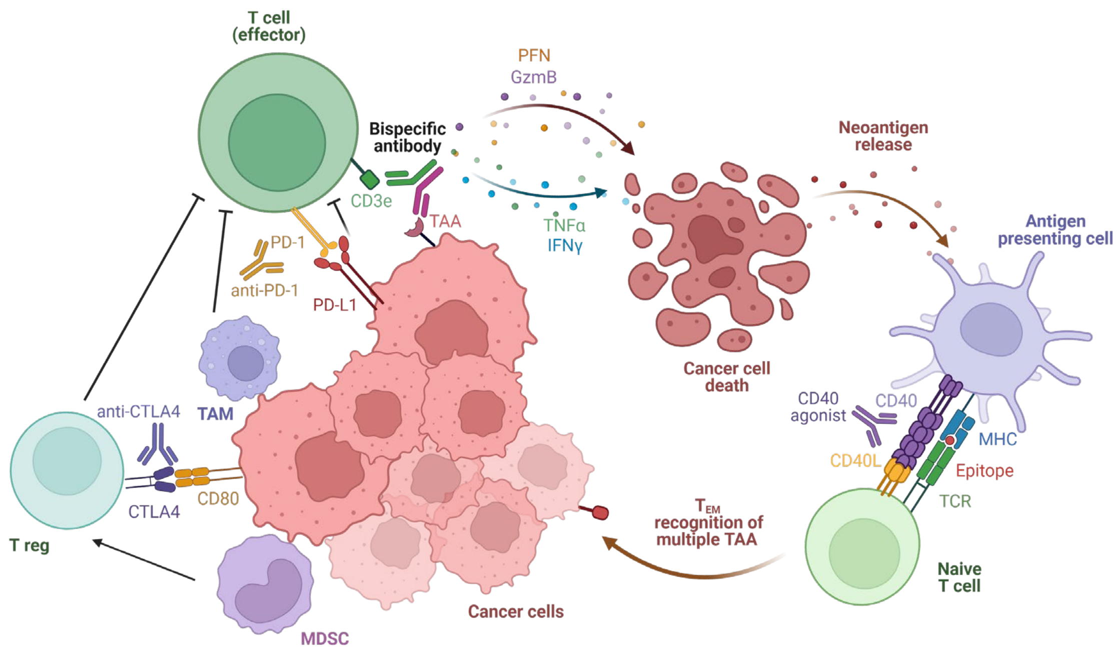

Figure 1. Working model

CD3 redirection bispecific antibodies binding tumor associated antigens on cancer cells and CD3 on recruited T cells to elicit cancer cell cytotoxicity and durable antitumor immunity.

CD3 redirection bispecific antibodies recruit and activate T cells independent of T cell receptor specificity. Activated CD8+ T cells release perforin and granzyme B as well as IFNγ and TNFα cytokines, resulting in target cancer cell killing and release of cancer neoantigens. When antigen presenting cells present neoantigen epitopes to naïve T cells, epitope spreading may occur, ultimately leading to enhancement of the T cell effector memory subset, and T_{EM} recognition and targeting of TAA+/- cancer cells to elicit durable antitumor immunity.

Resistance mechanisms observed clinically with T cell redirection therapies. Resistance mechanisms include a lack of tumor infiltrating T cells, low baseline level/

upregulation of PD-L1 on tumors in response to treatment, and increased tumor-infiltrating Tregs. In addition to Tregs, T cell activation may also be inhibited by other suppressive immune cell types such as MDSCs and TAMs in the TME.

Combination therapies may address an immune-suppressive TME and increase the T_{EM} population for durable antitumor immunity. Checkpoint blockade inhibition can elicit T cell effector memory phenotypes through epitope spreading and cancer neoantigen-specific TCR priming. Combination with PD-1 blockade therapy overcomes T cell suppression elicited by PD-L1 upregulation on tumor cells, anti-CTLA4 therapy depletes Tregs, and CD40 agonistic antibody therapy enhances antigen presentation and T cell effector function. In prostate cancer, multiple combination approaches may be required for achieving optimal antitumor immunity.

CD3e, Cluster of differentiation 3 epsilon; CD40(L), Cluster of differentiation 40 (ligand); CD80, Cluster of differentiation 3; CTLA4, Cytotoxic T-lymphocyte-associated protein 4; IFNγ, interferon gamma; IL2, interleukin 2; GzmB, granzyme B; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; PFN, perforin; PSMA, prostate-specific membrane antigen; TAM, tumor associated macrophage; TAA, tumor associated antigen; TCR, T cell receptor; T_{EM}, effector memory T cell; TNFα, Tumor necrosis factor alpha.. Created with BioRender.com

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This is to Certify that the Dissertation Prepared by

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Titled

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BY CD3 REDIRECTION THERAPIES TARGETING PROSTATE CANCER

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DEDICATION

I dedicate this thesis to my loving husband, Justin; my children, Joel and Ander; and my late brother-in-law, Mark.

Justin, thank you for your support and encouragement and for the sacrifice that you were willing to make with me to achieve this milestone. I couldn’t have accomplished this without your love, patience, and continual support.

Joel and Ander, you have been on this adventure with me from the womb through your first few years. Hopefully you will have witnessed and learned that determination and hard work can lead to great reward and big accomplishments which doesn’t have to be at the sacrifice of family and personal life. With hard work and lots of support, you too can try to change the world. I’m excited to use my extra time on fun adventures with you both.

Mark, in so many ways you were a rock in our family, and it broke our hearts to see you pass away from cancer so quickly and with such a young family. I hope that my efforts to research how to make immune-Oncology therapeutics will honor your memory and help patients like you to have more life-changing therapy options to give them more time to love and be loved by their families.

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Figure 28. Three avenues of research following these studies include further evaluating effects of PSMAxCD3 treatment on regulatory immune cells, exploring combinations with inhibitors of CXCR2 or CXCR4 to enhance T cell trafficking to the tumor, and exploring resistance mechanisms to treatment in the TRAMP.C2 syngeneic model using immune signatures. 109

LIST OF ABBREVIATIONS

4-1BB	activation-induced costimulatory molecule (CD137)
ABC	antibody bound per cell
AML	acute myeloid leukemia
ALL	acute lymphocytic leukemia
APC	antigen presenting cell
BCMA	B cell maturation antigen
BiTE	Bi-specific T-cell Engager
CAF	cancer associated fibroblast
CAR	chimeric antigen receptors
CBI	checkpoint blockade inhibitor
CEA	carcinoembryonic antigen
CD	cluster of differentiation
CDX	cell line-derived xenograft
CM	central memory
CR	complete response
CRS	cytokine release syndrome
CSF1R	colony stimulating factor 1 receptor
CTLA-4	cytotoxic T-lymphocyte associated protein-4
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DC	dendritic cell

DLBCL diffuse large B cell lymphoma

DPBS Dulbecco's phosphate-buffered saline

EM effector memory

EpCAM epithelial cell adhesion molecule

Fab fragment antigen-binding

Fc fragment crystallizable

GM-CSF granulocyte-macrophage colony stimulating factor

GPRC5D G protein–coupled receptor, class C, group 5, member D

GvHD graft versus host disease

Ig immunoglobulin

IHC immunohistochemistry

IL interleukin

IFNy interferon gamma

KI knock-in

LAG-3 lymphocyte activation gene 3

mCRPC metastatic castrate resistant prostate cancer

MDSC myeloid-derived suppressor cells

MHC Major histocompatibility complex

MM multiple myeloma

N naive

NK natural killer

NOD non-obese diabetic

NSG NOD *scid* gamma or NOD.*Cg-Prkdc*^{scid} *IL2rg*^{tmlWjl}/SzJ gamma

PD	pharmacodynamic
PD-1	programmed death receptor 1
PD-L1	programmed death ligand 1
PDX	patient-derived xenograft
PK	pharmacokinetic
PSMA	prostate specific membrane antigen
RPMI	Roswell Park Memorial Institute
RT	room temperature
ORR	overall response rate
SCF	stem cell factor
scFv	single-chain fragment variable
scid	severe combined immunodeficiency
TAA	tumor associated antigen
TAM	tumor associated macrophage
TCR	T cell receptor
TEMRA	terminally differentiated
TGFβ	transforming growth factor beta
TGI	tumor growth inhibition
TIM-3	T cell immunoglobulin and mucin domain-containing protein-3
TME	tumor microenvironment
TNFa	tumor necrosis factor alpha
Treg	regulatory T cell

CHAPTER 1: INTRODUCTION

1.1. T CELL-DIRECTED IMMUNOTHERAPIES

Over the past decade, immunotherapies have emerged as a new pillar in the standard of care for cancer treatment. Recent successes have been achieved in the clinic with immunological targeting of cancers by mobilizing patients' T cells, with different approaches each providing unique opportunities and challenges. These approaches include T cell checkpoint blockade, autologous T cell therapy, and CD3 redirector antibody therapy.

T cell checkpoint blockade inhibitors (CBI) such as anti-programmed death receptor-1 (PD-1), anti- programmed death receptor ligand-1 (PD-L1) and anti-cytotoxic T-lymphocyte associated protein-4 (CTLA-4) antibodies promote anti-tumor T cell responses by reducing T cell suppression pathways, thereby activating an antitumor immune response.[24,26,66,74] CBIs have demonstrated clear survival benefits in melanoma and non–small cell lung cancers, amongst others, but tend to be most successful in patient populations where tumors have a high mutational burden and are enriched with T cell infiltrates (i.e. immunologically "hot").[25,39,48,57]

Another immunological targeting approach, autologous cell therapy, utilizes patients' own T cells that are genetically engineered ex-vivo with chimeric antigen receptors (CAR) to target a tumor associated target antigen (TAA). CAR Ts have become a standard therapy for aggressive lymphomas and acute lymphoblastic leukemia, and are currently being explored clinically in solid tumors.[52,68,76] However, a drawback is that CAR T generation takes approximately 2-4 weeks before they can be administered.

One of the newest immunotherapeutic approaches is CD3 redirection, whereby bispecific antibodies targeting cluster of differentiation 3 (CD3) on T cells and a TAA on tumor cells elicit cytotoxicity independent of T cell receptor (TCR) specificity. Low levels of tumor neoantigens (i.e. poor antigenicity) or a lack of antigen-presenting cells such as dendritic cells have been shown to limit CBI efficacy, and thus the ability of bispecific CD3 engagement to enable T cell activation in the absence of antigen presentation could potentially overcome these challenges. Such therapeutics have shown great potential to effectively target cancers with patient's innate T cells in a more direct and specific manner than CBIs and offer a more universal and immediate (i.e. off-the-shelf) treatment as compared to autologous cell therapies. Notably, Teclistamab (targeting BCMA) and Talquetamab (targeting GPRC5D) have shown unprecedented overall response rates (ORR) in relapsed/refractory (R/R) multiple myeloma in Phase 2 clinical trials with 63% and 74% ORR respectively.[20,49]

1.2. CD3 REDIRECTION

Activation and cytotoxicity by T cells normally occur after primary signal binding through the TCR and major histocompatibility complex (MHC), which determines signal specificity. Then secondary signal binding through a co-stimulatory domain determines the magnitude of response.[24] Binding of CD3 redirection antibodies to a TAA on cancer cells and CD3 in the TCR on T cells also recruits T cells to kill cancer cells, but not through MHC recognition.[7,17,24] T cell activation results in the differentiation of naïve T cells into CD8+ cytotoxic T cells as well as proliferation through the release of cytokines such as tumor necrosis factor alpha (TNFα), interferon gamma (IFNy), interleukin-2 (IL-2) and others.[72] Target cell cytotoxicity is achieved through T cell release of perforins

which form pores in the membranes of target cells by which granzymes released from T cells gain access to the target cell, triggering apoptosis through caspase pathway induction (Figure 2).[24,72] A strong TCR primary signal has been shown to elicit T cell activation independent from co-stimulatory signaling thus explaining how CD3 bispecific antibodies achieve target killing in the absence of co-stimulatory binding.[72]

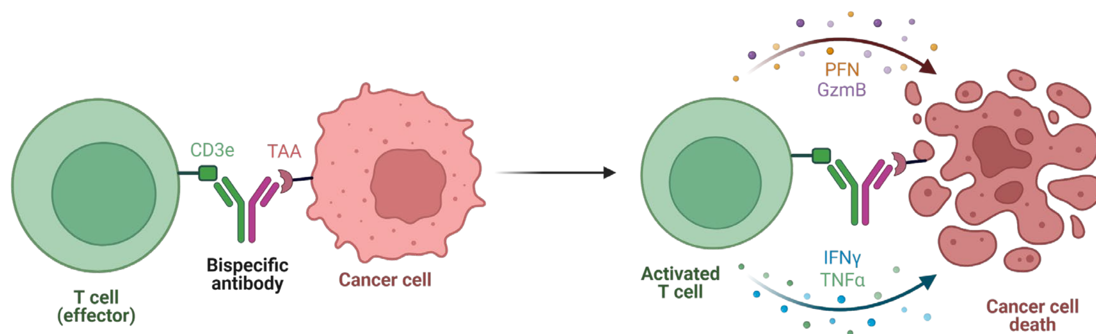

Figure 2. Mechanism of antitumor effects of CD3 redirection bispecific antibodies.
CD3e, Cluster of differentiation 3 epsilon; IFNγ, interferon gamma; GzmB, granzyme B; PFN, perforin; TAA, tumor associated antigen; TNFα, Tumor necrosis factor alpha. Created with BioRender.com
CD3 redirection bispecific antibodies bind tumor associated antigens on cancer cells and CD3 on recruited T cells to elicit cancer cell cytotoxicity independent of T cell receptor specificity. Cancer cell cytotoxicity occurs through perforin and granzyme B release resulting in apoptosis through caspase pathway induction. CD3 binding of bispecific antibody also results in release of T cell activating IFNγ and TNFα cytokines.

Technical advances in antibody engineering have made dual-targeting possible, with different antibody formats explored including Bi-specific T-cell Engager (BiTE) and bispecific antibodies (bispecifics).[36] Some bispecifics have utilized fragments of antibodies such as single-chain variable fragments (scFv) attached through a linker without the fragment crystallizable (Fc) region on an antibody, while others have utilized two different antigen-binding fragment (Fab) arms on a single immunoglobulin (Ig) antibody.[36,63] The former approach has been utilized by BiTEs and dual-affinity re-targeting therapies which have a lower molecular weight and thus a shorter serum half-life, while DuoBody and knob in hole use the latter approach and have a longer half-

life.[33,36,63] Additionally, these antibodies are engineered with an Fc silencing mutation to prevent Fc-mediated clearance of the effector T cells (via interaction with Fcγ receptors on phagocytic cells), and to ensure that cross-linking-driven activation of T cells only occurs when TAA is present.[7,33,36]

Selection of the TAA is a critical part of therapeutics design, as specificity between cancerous and normal tissue must be achieved to have a therapeutic window where efficacy is maximized, and toxicity is manageable. Hematologic TAA targets such as CD19, CD20, and B cell maturation antigen (BCMA) have demonstrated promise in lymphoma and multiple myeloma settings due to the broad expression of the TAA in these cancers.[47] Additionally, CD3 bispecific treatment can be tolerated despite on-target, off-tumor expression of the TAAs on normal B and plasma cells. Temporary immune suppression occurs during treatment and eventually resolves with reconstitution of normal cells once treatment is withdrawn.[47] This has been more challenging in solid tumors where on-target, off-tumor targeting of normal cells can lead to more serious toxicities.[1] Thus TAAs must be more cancer-restricted or there must be significantly elevated levels in cancer compared to normal tissues to establish a therapeutic safety window.

1.3. CLINICAL TRIALS AND T CELL RESPONSES

CD3 redirection agents targeting either hematologic or solid tumor cancers are currently being pursued preclinically or clinically for multiple tumor types.[7,47,64,73] Catumaxomab was the first clinically approved T cell redirector targeting epithelial cell adhesion molecule (EpCAM) in gastric and ovarian cancers in 2009 but could only be given intraperitoneally to treat ascites due to toxicity observed with systemic

administration.[33,45] Strong binding of catumaxomab to CD3 in addition to an active Fc resulted in adverse events including cytokine release syndrome (CRS) in patients.[33,45] CRS results from the release of pro-inflammatory cytokines causing a cytokine storm wherein patients experience flu-like symptoms including fever, hypotension, hypoxia, and in extreme cases multi-organ failure which requires immune suppression treatment to mitigate symptoms as well as possible suspension of treatment.[40,47] CRS has been dose limiting to many clinically tested redirectors including catumaxomab. Catumaxomab was later removed from the market in 2013 for commercial reasons.[45]

The first clinical success with a CD3 redirector was observed with blinatumomab, an anti-CD19xCD3 BiTE, which was approved by the FDA for the treatment of R/R B-cell acute lymphocytic leukemia (ALL) in 2014.[45,47] The rate of complete remission was 40-50% in B-lineage ALL, 37% R/R lymphoma and 19% in R/R diffuse large B cell lymphoma (DLBCL).[22,67,69] The ORR was 69% for R/R lymphoma and 43% for DLBCL with durable responses in some patients without further treatment.[22,69] A range of severity of CRS was observed with blinatumomab as well as neurologic findings thought to be associated with increased cytokines in the central nervous system; however, these were dose-dependent, resolved with discontinuation of treatment, and stepwise intra-patient dose escalation was shown to prevent these adverse findings.[22,64,67,69] Blinatumomab became the first CD3 redirection bispecific to achieve clinical activity that could be given safely.

Although blinatumomab has shown efficacy in the clinic, only subsets of patients respond. Relapse after initial response occurs in approximately 20% of cases, suggesting the need to further enhance these therapies and understand mechanisms of resistance.[67]

When blinatumomab responder versus non-responder patients were compared, patients with the best responses had expansion of CD8+ T-cells, with an increase in effector memory subsets.[67] In contrast, poor responders had elevated recruitment of regulatory T cells that suppress T cell effector function.[67] Similarly, responder patients treated with a BCMA targeting CAR-T cell therapy also had an enriched effector memory cell population of T cells.[77] Thus, durable anti-tumor responses may be best achieved if memory T cell subsets are preferentially expanded. It has been shown that upon activation a naïve T cell will undergo differentiation into central memory, then effector memory, and finally terminally differentiated effector memory T cell phenotypes that correspond to up and down-regulation of cell surface antigens CD45RA, CD62L, CCR7 and CD44 (Figure 3).[21,46] As T cells differentiate, they lose self-renewal capacity and have a shorter life span but have increased cytotoxic potential (i.e. effector function).[21,46,58] Thus it is likely optimal for effector memory subtypes to be preferentially expanded after treatment as they have a greater self-renewal capacity and lifespan compared to terminally differentiated T cells, yet still maintain effector functionality.

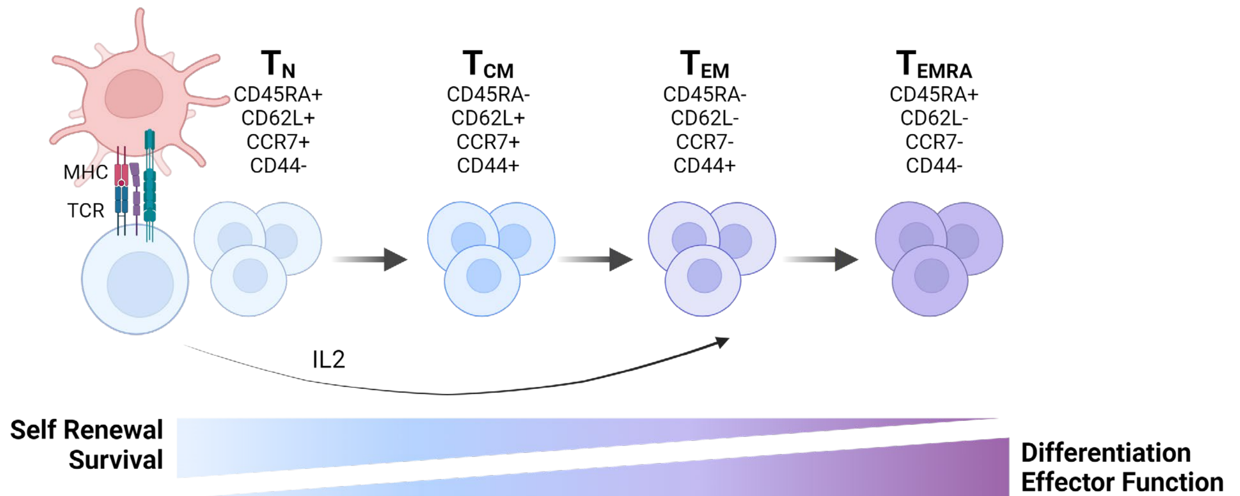

Figure 3. T cell Differentiation and Memory Phenotype After Activation Through TCR and MHC Binding.

IL2, interleukin 2; MHC, major histocompatibility complex; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{EMRA}, terminally differentiated effector memory T cell T cell; T_N, naïve T cell; TCR, T cell receptor. Created with BioRender.com

When naïve T cells are activated through MHC and TCR complex binding, they undergo differentiation into central memory, then effector memory, and finally terminally differentiated effector memory T cells. These T cell memory phenotypes can be characterized by modulation of cell surface antigens CD45RA, CD62L, CCR7 and CD44. As T cells differentiate, they lose self-renewal capacity and have a shorter life span but have increased cytotoxic effector function.

More recently teclistamab, a BCMA-targeting CD3 redirector was approved by the FDA for the treatment of R/R multiple myeloma (MM). Teclistamab treatment demonstrated an ORR of 63% with approximately 40% complete responders.[20] Additionally there are several CD3 bispecific antibodies in phase 3 clinical trials or that have received expedited breakthrough therapy designation including glofitamab, eporitamab, and mosunetuzumab targeting CD20 in lymphomas as well as flotetuzumab and APVO436 targeting CD123 in acute myeloid leukemia (AML) and TNB383B targeting BCMA in MM.[73] Although there are no approved CD3 redirection therapies for the treatment of solid tumor cancers, there are many ongoing clinical trials.[47]

Currently, CD3 redirection bispecifics are being evaluated in clinical trials in a range of solid tumor cancers targeting TAAs including carcinoembryonic antigen (CEA),

EpCAM, human epidermal growth factor 2, and prostate-specific membrane antigen (PSMA) among others.[47] Early reports from clinical trials with CD3 bispecifics targeting CEA or EpCAM showed limited efficacy (up to stable disease) but were hampered by dose-limited toxicities consisting of CRS, target tissue damage, and tumor lesion inflammation.[47] The most promising results have been observed in a trial with pasotuxizumab targeting PSMA in castrate-resistant prostate cancer, where two patients had long-term responses.[27,28] This clinical trial demonstrated CD3 targeting of PSMA positive (PSMA+) prostate cancer can offer a therapeutic advantage; however, anti-drug antibodies were observed with subcutaneous (SC) dosing and continuous intravenous infusion can be burdensome to patients over long periods of treatment.[28] These clinical trial results offer some promise for targeting solid tumors with CD3 redirectors; however less efficacy has been seen compared to hematologic cancers to date most likely due to a combination of immune-suppressive tumor micro-environment and higher hurdle for therapeutics and immune cell infiltration into solid tumors.

1.4. CD3 TARGETING OF PSMA IN PROSTATE CANCER

Prostate cancer is the leading cause of cancer-related death in men and 30-40% of patients become hormone refractory and no longer respond to standard treatments.[3,4,65,70] PSMA is a transmembrane protein normally expressed on epithelial cells in prostate tissue that is overexpressed in prostate cancer.[3,4,70] PSMA is upregulated following androgen deprivation therapy.[75] Increasing PSMA expression correlates with increasing stage/grade of disease which makes it an ideal target for advanced castrate-resistant prostate cancer (CRPC).[3,70] Radioligand tracers that detect PSMA including the positron emission tomographic imaging tracer ^{68}Ga-PSMA-11 are used clinically to

detect PSMA+ prostate cancer cells for diagnostic purposes.[12,70] Additionally, there are radioligand molecules currently being evaluated for therapeutic PSMA-targeting including lutetium (^{177}Lu-PSMA) and actinium (^{225}Ac-PSMA-617) which have shown greater than 50% decreases in prostate-specific antigen (PSA) levels (a soluble tumor antigen indicative of relative tumor burden) in more than half of patients demonstrating proof of concept for therapeutic targeting of PSMA.[15,70] Although PSMA-targeting antibodies have proven useful in imaging and diagnostics, when labeled with beta-emitting radionuclides, anti-tumor responses were minimal and when used as an antibody-drug conjugate anti-tumor effects were limited by toxicity associated with the drug payload.[70] These data support the therapeutic targeting of PSMA and suggest that an alternative approach such as immunological T cell approaches may also achieve efficacy and avoid toxicity related to the drug conjugate or radiotherapy.

More recently, bispecific antibody approaches have been used to enhance anti-tumor T cell responses to tumors by targeting PSMA.[7] Preclinically, PSMA-targeting CD3 redirectors including pasotuxizumab and the half-life extended BiTE AMG 160 have demonstrated dose-dependent and human T cell-dependent cytotoxicity of PSMA+ prostate cancer cell lines such as LNCaP, 22Rv-1 and C4-2.[10,17,27] *In vitro* cytotoxicity was shown to correlate with T cell activation as indicated by increased T cell cytokine levels such as interleukin (IL) 2 (IL2), TNFα and IFNγ as well as increased membranous activation markers such as CD25, CD69, 4-1BB and PD-1.[17,27] Efficacy was also demonstrated in mouse models with PSMA+ human xenograft tumors co-injected with human T cells, where investigators observed prevention of tumor establishment.[17] Additionally, investigators observed efficacy against established

PSMA+ SC xenografts naturally expressing PSMA (22Rv-1) or engineered to overexpress PSMA (PC-3 with engineered PSMA expression) with adoptive transfer of human T cells.[17,27] Treatment demonstrated statistically significant inhibition of tumor growth with up to 91-99% tumor growth inhibition with complete responses (cures) observed in some animals.[17,27] Overall, anti-tumor efficacy correlated with relative PSMA levels.

In addition to the first clinically evaluated CD3 targeting BiTE pasotuxizumab, there are several ongoing clinical studies with additional PSMA-targeting CD3 redirectors in prostate cancer.[7,47] JNJ-63898081, a CD3 redirecting bispecific antibody that binds PSMA and has a longer serum half-life than the BiTE, has been evaluated clinically in metastatic CRPC (mCRPC) patients.[43] Overall, limited efficacy was observed in response to treatment with transient PSA decreases but not radiographic responses.[43] Investigators found that CRS could be successfully managed with step-up dosing, although dose-limiting toxicity was still observed at the highest doses tested.[43] Anti-drug antibodies were found in most of the patients receiving SC administration, resulting in loss of exposure that may have limited responses in some patients.[43] Although recent clinical experience suggests PSMA targeting CD3 redirectors may offer clinical benefit, responses have so far been suboptimal, and clinical trials have not progressed far enough to determine durability of the T cell responses elicited. A primary goal of the current dissertation research was to determine if durable T cell responses are elicited with PSMAxCD3 therapeutics in clinically relevant animal models, and if so, to evaluate if combination strategies could enhance these responses (see section 1.7).

