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14. ABSTRACT

Invariant natural killer T cells (iNKT cells), through their ability to mature dendritic cells (DCs) and provide help for CD4 and CD8 T cell responses, can play a major role in regulation of the cellular adaptive immune response. Our major objective in this proposal is to identify an optimal agent and method of delivery for iNKT cell activation and enhancement of cytolytic T cell responses against prostate/PCa associated antigens. Our findings with respect to peptide based vaccines are not yet conclusive due largely to modest and inconsistent responses to peptides. It is well established that peptides (alone or pulsed onto DCs) are not optimal immunogens, and it is not yet clear whether or to what extent we can boost their efficacy using iNKT cell ligands. Over the next year we will assess some alternate approaches including the use of longer or modified peptides and other modes of administration, as the use of peptide or peptide pools still offers substantial advantages with respect to human clinical trials. In contrast to peptides, the data indicate that iNKT cell ligands can enhance responses to cellular vaccines. Therefore, in parallel with the peptide efforts, we will use this approach to assess alternative iNKT cell ligands and begin to assess efficacy in tumor bearing mice.

15. SUBJECT TERMS
prostate cancer, immunotherapy, NKT cells

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INTRODUCTION

Invariant natural killer T cells (iNKT cells), play a major role in regulation of the cellular adaptive immune response through their ability to mature dendritic cells (DCs) and provide help for CD4 and CD8 T cells. The iNKT cell activating ligand αGalCer and relatives can stimulate potent anti-tumor immune responses, and in combination with other immune stimuli can eradicate even established tumors in multiple mouse models. Successful treatment in humans will probably also require a combination of modalities. Therefore, it is critical to both optimize iNKT cell based therapies and to determine how they may synergize with other modalities. Our major objective in this proposal was to identify an optimal agent and method of delivery for iNKT cell activation and enhancement of cytolytic T cell responses against prostate/PCa associated antigens. Further objectives are to assess synergy between iNKT cell activation and castration, to validate candidate PCa associated antigenic peptides for human clinical trials, and to establish assays for assessing immune responses to these peptides in clinical trials. **Aim 1** was to directly compare αGalCer and analogues based on their ability to break tolerance and stimulate CTL responses to a prostate expressed self antigen (prostate stem cell antigen, PSCA) in healthy mice. **Aim 2** was to compare these agents for their ability to similarly stimulate CTL responses to PSCA expressed by tumor cells in the TRAMP model. **Aim 3** was to more extensively characterize and optimize responses to the optimal agent. Finally, **Aim 4** was to conduct preclinical studies of our lead iNKT cell activating agent in mice expressing human HLA-A2.1.

Year 2 has been focused on determining whether a combination PCa tumor cell-based vaccine which was most active in normal mouse (**Year 1**) is capable of elliciting an immune response in TRAMP mice bearing tumors, a more stringent test. Here, we report that not only is the combination vaccine able to 'break tolerance' to TRAMP tumor cells, but the vaccine appears to be protective in late stage TRAMP. Goals for **Year 3** are to validate our preliminary findings, determine minimal components of active vaccine, and to define the vaccine utility in the spectrum of model PCa disease, in order to aid in clinical trial design. Aims were as follows:

Aim 1. Compare the activity of αGalCer analogues loaded onto peptide/DC vaccines to elicit cytolytic T cell responses against prostate specific antigens in healthy mice.

Aim 2. Compare the ability of αGalCer analogue/peptide/DC vaccines to elicit cytolytic T cell responses in TRAMP mice.

Aim 3. Further characterize and optimize the anti-tumor effects of the lead agent.

