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14. ABSTRACT

The role of innate immunity in prostate cancer tumorigenesis is unclear. Toll-like receptors (TLRs) are key signaling molecules that regulate innate and adaptive immune responses in the presence of pathogens and endogenous ligands. We have generated and begun to characterize TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice. Initial results reveal that *de novo* prostate cancers in absence of MyD88 develop higher grade adenocarcinomas than wild-type controls at 30 weeks of age. Analysis of tumor infiltrating cells at 24 and 30 weeks of age already reveal increased infiltration of macrophage lineage cells, decreased CD8 T lymphocytes, NK cells, as well as decreased activation of canonical NF-κB levels and increased non-canonical NF-κB levels in the MyD88-deficient TRAMP Tg$^{+/-}$ mice. We have also observed decreased splenocytic NK cells in MyD88$^{-/-}$ animals with and without the presence of prostate cancer. From these data, we will determine the role of the MyD88 pathway of innate immunity in prostate cancer tumorigenesis. However, we have noted several limitations of our current system in achieving our initial aims. Thus, we have also proposed and now have been approved a revised SOW.

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Prostate cancer, tumor microenvironment, Toll-like receptors

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Introduction

Prostate cancer is the most prevalent non-skin solid malignancy and the second-leading cause of cancer-related mortality in men in the U.S.[1]. Treatment of metastatic prostate cancer with androgen-deprivation therapy ultimately leads to development of castration-resistant disease, where cancer cells become more responsive to even minute quantities of testosterone. Emerging therapies are available for castration-resistant prostate cancer (CRPC), including chemotherapy, immune-based therapies, and second line hormone therapies, all with limited efficacy. Improved and likely combinatorial therapies will be necessary.

Inflammation has long been associated with the prostate cancer microenvironment, and may facilitate tumor growth or promote an anti-tumor immune response. Evidence suggests that cancer cells can be hijacking inflammatory pathways to promote angiogenesis and proliferation[2]. Conversely, inflammation can trigger the infiltration of cytotoxic immune effector cells, resulting in the production of clonal CD8^{+} T cells[3]. However, the contribution of the tumor infiltrating lymphocytes (TILs) to prostate cancer development, growth, and metastasis is unclear. We are interested in understanding the mechanisms for development of TILs and how they modulate prostate cancer. Our hypothesis is that the innate immune response can program TILs and play a key role in tumor surveillance, are important in generation of tumor-specific immunity, and that by modulating the composition of TILs through innate immunity, we can alter tumor growth.

Body

Pathogens or cancerous cells alike can produce danger signals that elicit the activation of immune responses. These signals in the form of conserved molecules termed pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) can be discriminated from self-antigens by a family of pattern-recognition receptors of innate immunity, including Toll-like receptors (TLRs). Thirteen mammalian TLRs have been identified to date with ligands ranging from lipopolysaccharide (LPS) found in gram-negative bacterial walls recognized by TLR4, double stranded RNA produced by many viruses for TLR3, viral CpG motifs with TLR9, to endogenous ligands such as heat-shock protein 70 and chromatin component HMG-B1. Activation of these receptors leads to induction of multiple inflammatory pathways, including nuclear factor-kappa B (NF-κB) and interferon regulatory factors (IRFs), which may mediate the development of cytotoxic T lymphocytes (CTLs) and dendritic cell (DC) maturation[4]. Although TLRs have been shown to inhibit negative regulatory cells such as Tregs, the relationship between TLRs and myeloid-derived suppressor cells (MDSCs) is less clear[4-5].

TLRs recruit adaptor proteins such as MyD88 and serine kinase IL-1 receptor-associated kinase (IRAK), leading to activation of MAP kinases, NF-κB, and expression of inflammatory genes. Most TLRs utilize the MyD88 pathway. The role of TLRs in modulating cancer is conflicting, as prior reports have suggested tumor promoting as

well as suppressing effects. Deficiency in MyD88 confers decreased development of tumors in a mouse model of spontaneous intestinal tumorigenesis and diethylnitrosamine-induced hepatocellular tumors[6-7]. In contrast, a recent report suggested that MyD88 inhibition promoted pancreatic cancer growth through dendritic cell and Th2 activation[8].