1.5. RESISTANCE MECHANISMS AND COMBINATION RATIONALE

The success of CD3 redirectors in hematological cancers may in part be due to the location of disease in the bone marrow and lymph system being easily accessible by T cells. The treatment of solid tumors poses more challenges to T cell mediated treatments due to an immune suppressive tumor micro-environment and the need for T cell infiltration into a solid tumor mass. Solid tumors are often broadly characterized in terms of relative amount of immune cell infiltration, with terms used for high/ low immune infiltrate status such as "hot/cold", "immune inflamed/immune desert", and ranging from some infiltrates observed to a lack of immune cells entirely with stromal components blocking all infiltration (i.e. "immune excluded").[19,47] Clinical sensitivity to CBI therapies has been correlated with immune infiltrate in solid tumors, both at baseline and post-treatment, suggesting that CD3 redirectors will require T cell trafficking to the tumor site for optimal efficacy.[7,47,48] Secretion of soluble factors such as transforming growth factor-β (TGF-β) and C-X-C motif chemokine ligand (CXCL) 12 (CXCL12) can limit trafficking of T cells into the tumor.[45] Alternately, the CXCL10 and C-X-C motif chemokine receptor (CXCR) 3 (CXCR3) interaction has been shown to enhance tumor recruitment of T cells.[47] In clinical patient samples treated with tebentafusp, a TCR/anti-CD3 bispecific fusion protein targeting gp100 for the treatment of advanced uveal melanoma, increased CXCL10 levels in serum correlated longer overall survival and enhanced elimination of tumor burden.[47]

Additionally, the tumor immune-microenvironment (TME) has been shown to contain immune-suppressive regulatory cells such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSC), as well as immunosuppressive cytokines and the

upregulation of T cell suppressive receptors such as programmed death-ligand 1 (PD-L1) (Figure 4).[19,34,56] T cells from solid tumors have been shown to have upregulated T cell co-inhibitory immune checkpoint molecules such as PD-1, CTLA-4, T cell immunoglobulin, and mucin domain-containing protein 3 (TIM-3) and lymphocyte activation gene 3 (LAG-3).[34,35,56,61,67] The T cell inhibitory nature of solid tumors suggests many possible resistance mechanisms against CD3 redirection therapies and combinations will likely be needed in the clinic to optimize response and circumvent resistance to therapy.

Two resistance mechanisms have been observed in response to blinatumomab treatment in the clinic including increased Tregs and upregulation of PD-L1 on B-precursor ALL cells (Figure 4).[11,14,32] Elevated frequencies of Tregs were observed in blinatumomab non-responders in a trial in B-precursor ALL.[11] Tregs are CD4 positive T cells characterized by high levels of CD25 and intracellular transcription factor FoxP3 and can suppress T cell activation by secreting anti-inflammatory cytokines, blocking co-stimulatory signals like CTLA-4 and expressing checkpoint receptors.[11,31,42] Elevated Treg levels produced increased IL-10 and were shown to suppress CD8 T cell proliferation and cytotoxicity.[11]

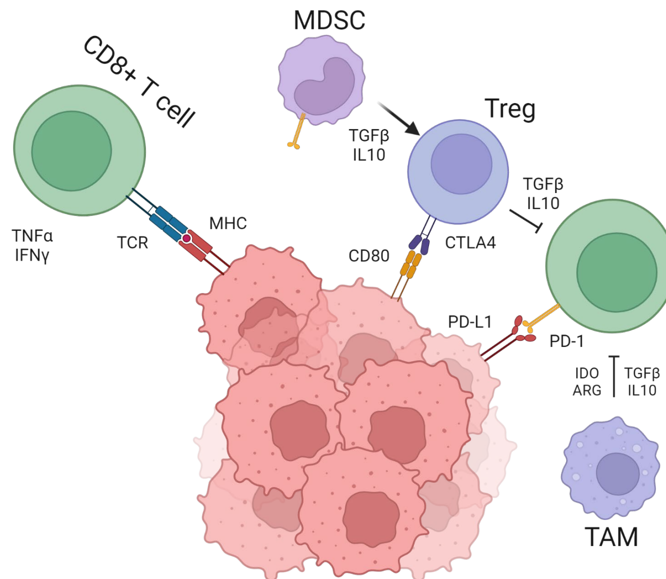

Figure 4. T cell inhibition in TME can be regulated by MDSCs, Tregs and TAMs.

ARG, arginase; IDO, indoleamine 2,3-dioxygenase; IFNγ, interferon gamma; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; TAM, tumor associated macrophage; TCR, T cell receptor; TME, tumor microenvironment; TNFα, Tumor necrosis factor alpha Treg, T regulatory cell. Created with BioRender.com

CD8+ effector T cell binds tumor cells through TCR recognition of MHC complex resulting in secretion of pro-T cell cytokines TNFα and IFNγ. The TME contains immune-suppressive regulatory cells such as regulatory Tregs, MDSCs, and TAMs which can secrete immunosuppressive cytokines such as TGFβ and IL10 and metabolizing enzymes IDO and ARG and suppress T cell effector function. Tumor cells can also upregulate T cell suppressive receptors such as PD-L1 that can bind PD-1 on T cells and lead to T cell exhaustion or CD80 which binds CTLA-4 and enhances Treg function.

Additionally, a case study of non-responding ALL patient treated with blinatumomab showed that although T cell infiltrates were observed in the bone marrow, a treatment-induced increase in PD-L1 on the cancer cells may have led to a reduced response.[32] Subsequent in vitro studies demonstrated blockade of inhibitory PD-1/PD-L1 interactions restored the anti-tumor effects of blinatumomab.[14] This was further enhanced by promoting interactions between CD80/86 and CTLA-4.[14] Combinations of checkpoint blockade and CD3 redirection are currently being investigated clinically with pembrolizumab or nivolimumab (anti-PD-1 therapy), atezolizumab (anti-PD-L1 therapy) or ipilimumab (anti-CTLA-4 antibody).[7,31,45,47,63]

In addition to T cell suppression by Tregs or upregulation of inhibitory T cell receptors, T cell functionality can also be modulated by other suppressive immune cells in the TME including cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAMs). These cells produce cytokines such as TGF-β or IL-10, or metabolic products such as adenosine or indoleamine 2,3-dioxygenase that suppress T cell metabolism and activation.[7,45,47,63] Combinations with therapies that target MDSCs/ MDSC-derived chemokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), colony stimulating factor 1 receptor (CSF1R), or TGF-β may help CD3 redirectors overcome suppressed TME in solid tumors.[10,46,49]

Although combinations are being initiated clinically with CD3 redirector therapies both in hematological and solid tumor settings, it is not yet understood if these combinations will enhance the durability of T cell responses. Although CD3 bispecifics do not induce a traditional antigen-specific T cell response, an inflammatory response is initiated that can result in antigen presenting cell (APC) recruitment against tumor neoantigens released during T cell lysis suggesting the possibility of epitope spreading whereby T cells are presented with these neoantigens through MHC binding and then lyse the cancer cells by recognizing additional antigens other than the initial target antigen.[7,31] Epitope spreading is observed with CBI therapy and patients with a higher mutational load are more sensitive to treatment likely due to a greater immune response against the neoantigens released during tumor lysis.[31] These clinical findings suggest CD3 redirecting therapies could also elicit epitope spreading and memory immune

responses. Additionally, combination approaches may enhance these memory effector T cell phenotypes and increase epitope spreading.

1.6. PRE-CLINICAL MOUSE MODELS AND LIMITATIONS

Preclinically, CD3 redirection agents are often tested in xenotransplantation models, whereby immunodeficient mice are transplanted with human effector cells and human tumors.[7,8,17] In the majority of preclinical in vivo studies assessing the efficacy of CD3 redirector therapies, immune compromised mice are adoptively transferred with either peripheral blood mononuclear cells (PBMCs) or pan CD3 T cells, and human tumor cells are either implanted separately or co-implanted with these effector cells.[1,5,8,17,30,50] Investigators have demonstrated CD3 redirector treatment given intravenous (IV) or intraperitoneally (IP) inhibits tumor growth in these models that correlates with T cell infiltration and activation. [1,5,8,17,30,50] Preclinically, a PSMA-targeting BiTE demonstrated efficacy in both co-implantation models as well as established SC models using human PBMCs, thus demonstrating potential utility for this therapy in the clinic.[17] While useful for assessing T cell-mediated anti-tumor responses, these models have limitations including a lack of a complete immune system (including a lack of a myeloid compartment) and low peripheral engraftment and cytokines.

Without additional human cytokine support, human myeloid cells do not engraft for more than a week when injecting PBMCs, therefore preventing the assessment of any APC interactions with T cells in the context of CD3 redirector treatment.[62] Additionally, severely immunocompromised mice lacking functional mouse myeloid cells are needed to support engraftment of human T cell populations resulting in a model devoid of either human or mouse myeloid components.[61,62] Although these mice are reconstituted with

human T cells, the number and composition of these cells do not accurately reflect the human system and ultimately graft versus host (GvHD) results in an activation of the human T cells against mouse antigens independent of CD3 redirector treatment.[60,61] This leads to an artificial activation of all T cells and prevents a true assessment of immune-suppressed environments. Due to the relatively rapid onset and severity of GvHD symptoms such as body weight loss and poor overall body condition, the time to evaluate durable responses in such xenotransplant models is limited. Without an intact immune repertoire in the mouse host, adaptive memory cell responses as well as the durability of T cell responses cannot be evaluated adequately.

Recent advances to the xenotransplantation model have been made using immune compromised mice that have transgenic expression of human cytokines including IL3, GM-CSF, and stem cell factor (SCF) which promote enhanced human immune reconstitution from CD34+ human cord blood cells.[6,53,64,71] NSG-SGM3 or NOG-EXL mice 3 to 4 weeks old are sub-lethally irradiated and engrafted with CD34+ cord blood cells which results in reconstitution of human immune cells including T cells as well as some myeloid cells including B cells, NK cells and monocytes (unpublished data, Table 1). Evaluation of CBI therapies in these models has demonstrated inhibition of xenograft tumor growth as well as T cell infiltration.[6,9,55] These mice may offer a model system to test the effects of the tumor microenvironment and adaptive immune responses by CD3 redirection therapy using the same bispecific antibodies that are used clinically.

Table 1. Preliminary peripheral CD34+ engraftment in NSG-SGM3 and NOG-EXL mice

Immune Cells	NSG-SGM3	NOG-EXL	NOG-EXL
Counts/100 uL blood	**15 weeks post-engraftment**	**15 weeks post-engraftment**	**21 weeks post-engraftment**
%CD45+	54	50-72	41-51
CD3+ T cells	1290 - 2770	260 - 810	2580 - 4330
CD4+ T cells	900 - 2270	170 - 510	1890 - 1340
CD8+ T cells	620 - 2250	170 - 760	660 - 1270
CD14+ Monocytes	80 - 330	190 - 470	150 - 260
CD16/CD56+ NK cells	230 - 250	370 - 520	15 - 100
CD19+ B cells	410 - 840	1500 - 3100	1250 - 2170

CD3, cluster of differentiation 3; hGM-CSF, human granulocyte-macrophage colony-stimulating factor; hIL-3, human interleukin 3; NK, natural killer; NOD, non-obese diabetic; NSG, NOD SCID gamma; NOG-EXL, hGM-CSF/hIL-3 NOD SCID gamma; hSCF, human stem cell factor; SCID, severe combined immunodeficiency; SGM3, hGM-CSF/hIL-3/hSCF

A syngeneic murine model system with an intact mouse immune system and tumors bypasses many of the limitations described above for xenotransplantation, and thus represents a relevant model to evaluate the anti-tumor efficacy, survival, and mechanism of action of CD3 bispecifics.[2,5,8,30] A bispecific targeting mouse CD3 on the 2C11 epitope on T cells as well as the mouse tumor antigen TA99 (TYRP1/gp75) delayed tumor growth of mouse melanoma B16F10 tumors in immune competent mice by recruiting and activating mouse T cells, validating the mouse as an analogous model system to human.[2] Mouse syngeneic tumors also reflect the variability seen in human tumors with a range of immune infiltration including immune "hot" and "cold" tumors that respond to CBI similarly to humans.[41,51,59] One obvious limitation with a fully mouse syngeneic model is the inability to test clinical therapeutics directed against human antigens (although recombinant expression of human antigens on murine tumor cells has been frequently employed as solution to that problem, with some caveats discussed later in Chapter 3.) Additionally, the mouse surrogate CD3 binder clone 2C11 on T cells may not have the same affinity or ability to activate mouse T cells as clinical human CD3 binders in the clinical therapeutic bispecifics.

The development of human CD3 transgenic knock-in (hCD3) mouse models provides a fully immune-competent mouse model system to assess human CD3 redirection binders.[2,13,37] CD3 bispecifics targeting Mucin 16, HER2, and PSMA have shown inhibition of mouse tumors engineered to express these human targets as well as intra-tumoral infiltration of mouse T cells expressing human CD3.[2,13,37] Additionally, a combination of HER2xCD3 treatment with anti-PD-1 or anti-PD-L1 antibodies prolonged survival in mice bearing CT26 tumors expressing human HER2 in hCD3 mice.[30] Combination of PSMAxCD3 and a 4-1BB co-stimulatory antibody demonstrated enhancement of anti-tumor efficacy and survival in hCD3 mice bearing mouse prostate TRAMP.C2 tumors expressing human PSMA.[5] This combination treatment also prevented tumor establishment in mice that were previously cured with PSMAxCD3 and 4-1BB treatment and were re-challenged with new tumors. These recent studies suggest that the hCD3 mice provide a model system for evaluating combination approaches targeting immune-suppressed TME in mouse tumors, as well as testing the durable and memory T cell responses.

Overall, both the human xenotransplantation and murine syngeneic models each recapitulate different features of CD3 redirection biology, and collectively can be used to explore anti-tumor efficacy, characterize T cell responses to CD3 redirection therapy, and to test specific approaches to enhance these responses.

1.7. DISSERTATION RESEARCH OBJECTIVES

The primary goals of this research were to develop and utilize clinically relevant preclinical mouse models to characterize anti-tumor efficacy and T cell responses to CD3 redirection therapy, and secondarily to test specific combination therapeutic approaches

to enhance the durability of T cell responses. It was hypothesized that CD3 redirection antibodies would eliminate or reduce tumors in mice due to T cell killing of tumor cells, and that this would result from enhanced activation and tumor infiltration of T cells. It was further hypothesized that durable anti-tumor responses would occur as a result of the establishment of T cell memory subsets, potentially as a consequence of epitope spreading after TAA+ tumor cells are lysed and thereby presenting additional tumor-associated antigens for immune surveillance recognition.[44] Additionally, it was postulated that combination therapies targeting immune-suppressive TME could enhance durable T cell responses as well as epitope spreading, particularly in solid tumors (Figure 5).

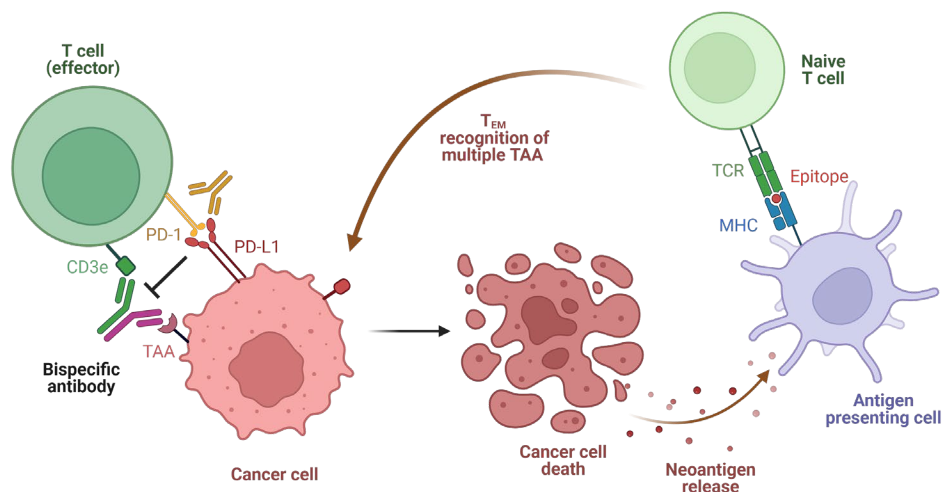

Figure 5. Proposed model for combination therapy with PSMAxCD3 redirection antibodies and CBI to elicit T cell memory phenotypes through epitope spreading and prostate cancer neoantigen-specific TCR priming.

CBI, checkpoint blockade inhibition; CD3e, cluster of differentiation 3 epsilon; MHC, major histocompatibility; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; PSMA, prostate-specific membrane antigen; TAA, tumor associated antigen; TCR, T cell receptor; T_{EM}, effector memory T cell. Created with BioRender.com.

CD3 redirection antibodies will inhibit tumor growth in mice due to T cell killing of tumor cells through binding of CD3 on T cells and TAA on tumor cells, resulting in tumor cell lysis and release of tumor neoantigens. It was hypothesized that durable anti-tumor responses would occur due to the establishment of T cell memory subsets, potentially recognizing multiple TAAs. T cell reponses to new TAA could occur as a consequence of epitope spreading where additional tumor-associated antigens are released during cell lysis which are taken up by antigen presenting cells and presented to T cells through MHC interactions. Additionally, it was postulated that combination therapies targeting immune-suppressive TME could enhance durable T cell responses as well as epitope spreading, particularly in solid tumors.

Dissertation research objectives:

1. Develop a PSMA+ human prostate cancer xenotransplantation model in CD34+ cord blood humanized mice and characterize the anti-tumor efficacy and T cell responses of PSMAxCD3 bispecific antibody therapy.
2. Further characterize the anti-tumor efficacy and T cell responses of PSMAxCD3 redirection in the context of tumor PD-L1 overexpression as a potential resistance mechanism.
3. Develop PSMA+ mouse syngeneic tumor models with immune “hot/cold” and CBI sensitive/insensitive characteristics and evaluate anti-tumor efficacy and T cell responses of PSMAxCD3 bispecific antibody therapy.
4. Evaluate combination strategies with CD3 redirection to enhance T cell responses in human xenotransplantation and mouse syngeneic tumor models.

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CHAPTER 2: DURABILITY OF T CELL RESPONSES ELICITED FROM PSMAXCD3 TREATMENT AND RESISTANCE MECHANISMS IN CD34+ ENGRAFTED MICE BEARING HUMAN PROSTATE XENOGRAFTS

2.1. ABSTRACT

Prostate cancer is the second leading cause of death among men in the United States and although initial responses to standard of care are good, approximately a third of patients relapse. PSMA has been shown to be overexpressed on prostate cancer with increasing levels correlating to disease severity making it an ideal target for CD3 redirection targeting metastatic castrate resistant prostate cancer. Clinical trials evaluating CD3 redirection antibodies in solid tumors are still in early phases and it is not understood if these therapies will be able to generate durable T cell responses. The low mutational burden, infiltrating immune cells, and PD-L1 levels in prostate cancer have led to minimal responses to CBI therapy and thus may inhibit CD3 redirection therapies as well. In this study we demonstrate efficacy of PSMA targeted CD3 redirection of T cells in human xenografts in CD34+ engrafted immune incompetent mice results in T cell infiltration with an activated phenotype and an enrichment of effector memory T cells. Overexpression of PD-L1 on the tumor cells resulted in resistance to PSMAxCD3 treatment with lack of immune infiltration. Combination of PSMAxCD3 with PD-1 blockade restored anti-tumor efficacy but did not significantly alter T cell phenotype. Combination therapy resulted in complete tumor responses and elicited immune responses that protected against but did not prevent growth of rechallenged tumors suggesting the establishment of durable T cell responses may need additional combinations in the clinic.

2.2. INTRODUCTION

Bispecific antibodies targeting cluster of differentiation 3 (CD3) on T cells and a TAA on hematological tumor cells, such as blinatumomab, which targets CD19, have shown impressive improvement to overall survival rates in hematological malignancies.[17,49,51,52] Despite promising data in the clinic, only subsets of patients respond to blinatumomab and 20% of responding patients relapse suggesting enhancements or combinations may be necessary to improve durability of T cell responses.[51] When comparing T cell responses in blinatumomab responding versus non-responding patients, investigators found expansion of CD8+ T-cells, with an increase in effector memory subsets in patients with the best responses, whereas, poor responders had elevated recruitment of regulatory T cells and upregulation of programmed death ligand 1 (PD-L1) levels that both suppress T cell effector function.[25,51] New CD3 bispecifics are being evaluated preclinically and clinically to target solid tumor cancers. In addition to facing these T cell suppressive resistance mechanisms observed with currently approved CD3 bispecifics, solid tumors may be relatively more difficult to treat due to a more immune suppressive environment and the additional hurdle of T cell infiltration into a solid tumor mass.[7,14,24,32,35,48]

T cell redirection bispecific antibodies targeting prostate specific membrane antigen (PSMA) are being explored as a potential treatment for prostate cancer where despite standard of care, 30-40% of patients become hormone refractory and relapse.[3,4,50,53] PSMA has been shown to be overexpressed in prostate cancer with expression correlating to severity of disease making it an ideal target for T cell redirected therapies in castrate resistant metastatic prostate cancer.[3,56] PSMA targeting CD3

bispecifics have shown preclinical antitumor efficacy in xenograft models using engraftment of human effector PBMC or T cells.[9,13,19] Although preclinical data looked promising, limited anti-tumor efficacy has been observed clinically with only some patients responding.[20,30,31,35] Lack of translatability of preclinical data to the clinic could be due to a lack of suppressive tumor microenvironment and T cell suppression in xenograft models with adoptive transfer of human PBMCs or T cells.

Preclinical human immune xenotransplantation models often only reconstitute human T cells due to the lack of human cytokine support needed to elicit engraftment of human myeloid cells for more than a week.[47] Without engraftment of the myeloid compartment, which makes up approximately 50-70% of the lymphocyte population in peripheral blood and bone marrow, antigen presenting cell (APC) interactions with T cells in the context of CD3 redirector treatment cannot be assessed.[47] Additionally, in order to sustain engraftment of human T cells needed as effector cells for CD3 redirection activity, severely immunocompromised mice lacking functional mouse myeloid cells are utilized, thus generating a model devoid of either human or mouse myeloid component.[46,47] In addition to a lack of myeloid engraftment, human T cells activate, expand, and target murine antigens, ultimately leading to graft versus host (GvHD). This non-physiological and chronic activation and expansion of human T cells occurs irrespective of treatment, and could potentially lead to T cell exhaustion.[6,46,47,54]

More recently, advances have been made to enhance human immune reconstitution using immune compromised mice that have transgenic expression of human cytokines including IL3, GM-CSF, and stem cell factor, and which are engrafted with CD34+ human cord blood cells.[6,23,39,41,47] These mice are sub-lethally irradiated and

engrafted with CD34+ cord blood cells resulting in reconstitution of human immune cells including T cells as well as myeloid cells.[39,47] Evaluation of clinically approved checkpoint blockade inhibitor (CBI) therapies, such as anti-PD-1 antibodies, in these models have demonstrated inhibition of xenograft tumor growth as well as T cell infiltration.[8,55] These human immune reconstituted mice with lymphocyte and myeloid compartments may offer a model system to test the effects of the tumor microenvironment and adaptive immune responses to CD3 redirection therapy using clinical human bispecific antibodies.

In these studies, we investigated the durability of T cells elicited from PSMAxCD3 treatment against prostate xenografts in CD34+ immune reconstituted mice. We demonstrated that PSMAxCD3 treatment could inhibit prostate patient-derived xenografts (PDX) and induce effector memory T cells that protected against tumor re-challenge. Additionally, we observed that overexpression of immune suppressive PD-L1 on LNCaP xenografts conferred resistance to PSMAxCD3 treatment. The combination of PSMAxCD3 with PD-1 blockade overcame PD-L1-mediated resistance, elicited T cell activation and effector memory phenotype and protected against tumor re-challenge.

2.3. MATERIALS AND METHODS

PSMAxCD3 Bispecific Duobody® antibody

PSMAxCD3 is a IgG4-proline-proline-alanine (PAA) bispecific DuoBody® antibody (PAA mutation for reduced affinity to Fc gamma receptors and nearly silent Fc-effector functionality) that binds to PSMA and CD3.[27] The anti-PSMA Fc monovalent arm PS3B25 was directed against the extracellular domain of PSMA, and was discovered by phage display panning. The anti-CD3ε monovalent Fc arm B219, was derived from a

SP34 antibody clone.[42] Both PSMA and CD3 mono-targeting arms have tight binding to cells, with low nM affinities that were comparable to clinical molecule JNJ-63898081.[30,31] Both the PSMA and CD3-mono-targeting arms lack cross-reactivity to mouse proteins.

Tumor cell lines and Patient-Derived Xenografts

The human prostate tumor cell line LNCaP.AR was obtained from Aragon Pharmaceuticals. It was generated through transfection of LNCaP cells with human androgen receptor (AR) cDNA introduced via retrovirus infection, resulting in a 3 fold increase in AR levels that confers anti-androgen therapy resistance.[3] LNCaP.AR cells express PSMA, and levels are increased upon androgen deprivation.[11] LNCaP.AR cells were lentivirally transduced with PD-L1 cDNA to stably express cell surface PD-L1. Cell lines were authenticated and tested by Analytical Biological Services, Inc. and all infectious panel testing was negative.

The prostate PDX LuCaP 86.2 model was obtained from the University of Washington and was derived from a 65 year-old cancer patient with bladder metastasis from prostate adenocarcinoma approximately 14 years post initial diagnosis. The LuCaP86.2 model has been genomically and phenotypically characterized as containing amplification of the androgen receptor, PTEN loss, high PSMA expression and BRCA2 heterozygous loss, thus offering a clinically relevant metastatic androgen-resistant prostate cancer model.[50]

Xenograft tumor studies

Female NOG-EXL (NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac with transgenic human GM-CSF and IL-3 (Taconic) or Female NSG-SGM with transgenic human GM-

CSF, SCF and IL-3, (The Jackson Laboratory) mice were used when they were approximately 4 weeks of age. All animals were allowed to acclimate and recover from any shipping-related stress for a minimum of 5 days before experimental use. Autoclaved water and irradiated food were provided ad libitum, and the animals were maintained on a 12-hour light and dark cycle. Cages, bedding, and water bottles were autoclaved before use and changed weekly. All experiments were carried out in accordance with *The Guide for the Care and Use of Laboratory Animals,*[38] and the USA Animal Welfare Act. Protocols were approved by the local ethics committees of Janssen Pharmaceuticals, Spring House, PA.

To humanize the immune system of the mice, human cord blood derived CD34+ isolated cells (~50,000 cells/mouse) were implanted after sublethal irradiation (100 centiGray). Mice were retro-orbitally bled and flow cytometry was conducted to evaluate peripheral engraftment at approximately 14 weeks post-engraftment.

