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14. ABSTRACT

Recent discoveries have demonstrated that the expression of LIGHT molecules within the tumor milieu counteracts cancer immune-evasion mechanisms and instigates activation and migration of T-cells into the tumor; however, the delivery of LIGHT to the tumor microenvironment has been a challenge. The overall goal of this project is to develop a targeted therapy that will deliver LIGHT to the tumor microenvironment by generating bispecific fusion proteins that are one-part LIGHT and one-part tumor-vasculature targeting antibodies (specifically targeting Delta-Like Ligand 4 (DLL4) and EphB4). In addition to LIGHT delivery, treatment with these antibodies alone has been shown to reduce tumor burden, which may enhance the effects of targeted LIGHT therapy. These bi-specific fusion proteins will be used, as proof-of-concept, to show that they lead to tumor eradication in both primary and advanced stage prostate cancer. In this annual report we summarize where we are in the production cycle and validation of these fusion proteins as well as provide a timeline for *in vivo* experiments.

15. SUBJECT TERMS
Regulatory T cells, prostate cancer, immunosuppression, tumor microenvironment, immunotherapy

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INTRODUCTION

Prostate cancer is the second most common cause of cancer related deaths in men, with approximately 32,000 deaths expected annually in the United States and 258,000 deaths expected annually worldwide (1). While treatments for primary prostate cancer patients exist, many lead to devastating side effects such as impotence or incontinence and there are limited options for patients with advanced disease (2, 3). Thus, there is an obvious need for new therapies and immunotherapy has been proposed as a solution. The ultimate goal of cancer immunotherapy is to stimulate the patient's immune system to eradicate malignant tumors. However, the use of cancer vaccines alone has had limited curative success. A highly vascularized tumor microenvironment being advantageous for tumor growth, lack of T cell homing to solid tumors and tumor-mediated immune suppression if the T cells do infiltrate are critical barriers to achieving complete tumor eradication (4). Delta-like Ligand 4 (DLL4) and EphB4 are two proteins involved in angiogenesis that are upregulated in tumor vasculature and tumors. Their expression in the tumor microenvironment allows specific targeting by antibodies or antibody fragments. Anti-DLL4 antibodies target the tumor vasculature and have been demonstrated to induce non-functional angiogenesis and in turn reduce tumor burden (5). Anti-EphB4 antibodies target EphB4 expressed on tumor cells and likewise mediate tumor cell death (6). LIGHT is a costimulatory molecule that we have recently shown inhibits regulatory T cell (Treg) mediated immunosuppression and synergizes with prostate cancer vaccines when expressed in mouse prostate tumors when delivered intra-tumorally (7). Bispecific fusion proteins that engage both the tumor and T cells are promising candidates for cancer therapy because they guide T cells to the tumor site and enhance anti-tumor immunity. Expression of LIGHT in the tumor microenvironment through DLL4 or EphB4 antibody targeting is a novel approach to modify the primary tumor microenvironment and any distant metastases. This translational project proposes the development of two novel bi-specific fusion proteins – a single chain variable fragment of a DLL4 antibody (scFv-DLL4) fused to LIGHT and a single chain variable fragment of EphB4 (scFv-EphB4) fused to LIGHT – which will permit targeting of LIGHT to highly vascularized and advanced prostate tumors. These fusion proteins are expected to result in LIGHT expression in tumors converting an immunosuppressive tumor microenvironment to an immunostimulatory environment. Combining this with an immunotherapeutic prostate cancer vaccine will result in tumor antigen associated (TAA) specific T cells being able to migrate to tumors and receive costimulation by LIGHT and avoid T cell inhibitory mechanisms (eg. Tregs).