We have currently been focused on studying the phenotype of TRAMP Tg^{+/-} x MyD88^{-/-} mice, work described in **Specific Aim 2**. When compared to TRAMP Tg^{+/-} animals at 24 weeks, we initially observed larger *de novo* prostate tumors and more aggressive histology (Figure 1). We have also examined expression of the T lymphocyte antigen CD3, macrophage antigen F4/80, and proliferative marker Ki-67 in 24 week wild-type and MyD88^{-/-} prostate tumors to examine TILs in the microenvironment. We show decreased infiltration of CD3 lymphocytes with concomitant increase in infiltrating F4/80 cells (Figure 2).
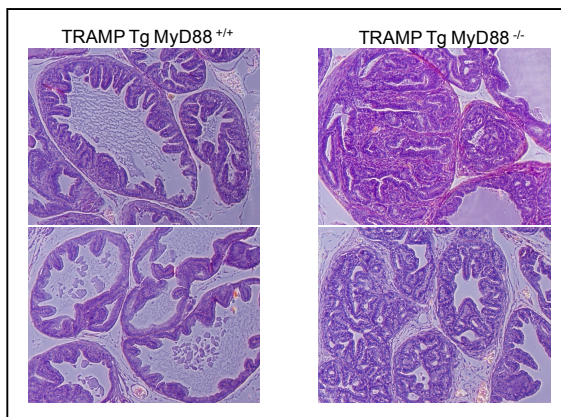

Figure 1. Larger and more aggressive adenocarcinoma in 24 wk-old C57Bl6 TRAMP Tg^{+/-} x MyD88^{-/-} transgenic mice compared to age matched C57Bl6 TRAMP Tg^{+/-} mice with H&E staining at 20x magnification.
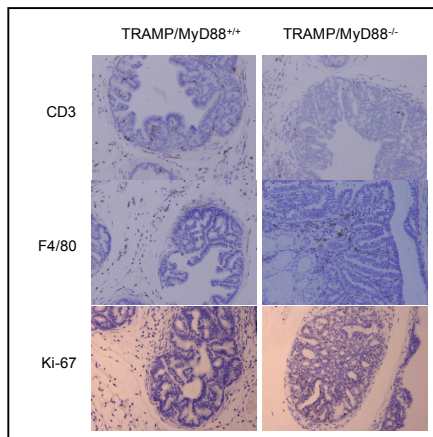
Figure 2. Inflammatory infiltrate in autochthonous prostate cancer in absence of MyD88. Infiltration of CD3, F4/80, and Ki-67 examined by immunohistochemistry in 24 wk-old C57Bl6 TRAMP Tg^{+/-} transgenic mice compared to age matched C57Bl6 TRAMP Tg^{+/-} x MyD88^{-/-} mice.
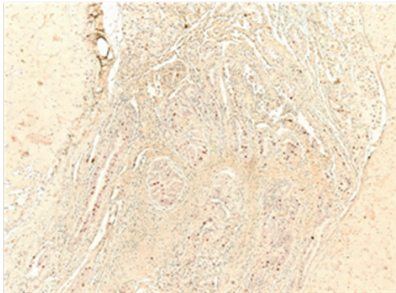
This suggests the infiltration of myeloid derived suppressor cells that can negatively regulate T cells. We further examined the expression of canonical as well as non-canonical NF-*k*B subtypes using antibodies against phosphorylated I*k*B for p65

activation and NIK for p100 activation and observed decreased p65 activation and increased p100 activation in the absence of MyD88 (Figure 3-4).
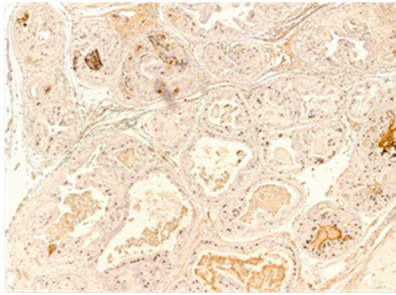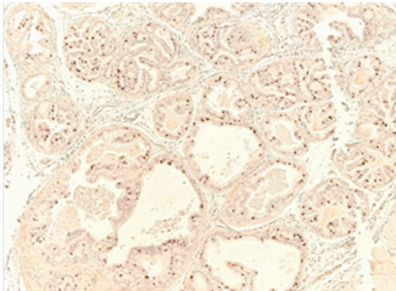