Aim 4. Assess immune responses to αGalCer analogue/DC vaccine using human *SIM2* and *ERG* peptides in HLA-A2.1 transgenic mice.
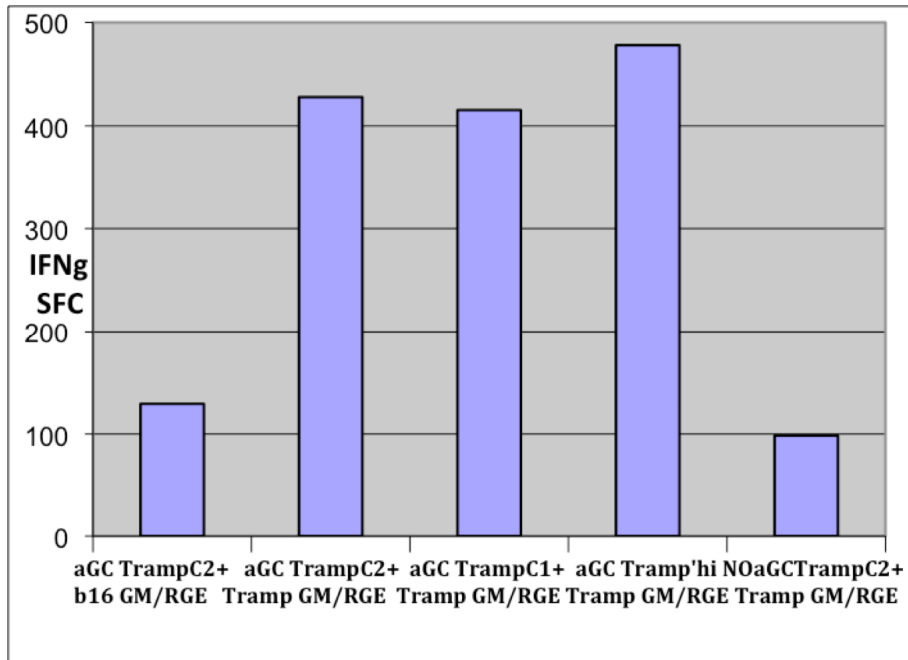
BODY

Aim 1. Compare the activity of αGalCer analogues loaded onto peptide/DC vaccines to elicit cytolytic T cell responses against prostate specific antigens in healthy mice.

We previously established that immunization with dendritic cells loaded with peptide and αGalCer could augment immune responses. As outlined in the proposal, we used a panel of prostate stem cell antigen (PSCA) derived peptides as well as a control peptide from SV40 T Ag (TagIV) and *in vitro* matured DCs. Healthy male C57BL/6 mice were immunized subcutaneously and splenocytes harvested. As shown previously, we found that DCs that were pulsed with a pool of PSCA derived peptides and αGalCer increased subsequent *in vitro* IFNγ ELISPOT responses in some experiments, but the effect was variable and not consistent. Further experiments using individual peptides similarly yielded variable results, and further controls showed that DCs loaded with αGalCer alone (without peptide) could nonspecifically enhance subsequent *in vitro* responses (previous report). As outlined below, given these initial challenges in consistently augmenting specific peptide responses using DCs, we focused further studies on intact tumor cells (**Year 1** report, new data in **Fig. 1**; an alternative originally planned in **Aim 3**).

Aim 2. Compare the ability of αGalCer analogue/peptide/DC vaccines to elicit cytolytic T cell responses in TRAMP mice. While immune responses to a variety of tumor vaccines can clearly be elicited in healthy mice, data from our and others outlined in the proposal have shown that mice bearing prostate cancers in the TRAMP model (prostate specific expression on SV40 T antigen, Tag, oncogene) do not respond. Therefore, in

conjunction with our initial focus on *in vivo* optimization of vaccines in healthy mice, we previously completed basic *in vivo* and *in vitro* studies to understand the molecular basis for lack of T cell responses (anergy) in TRAMP mice. These studies, which were published (Nowak et al., 2010), showed that iNKT cells migrate into the prostate cancers in the TRAMP mouse model, which express CD1d, as does human prostate epithelium. Also, we found that TRAMP derived tumor cells (TRAMP C-2 cells) induced an immune deviated state in iNKT cells, in that they became refractory to making IFNγ in response to stimulation, even in the presence of IL-12 and despite IL-12 receptor expression. Importantly, however, we found that IFNγ could be induced by the inclusion of αGalCer on TRAMP C2 cells. These findings provided a basis for aggressively pursuing the use of αGalCer and other iNKT cell activating ligands in conjunction with tumor cell based vaccines (see also **Aim 3**).

