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**Light: A Novel Immunotherapy for Primary and Metastatic Prostate Cancer**

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**Abstract:**

Over-expression of LIGHT has been shown in various tumor models to induce tumor regression and tumor immunogenicity. However, the models are based on transplanted tumors that express artificial foreign antigens that function as tumor antigens. LIGHT has never been evaluated in prostate cancer, where self-antigens likely exist. We have provided the first evidence that LIGHT-induced T cells are specific for at least one relevant prostate expressed self-antigen, PSCA. We have also demonstrated that LIGHT treatment in prostate cancer has a positive effect on the tumor microenvironment, which suggests a strong likelihood that combination treatment with LIGHT and immunotherapeutic vaccination will have an impact against primary and possibly metastatic prostate cancer. Thus, therapeutic intervention by delivering LIGHT to the tumors may serve the dual purpose of inhibiting immune-suppression mediated by regulatory T cells while simultaneously activating tumor-specific immune responses, which we hope to demonstrate can be boosted by vaccination. This study may potentially provide a practical means of overcoming tumor-mediated immunosuppressive mechanisms in a variety of solid human tumors, including those of the prostate, which would have important implications for patients who are diagnosed at the later stages of disease and currently have no recourse for treatment.

**Subject Terms:**

Regulatory T cells, prostate cancer, immunosuppression, tumor microenvironment

**Security Classification:**

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INTRODUCTION
Prostate cancer is the second leading cause of cancer-related death in the United States and according to the American Cancer Society’s most recent estimates, will affect almost 200,000 men in 2009. Of these, almost 30,000 men are estimated to die [1, 2]. Much of the focus of past and current research aims to improve methods to detect the disease at the very earliest stage of carcinogenesis. However, treatment options remain limited [3]. In many cases, expectant management or “watchful waiting” is the standard of care. The current modalities available for treatment have debilitating side effects which include, but are not limited to, urinary, bowel and erectile dysfunction, loss of fertility, effects due to the loss of testosterone (including fatigue, decreased sexual desire, weight gain, loss of muscle mass and osteoporosis) and the well-known devastating side effects of chemotherapy [4, 5]. Metastatic prostate cancer is a death sentence as it is infeasible to remove metastasis by radiation or surgery or any other existing modality. There is no cure for advanced prostate cancer, and thus, there is a significant need to focus research efforts on developing new therapeutic strategies.

While surgery or radiation therapy may be used to treat primary tumors, once the disease spreads beyond the prostate, immunotherapy may be the only way to treat it [6, 7]. A majority of clinical trials for the immunotherapy of prostate cancer have yielded results similar to those seen for most other cancers, which is the induction of tumor-specific immune responses yet limited success in terms of regression or survival. Despite the 2009 U.S Food and Drug Administration (FDA) approval of PROVENGE, the first immunotherapeutic cell-based vaccine that can be prescribed for hormone-refractory prostate cancer patients, excitement is dampened because there have been no objective cures [8]. The failure to clear tumors despite successful induction of immunity in the majority of clinical trials may, in part, be attributed to the suppressive environment within the tumor that disables function of the immune system. Thus, it is essential to develop therapeutic modalities that aim to generate tumor-specific immunity and simultaneously inhibit local immune suppression [9]. Since regulatory T cells appear to be central to inhibiting anti-tumor immunity, the goal of our proposal is to establish a therapeutic intervention that can overcome the suppressive activity of regulatory T' cells while simultaneously inducing prostate cancer-specific immunity.

LIGHT, a ligand for Herpes Virus Entry Mediator (HVEM) and Lymphotoxin beta-receptor (LTβR), is predominantly expressed on activated immune cells, signaling via LTβR is required for the formation of organized lymphoid tissues while signaling via HVEM induces costimulation [10-13]. Although LIGHT has not been extensively studied in the prostate cancer setting and has not been associated with the inhibition of Treg development or function, our previous experience using LIGHT in a virally-induced tumor model suggests a strong connection between forced LIGHT expression in tumors with a survival benefit and change in tumor milieu [14-16]. Therefore, we hypothesize that Treg formation and function within the tumor microenvironment can be inhibited by the forced expression of the costimulatory molecule, LIGHT, thereby improving the efficacy of therapeutic vaccines in the absence of a suppressive tumor microenvironment where strong antitumoral response may emerge, resulting in an increase survival and tumor specific immunogenicity. Thus we have proposed the following aims: Aim 1) To determine whether forced expression of LIGHT can inhibit prostate tumor-induced differentiation and function of CD4+ regulatory T cells; Aim 2) To determine whether forced expression of LIGHT can alter the pattern of infiltration and maturation of immune cells, other than T cells, within the tumor microenvironment; Aim 3) To determine whether forced expression of LIGHT in combination with vaccination can induce regression of well-established primary and metastatic prostate tumors.
BODY

