THE IMMUNOLOGICAL EFFECTS OF CHECKPOINT BLOCKADE IN COMBINATION WITH A TYROSINE KINASE INHIBITOR IN A MURINE MODEL OF RENAL CELL CARCINOMA

By

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ABSTRACT

Renal Cell Carcinoma (RCC) is responsible for approximately 13,500 deaths in the US each year and 65,000 new cases are diagnosed here annually. Despite the approval of multiple new drugs targeting mTOR and angiogenic pathways, there is still no curative treatment. Only 20-30% of patients respond to these targeted therapies; all eventually become resistant and progress. One of the most widely used targeted therapies is sunitinib, a tyrosine kinase inhibitor (TKI) primarily targeting the vascular endothelial growth factor receptors (VEGFRs). There is some evidence that sunitinib has off-target effects on the immune system, including reducing the number of myeloid derived suppressor cells (MDSCs) but also causing lymphopenia in patients. Recently completed and ongoing clinical trials of monotherapy with antibody blockade of Programmed Death 1 (PD-1) have demonstrated efficacy in RCC patients. We explored the therapeutic potential, as well as the immunomodulating effects, of combined sunitinib and anti-PD-1 antibody therapy using the orthotopic murine Renca model of RCC. Though short-term experiments did not reveal a significant increase in therapeutic potential over sunitinib alone, we did observe significantly more IFNγ+ CD8 T cells in the tumor-infiltrating lymphocytes (TIL) of combination-treated mice compared to those who received sunitinib alone. We also examined the effect of sunitinib therapy on the *in vivo* expression of B7-H1/PD-L1, a known ligand for PD-1, on tumor cells harvested from treated, tumor-bearing mice. In wild-type Balb/c mice, sunitinib therapy significantly reduced B7-H1 expression. This decrease in B7-H1 expression, as well as the dramatic therapeutic effect of sunitinib, were not seen in Balb/c RAG knockout mice, however. While the combination of sunitinib and anti-PD-1 antibody was not effective in our orthotopic Renca model, the rationale behind

combining anti-angiogenic therapy, particularly TKIs, with immune checkpoint blockade is supported by an extensive body of literature and worthy of continued investigation.

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INTRODUCTION

The American Cancer Society estimates there will be 61,560 new cases of all types of kidney cancers and 14,080 deaths attributable to kidney cancer in the United States alone in 2015 (1). In their earliest stages, when the chance for complete cure through surgical resection is highest, renal cancers are frequently asymptomatic. Unless diagnosed early due to incidental diagnostic scans, many patients present with metastatic disease. While 5 year survival rates are now above 70% for patients diagnosed at Stage I or II, survival declines to just over 50% for Stage III and drops to less than 10% for those who present with Stage IV disease (2). With traditional cytotoxic chemotherapies and radiation providing little or no benefit, there has long been a significant unmet need for curative therapies, particularly in the setting of advanced and metastatic disease.

Renal cancers are unique in that they are one of only a handful of naturally "immunogenic" cancers, as evidenced by spontaneous regression of metastatic disease following surgical resection of the primary tumor (3,4). This immunogenic potential led to the introduction of high-dose interleukin-2 (IL-2) therapy more than thirty years ago, followed by interferon-α (IFN-α). Neither of these cytokines is particularly effective – historically only 5-10% of treated patients achieved remission. Even with more recent selection for the most favorable histological sub-types, only 25% of patients treated with IL-2 achieve durable responses (5). Many patients are unable to tolerate the potentially life-threatening side effects of cytokine therapy but for more than twenty years it represented the only hope for those with metastatic disease.

The 2005 approval of sorafenib, an oral kinase inhibitor, marked a turning point for the treatment of kidney cancer. Sorafenib and the numerous targeted therapies that have followed arose from a better understanding of the underlying genetics and angiogenic characteristics of renal malignancies. Clear Cell Renal Cell Carcinoma (RCC) accounts for 66 – 75% of all kidney cancer cases and many of these cancers have a characteristic genetic change in a tumor suppressor gene – the von Hippel-Lindau (VHL) mutation(6). The von Hippel-Lindau protein negatively regulates hypoxia inducible factor 1a (HIF1a) through ubiquitination and subsequent degradation. Without this regulation, HIF1a induces angiogenic genes including vascular endothelial growth factor (VEGF) (7). Sorafenib actually targets multiple kinases, including the serine/threonine kinases C-RAF and B-RAF as well as the tyrosine kinases of the VEGF receptor (VEGFR) and platelet-derived growth receptor (PDGFR) families (8). This dual action may curb tumor growth by inhibiting tumor cell proliferation and survival (C-RAF and B-RAF) while simultaneously slowing angiogenesis via VEGFR/PDGFR.

Shortly after sorafenib's approval, sunitinib, an oral receptor tyrosine kinase inhibitor (TKI), received FDA approval in early 2006. Like sorafenib, sunitinib targets the VEGFR/PDGFR families but also demonstrates inhibition of KIT, FLT3 and colony-stimulating factor receptor type 1 (9). Initial response rates are between 30% and 40% but complete responses are rare and it is not considered a curative therapy (10–12). Though sunitinib, like so many targeted therapies, is administered orally, it is not without side effects. Commonly reported side-effects observed in a large, randomized Phase III trial comparing sunitinib to IFN-α were nausea, diarrhea, hypertension, fatigue, stomatitis and hand-foot syndrome (13). Laboratory abnormalities included increased lipase and uric acid

as well as multiple hematologic deficiencies - leukopenia, neutropenia, thrombocytopenia and lymphopenia. Cardiotoxicity and hypothyroidism occurred less frequently but at significantly higher rates in the sunitinib-treated patients. Trial participants taking sunitinib were almost twice as likely as the IFN-α cohort to require a dose reduction (50% vs 27%).

Dose reductions and interruptions are extremely common among patients taking sunitinib. This is unfortunate because sunitinib seems to be most effective at higher concentrations – increased sunitinib exposure is associated with a higher probability of a partial or complete response as well as lengthier time to progression and overall survival (14). The Phase III trial mentioned above also demonstrated increased response rates with longer duration sunitinib treatment (15). For the RCC patients who are able to remain on sunitinib therapy, many will develop resistance after a median 6-15 months of treatment (16). There are likely to be several factors contributing to this resistance, including alternate proangiogenic pathways that ultimately lead to the revascularization of the tumor and various factors in the tumor microenvironment (TME) and stroma, such as cytokines and immunosuppressive cells (16,17). The emergence of resistance has led to patients being treated sequentially with multiple targeted therapies, though simultaneous combinations have also been explored. Several of these combinations have demonstrated significant toxicities with little gain in efficacy (18).

Given the promiscuity of kinase inhibitors, it is not surprising that their use is associated with off-target effects, particularly of the immunologic variety. Sorafenib seems to interfere with immune activation by inhibiting dendritic cell functionality on multiple fronts, but sunitinib does not and may actually reduce the numbers of regulatory T cells (19). Sunitinib has also been shown to reduce myeloid derived suppressor cell (MDSC)

populations in mice bearing Renca tumors (20), as well as in metastatic RCC (mRCC) patients (21), and reverse suppression of IFNγ-producing T cells (22).

As numerous targeted therapies were being tested in clinical trials, there was a similar surge in cancer immunotherapy agents, particularly for immunogenic malignancies such as melanoma and RCC. Immunotherapy is especially attractive because it has the potential to induce a memory response through the adaptive immune system, something targeted therapies cannot offer. Tumors employ numerous strategies to evade destruction by the host immune system, which translate to multiple therapeutic opportunities to overcome these barriers (23). At the forefront of the possible immunotherapeutic approaches is immune checkpoint blockade (24,25). Our immune systems depend on a delicate balance between co-stimulatory and checkpoint molecules to control our immune responses, keeping pathogens at bay while simultaneously preventing autoimmunity. This exquisite control occurs largely through the various checkpoint molecules and their ligands which can modulate the amplitude, duration and breadth of T and B cell responses.

The most widely studied of these checkpoint molecules include cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1) along with its ligand PD-L1 (also called B7-H1). Ipilimumab, which blocks CTLA-4, was brought to market first, garnering FDA approval for unresectable metastatic melanoma in 2011 based on its improved overall survival in a randomized phase III trial (26). In a phase II trial in mRCC patients, ipilimumab demonstrated a response rate of 13% but 33% of patients experienced a grade III or IV immune-related adverse event (IRAE) (27). This high rate of IRAEs is not surprising given CTLA-4's expression profile and the phenotype of CTLA-4 knockout

mice, which develop lymphoproliferative disease leading to tissue destruction and death at only 3-4 weeks of age (28). PD-1 and PD-L1 targeted antibodies have a better safety profile than those blocking CTLA-4, with substantially fewer IRAEs. PD-1 and PD-L1 are primarily active in the tumor microenvironment rather than the lymph nodes, leading to more tumor-targeted immune activation rather than the systemic activation seen with CTLA-4 blockade. Even PD-1 knockout mice fare better than those lacking CTLA-4 – though not completely without an autoimmune phenotype, these mice tend to develop tissue-specific, rather than systemic, responses and live substantially longer (29,30).

In light of the history of immunotherapy in mRCC, there was much interest in developing new treatments that took advantage of our vastly expanded knowledge of the immune system and its interaction with the TME. Numerous different vaccine strategies continue to be explored (31) but previous links between tumoral B7-H1 expression and poor prognosis in RCC patients pointed to the potential of targeting the PD-1-PD-L1 interaction (32–38). Frigola et al found a soluble form of B7-H1 in the sera of RCC patients that was associated with larger tumors, advanced stage and grade as well as a higher risk of death (39). With an intact binding domain, this soluble B7-H1 is still capable of delivering pro-apoptotic signals to T cells. Additionally, PD-1 expression was seen on tumor-infiltrating immune cells of mRCC patients and was associated with larger tumors of a higher grade and an increased risk of cancer-specific death (40). The frequently observed expression of both tumor cell B7-H1 and immune cell PD-1 makes the PD-1-PD-L1 axis a logical target in mRCC, offering the potential for significant tumor regression without the serious immunological side effects seen with ipilimumab.

Multiple antibodies targeting PD-1 and PD-L1 were explored as monotherapies in mRCC patients. The first anti-PD-L1 antibody tested, BMS 936559, had an excellent safety profile but the objective response rate in mRCC was a disappointing 12% (41), prompting Bristol Myers to abandon it and focus their efforts on their simultaneously tested anti-PD-1 antibody, BMS 936558, now known as nivolumab. Nivolumab was tested for safety, tolerability and efficacy in Phase I and II trials. It has demonstrated efficacy in mRCC patients with objective response rates ranging from 17% to 29% and significantly fewer grade III or IV IRAEs than seen with ipilimumab (42–46). Genentech's MPDL3280A also targets PD-L1 and proved to be well-tolerated with no dose-limiting toxicities, pneumonitis, colitis or deaths attributed to treatment (47). Though the response rate was similar to BMS 936559, there are ongoing clinical trials of MPDL3280A in mRCC.