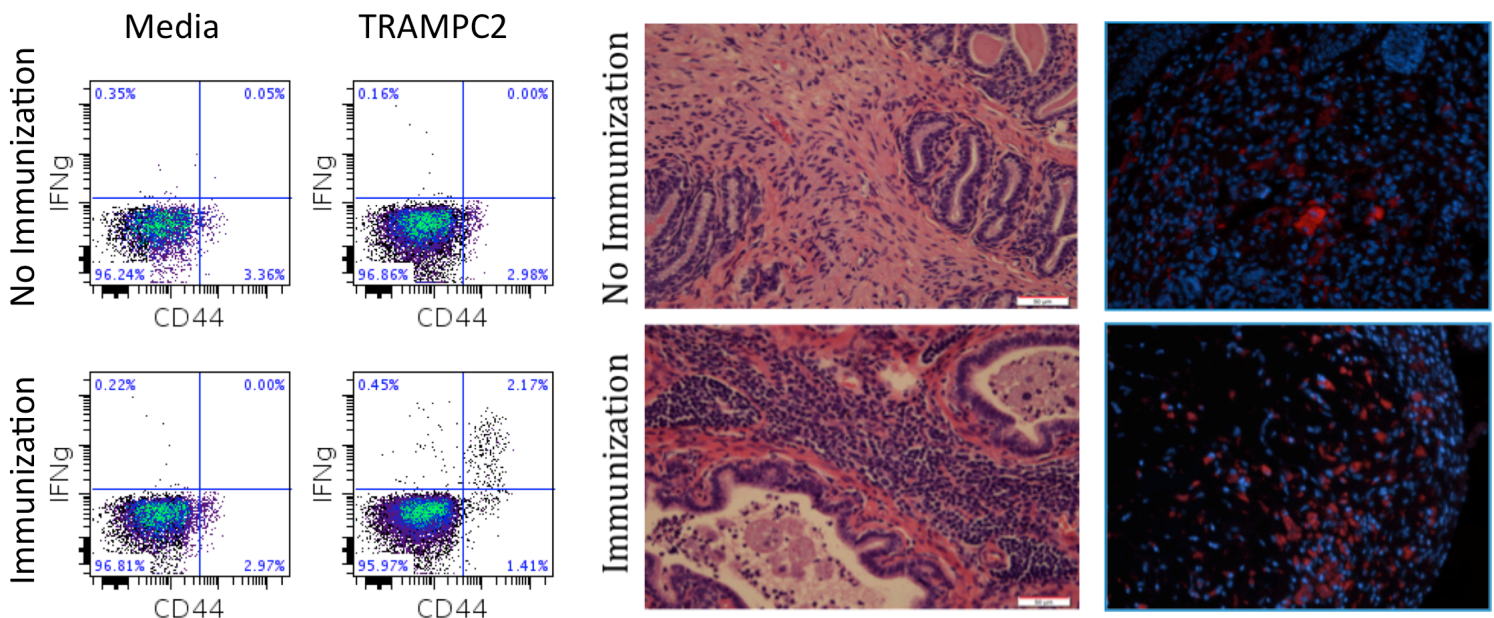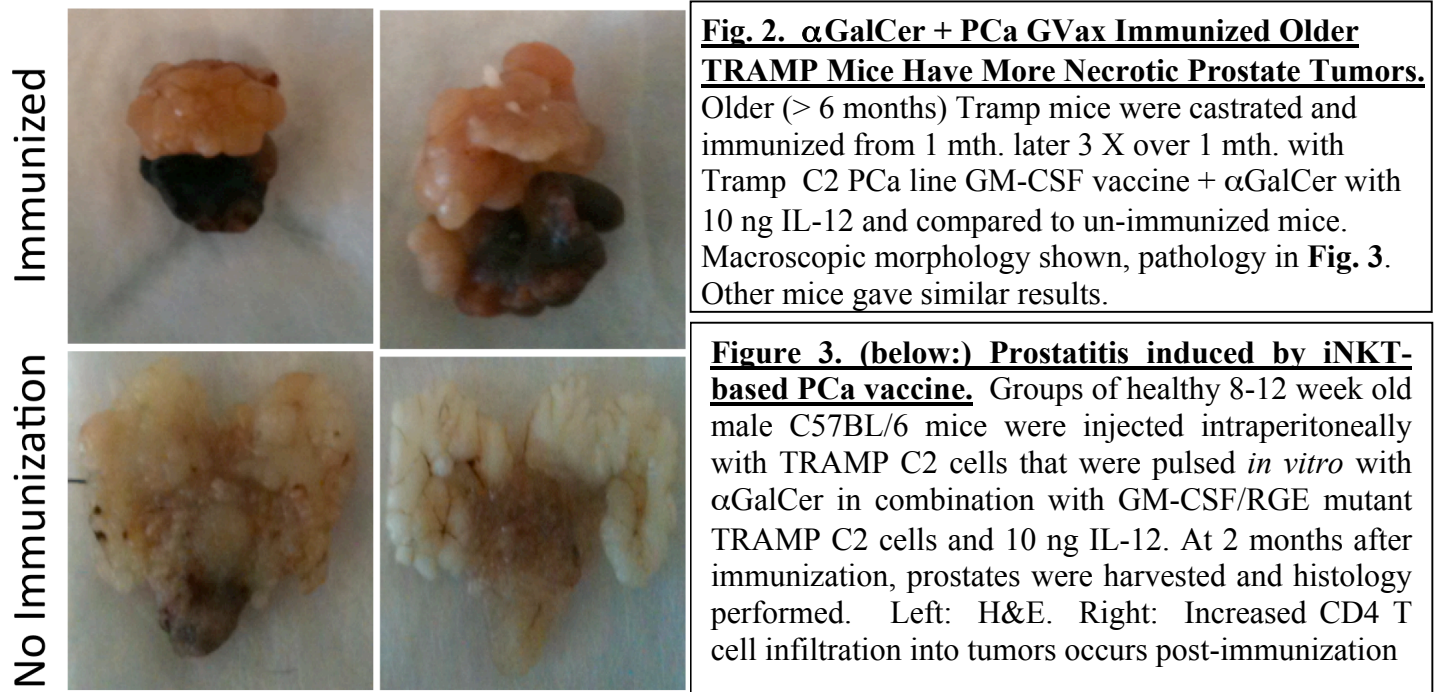

Fig. 1. IFNγ EliSpot of splenocytes from mice immunized with PCa cell vaccines. Tramp cell lines C2, C1, or a CD1d high C2 subline we derived ± GM-CSF/RGE vaccine ± αGalCer were compared to similar Tramp C2 + B16 melanoma as the GVax carrier (all + 10 ng IL-12) for a single vaccination and ELIspot determined IFNγ responses against 2 PCa peptides. αGalCer was required for optimal responses (see right bar). Other PCa peptide pairs gave similar results. Left bar shows some effect of αGalCer with B16 vax combination. Initial experiments revealed that IL-12 adjuvant was needed. Immune and tumor responses of castrated Tramp mice vaccinated in this way are being followed to complete this study.

Fig. 1 shows that αGalCer pulsed TRAMP C2 cells had some ability to induce IFNγ-producing CD8 T cells reactive with PCa antigens in combination with GM-CSF / RGE producing B16 vaccine ('GVax'), consistent with our **Year 1** data on αGalCer-pulsed TRAMP C2 in WT mice. However, this was markedly enhanced by combination αGalCer pulsed TRAMP C2 with GM-CSF / RGE produced by TRAMP-C2 rather than third party B16. 2 other TRAMP derived cell lines worked equally well as vaccine carriers, generalizing the effect. These were TRAMP C1, an independent TRAMP cell line, and a C2 subline with more aggressive growth characteristics we have obtained. Importantly, the beneficial effect of TRAMP-based vaccine combination was dependent upon αGalCer, and therefore iNKT cells (**Fig. 1**), confirming our overal strategy in TRAMP mice.