LNCaP-AR cells were propagated in log phase and enzymatically dissociated using TrypLE™ (Gibco by Life Technologies, Cat #12563-029) to be plated at 1e6 cells/well in 0.5 mL Cultrex (Trevigen, Cat #3433-005) in 24-well plates. RPMI 1640 media with 10% heat inactivated FBS was added in a drop-wise fashion on top of the Cultrex and replaced every other day. After 4 days of growth, when colonies were apparent under the microscope, spheroids were rinsed and implanted SC in the right flank of each mouse. The day of spheroid implantation was designated as Day 0 of the study.

LuCaP 86.2 tumors, harvested from donor mice, were used for Study ONC2016-087. LuCaP 86.2 tumors, approximately 500 mm^3 in size, were excised and cut into fragments approximately 2 mm × 3 mm in size, then implanted SC in the right flank of

experimental mice using 13gauge x 31/4" trocar needles. The implant day was designated as Day 0.

Animals were randomized onto study by tumor volume, T cell engraftment, and donor such that group means or distributions were similar. CD3xNull (Janssen R&D), PSMAxCD3 (Janssen R&D), PBS, and pembrolizumab (anti-human PD-1, Keytruda, NDC00006-3026-02) were administered IP twice a week according to body weight (10 mL/kg).

Body weight and SC tumor volume were measured for each animal twice a week throughout the study. Animals were monitored daily for clinical signs and tumor burden. When individual animals exhibited negative clinical signs, such as lethargy, ruffled and matted coat, hunched posture, cyanotic extremities, or dyspnea, or reached 20% body weight loss as compared with initial body weights, they were removed from the study and humanely euthanized. Animals were removed when a maximum tumor volume of ≥1,500 mm^3 was reached, or when adverse clinical signs were noted.

Tumor and tissue processing

Tumors from Study were placed in C tubes containing 5 mL of RPMI 1640 medium for processing. Tumors were cut into 1 to 2 mm pieces in the C tubes, and 162.5 µL of an enzyme cocktail from the Tumor Dissociation Kit (Cat# 130-096-730) was added to each tube. Tubes were placed in the Gentle MACS Octo Dissociator with Heaters (Miltenyi) and processed using the manufacturer's setting "37C_h_TDK_2". Tumor cell suspensions were filtered through 70-µm cell strainers into media. Similarly, spleens were filtered through 70-µm cell strainers with syringe plungers into media. Tubes were centrifuged at 1,500 revolutions per minute (rpm) for 5 minutes, cell

supernatant was aspirated, and cell pellets were resuspended in complete culture medium. Samples were lysed for red blood cells using ACK lysis buffer (Gibco) with 1-3 rounds of 3-minute incubation of 200 µL per well followed by 3 cycles of washing and centrifuging. Cell count and viability of each sample were determined using a Vi-Cell counter (Beckman Coulter). Samples were plated with 1×10^6 viable cells in a 96-well round-bottom plate and centrifuged at 1,500 rpm for 3 minutes. Cell supernatant was discarded, and then cells were stained for flow cytometry analysis.

Flow cytometry and antibodies

Processed tumors or spleens were stained with 50 µL per well of PBS containing Fc block and LIVE/DEAD™ stain. Plates were incubated in the dark for 15 minutes at RT. Samples were washed with 150 µL of cold stain buffer and centrifuged for 3 cycles, as described above for tumor cell preparation. Stain buffer (50 µL) containing pre-incubation antibodies specific for immune cell markers (CC47 and CD45RA,

Table 2) was added to each sample well and the plates were incubated in the dark for at least 30 minutes on ice. Plates were then washed and centrifuged for 3 cycles, as described above Stain buffer (50 µL) containing antibodies specific for immune cell markers (Table 2) was added to duplicate sample wells for each panel (T cell or Myeloid) and the plates were incubated in the dark for at least 30 minutes on ice. Plates were then washed and centrifuged for 3 cycles, as described above.

Table 2: Human Flow Cytometry Panel Reagents.

Marker/fluorochrome	Clone	Source	Catalog number	Dilution
CD45/PED 594	HI30	Biolegend	304052	1:300
CD3/BV711	UCHT1	BD Biosciences	563725	1:200
CD4/APC-H7	RPA-T4	BD Biosciences	560158	1:200
CD8/PerCP-Cy5.5	RPA-T8	BD Biosciences	560662	1:200
CCR7/BV421	G043H7	Biolegend	353208	1:10
CD45RA/AF488	HI100	Biolegend	304114	1:300
CD127/AF700	A019D5	Biolegend	351344	1:10
CD137/BV605	4B4-1	Biolegend	309822	1:200
CD25/BV650	BC96	Biolegend	302634	1:200
HLA-DR/BV785	G46-6	BD Biosciences	564041	1:300
ICOS/PE-Cy7	ISA-3	ThermoFisher	25-9948-42	1:300
PD-1/PE	MIH4	BD Biosciences	557946	1:300
TIM-3/APC	344823	R&D Systems	FAB2365A	1:5
CD45/PED 594	HI30	Biolegend	304052	1:300
CD3/BV711	UCHT1	BD Biosciences	563725	1:200
CD14/APC-H7	MOP9	BD Biosciences	560180	1:300
CD19/BV421	HIB19	BD Biosciences	562440	1:300
CD11b/AF700	ICRF44	BD Biosciences	557918	1:200
CD16/BUV395	3G8	BD Biosciences	563785	1:300
CD56/PE-Cy7	B159	BD Biosciences	557747	1:200
HLA-DR/BV785	G46-6	BD Biosciences	564041	1:300
PD-L1/APC	MIH2	BioLegend	393610	1:200
PD-L2/PE	MIH18	BioLegend	345506	1:200
PSMA/AF488	LNI-17	BioLegend	342506	1:300
LIVE/DEAD™ Aqua	NA	Thermo Fisher	L34957	NA

AF, Alexa Fluor; APC, allophycocyanin; BB, brilliant blue; BUV, brilliant ultraviolet; BV, brilliant violet; CCR7, C-C motif chemokine receptor 7; CD, cluster of differentiation; CTLA4, cytotoxic T-lymphocyte associated protein 4; Cy, cyanine; HLA-DR, human leukocyte antigen – DR isotype; ICOS, Inducible costimulator; NA, not applicable; PD-1, programmed cell death 1; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2; PE, phycoerythrin; PED, phycoerythrin dazzle; PerCP-Cy5.5, peridinin chlorophyll- cyanine 5.5; PSMA, prostate specific membrane antigen; TIM-3, T-cell immunoglobulin and mucin-domain containing-3
Flow cytometry panels were created using selected reagents from the above table.

All flow cytometry samples were resuspended in stain buffer (200 µL/well) and analyzed using the Fortessa flow cytometer (BD Biosciences). Raw flow cytometry data were further analyzed using FlowJo software (BD Biosciences, Version 10). The gating strategy was the following: cells→ singlets→ live cells→ human $CD45^+$ tumor infiltrating immune cells. $CD45^+$ cells were gated further on $CD4^+$ or $CD8^+$ T cells. $CD4^+$ or $CD8^+$ T cells were further gated on subsequent T cell markers (ie. CD25, PD-1, TIM3, CD137, ect.). CD45- tumor cells were gated on PD-L1 and PSMA expression. $CD45^+$

tumor infiltrating immune cells were also further gated on non-T cell immune infiltrating cells (CD14, CD19, CD11b, ect.). The percentage or counts of immune cells in each sample was graphed in Prism.

Immunohistochemistry (IHC)

Xenograft tumor samples were formalin-fixed for IHC for approximately 24 hours and then transferred into 70% ethanol. Tissues were then processed and paraffin-embedded. All formalin-fixed, paraffin-embedded samples were sectioned at 4 µm and placed on positively charged glass slides. PSMA (clone 3E6), CD8 (clone SP57), CD4 (EPR6855) and PD-L1 (clone 28-8) IHC staining was performed with Hematoxylin counterstain.

Calculations and Statistics

Body weights and tumor volumes were collected twice weekly. SC tumor volume was calculated using the formula: Tumor volume (mm^3) = ($D \times d^2/2$); where 'D' represents the larger diameter, and 'd' the smaller diameter of the tumor as determined by caliper measurements. Body weight changes of individual mice were calculated using the formula: $([W-W_0]/W_0) \times 100$, where 'W' represents body weight on a particular day, and 'W_0' represents body weight at initiation of treatment. Tumor volume and body weight data were graphed while at least two thirds of the animals remained in each group. All data were graphed using Graph Pad Prism.

The percent TGI was defined as the difference between mean tumor volumes of the treated and control groups, calculated as % TGI = $((TV_c-TV_t)/TV_c) \times 100$ where 'TV_c' is the mean tumor volume of the control group and 'TV_t' is the mean tumor volume of

the treatment group. A complete response (CR) was defined as complete tumor regression, with no palpable tumor.

All data were graphed using Prism. Statistical significance for tumor growth inhibition was evaluated for treatment groups compared with the CD3xNull or CD3xNull and Isotype control treated groups or for combination treatment groups compared to single agent therapies. Differences between groups were considered significant when $p \leq 0.05$. Statistical significance for was calculated either using the linear mixed-effects (LME) analysis, with treatment and time as fixed effects and animal as random effect, or using post-hoc analysis from a mixed model for repeated measures (MMRM) with the fixed effects group, time (as a factor), and the interaction between group and time and a random effect for subject comparing mean tumor burdens at each measurement timepoint in R software version 3.4.2 (using Janssen's internally developed Shiny application version 4.0). Logarithmic transformation (base 10) was performed if individual longitudinal response trajectories were not linear. The information derived from this model was used to make pairwise treatment comparisons of animal body weights or tumor volumes to that of the control group or for combination treatments compared to the single agent treatments.

Statistical significance of terminal tumor volumes following rechallenge were evaluated for rechallenged group compared with naïve tumor challenge by unpaired T test. Statistical significance of T cell phenotypes was evaluated for treatment groups compared with tumors or spleens from the control treated group by one-way ANOVA with Dunnett's multiple comparisons test.

2.4. RESULTS

PSMAxCD3 treatment inhibits growth of LuCaP86.2 PDX

To assess durability of T cell responses elicited from PSMAxCD3 treatment in the context of a human immune system, castrate-resistant LuCaP 86.2 PDX tumors that express high levels of PSMA were established in CD34+ cord blood humanized NOG-EXL mice that express human cytokines supporting immune cell engraftment. Female NOG-EXL mice were injected with 10e5 CD34+ cord blood cells from 6 donors one day post sublethal irradiation dose of approximately 100 centigray (cGy). PSMAxCD3 treatment was initiated when tumors were established in mice where engraftment of the human immune system was verified both by human CD45+ cells and T cells in the peripheral blood 33 days post PDX fragment implant and 14 weeks post CD34+ engraftment.

Statistical significance of PSMAxCD3 treatment on LuCaP 86.2 PDX was assessed up to Day 63 when at least 8 animals remained in each group. Although growth of LuCaP 86.2 PDX tumors was slower in the CD34+ engrafted mice, statistically significant antitumor efficacy was observed at 0.5 mg/kg of PSMAxCD3 (n=20) as assessed by change in growth rate over time compared to untargeted CD3 control bispecific antibody NullxCD3 treatment (n=15) with 55% tumor growth inhibition (TGI) on Day 63 ($p<0.001$; Figure 6). PSMAxCD3 treatment delayed outgrowth of LuCaP 86.2 tumors in most mice with some mice experiencing complete tumor control (Figure 7). Cord blood donors varied in response to treatment with complete responses observed in 2 of the 3 donors. Some animals were removed from study early due to macrophage induced anemia resulting from the CD34+ engrafted cells causing body weight loss and

poor body condition score; however, no body weight loss was associated with treatment (data not shown).

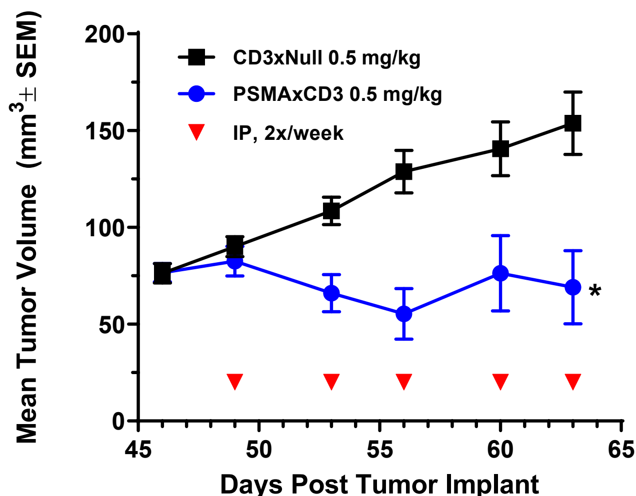


Figure 6. Effect of PSMAxCD3 on Growth of LuCaP86.2 Human Patient-Derived Xenografts in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; IP, intraperitoneally; LME, linear mixed effects; PSMA, prostate specific membrane antigen; SEM, standard error of the mean; TGI, tumor growth inhibition. Group tumor volumes are graphed as the mean ± SEM (n=15-20, mean data graphed when at least 8 mice remained in each group). Tumor fragments were implanted on Day 0. Treatment intraperitoneally twice weekly on Days 49, 53, 56, 60, and 63, as represented by red triangles. *Denotes significant difference (p=0.0010) of growth rates of PSMAxCD3 treatment compared with CD3xNull group by LME with 55% TGI on Day 63.

Durable T cell responses were assessed in animals whose treatment elicited complete responses by evaluating immune rejection of re-challenged tumors. After 2 doses of PSMAxCD3, 4 animals that were tumor-free and 1 with a tumor below 20 mm3 on Day 56, were removed from treatment for 1 week to ensure antibody washout time and then rechallenged with LuCaP 86.2 fragments on Day 60 (Figure 7). As a control for tumor growth, 5 tumor-naïve CD34+ humanized NOG-EXL mice were implanted with fragments on Day 60. On Day 67, 1 week post rechallenge, mice previously receiving PSMAxCD3 had significantly smaller tumor volumes compared to the tumor-naïve mice

suggesting lasting T cell responses against PSMA expressing tumors (p=0.0078, Figure 8).

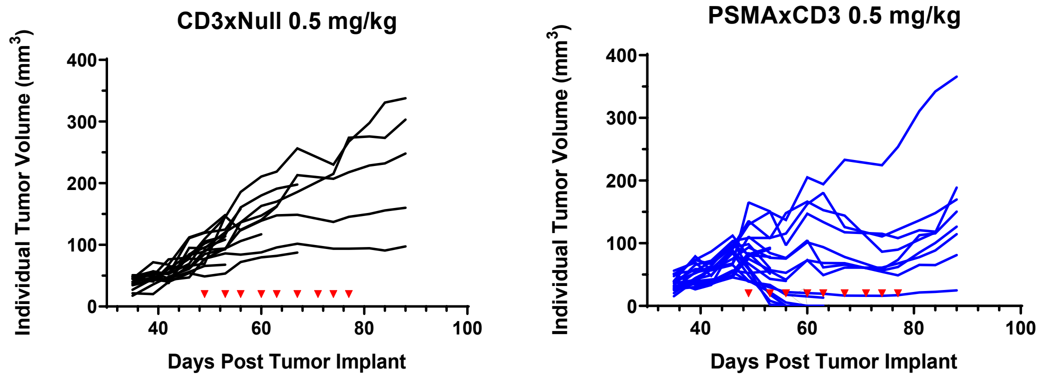


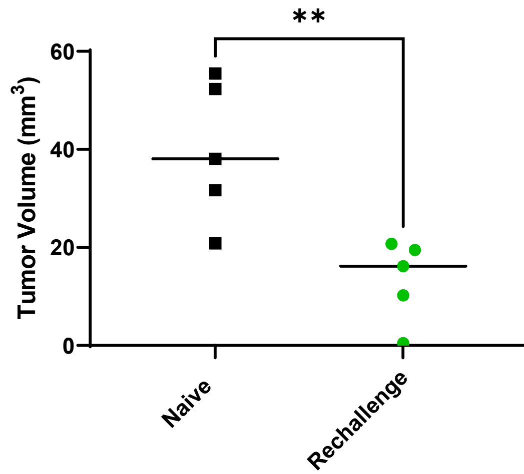
Figure 7. PSMAxCD3 Treatment Resulted in Several Complete Responses of LuCaP86.2 Human Patient-Derived Xenografts in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; PSMA, prostate specific membrane antigen. Individual tumor volumes are graphed (n=15-20). Tumor fragments were implanted on Day 0. Treatment intraperitoneally twice weekly on Days 49, 53, 56, 60, and 63, 67, 71, 74 and 77 as represented by red triangles. Complete responders were re-challenged with LuCaP86.2 tumor fragments on Day 60 (black triangle) after last dose on Day 53.

PSMAxCD3 elicits durable anti-tumor T cell responses against LuCaP86.2 PDX

Tumors from control rechallenged and previously treated PSMAxCD3 animals were pooled and assessed for T cell infiltration and phenotype. Mice who previously received PSMAxCD3 treatment showed an almost 3-fold increase of CD3+ T cell infiltration with a 4.5-fold increase in CD8+ T cells into the tumor. Additionally, the tumor re-challenge resulted in an enhancement of CD45- CCR7- effector memory T cell phenotype in pooled tumors by flow cytometry compared to control tumors in treatment-naïve mice (Figure 8). The tumor rechallenge protection and enhancement of effector memory T cell responses suggest that PSMAxCD3 treatment can result in durable T cell memory responses, the phenotype observed in the clinic in the responding patients.[48]

A.



B.

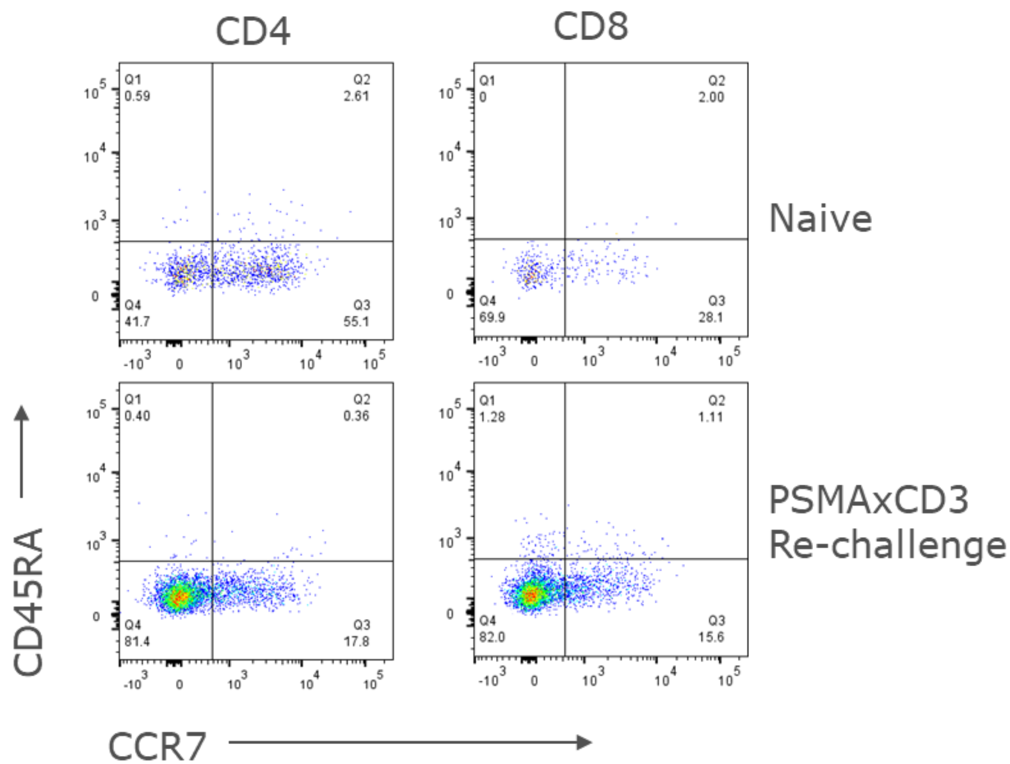


Figure 8. T cell Responses Elicited from PSMAxCD3 Treatment Protect Against LuCaP86.2 Re-challenge Tumors in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; PSMA, prostate specific membrane antigen. A. Individual tumor volumes are graphed with lines denoting medians (n=5). LuCap86.2 PDX fragments were implanted on Day 60. A. Previous PSMAxCD3 treatment significantly protected against tumor rechallenge (p=0.0078 by T test) and B. Staining for CD45RA and CCR7 on CD4 or CD8 T cells in pooled tumors from naïve or rechallenged

PSMAxCD3 treated animals shows enhanced CD45RA- CCR7- effector memory T cell responses in pooled tumors by flow cytometry.

PD-L1 Expression on LNCaP xenografts confers resistance to PSMAxCD3 treatment but combination with pembrolizumab restores anti-tumor activity

Low baseline tumor PD-L1 levels, such as in prostate cancer, have been associated with an immune "cold" tumor microenvironment, and are a negative predictor for anti-PD-1 therapy responses.[18,36] Treatment with CD3 bispecifics in the clinic has shown upregulation of inhibitory T cell mechanisms such as PD-1 on T cells as well as PD-L1 on tumors that could mediate resistance.[25] To model PSMAxCD3 treatment in an immune repressed tumor microenvironment, we evaluated the treatment in PSMA+ LNCaP xenografts with innate PD-L1 and upregulated PD-L1 expression in the context of a human immune system with CD34+ engraftment in NSG-SGM3 or NOG-EXL mice. We hypothesized that a combination of checkpoint blockade of PD-1 with pembrolizumab would enhance PSMAxCD3 responses in tumors with increased PD-L1.

Female NSG-SGM3 or NOG-EXL mice were injected with CD34+ cord blood cells from 2 donors one day post sublethal irradiation. PSMAxCD3 treatment was initiated when tumors were established in mice where engraftment of the human immune system was verified both by human CD45+ cells and T cells in the peripheral blood 17-20 days post LNCaP spheroid implant and 14-15 weeks post CD34+ engraftment. Statistical significance of PSMAxCD3 treatment on LNCaP tumors was assessed up to Day 63 when at least 7 animals remained in each group. In parental LNCaP tumors expressing androgen receptor, PSMAxCD3 treatment at 5 mg/kg elicited statistically significant antitumor efficacy assessed by change in growth rate over time ($p<0.005$) with 46% TGI

compared to PBS treated controls in parental LNCaP xenografts and the combination with anti-PD-1 therapy pembrolizumab offered no additional tumor growth inhibition (Figure 9A). In contrast, LNCaP xenografts with overexpressed PD-L1 demonstrated resistance to PSMAxCD3 therapy with a reduced tumor growth inhibition of 23% (Figure 9B); however, combination treatment with pembrolizumab resulted in enhanced, statistically significant antitumor effect as assessed by growth rate over time compared to CD3xNull as well as both single agents ($p<0.005$) with 62% TGI on Day 38 (Figure 9B).

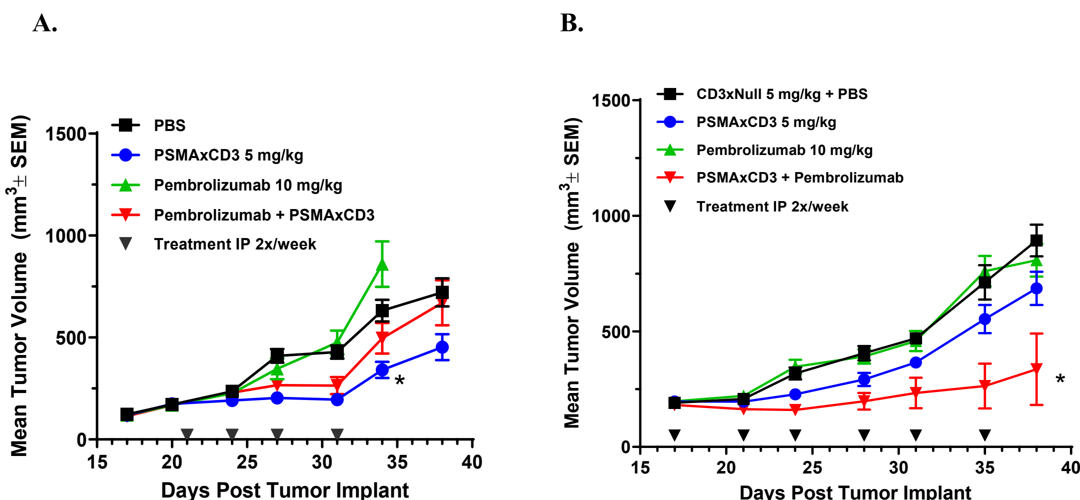


Figure 9. Effect of PSMAxCD3 and in Combination with Pembrolizumab on Growth of LNCaP Xenografts without and with overexpression of PD-L1 in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; IP, intraperitoneally; LME, linear mixed effects; PSMA, prostate specific membrane antigen; SEM, standard error of the mean; TGI, tumor growth inhibition. Group tumor volumes are graphed as the mean ± SEM (n=12-15, 3-5 removed for TIL evaluation, mean data graphed when at least 7 mice remained in each group). LNCaP parental (A) or overexpressing PD-L1 (B) tumor spheroids were implanted on Day 0. Treatment intraperitoneally twice weekly on Days 21, 24, 27 and 31, for A and Days 17, 21, 24, 28, 31 and 35 for B. as represented by triangles. *Denotes significant difference ($p<0.005$) of growth rates of PSMAxCD3 treatment compared with PBS or CD3xNull group by LME Day 34 or 38.

PSMAxCD3 treatment elicits T cell infiltration and expansion of memory T cells

Tumors were collected 24 hours post-4th dose from LNCaP tumor bearing animals engrafted with two HSC donors (n=3-5 per treatment group) to assess immune infiltration and phenotype. PSMAxCD3 treatment resulted in infiltration of human CD45+ immune and CD3+ T cells into LNCaP xenografts with a trend towards enhanced infiltration in

combination with pembrolizumab (Figure 10). PD-L1 overexpression in LNCaP xenografts did not markedly affect immune or T cell infiltration, although the variability of lymphocyte infiltration was high within groups with some animals having less overall immune infiltration. The variability in T cell infiltration suggests that some animals are having more robust T cell responses to treatment than others which was observed in the tumor growth inhibition data as well with one animal completely responding, several with tumor growth stasis while two continued to grow out in the combination treatment group. This is also representative of what has been observed clinically with immune therapies where some patients have a more robust antitumor immune response, suggesting this model may be more clinically relevant.

