Award Number: W81XWH-11-1-0518

TITLE: LIGHT: A Novel Immunotherapy for Primary and Metastatic Prostate Cancer

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REPORT DATE: November 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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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 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.

Regulatory T cells, prostate cancer, immunosuppression, tumor microenvironment
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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 in the United States [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 prostate cancer 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, 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 anti-tumoral 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: 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 1 A) and flow cytometry (Figure 1 B).

To compare the effects of Ad-LIGHT on the frequency and function of CD4+ T cells, C56BL6 mice were first challenged with 5x10^5 TRAMP-C2 cells, normalized when tumor volumes were approximately 300mm^3 and treated with the appropriate vaccination scheme according to each group. 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

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.
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.

![Treg Suppression Assay](image)

**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 from 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

Since we were unable to generate a proper DEREG mouse model to study adoptive transfer, we focused on the ratio of Tregs to total TILs within the tumor microenvironment in the TRAMP-C2-challenged C57Bl6 mouse model as it is indicative of successful tumor immunotherapy. C57Bl6 mice were first challenged with $5 \times 10^5$ TRAMP-C2 cells, normalized when tumor volumes were approximately 300mm³ and treated with the
vaccination scheme outlined in the attached manuscript (Yan el at). 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 3A, 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. Additionally, after examination of Treg population isolated from the spleens of these mice we found no significant difference in the percentage of splenic Tregs between treatment groups (Figure 3B). Further investigation into the phenotypes of immune cells was carried out, specifically macrophage, natural killer cells, CD8+ T cell, Th1 T cell, Th2 T cell, and dendritic cell populations were examined in Task 2.1.

Figure 3. Increase in ratio of tumor infiltrating lymphocytes to Tregs with LIGHT treatment while no change in percentage of Tregs within spleen. A. Tumor infiltrating lymphocytes were collected from treated tumors 7 days after Ad-Ctrl or Ad-LIGHT injection or no treatment (NIL). Cells were stained for CD3 and intracellular transcription factor Foxp3 and analyzed by flow cytometry. The mean number of CD3+ T cells was significantly higher in Ad-LIGHT treated mice compared to untreated, while the mean number of Foxp3+ Tregs was not significantly differently, despite the increase in total number of infiltrating lymphocytes. (p<0.05, two-tailed students t-test). B. Splenocytes were isolated from the same mice bearing TRAMP-C2 tumors and stained for CD3, CD4, CD25, and FoxP3. Tregs were defined as CD4+, CD25+, and FoxP3+ within the CD3+ gate (total lymphocyte population). The data suggests that there is no significant difference in the total percentage of Tregs isolated from spleens between treatment groups (One-way ANOVA, p=0.66).
**Task 1.3:** Determine whether forced expression of LIGHT in tumor can prevent the differentiation of naïve CD4+ T cells into Tregs.

Initial investigation into task 1.3 required 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 [19]. However, we were unable to generate this mouse line due to breeding difficulties and because of this we did not have the proper mouse strain required for the proposed adoptive transfer experiments. Therefore, as an alternative we utilized the TRAMP-C2 challenge model in C57BL6 mice. Specific methodology of these experiments including treatment strategy is outlined in the manuscript by Yan et al provided with this report.

In the first progress report, we demonstrated that the expression of LIGHT alters the tumor microenvironment by drawing in TILS with an increased ratio of T responder (CD3+) cells versus Tregs. However, we were unable to distinguish the true phenotype of the TILS due to the number of tumor cells and debris that masked the lymphoid markers. Therefore the sensitivity of detecting surface markers from the minor population of TILs was obstructed. We mitigated this problem by using the GentleMACS dissociator (Miltenyi, Auburn, CA), a small bench top instrument for the automated dissociation of tissues into single-cell suspensions, and isolating TILS from tumor debris with a sugar based gradient, Lympholyte-M.

![Frequency of TILs](image)

**Figure 4. Ad-LIGHT draws in CD8+CD3+ and CD4+3+ T cells.** TRAMP-C2 tumors harvested from C57Bl6 mice that were either untreated, given Ad-Control or Ad-LIGHT were analyzed for infiltrating CD4+ and CD8+ T cell populations. Tumors were isolated, minced, and processed with the GentleMACS dissociator. TILS were separated in a Lympholyte-M dissociation gradient prior to being phenotyped via flow cytometry.