With sunitinib already approved for mRCC and such promising responses in phase I and II trials of nivolumab monotherapy with manageable IRAEs, we wondered if there would be a synergistic therapeutic effect between the two. We chose to use the orthotopic Renca murine model of RCC for our studies, believing it to be more physiologically relevant than the more frequently employed subcutaneous model. Renca is among the "highly immunogenic" murine models, based on immunoprofiling, slow tumor growth *in vivo* and response to an immunotherapy regimen (which did not involve checkpoint blockade) (48). The highly immunogenic tumors identified in this study were also marked by large numbers of suppressor cells but very few infiltrating cytotoxic T cells. These observations suggest that an approach that reduces suppressor cell populations while increasing tumor vasculature permeability and simultaneously enhancing the activity and number of tumor-specific T cells would be most effective in a highly immunogenic tumor such as Renca.

Based on the literature, the combination of sunitinib and anti-PD-1 antibody should provide these advantages and hopefully, a therapeutic benefit as well.

MATERIALS AND METHODS

Cell Lines: Renca cells were purchased from ATCC (catalog # CRL-2947) and grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum, Non-Essential Amino Acids (0.1 mM), Sodium Pyruvate (1 mM), L-glutamine (2 mM) and penicillin/streptomycin. Mycoplasma testing of cultured cells was conducted with R&D Systems' MycoProbe Mycoplasma Detection Kit according to the kit directions.

Mice: Female BALB/cJ mice (8-10 weeks of age) were ordered from The Jackson Laboratory (stock number 000651). Female BALB/c $RAG^{-/-}$ mice (8-10 weeks of age) were originally a generous gift from Hyam Levitsky but were later maintained and bred by our laboratory. Mice were housed in specific-pathogen-free facilities. All experimental procedures were performed in accordance with protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

Tumors: For orthotopic tumors, cells were harvested with Trypsin-EDTA (0.25%) which was neutralized with complete medium, spun down @ 800 RPM then washed twice in RPMI alone. After counting, the cells were resuspended in 50% RPMI/50% Matrigel (BD Biosciences) at the appropriate concentration. Mice were anesthetized with a Ketamine (100 mg/kg)/Xylazine (20 mg/kg) combination via intraperitoneal injection. Once adequate depth of anesthesia was confirmed, the pre-shaved area of the right flank was cleansed with an isopropanol wipe and a 2 cm incision was made through the skin. A slightly smaller incision was made in the peritoneum and the kidney was gently brought out of the abdominal cavity. An 18 gauge needle was inserted into the bottom of the kidney and pushed up through the cortex of the kidney then carefully removed. Using a 50 µl glass

Hamilton syringe with a 26 gauge 5/8 inch blunt needle, 30 µl of cell suspension was injected into the kidney capsule at the top of the kidney. After withdrawing the needle, the kidney was returned to the abdominal cavity and the peritoneum was closed with 1-2 stitches. The skin incision was then closed with three 9 mm surgical clips and triple antibiotic ointment was applied. Mice received 5 mg/kg Ketoprofen intraperitoneally and were kept warm while recovering from anesthesia. For subcutaneous tumors, cells were harvested with Trypsin-EDTA (0.25%) which was then neutralized with complete medium, spun down @ 800 RPM then washed twice in RPMI alone. After counting, the cells were resuspended in RPMI alone at the appropriate concentration. Tumors were established by subcutaneous injection of 50 µl of cell suspension in the mouse's flank. Tumors were measured 2-3 times weekly using digital calipers and tumor volume was calculated using the modified ellipsoid formula of 1/2 (Length x Width^2) (49,50). Tumor-bearing mice were sacrificed when tumor volume exceeded 1500 mm^3 or tumors became ulcerated.

Sunitinib: For *in vitro* experiments, Free Base Sunitinib/Sutent/SU-11248 (S-8877) was purchased from LC Laboratories and dissolved in DMSO to yield a 5mM stock solution. All *in vivo* experiments utilized Sunitinib Malate Salt/Sutent/SU-11248 (S-8803, LC Laboratories) which was resuspended in Ora-Plus (Paddock Laboratories) at a concentration of 10.4 mg/ml. Unless otherwise specified, mice were dosed daily with 40 mg/kg via oral gavage, a dose recommended by our colleague Hans Hammers to most closely model the effect of sunitinib in renal cell carcinoma patients but not completely eradicate or prevent the growth of tumors in mice.

Anti-PD-1 Antibodies: Anti-PD-1 antibody was purified from a hamster monoclonal antibody (mAb)-producing hybridoma (clone G4, a gift from Leiping Chen) supernatant

produced in our laboratory for the earliest experiments and later provided by CoStim Pharmaceuticals (clone 8H3). Mice not receiving anti-PD-1 antibody were given the appropriate isotype-matched control. Antibodies and isotype controls were dosed 3 times at 10 mg/kg in sterile PBS, administered every 3 days via intraperitoneal injection.

Cell/Tissue Harvest: Mice were first anesthetized with a Ketamine (150 mg/kg)/Xylazine (30 mg/kg) combination via intraperitoneal injection. Once an extremely deep state of anesthesia was confirmed, mice were perfused with 30 ml of 1x PBS via transcardial perfusion using a 21 G Vacutainer Safety-Lok Blood Collection Set (BD) inserted into the left ventricle and then severing the inferior vena cava. Spleens, lymph nodes, normal kidneys and tumors/tumor-bearing kidneys were all harvested into harvest media. A single cell splenocyte suspension was generated by mashing spleens through a 100 micron cell strainer which was rinsed with harvest media, then centrifuging at 1200 rpm for 5-7 minutes, decanting and resuspending in 2.5 ml ACK Lysing Buffer for 2 minutes before dilution with 1x PBS. They were again spun down, decanted and the resulting cell pellets were resuspended in 10 ml harvest media for counting and eventual staining or plating for stimulation. Lymph nodes were mashed in a 24 well dish, then transferred to a microcentrifuge tube for pelleting at 1200 rpm for 5 minutes in a benchtop centrifuge then resuspending in 500 µl – 1 ml of harvest media (depending on the size of the pellet). Kidneys and tumor-bearing kidneys were typically weighed and photographed prior to processing. For isolating infiltrating lymphocytes, kidneys and tumors/tumor-bearing kidneys were coarsely chopped with scissors, mashed through a 100 micron cell strainer, washed, pelleted and then subjected to a 44%/67% Percoll (GE Healthcare, 17-0891-01) gradient. The buffy coat was removed, washed, pelleted and resuspended in harvest media

for counting and eventual plating for staining or stimulation. When the kidney or tumor cells themselves were desired for analysis, non-necrotic tissue was selected and finely minced with razor blades before digestion in a shaker for 2 hours at 37°C in Collagenase I (1 mg/ml, Life Technologies, 17100-017). Following digestion, the samples were pelleted, decanted and resuspended in 3 ml warm Trypsin-EDTA (0.25%) followed by a 5 minute incubation at 37°C. The Trypsin was neutralized with 3 ml harvest media containing 500 U DNase I (Roche, 10104159001) before filtering the samples through a 70 micron cell strainer. After pelleting and decanting, the digested cells were resuspended in media to be plated for surface staining for flow cytometry.

Flow Cytometry/Intracellular Cytokine Staining: Before staining, samples intended for intracellular cytokine analysis were stimulated for 5-6 hours in harvest media supplemented with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml), Ionomycin (500 ng/ml) and Protein Transport Inhibitor Cocktail (1:500, eBioscience). For experiments with more than four colors, cells were first incubated with LIVE/DEAD Fixable Aqua (Invitrogen) diluted 1:5000 in 1x PBS for 30 minutes at room temperature in the dark, then washed with 1x PBS or FACS buffer (1x PBS and 5% FBS). Flow antibodies (purchased from BD Pharmingen, eBiosciences, Biolegend or Invitrogen) were diluted in PBS or FACS buffer for surface staining or Permeabilization Buffer (eBioscience, 00-8333-56) for intracellular staining. For surface staining, cells were incubated in the diluted antibody cocktails for 20-30 minutes at room temperature in the dark, then washed with 1x PBS or FACS buffer. If no intracellular staining was to be performed, the samples were either resuspended in PBS or FACS buffer for immediate analysis or subjected to a gentle 30 min fixation in 2% paraformaldehyde at 4°C for later analysis. For intracellular staining, cells

were incubated in Fixation/Permeabilization solution (eBioscience, 88-8824-00) for 45 minutes at room temperature in the dark, then washed with Permeabilization Buffer before incubation in the intracellular antibody cocktail for 20-30 minutes at room temperature in the dark. After staining, the cells were washed with Permeabilization Buffer and resuspended in PBS. Samples were analyzed using either a FACSCalibur (BD) or LSR II (BD) and flow cytometric data were exported and analyzed using FlowJo software (Tree Star, Inc.). Graphing and statistical analyses (unpaired, two-tailed Student's t-test) were performed using Prism software (GraphPad Software, Inc.).

RESULTS

Combination therapy in orthotopic tumors from *Mycoplasma*-contaminated cell cultures Preliminary in vivo experiments in our laboratory examined the anti-tumor and immunologic effects of sunitinib and anti-PD-1 antibody alone or combined in a small number of mice orthotopically implanted with 5 x 10^5 Renca cells. After five days of tumor growth, anti-PD-1 antibody was administered every three days for a total of five doses. Sunitinib treatment was started after eight days and continued daily through the end of the experiment (day 20). While the combination therapy showed only a modest increase in anti-tumor effect compared to sunitinib alone (**Figure 1A**), the numbers of IFNγ+ CD8 T cells and IL-2+ CD4 T cells in the tumor draining lymph nodes (TDLN) increased dramatically (49-fold and 14-fold respectively) (**Figure 1B-C**).

Unfortunately, routine testing of the Renca cells used in these early experiments revealed that the frozen stocks were all contaminated with *Mycoplasma* bacteria. Newly purchased Renca cells from ATCC were expanded to make new frozen stocks for future experiments. These stocks were *Mycoplasma*-negative in all subsequent tests.

Combination therapy in orthotopic tumors from *Mycoplasma*-negative cell cultures

Multiple in vivo experiments using the same number of implanted cells with the same treatment regimen were conducted with these new, uncontaminated Renca cells. No individual experiment demonstrated a difference in anti-tumor effect between sunitinib alone and combination therapy. Combined tumor burden data from four orthotopic experiments (5-10 mice per treatment group in each experiment) clearly illustrates that

adding anti-PD-1 antibody to sunitinib does not reduce the tumor burden compared to sunitinib treatment alone (p=0.8184) (**Figure 2**) though both regimens are significantly better (p<0.0001) than no treatment or anti-PD-1 alone. Somewhat surprisingly, we found that anti-PD-1 monotherapy was no more effective than vehicle/IgG control (p=0.6533). On more than one occasion, the mice who received anti-PD-1 antibody alone actually fared slightly worse than their untreated counterparts (data not shown), though not to the extent shown in Figure 1.