Aim 3. Further characterize and optimize the anti-tumor effects of the lead agent.
Our initial approach was to establish the lead agent based on studies with peptide vaccines, and then to evaluate them in settings including tumor cell vaccines. However, based on results outlined above, we have carried out studies using tumor cell vaccines in parallel with the peptide vaccine studies. Taking advantage of CD1d expression on TRAMP-C2 cells, we carried out studies to determine whether pulsing αGalCer onto TRAMP C2 would enhance their ability to induce T cell responses. In addition, we assessed whether responses could be further enhanced by the inclusion of cells secreting GM-CSF to enhance DC recruitment. The GM-CSF transduced cells used for these studies were previously generated B16 melanoma cells that also expressed a dominant negative version of MFG-E8 protein (RGE) that may further enhance Th1 type DC responses. Finally, based on data cited above (Nowak et al., 2010), we included low dose IL-12 in the stimulation. As shown in **Fig. 1**, vaccination with TRAMP C2 plus the GM-CSF/RGE transduced cells and IL-12 resulted in modest activation as assessed by subsequent in vitro stimulation with a panel of PSCA peptides (TRAMP C2 cells express PSCA) and IFNγ ELISPOT assays. However, this response was markedly enhanced by the addition of αGalCer. These experiments also indicated that GM-CSF/RGE cells contributed to this response.
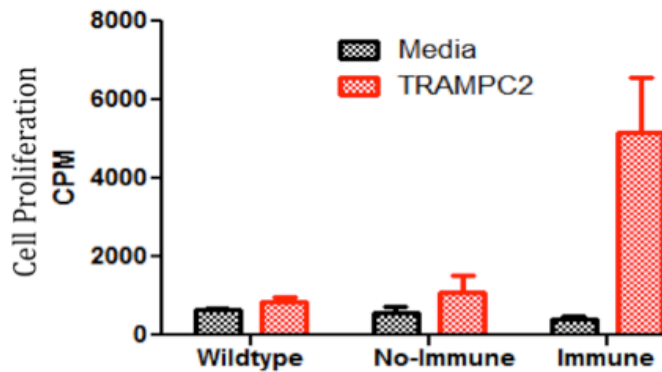
iNKT + GVax in older TRAMP mice: Older > 6 month old TRAMP mice have well-developed primary in situ PCa tumors (39). Castration can enhance immune responses and potentially delay progression, even this late, but is not curative in this model much beyond ~ 12 weeks of age, rather as in clinical PCa in more advanced disease. We chose to first test response in such animals, since they reflect worse-case scenarios for phase 1 trial settings. Remarkably, even in this advanced group, we detected strong anti-PCa responses, seen as necrotic tumors in all vaccinated animals examined and not controls (**Fig. 2**), showing that we can therapeutically 'break tolerance' to PCa even at this late stage of disease in this established PCa model. Consistent with these results, immunized mice also showed a remarkable prostate mono-nuclear cell infiltrate (**Fig. 3**). Vaccination timing is now being optimized with immuno-monitoring and determining the nature of this potentially protective prostatitis with additional use of HLA-A2.1/Pb-ERG transgenic mice (**Aim 4**).
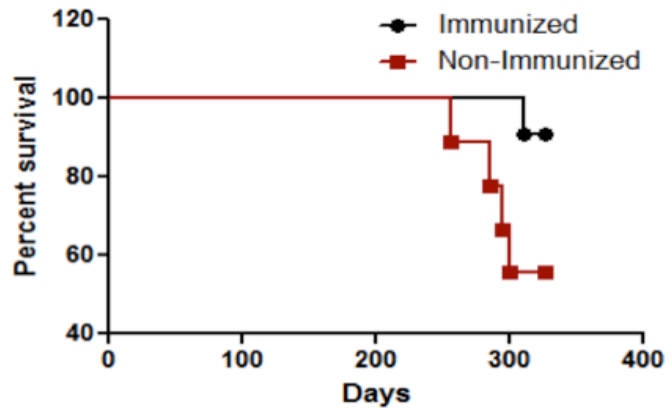

Fig. 2. αGalCer + PCa GVax Immunized Older TRAMP Mice Have More Necrotic Prostate Tumors. Older (> 6 months) Tramp mice were castrated and immunized from 1 mth. later 3 X over 1 mth. with Tramp C2 PCa line GM-CSF vaccine + αGalCer with 10 ng IL-12 and compared to un-immunized mice. Macroscopic morphology shown, pathology in **Fig. 3**. Other mice gave similar results.

Figure 3. (below:) Prostatitis induced by iNKT-based PCa vaccine. Groups of healthy 8-12 week old male C57BL/6 mice were injected intraperitoneally with TRAMP C2 cells that were pulsed *in vitro* with αGalCer in combination with GM-CSF/RGE mutant TRAMP C2 cells and 10 ng IL-12. At 2 months after immunization, prostates were harvested and histology performed. Left: H&E. Right: Increased CD4 T cell infiltration into tumors occurs post-immunization

Fig. 4 (above left). IFNγ FACS of splenocytes from mice immunized with PCa cell vaccines. Tumor-bearing TRAMP mice were castrated and vaccinated as in **Figs. 2 - 3**. TRAMP C2 GM-CSF/RGE vaccine + αGalCer induced higher IFNγ responses against PCa. Cytokine FACS of CD8+CD44+ T cells from immunized mice versus controls, which produce lower levels of IFNγ upon restimulation with tumor antigen.