We show in **Figure 4** the results of our phenotype analysis of TILs utilizing the GentleMACS and Lympholyte-M strategy. Compared to untreated control and Ad-Control population, Ad-LIGHT induced a higher frequency of infiltrating effector T cells into the tumor. Specifically, a majority of the TILs detected in Ad-LIGHT treated tumors were CD8+CD3+ (21.7 %) and CD4+CD3+ (11.2 %) T cells. These results agree with and extend our previously reported results where a massive infiltration of CD3+ T cells was detected with Ad-LIGHT treatment. Additionally, the increased frequency of cytotoxic CD8+ T cells and helper CD4+ T cells support and potentially explain our original preliminary findings, where Ad-LIGHT treatment induced mPSCA specific CD8+ T cells that resulted in a delay in tumor growth and an increase in survival.
As seen in Figure 5, there was an increase in intratumoral CD4+ and CD8+ T cells following forced expression of membrane bound LIGHT in a prostate cancer tumor model. (A) Tumor infiltrating lymphocytes were isolated from untreated or treated tumors 7 days after Ad-Control or Ad-LIGHT injection. Cells were stained with CD4, CD8 and CD3 Ab and analyzed via flow cytometry. The number of TIL/gram of tumor from CD8+/CD3+ and CD4+/CD3+ T cells were significantly higher in Ad-LIGHT treated mice compared to untreated. (p<0.05, one-way ANOVA). (B) The number of CD4+/CD25+/Foxp3+ Tregs per gram of tumor were not significantly different, despite the increase in total number of infiltrating lymphocytes in the Ad-LIGHT samples. Shown is the average number of FoxP3+ TIL (±SD) from 5 treated mice/group. Data are representative of two individual experiments.

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

During the no-cost extension year we continued to work on task 1.4. While we initially proposed to breed TRAMP mice with (Depletion of Regulatory T cell) Dereg mice to generate a model that would spontaneously develop prostate cancer while having their regulatory T cells be depleted via administration of diphtheria toxin, generation of this model failed due to breeding difficulties [19] As an alternative to this we started breeding TRAMP mice with FOXP3<sup>DTR</sup> mice (purchased from Jackson Laboratories) to generate a new TRAMP-FOXP3<sup>DTR</sup> strain, allowing us to directly regulate depletion of Tregs through treatment with diphtheria toxin (DT) in a mouse strain that will spontaneously develop prostate cancer. We were able to examine two groups of three 12-14-week-old TRAMP-FOXP3<sup>DTR</sup> mice for effects of spontaneous prostate cancer development with and without Treg depletion. Group 1 was left untreated while group 2 received two doses of DT at 25 µg/kg spaced seven days apart through i.p. injection. Occurrence of Tregs in each group were analyzed through flow cytometric analysis of CD4+FOXp3+CD25+ cells at week 25 and verified to be depleted in DT-treated groups. Prostates were isolated from both groups, sectioned and stained with hematoxylin and eosin, and assigned Gleason scores.
These results may indicate that elimination of Tregs results in a higher rate of spontaneous prostate cancer development in this model, or that multiple treatments with diphtheria toxin (DT) may accelerate this transformation.

**SPECIFIC AIM 2: 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 TRAMP-C2 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 6**), specific methods are outlined within the manuscript submitted with this report (Yan et al 2015). The following cytokines/chemokines were analyzed: MIP 1a, MIP 1b, VEGF, TGFβ1, TGFβ2, TGFβ3, IL-12(p70), GM-CSF, IFNγ, IL1α, IL1β, IL2, IL4, IL5, IL6, IL9, IL10, IL13, IL15, IL17, KC, MCP1, M-CSF, MIP2, TNFα.
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.

Figure 6. LIGHT treatment results in a reduced suppressive cytokine microenvironment. TRAMP-C2 tumors from untreated (NIL), Ad-GFP, or Ad-LIGHT treated C57Bl6 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.
Task 2.2 Compare the frequency and phenotype of tumor-infiltrating cells.

Figure 7. LIGHT does not alter the frequency of NK or MDSCs. Tumors were isolated from the TRAMP-C2 challenged mice from the untreated, Ad-Control, Ad-LIGHT, Ad-Control/mPSCA Vaccine, or Ad-LIGHT/mPSCA Vaccine, or mPSCA Vaccine alone. TILS were extracted from tumors and MDSCs (CD11b+GR1+) and NK cells (CD11b+NK1.1+) were stained and phenotyped via flow cytometry.