Flow cytometric analysis of the IFNγ+ CD8 T cells from these experiments did not yield the same dramatic differences as the earlier studies with contaminated tumor cultures. The following p values and graphs are from a single experiment but are representative of all results. In the TDLN, mice receiving combination therapy did not have significantly greater numbers (p=0.6467) or percentages (p=0.0635) of IFNγ+ CD8 T cells when compared to mice treated with sunitinib alone **(Figure 3A-B)**. There was, however, a difference in the numbers of IFNγ+ CD8 T cells in the tumor-infiltrating lymphocytes (TIL) of combination-treated mice compared to sunitinib alone (p=0.0363) **(Figure 3C)**. Even when tumor burden is taken into account, the number of IFNγ+ CD8 T cells per gram of tumor was still significantly different between the two groups (p=0.0347) **(Figure 3D)**. The difference in percentage of IFNγ+ CD8 T cells in the TIL was not statistically significant (p=0.2704) **(Figure 3E)**.

We saw none of the differences in IL-2+ CD4 T cells that were observed with the *Mycoplasma*-contaminated Renca cells. In the TDLN, the number of these cells was roughly equivalent for all four groups of mice **(Figure 4A)**. When we examined the percentage of IL-2+ CD4s, mice receiving the combination treatment did have a

significantly greater proportion than the mice who received anti-PD-1 alone (p=0.0379) but not those given sunitinib alone (p=0.1412) **(Figure 4B)**. In the TIL, the only significant difference was between the numbers of IL-2+ CD4s in the anti-PD-1 only and combination mice. Unlike the percentage in the TDLN, here the anti-PD-1 treated mice had significantly greater (p=0.0212) numbers of IL-2+ cells **(Figure 4C)** but a slightly smaller percentage **(Figure 4E)**. When tumor weight was taken into consideration, there were no significant differences between any of the treatment groups.

We then looked at a population of CD4 T cells that could potentially be involved in down-regulating the anti-tumor immune response. FoxP3+ CD4 T cells can be "regulatory" T cells and suppress T helper functions during an immune response or play a role in the maintenance of tolerance. FoxP3 can also serve as an activation marker though, so without a functional suppression assay we cannot say with certainty that the FoxP3+ cells we observed were responsible for, or capable of, suppressing the anti-tumor immune response. The issue of the validity of FoxP3 as a marker of regulatory T cells is irrelevant in this case because there were no significant differences in the numbers or percentages of FoxP3+ CD4 T cells in the TDLN or TIL between the sunitinib alone and combination treated mice. Mice who received anti-PD-1 plus sunitinib did have somewhat lower numbers of FoxP3+ cells in their TDLN **(Figure 5A)** but the percentages were essentially the same for all of the regimens **(Figure 5B)**. In the TIL, the only statistically significant difference we noted was between the percentage of FoxP3+ cells of untreated and combination mice **(Figure 5E)** where combination mice had a significantly higher proportion of FoxP3+ cells (p=0.0425).

Sunitinib treatment has been shown) to decrease the population of myeloid-derived suppressor cells (MDSC) in mouse models as well as in RCC patients (51). We observed the same phenomenon in our orthotopic Renca experiments, with MDSC defined as GR1+ CD11b+ cells by flow cytometric analysis. When examining the TIL, mice treated with sunitinib alone or in combination with anti-PD-1 have significantly lower percentages of MDSC ($p = 0.0001$ and $p = 0.0003$, respectively) when compared to untreated mice **(Figure 6A)**. For mice treated with anti-PD-1 alone, there is a marked, but not statistically significant ($p=0.0540$), reduction relative to the untreated mice. The same holds true for the number of MDSC in the TIL ($p=0.0323$ for sunitinib, $p=0.0294$ for combination and $p=0.4346$ for PD-1 alone) **(Figure 6B)** and per gram of tumor ($p=0.0248$, $p=0.0412$ and $p=0.3135$ respectively) **(Figure 6C)**. Though present, the differences were not as significant for the number of MDSC in the TDLN ($p=0.0569$, $p=0.0953$ and $p=0.8311$ respectively) **(Figure 6D)**.

In vitro surface expression of B7-H1, MHC II and B7-DC in Renca cells

In order to better understand the lack of additional anti-tumor effect conferred by adding anti-PD-1 antibody to sunitinib treatment, we examined Renca cells' *in vitro* expression of three common co-stimulatory molecules – B7-H1 (PD-L1), MHC Class II (the ligand of another checkpoint molecule with therapeutic potential, lymphocyte activation gene 3 (LAG-3)) and B7-DC (PD-L2, another ligand of PD-1) via flow cytometry under different culture conditions. At baseline (standard culture media), only B7-H1 expression was detected **(Figure 7A)**. In order to simulate how expression might change in the setting of

an *in vivo* anti-tumor immune response, we cultured Renca cells with 100 units/mL (10ng/mL) IFNγ. After 24 hours in the presence of IFNγ, B7-H1 expression increased more than five-fold from baseline. Median fluorescence intensity (MFI) was 68 at baseline compared to 365 when cultured with IFNγ **(Figure 7B)**. MHC II expression was also affected - MFI increased from 4.3 to 39 in the presence of IFNγ. B7-DC expression was not induced by IFNγ **(Figure 7C)**.

We then interrogated the direct effect of sunitinib on B7-H1 expression in Renca cells cultured with IFNγ. Assuming that 100nM represents a physiologically relevant dose, we examined doses of sunitinib ranging from 10nM to 1μM for either 24 or 48 hours but saw no effect on the MFI (data not shown) or the percentage of B7-H1 positive cells **(Figure 7D)**.

In vivo surface expression of B7-H1 and MHC II in harvested tumor cells

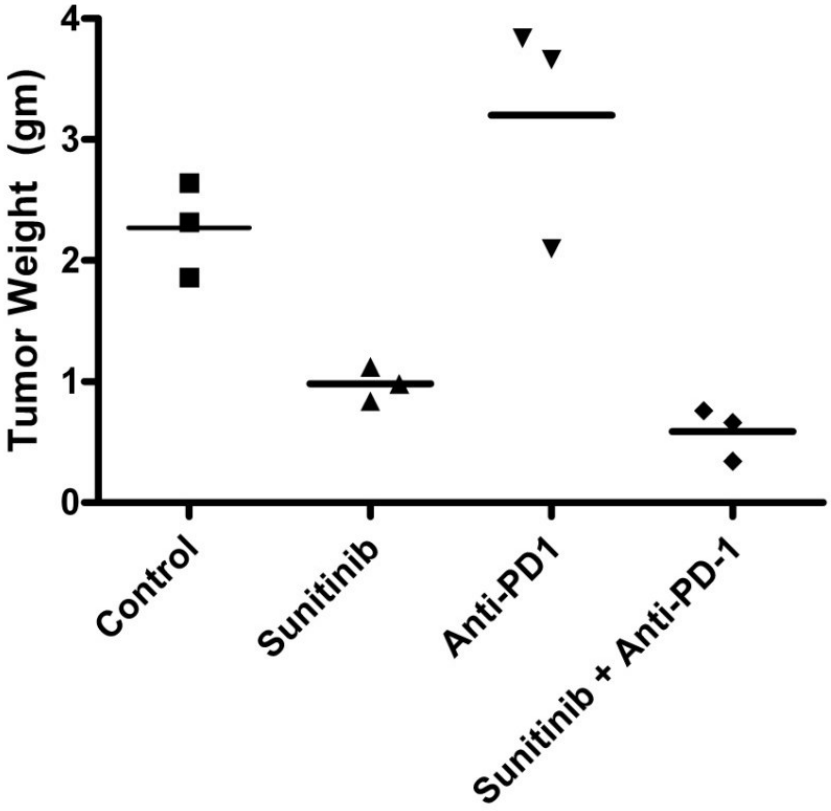
Though sunitinib did not change B7-H1 expression *in vitro*, we wondered if treatment with sunitinib would affect *in vivo* expression. Orthotopic Renca tumors were implanted in wild-type (WT) BALB/c mice and allowed to grow for five days before treatment was initiated. Mice received vehicle or sunitinib (20 mg/kg) via oral gavage for seven days. Note that this sunitinib dose is half our normal dose. It was necessary to use a less effective dose in order to yield sufficient tumor tissue for analysis. The tumors were harvested and digested to yield a single cell suspension suitable for fluorescent antibody staining and flow cytometric analysis. Mice treated with sunitinib had significantly lower percentages of B7-

H1 positive cells (p=0.0006) **(Figure 8A)**. While not as dramatic a decrease, treated mice had significantly reduced proportions of MHC II+ cells (p=0.0116) **(Figure 8B)**.

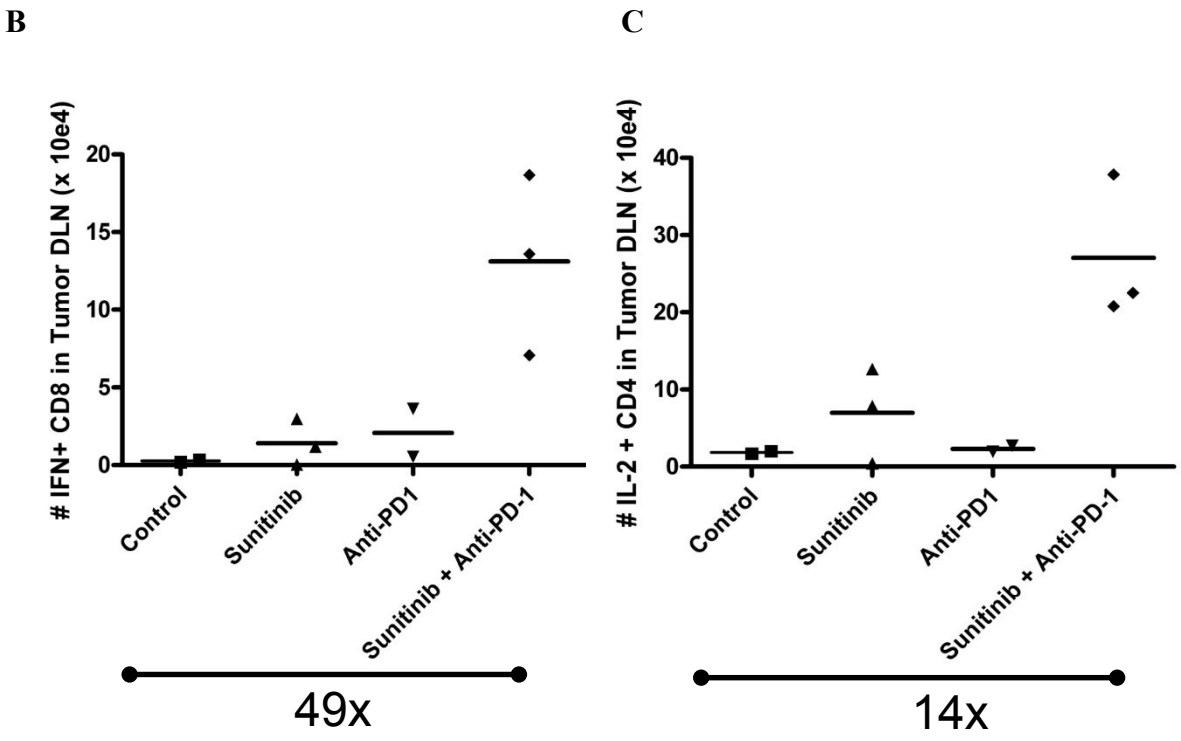
Sunitinib effects in RAG1$^{-/-}$ tumor-bearing mice