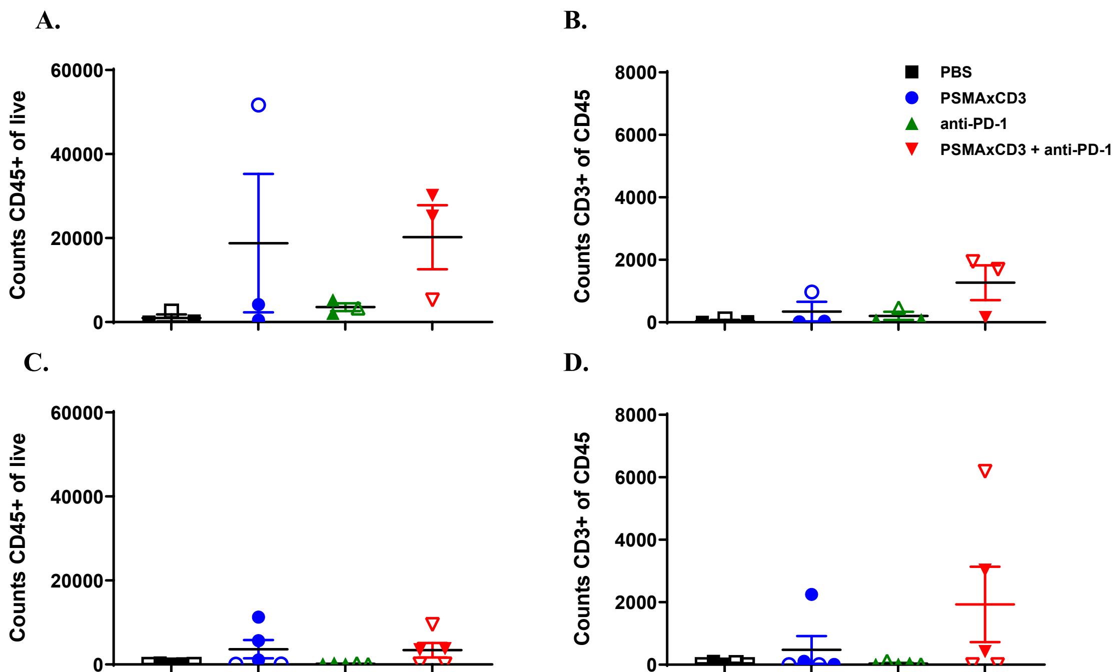


Figure 10. CD45+ immune and CD3+ T cell Infiltration Elicited from PSMAxCD3 Treatment Alone or in Combination With Anti-PD-1 Pembrolizumab in LNCaP Tumors with innate (A, B) or overexpression (C, D) of PD-L1 in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; PSMA, prostate specific membrane antigen; SEM, standard error of the mean. Individual counts of CD45+ or CD3+ immune cells in the tumor are graphed indicated as open or closed symbols for different CD34+ donors with means ± SEM represented by lines and error bars (n=3-5) for A-B LNCaP parental and C-D LNCaP overexpressing PD-L1. PSMAxCD3 treatment trended toward

enhanced immune and T cell infiltration into tumors. All treatments p=ns compared to PBS control by ANOVA.

Immunohistochemistry of LNCaP tumors treated with PSMAxCD3 demonstrated treatment-dependent infiltration of CD4 and CD8 T cells as well as an upregulation of PD-L1 (Figure 11). Treatment with PSMAxCD3 in LNCaP tumors was able to elicit T cell mediated elimination of tumors despite PD-L1 upregulation; however, there were lower levels of PD-L1 on tumors at the start of treatment. In the LNCaP tumors overexpressing PD-L1, high levels of PSMA and PD-L1 in control treated samples was observed with minimal CD4 and CD8 infiltration (Figure 12). Treatment with PSMAxCD3 and anti-PD-1 pembrolizumab as single agent therapies have minimal effects on infiltration of CD4+ or CD8+ T cells; however, combination treatment resulted in enhanced T cell infiltration and marked tumor size reduction without downregulation of the PSMA target.

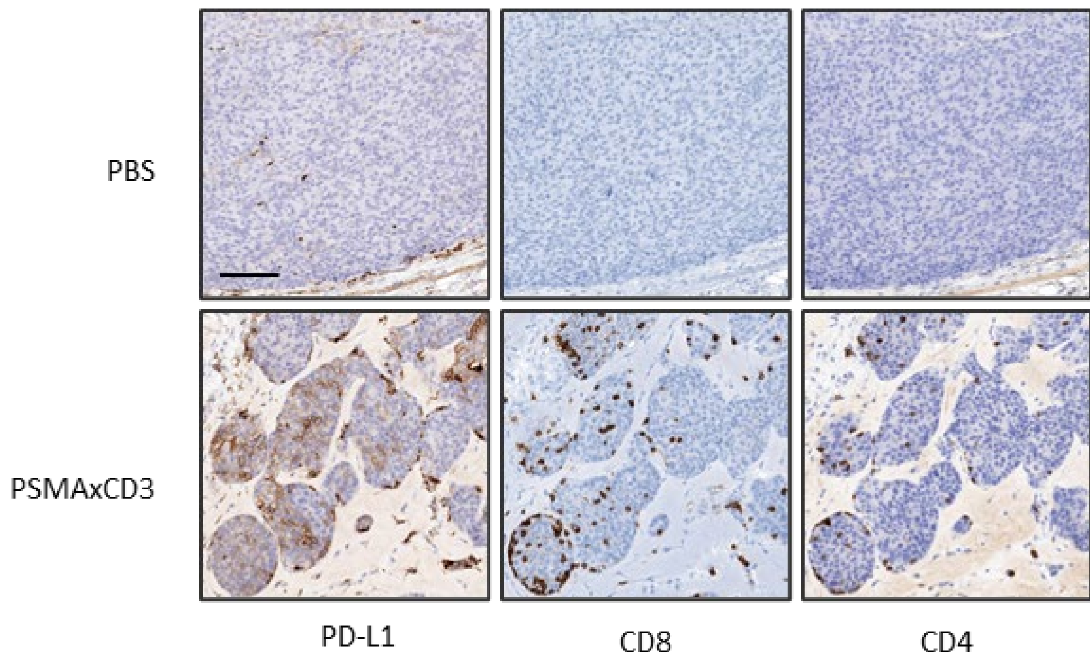


Figure 11. Immunohistochemistry of PD-L1, CD8 and CD4 in LNCaP Xenografts Treated With PSMAxCD3 Treatment in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; PSMA, prostate specific membrane antigen. Representative tumors are shown for each group with scale bar=100 microns. PSMAxCD3 treatment demonstrated enhanced CD4 and CD8 T cell infiltration into tumors and resulted in increased levels of PD-L1.

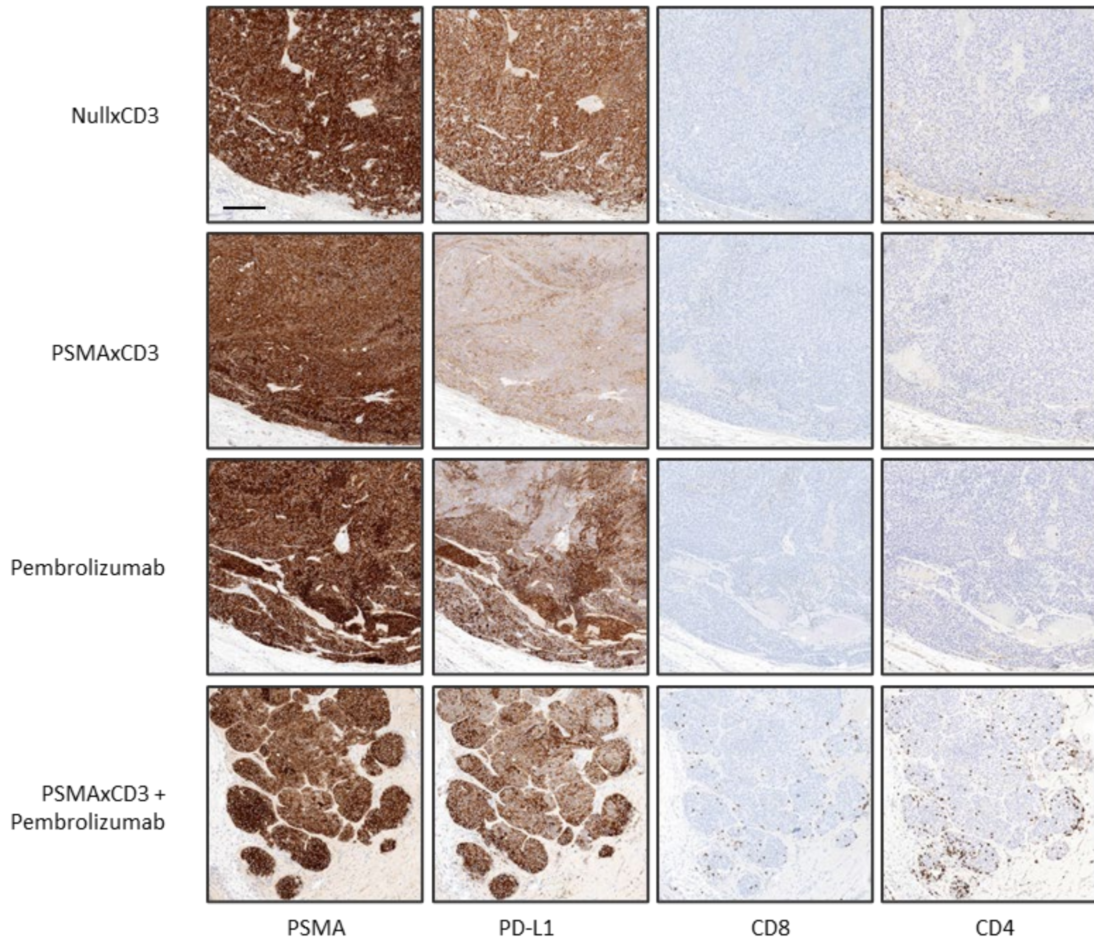


Figure 12. Immunohistochemistry of PSMA, PD-L1, CD8 and CD4 in LNCaP Xenografts Overexpressing PD-L1 Treated With PSMAxCD3 Treatment Alone or in Combination With Anti-PD-1 Pembrolizumab in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; PSMA, prostate specific membrane antigen. Representative tumor shown for each group with scale bar=250 microns. PSMAxCD3 treatment demonstrated enhanced CD4 and CD8 T cell infiltration into tumors without downmodulation of PSMA or PD-L1.

Due to the high variability of infiltration in the first assessment of treated LNCaP xenografts overexpressing PD-L1, the study was repeated with a larger sample size per timepoint (n=10). PSMAxCD3 treatment again resulted in immune and T cell infiltration in a subset of animals with a greater number of animals having enhancement of immune

cell infiltration in combination with pembrolizumab (Figure 13). Tumors having greater than 100 counts of infiltrating T cells were further gated on phenotype to explore if PSMAxCD3 treatment resulted in durable T cell phenotypes and if the combination with pembrolizumab further enhanced the phenotype or simply resulted in greater infiltration.

PSMAxCD3 treatment as a single agent or in combination with pembrolizumab resulted in CD4 and CD8 T cell infiltration with all animals demonstrating infiltration in the combination group (Figure 13). PSMAxCD3 treatment elicited an activated T cell phenotype with MHCII cell surface receptor DR (HLA-DR), inducible costimulatory molecule (ICOS), and PD-1 expression on CD4 and CD8 T cells (Figure 13). Again, combination with pembrolizumab resulted in a more consistent activated phenotype but not different than single agent PSMAxCD3 treatment. PSMAxCD3 treatment elicited a response from CD45- CCR7- effector memory T cells that was significantly higher than the population present in spleens of the control mice ($p<0.0005$, Figure 14). The expansion of effector memory T cells was also present in the animals receiving combination treatment with pembrolizumab and was also significant compared to control spleens. The T cell phenotyping results suggest that the enhancement in anti-tumor activity with the combination of PSMAxCD3 and pembrolizumab in PD-L1 overexpressing LNCaP xenografts results from an overall enhancement in infiltrating T cells and not a change in T cell phenotype.

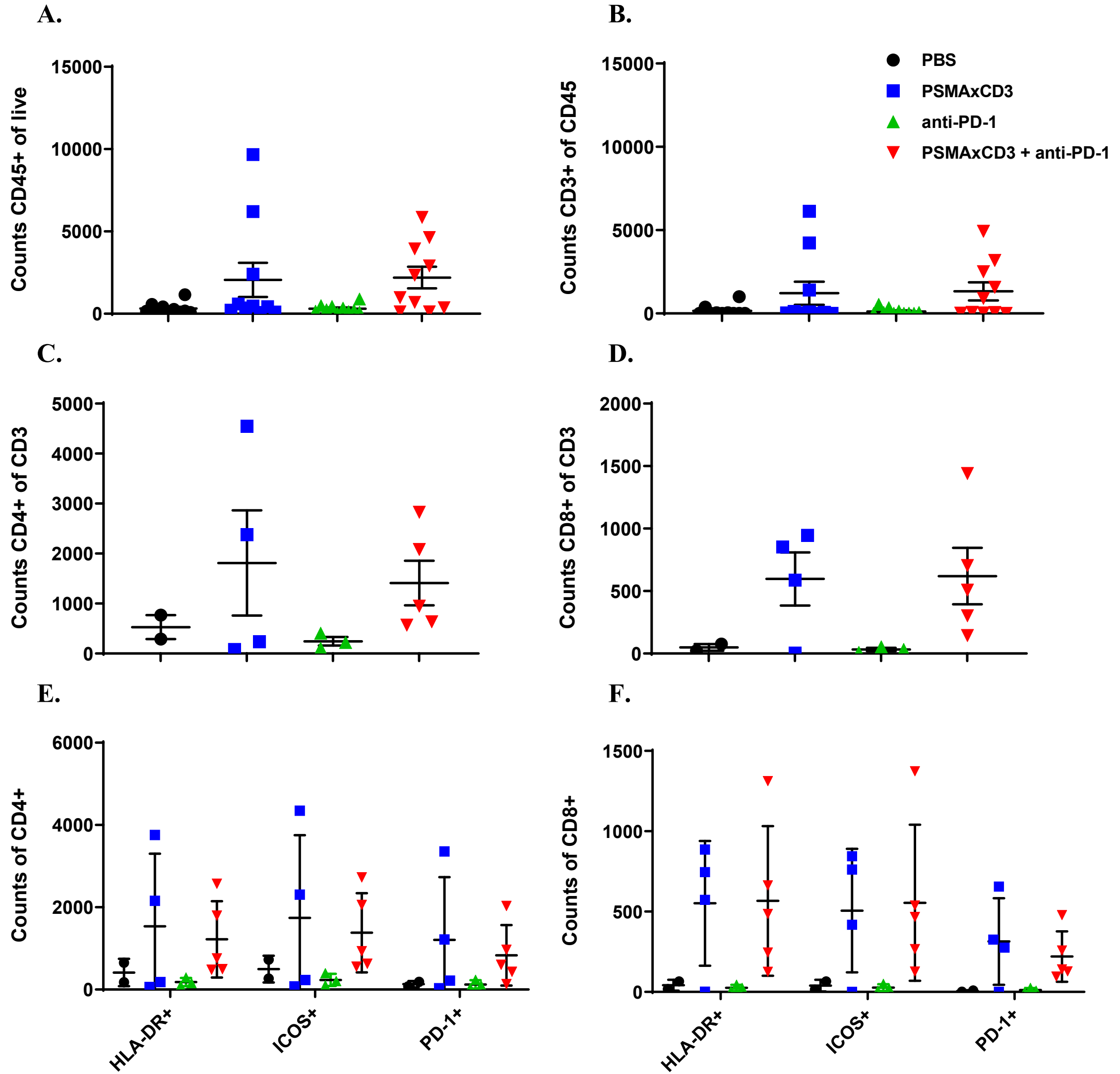


Figure 13. T Cell Infiltration (A-D) and Phenotype (E-F) Elicited from PSMAxCD3 Treatment Alone or in Combination With Anti-PD-1 Pembrolizumab in LNCaP Tumors with overexpression of PD-L1 in CD34+ HSC Humanized Mice.

HLA-DR, MHCII cell surface receptor DR ; HSC, hematopoetic stem cell; ICOS, inducible costimulatory molecule; PSMA, prostate specific membrane antigen; SEM, standard error of the mean. Individual counts of A-B CD45+ and CD3+ immune cells C-D CD4+ or CD8+ T cells and E-F HLA-DR+, ICOS+ and PD-1+ T cell subsets in the tumor are graphed with means ± SEM indicated by black lines (n=2-5). PSMAxCD3 treatment trended toward enhanced immune and T cell infiltration into tumors with an activated phenotype. All treatments p=ns compared to PBS control by ANOVA.

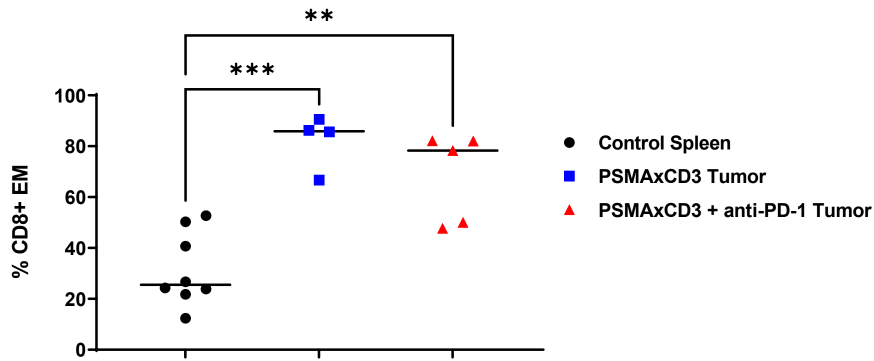


Figure 14. PSMAxCD3 Treatment Alone or in Combination With Anti-PD-1 Pembrolizumab Elicited a CD8+ Effector Memory T cell Response in LNCaP Tumors with overexpression of PD-L1 in CD34+ HSC Humanized Mice.

EM, effector memory; HSC, hematopoietic stem cell; PSMA, prostate specific membrane antigen. Individual percent CD8+ CD45- CCR7- are graphed with medians indicated by black lines (n=4-8). PSMAxCD3 treatment significantly increased CD8+ effector memory phenotype for PSMAxCD3 or PSMAxCD3 + pembrolizumab compared to control spleen (***p=0.0001, **p=0.0014, respectively by ANOVA).

Effects of PSMAxCD3 treatment on myeloid cell infiltration into LNCaP xenografts overexpressing PD-L1 were also assessed by flow cytometry (Figure 15). PSMAxCD3 treatment resulted in myeloid cell infiltration of CD19+ B cells, CD56+ natural killer (NK) cells as well as dendritic cells (DC) in a subset of animals. Combination with pembrolizumab demonstrated a trend of enhanced myeloid infiltration suggesting treatment can reverse an immune “cold” tumor and possibly recruit immune cells capable of eliciting tumor antigen specific immune responses.

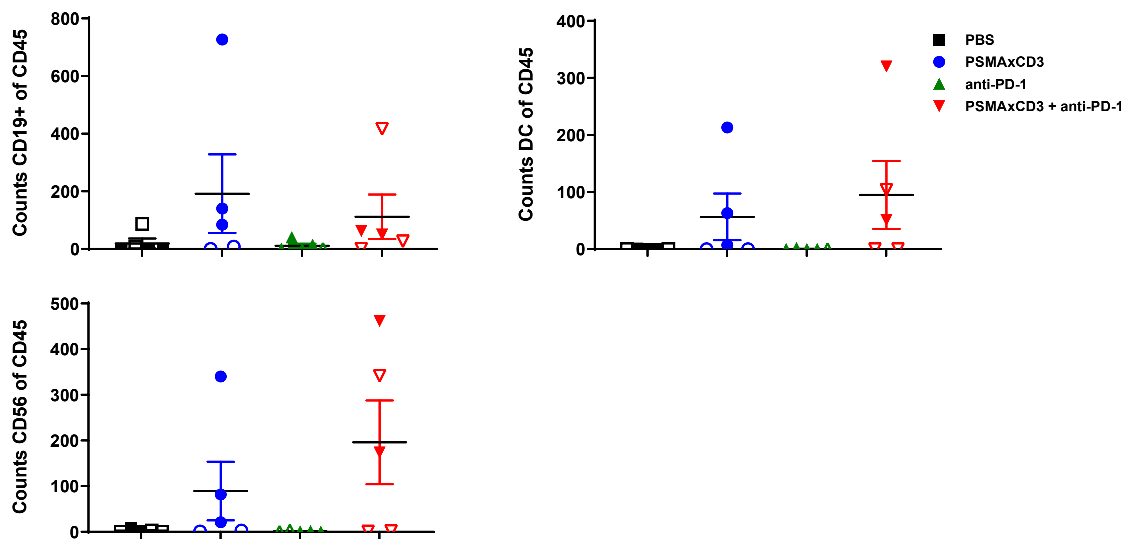


Figure 15. PSMAxCD3 Treatment Alone or in Combination With Anti-PD-1 Pembrolizumab Elicited Myeloid cell Infiltration in LNCaP Tumors with overexpression of PD-L1 in CD34+ HSC Humanized Mice.

DC, dendritic cell; HSC, hematopoietic stem cell; PSMA, prostate specific membrane antigen; SEM, standard error of the mean. Individual counts of CD19+ B cells, CD56+ NK cells or Dendritic cells were graphed with means ± SEM indicated by black lines (n=5). PSMAxCD3 treatment resulted in as trend of enhanced myeloid infiltration into tumors. All treatments p=ns compared to PBS control by ANOVA.

Durable T cell responses were assessed in animals whose treatment elicited complete responses by evaluating immune rejection of re-challenged tumors. To enhance the number of complete responders, groups of 30 animals were treated with PSMAxCD3 alone or in combination with pembrolizumab after animals were injected with 10e5 CD34+ cord blood cells from 3 donors one day post sublethal irradiation dose of approximately 100 centigray (cGy). PSMAxCD3 treatment was initiated when tumors were established in mice where engraftment of human immune system was verified both by human CD45+ cells and T cells in the peripheral blood 16 days post LNCaP spheroid implant and 14 weeks post CD34+ engraftment. After 4 doses of PSMAxCD3 in combination with pembrolizumab, statistically significant inhibition of tumors as assessed by change in growth rate over time was observed compared to control treatment

resulting in 56% TGI ($p<0.0001$, Figure 16). Single agent treatment of PSMAxCD3 did not result in any complete tumor responses but did result in long term tumor growth stasis in 4 animals whose tumors did not exceed 600 mm^3 at the end of the study. Of 30 animals treated with the combination of PSMAxCD3 and pembrolizumab, 6 animals were tumor free and 1 had a tumor below 70 mm3 on Day 45.

These responding animals were removed from treatment for 2 weeks with their last dose on Day 28 to ensure antibody washout time and then rechallenged with LNCaP spheroids overexpressing PD-L1 on Day 45. As a control, 15 tumor-naïve, donor-matched CD34+ engrafted NOG-EXL mice were implanted with LNCaP spheroids overexpressing PD-L1 on Day 45. Mice previously receiving combination treatment of PSMAxCD3 and pembrolizumab had statistically significant inhibition of tumor re-challenge as assessed by change in mean tumor burden on Day 77 compared to the tumor-naïve mice with 48% TGI suggesting lasting T cell responses against PSMA expressing tumors ($p=0.0017$, Figure 16A-B).

Re-challenged mice were also assessed for durability of primary tumor responses wherein 2 animals maintained complete tumor inhibition for over 35 days, 2 animals tumors outgrew but were declining at the end of the study and 2 animals whose tumors were actively outgrowing (Figure 16C-D). Similarly, there were a range of responses against the re-challenged tumors where some animals exhibited long-term control of tumor growth while others' tumors outgrew similarly to tumor-naïve controls (Figure 16E-F). These results demonstrate an advantage in long-term durable antitumor responses with combination therapy of PSMAxCD3 and pembrolizumab; however, previously

treated mice did not show a complete immune response against re-challenged tumors suggesting additional combinations may be needed in the clinic.

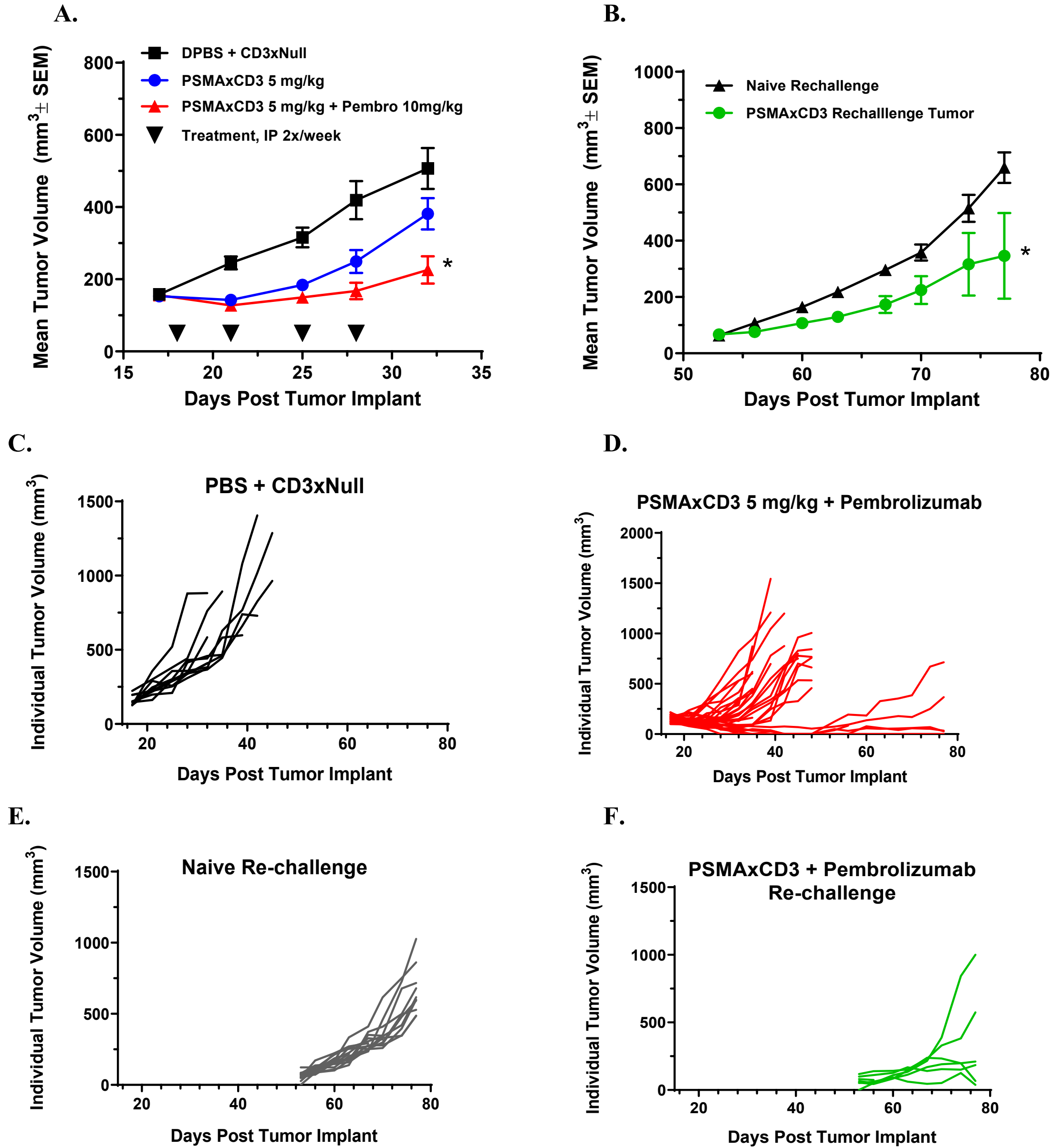


Figure 16. Antitumor Responses Elicited from Combination Treatment with PSMAxCD3 and Pembrolizumab Demonstrate Protection Against Tumor Re-challenge in CD34+ HSC Humanized Mice.