Tg (M2925)

MyD88 KO/Tg (M2915)

Figure 3. Increased staining of phosphorylated IkB indicative of activated NK-κB in 24 wk-old C57Bl6 TRAMP Tg$^{+/-}$ mice compared to age matched C57Bl6 TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice.
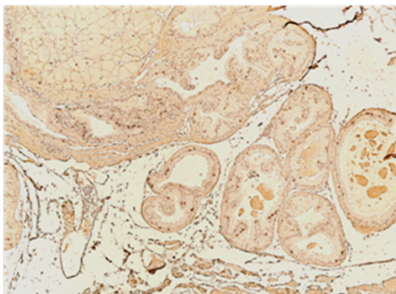

Tg (M2925)

MyD88 KO/WT (M2935)

Figure 4. Decreased staining of NIK indicative of decreased p100 activation in 24 wk-old C57Bl6 TRAMP Tg$^{+/-}$ transgenic mice compared to age matched C57Bl6 TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice.
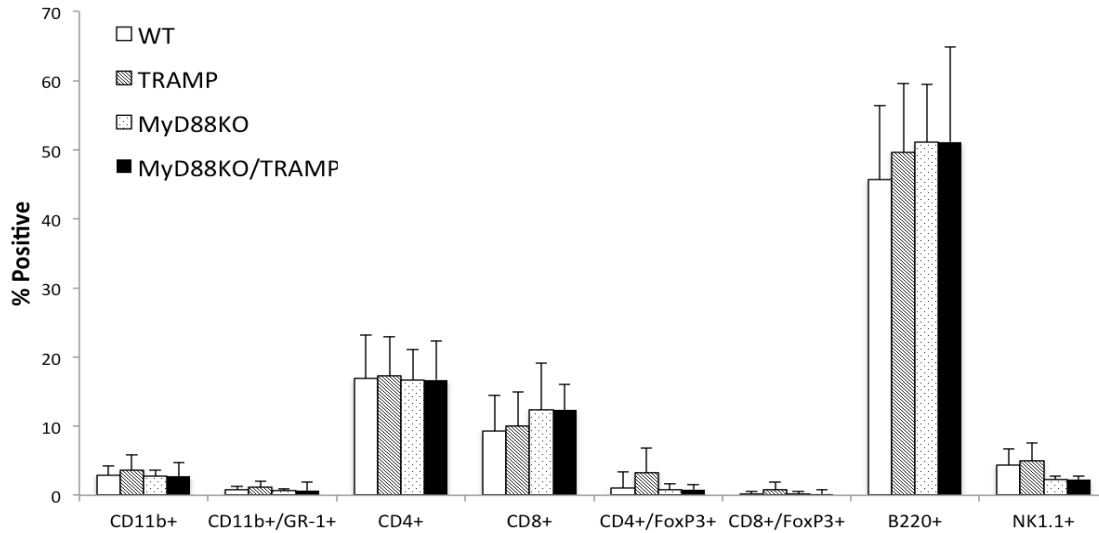
We examined the splenocytes from six mice in each group of wild-type, TRAMP Tg$^{+/-}$, MyD88$^{-/-}$. TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice and found a significant difference in splenic NK development in MyD88$^{-/-}$ and TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice (Figure 5).
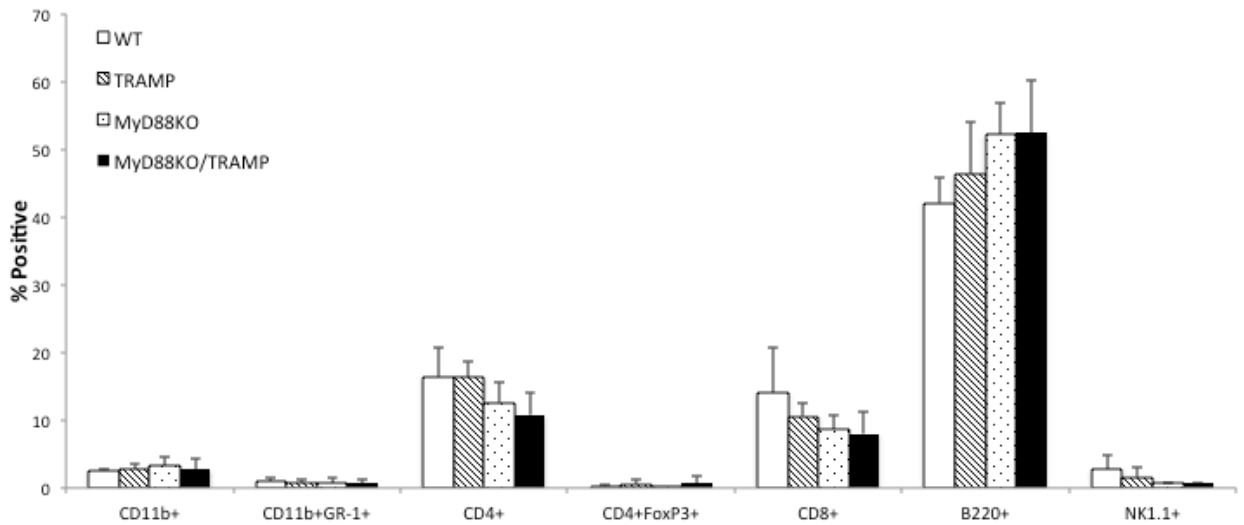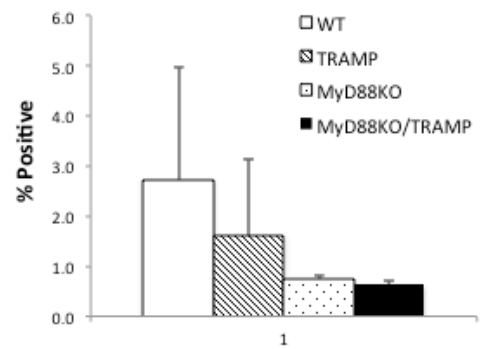