KEYWORDS

- Prostate Cancer
- Immunotherapy
- Tumor Microenvironment
- LIGHT
- T regulatory cells
- Fusion proteins

ACCOMPLISHMENTS

Major goals of the project

Specific Aim 1: To produce and validate scFv-DLL4-LIGHT protein and scFv-EphB4-LIGHT protein for in vivo studies.			
Aim 1.1: Produce a sufficient amount of scFv-DLL4-LIGHT protein and validate product for in vivo studies.	**Months**	**PI**	**Status**
Task 1: Establish a stable CHO cell line for scFv-DLL4-LIGHT expression	1-3	Gill	*Revised*
Task 2: Produce and purify a large amount of scFv-DLL4-LIGHT for animal experiments	4-6	Gill	*Revised*
Aim 1.2: Design and produce a sufficient amount of scFv-EphB4-LIGHT protein and validate product for in vivo studies.			
Task 1: Design and clone the scFv-EphB4-LIGHT plasmid construct	1-2	Kast	*Complete*
Task 2: Establish a stable CHO cell line for scFv-EphB4-LIGHT expression	4-6	Gill	*Revised*
Task 3: Produce and purify a large amount of scFv-EphB4-LIGHT for animal experiments	4-6	Gill	*Revised*
Milestone(s) Achieved: Production of two bi-specific fusion proteins that may target tumor vasculature and activate/recruit T cells.			*Revised*

Specific Aim 2: To determine the efficacy of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in TRAMP-C2 tumor bearing mice.			
Aim 2.1: Determine the dosage and duration of treatment for both scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in TRAMP-C2 challenged mice via tumor growth and survival.			
Task 1: Obtain IACUC/ACURO approval for planned mouse experiments	1-4	Kast	*Complete*
Task 2: Optimization of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT treatment schedule determined by survival and tumor burden.	6-12	Kast	*Revised*
Aim 2.2: Assess immunological mechanisms of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT treated TRAMP-C2 challenged mice and elect the more efficacious treatment.			
Task 3: Induction of tumor specific T cells against Tumor Associated Antigens.	13-17	Kast	*Revised*
Task 4: Isolation, frequency and phenotype of tumor-infiltrating cells (Th1, Th2, Tregs, NK, Th17, MDSC, macrophages).	17-20	Kast	*Revised*
Task 5: Compare the intra-tumoral cytokine and chemokine profile following treatment	17-20	Kast	*Revised*
Task 6: Evaluate Treg functionality post treatment.	17-20	Kast	*Revised*
Aim 2.3: Determine whether scFv-DLL4-LIGHT <u>or</u> scFv-EphB4-LIGHT treatment in combination with therapeutic prostate cancer vaccine can induce complete regression in TRAMP-C2 challenged mice.			
Task 7: Determine the most optimal vaccination scheme that will result in complete tumor control after combination treatment in TRAMP-C2 challenged mice.	20-24	Kast	*Revised*
Milestone(s) Achieved: IACUC/ACURO approval. Identification of the optimal dosage and duration, immunological mechanisms of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in the TRAMP-C2 challenged setting. The more efficacious fusion protein (based on survival, tumor burden and immunological mechanisms) will be elected for further testing in combination treatments.			*Revised*

Specific Aim 3: Determine whether scFv-DLL4-LIGHT or scFv-EphB4-LIGHT treatment in combination with a therapeutic prostate cancer vaccine can induce complete regression of primary and advanced prostate tumors in the TRAMP model.			
Aim 3.1: Determine survival and tumor burden of primary prostate tumors in the TRAMP model after combination treatment.			
Task 1: Examine survival and tumor burden in combination treatment with MPSP TriVax and the elected bi-functional protein in primary prostate cancer (determined by Aim 2).	24-31	Kast	*Revised*
Aim 3.2: Determine survival and tumor burden of primary prostate tumors in the TRAMP model after combination treatment.			
Task 2: Examine survival and tumor burden in combination treatment with MPSP TriVax and the elected bi-functional protein in advanced prostate cancer (determined by Aim 2).	31-36	Kast	*Revised*
Task 3: Prepare and submit manuscript for publication.	35-36	Kast/ Gill	*Revised*
Milestone(s) Achieved: Efficacy of the elected bi-functional protein in combination with MPSP TriVax will be determined in primary and advanced disease. Manuscript submitted to high impact journal.			*Revised*

Accomplished goals during reporting period:

Applicable to all aims of the study:

1). Small amounts of scFv-EphB4-LIGHT and scFv-DLL4-LIGHT have been produced, however the quantity was not applicable for *in vivo* applications. Collaborator (Dr. Gill) was unable to scale up production of the fusion proteins.