Further immuno-monitoring demonstrated that specific anti-tumor responses were measurable in αGalCer-GVax tumor cell vaccinated mice. Specifically, intracellular cytokine FACS demonstrated memory CD8 T cells producing large amounts of IFNγ in response to tumor cells (**Fig. 4**). The scale of this response, presumably to multiple antigens, may help explain the greater promise relative to pooled peptide vaccines. Significantly, tumor cells also stimulated T cell proliferation in immunized mice (**Fig. 5**), a measure to CD4 T cell help. Finally, immunization produced a clear prolongation of life span in older castrated and immunized TRAMP mice (**Fig. 6**), confirming the clinically relevant basis for the tumor pathology results (**Figs. 2, 3**) and consistent with the strong immunity generated by the vaccination combinations (**Figs. 1, 4, 5**).

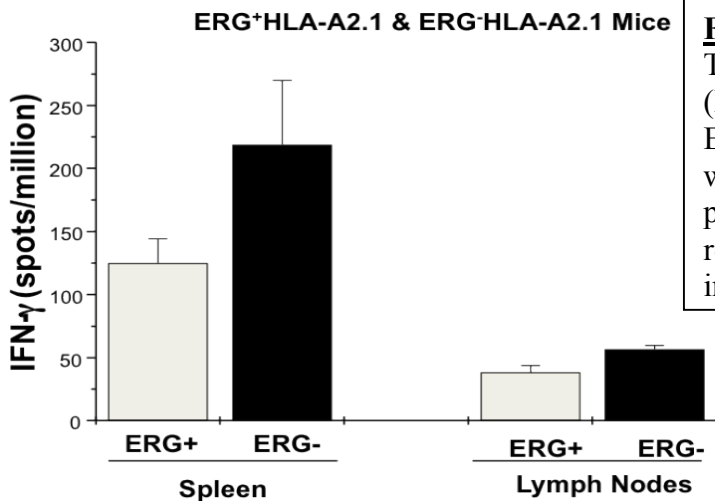| **Fig. 5. Proliferative response of splenocytes from mice immunized with PCa cell vaccines.** Tumor-bearing TRAMP mice were castrated and vaccinated as above with TRAMP C2 GM-CSF/RGE vaccine + αGalCer.. Immune cells from immunized mice proliferate upon restimulation with tumor antigen.	**Fig. 6. Survival curves of mice immunized with PCa cell vaccines and controls.** Tumor-bearing TRAMP mice were castrated and vaccinated as above with TRAMP C2 GM-CSF/RGE vaccine + αGalCer. A significant increase in survival was detected ($p < 0.04$). Surviving mice were sacrificed at 330 days for immuno-monitoring.

We have also compared the efficacy of αGalCer versus αC-GalCer. Preliminary studies have shown comparable responses, but suggest that IL-12 may not be required to obtain optimal responses with αC-GalCer (**Fig. 5; Year 2 Report**). However, further experiments are clearly needed in tumor-bearing mice during Year 3 to determine whether there are marked differences between iNKT cell ligands.

Aim 4. Assess immune responses to αGalCer analogue/DC vaccine using human *SIM2* and *ERG* peptides in HLA-A2.1 transgenic mice.
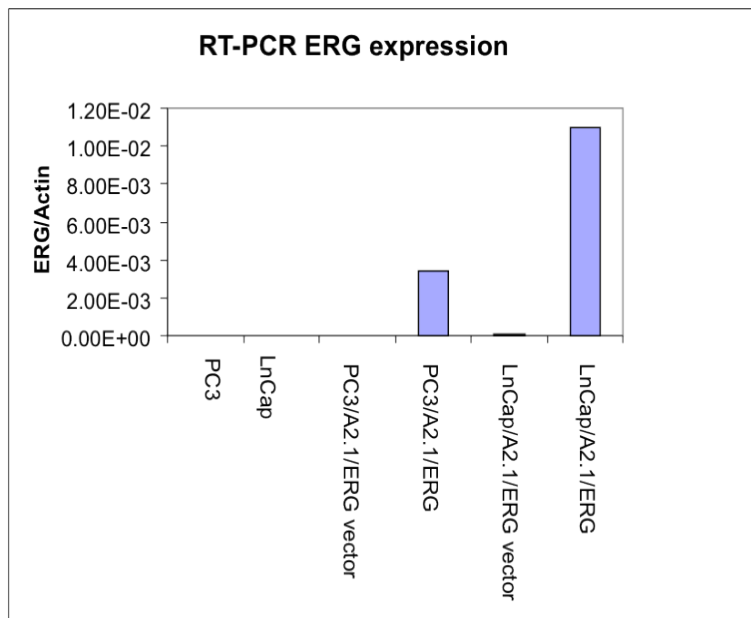
In preliminary experiments we reported the effects of αGalCer on responses to ERG and SIM2 peptides (**Figs. 5, 6; Year 2 Report**). Mice were vaccinated subcutaneously with WT19 cells pulsed with peptide, minus or plus αGalCer, and splenocytes were assessed for peptide responses by ELISPOT. Responses to an ERG peptide were increased, but we did not observe an increase in the response to a SIM2 peptide (not shown). Therefore, we will instead test the cell-based vaccine in HLA A2.1 transgenic mice.