SPECIFIC AIM 1

Predicted Outcome: Determine whether forced expression of LIGHT can inhibit prostate tumor-induced differentiation and function of CD4+ regulatory T cells.

Task 1.1: Compare the effect of treatment with Ad-LIGHT on frequency and function of CD4+ T cells.

LIGHT is predominantly expressed on activated immune cells. Signaling via LTβR is required for the formation of organized lymphoid tissues while signaling via HVEM induces costimulation \[17\]. One of the many well studied immune escape mechanisms includes the suppressive capacity of regulatory T cells (Tregs). The development of induced Tregs (iTregs) from naïve CD4+ cells within the tumor microenvironment remains a mystery \[16\]. Here, we hypothesize an interesting connection between LIGHT and immune escape involving the interactions between LIGHT, HVEM, and a receptor B and T lymphocyte attenuator (BTLA). BTLA, a molecule closely resembles CTLA4, inhibits T cell activation when bound to the ligand HVEM \[12\]. LIGHT is capable of disrupting BTLA-HVEM interaction through competitive binding \[18\]. Given two possible interactions with HVEM, naïve T cell fate may be determined depending on the stimulation received. Since the absence of costimulation leads to the development of Tregs, conversely, co-stimulation with LIGHT may prevent naïve T cells from becoming inhibitory immune modulators in a tumor microenvironment. In establishing our prostate cancer tumor model, we show that forced expression of LIGHT via an adenovirus vector in TRAMP-C2 prostate cancer cells express high levels of LIGHT on the cell surface within 24 and 48 hours as shown by quantitative PCR (Figure 1a) and flow cytometry (Figure 1b).

To compare the effects of Ad-LIGHT on the frequency and function of CD4+ T cells, C57BL6 mice were challenged with TRAMP-C2 cells to establish palpable tumors. Tumors were then sized and normalized between treatment groups, tumors were treated with 10^12 Ad-LIGHT virus or Ad-Control virus. We began to investigate the effects of Ad-LIGHT on a specific cell type, Tregs. Two treatments of Ad-LIGHT and Ad-Control were injected intratumorally in TRAMP-C2 challenged mice. A week subsequent to the second treatment, tumor draining lymph nodes were pooled together from the treatment groups, CD4^+CD25^{hi} population were isolated representing the Treg population. Tregs were co-cultured in decreasing ratios with CD4^+CD25^{-} responder cells (Tresp) isolated from the treatment groups. Successful Ad-LIGHT Infection (Flow Cytometry) was measured using the LTβR-Fc antibody.

![Figure 1. TRAMP-C2 infected cells are capable of expressing membrane bound LIGHT. A. 5x10^5 TRAMP-C2 cells were infected with 10^3 adeno-LIGHT viral particle per cell. mRNA was isolated and demonstrates a 10 fold increase in expression of LIGHT compared to adeno-control infected TRAMP-C2 cells. Expression of LIGHT weakens after 24 hours. B. Membrane bound LIGHT was detected via flow cytometry with LTβR-Fc antibody. Expression of LIGHT correlates with the mRNA expression level, where 24 hours shows the highest levels of LIGHT expression.](image)
from naïve C57BL6 mice. Proliferation of responder cells were measured via the addition of radioactive thymidine to each co-culture. Proliferation is directly correlated to the suppressive capacity of Tregs; increased proliferation equates to minimal suppressive functions, and vice versa. **Figure 2** demonstrates that untreated, Ad-control, and B6 Tregs showed expected results; with decreasing ratios of Tregs to Tresp cells (decreasing suppression) there was an increase in proliferation from Tresp. However, Tregs from the LIGHT treated mice completely lose their suppressive capacity even at a high Treg to Tresp ratio. This data suggests an unknown mechanism in which forced LIGHT expression in tumors indirectly, or directly, affects Treg functionality, supporting our hypothesis.