For task 2.2, we explored the frequency of NK cells and MDSCs in TILs after Ad-LIGHT treatment followed by mPSCA vaccine utilizing the same TRAMP-C2 tumor challenge in C57Bl6 mice and treatment schedule reported in the Yan et al manuscript provided with this progress report. The results show a trend in Figure 7, an increased frequency of NK cells and MDSC’s are found in the tumor subsequently to mPSCA vaccination. Ad-LIGHT did not contribute to any additional infiltration of these cells. The infiltration of NK cells, also known as cytotoxic lymphocytes of the innate immune system play an important role in tumor killing and immunological control. The mPSCA vaccine shows great success in recruiting NK cells to the tumor microenvironment. However, the addition of LIGHT expression on prostate tumors does not affect theses frequencies. Interestingly, the frequency of MDSC’s increased with mPSCA vaccination alone. The addition of Ad-LIGHT or Ad-LIGHT alone does not alter the frequency of MDSC’s as compared to the untreated control. MDSC’s are early progenitor cells that are known to have suppressive functions in inhibiting NK, CD4+ and CD8+ T cells. Additionally, MDSC’s are known to induce the production of Arginases (Arg) that inhibits T cell proliferation and induces T cell apoptosis. Since Arg and other gene targets, including nitric oxide synthase (NOS) and indolamine (IDO) [20], may be expressed by activated MDSC’s, we began to explore these genes in tumors subsequent to treatment. The current data suggest that the mPSCA vaccine seems to induce favorable (NK cells) and unfavorable cells (MDSCs) while LIGHT does not affect these frequencies.
Figure 8. LIGHT alters the suppressive tumor microenvironment to a pro-inflammatory setting. Tumors were isolated from TRAMP-C2 challenged mice who were given either no treatment, Ad-Control, or Ad-LIGHT (treatment regimen provided in detail with attached Yan et al manuscript). Various gene targets were examined including LIGHT, NOS, IDO, and Arg2. (Two-way ANOVA, p<0.05)

We then isolated TRAMP-C2 tumors from untreated control, Ad-Control and Ad-LIGHT treated C57Bl6 mice and looked for various gene targets including LIGHT, NOS, IDO and Arg2 (Figure 8). As expected, LIGHT treated tumors show an increase in LIGHT expression as compared to Ad-Control and untreated control. NOS mRNA expression is elevated with Ad-LIGHT treatment. High level of NOS expression has been shown in literature to inhibit tumor growth and induce tumor apoptosis [21], whereas lower levels of NOS have been associated with promotion in tumor survival. Although LIGHT induces NOS expression, the reduction in IDO and Arg2 is not LIGHT specific but adeno-vector specific. IDO and Arg2 mRNA expression are lower in Ad-Control and Ad-LIGHT treated tumors as compared to untreated control. These genes are known to impair T cell responses and consequently induce T cell apoptosis [22-24]. These results suggest that reduced tumor burden occurs as a result of LIGHT altering the tumor microenvironment by increasing NOS expression.
SPECIFIC AIM 3: 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.

![Survival Surgery with Ad-Treatment](image)

**Figure 9.** A single dose of Ad-LIGHT via intraprostatic injections does not reduce tumor burden in TRAMP mice as compared to untreated and Ad-CTRL.

To address task 3.1, 10-12 week old TRAMP mice treated with Ad-LIGHT via intra prostatic injections are not significantly different than Ad-Control or Untreated mice when comparing its tumor burden, harvested at 20 weeks of age. These results indicate that the single treatment of Ad-LIGHT does not induce a strong antitumoral response to reduce tumor burden in autochthonous primary tumors (Figure 9). This suggest that a single dose of LIGHT may not be effective in inducing an immune response to eradicate the existing tumor. Potentially, the effects of LIGHT may have been diminished by the time tumor weights were measured since the length of time between treatment and harvest are approximately 8 weeks apart.