2). New constructs have been developed to hone LIGHT to target tumor vasculature as explained in justifications section (figure 1).

Figure 1: Visual representation of LIGHT-VTP protein sequence. DNA sequences from left to right include: 6x histidine tag, tobacco etch protease site, LIGHT, 3x glycine linker, and vascular targeting peptide.

3). The constructs were both generated and cloned into a PET28a vector and delivered to Stbl3 and BL21 bacteria.

4). Transformation of BL21 *E. coli* with construct followed by IPTG induction and LIGHT-VTP production verification is underway. We have been able to identify protein production of a 6xHis-containing protein at the size we expect via western analysis of bacterial lysates (figure 2).

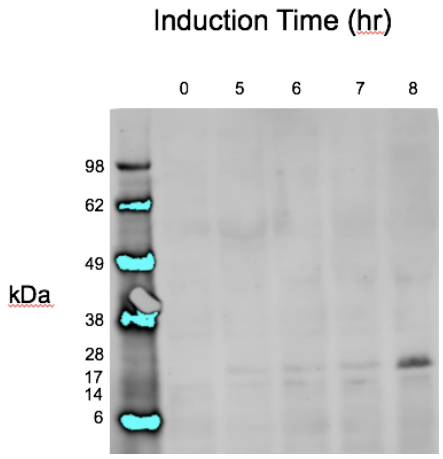

Figure 2: Western analysis of bacterial lysates probing for 6x poly-histidine sequence. LIGHT-VTPa size estimated to be 19kDa. Seeblue Plus 2 ladder used in 1x MES buffer.

Opportunities for training and professional development:

Nothing to report

How were the results disseminated to communities of interest:

Nothing to report

What do we plan on doing to accomplish our goals during the next reporting period:

IMPACT

Impact on the development of the principal discipline of the project:

Nothing to report

Impact on other disciplines:

Nothing to report

Impact on technology transfer:

Nothing to report

Impact on society beyond science and technology:

Nothing to report

CHANGES/PROBLEMS

Changes in approach and reasons for change:

The primary purpose of our grant was to deliver LIGHT protein into prostate tumors through LIGHT-DLL4 and LIGHT-EphB4 fusion proteins. However, our collaborator (Dr. Gill) was unable to generate sufficient amount of these fusion proteins in mammalian culture systems.

Because of this, we are adjusting our LIGHT fusion protein constructs to match ones that were used by Johansson-Percival et al. (9). That group learned from our published work that expressing LIGHT in prostate tumors resulted in T cell infiltration, loss of T regs, and induced a significant therapeutic effect (7). That group then designed and produced LIGHT fused to two separate vascular targeting peptides (VTP) in order to overcome in vivo delivery problems as we had also aimed for in our original proposal. They showed that when injected intravenously, the LIGHT-VTP proteins were able to induce T cell infiltration, formed intra-tumoral tertiary lymphoid structures, normalized tumor vasculature, and increased lifespan in pancreatic tumor models, just as we had hypothesized would be the case in our prostate cancer model. We now plan to utilize LIGHT

fused to the two VTP sequences (which we will refer to as VTPa and VTPb) to test their treatment efficacy against our prostate tumor model.