HSC, hematopoietic stem cell; IP, intraperitoneally; LME, linear mixed effects: MMRM, mixed model repeated measures; PSMA, prostate specific membrane antigen. Group tumor volumes of (A) original LNCaP xenograft overexpressing PD-L1and (B) re-challenged xenografts implanted on Day 0 and 45, respectively, are graphed as the mean ± SEM (A n=10-30, B n=7-15). Treatment IP twice weekly on Days 18, 21, 25, and 28 as represented by black triangles. *Denotes significant difference ($p<0.005$) of growth rates of PSMAxCD3 compared to controls by LME or MMRM on Day 34 or 38, respectively. **(**C-D) Individual tumor volumes are graphed. Previous PSMAxCD3 treatment significantly protected against tumor rechallenge.

2.5. DISCUSSION

Clinical trials with CD3 redirectors in solid tumors are still in early stages and the durability of T cell responses remains to be demonstrated. Additionally, it has been reported that solid tumors have tumor microenvironments that can suppress T cell responses, including tumor upregulation of PD-L1 in response to therapy.[26,27] Clinical data with the CD3 redirector blinatumomab has shown upregulation of PD-L1 in response to treatment, suggesting a resistance mechanism.[12] In the current studies, we investigated the potential for PSMAxCD3 treatment to elicit durable T cell responses in PSMA+ xenografts in CD34+ engrafted immunocompromised mice. We demonstrated anti-tumor efficacy of PSMAxCD3 against LuCaP86.2 PDX and LNCaP xenograft tumors that elicited T cell infiltration and change in T cell phenotype. Additionally, we showed that overexpression of PD-L1 on LNCaP prostate xenografts resulted in resistance to PSMAxCD3 treatment and tumor infiltration of T cells, but that efficacy was restored when combined with PD-1 blockade therapy.

Our studies demonstrated that treatment with PSMAxCD3 elicited a range of antitumor responses with some animals exhibiting complete responses. Unlike previous studies utilizing mice engrafted with human PBMCs or T cells, the CD34 engrafted model demonstrates donor variability in immune responses that may be more clinically relevant. We showed that in the most robust antitumor responses elicited by PSMAxCD3 against LuCaP86.2 tumors, complete responders elicited effector memory T cell responses that protected against rechallenge of tumors. This result is consistent with clinical data that has previously shown correlation between treatment response and effector memory T cell expansion.[51]

Similar to the antitumor efficacy observed in LuCaP86.2 tumors, PSMAxCD3 treatment also inhibited growth of LNCaP tumors resulting in T cell infiltration. Although we observed increased levels of PD-L1 on tumors after treatment with PSMAxCD3, we observed no enhanced antitumor effects when combined with PD-1 blockade. In contrast, when LNCaP tumors were transduced to overexpress PD-L1, we observed reduced anti-tumor activity with PSMAxCD3 as well as decreased number of T cells in the tumor confirming this as a model resistant to T cell mediated treatments. Combination treatment of PSMAxCD3 with PD-1 blockade restored antitumor efficacy and T cell infiltration. These results are consistent with other studies showing enhancement of antitumor effects of CD3 redirecting bispecifics and PSMA-targeting chimeric antigen receptor cell therapies with CBI therapy.[1,40,45] A recent clinical case report regarding PSMAxCD3 bispecific JNJ-63898081 treatment re-sensitizing a metastatic CRPC to pembrolizumab suggests that the CBI combination treatment approach holds promise.[43]

Our results demonstrated that in addition to increasing numbers of tumor infiltrating immune cells, treatment with PSMAxCD3 could impact phenotype of T cells. We observed increases in activation-associated markers including HLA-DR, ICOS and PD-1 on both CD4 and CD8 T cells. Upregulation of PD-1 and HLA-DR has been shown to occur after activation of T cells and inducible costimulatory molecule ICOS expression has been associated with T cell activation and enhanced survival.[21,22] Additionally, significant increases in effector memory T cells were observed in the LNCaP xenografts overexpressing PD-L1. Although the combination of PD-1 blockade with PSMAxCD3 resulted in enhanced tumor growth control, no changes in T cell phenotype were

observed. This suggests the tumor control was not associated with enhanced activation or memory phenotype. These findings are consistent with other efforts to combine immunotherapies such as a DNA encoding vaccine targeting prostatic acid phosphatase (PAP) with PD-1 blockade in metastatic castrate resistant prostate cancer where treatment enhanced T cell responses but did not result in a phenotype change.[34] Other studies have shown that combinations of CD3 redirectors with CBI may also reduce regulatory T cells which could be an explanation for the enhanced efficacy.[5] The induction of regulatory T cells has also been demonstrated as a clinical resistance mechanism and thus this could provide good rationale for combining T cell redirectors with CBI clinically.[10] Combination of PSMAxCD3 also resulted in some complete tumor responses in the PD-L1 overexpressing tumor model and although suppression of growth of tumor re-challenge was observed, the T cell responses elicited did not prevent tumor take suggesting additional combinations may be needed for durable clinical responses. Recent studies combining CD3 redirectors with antibodies targeting T cell co-stimulatory pathways have recently shown success at completely regressing tumors and subsequently preventing tumor re-challenge.[1,5]

In addition to modulating T cell infiltration, activation, and phenotype, we showed that PSMAxCD3 treatment also elicited tumor infiltration of myeloid cells including B cells, NK cells and dendritic cells. Evaluation of myeloid immune infiltration has not previously been reported in human immune cell engrafted models due to lack of myeloid cell reconstitution. This suggests that PSMAxCD3 treatment can modify immune “cold” prostate cancers with low mutational burden to allow for enhanced immune function against the tumor, and allowing for combination efficacy with CBI.

Further characterization of myeloid responses might reveal if the infiltrating myeloid cells are also modified towards a less suppressive phenotype and if infiltrating dendritic cells are recognizing cancer-associated neoantigens and resulting in efficacy from epitope spreading.

The present studies demonstrated tumor control and durable anti-tumor responses in prostate xenograft models treated with PSMAxCD3 which elicited immune responses that could inhibit growth of rechallenged tumors. We demonstrated that tumor upregulation of PD-L1 inhibited T cells responses elicited by PSMAxCD3 treatment and that combination with PD-1 blockade could restore antitumor activity. Although the combination of PSMAxCD3 with PD-1 blockade enriched for effector memory T cells with activated T cell responses that suppressed regrowth of tumors, rechallenged tumors were not completely inhibited suggesting further combinations may be needed for robust durable T cell responses in the clinic.

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CHAPTER 3: EFFECT OF TUMOR MICROENVIRONMENT ON T CELL RESPONSES ELICITED BY PSMAXCD3 BISPECIFIC ANTIBODY IN HUMAN CD3E KNOCK-IN MICE

3.1. ABSTRACT

PSMA is overexpressed on prostate cancer, the most common cancer in men, with increasing levels corresponding to worsening disease. CD3 redirection bispecific antibodies targeting mCRPC are currently being evaluated clinically; however, minimal antitumor responses have so far been observed. This may be due to possible suppressive tumor microenvironments and lack of immune cell infiltration into solid tumor masses. It is unclear whether bispecific antibodies redirecting T cells can elicit durable antitumor responses as a monotherapy or whether combination strategies will be needed to overcome suppressive TME. In the current studies we demonstrate more robust efficacy of PSMA targeted CD3 redirection of T cells in immune "hot" CT26 tumors expressing human PSMA than in immune "cold" TRAMP.C2 tumors expressing mouse PSMA. PSMAxCD3 elicited some durable responses against immune "hot" tumors with T cell infiltration of activated, effector memory CD8+ T cells with active effector function which remained mostly unchanged with CBI combination, although more complete responses were observed with greater T cell infiltration. In contrast, minimal intratumor T cell infiltration was observed, even in combination with CBI therapy, despite expansion of CD8+ T cell effector memory cells suggesting suppressive TME may require further combination with therapies that can improve T cell trafficking to tumors.

3.2. INTRODUCTION

Prostate cancer is the most common cancer in men and despite standard of care treatments, 30-40% of patients become hormone refractory and relapse.[2,3,49,52] PSMA is a transmembrane protein normally expressed on epithelial cells in prostate tissue that is overexpressed in prostate cancer.[2,3,49,52] Expression of PSMA expression correlates with increasing stage/grade of disease thus making it an ideal target to explore for immunological therapies targeting advanced castrate-resistant prostate cancer (CRPC).[55] T cell redirection bispecific antibodies targeting PSMA on prostate cancer cells and CD3 on T cells have shown robust antitumor activity preclinically in xenograft models using engraftment of human effector PBMC or T cells.[7,13,18] Although preclinical data looked promising, limited anti-tumor efficacy has been observed clinically and overall targeting of solid tumors with CD3 bispecifics has proven challenging.[5,19,29,34,52]

CD3 redirection antibodies have shown impressive overall survival rates in hematological malignancies and patients with the best responses had expansion of effector memory CD8+ T-cells, whereas poor responders had elevated recruitment of regulatory T cells that suppress T cell effector function.[8,11,16,48,50,51] Unlike hematological cancers, solid tumors may be more difficult to treat with T cell redirectors due to an immune suppressive environment and lack of T cell infiltration into the solid tumor mass.[6,14,22,30,34,35,47] The tumor microenvironment in solid tumors can be characterized in terms of immune cell infiltration status from immune "hot" with more immune infiltration to "cold" or "immune excluded" with a lack of immune cells and stromal components blocking infiltration.[14,39] Sensitivity to T cell checkpoint blockade inhibitor (CBI) therapies in the clinic has been correlated with immune infiltrate in solid tumors

suggesting the need for T cell trafficking to the tumor site for CD3 redirectors as well.[32,39,41]

Syngeneic models representing immunologically "hot" and "cold" tumor environments can be used to assess the contribution of an immune suppressive TME on prostate targeting CD3 redirection treatment. The colon CT26 model has been historically well characterized as responsive to CBI therapy and thus provides an ideal model representing an immune-responsive tumor model.[27,36,44] In contrast, TRAMP.C2 tumors generated from a cell line derived from the transgenic adenocarcinoma of the mouse prostate (TRAMP) model represent immunologically cold prostate tumors that do not respond well to most CBI therapies.[12,36,45,56] These tumor models will help model potential responder and non-responder patient subsets and allow for the comparison of efficacy and T cell phenotype in different TME settings.

In the current studies, we assessed the anti-tumor effect and T cell phenotype elicited from PSMAxCD3 treatment in immunologically "hot" colon CT26 syngeneic tumors expressing human PSMA (CT26/hPSMA) as well as "cold" mouse prostate TRAMP.C2 syngeneic tumors expressing mouse PSMA (TRAMP.C2/mPSMA). We investigated whether the combination of PSMAxCD3 with CBI would enhance efficacy compared to monotherapy and elicit durable T cell responses. PSMAxCD3 treatment elicited more robust tumor control in the immunologically "hot" CT26 model which elicited greater immune infiltrate. In contrast, minimal efficacy was observed in the immune "cold" prostate TRAMP.C2 model with lower tumor immune infiltrate. Combination with CBI therapies anti-PD-1 and anti-CTLA-4 did not confer enhanced anti-tumor control in the CBI sensitive CT26 model as compared to CBI treatment alone

but did confer protection against tumor re-challenge. Some combination benefit was observed with combination with anti-CD40 treatment in the TRAMP.C2 model with respect to tumor volume inhibition during treatment; however, this ultimately did not translate into a survival benefit.

3.3. MATERIALS AND METHODS

PSMAxCD3 Bispecific Duobody® antibody

The PSMAxCD3 antibody, JNJ-63898081, is a IgG4-proline-proline-alanine (PAA) bispecific DuoBody® antibody (PAA mutation for reduced affinity to Fc gamma receptors and nearly silent Fc-effector functionality) that binds to PSMA and human cluster of differentiation CD3.[25] The mouse PSMAxCD3 is a mouse silent IgG2a antibody (AAS mutation for reduced affinity to Fc gamma receptors and nearly silent Fc-effector functionality) targeting mouse PSMA and human CD3. The anti-PSMA Fc monovalent arms were directed against the extracellular domain of either human or mouse PSMA and were discovered by phage display panning. The anti-CD3ε monovalent Fc arm B219, was derived from a SP34 antibody clone.[39] The human PSMA, mouse PSMA, and human CD3 mono-targeting arms have tight binding to cells, with low nM affinities and the mouse PSMA arm had comparable binding to JNJ-63898081.[29,30] The human PSMA and CD3-mono-targeting arms lack cross-reactivity to mouse proteins.

Tumor cell lines

The mouse colorectal tumor cell line CT26 was obtained from ATCC. Human PSMA expression was achieved through transduction of CT26 cells with lentivirus containing PSMA DNA resulting in a low expression of about 1430 PSMA molecules per

cell based on Quantibrite bead assessment by flow cytometry (unpublished data not shown). The mouse prostate TRAMP.C2 model was obtained from the University of Texas M.D. Anderson Cancer Center. Mouse PSMA expression was achieved through transduction of TRAMP.C2 cells with lentivirus containing PSMA DNA resulting and PSMA expression was confirmed by ex vivo bivalent mPSMA binding on dissociated tumor cells (data not shown). Cell lines were authenticated and tested by Analytical Biological Services, Inc. and all infectious panel testing was negative.

Syngeneic Tumor Models

Female or male CD3 transgenic knock-in (KI) mice were used when they were approximately 5-10 weeks of age. To generate the CD3 KI transgenic mice, two approaches were used; first the human extracellular N-terminal binding domain of CD3e was inserted to replace the mouse exon 1 sequence in Balb/c mice (hCD3e), and second, exons 1-7 were inserted to replace exons 3-5 of the mouse CD3e in C57Bl/6 mice (Biocytogen, B-hCD3e) (Figure 17). The mouse T cells in these mice have been shown to express the human CD3e and are capable of binding to a human bispecific CD3 redirection antibody with an N-terminal CD3 binder (data not shown).

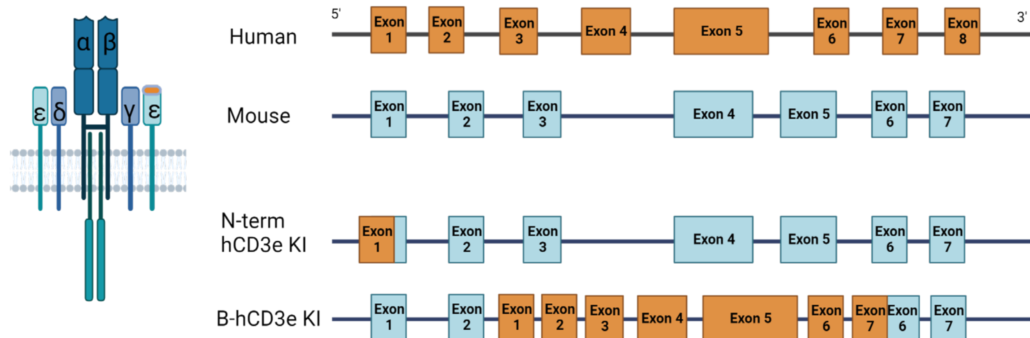

Figure 17. Development of CD3e KI Mice. Schematic of transgenic modifications to express human CD3e on mouse T cells either by expressing only the human (orange) N-terminus region by replacing mouse (blue) exon 1 as in the N-term hCD3e KI schematic and the T cell receptor schematic on the left or by expressing the full length CD3e as in the Biocytogen (B)-hCD3e KI. Created with BioRender.com

All animals were allowed to acclimate and recover from any shipping-related stress for a minimum of 5 days before experimental use. Autoclaved water and irradiated food were provided ad libitum, and the animals were maintained on a 12-hour light and dark cycle. Cages, bedding, and water bottles were autoclaved before use and changed weekly. All experiments were carried out in accordance with *The Guide for the Care and Use of Laboratory Animals,*[38] and the USA Animal Welfare Act. Protocols were approved by the local ethics committees of Janssen Pharmaceuticals, Spring House, PA.

CT26 cells expressing human PSMA and TRAMP.C2 cells expressing mouse PSMA were propagated in log phase in RPMI 1640 media with 1X sodium pyruvate, 1X non-essential amino acids, and 10% heat inactivated FBS and RPMI 1640 media with 1X Glutamax and 10% heat inactivated FBS, respectively and enzymatically dissociated using TrypLE™ (Gibco by Life Technologies, Cat #12563-029). 1e6 CT26/hPSMA or 5e6 TRAMP.C2/mPSMA cells were injected on Day 0 of the study.

Table 3: **Syngeneic Model Systems.**

Bispecific	Tumor Line	Model	Mouse Strain	CD3e KI mice
hPSMAxhCD3 on human IgG4 PAA	CT26/hPSMA	Mouse colon adenocarcinoma	Balb/c (female)	N-term hCD3e
mPSMAxhCD3 on mouse IgG2a AAS	TRAMP.C2/mPSMA	Mouse prostate adenocarcinoma	C57Bl/6 (male)	B-hCD3e

Animals were randomized into study by tumor volume, such that group means or distributions were similar. CD3xNull (Janssen R&D), PSMAxCD3 (JNJ-63898081, Janssen R&D), Isotype Control (Janssen R&D), anti-mouse PD-1 (mIgG2a PAA, Janssen R&D), anti-mouse CTLA-4 (mIgG2a, InvivoGen, mctla4-mab10-b), or anti-mouse CD40 (BioXCell, clone FGK4.5) were administered IP twice a week according to body weight (10 mL/kg).

Body weight and SC tumor volume were measured for each animal twice a week throughout the study. Animals were monitored daily for clinical signs and tumor burden. When individual animals exhibited negative clinical signs, such as lethargy, ruffled and matted coat, hunched posture, cyanotic extremities, or dyspnea, or reached 20% body weight loss as compared with initial body weights, they were removed from the study and humanely euthanized. Animals were removed when a maximum tumor volume of ≥1,000 mm^3 was reached, or when adverse clinical signs were noted.

Tumor and tissue processing

Tumors from Study were placed in C tubes containing 2.35 mL of RPMI 1640 medium for processing. Tumors were cut into 1 to 2 mm pieces in the C tubes, and 122.5 µL of an enzyme cocktail from the Tumor Dissociation Kit (Cat# 130-096-730) was added to each tube. Tubes were placed in the Gentle MACS Octo Dissociator with Heaters (Miltenyi) and processed using the manufacturer's setting "37C_m_TDK_1".

Tumor cell suspensions were filtered through 70-µm cell strainers into media. Similarly, spleens were filtered through 70-µm cell strainers with syringe plungers into media. Tubes were centrifuged at 1,500 revolutions per minute (rpm) for 5 minutes, cell supernatant was aspirated, and cell pellets were resuspended in complete culture medium. Samples were lysed for red blood cells using ACK lysis buffer (Gibco) with 1-3 rounds of 3-minute incubation of 200 µL per well followed by 3 cycles of washing and centrifuging. Cell count and viability of each sample were determined using a Vi-Cell counter (Beckman Coulter). Samples were plated with 1×10^6 viable cells in a 96-well round-bottom plate and centrifuged at 1,500 rpm for 3 minutes. Cell supernatant was discarded, and then cells were stained for flow cytometry analysis.

Flow cytometry and antibodies

Processed tumors or spleens were stained with 50 µL per well of PBS containing Fc block and LIVE/DEAD™ stain. Plates were incubated in the dark for 15 minutes at RT. Samples were washed with 150 µL of cold stain buffer and centrifuged for 3 cycles, as described above for tumor cell preparation. Stain buffer (50 µL) containing antibodies for cell surface antigens (Table 4) was added to each sample well and the plates were incubated in the dark for at least 30 minutes on ice. Samples were washed with cold stain buffer and centrifuged for 3 cycles and then fixed and permeabilized for 20 min on ice (T cell or Myeloid). For the T cell panel, samples were washed with cold 1X BD Perm/Wash buffer and centrifuged for 3 cycles and then 1X BD Perm/Wash buffer (50 µL) containing antibodies specific for intracellular markers (Granzyme B, CTLA-4, see Table 4) was added to sample wells for each panel and the plates were incubated in

the dark for at least 30 minutes on ice. Plates were then washed and centrifuged for 3 cycles, as described above.

Table 4: Mouse Flow Cytometry Panel Reagents.

Marker/fluorochrome	Clone	Source	Catalog number	Dilution
CD45/BUV395	30-F11	ThermoFisher	564279	1:50
CD4/BV650	GK1.5	BD Biosciences	100469	1:50
CD8/FITC	53-6.7	Biolegend	11-0081-85	1:50
CD90.2/BV605	53-2.1	eBio	140317	1:50
PD-1/BV785	29F.1A12	Biolegend	135225	1:50
TIM-3/APC	8B.2C12	Biolegend	17-5871-82	1:50
CD25/AF700	PC61	eBio	102024	1:50
CD137/PE	17B5	Biolegend	106106	1:50
CD44/PerCP-Cy5.5	IM7	Biolegend	103032	1:50
CD62L/PE-Cy7	MEL-14	Biolegend	104418	1:50
GzB/PE-CF594	QA16A02	BD Biosciences	372216	1:15
CTLA-4/BV421	UC10-4B9	Biolegend	106312	1:50
CD11c/BV711	N418	Biolegend	117349	1:50
NKp46 (CD335)/BV711	29A1.4	Biolegend	108745	1:50
CD11b/BV711	M1/70	Biolegend	101242	1:50
CD19/BV711	1D3	BD Biosciences	563157	1:50
CD45/BUV395	30-F11	BD Biosciences	564279	1:50
CD11b/BV650	M1/70	Biolegend	101239	1:20
NKp46/FITC	29A1.4	Biolegend	137606	1:100
B220/BV605	RA3-6B2	BD Biosciences	563708	1:50
CD11c/BV785	N418	Biolegend	117335	1:15
CD86/APC	GL-1	Biolegend	105012	1:50
Ly6C/AF700	HK1.4	Biolegend	128024	1:100
PD-L1/PE	10F.9G2	Biolegend	124308	1:50
CD80/PerCP-Cy5.5	16-10A1	Biolegend	104722	1:50
CD86/PE-Cy7	GL-1	Biolegend	105014	1:50
Ly6G/BV421	1A8	Biolegend	127628	1:50
CD90.2/BV711	53-2.1	BD Biosciences	740647	1:50
LIVE/DEAD™ Aqua	NA	Thermo Fisher	L34976	NA

AF, Alexa Fluor; BB, brilliant blue; BUV, brilliant ultraviolet; BV, brilliant violet; CD, cluster of differentiation; CTLA4, cytotoxic T-lymphocyte associated protein 4; Cy, cyanine; human; not applicable; GzB, Granzyme B; Ly6, lymphocyte antigen 6; NK, natural killer; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; PE, phycoerythrin; PE-CF594, phycoerythrin-Clear Fluor 594; TIM-3, T-cell immunoglobulin and mucin-domain containing-3

Flow cytometry panels were created using selected reagents from the above table.

All flow cytometry samples were resuspended in stain buffer (200 µL/well) and analyzed using the Fortessa flow cytometer (BD Biosciences). Raw flow cytometry data were further analyzed using FlowJo software (BD Biosciences, Version 10). The gating strategy for the T cell panel was the following: cells→ singlets→ live cells→ mouse

$CD45^+$ tumor infiltrating immune cells or mouse CD45- non-immune tumor cells excluding the myeloid dump channel BV711. $CD45^+$ cells were gated further on CD90.2+ and then on $CD4^+$ or $CD8^+$ T cells. $CD4^+$ or $CD8^+$ T cells were further gated on subsequent T cell markers (ie. CD25, PD-1, TIM3, CD137, ect.). CD45- tumor cells were gated on PD-L1 and PSMA expression. The gating strategy for the myeloid panel was the following: cells→ singlets→ live cells→ mouse $CD45^+$ tumor infiltrating immune cells or mouse CD45- non-immune tumor cells excluding the T cell dump channel BV711. $CD45^+$ $CD90.2^-$ cells were gated further on $B220^+$, $CD11b^+$, $CD11c^+$, and NKp46. Tumor associated MSDCs, TAMs and Neutrophils were gated from $CD11b^+$ on $Ly6C^{hi}$, $Ly6C^{lo}$, and $Ly6G^+$, respectively, and were further gated on $CD80^+$, $CD86^+$, and $PD\text{-}L1^+$. CD45- tumor cells were gated on $PD\text{-}L1^+$. We used a gating strategy previously described in the literature to distinguish naïve/memory T cell subsets.[37] The percentage or counts/mg of tumor of immune cells in each sample was graphed in Prism.

Calculations and Statistics

Body weights and tumor volumes were collected twice weekly. SC tumor volume was calculated using the formula: Tumor volume (mm^3) = ($D \times d^2/2$); where 'D' represents the larger diameter, and 'd' the smaller diameter of the tumor as determined by caliper measurements. Body weight changes of individual mice were calculated using the formula: $([W-W_0]/W_0) \times 100$, where 'W' represents body weight on a particular day, and 'W_0' represents body weight at initiation of treatment. Tumor volume and body weight data were graphed while at least two thirds of the animals remained in each group. All data were graphed using Graph Pad Prism.

The percent TGI was defined as the difference between mean tumor volumes of the treated and control groups, calculated as % TGI = $((TV_c-TV_t)/TV_c)\times100$ where 'TV_c' is the mean tumor volume of the control group and 'TV_t' is the mean tumor volume of the treatment group. A complete response (CR) was defined as complete tumor regression, with no palpable tumor.

Statistical significance for tumor growth inhibition was evaluated for treatment groups compared with the CD3xNull and Isotype control treated groups or for combination treatment groups compared to single agent therapies. Differences between groups were considered significant when $p\leq0.05$. Statistical significance for was calculated either using the linear mixed-effects analysis, with treatment and time as fixed effects and animal as random effect, or using post-hoc analysis from a mixed model for repeated measures (MMRM) with the fixed effects group, time (as a factor), and the interaction between group and time and a random effect for subject comparing mean tumor burdens at each measurement timepoint in R software version 3.4.2 (using Janssen's internally developed Shiny application version 4.0). Logarithmic transformation (base 10) was performed if individual longitudinal response trajectories were not linear. The information derived from this model was used to make pairwise treatment comparisons of animal body weights or tumor volumes to that of the control group or for combination treatments compared to the single agent treatments.

Statistical significance of terminal tumor volumes following rechallenge were evaluated for rechallenged group compared with naïve tumor challenge by unpaired T test. Statistical significance of T cell phenotypes was evaluated for treatment groups compared with tumors or spleens from the control treated group by one-way ANOVA followed by Dunnett's multiple comparisons test.