**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.

![Combined Ad-LIGHT and PSCA TriVax vaccination](image)

**Figure 10.** Combined Ad-LIGHT and PSCA TriVax vaccination shows an increasing trend in TAA specific T cells as compared to PSCA TriVax, Ad-LIGHT alone shows no TAA specific response. Specific group treatments are outlined in the attached manuscript. A. No significant difference is seen in the number of TAA-specific T cells within groups treated with PSCA TriVax alone or combined with Ad-LIGHT treatment (One-way ANOVA followed by Tukey’s multiple comparison p>0.05). B. No significant number of TAA specific T cells are generated by Ad-LIGHT or Ad-Control (One-way ANOVA, p>0.05).
Ad-LIGHT treatment alone in mice does not induce TAA specific T cells, and combination treatment of PSCA TriVax with Ad-LIGHT results in a increase in number of specific T cells that is trending towards significance (ns, p=0.165) (Figure 10 A and B). This result indicates the the mechanism of action for Ad-LIGHT is merely the recruitment of T cells into the tumor microenvironment and the reduction in functionality of Tregs. For assay examining potential induction of mPSCA-specific T cells C57Bl6 mice harboring ~300 mm³ TRAMP-C2 tumors were given 2 adenovirus treatments (2x10¹⁰ viral particles per treatment) spaced three days apart. Mice were euthanized 7 days after the last treatment, and analysis of mPSCA-specific splenic T cells was carried out. For specific details on PSCA Trivax treatments and ELISSPOT conditions, refer to manuscript included with submission (Yan et al).

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

During the time period of this grant our original collaborator, Alphavax Inc. went out of business due to lack of investor funds. Therefore, we explored other vaccination platforms that have been shown to induce an immune response towards a prostate-associated tumor antigen. We show in figure 11 that mPSCA synthetic peptide vaccine, mPSCA TriVax, containing mPSCA₈₃₋₉₁, anti-CD40 antibody and Poly-ICLC, improves survival and reduces tumor burden. After evaluating this alternative vaccination platform we were able to make progress into the effect of Ad-LIGHT and PSCA therapeutic vaccination on survival.

![Figure 11. Ad-LIGHT and mPSCA TriVax delays tumor growth and increases survival.](image)

Mice were first treated with two doses of Ad-LIGHT (or Ad-control) prior to receiving mPSCA TriVax. **A.** 2 weeks post treatment, animals who received Ad-LIGHT followed by mPSCA TriVax showed a delay in tumor growth. **B.** Tumor bearing mice treated with Ad-LIGHT followed by mPSCA TriVax had longer survival than mPSCA TriVax alone or untreated alone. Experiments were repeated once and representative data is shown (Two-way ANOVA on a single time-point, p<0.001)

Additionally, a synergistic effect was found in mice that received Ad-LIGHT followed by the mPSCA TriVax. Specifically, combination of these two reduces the tumor burden and increases survival of tumor bearing animals as compared to mPSCA TriVax alone. This approach is capable of reducing the tumor volume by more than 50% as compared to untreated control. The data suggests that Ad-LIGHT and mPSCA TriVax reduces the tumor burden and increases survival in tumor bearing animals.
**Task 3.4** Determine whether combined treatment of Ad-LIGHT followed by PSCA vaccination induces regression of metastatic tumors in mice challenged with TRAMP-C2 cells.

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<th>TRAMP-C2 re-Challenge 1x10^6 cells</th>
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**Table 2.** TRAMP-C2 challenged mice were treated as seen in the groups above. Tumors were resected 1 week post treatment and the mice were rechallenged with 1x10^6 cells 2 weeks post resection. The % tumor regrowth evaluates whether the rechallenged flank had tumor growth.

To address task 3.4, C56BL6 mice were first challenged with 5x10^5 TRAMP-C2 cells, normalized when tumor volumes were approximately 30 and treated with the appropriate vaccination scheme according to each group. Tumors were then resected 1-week post treatment and re-challenged 2-weeks post resection. Mice with resected tumors that grew back were censored in the study as full resection of primary tumor was unsuccessful. The induction of memory T cells plays an essential role in cancer-fighting properties. The idea of a successful vaccine provides an advantage to the immune system in mounting an immune response against invasive diseases and pathogens. Memory T cells plays a crucial role in orchestrating these immune responses, they have distinct activation and intracellular markers with a lower threshold and a diverse cytokine, profile against specific antigens [25-27]. Some studies show that in-situ tumor destruction of melanoma or fibrosarcomas (excision of the primary tumor), aid the immune system in mounting an anti-tumoral response against the tumor re-challenge [28, 29]. In this study, we show that the excision of the primary tumor did not protect against the tumor re-challenge (untreated group). However, we demonstrate that the vaccination with PSCA TriVax and subsequently the removal of the primary tumor, protected against the tumor re-challenge. This data suggest that PSCA TriVax induced tumor antigen specific immune response that were capable of mounting an immune response against TRAMP-C2 cells. In a translational sense, patients diagnosed with prostate cancer may opt for a prostatectomy in combination with a therapeutic vaccine that will further control the future progression of the malignant disease [30]. LIGHT was not capable of inducing memory against TRAMP tumors, although this was not surprising due to the lack of TAA specific T cells in Ad-LIGHT vaccinated mice as seen in figure 10. (Table 2).
**Task 3.5** Determine whether combined treatment of Ad-LIGHT followed by PSCA vaccination prevents the outgrowth of spontaneous metastatic tumors in TRAMP mice