Figure 5. Immune populations in TRAMP mouse splenocytes. TRAMP transgenic mice with and without wild type MyD88 were sacrificed at 25 weeks. Splenocyte immune populations were measured using flow cytometry and staining with FITC-CD11b, PE-Gr-1, FITC-CD8, APC-CD4, PE-FoxP3, FITC-B220, and PE-NK1.1.

However, with increasing animal numbers, we did not consistently see a significant increase in prostate size in 24 week TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ versus TRAMP Tg$^{+/-}$ mice. In human prostate cancer, tumor size is often not indicative of prostate cancer aggressiveness and ability for metastasis, and TRAMP tumors at 24 weeks in both wild-type and MyD88-deficient animals were at too early a stage to convince us of the phenotype. Thus, we elected to examine a cohort of animals at 30 weeks. These results have been completed with six mice in each group of wild-type, TRAMP Tg$^{+/-}$, MyD88$^{-/-}$. TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice. Interestingly, our results show no significant difference in weight of prostate tumors in TRAMP Tg$^{+/-}$ versus TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice (Figure 6). Similarly, we found a significant difference in splenic NK development in MyD88$^{-/-}$ and TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice (Figure 7). Histologically, the tumors from TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice appear to have a more aggressive adenocarcinoma (Figure 8). We proceeded to stain by immunofluorescence and immunohistochemistry. Although the immunohistochemistry of IkB, NIK, and F4/80 are pending, we did perform immunofluorescence examining NK cells (CD49), p63, CD8, and AR to date, with decreased NK$^+$ and CD8$^+$ cells in TRAMP Tg$^{+/-}$ compared to TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ 30 week mice, and decreased p63 staining consistent with histologically more aggressive tumors (Figure 9).
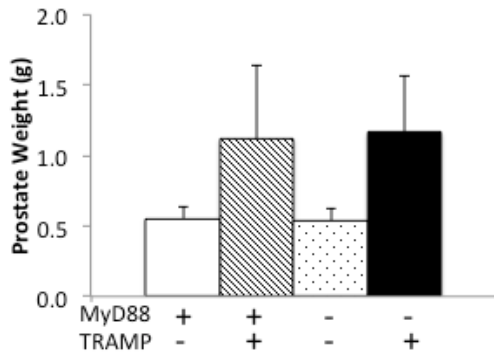
Figure 6. Similar sized tumors by weight (grams) in 30 wk-old C57Bl6 TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ transgenic mice compared to age matched C57Bl6 TRAMP Tg$^{+/-}$ mice.