As a first step towards elucidating the mechanism by which sunitinib was able to reduce the proportion of B7-H1 positive tumor cells, we turned to the RAG1$^{-/-}$ mouse model (RAG KO) on a Balb/c background. RAG KO mice are unable to perform V(D)J recombination and lack mature T and B cells (52). Renca tumors were orthotopically implanted and sunitinib or vehicle was administered via oral gavage for nine days, from Day 7 until Day 15. Tumors were harvested, weighed and digested to facilitate fluorescent antibody staining and flow cytometric analysis. Unlike in WT mice, sunitinib treatment of tumor-bearing RAG KO mice did not result in a significant reduction in tumor burden (p=0.2888) **(Figure 9A)**. Examination of B7-H1 expression via flow cytometry showed that RAG KO mice did not exhibit the significant reduction in percentage of B7-H1 positive cells seen in WT mice (p=0.0952 vs 0.0006 for WT) **(Figure 9B)**. For MHC II, there was a significant reduction with sunitinib treatment in RAG KO mice (p=0.0118), just as in WT mice **(Figure 9C)**. Though we do not have B7-DC data for WT mice, in RAG KO mice there were significantly lower proportions of B7-DC+ cells (p=0.0023) **(Figure 9D)**.

A



<u>Figure 1:</u> Sunitinib + anti-PD-1 combination treatment of *Mycoplasma* contaminated Renca tumors. Sunitinib + anti-PD-1 combination therapy shows a modest increase in anti-tumor effect but significantly improves the immunological response to *Mycoplasma* contaminated Renca tumors**.** Renca cells that were later determined to be contaminated with *Mycoplasma* were orthotopically implanted and, once established, treated with Sunitinib, anti-PD-1 monoclonal antibody or a combination of the two. **(A)** Tumor burden of mice in the four treatment groups.



(B) Number of IFNγ+ CD8 T cells in the tumor draining lymph nodes. **(C)** Number of IL-2+ CD4 T cells in the tumor draining lymph nodes.

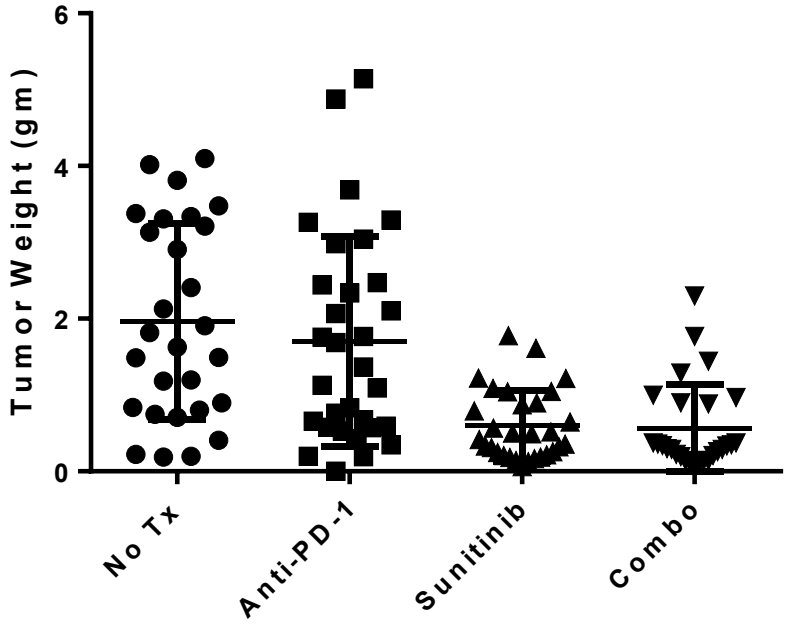


Figure 2: Sunitinib + anti-PD-1 combination treatment of *Mycoplasma*-negative Renca tumors. The combination of sunitinib and anti-PD-1 does not increase anti-tumor effect compared to sunitinib alone in non-*Mycoplasma*-contaminated orthotopic Renca tumors. Combined tumor burden from four experiments.

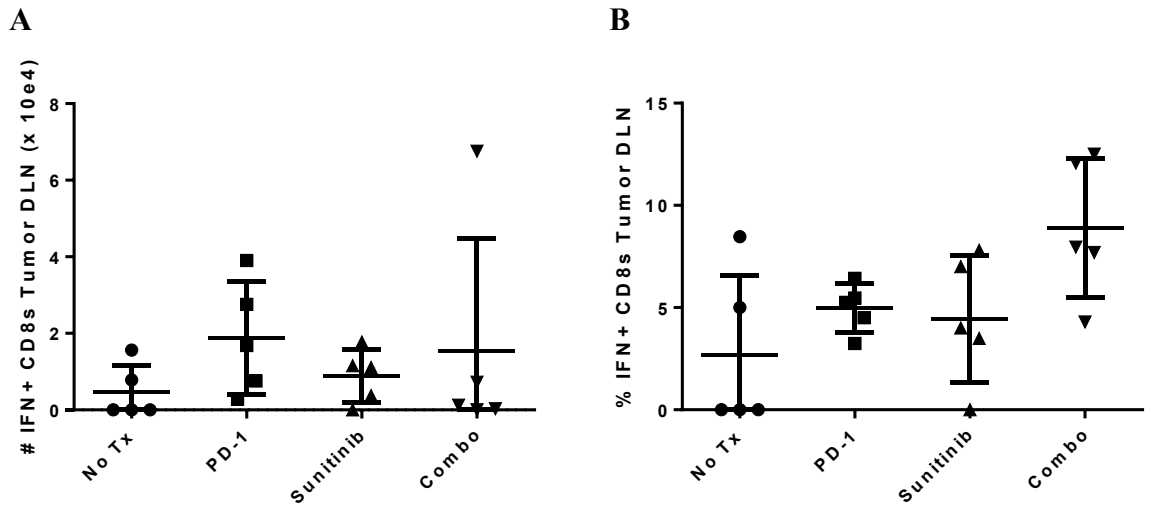
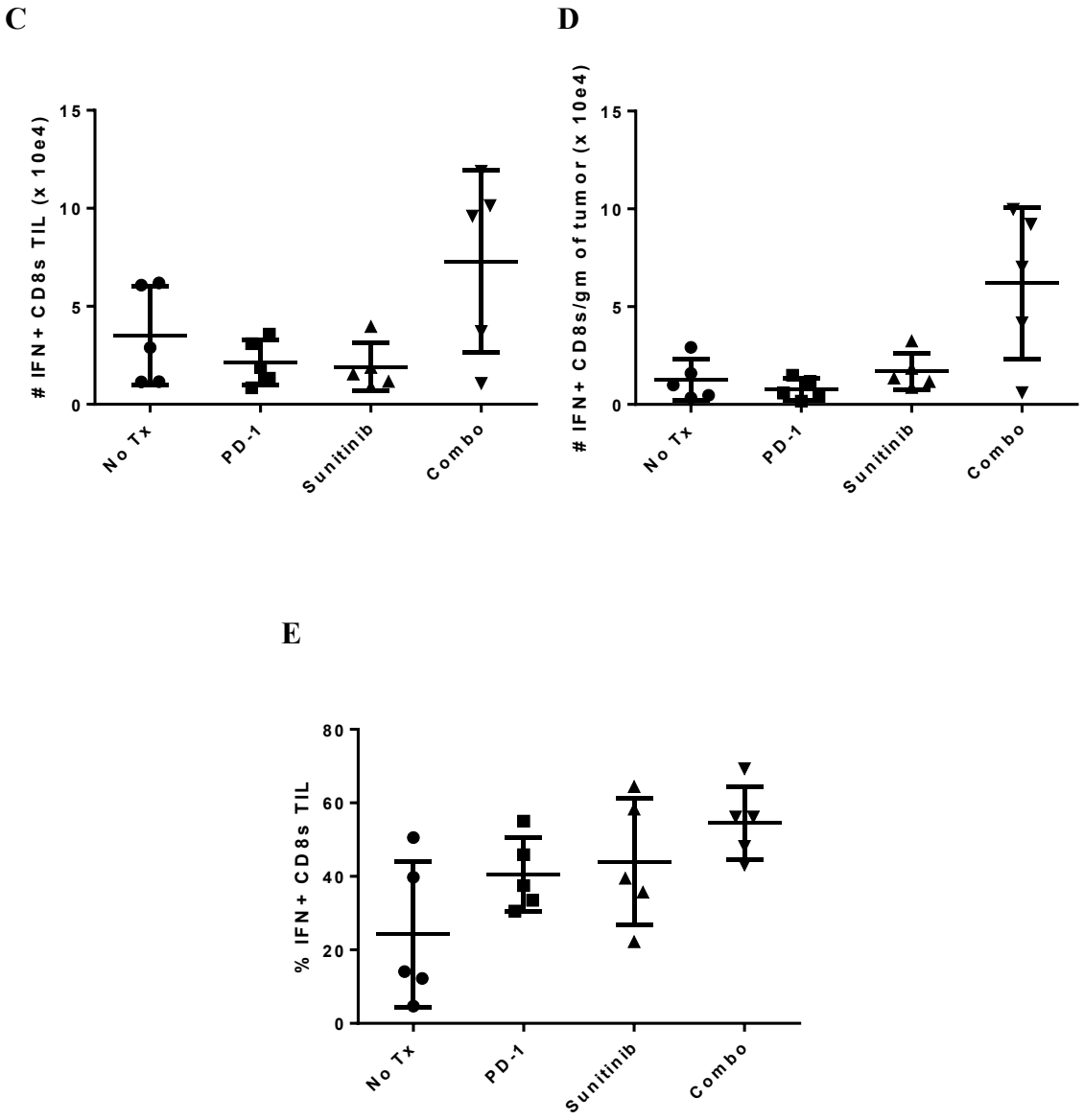


Figure 3: IFNγ+ CD8 T cells in mice treated with combination therapy. IFNγ+ CD8 T cells are increased in the TIL, but not TDLN with combination therapy. **(A)** Number of IFNγ+ CD8 T cells in the TDLN. **(B)** Percentage of IFNγ+ CD8 T cells in the TDLN.