![Graph showing tumor burden in different treatment groups](image)

**Figure 12.** Adenovirus synergistically reduces tumor burden in PSCA vaccinated TRAMP mice. LIGHT itself does not contribute to this effect (one-way ANOVA followed by Tukeys multiple comparison test, p<0.001).

For task 3.5, 10-week old TRAMP mice were vaccinated and treated with either Ad-LIGHT, Ad-Control or no treatment via intra-prostatic injections (Figure 12). The mice were then sacrificed and tumor weights were harvested at 20 weeks of age to evaluate tumor burden. The TRAMP model is widely used in the field of prostate cancer research, since the progression of disease mirrors a subset of patient cases. In this study, the vaccination of TRAMP mice with PSCA TriVax, Ad-Control and Ad-LIGHT alone did not improve the disease status. However, combining the PSCA TriVax with an adenovirus vector (either Ad-Control or Ad-LIGHT) induced an immunogenic response and resulted in a lower tumor burden as compared to single treatments, suggesting an adenovirus effect. Our results propose that the use of an immunogenic vaccine with an inflammatory response via intraprostatic injections may reduce tumor burden. The adenovirus effect parallels our results from the mRNA of IDO, NOS and Arg2 (From Task 2.2) where an adeno-inflammation effect was detected. (**p<0.001, one-way ANOVA).
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-\(\gamma\) releasing CD8+ cells.
- Intratumoral LIGHT expression results in a delay in tumor growth and extended survival.
- Ad-LIGHT induced a higher frequency of infiltrating effector T cells into the tumor, specifically CD8+CD3+ and CD4+CD3+ T cells, as compared to the untreated and Ad-Control populations.
- LIGHT interaction directly affects the induction of Tregs from a naïve CD4+ T cell population.
- mPSCA TriVax induces infiltration of NK and MDSCs, whereas LIGHT does not affect the frequency of these cells.
- The reduction in IDO and Arg2 is not LIGHT specific but adeno-vector specific.
- Ad-LIGHT and mPSCA TriVax reduces tumor burden and increases survival in tumor bearing animals.
- LIGHT treatment may contribute to reducing tumor burden by altering the tumor microenvironment by increasing NOS expression and compromising tumor immunosuppression via Tregs.
- The number of T cells is significantly higher in Ad-LIGHT treated tumors than Ad-Control or Untreated. Interestingly, the number of Tregs is not statistically different in each treatment group.
- PSCA TriVax induces TAA specific T cells, LIGHT aids in the recruitment of naïve T cells to the tumor microenvironment.
- PSCA TriVax induces memory T cells in a tumor re-challenge experiment.
- TRAMP mice have a reduced tumor burden when treated with PSCA TriVax and Ad-LIGHT or Ad-Control, indicating an adeno inflammation effect.
- Generated a TRAMP-FOXP3\(^{DTR}\) mouse model to study the effect of spontaneous prostate cancer development in the controllable absence of Tregs as an alternative to the proposed TRAMP-DEREG model.

REPORTABLE OUTCOMES

Manuscripts:


Conference Abstracts (See appendices for full text of submitted abstracts):

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

**Patents and licenses applied for and/or issued:**

None

**Degrees obtained:**


**Development of cells lines, tissue or serum repositories:**

None

**Database and animal models generated:**

TRAMP-FOXP3DTR mouse model: xmodel that spontaneously develops prostate cancer and can have its Treg cell population depleted by treatment with diphtheria toxin.