Figure 7. Immune populations in TRAMP mouse splenocytes. TRAMP transgenic mice with and without wild type MyD88 were sacrificed at 30 weeks. Splenocyte immune populations were measured using flow cytometry and staining with FITC-CD11b, PE-Gr-1, FITC-CD8, APC-CD4, PE-FoxP3, FITC-B220, and PE-NK1.1. Top right graph shows expanded view of NK1.1 staining.
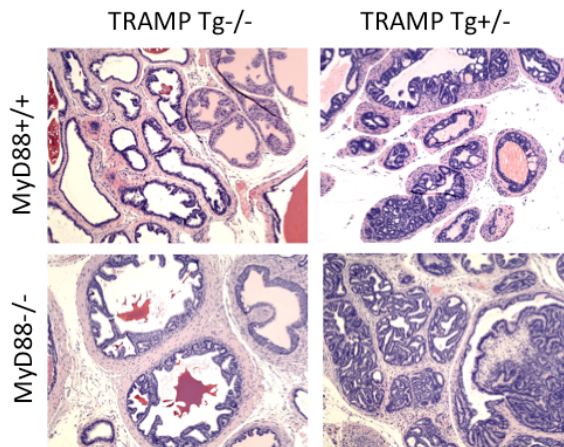
Figure 8. Histologically more aggressive adenocarcinoma in 30 wk-old C57Bl6 TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ transgenic mice compared to age matched C57Bl6 TRAMP Tg$^{+/-}$ mice with H&E staining at 20x magnification.
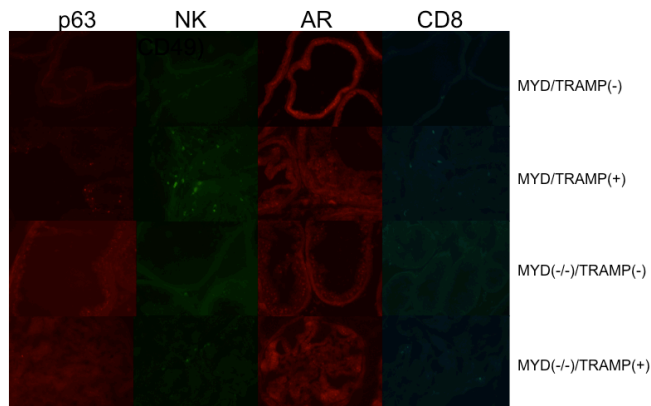

Figure 9. Staining of p63, CD49, AR, CD8 in C57Bl6, TRAMP Tg$^{+/-}$, MyD88$^{-/-}$, and MyD88$^{-/-}$ x TRAMP Tg$^{+/-}$ 30 wk-old mice by immunofluorescence at 40 x magnification.

We were prepared to stain our human tumor microarray to examine the contribution of various TILs specific as described in **Specific Aim 3** with IRB approved. However, prior to doing so, we examined the literature and identified several recent reports staining prostate cancer TMAs. This includes identifying increased expression of NK-kB subunit p65 with more aggressive tumors, but a modest difference on multivariate analysis, and increased CD8 positive, but not CD4 and CD20 positive TILs in areas of prostate cancer[9-10]. With these marginal results, our Aim 3 examining TILs in a tumor microarray seem less fruitful.