(**C)** Number of IFNγ+ CD8 T cells in the TIL. **(D)** Number of IFNγ+ CD8 T cells in the TIL per gram of tumor. **(E)** Percentage of IFNγ+ CD8 T cells in the TIL.

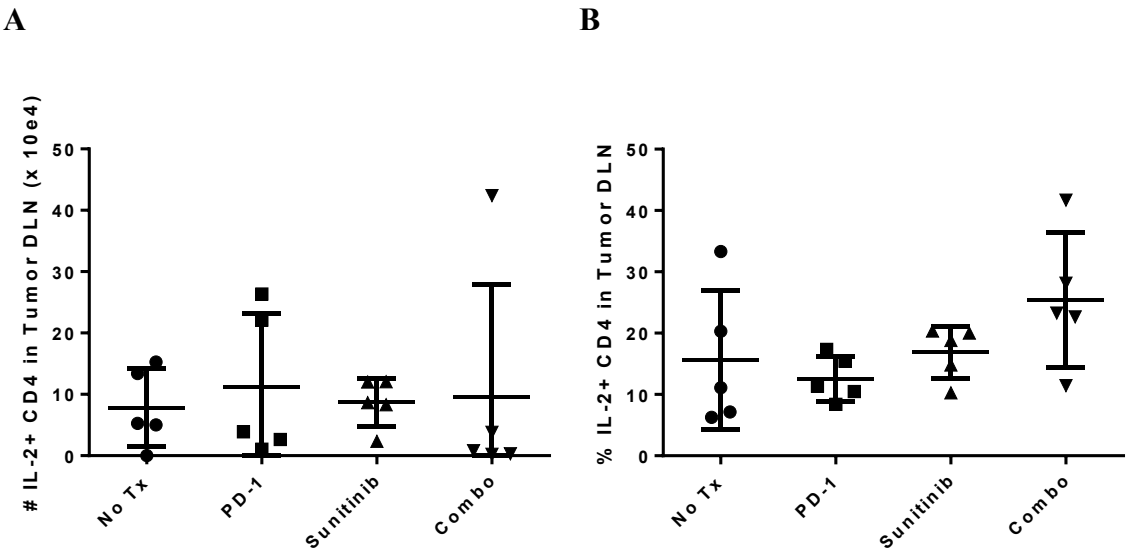
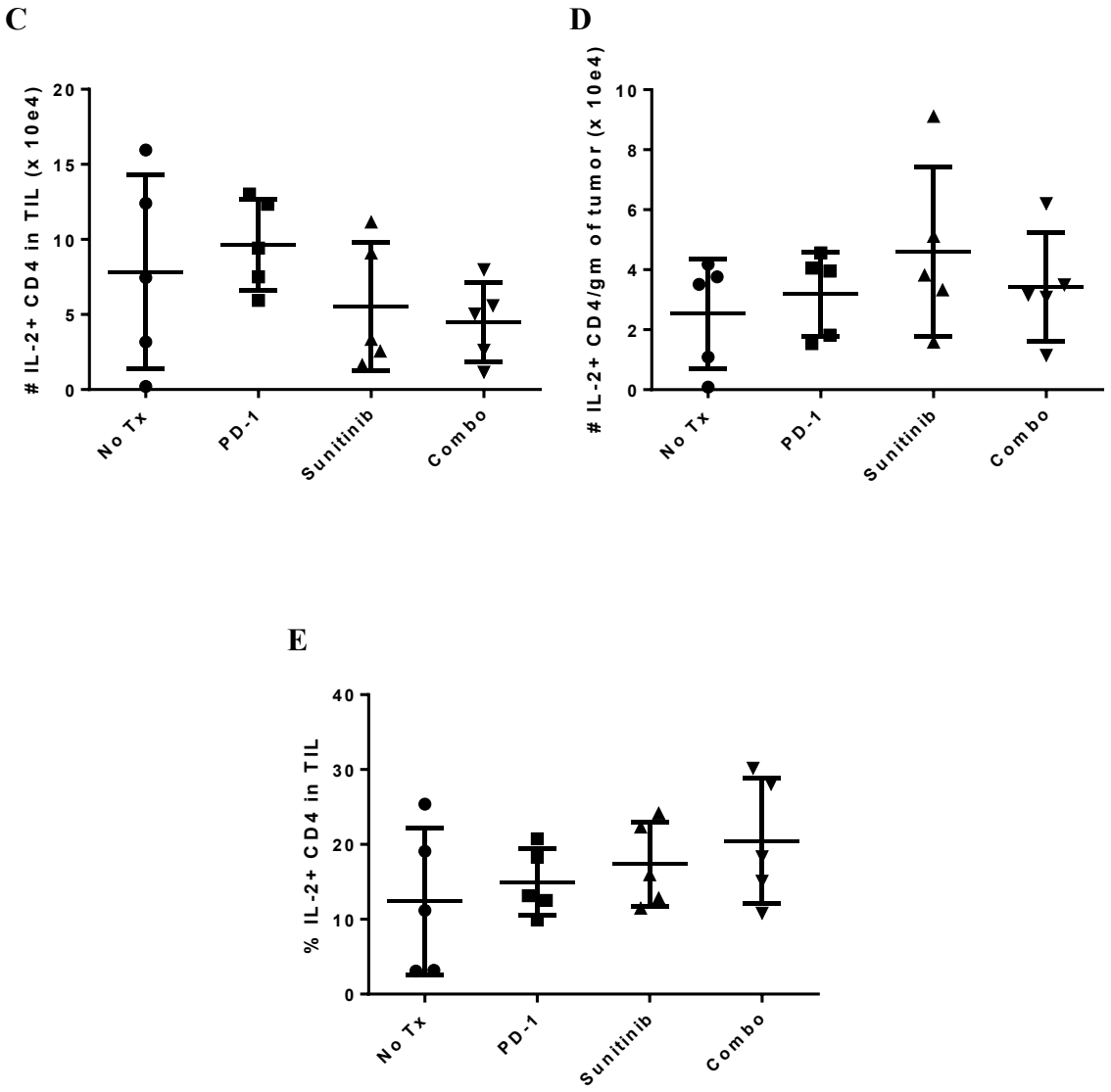


Figure 4: IL-2+ CD4 T cells in mice treated with combination therapy. Mice treated with combination therapy do not have significantly greater numbers or percentages of IL-2+ CD4 T cells in their TDLN or TIL compared to sunitinib treated mice. **(A)** Number of IL-2+ CD4 T cells in the TDLN. **(B)** Percentage of IL-2+ CD4 T cells in the TDLN.



(C) Number of IL-2+ CD4 T cells in the TIL. **(D)** Number of IL-2+ CD4 T cells in the TIL per gram of tumor. **(E)** Percentage of IL-2+ CD4 T cells in the TIL.

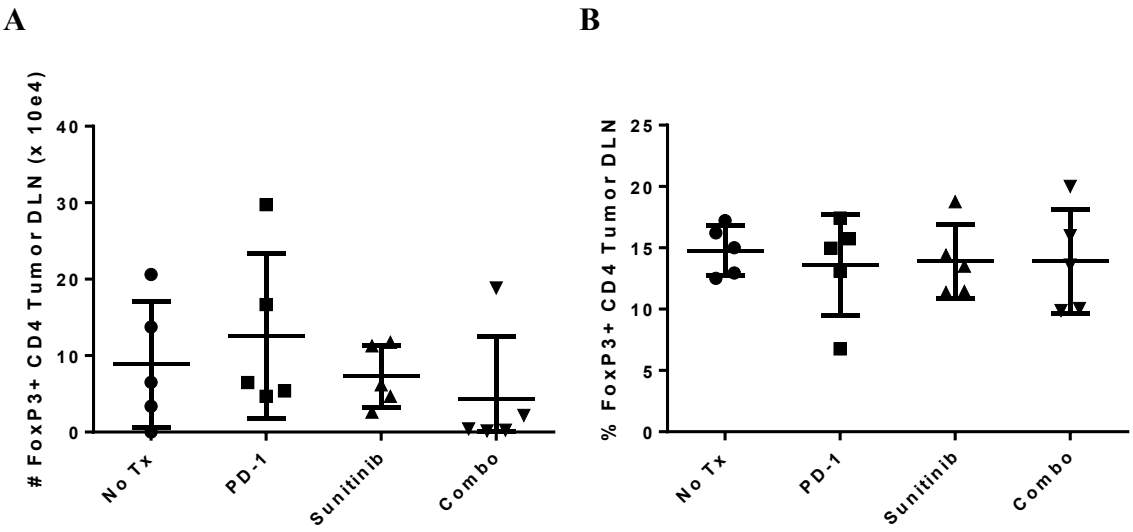
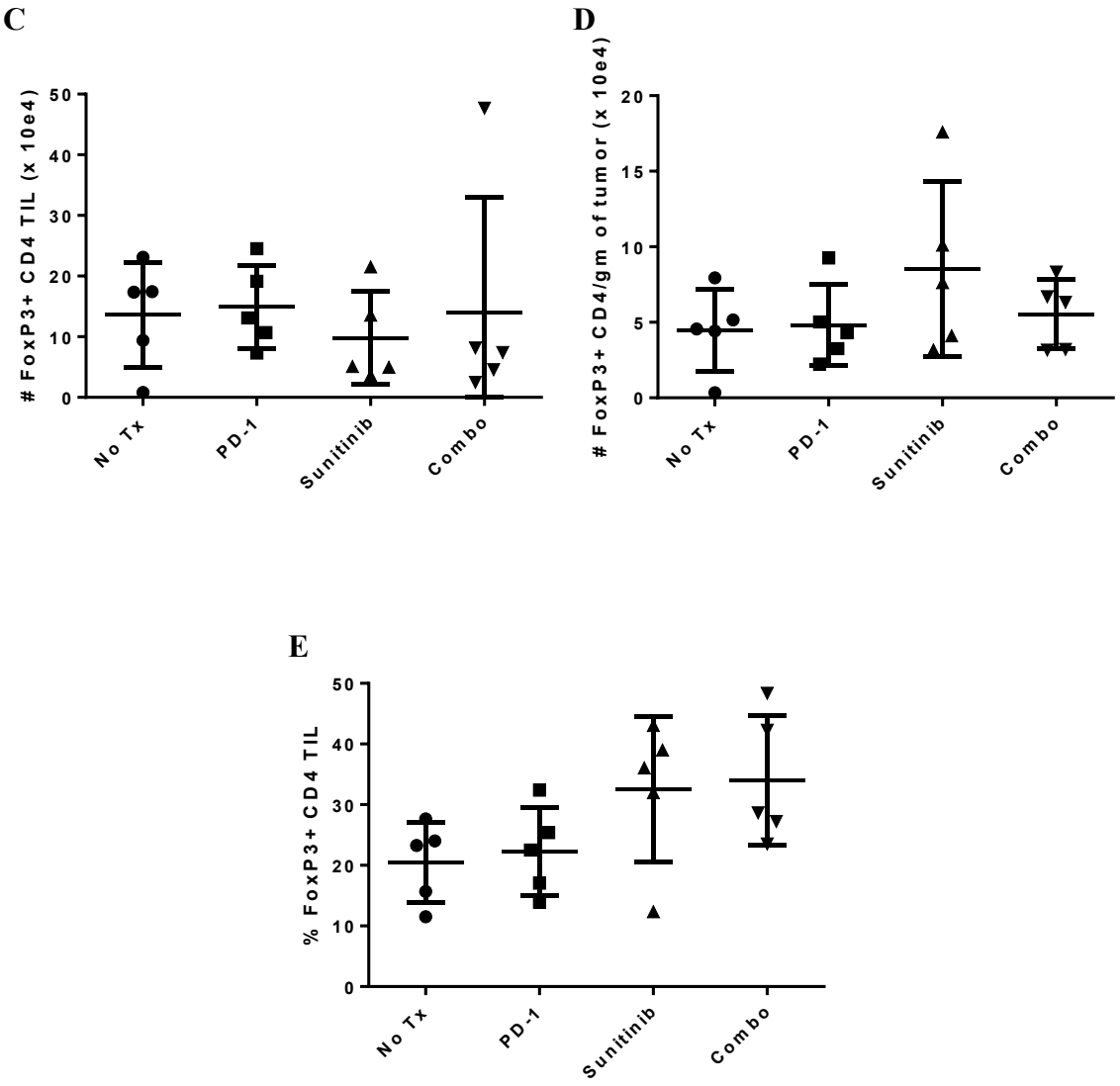