3.4. RESULTS

Efficacy of PSMAxCD3 in CBI sensitive murine colon CT26 tumor model expressing human PSMA

To assess durability of T cell responses elicited from PSMAxCD3 treatment in the context of a complete innate immune system, CT26 mouse colon tumor cells expressing human PSMA (CT26/huPSMA) were established in female hCD3e KI transgenic mice. CT26 syngeneic tumors have been described as immune checkpoint inhibition responsive with infiltration of cytotoxic immune cells including T cells and NK cells, thus representing a "hot" or immune responsive tumor setting.[27,36,44] In this immune responsive setting, effect of PSMAxCD3 treatment as a single agent therapy or in combination with anti-mouse PD-1 or anti-mouse CTLA-4 antibody therapy was evaluated on tumor growth and T cell phenotype.

Statistical significance of PSMAxCD3, JNJ-63898081, treatment alone or in combination with anti-PD-1 on CT26/huPSMA was assessed up to Day 21 when at least 9 of 10 animals remained in each group. Treatment with JNJ-63898081 at 5 or 10 mg/kg alone or in combination with anti-PD-1 at 10 mg/kg demonstrated significant inhibition of CT26/huPSMA tumor growth as assessed by change in growth rate over time compared to the control group treated with untargeted CD3 control bispecific antibody NullxCD3 and isotype control ($p<0.05$, Figure 18A). JNJ-63898081 treatment at 5 or 10 mg/kg resulted in 83% and 54% TGI, respectively, on Day 21 compared to controls with tumors regressing after the first 2 doses but ultimately continuing to grow out, possibly due to mouse anti-drug antibodies generated against the human Fc in human JNJ-63898081 (Figure 18C). Treatment with anti-PD-1 resulted in suboptimal 44% TGI

compared to controls. Although combination of JNJ-63898081 at 10 mg/kg with anti-PD-1 demonstrated a slightly enhanced anti-tumor effect, this was not significant, and the combination of JNJ-63898081 at 5 mg/kg with anti-PD-1 offered no advantage over single agent treatment.

In addition to inhibiting tumor growth up to day 21 when control animals remained, JNJ-63898081 treatment alone or in combination with anti-PD-1 statistically enhanced survival to tumor burden of 1000 mm3 ($p<0.05$, Figure 18B). JNJ-63898081 treatment at 5 or 10 mg/kg resulted in 33% and 24% increased life span (ILS), respectively, while anti-PD-1 treatment resulted in a suboptimal 14% ILS. Combination of JNJ-63898081 at either 5 or 10 mg/kg with anti-PD-1 did not demonstrate enhanced survival compared to single agent treatments; however, 1 complete response was observed with the high dose treatment of JNJ-63898081 and with combination of JNJ-63898081 at either dose with anti-PD-1 suggesting a trend of enhanced responses. No adverse effects of any treatment were observed on body weight (data not shown).

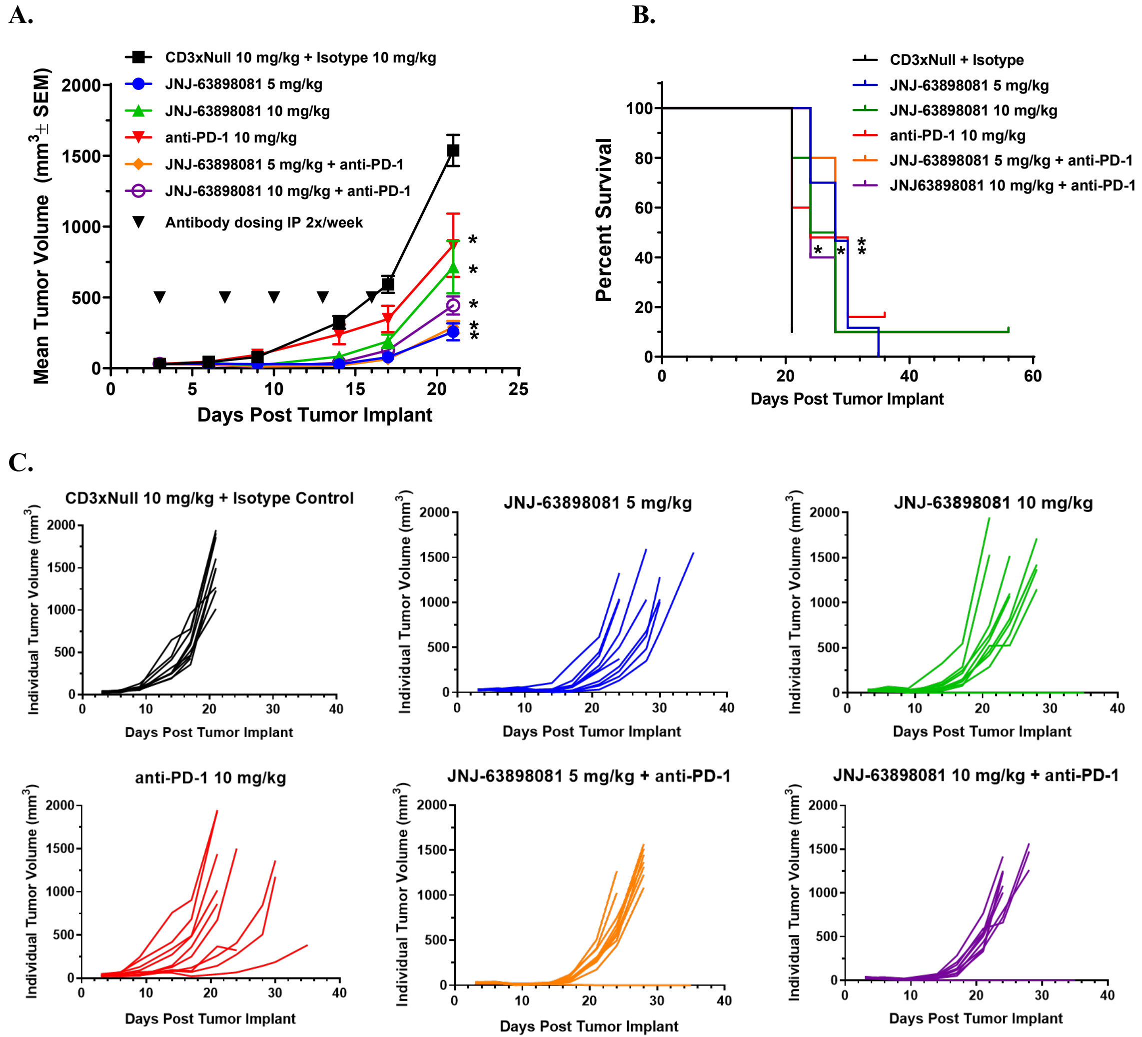

Figure 18. Effect of PSMAxCD3 alone or in combination with anti-PD-1 on Growth of CT26 Mouse Syngeneic Tumors Expressing Human PSMA in hCD3e KI Balb/c mice.

IP, intraperitoneally; KI, knock in; LME, linear mixed effects; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; SEM, standard error of the mean; TGI, tumor growth inhibition. A. Group tumor volumes are graphed as the mean ± SEM (n=10, mean data graphed when at least 9 mice remained in each group). Tumor cells were implanted on Day 0. Treatment IP twice weekly on Days 3, 6, 9, and 13, as represented by black triangles. All treatment groups significantly inhibited tumor growth (*p<0.05 by LME) as assessed by change in growth curves compared with CD3xNull + Isotype Control (control) group. B. Survival was plotted by Kaplan Meier. All treatment groups significantly enhanced survival (*p<0.05) as assessed by log rank test compared to control group. C. Individual tumor growth plots show delayed tumor growth in all groups with 1 complete regression each in groups treated with JNJ-63898081 at 10 mg/kg, JNJ-63898081 5 mg/kg + anti-PD-1 and JNJ-63898081 10 mg/kg + anti-PD-1.

Combination of PSMAxCD3 with anti-PD-1 and anti-CTLA-4 in CBI sensitive murine colon CT26 tumor model expressing human PSMA

Since the combination of JNJ-63898081 with anti-PD-1 did not elicit complete control of CT26/huPSMA tumors, anti-CTLA-4 was added to the double combination. Combination of anti-PD-1 and anti-CTLA-4 has been evaluated clinically and also reported to enhance memory T cell responses in combination with vaccine therapy and thus this approach was investigated here for combination with CD3 redirection.[9,54] Statistical significance of JNJ-63898081 treatment alone at 5 mg/kg or in combination with anti-CTLA-4 at 2.5 mg/kg or in triple combination with anti-PD-1 at 5 mg/kg and anti-CTLA-4 at 2.5 mg/kg on CT26/huPSMA was assessed up to Day 21 when at least 10 of 10 animals remained in each group. Treatment with JNJ-63898081 alone or in combination with anti-CTLA-4 or in triple combination with anti-PD-1 and anti-CTLA-4 demonstrated significant inhibition of CT26/huPSMA tumor growth as assessed by change in growth rate over time compared to the control group treated with untargeted CD3 control bispecific antibody NullxCD3 and isotype control ($p<0.003$, Figure 19A). JNJ-63898081 treatment resulted in 58% TGI compared to controls consistent with the previous study. Treatment with anti-CTLA-4 or anti-PD-1 and anti-CTLA-4 resulted in 88% and 96% TGI compared to controls. Combination of JNJ-63898081 with anti-CTLA-4 or the triple combination with anti-PD-1 and anti-CTLA-4 did not elicit enhanced activity when compared to treatment with anti-CTLA-4 or anti-PD-1 and anti-CTLA-4 and resulted in fewer complete responses (1 and 4 of 10 compared to 8 and 9 of 10, respectively) suggesting possible overactivation of T cells resulting in T cell death known as activation induced cell death (AICD) (Figure 19C).[17]

JNJ-63898081 treatment alone or in combination with anti-CTLA-4 or in triple combination with anti-PD-1 and anti-CTLA-4 statistically enhanced survival to tumor burden of 1000 mm3 ($p<0.005$, Figure 19B). Treatment with anti-CTLA-4, both double combinations and the triple combination resulted in greater than 63% increased life span (ILS). Similar to the effect on tumor growth inhibition, combination of JNJ-63898081 with anti-CTLA-4 or triple combination with anti-PD-1 and anti-CTLA-4 did not demonstrate enhanced survival compared to single agent treatments. No adverse effects of any treatment were observed on body weight (data not shown).

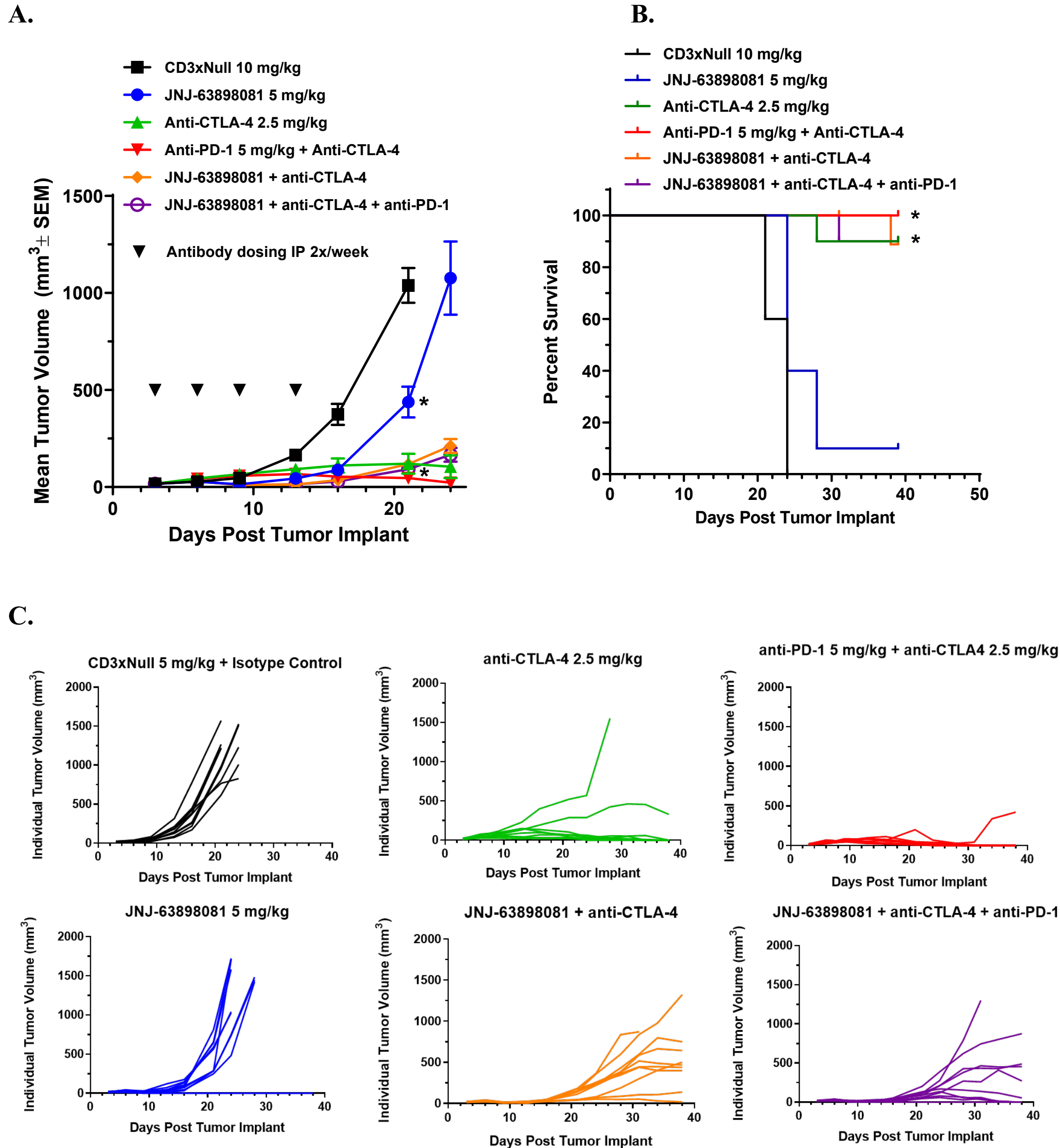

Figure 19. Effect of PSMAxCD3 alone or in combination with anti-CTLA-4 or in triple combination with anti-PD-1 and anti-CTLA-4 on Growth of CT26 Mouse Syngeneic Tumors Expressing Human PSMA in hCD3e KI Balb/c mice.

CTLA-4, cytotoxic T-lymphocyte associated protein-4; IP, intraperitoneally; KI, knock in; LME, linear mixed effects; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; SEM, standard error of the mean; TGI, tumor growth inhibition. A. Group tumor volumes are graphed as the mean ± SEM (n=10, mean data graphed when at least 10 mice remained in each group). Tumor cells were implanted on Day 0. Treatment IP twice weekly on Days 3, 6, 9, and 13, as represented by black triangles. All treatment groups significantly inhibited tumor growth (*p<0.003 by LME) as assessed by change in growth rates compared with CD3xNull + Isotype Control (control) group. B. Survival was plotted by Kaplan Meier. All treatment groups except JNJ-63898081 single agent significantly increased survival (*p<0.005) as assessed by log rank test compared with control group. C. Individual tumor growth plots show delayed tumor growth in all groups with 1 complete regression with JNJ- 63898081, 8/10 with anti-CTLA-4, 9/10 with anti-PD-1 + anti-CTLA-4, 1/10 with JNJ-63898081 + anti-CTLA-4 and 4/10 with JNJ-63898081 + anti-PD-1 + anti-CTLA-4.

PSMAxCD3 and combination of PSMAxCD3 with anti-PD-1 and anti-CTLA-4 induce memory T cell responses that protect against tumor rechallenge

To assess durability of T cell responses, animals treated with PSMAxCD3 monotherapy or double combination with anti-CTLA-4 or triple combination with anti-PD-1 and anti-CTLA-4 with complete responses of the initial CT26/hPSMA tumors were rechallenged with CT26/hPSMA cells on Day 52, 39 days post last treatment. The 1, 1 and 4 animals previously treated with PSMAxCD3 single agent, PSMAxCD3 in combination with anti-CTLA-4 and the triple combination of PSMAxCD3 with anti-CTLA-4 and anti-PD-1, respectively, rejected the CT26/hPSMA rechallenge demonstrating PSMAxCD3 alone or in combination with CBI can successfully form memory immune responses against the tumor (Figure 20). Treatment with anti-CTLA-4 and the combination of anti-PD-1 (n=5) also demonstrated anti-tumor immunity with complete rejection of rechallenged tumor suggesting the memory responses in combination groups could be elicited despite PSMA targeting.

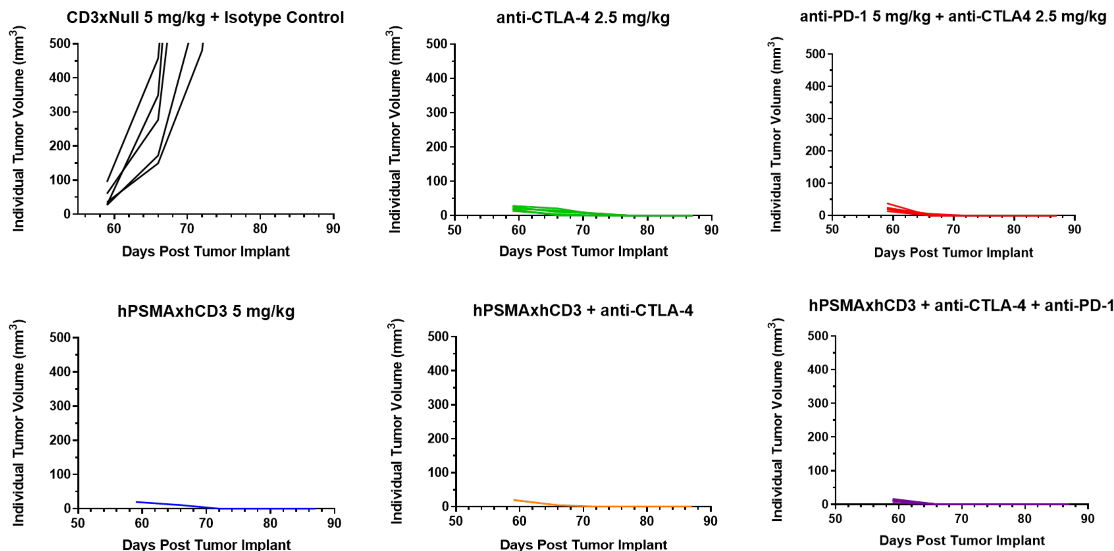

Figure 20. PSMAxCD3 alone or in combination with anti-CTLA-4 or in triple combination with anti-PD-1 and anti-CTLA-4 protects against rechallenge of CT26 Mouse Syngeneic Tumors Expressing Human PSMA in hCD3e KI Balb/c mice.

CTLA-4, cytotoxic T-lymphocyte associated protein-4; KI, knock in; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen. Individual tumor volumes graphed (n=1-5). Rechallenged tumor cells were implanted on Day 52. All treatment groups inhibited tumor growth of rechallenged tumors compared with tumor naïve control group.

Treatment with PSMAxCD3 results in T cell infiltration, activation and expansion of effector memory T cell phenotype in CT26/hPSMA tumors

Effect of treatment of PSMAxCD3 alone or in triple combination with anti-PD-1 and anti-CTLA-4 on T cell infiltration was assessed in CT26/hPSMA tumors. T cell phenotype was evaluated by activation and inhibition T cell markers (PD-1, CD-137, TIM3, CTLA-4), effector function (Granzyme B) and memory markers (CD62L, CD44).[20,28,37,43] Variable immune and T cell infiltration as assessed by CD45+ and CD3+ cells, respectively, was observed in response to PSMAxCD3 treatment alone or in combination with CBI (Figure 21A). Infiltration correlated to efficacy response with better T cell infiltration in animals with smaller tumors. A trend towards increased T cell infiltration was observed with PSMAxCD3 treatment alone or in combination with CBI

with an increase in both CD4+ and CD8+ T cells (Figure 21B). PSMAxCD3 treatment resulted in a trend of CD8+ T cell activation as assessed by CD137 and PD-1 and increased intracellular cytokine granzyme B (GzB) with a significant increase in intracellular CTLA-4 and T cell inhibitory marker TIM-3 as compared to NullxCD3 + Isotype control (Figure 21C). Combination of PSMAxCD3 with anti-PD-1 and anti-CTLA-4 resulted in significant CD8+ T cell intracellular GzB and otherwise had a similar phenotype as PSMAxCD3 alone treatment (Figure 21E). Similar trends were observed in CD4+ T cells with significant CD137+ activation and increased intracellular CTLA-4 as compared to NullxCD3 + Isotype control (Figure 21C). T cell phenotypes demonstrate activation of T cells with upregulation of inhibitory markers in response to PSMAxCD3 treatment and combination with CBI did not dramatically alter these phenotypes. PSMAxCD3 treatment increased the number of terminally differentiated effector memory (TEMRA) and effector memory (EM) CD8+ T cells in CT26/hPSMA tumors (Figure 22). Although not significant, CD8+ EM T cells were enhanced with PSMAxCD3 treatment with EM T cell responses correlating with reduced tumor volume.

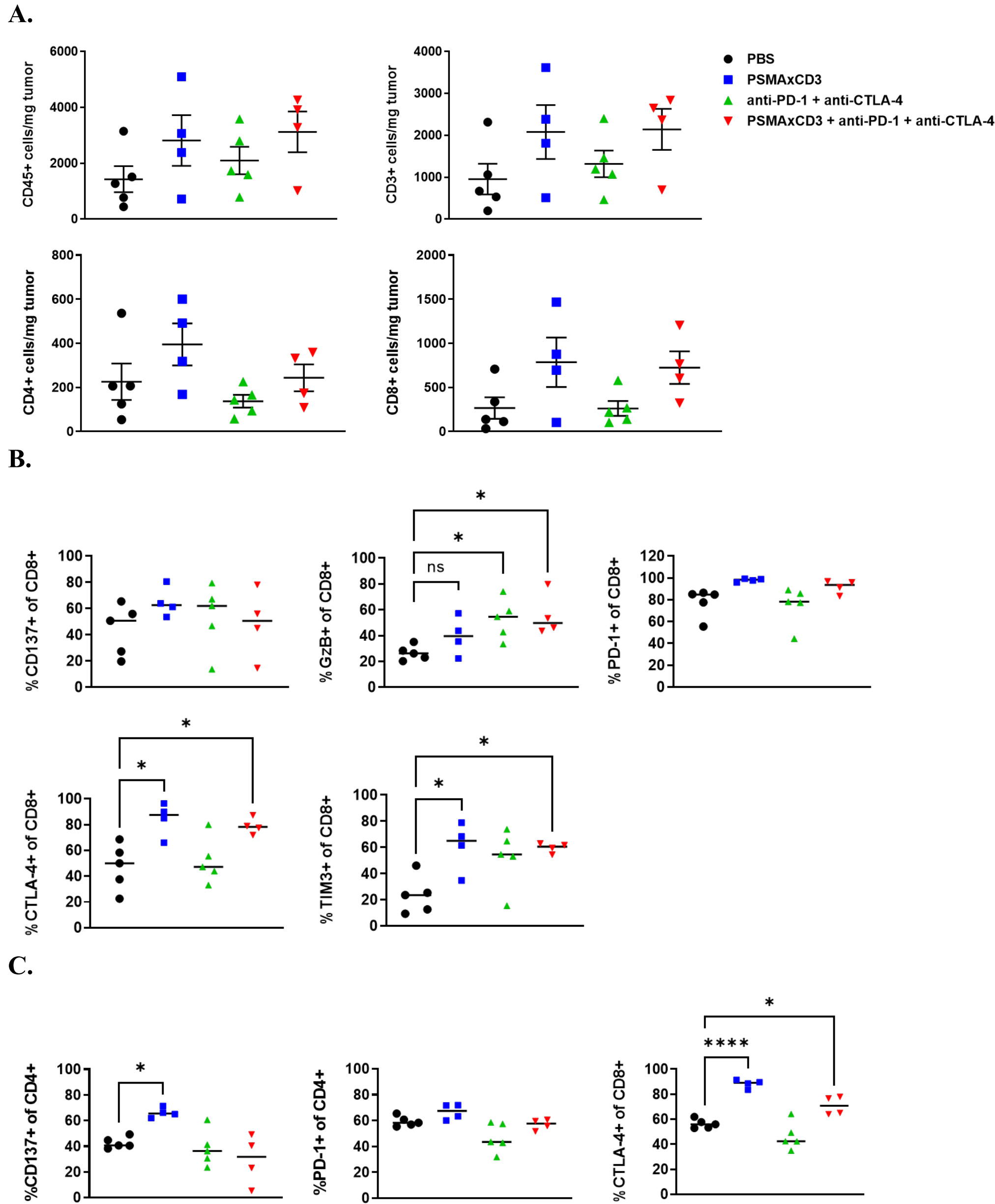

Figure 21. T Cell Infiltration (A) and Phenotype (B-C) Elicited from PSMAxCD3 Treatment Alone or in Combination With Anti-PD-1 and anti-CTLA-4 in CT26 Tumors With Human PSMA in hCD3e KI Balb/c mice.

ANOVA, analysis of variance; CTLA-4, cytotoxic T-lymphocyte associated protein-4; GzB, granzyme B; KI, knock in; ns, not significant; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; TIM3, T-cell immunoglobulin mucin-3. Individual cells/mg in each tumor tumor of A. CD45+, CD3+, CD4+, and CD8+ immune infiltration and B. CD8+ T cell subsets or C. CD4+ T cell subsets are graphed with means ± SEM represented by lines and error bars (n=4-5). *Denotes significance by ANOVA compared to PBS with *p<0.05, ****p<0.0001. PSMAxCD3 treatment alone or in combination with anti-PD-1 and anti-CTLA-4 trended toward enhanced immune and T cell infiltration into tumors (p=ns). PSMAxCD3 treatment significantly increased CD8+ and CD4+ inhibitor molecules as assessed by CTLA4 and TIM3 and CD4+ activation as assessed by CD137. Combination of anti-PD-1 with PSMAxCD3 did not alter T cell phenotype but did significantly increase CD8+ effector function as assessed by GzB compared to PBS control.

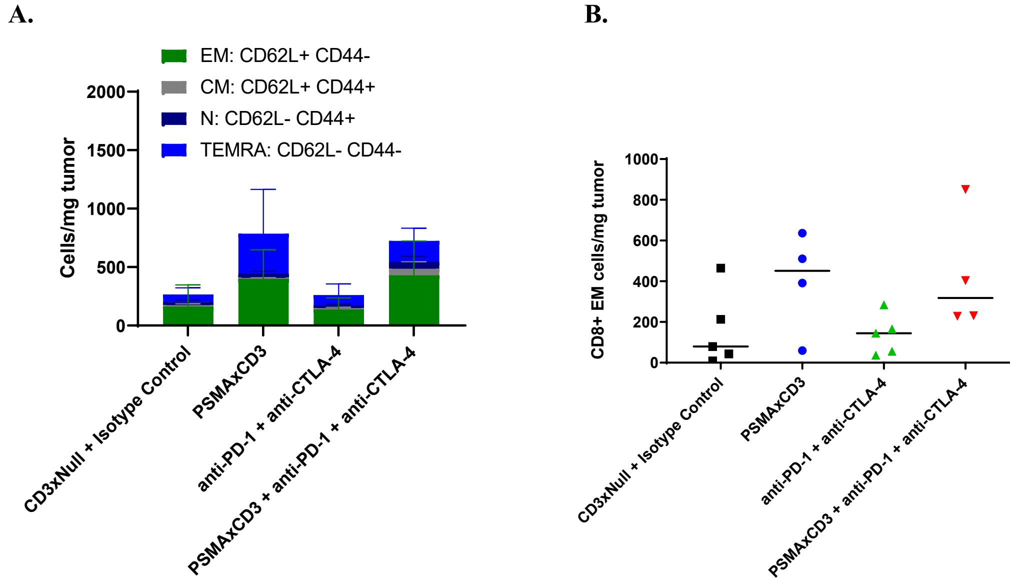

Figure 22. Effector Memory CD8+ T Cells Expanded in CT26 Mouse Syngeneic Tumors Expressing Human PSMA Treated With PSMAxCD3 Alone or in Combination With Anti-PD-1 and anti-CTLA-4 in hCD3e KI Balb/c mice.