Both VTPs can specifically bind tumor vasculature. VTPa is a 5-amino acid sequence consisting of CGKRK and has the ability to bind tumor endothelial cells and VEGF positive extracellular matrices (10). VTPb is a 7-amino acid sequence consisting of CRGRRST that has the ability to bind to platelet derived growth factor receptor-β (PDGFR-β), which is found on angiogenic pericytes and endothelial cells (11,12). Because VTPa and VTPb have the ability to exclusively bind to tumor angiogenic tissue, we hypothesize that attaching VTPa and VTPb onto the LIGHT C-terminus will induce an immunogenic response to TRAMP/C2 prostate tumor models.

As these two LIGHT-VTP fusion proteins have successfully been produced in a bacterial expression system as shown in the literature (9), we are confident that we can produce sufficient protein for in vivo use and therefore created a revised statement of work proposal with an accelerated timeline so that the project can be carried out within the no cost extension period (see SOW, table below).

Specific Aim 1: To produce and validate LIGHT-VTPa protein and LIGHT-VTPb protein for in vivo studies.			
Aim 1.1: Produce a sufficient amount of LIGHT-VTPa protein and validate product for in vivo studies.			
Task 1: Design and clone the LIGHT-VTPa plasmid construct	1-1	Kast	*Complete*
Task 2: Establish competent Stbl3 and BL21 *E. coli* stocks for LIGHT-VTPa expression	1-2	Kast	*Complete*
Task 3: Produce and purify a large amount of LIGHT-VTPa for animal experiments	2-2	Kast	*Ongoing*
Aim 1.2: Design and produce a sufficient amount of LIGHT-VTPb protein and validate product for in vivo studies.			
Task 1: Design and clone the LIGHT-VTPb plasmid construct	1-1	Kast	*Complete*
Task 2: Establish competent Stbl3 and BL21 *E. coli* stocks for LIGHT-VTPb expression	1-2	Kast	*Complete*
Task 3: Produce and purify a large amount of LIGHT-VTPb for animal experiments	2-2	Kast	*Ongoing*
Milestone(s) Achieved: Production of LIGHT proteins that may target tumor vasculature and activate/recruit T cells.			

Specific Aim 2: To determine the efficacy of LIGHT-VTPa and LIGHT-VTPb in TRAMP-C2 tumor bearing mice.			
Aim 2.1: Determine the dosage and duration of treatment for both LIGHT-VTPa and LIGHT-VTPb in TRAMP-C2 challenged mice via tumor growth and survival.			
Task 1: Submit ACURO approval for planned mouse experiments. IACUC has already been approved.	1-2	Kast	*Pending*
Task 2: Optimization of LIGHT-VTPa and LIGHT-VTPb treatment schedule determined by survival and tumor burden. • Challenge C56BL/6 mice with TRAMP-C2 cells. • Treat animals with either negative control (no treatment), mouse recombinant LIGHT, LIGHT-VTPa, or LIGHT-VTPb in tumor bearing mice. LIGHT-VTP dosages will range from 20ng to 2,000ng as established by Johansson-Percival et al. • Measure tumor volume and survival. • Optimized LIGHT-VTPa and LIGHT-VTPb dosages will be utilized for subsequent tasks and aims. Mouse Strain: C57BL/6 Cell line: TRAMP-C2 cells/Kast Lab Total number of Animals required: n=12 /group, 16 groups with controls. **96** C57BL/6 mice per experiment. (**192** C57BL/6 mice required for 2 studies)	2-5	Kast	*Pending*
Aim 2.2: Assess immunological mechanisms of LIGHT-VTPa and LIGHT-VTPb treated TRAMP-C2 challenged mice and elect the more efficacious treatment.			
Task 3: Induction of tumor specific T cells against Tumor Associated Antigens. • Challenge C57BL/6 mice with TRAMP-C2 tumors. • Treat animals with either negative control (no treatment), mouse recombinant LIGHT, LIGHT-VTPa, or LIGHT-VTPb. • Harvest tumors and lymphoid organs to assess TAA specific T cells via ELISpot assay. Mouse Strain: C57BL/6 Cell line: TRAMP-C2	5-7	Kast	*Pending*