Figure 5: FoxP3+ CD4 T cells in mice treated with combination therapy. The number and percentages of FoxP3+ CD4 T cells in the TDLN and TIL of combination and sunitinib-treated mice were not significantly different. **(A)** Number of FoxP3+ CD4 T cells in the TDLN. **(B)** Percentage of FoxP3+ CD4 T cells in the TDLN.



(C) Number of FoxP3+ CD4 T cells in the TIL. **(D)** Number of FoxP3+ CD4 T cells in the TIL per gram of tumor. **(E)** Percentage of FoxP3+ CD4 T cells in the TIL.

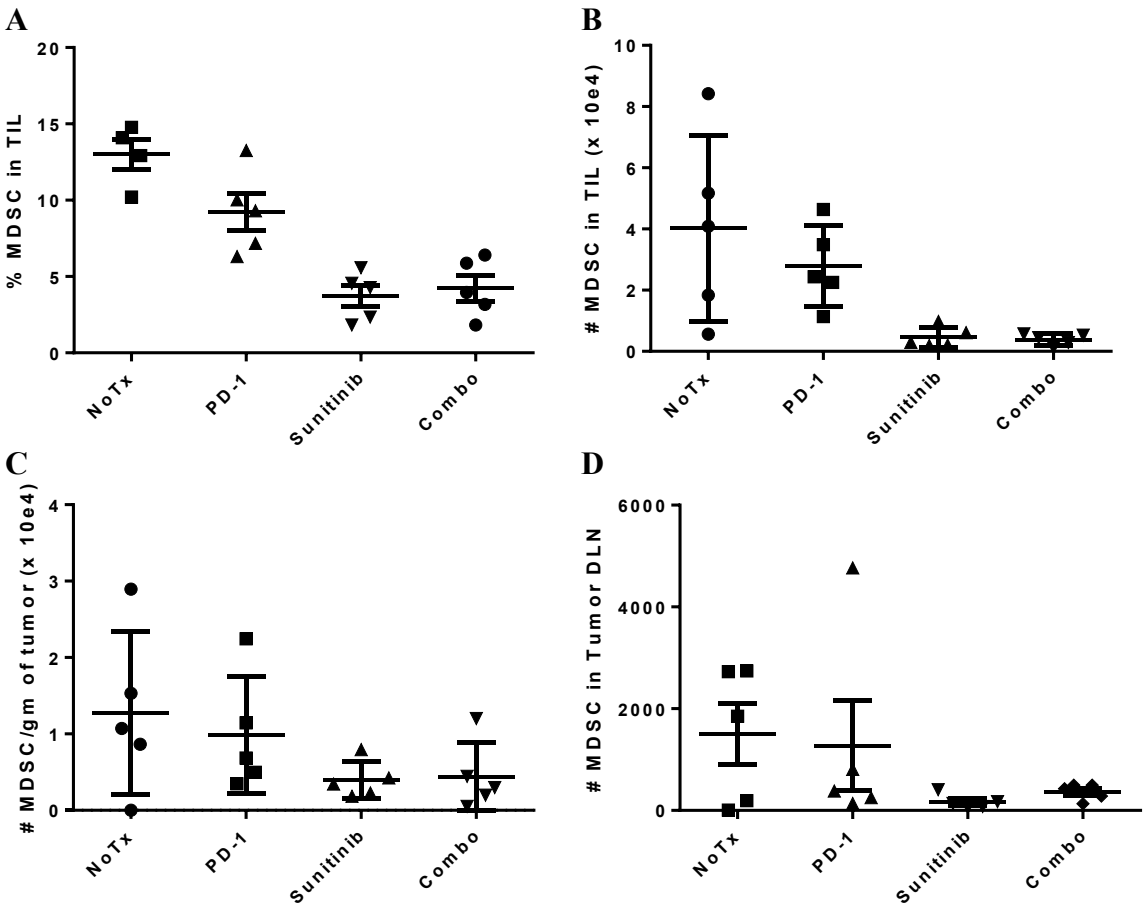


Figure 6: Sunitinib and myeloid derived suppressor cells (MDSC). Treatment with sunitinib, alone or in combination with anti-PD-1 antibody, reduces myeloid derived suppressor cells (MDSC) numbers and percentages. **(A)** Mice treated with sunitinib alone or sunitinib in combination with anti-PD-1 antibody have significantly lower percentages and **(B)** numbers of MDSC in their TIL compared to untreated or anti-PD-1 only mice, as well as **(C)** significantly fewer MDSC per gram of tumor. **(D)** In the TDLN of mice treated with sunitinib alone or the combination therapy, the numbers of MDSC are reduced for both groups, but not significantly.

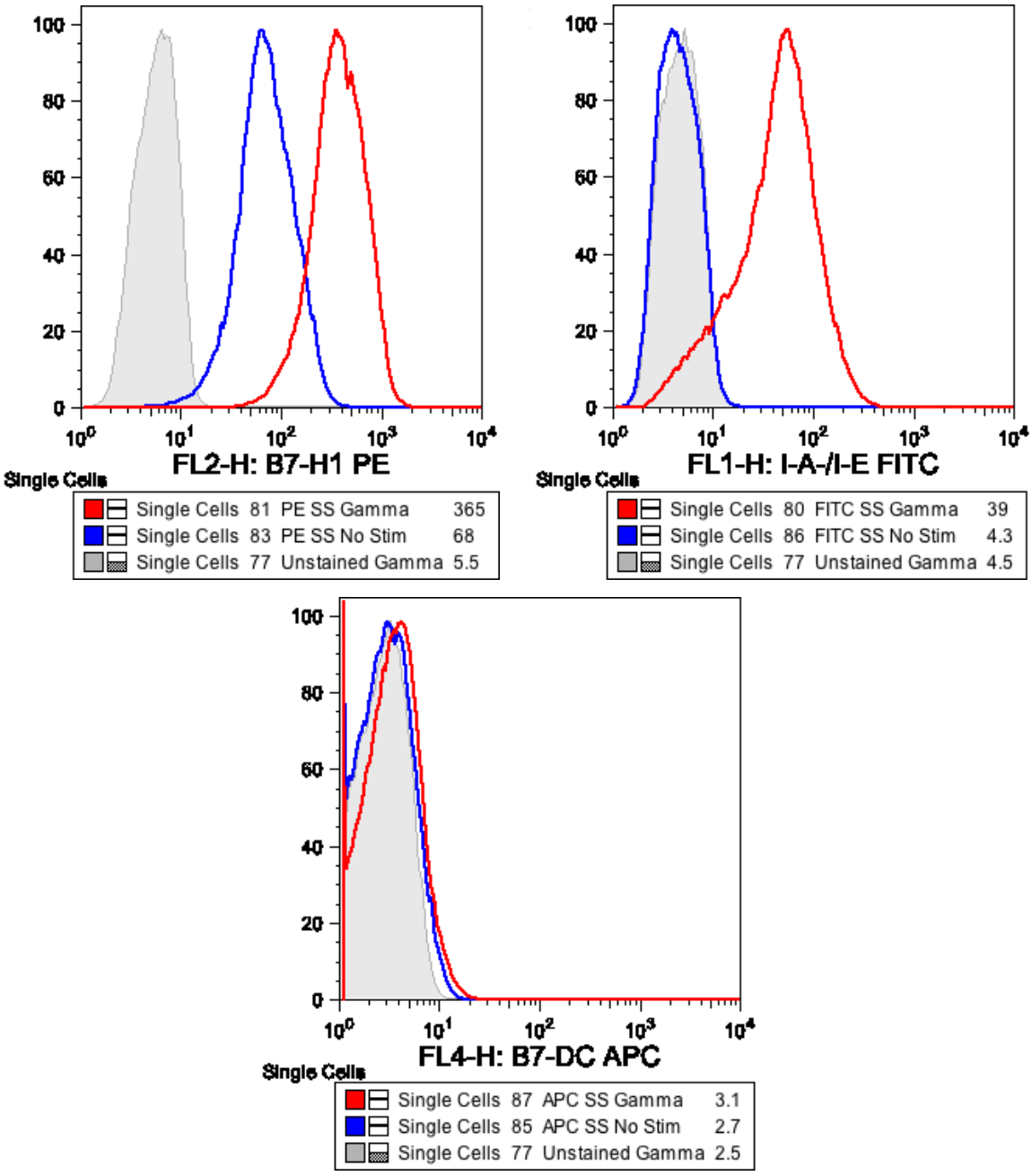
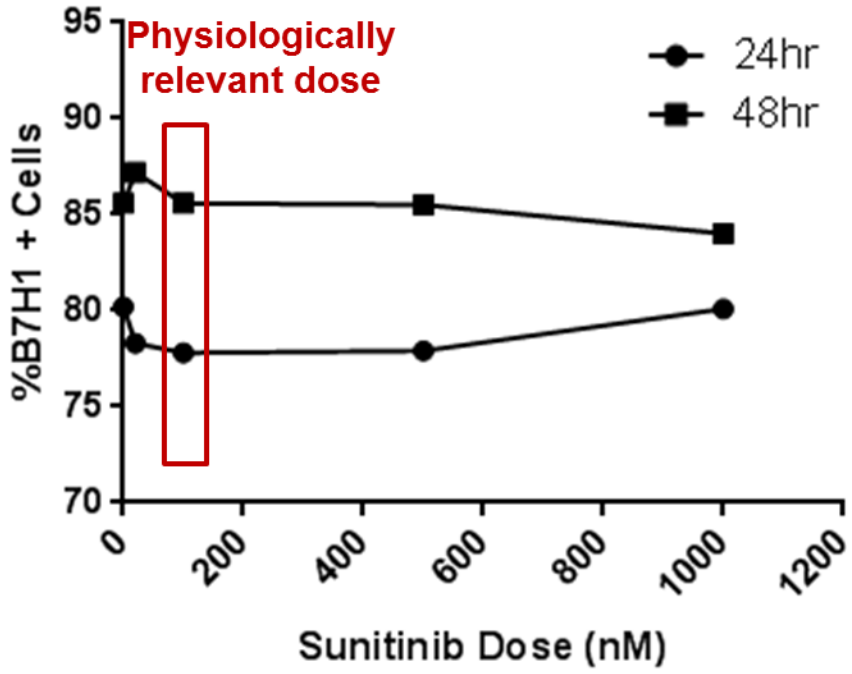