**Figure 2.** Regulatory T cells from LIGHT-treated mice lose their suppressive abilities. CD4-CD25-responder T cells (Tresp) from naïve (B6) mice were co-cultured with CD4+CD25hi Tregs isolated form tumor draining lymph nodes in various decreasing ratios for 3 days. 3H-thymidine was added to cultures on the last day to measure Tresp proliferation of Tresp cells alone (1:0 Tresp:Treg ratio) was taken as 100% proliferation. Tregs isolated from Ad-Ctrl treated mice suppressed Tresp proliferation at all co-culture ratios. Tregs isolated from Ad-LIGHT treated mice lose the ability to suppress Tresp proliferation. Tregs isolated from untreated tumor-bearing mice or naïve mice showed statistically similar suppressive capacity to Ad-Ctrl treated mice. (3 experiments, n=10/experiment, two-tailed T-test).

**Task 1.2:** Determine whether tumors induce differentiation of naïve CD4+ T cells into Tregs

In addressing Task 1.2, whether forced LIGHT expression will hinder the differentiation of naïve cells to Tregs, treated tumors were isolated where tumor infiltrating lymphocytes were cell surface phenotyped via flow cytometry (**Figure 3**). In comparing the three treatment groups, there was an increase in the overall number of CD3+ T cells (white bars) in Ad-LIGHT treated tumors. Although the vector control shows slight immunogenicity as seen by the increase in infiltrating T cells, the additional effects of LIGHT expression are beyond that of the control and untreated groups. Thus, LIGHT is shown to alter the tumor microenvironment by
drawing in TILs, suggestive of an active immune response taking place within the tumor. More interestingly, despite the increase in infiltrating T cells (CD3+ population) there is an increased ratio of Tresp versus Tregs (Figure 3, black bars). The data suggest a more immunostimulatory tumor microenvironment with increased TILs and possibly a less immunosuppressive milieu as seen by increased ratio of Tresp versus Tregs. Thus, expression of LIGHT changes the microenvironment such that either infiltration of natural Tregs from the periphery or differentiation of induced Treg/FoxP3+ cells within the tumor is suppressed. Detection of CD4 and CD8 staining was not successful in this primary analysis, therefore optimization of TIL isolation and the staining protocol is ongoing to further define the identity of the infiltrating T cells. Further investigation of various types of immune cells, macrophages, natural killer cells, CD8+ T cells, Th1 T cells, Th2 T cells, dendritic cells, amongst many, will be examined in Task 2.1.

**Task 1.3:** Determine whether forced expression of LIGHT in tumor can prevent the differentiation of naïve CD4+ T cells into Tregs.

Task 1.3 requires the breeding of TRAMP mice with (Depletion of Regulatory T cell) DEREG mice, to generate a model that will spontaneously develop prostate cancer but their regulatory T cells may be depleted via administration of diphtheria toxin. The advantage of using this transgenic mouse model, DEREG mice, is to allow us to selectively deplete Tregs at any given point [19]. The generation of these mice will help us investigate the effects of Ad-LIGHT on Treg development from naïve T cells. A setback we encountered is that our DEREG colony of mice could not sustain itself due to aged breeders and lack of sufficient offspring with the correct phenotype, therefore no new DEREG mice have been bred. To remedy this we are currently obtaining new DEREG breeder mice from a different source (NIH NIAID, Bethesda, MD) where the newly transferred young DEREG males will help reestablish our colony. The animal transfer is currently in process between our two institutions. We have been notified that new DEREG mouse breeders will arrive next week, after which they will immediately be placed into breeder cages to expand the colony. With the new population of mice, the crossing of TRAMP and DEREG mice will progress and we will be able to complete task 1.3.

**Task 1.4:** Determine the effect of forced expression of LIGHT on the differentiation and activation state of tumor-infiltrating CD4+ T cells.