Problems, Limitations, and Submission of Revised SOW
At the last annual summary, we presented limitations to our initial aims using TRAMP Tg$^{+/-}$ animals. This includes the length of time for development of tumors from 24 to 30 weeks of age, the ubiquitous presence of our gene knockout in prostate epithelium, stroma, as well as immune system, and the fixed nature of the prostate model with expression of the large T antigen, which may have limited translational implications. We have combined TRAMP cell lines with urogenital sinus mesenchymal

cells and implanted the cells subcutaneously to develop a more versatile system. However, no prostate cancer glandular architecture formed (Figure 10).


TRAMP cell tumor   TRAMP cell + UGSM tumor
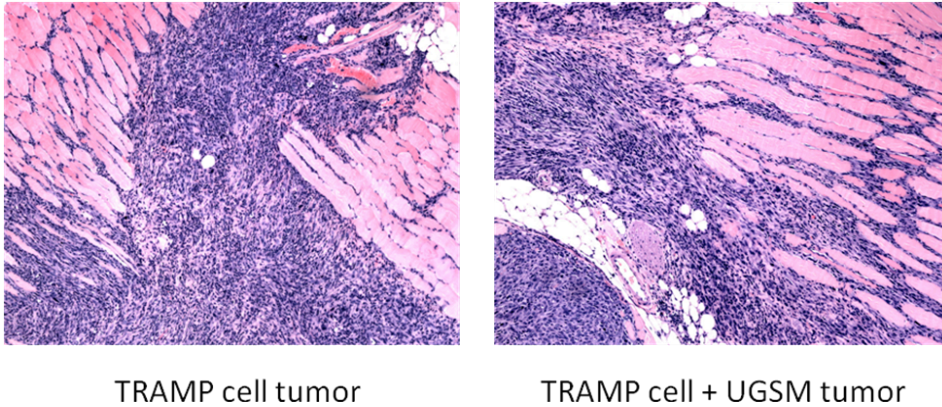
Figure 10. Subcutanous TRAMP Model to Recapitulate Prostate Cancer. TRAMP C2 cells with and without urogenital sinus mesenchymal cells were implanted in the presence of Matrigel and allowed to grow for 2 weeks prior to sacrifice. Paraffin embedded sections were stained by H&E at 20x magnification.

Reflecting on the key determinants in prostate cancer progression, development of CRPC and expression of the androgen receptor, we proposed a change in our model system and submitted a revised SOW which was submitted 8/1/2013 and we have just received approval on 4/2/2014 to proceed.

Briefly, we propose a model of disease progression in prostate cancer, where damage-associated molecular patterns (DAMPs) released by the tumor stimulate the innate immune pathways through TLRs. As NF-κB subunits have been previously shown to modulate AR expression and activity, NF-κB signaling through TLR activation may promote the development of castration-resistant disease. We further propose to define the contribution of innate immune signaling in the tumor, stromal, and immune compartments, specifically with regard to the modulation of AR, which is unknown. The ultimate goal is to understand the function of these pathways to effectively modulate the inflammatory response. The proposed project targets inflammatory signaling through pattern-recognition receptors as a possible mechanism for the development of castration-resistant prostate cancer. To examine this question, we need a more dynamic model to be able to alter the aggressiveness of the tumor and specifically modulate the TLR signaling pathway in prostate epithelium, stroma, and immune system.

To parse out the role of TLR signaling in various compartments, we propose adapting a previously published subcutaneous prostate tumor model based on lentiviral transfection of primary prostate epithelium[11-12]. This model has been developed in Owen Witte's laboratory at UCLA, which we will collaborate with. Prior reports have been performed using both human and murine prostate epithelium on an