ANOVA, analysis of variance; CM, central memory; CTLA-4, cytotoxic T-lymphocyte associated protein-4; EM, effector memory; KI, knock in; N, naïve; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; SD, standard deviation; TEMRA, terminally differentiated. A. Mean ± SD of cells/mg tumor of N, CM, EM or TEMRA CD8+ T cells (n=4-5) B. Individual cells/mg in each tumor of CD8+ effector memory T cells are graphed with medians denoted by black lines (n=4-5). PSMAxCD3 treatment alone or in combination with anti-PD-1 and anti-CTLA-4 trended toward enhanced immune and T cell infiltration into tumors with an activated phenotype (p=ns by ANOVA).

PSMAxCD3 minimally inhibits growth of TRAMP.C2 syngeneic tumors and combination with CBI does not enhance anti-tumor effects

In contrast to the CT26 model, TRAMP.C2 is a prostate tumor model derived from a transgenic mouse model that has been shown to have lower mutational burden and lower cytotoxic infiltrating immune cells than CT26.[12,36] Additionally, TRAMP.C2 tumors are insensitive to immune checkpoint blockade treatments such as anti-PD-1 and anti-CTLA-4 (unpublished data, not shown). This model was chosen to assess durability of T cell responses elicited from PSMAxCD3 treatment in the context of a complete innate immune system in an immune insensitive or “cold” tumor setting. Additionally, in contrast to the CT26 model, TRAMP.C2 mouse prostate tumor cells were transduced

with lentivirus to express mouse PSMA (TRAMP.C2/muPSMA). This tumor model system allows for the evaluation of the human CD3 binder on the bispecific antibody but targets mouse PSMA thereby eliminating any possibility of a "vaccine effect" from introducing a human antigen that may be immunoreactive. PSMAxCD3 was assessed as a single agent therapy or in combination with anti-mouse PD-1, anti-mouse CTLA-4, or anti-mouse CD40 antibody therapy on established TRAMP.C2/muPSMA tumors in male B-hCD3e KI transgenic mice.

Statistical significance of PSMAxCD3, treatment alone or in combination with anti-PD-1 on TRAMP.C2/muPSMA was assessed up to Day 32 when at least 8 of 10 animals remained in each group. Treatment with PSMAxCD3 at 10 mg/kg demonstrated significant inhibition of TRAMP.C2/muPSMA tumor growth as assessed by change in growth rate over time compared to the control group treated with untargeted CD3 control bispecific antibody NullxCD3 and isotype control resulting in 43% TGI ($p<0.05$, Figure 23A).

In addition to inhibiting tumor growth up to day 32 when control animals remained, PSMAxCD3 treatment alone or in combination with anti-PD-1 resulted in a non-statistically significant survival to tumor burden of 1000 mm3 ($p=0.09$ at 10 mg/kg, Figure 23B) with 8% and 16% ILS at the 5 mg/kg and 10 mg/kg dose levels, respectively. Similar to the observations on effect on tumor growth, combination of PSMAxCD3 at either 5 or 10 mg/kg with anti-PD-1 did not demonstrate enhanced survival compared to single agent treatments. No adverse effects of any treatment were observed on body weight (data not shown).

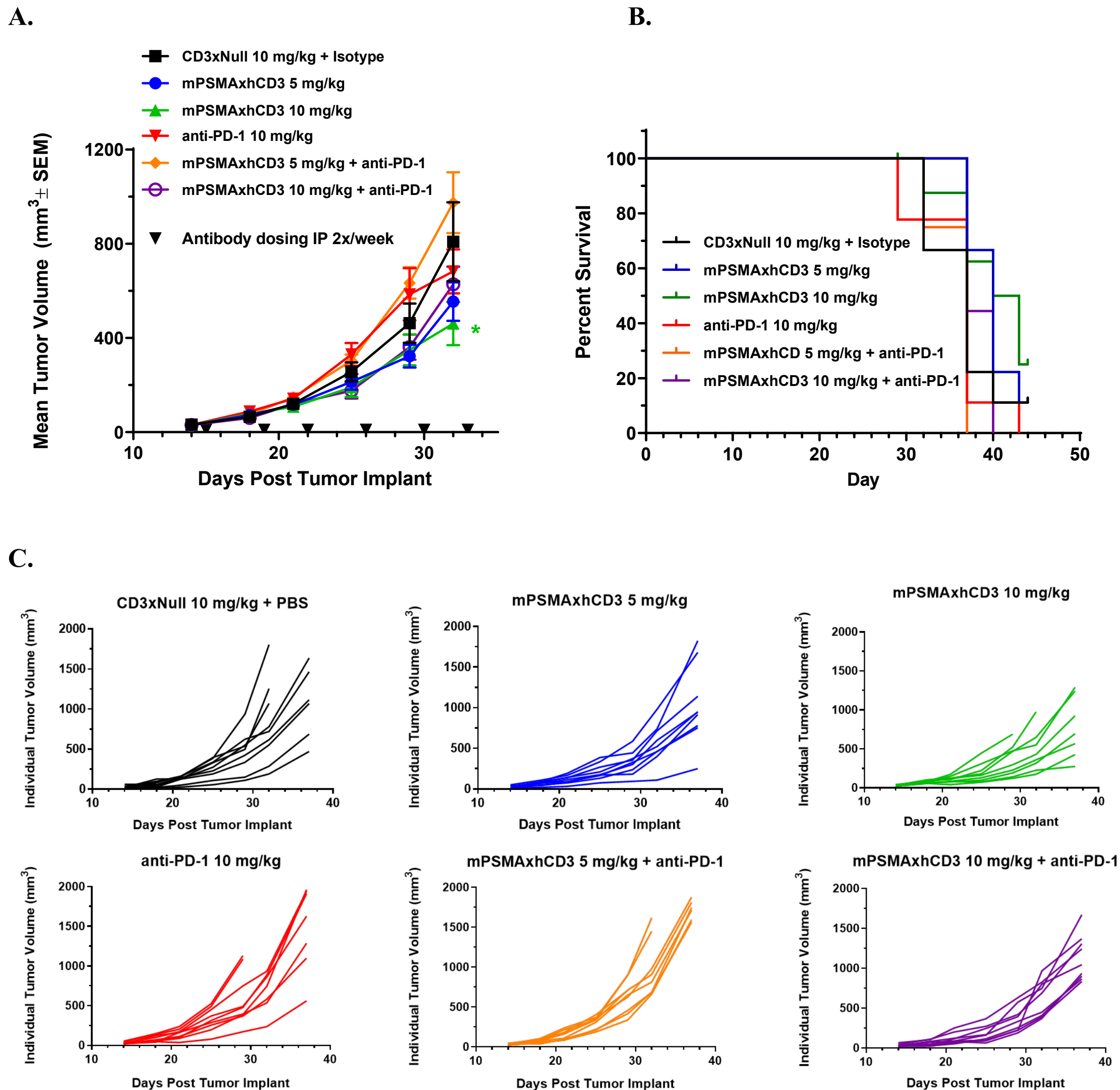

Figure 23. Effect of PSMAxCD3 alone or in combination with anti- PD-1 on Growth of TRAMP.C2 Mouse Syngeneic Tumors Expressing Mouse PSMA in B-hCD3e KI C57Bl/6 mice.

CTLA-4, cytotoxic T-lymphocyte associated protein-4; IP, intraperitoneally; KI, knock in; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; SEM, standard error of the mean; TGI, tumor growth inhibition. A. Group tumor volumes are graphed as the mean ± SEM (n=10, mean data graphed when at least 8 mice remained in each group). Tumor cells were implanted on Day 0. Treatment IP twice weekly on Days 15, 19, 22, 26, 30 and 33, as represented by black triangles. PSMAxCD3 at 10 mg/kg significantly inhibited tumor growth ($p<0.05$ by LME) as assessed by change in growth rate compared with CD3xNull + Isotype Control (control) group. B. Survival was plotted by Kaplan Meier. PSMAxCD3 at 10 mg/kg did not significantly increase survival ($p=0.09$, ns) as assessed by log rank test compared to control group. C. Individual tumor growth plots show delayed tumor growth in all groups except anti-PD-1 or PSMAxCD3 5 mg/kg + anti-PD-1.

Triple combination of PSMAxCD3 with anti-CTLA-4 and anti-PD-1 does not inhibit growth of TRAMP.C2 syngeneic tumors while combination with anti-CTLA4 shows some enhanced activity

As previously observed, TRAMP.C2 tumors were non-responsive to anti-PD-1 checkpoint blockade treatment and no enhanced efficacy was observed with the combination of PSMAxCD3 and anti-PD-1 in this model. The triple combination of PSMAxCD3, anti-PD-1 and anti-CTLA-4 was explored in the immune "cold" environment of TRAMP.C2 tumors with mouse PSMA overexpression to assess if it would generate more durable T cell responses in the absence of a "vaccine effect" which was observed in the immune "hot" CT26 syngeneic tumor model with human PSMA overexpression. Additionally, PSMAxCD3 was combined with anti-CD40 to explore if tumor control could be achieved with an agent known to decrease tumor-associated macrophages and regulatory T cells as well as enhance APC presentation of tumor antigens.[33,42,53,56]

Statistical significance of PSMAxCD3 treatment alone at 10 mg/kg, or in triple combination with anti-PD-1 at 10 mg/kg and anti-CTLA-4 at 2.5 mg/kg, or combination with anti-CD40 at 10 mg/kg on TRAMP.C2/mPSMA was assessed up to Day 28 when at least 9 of 10 animals remained in each group. Treatment with PSMAxCD3 alone or in triple combination with anti-PD-1 and anti-CTLA-4 did not demonstrate inhibition of TRAMP.C2/mPSMA tumor growth (Figure 24A) and did not prolong survival to maximal tumor burden (Figure 24C).

PSMAxCD3 treatment in combination with anti-CD40 resulted in enhanced anti-tumor effect with 60% TGI on Day 28 compared to control treatment whereas anti-CD40

treatment alone elicited 45% TGI (Figure 24B). Treatment with anti-CD40 alone or in combination with PSMAxCD3 significantly inhibited mean tumor volumes compared to control treated animals ($p<0.001$). Although there was an increased inhibition of tumor growth in the combination group, it was not statistically significant and the combination of PSMAxCD3 with anti-CD40 did not elicit a survival advantage compared to anti-CD40 treatment alone with both treatments resulting in a 17% ILS ($p<0.05$ for both treatments compared to control, Figure 24D). Anti-CD40 was only given for 3 doses due to body weight loss observed with treatment (data not shown) and tumors in the combination group rapidly regrew after Day 24 (Figure 24E) suggesting tumor control may have been prolonged if anti-CD40 treatment was continued.

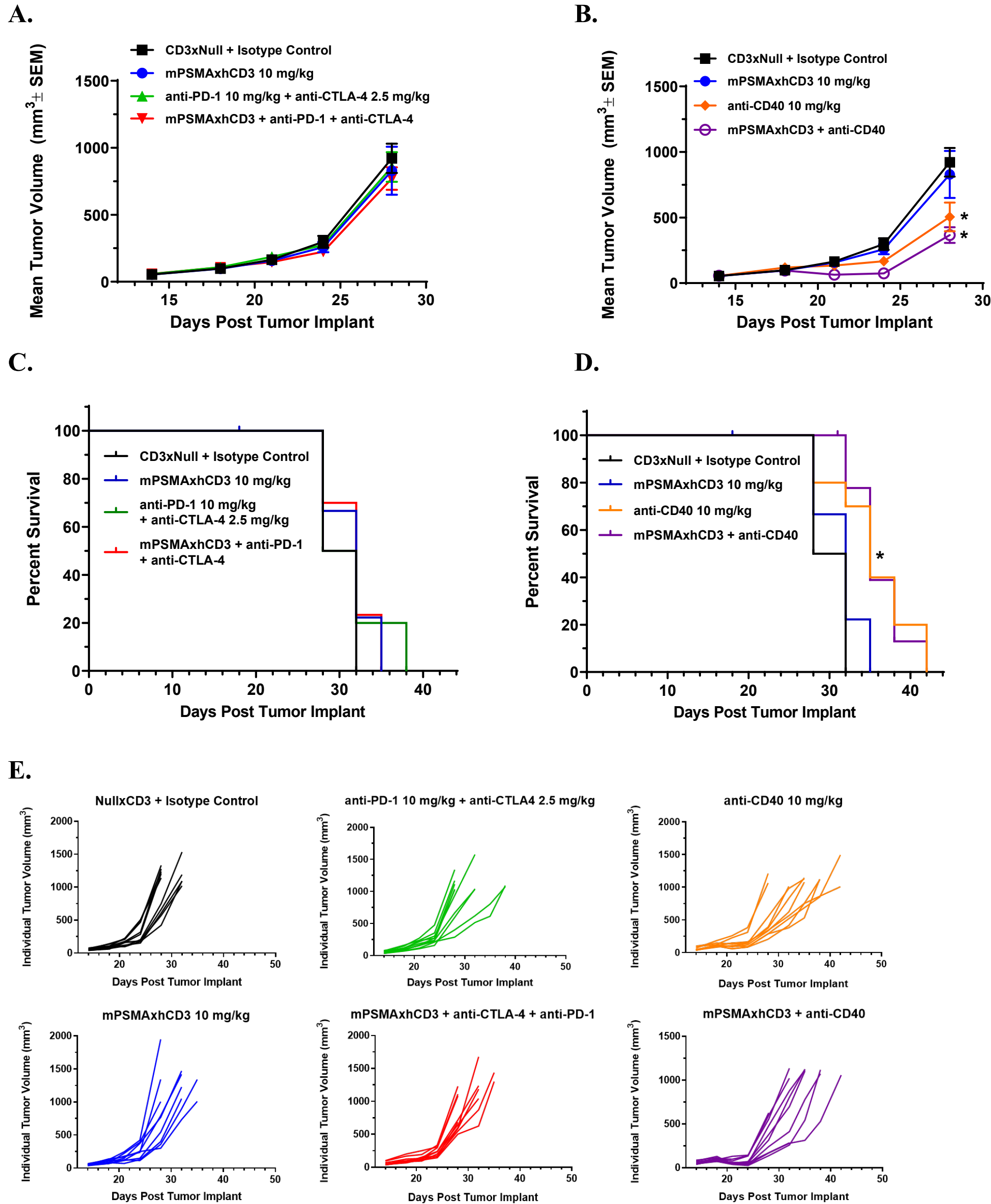

Figure 24. Effect of PSMAxCD3 alone or in combination with anti- PD-1 and anti-CTLA-4 or combination with anti-CD40 on Growth of TRAMP.C2 Mouse Syngeneic Tumors Expressing Mouse PSMA in B-hCD3e KI C57Bl/6 mice.

CTLA-4, cytotoxic T-lymphocyte associated protein-4; IP, intraperitoneally; KI, knock in; MMRM, mixed model for repeated measures; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; SEM, standard error of the mean; TGI, tumor growth inhibition. A-B. Group tumor volumes graphed as the mean ± SEM (n=10). Tumor cells were implanted on Day 0. Treatment IP twice weekly on Days 15, 18, 21, 24, 28 and 31, except anti-CD40 on Days 15, 18 and 21. Anti-CD40 10 mg/kg alone or in combination with PMSAxCD3 10 mg/kg inhibited tumor growth (*p<0.001 by MMRM) as assessed by change in tumor burden compared with control group. C-D. Survival was plotted by Kaplan Meier. Anti-CD40 alone or in combination with PSMAxCD3 increased survival (*p<0.05) as assessed by log rank test compared to control group. E. Individual tumor growth plots show delayed tumor growth in groups treated with anti-CD40 alone, or anti-PD-1 + anti-CTLA4, or in in combinations with PSMAxCD3.

Minimal T cell infiltration observed with PSMAxCD3 treatment of TRAMP.C2 tumors expressing mouse PSMA; however, activation and expansion of effector memory T cell phenotype was observed

Effect of treatment of PSMAxCD3 alone, in triple combination with anti-PD-1 and anti-CTLA-4 or in double combination with anti-CD40 on T cell infiltration and phenotype was assessed in TRAMP.C2/mPSMA tumors. In contrast to treatment with PSMAxCD3 in immune "hot" CT26 tumors, minimal T cell infiltration was observed in immune "cold" TRAMP.C2 tumors which explains lack of anti-tumor effect (Figure 25A). Combination with anti-PD-1 and anti-CTLA-4 resulted in more T cell infiltration, although not significant (Figure 25A). No enhancement of T cell infiltration was observed with PSMAxCD3 combination with anti-CD40 (Figure 26A). Minimal CD8+ T cell activation as assessed by CD137 and intracellular GzB was observed with PSMAxCD3 alone or in combination with anti-PD-1 and anti-CTLA-4; however, statistically significant increase in PD-1 and intracellular CTLA-4 was observed ($p<0.05$ compared to control, Figure 25B). PSMAxCD3 in combination with CBI reduced TIM3 expression on CD8+ T cells suggesting reduction in T cell exhaustion (Figure 25B). Interestingly, the combination of PSMAxCD3 with anti-CD40 increased intracellular GzB levels suggesting enhanced CD8+ effector function as compared to control treated tumors ($p<0.05$, Figure 26B). Similar trends were observed with PSMAxCD3 treatment alone or in combination with CBI treatment with enhancement of EM CD8+ T cells; although numbers of cells per mg of tumor were greatly reduced compared to treatment in CT26 tumors (Figure 27A-B). Treatment with PSMAxCD3 with anti-PD-1 and anti-CTLA-4 had the greatest, statistically significant expansion of EM CD8+ T cells

demonstrating similar effects on T cells as was observed in CT26 tumors (Figure 27B). Together this data demonstrates that T cell trafficking into the immune "cold" TRAMP.C2 tumors prevents robust and durable T cell responses against the tumor despite

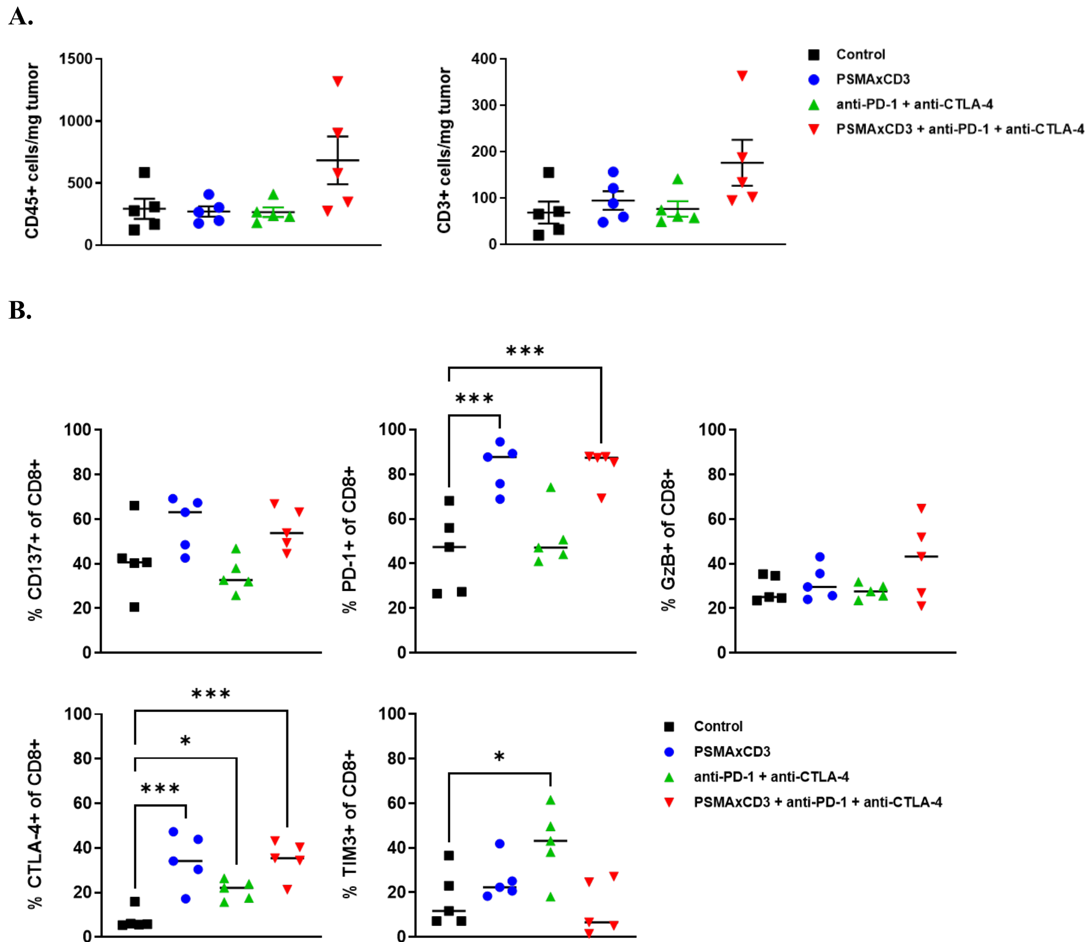

Figure 25. T Cell Infiltration (A) and Phenotype (B) Elicited from PSMAxCD3 Treatment Alone or in Combination With anti-PD-1 and anti-CTLA-4 in TRAMP.C2 Mouse Syngeneic Tumors Expressing Mouse PSMA in B-hCD3e KI C57Bl/6 mice.

ANOVA, analysis of variance; CTLA-4, cytotoxic T-lymphocyte associated protein-4; GzB, granzyme B; KI, knock in; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; TIM3, T-cell immunoglobulin mucin-3. Individual cells/mg tumor of A. CD45+ immune or CD90.2+ T cells and B. CD8+ T cell phenotypes in the tumor are graphed per tumor with means ± SEM represented by lines (n=5). *Denotes significance by ANOVA with *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Although not significant, a trend of enhanced infiltration of CD45+ immune cells and CD3 T cells was observed with combination PSMAxCD3 + anti-PD-1+ anti-CTLA-4 treatment (p=0.0539, p=0.0601, respectively). All treatments resulted in a significant increase of CTLA-4 on CD8 T cells while PSMAxCD3 alone or in combination with anti-PD-1+ anti-CTLA-4 resulted in a significant increase in PD-1 and TIM3.

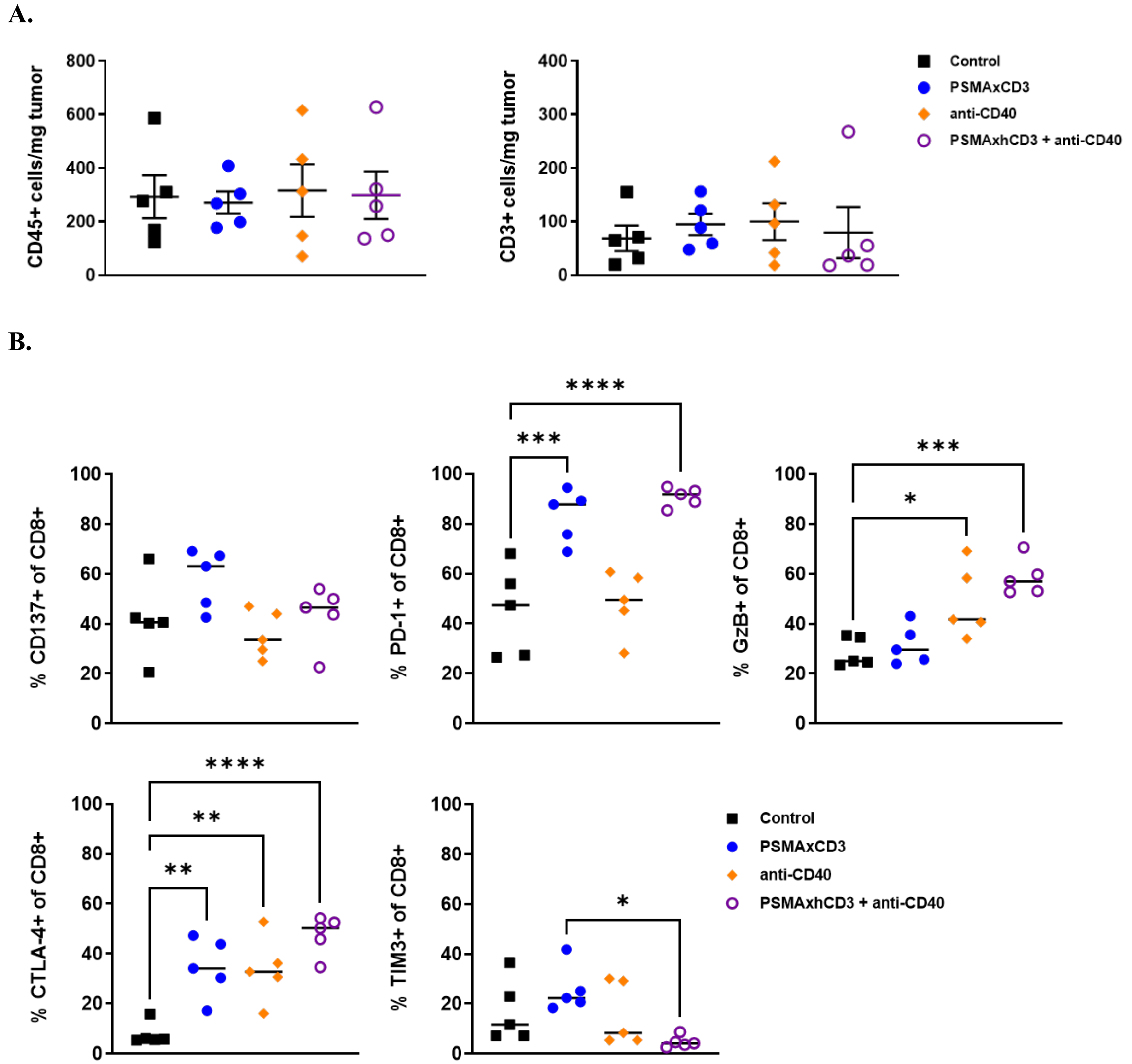

Figure 26. T Cell Infiltration (A) and Phenotype (B) Elicited from PSMAxCD3 Treatment Alone or in Combination With anti-CD40 in TRAMP.C2 Mouse Syngeneic Tumors Expressing Mouse PSMA in B-hCD3e KI C57Bl/6 mice.