Figure 7: ***In vitro*** **expression of co-stimulatory molecules on Renca cells.** Renca cells were cultured with and without IFNγ **(A)** B7-H1 was expressed at baseline and increased more than five-fold when cultured with IFNγ. **(B)** MHC II was not expressed at baseline but did respond to IFNγ stimulation. **(C)** No B7-DC expression was observed at baseline or with IFNγ.

D



(D) B7-H1 expression after 24 or 48 hours of culture with a range of sunitinib concentrations.

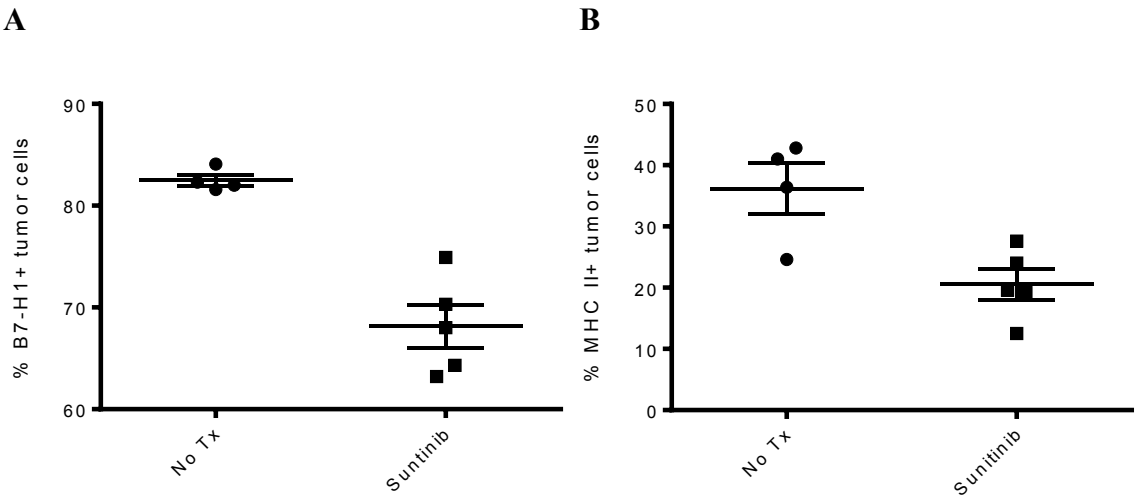
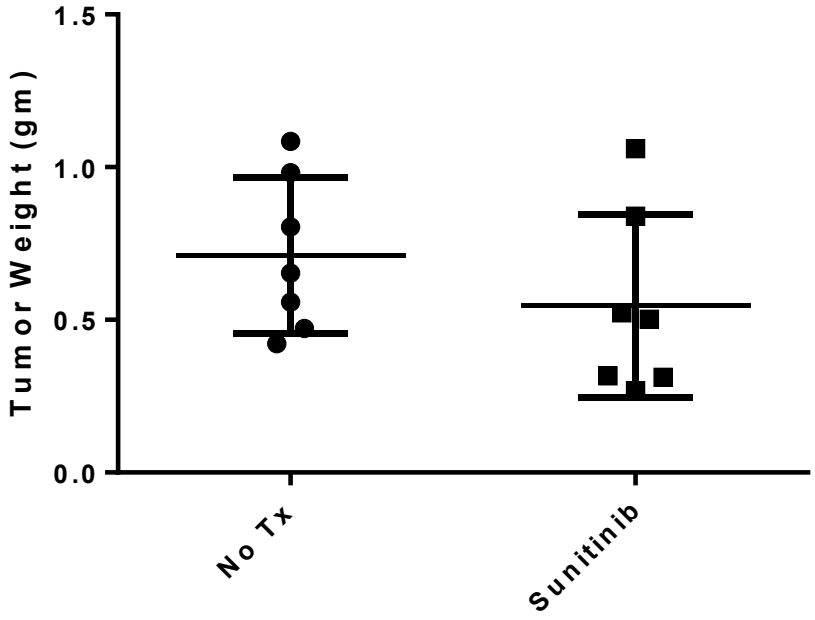
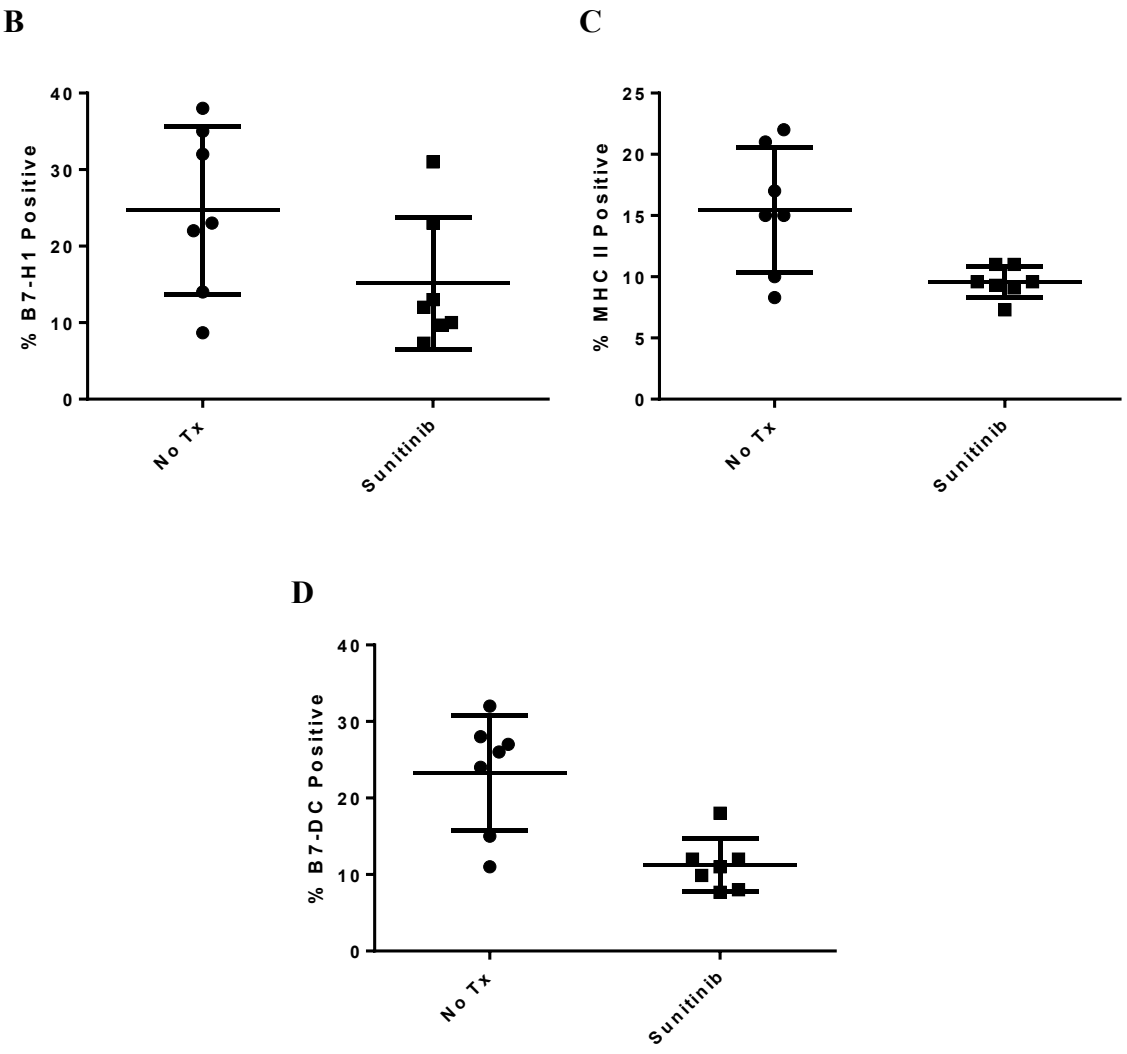


Figure 8: *In vivo* sunitinib treatment and co-stimulatory molecules. *In vivo* sunitinib treatment reduces B7-H1 and MHC II positivity in harvested tumor cells. Renca tumors were implanted orthotopically and allowed to grow for 5 days before commencing treatment. Mice received vehicle (No Tx) or 20 mg/kg sunitinib via oral gavage for 7 days before tumors were harvested. **(A)** A significantly lower proportion of tumor cells were B7-H1+ in mice treated with sunitinib. **(B)** The percentage of MHC II+ cells was also significantly lower in treated mice.

A



<u>Figure 9</u>: Effects of sunitinib treatment in T and B cell deficient RAG knockout mice. The effects of sunitinib were blunted in RAG KO mice. RAG KO mice with established orthotopic Renca tumors were given vehicle or sunitinib via oral gavage. (A) After nine days of treatment, there was no significant difference in tumor weights between mice who received sunitinib and those given vehicle (No Tx).