immunocompromised background. We propose, developing a model using murine prostate epithelium on a syngenic immunocompetent host. The flexibility of the model allows a variation in the oncogenic drivers of the tumors (Figure 11), which subsequently produces disease ranging from PIN (AKT/ERG and TRAMP) to castration-resistant prostate cancer (AKT/ERG + AR). As the tumor cells are combined with fetal mesenchymal cells for implantation, this model allows for the genotypic manipulation of both the tumor and its surrounding stroma. We currently possess knockouts of MyD88, TRIF, and NIK on a C57Bl/6 background, which represent various steps in TLR signaling. By altering the genotype of the tumor and stromal cells in the tumor grafts, we can more clearly define the role of innate immunity in each compartment.
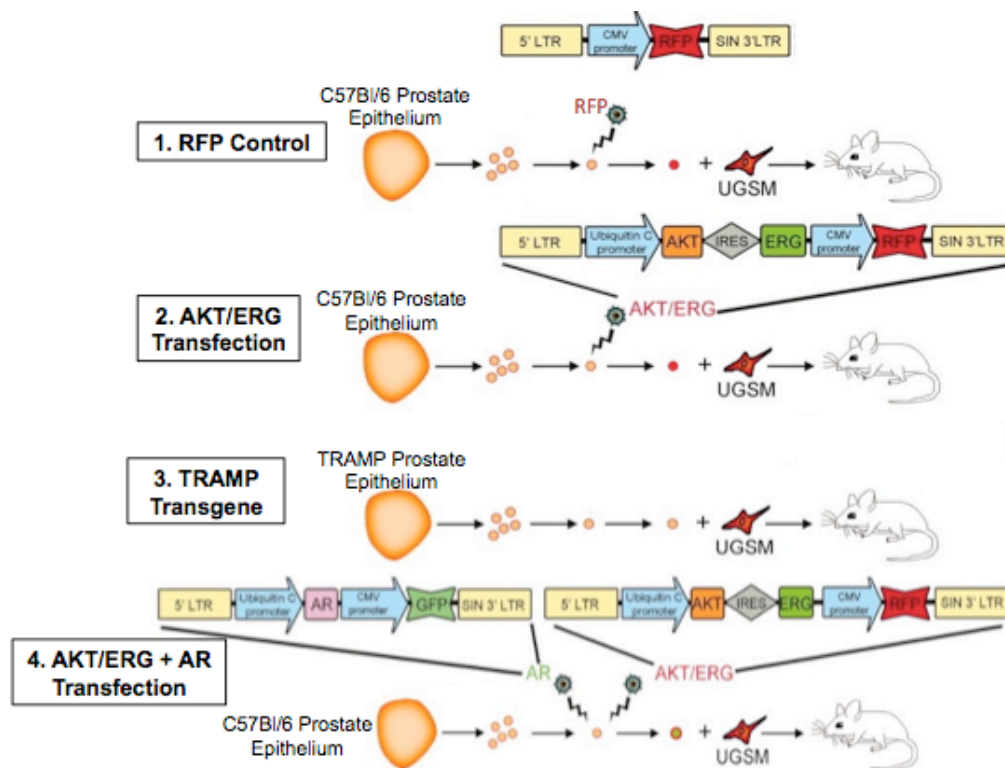

Figure 11. Diagram of subcutaneous prostate tumor model. (Figure adapted from Goldstein et al. *Science*, 2010) The four oncogenic conditions of the model are listed 1-4. 1 serves as a control for the lentiviral transfection. In conditions 2 and 4, benign prostate epithelium from C57Bl/6 mice is transfected with either a lentivirus containing constitutively expressed Akt/ERG (2) or with both the Akt/ERG lentivirus and another containing constitutively expressed AR (4). In condition 3, prostate epithelium is extracted from TRAMP transgenic C57Bl/6 mice, which contains the SV40 large T antigen driven by a prostate-specific promoter. The prostate epithelium from all four conditions is combined with murine urogenital sinus mesenchyme (UGSM) and implanted into the kidney capsule of recipient mice.

To measure the effect of signaling, we will monitor tumor size and metastasis, TILs, splenic immune populations, serum and tumor cytokines, and various tumor-

derived RNA levels. Mice with established tumors will also be challenged with chemical or surgical castration to stimulate the development of castration-resistant disease. AR expression and activity will be measured in tumors of varied genotypes, as well as the expression of AR target genes.