As stated in Task 1.2, we are currently in the process to modifying/troubleshooting our protocols to maximize the efficacy of CD4+ staining. Our troubleshooting includes modifying the enzyme concentrations and combinations used to dissociate the tissue and the time the tissue is incubated with those dissociating enzymes such that surface molecule expression is not affected. We have also been evaluating the use of the GentleMACS dissociator (Miltenyi, Auburn, CA), a small benchtop instrument for the automated dissociation of tissues into single-cell suspensions. The advantage of this system is to standardize tissue dissociation and homogenization...
procedures to enable more reliable and reproducible results. This task also requires the use of DEREG mice. No data have been acquired for this task thus far, though we do not anticipate any difficulties in completing this task as per the original timeframe.

SPECIFIC AIM 2

Predicted Outcome: Determine whether forced expression of LIGHT can alter the pattern of infiltration and maturation of immune cells, other than T cells, within the tumor microenvironment.

Task 2.1: Compare the intra-tumoral cytokines and chemokine profile following treatment with Ad-LIGHT

For Task 2.1, tumors treated with Ad-LIGHT, Ad-Control, or left untreated were isolated from challenged C57BL6 mice 3 days subsequent to the second LIGHT injection. Tumors were homogenized and supernatant was collected for a multiplex ELISA, Bioplex Assay (Figure 4). The following cytokines/chemokines were analyzed: MIP 1a, MIP 1b, VEGF, TGFβ1, TGFβ2, TGFβ3, IL-12(p70), GM-CSF, IFNγ, IL1a, IL1b, IL2, IL4, IL5, IL6, IL9, IL10, IL13, IL15, IL17, KC, MCP1, M-CSF, MIP2, TNFα.

Figure 4. LIGHT treatment results in a reduced suppressive cytokine microenvironment. Tumors from untreated (NIL), Ad-GFP, or Ad-LIGHT treated mice (n=4/group) were isolated 7 days after the second Ad injection, weighed and homogenized in PBS supplemented with protease inhibitors. Clarified supernatants were tested for a panel of cytokines pertinent to prostate tumors. Shown is the concentration of cytokine in pg/mL per gram of tumor (± SEM). A trend in reduction in both TGFβ1 and TGFβ2 is seen in Ad-LIGHT treated mice compared to untreated or vector control treated mice while a massive increase in MIP1a and MIP1b was seen in Ad-LIGHT treated tumors.

Ad-LIGHT treated tumors display more inflammatory cytokines (MIP 1a/MIP 1b) compared to control and untreated groups. In addition, there is a trend (though non-statistically significant) towards decreased suppressive cytokines such as TGFβ1 and TGFβ2. One of the major inconsistencies we have encountered with cytokine profiling is the variability in LIGHT injections, since there is no measure of the number of viral particles that were actually taken up. Hence, an increase in sample size is needed to analyze statistical differences between Ad-LIGHT and Ad-control treated tumors. In addition to increased sample sizes, we are also separately evaluating a non-ionic surfactant co-polymer called polaxomer that becomes more viscous at
higher temperatures which we would use to enhance retention of the adenovirus particles within the tumor after injection. If proven to enhance vector retention and LIGHT expression, we will consider using polaxomer as the vehicle for future experiments instead of standard saline.

**Task 2.2:** Compare the frequency and phenotype of tumor-infiltrating cells.

Task 2.2, similar to Task 1.4, requires modification/troubleshooting of TIL staining protocol, and no data have been collected for this task yet.

**SPECIFIC AIM 3**

**Predicted Outcome:** Determine whether forced expression of LIGHT in combination with vaccination can induce regression of well-established primary and metastatic prostate tumors.

**Task 3.1:** Determine efficacy of treatment with Ad-LIGHT on inducing prostate cancer associated antigen-specific CD8+ T cells and regression of autochthonous primary prostate tumors in TRAMP mice.

Work on Task 3.1 will start during the current period of performance.

**Task 3.2:** Determine efficacy of treatment with Ad-LIGHT on inducing prostate cancer associated antigen-specific CD8+ T cells and regression of primary tumors in mice challenged with TRAMP-C2 cells.