(B) Percentage of B7-H1+ tumor cells in untreated or sunitinib-treated RAG KO mice. **(C)** A significantly lower proportion of tumor cells were MHC II+ in RAG KO mice treated with sunitinib. **(D)** The percentage of B7-DC+ cells was also significantly reduced.

DISCUSSION

Despite the observed efficacy of anti-PD-1 monotherapy in a subset of renal cell carcinoma patients, we found that monotherapy antibody blockade of PD-1 does not reduce tumor burden in the orthotopic Renca murine model. As expected, sunitinib monotherapy has significant anti-tumor effects in this model. Though it provided a significant therapeutic benefit similar to that seen with sunitinib alone, the combination of sunitinib and PD-1 blockade offered no treatment advantage, at least in the short-term. While pilot experiments showed a dramatic immunological advantage to combination therapy, these results were only seen when *Mycoplasma*-contaminated tumor cell cultures were used to establish the orthotopic tumors. The increased numbers of IFNγ+ CD8 T cells and IL-2+ CD4 T cells observed in the TDLN of mice given combination therapy could not be replicated with *Mycoplasma*-negative cells.

In the tumor itself, combination therapy does seem to provide some modest immunological benefit over treatment with sunitinib alone. We observed increased numbers of IFNγ+ CD8 T cells in the TIL of mice given both agents. Despite seeing no short-term therapeutic advantage to the combination of sunitinib and anti-PD-1, it may be that in the longer term or combined with other immunotherapies, co-administration could prove to be advantageous. Sunitinib is known to induce leukopenia and in some of our experiments, the combined therapy resulted in greater overall numbers of total cells and of CD4 and CD8 T cells specifically in the spleens and lymph nodes (data not shown). This boosting of cell numbers could improve the long-term immune response in the setting of sunitinib therapy.

Though Renca has been classified as “immunogenic”, the authors of this paper are describing the immune infiltrates in subcutaneous Renca tumors and an anti-tumor response to their own combinatorial immunotherapy regimen which includes a dendritic cell (DC) vaccine and toll-like receptor agonist, not checkpoint blockade (48). It is also important to note that the immunosuppression in orthotopic Renca tumors seems to be distinct from that seen in subcutaneous tumors, namely that Treg depletion was followed by tumor regression in subcutaneous, but not orthotopic, tumors (53). Perhaps an immunological “boost” is required for checkpoint blockade to achieve its full therapeutic potential in the orthotopic Renca model. The observation that combination therapy makes such an enormous difference in the setting of *Mycoplasma*-contaminated tumor cells speaks to the potential for renal cell carcinoma therapeutic vaccines in concert with checkpoint blockade and a TKI or other VEGF-targeted therapy. A study utilizing irradiated Renca cells as a vaccine showed that while an antitumor memory response was generated with the vaccine alone, it was only with the addition of PD-L1 blockade and CD4 depletion (targeting Tregs) that tumor regression and long-term protective immunity were observed (54). Multiple trials are underway to test RCC vaccines in combination with targeted therapies, including sunitinib. The timing of vaccine administration in relation to sunitinib therapy can have a significant effect on efficacy however, due to potential depletion of CD11b+ CD11c+ antigen presenting cells (55). Historically, vaccines targeting mRCC have failed to produce the desired results and none of them have been brought to market (56). The past few years have seen a new crop of promising candidates but to date no clear winner has emerged (31).

One of these is IMA901 which employs 9 tumor-associated peptides (TUMAPS) identified through analysis of the mRNA and HLA ligands of 32 RCC patients (57–59). The peptides are given intradermally with GM-CSF as an adjuvant and a single low-dose cyclophosphamide infusion prior to the first vaccination. IMA901 has completed Phase I and II trials; there is an ongoing Phase III randomized trial in combination with sunitinib (NCT01265901). Also in a randomized Phase III trial with sunitinib is AGS-003, an autologous DC vaccine. This approach requires isolating RNA from patient tissue obtained during resection and amplifying the RNA for electroporation (along with synthetic CD40L RNA) into activated monocyte-derived DCs for infusion. Like other autologous vaccines, it is time consuming and very expensive. An open label Phase II trial explored AGS-003 in combination with sunitinib in treatment-naïve intermediate and poor risk patients. Out of 21 patients only 9 experienced partial responses and there were no complete responses (60). A randomized Phase III trial comparing AGS-003 plus sunitinib to sunitinib alone as a first-line therapy is currently enrolling (NCT01582672).

A novel approach to therapeutic cancer vaccines involves allogeneic tumor cells modified to express the murine α(1,3)galactosyltransferase epitopes (αGal) which induce a robust xenoantigen response (61). HyperAcute Renal (HAR) seeks to exploit humans' pre-existing antibodies that bind these epitopes, leading to opsonization, complement activation and cell lysis. Antigen presenting cells will be recruited for antigen uptake and processing which should lead to activation and proliferation of CD4 and CD8 T cells. Vaccines for pancreatic, lung, breast and prostate cancer as well as melanoma are currently in development. Results to date look promising and Phase II and III trials, including in

combination with checkpoint blockade or approved chemotherapy regimens, are already underway for some of these indications.

Our observations concerning the decreased B7-H1/PD-L1 expression seen on tumor cells of sunitinib treated mice as well as the blunted effect of sunitinib in RAG KO mice are both noteworthy and merit further investigation. The results in RAG KO mice speak to the role the immune system plays in sunitinib's therapeutic effect as well as in the expression of B7-H1 and its response to sunitinib. The full extent and mechanism(s) of sunitinib's immunological effects has yet to be completely elucidated. As mentioned earlier, tumor PD-L1 expression has historically been associated with poor prognosis in RCC patients. A recent large, randomized phase III trial (COMPARZ) comparing sunitinib to pazopanib, another VEGF-targeted TKI, as a first-line therapy in patients with mRCC provided further evidence of this (62). Both agents demonstrated comparable efficacy, albeit with different safety profiles and quality-of-life indicators. Pre-treatment biopsies showed a significant negative correlation between tumoral PD-L1 expression and overall survival. The differences in overall survival were even more dramatic for patients with high PD-L1 expression and large numbers of intra-tumoral CD8 T cells. Patients with these findings upon initial biopsy may derive benefit from combining PD-1 or PD-L1 blockade with VEGF-targeted therapy.

Expression of PD-L1 post-TKI therapy has not been explored as extensively. A recent study reported that mRCC patients who received anti-angiogenic therapy before resection of their primary tumor had more infiltrating CD4 and CD8 T cells, which was inversely correlated with progression-free survival as well as overall survival (63). These samples also had increased CD4+ FoxP3+ regulatory T cells and higher expression of PD-L1, both

of which were associated with decreased patient survival. A more informative approach would involve examination of paired pre- and post-treatment biopsies. Sharpe et al used sequential primary tumor tissue samples from patients enrolled in three phase II trials looking at the effects of VEGF-targeted TKI therapy before resection (64). Patients in two of the trials received sunitinib while those on the third trial were treated with pazopanib. After 12-16 weeks of therapy, PD-L1 expression and the percentage of FoxP3+ cells both declined significantly. No significant differences were observed for these two biomarkers between patients who had early or delayed progression or no progression.

Ongoing studies continue to explore the potential of checkpoint blockade in RCC patients. In a clinical trial of ipilimumab and nivolumab as single agents or in combination, the combination showed an increased overall response rate with manageable toxicities (65). A phase II study is planned to test the same combination against sunitinib monotherapy (NCT 02231749). A phase Ib trial combining sunitinib and nivolumab found an unacceptably high rate of grade 3/4 adverse events (AEs) (73% of patients), leading to over one fourth of the participants discontinuing due to AEs (66). This was particularly disappointing given that the objective response rate was 52% with median progression-free survival of 48.9 weeks. After promising clinical activity in a phase Ib trial (67), an ongoing randomized phase II study is comparing Genentech's MPDL 328A anti-PD-L1 antibody alone or in combination with bevacizumab (a VEGF-targeted mAb) with sunitinib monotherapy (NCT 01984242). The number of ongoing and planned trials looking at ipilimumab, nivolumab or other PD-1/PD-L1 targeted agents in RCC patients is too large to list them all here. Suffice it to say, there is great interest in the observed efficacy to date and possible combinations that might yield an increased benefit.

CTLA-4 and the PD-1-PD-L1 axis are not the only immune checkpoints with therapeutic potential. Exploring blockade of other checkpoint molecules using the Renca orthotopic model, either alone or in combination would certainly be worthwhile. Pre-clinical work has shown co-expression of lymphocyte activating gene 3 (LAG3) and PD-1 on tumor-infiltrating CD4s and CD8s. In three transplantable murine models, dual blockade cleared tumors that were largely resistant to either monotherapy (68). Mice receiving the combination also had increased percentages of IFNγ+ CD8 T cells in their TDLN and TIL. Similar results were seen with combining PD-1 and T-cell immunoglobulin and mucin domain-3 (TIM-3) antibody blockade in murine tumor models (69). While either antibody alone did show some improvement in IFNγ+ CD8 T cell populations, neither resulted in complete regression and the anti-tumor effect was limited and variable. The combined blockade resulted not only in more robust IFNγ production by CD8 T cells but also a more consistent anti-tumor response with 50% of mice achieving complete and lasting regression. Though the study did not look at a murine RCC model, it would be reasonable to expect similar results.

While the combination of sunitinib and anti-PD-1 antibody was not effective in our orthotopic Renca model or well tolerated in clinical trials, the rationale behind combining anti-angiogenic therapy, particularly TKIs, with immune checkpoint blockade is supported by an extensive body of literature and should continue to be explored (70). Efforts to identify biomarkers associated with response and resistance to both targeted therapies and immunotherapies could help to advance the potential of such combinations (17,71,72). The promise of a cure lies in combination therapies targeted to an individual's tumor, not a handful of single agents used sequentially for all patients in a particular disease group.

With so many exciting developments in both types of treatment, one hopes we will soon find the key to unlocking their synergistic potential.

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CURRICULUM VITAE

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Educational History:

MS expected	2015	Immunology	Johns Hopkins School of Medicine
BS	1998	Biological Science	California State University
BA	1997	Anthropology	Rice University

Professional Experience

Clinical Research Coordinator	2014-present	Johns Hopkins School of Medicine
Oncology Research Specialist	2007 – 2010	Johns Hopkins School of Medicine
Cytogenetic Technologist	2006 – 2007	Genzyme Genetics
Cytogenetic Technologist	2006	Baylor College of Medicine
Staff Research Associate	1996 – 1999	Livermore National Laboratory

Publications

Buckheit R, Allen T, **Alme A**, Salgado M, O'Connell K, Huculak S, Falade-Nwulia O, Williams T, Gallant J, Siliciano R, Blankson J (2012) Host factors dictate control of viral replication in two HIV-1 controller/chronic progressor transmission pairs. Nat Commun. 3:716.

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