Through this modification, we intend to elucidate the precise function of innate immune signaling in prostate cancer. The ultimate goal is to modulate the inflammatory response to produce a potent anti-tumor effect and prevent the development of castration-resistant prostate cancer. The release of certain DAMPs or the activation of certain TLRs may provoke an increase in tumor killing or a decrease in the function of regulatory cells. These may be exploitable as a drug target for the treatment of prostate cancer. However, this effect may be opposed by the development of castration resistance, so the relative contributions of each side must be elucidated.

We were recently awarded a small grant from the Concern Foundation which led to exciting preliminary data showing that just the presence of an intact immune system greatly altered tumor growth using the proposed reconstitution system and in this case larger tumors in a C57Bl6 versus SCID background (Figure 12).
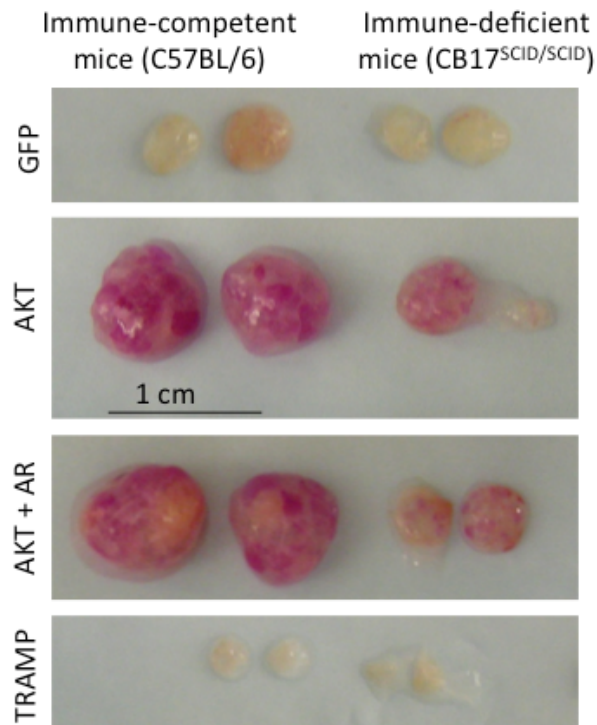
Figure 12. Larger C57Bl6 tumors generated by retroviral infection by AKT and AKT + AR in a C57Bl6 versus CB17scid/scid background.

Key Research Accomplishments

- We have generated groups of C57Bl6, C57Bl6 x TRAMP Tg$^{+/-}$, MyD88$^{-/-}$, MyD88$^{-/-}$ x TRAMP Tg$^{+/-}$ mice at 24 and 30 weeks
- We have shown that absence of MyD88 does not lead to increased size of TRAMP prostate cancer tumors, but increased grade of carcinoma at 30 weeks
- We have shown that MyD88-deficient mice with and without TRAMP autochthonous tumors have decreased splenocytic natural killer cells at 24 and 30 weeks.
- We have shown that absence of MyD88 leads to increased tumor infiltration of CD11b^{+} cells, decreased CD8^{+} T lymphocytes, and NK cells.
- We have shown that absence of MyD88 while leads to decreased activation of canonical NF-κB with decreased p65 activation, but appears to lead to increased non-canonical NF-κB with increased NIK activation.
- We have realized that there are severe limitations to crossing the TRAMP transgenic mice to various TLR signaling component-deficient mice and have submitted a revised SOW on 8/1/2013 and approved on 4/2/14

Reportable Outcomes

We have presented this work in yearly seminars at the UCLA Prostate SPORE Lecture Series, and this year a portion of this work was presented at a seminar series for the Jonsson Comprehensive Cancer Center Tumor Immunology Section as well as an invited talk to the UCLA Radiation Oncology Department.

Conclusions

We have shown that TRAMP Tg$^{+/-}$ x MyD88-deficient mice result in accelerated prostate cancer development with increased infiltration of CD11b^{+} myeloid cells, decreased T lymphocytes, and a skewing of canonical and non-canonical NF-κB activation. We are preparing our initial manuscript describing this phenotype once we complete the immunofluorescence and immunohistochemistry at 30 weeks. We have also identified limitations to our current aims and have suggested changes that will refocus the hypothesis to examine the role of innate immunity using a more dynamic model.

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