We have previously shown in our preliminary data that LIGHT therapy is capable of inducing tumor specific immunity towards PSCA. CD8+ IFNγ releasing T cells showed a strong response against PSCA peptide as compared to control treated mice. In addition to inducing PSCA specific immunity, challenged mice treated with Ad-LIGHT also show a delay in tumor growth and an extended survival (**data not shown**). These results demonstrate the ability of LIGHT to induce CD8+ IFNγ releasing T cells, a possible mechanism that results in the delay in tumor growth and extended survival. We have repeated this task several times but were not able to demonstrate the same degree of effect shown in the preliminary results. We believe the expression of LIGHT is not optimally expressed due to lack of retention and fluidity of treatment within the tumor. As mentioned in Task 2.1, we are currently attempting to mitigate the retention of LIGHT within the tumor environment with the non-ionic surfactant co-polymer, polaxomer. If proven to enhance vector retention and LIGHT expression, we will consider using polaxomer as the vehicle for future experiments instead of standard saline.

**Task 3.3:** Compare efficacy of treatment with Ad-LIGHT and combined treatment of Ad-LIGHT followed by vaccination with VRP on inducing regression of primary tumors in mice with TRAMP-C2 tumors.

As Alphavax, Inc., our original collaborator, that produced our VRP vaccine went out of business due to lack of investor funds we are currently evaluating other vaccination platforms, namely a
lentiviral vector vaccine and a peptide based vaccine expressing PSCA as an alternative to VRP (VEE replicon particles expressing mouse PSCA) [20-22]. Therefore, Tasks 3.1, 3.3, 3.4, and 3.5 have been delayed. We did evaluated the efficacy of an old batch of VRPs in a scaled experiment where we immunized mice with a heterologous PSCA-DNA prime and PSCA-VRP boost and assessed PSCA T cell specificity via an Elispot assay (Figure 5). The data suggest no statistical significance between the untreated and vaccinated mice, demonstrating the contracted quality of VRPs. With the lack of a follow-up vaccination after LIGHT treatment, we sought alternative heterologous/homologous vaccination platforms. It appears that amphotrophic (via VSV-G protein) lentiviral vector vaccination against PSCA shows promising results in inducing PSCA specific T cells and shows a slight delay in TRAMP-C1 tumor growth and extends survival. In assessing a peptide vaccine platform, literature has shown that TriVax, combination of peptide, anti-CD40 antibody (BioXcell) and PolyIC (Hiltinol) are capable of inducing tumor specificity and delaying tumor growth [25]. We are currently in the process of evaluating TriVax peptide vaccination against PSCA, in a pilot study with LIGHT treatment and various heterologous/homologous peptide vaccinations we show that the TriVax method is a potential vaccination replacement for VRPs (Figure 6). Although the use of Lentivirus vectors and peptide based vaccinations were not part of the original proposal, there are many advantages of evaluating these vector platforms for our project:

1. Lentiviral vectors have been show in literature to induce tumor-specificity in many tumor models [23, 24].
2. The vector is currently being produced by one of our other collaborators at USC, Dr. Pin Wang, Associate Professor of Biomedical Engineering. Vaccine production will be transferred to our lab, eliminating dependence on another lab or company for vaccine production.
3. Collaborators have shown partial protective efficacy of PSCA VSVG-lentivirus in the TRAMP-C1 model (data not shown, but submitted for publication)
4. Optimized peptide vaccination (TriVax) has been show in the literature to induce tumor specificity and delay in tumor growth [25].

Figure 6. Ad-LIGHT and peptide vaccinated mice displays a delay in tumor growth as compared to untreated mice. Mice were first treated with two doses of Ad-LIGHT (or Ad-control) prior to receiving various vaccinations against PSCA. A two-way Anova was performed in comparing all treatment groups untreated, on Day 48, Ad-LIGHT & Pep Prime/Pep boost showed statistical significance with a p-value <0.001. Ad-LIGHT & Pep Prime/Pep Boost vs Ad-Con. & Pep Prime/Pep Boost had a p-value <0.05 (Paired T-test, two tailed 95% CI). There was no significant difference between Ad-LIGHT& DNA Prime/Pep Boost compared to Ad-Con & DNA Prime/Pep Boost.
Thus, after evaluating these alternative vaccination platforms we will be able to make progress into the effect of Ad-LIGHT and PSCA therapeutic vaccination.