ANOVA, analysis of variance; CTLA-4, cytotoxic T-lymphocyte associated protein-4; GzB, granzyme B; KI, knock in; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; TIM3, T-cell immunoglobulin mucin-3. Individual cells/mg tumor of A. CD45+ immune or CD90.2+ T cells and B. CD8+ T cell phenotypes in the tumor are graphed per tumor with means ± SEM represented by lines (n=5). *Denotes significance by ANOVA with *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Treatment with PSMAxCD3 and anti-CD40 or the combination did not elicit infiltration of CD45+ immune cells and CD3 T cells. All treatments resulted in a significant increase of CTLA-4 on CD8 T cells while PSMAxCD3 alone or in combination with anti-CD40 resulted in a significant increase in PD-1. Treatment with anti-CD40 alone or in combination with PSMAxCD3 significantly increased GzB in CD8 T cells. Combination of PSMAxCD3 + anti-CD40 significantly decreased TIM3 as compared to PSMAxCD3 monotherapy.

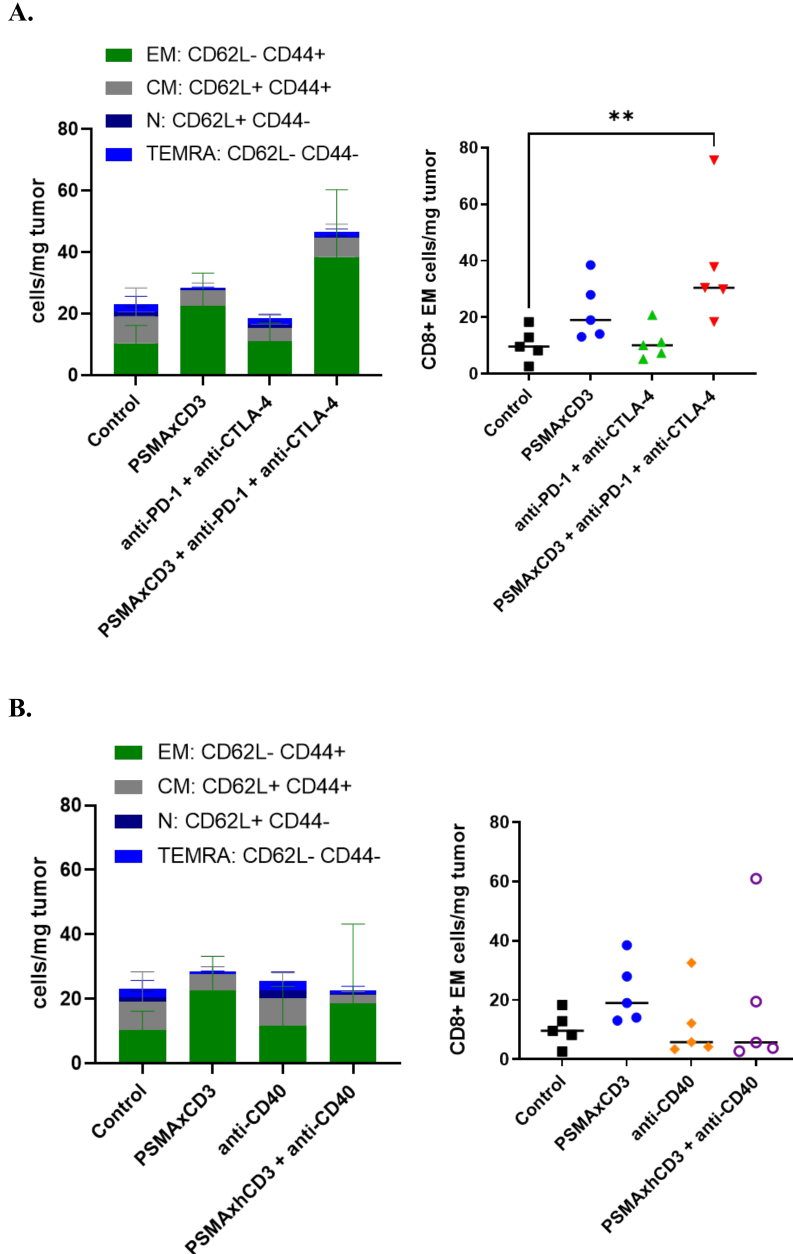

Figure 27. Effector Memory CD8+ T Cells Expanded in TRAMP.C2 Mouse Syngeneic Tumors Expressing Mouse PSMA Treated with PSMAxCD3 Alone or in Combination with anti-PD-1 and anti-CTLA-4 or in Combination with anti-CD40 in B-hCD3e KI C57Bl/6 mice

ANOVA, analysis of variance; CM, central memory; CTLA-4, cytotoxic T-lymphocyte associated protein-4; EM, effector memory; KI, knock in; N, naïve; PD-1, programmed death receptor 1; PSMA, prostate specific membrane antigen; SD, standard deviation; TEMRA, terminally differentiated. Mean ± SD of cells/mg tumor of N, CM, EM or TEMRA CD8+ T cells (left) and individual cells/mg in each tumor of CD8+ effector memory T cells are graphed with medians denoted by black lines (right) of A. PSMAxCD3, anti-PD-1 and anti-CTLA-4 or combination treatment and B. PSMAxCD3, anti-CD40 or combination treatment (n=5). PSMAxCD3 treatment trended towards an increase in effector memory T cells, although not significant. Combination of PMSAxCD3 + anti-PD-1 + anti-CTLA-4 resulted in significant increase in effector memory CD8 T cells compared to Control (*p<0.01 by ANOVA) while combination with anti-CD40 did not affect T cell memory phenotypes.

3.5. DISCUSSION

In these studies, we investigated the effects of immune “hot” and cold” syngeneic tumors on efficacy of PSMAxCD3 alone or in combination with PD-1 blockade and CTLA-4 depleting antibody or CD40 agonism in human CD3e KI mice. We demonstrated more robust antitumor efficacy and increased intra-tumoral T cell infiltration with PSMAxCD3 treatment in the immune “hot” model as compared to the immune “cold” model that exhibited minimal efficacy and poor T cell infiltration that was unchanged in response to therapy. PSMAxCD3 treatment also resulted in changes to T cell phenotype including both activation and upregulation of inhibitory markers as well as expansion of effector memory T cells.

We investigated the potential for PSMAxCD3 treatment to elicit durable T cell responses in the immune “hot” CT26 model expressing human PSMA in CD3e KI mice. PSMAxCD3 significantly inhibited CT26 tumor growth and enhanced life span. In both studies performed, one animal each developed a complete immunological response against the tumor. Treatment was able to elicit an immune response that protected against re-challenge of the PSMA+ tumor. PSMAxCD3 treatment elicited an immediate durable response; however, regrowth occurred in all but one mouse suggesting anti-drug antibodies (ADAs) may be forming against the human framework of the JNJ-63898081 antibody. If ADAs did indeed contribute to lack of durable responses, murinizing the Fc of the antibody could result in longer duration of serum drug exposure that could lead to more complete responses.[24] Although not significant, PSMAxCD3 treatment enhanced T cell infiltration into the tumor with a correlation between tumor size reduction and amount of infiltration. Infiltrating tumors had a trend of increased activation markers

CD137 and PD-1 and enhanced effector function by GzB production and a significant upregulation of T cell exhaustion and inhibitory markers TIM3 and CTLA-4. Similar to the infiltration and activation status, there was also a trend for enhanced effector memory T cells. The variability within the tumors suggests a similarity to what T cell responses have been observed clinically, with the best responders having the highest T cell expansion with an enrichment of effector memory CD8+ T cells.[50]

In contrast, PSMAxCD3 treatment of the immune "cold" TRAMP.C2 model expressing mouse PSMA resulted in suboptimal anti-tumor efficacy with minimal survival advantage and no complete responses. PSMAxCD3 treatment did not enhance T cell infiltration into the immune "cold" tumors and T cell levels in tumors receiving treatment were 20-fold lower than in the immune "hot" CT26 model. T cells in PSMAxCD3 treated tumors had slightly elevated CD137 but no enhanced granzyme B production. PSMAxCD3 treatment did enhance PD-1 and CTLA-4 expression on T cells suggesting upregulation of T cell inhibition probably due to a TME response. Effector memory CD8+ T cells were slightly elevated after PSMAxCD3 treatment, although not significantly.

Effect of the combination of PSMAxCD3 with PD-1 blockade was significant compared to PD-1 blockade alone but not compared to PSMAxCD3 alone while the triple combination of PSMAxCD3 with PD-1 blockade and CTLA-4 depleting antibody treatment was significantly enhanced compared to PSMAxCD3 but not to CBI monotherapy in the immune "hot" CT26 model. We observed complete responses to the initial CT26 tumor with monotherapy and combination treatment and all treatments elicited immune responses that protected against tumor rechallenge. We would expect

that CTLA-4 treatment could elicit immune surveillance against the tumor by priming and activating T cell responses with antigen presenting cells; however, we would not expect to see this in the PD-1 treated groups.[10] This supports our findings that the optimal protection against rechallenge was elicited in the combinations including the CTLA-4 depleting antibody and coincides with previous reports that this CBI combination with vaccine therapy elicited durable T cell responses.[8] These findings are in contradiction to previous reports; however, that dataset used the mouse 2C11 CD3 binder which may have weaker binding affinity than the CD3 binder reported here.[1]

Unfortunately, our CTLA-4 dose as a monotherapy or in combination with PD-1 blockade was extremely active and did not leave a window by which to see improvement with PSMAxCD3. In the combinations with the CTLA-4 depleting antibody, PSMAxCD3 addition appeared to lessen the effect of the CBI treatment. Additionally, previous reports have shown combination benefit with CD3 redirectors and CBI treatment in syngeneic models.[6,21] One possible explanation for these results is that the high affinity binder on the CD3 bispecific in combination with strong CBI may be causing T cells to undergo AICD.[17] To understand the ideal CD3 combination with CBI, future investigations could evaluate efficacy and T cell responses of a PSMAxCD3 bispecific with a lower binding affinity to CD3 in combination with a lower, more sub-optimal dose of CTLA-4. Additionally, it is possible that the current model may be overestimating immune responses due to the presence of human PSMA which may be increasing innate T cell infiltration due to a vaccine-effect of a non-mouse antigen in the tumor. It has been shown that using genetically modified mice that express human CD3

as well as the human target antigen have overcome this vaccine-effect and future studies could pursue this strategy or target mouse PSMA instead.[4,45]

In contrast to the immune "hot" model, combination of PSMAxCD3 with PD-1 blockade and CTLA-4 depleting antibody did not result in any enhanced efficacy in the immune "cold" TRAMP.C2 model. T cell activation, effector function and exhaustion were not significantly changed compared to PSMAxCD3 monotherapy. We also investigated the combination of PSMAxCD3 and CD40 agonism as it has previously been reported that TRAMP.C2 tumors are sensitive to CD40 agonism and CD40 agonism may play a role in antigen presentation to APCs, thus possibly eliciting better responses to the PSMA antigen as well as epitope spreading whereby APCs could recognize new tumor-specific antigens.[10,22,42] A trend towards enhanced efficacy with the combination treatment was observed early in the treatment; however, tumors outgrew at a similar rate as the CD40 agonism treatment alone. No enhancement of T cell infiltration was observed in tumors treated with the combination; however, combination treatment did statistically enhance the T cell effector functionality as assessed by GzB production. Additionally, combination treatment significantly reduced TIM3 expression on the surface of CD8 T cells compared to PSMAxCD3 monotherapy suggesting that the combination was preventing T cell exhaustion. No significant differences in T cell memory phenotype were observed with the combination compared to PSMAxCD3 monotherapy; however, effector memory T cells comprised most of the CD8 compartment suggesting a lack of overall T cells rather than an inferior memory phenotype. Although T cell memory phenotype may not be altered by CD40 agonism, there could be changes in the myeloid compartment such as a suppression of tumor

associated macrophages and activation of antigen presenting cells which has previously been reported.[53]

The present studies demonstrated more robust tumor control and survival advantage leading to some durable anti-tumor responses in response to PSMAxCD3 treatment in the immune "hot" model while minimal efficacy and no survival advantage was observed in the immune "cold" model. PSMAxCD3 treatment elicited T cell infiltration, activation, and expansion of effector memory CD8+ T cells in the immune "hot model" a lack of T cell trafficking to the tumor resulted in lack of durable efficacy in the immune "cold" model. Taken together, these data suggest that combination with additional therapies aimed at enhancing T cell infiltration may be needed to fully control tumor growth in immune "cold" settings such as prostate cancer.

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CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1. SUMMARY

Despite advances in treatments targeting prostate cancer, roughly a third of patients progress to hormone refractory metastatic disease.[4,52] Prostate specific membrane antigen (PSMA) has been shown to be overexpressed on prostate cancer with levels correlating to disease severity, making it a promising target for immune targeting therapeutic approaches.[3,5,55,59] T cell engaging cluster of differentiation 3 (CD3) bispecifics targeting PSMA have been evaluated preclinically and clinically; however, clinical trials are in early stages and durable anti-tumor responses have yet to be realized.[9,14,21,22,31] Unlike hematological cancers where CD3 redirection antibodies have shown impressive overall survival rates, solid tumors have been more challenging for immunological therapeutics likely due to an immune suppressive tumor microenvironment (TME) and the need for therapeutics and immune cells to traffic into the solid tumor mass.[7,18,50,54] Efficacy of T cell checkpoint blockade inhibitor (CBI) therapies in solid tumors in the clinic has been correlated with immune infiltration and prostate cancers have shown minimal response due to their immunologically "cold" TME, suggesting this will also be a challenge for CD3 redirector bispecific antibodies.[15,17,20,25,28,36,39]

Although CD3 bispecifics have been successful in hematological indications, 20% of patients given the CD19 targeting CD3 redirector, blinatumomab, relapsed suggesting enhancements or combinations may be necessary to improve durability of T cell responses.[53] Patients with robust antitumor responses had expansion of effector memory CD8+ T-cells, whereas poor responders had elevated recruitment of regulatory T cells

and upregulation of programmed death ligand 1 (PD-L1) levels that both suppress T cell effector function.[10,12,26,53] These results suggest that expansion of effector memory T cells is important for lasting clinical responses and the observed resistance mechanisms prompt a need for combinations in the clinic that can boost and prolong T cell responses in patients.

Often preclinical responses in xenograft models using engraftment of human effector PBMC or T cells have demonstrated robust antitumor efficacy which hasn't always predicted clinical activity.[14,21,22] Lack of translatability of preclinical data is likely due to the lack of suppressive tumor microenvironment and T cell suppression in xenograft models with human immune transplantation. Human immune xenotransplantation models in mice result in reconstitution of T cells alone thereby limiting assessment of immune cell interactions.[48] The absence of the myeloid compartment prevents understanding of T cell suppression and antigen presenting cell interactions with T cells.[48] These models also inaccurately reflect the human immune system due to the graft versus host disease (GvHD) response of the human T cells against the mouse host which results in non-physiological activation and expansion of human T cells, irrespective of treatment.[6,47,48,56] Due to the lack of myeloid cell engraftment and the overactivation of T cells due to GvHD, xenograft tumors in immune compromised mice lack suppressive immune cell infiltration thus having limited suppressive TME.

To understand the T cell responses elicited by PSMAxCD3 bispecific antibodies in the context of a complete immune system as well as in the presence of potential resistance mechanisms, additional preclinical models are necessary. CD34+ humanized NOD/SCID/IL2Rγ or NOG mice transgenic for human IL3, GM-CSF and SCF may

provide a more relevant model to evaluate the range of T cell responses observed in the clinic with T cell mediated therapies.[6,23,56] Evaluation of CBI therapy in these models has demonstrated a donor-dependent T cell response with suboptimal overall responses similar to what has been observed in the clinic.[8,57] Additionally, immune "hot" and "cold" syngeneic tumor models that have been characterized as CBI sensitive and insensitive in immune competent transgenic mice expressing human CD3e also provides a mouse model system to evaluate T cell responses to clinical CD3 redirectors with relevant TME.[13,29,30,38,46] In these studies, we utilized these two mouse model systems to evaluate the efficacy and T cell phenotype elicited from PSMAxCD3 treatment against PSMA+ tumors.

In Chapter 2, we assessed the potential for PSMAxCD3 redirection antibody to elicit durable T cell responses against PSMA+ prostate xenograft tumors in immune compromised mice engrafted with CD34+ cord blood cells. In these studies, we demonstrated efficacy of PSMAxCD3 results in T cell infiltration with an activated phenotype and an enrichment of effector memory T cells. To study resistance mechanisms and suppressive TME, we evaluated efficacy of PMSAxCD3 in prostate xenograft tumors with overexpression of PD-L1. PD-L1 upregulation conferred resistance to PSMAxCD3 treatment which was overcome by combination with PD-1 blockade. Although combination treatment restored anti-tumor efficacy, T cell phenotype was not significantly altered. Complete responders to combination therapy also demonstrated T cell responses that protected against but did not prevent growth of rechallenged tumors. These data validate this model system as a more clinically translatable system to evaluate CD3 redirection therapies. The results suggest

combination strategies will be needed to establish durable T cell responses in patients and other combinations in addition to PD-1 blockade should be explored.

In Chapter 3, we evaluated efficacy of PSMAxCD3 treatment in immune "hot" and "cold" tumors using CT26 and TRAMP.C2 syngeneic tumor models expressing PSMA, respectively, in immune competent transgenic mice expressing human CD3ε. PSMAxCD3 treatment was more efficacious in immune "hot" CT26 tumors than in immune "cold" TRAMP.C2 tumors and efficacy correlated to immune cell infiltration. We demonstrated that in immune sensitive tumor models PSMAxCD3 treatment elicited some durable responses correlating with T cell infiltration of activated, effector memory CD8+ T cells that protected against tumor rechallenge. Combination with CBI resulted in enhanced complete responses and greater T cell infiltration; however, no change in T cell phenotype was observed. In contrast, treatment with PSMAxCD3 in the immune "cold" tumor model resulted in minimal efficacy and minimal intratumor T cell infiltration. Combination of PSMAxCD3 with CBI therapy did result in a greater number of tumor infiltrating T cells with an enrichment of CD8+ T cell effector memory cells; however, no enhancement of efficacy was observed suggesting suppressive TME may require further optimization of combination strategies.

Together, the findings of this work suggest that the two mouse models evaluated can evaluate T cell responses elicited from CD3 redirection therapies in a more clinically translatable manner where TME and resistance mechanisms can be evaluated. In these models we demonstrated that PSMAxCD3 treatment can elicit robust T cell responses that can regress tumor growth and surveil against reestablishment of disease; however, suppressive TME, poor immune infiltration, and upregulation of PD-L1 by prostate

cancers can inhibit T cell responses elicited by PSMAxCD3 treatment. Therefore, combination therapy strategies will likely be necessary to elicit durable T cell responses in the clinic. Although combination with CBI therapy demonstrated enhancement of efficacy, increased immune infiltration, and enrichment of effector memory T cells, tumor growth still progressed. This work suggests additional combinations will be needed in addition to CBI to overcome resistance mechanisms in solid tumors such as prostate cancer.

4.2. FUTURE DIRECTIONS

Evaluate effects of PSMAxCD3 on regulatory immune cells

In addition to modulating T cell responses and phenotypes, we showed that PSMAxCD3 treatment also elicited tumor infiltration of myeloid cells including B cells, NK cells and dendritic cells. At the end of Chapter 2 we suggested that PSMAxCD3 treatment can reverse immune "cold" prostate cancers with low mutational burden to enhance antitumor immune function. Myeloid infiltration and phenotype could be further assessed to see if, like activation of T cells, infiltrating myeloid cells show a less suppressive phenotype in response to PSMAxCD3 treatment.

It has been reported that prostate cancer is infiltrated with myeloid-derived suppressor cells (MDSCs) including an enrichment with polymorphonuclear (PMN) MDSCs which are associated with poor prognosis and disease progression.[27,40] Tumor associated macrophages (TAMs) also contribute to a suppressive TME and interfere with T cell responses.[34,51] Preclinical studies have shown that combination of anti-CD40 and anti-CSF-1R treatment resulted in decreased suppressive (Ly6C^{lo} MHC II^{hi} and MHC^{lo}) TAMs while increasing proinflammatory (Ly6C^{int} MHC II^{hi} Ly6C^{int}) TAMs with higher

expression of CD80 and CD86 costimulatory molecules, which in turn resulted in increased T cell activity.[16,58] If infiltrating MDSCs are found in the tumors and if PSMAxCD3 treatment in not able to reverse the suppression, combinations could be explored with MDSC-targeting therapies such as anti-CSF-1R or targeting the CXCR1/2 pathway to prevent MDSC trafficking to tumors.[19,44]

Another type of myeloid cells, dendritic cells that infiltrate the tumor or the tumor draining lymph nodes could be assessed for phenotype including an activated mature phenotype with increased MHC II, CD80 and CD86 as well as CD103 which would indicate possible processing of tumor antigens for T-cell priming.[44,45,] In addition to PSMAxCD3 effects on suppressive or activating myeloid cell subtypes, regulatory T cells could also be monitored to see if increased regulatory cells drive resistance to therapy. Vaccine strategies targeting prostate cancer such as sipuleucel-T have recently demonstrated some clinical responses; however, responses are rare.[35] Combination of vaccines with PSMAxCD3 may elicit enhanced efficacy by priming antitumor T cells and enhancing epitope presentation to dendritic cells and thus epitope spreading.

Evaluate additional combination strategies with PSMAxCD3 treatment in TRAMP.C2 syngeneic prostate cancer model

In our concluding remarks in Chapter 3, we discussed the need for additional combination strategies particularly in the immune “cold” TRAMP.C2 mouse syngeneic model. Immune signatures could be evaluated in tumors with and without treatment to identify drivers of resistance or immune suppression. Immune signatures elicited from PSMAxCD3 treatment could be compared between sensitive and resistant (hot/cold) syngeneic models to further identify immune dysfunction that may be contributing to

resistance. RNA sequencing and Nanostring analysis could be used to identify the immune responses and dysfunction as described in Jiang, P., et al.[24] Results of this analysis could help direct further combinatorial strategies.

Evaluate additional combination strategies with PSMAxCD3 treatment that increase trafficking of T cells into tumors

In Chapter 3 we observed minimal inhibition of TRAMP.C2 tumor growth in response to PSMAxCD3 treatment even though T cells were activated and enriched for effector cell phenotypes. Combinations with CBI did not statistically enhance tumor control. Intratumoral infiltration of T cells was markedly lower in this model compared to the immune "hot" CT26 tumor model suggesting that part of the lack of treatment response may be due to low T cell trafficking to the tumor. Future work could explore combinations of PSMAxCD3 with therapies that enhance T cell trafficking.

C-X-C chemokine receptor 4 (CXCR4) has been reported to play a role in tumor progression, metastasis, and immune cell trafficking.[1] CXCR4 was found to be expressed on 57% of clinical prostate cancer patients suggesting it may play a role in promoting MDSC trafficking while suppressing T cell tumor infiltration.[1,38] Plerixafor, a CXCR4 inhibitor, demonstrated increased intratumoral CD8+ T cell and natural killer cell accumulation in patients with solid tumors and other inhibitors are being evaluated in clinical trials with CBI.[1,2] In addition to CXCR4, the IL8/CXCR2 pathway has also been implicated in prostate cancer progression and immune cell trafficking.[33] Combinations of PSMAxCD3 and inhibitors of CXCR4 or CXCR2 could be evaluated preclinically to assess if increased trafficking could lead to more durable T cell responses.

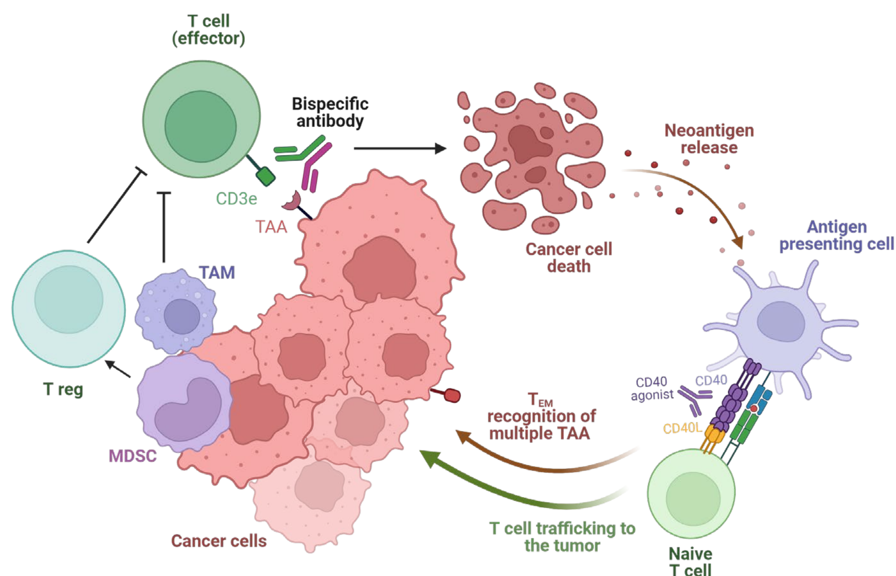

Figure 28. Three avenues of research following these studies include further evaluating effects of PSMAxCD3 treatment on regulatory immune cells, exploring combinations with inhibitors of CXCR2 or CXCR4 to enhance T cell trafficking to the tumor, and exploring resistance mechanisms to treatment in the TRAMP.C2 syngeneic model using immune signatures.

CD3e, Cluster of differentiation 3 epsilon; CD40(L), Cluster of differentiation 40 (ligand); CD80, Cluster of differentiation 3; MDSC, myeloid-derived suppressor cell; TAM, tumor associated macrophage; TAA, tumor associated antigen; T_{EM}, effector memory T cell; Treg, regulatory T cell. Created with BioRender.com

4.3. CONCLUDING REMARKS

The findings presented in this dissertation demonstrate that PSMAxCD3 therapy is able to elicit antitumor efficacy that is concomitant with establishment of activated effector functioning T cells with an enrichment for effector memory CD8 T cells in immune sensitive tumor models. In the presence of immune suppressive TME, such as overexpression of PD-L1 on tumor cells or such as a syngeneic immune “cold” model, therapy-driven T cell infiltration was suppressed, and antitumor activity was attenuated. Combination of PSMAxCD3 with CBI restored anti-tumor activity, resulting in some complete tumor responses in which mice exhibited durable T cell memory responses

against tumor rechallenge. Although combination of PSMAxCD3 demonstrated significant antitumor activity with durable memory T cell responses, rechallenged tumors were not completely irradicated suggesting incomplete epitope spreading and insufficient memory T cell responses for long term responses. This supports the need for additional combination strategies with PSMAxCD3 in the clinic. These results demonstrate that preclinical mouse models with immune suppressive TME may offer a more clinically translatable setting to evaluate solid tumor CD3 redirectors and can help guide combination strategies to overcome solid tumor challenges to immune therapies.

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