**Funding received based on work supported by this award:**

1. Grant PC140761 - Department of Defense – “LIGHT-ing up prostate cancer for immunotherapy.”

**Research opportunities applied for:**

None

**CONCLUSION**

Published data shows that in some tumor models, over-expressing LIGHT can induce tumor regression. However, the models have classically been 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. Specifically, we have demonstrated the effects of LIGHT upon tumor infiltrating lymphocytes and their ability in compromising the suppressive tumor microenvironment. Forced expression of LIGHT in tumors can prevent the differentiation of naïve CD4+ T cells into Tregs. We also demonstrate that LIGHT expression increases NOS expression which may contribute to the tumor growth delay and tumor apoptosis.

Our data shows the efficacy of combination treatment with LIGHT and mPSCA TriVax in reducing tumor burden and increasing survival, which suggests a future clinical impact for primary and possibly metastatic prostate cancer patients. Additionally, we provide evidence that the treatment of prostate tumors with LIGHT can synergize with a therapeutic cancer vaccine in enhancing the anti-tumor response through the recruitment of immune modulating T cells, while Tregs are not being recruited, resulting in a positive switch in the Teff/Treg balance. Interestingly, we found that LIGHT itself does not contribute to the induction of TAA specific T cells or the development of memory T cells. 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. Our work from this study establishes a foundation for usage of LIGHT as 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.

PERSONNEL

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REFERENCES

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+ IFNg-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.

Southern California Clinical and Translational Science Institute Oral Presentation- May 6, 2013

Forced LIGHT protein expression in TRAMP tumors induces prostate cancer specific immunity and increases survival.

Yan L; Da Silva D; Kanodia S; Verma B; Kast WM

Introduction: The ultimate goal of cancer immunotherapy is to stimulate the immune system to eradicate malignant tumors. One of the most common responses effective therapeutic vaccines elicit is tumor specific T cells; however, a suppressive tumor microenvironment counteracts the efficacy of these vaccines. We are in need of a therapeutic modality that will cure prostate cancer by activating immune response while eliminating tumor immunosuppression. In this study
we evaluate the ability of LIGHT (a ligand for lymphotoxin beta receptor and herpes virus entry mediator) to break self-tolerance against prostate cancer antigens while eliminating immunosuppressive modulators in the tumor microenvironment. In an HPV induced cervical cancer model, forced LIGHT expression induced naïve T cells recruitment into the tumor microenvironment, HPV-specific immunity and increased overall mice survival. Forced LIGHT expression has not been studied in a prostate cancer setting where tolerance to self-antigens exists; we hypothesize that forced LIGHT expression in murine prostate cancer will increase survival by inducing prostate cancer specific T cells and inhibiting suppressive T cell functionality.

**Methods:** We first performed an in-vitro experiment to evaluate the ability of murine prostate cancer cells to uptake adenovirus virus encoding LIGHT (Ad-LIGHT), a method to validate our delivery system of our ligand. LIGHT expression on cell surface was determined via flow cytometry and LIGHT mRNA was evaluated via quantitative real time PCR. A subsequent series of in-vivo mouse studies were then established to evaluate the effect of LIGHT in a therapeutic cancer setting. To assess LIGHT’s capacity to induce an immune response against prostate cancer, C57BL6 mice (n=10 per treatment group) were challenged with murine prostate cancer cells on day 0 and followed up to day 30 when their tumor volumes were approximately 100mm^3. Tumor volumes were normalized in each group prior to treatment to void tumor volume bias. Groups were assigned to either no treatment, adenovirus vector control or adenovirus LIGHT. We then analyzed for tumor specific T cells, infiltrating T cells, functionality of suppressive T cells, tumor growth status and survival post challenge and treatment.

**Results:** Murine prostate cancer cells took up Ad-LIGHT and highest levels of expression were detected within the first 48 hours, 11 fold increase compared to control. Forced LIGHT drew in effector T cells into the tumor microenvironment and inhibited the production or infiltration of suppressive T cells (t-test, p<0.05). It was also shown that the suppressive T cells that were present within the system had impaired suppressive capability subsequent to LIGHT treatment. LIGHT extended mean survival (t-test, p=0.0172) and induced prostate specific T cells (Log rank test, p<0.01).
Conclusion: Forced LIGHT treatment delays prostate cancer progression in cancer bearing mice by inhibiting tumor immunosuppression and inducing prostate cancer immune specificity. We propose LIGHT as strong candidate for single-therapy treatment in prostate cancer and for future experiments with combination therapies.