**Task 3.4:** Determine whether combined treatment of Ad-LIGHT followed by vaccination with VRP induces regression of metastatic tumors in mice challenged with TRAMP-C2 cells.

No data have been collected for Task 3.4 yet as the intent was to begin experiments in the latter half of the second year of the project and throughout year 3.

**Task 3.5:** Determine whether combined treatment of Ad-LIGHT followed by vaccination with VRP prevents the outgrowth of spontaneous metastatic tumors in TRAMP mice.

No data have been collected for Task 3.4 yet as the intent was to begin experiments in the latter half of the second year of the project and throughout year 3.
KEY RESEARCH ACCOMPLISHMENTS

- Ad-LIGHT inhibits the functionality of Tregs in Ad-LIGHT treated tumors. Tregs lose their suppressive capacity and fail to suppress the proliferation of responder T cells.
- A high frequency of CD3+ tumor infiltrating lymphocytes are recruited into tumors subsequent to LIGHT therapy, while the number of Tregs remains unchanged.
- Inflammatory cytokines were dramatically increased in LIGHT treated tumors while suppressive cytokines were unchanged or decreased.
- Intratumoral LIGHT expression alone is capable of inducing PSCA specific IFN-γ releasing CD8+ cells.
- Intratumoral LIGHT expression results in a delay in tumor growth and extended survival.

REPORTABLE OUTCOMES

2. Awarded California Clinical and Translational Science Institute TL1 Graduate Student Training Fellowship, 07/01/12 – 06/30/13.

CONCLUSION

Published data shows that in some tumor models, over-expressing LIGHT can induce tumor regression. However, the models are based on transplanted tumors that express artificial foreign antigens that function as tumor antigens. Moreover, even in these models, antigen-specificity of T cells induced by over-expressing LIGHT in tumors has not been demonstrated. We have provided the first evidence that LIGHT-induced T cells are specific for at least one relevant prostate expressed self-antigen, PSCA. We have also demonstrated that LIGHT treatment in prostate cancer has a positive effect on the tumor microenvironment, which suggests a strong likelihood that combination treatment with LIGHT and immunotherapeutic vaccination will have an impact against primary and possibly metastatic prostate cancer. Thus, therapeutic intervention by delivering LIGHT to the tumors may serve the dual purpose of inhibiting immune-suppression mediated by regulatory T cells while simultaneously activating tumor-specific immune responses, which we hope to demonstrate can be boosted by vaccination. This study may potentially provide a practical means of overcoming tumor-mediated immunosuppressive mechanisms in a variety of solid human tumors, including those of the prostate, which would have important implications for patients who are diagnosed at the later stages of disease and currently have no recourse for treatment.
REFERENCES

APPENDICES

*The American Association of Immunologists Conference 2012.*

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LIGHT expression in prostate cancer inhibits tumor growth and induces prostate antigen-specific immunity

Lisa Yan, Diane Da Silva, Shreya Kanodia, Andrew Gray, W. Martin Kast

An immunosuppressive tumor microenvironment has always been a hurdle for successful immunotherapy even in the presence of induced tumor-specific T cells. Regulatory T cells (Tregs) appear to be key regulators in local immune suppression. LIGHT, a ligand for lymphotoxin-β receptor (LTβR), is predominantly expressed on activated immune cells, signaling via LTβR is required for the formation of organized lymphoid tissues. Forced expression of LIGHT recruits naive T cells into tumors and is capable of establishing tumor specific immunity. However this has never been tested in prostate cancer models where tolerance to self-antigen likely exists. Here we test the hypothesis that forced expression of LIGHT in prostate tumors induces prostate cancer-specific immunity and results in tumor regression by altering the suppressive activity of Tregs and consequently enhancing a more persistent proinflammatory microenvironment. Our data show that intratumoral expression of LIGHT via adenovirus delivery in TRAMP-C2 tumor challenged mice develop de novo CD8+ IFNγ-secreting prostate antigen-specific T cells and display increased survival compared to control treated mice. LIGHT-treated mice also display an increase in ratio of tumor infiltrating lymphocytes to Tregs as well as decrease in Treg suppression activity. Our data suggest that LIGHT treatment can alter the microenvironment such that natural and vaccine-induced prostate tumor antigen specific T cells mediate tumor regression.