We have now generated further reagents to complete and extend the project. Specifically, we have new data showing that ERG-derived paptides can generate good CTL responses in HLA-A2.1/Pb-ERG TRAMP mice (**Fig. 7**), which indicates that prostatic expression pf ERG does not hinder peptide-induced responses. Probasin-driven expression of ERG in HHD mice results in a modest anti-ERG cytotoxic lymphocyte response upon immunization with ERG295 peptides. This indicates that overexpression of ERG in the prostate tissue does not compromise the ability of ERG-derived, HLA-A2.1-restricted peptides to overcome immune tolerance to ERG. IN Year 3, we will determine whether αGalCer enhances such responses in these new mice strains.

Finally, LNCap and PC3 cell lines that express both HLA-A2.1 and ERG have been generated (**Fig. 8**). These will be used in xenograft experiments in SCID mice and also in killing assays.

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Figure 7. ERG+HLA-A2.1+ Transgenic mice model.
The Probasin-ERG mouse was crossed to the HHD (HLA-A2.1) mouse to generate ERG+HLA-A2.1+ and ERG- HLA-A2.1+ hybrid offspring. These hybrid mice were immunized with ERG295 peptides and spleens and prostate-draining lymph nodes were tested for responsiveness to this peptide 10 days post-immunization.

Figure 8. Prostate cell lines expressing tumor antigen ERG.
LnCap and PC3 were transduced with ERG and HLA A2.1. ERG expression was determined by real time RT- PCR Cell surface HLA A2.1 expression was confirmed by FACS (not shown).

KEY RESEARCH ACCOMPLISHMENTS
- Characterized effects of iNKT cell ligands on peptide/DC vaccines
- Characterized effects of different iNKT cell ligands on cellular vaccines
- Demonstrated that prostate cancer cells could induce a state of anergy in iNKT cells
- Demonstrated that this anergy could be reversed by iNKT cell ligand
- Generated data showing that iNKT cell ligands can enhance T cell responses to cellular vaccines
- Generated data showing that iNKT cell ligands can enhance anti-tumor responses to cellular vaccines
- Generated data showing that iNKT cell ligands can enhance prostate infiltration to cellular vaccines
- Generated improved TRAMP HLA-A2.1/Pb-ERG transgenic mice to complete vaccine studies

REPORTABLE OUTCOMES
Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, Exley MA. (2010). Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha-galactosylceramide. PLoS One. 5, e11311.

CONCLUSIONS
We have obtained better results with cell-based rather than peptide-based vaccines. It is well established that peptides (alone or pulsed onto DCs) are not optimal immunogens, and although we can boost their efficacy using iNKT cell ligands, results do not appear as promising as our GM-CSF – αGalcer combination presented on prostate tumor cells, which shows protective anti-tumor effects as well as generating tumor-specific immunity. Over the next year we will assess the role of each component of our vaccine in PCa tumor-bearing

mice, including a second model through a new collaboration with Dr. P. Pandolfi (BIDMC). We will also define the critical cellular immune elements involved in protection downstream of iNKT, which could aid in clinical trial immuno-monitoring. This work will be written up and submitted for publication. A major manuscript is being outlined at the moment and a second may develop from results with the second model. Finally, we will coordinate with collaborators including Dr. G. Dranoff (DFCI, Boston) and C. Drake (Johns-Hopkins) who is planning clinical trials of prostate cancer GVax and considering αGalCer as adjuvant, based on our data.

REFERENCES
Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, Exley MA. (2010). Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha-galactosylceramide. PLoS One. 5, e11311.