Total number of animals required: : n=12 /group, 6 groups with controls. **77 (72 for treatment groups + 5 naïve mice for Treg Suppression Assay)** C57BL/6 mice per experiment. (**154** C57BL/6 mice required for 2 experiments) *****Animals numbers listed here are used for Aim 2 Task 3-Task 5; different organs will be harvested for different tasks.			
Task 4: Isolation, frequency and phenotype of tumor-infiltrating cells (Th1, Th2, Tregs, NK, Th17, MDSC, macrophages). • Challenge C57BL/6 mice with TRAMP-C2 tumors. • Treat animals with either negative control (no treatment), recombinant mouse LIGHT, LIGHT-VTPa, or LIGHT-VTPb. • Harvest tumors and lymphoid organs. • Immune modulatory cells will be analyzed via flow cytometry for markers typically expressed by Th1 effectors (CXCR3, CCR5, IL-12Rβ2 and IL-18R), Th2 effectors (T1/ST2, CCR3, CCR4, CCR8 and CRTh2), Tregs (CD25, CTLA4, CD103, CD127 and *Foxp3*), MDSC (CD11b, GR-1), granulocytic and monocytic (CD11b, GR-1, Ly6C, Ly6G), NK cells (CD122, CD27, $CD11b^{hi}$, $CD43^{hi}$, $DX5^{hi}$) and Th17 pro-inflammatory cells (IL-12Rb2, IL-23R, IL-17, IL-22, IL-2 and TNFα.	5-7	Kast	*Pending*
Task 5: Compare the intra-tumoral cytokine and chemokine profile following treatment • Challenge C57BL/6 mice with TRAMP-C2 tumors. • Treat animals with either negative control (no treatment,) recombinant mouse LIGHT, LIGHT-VTPa, or LIGHT-VTPb. • Harvest tumors and measure intra-tumoral cytokines/chemokines via multiplex assay.	5-7	Kast	*Pending*
Task 6: Evaluate Treg functionality post treatment. • Challenge C57BL/6 mice with TRAMP-C2 tumors. • Treat animals with either negative control (no treatment), recombinant mouse LIGHT, LIGHT-VTPa, or LIGHT-VTPb. • Harvest lymph nodes and measure proliferation of T-responder cells.	5-7	Kast	*Pending*
Aim 2.3: Determine whether LIGHT-VTPa or LIGHT-VTPb treatment in combination with therapeutic prostate cancer vaccine MPSP Trivax can induce complete regression in TRAMP-C2 challenged mice.			
Task 7: Determine optimal vaccination scheme that will result in complete tumor control after combination treatment in TRAMP-C2 challenged mice. • Challenge C57BL/6 mice with TRAMP-C2 tumors. • Treat animals with either negative control (no treatment), MPSP Trivax, recombinant mouse LIGHT, LIGHT-VTPa, LIGHT-VTPb, or MPSP Trivax in combination. • Monitor tumor growth and survival. Mouse Strain: C57BL/6 Cell line: TRAMP-C2 cells Total number of Animals required: n=12 /group, 8 groups with controls. **96** C57BL/6 mice per experiment. (**192** C57BL/6 mice required for 2 experiments)	7-11	Kast	*Pending*
Milestone(s) Achieved: IACUC/ACURO approval. Identification of the optimal dosage and duration, immunological mechanisms of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in the TRAMP-C2 challenged			

setting. The more efficacious fusion protein (based on survival, tumor burden and immunological mechanisms) will be elected for further testing in combination treatments.			
Task 3: Prepare and submit manuscript for publication	10-11	Kast	*Pending*

Actual or anticipated problems or delays and actions or plans to resolve them:

To prevent issues with LIGHT-VTPa or b production we have ordered these fusion proteins from Thermo Fischer to ensure we have enough protein for *in vivo* experiments by December of this year.

Changes that had a significant impact on expenditures:

Nothing to report

Significant changes in use or care of vertebrate animals, biohazards, or other agents:

Nothing to report

PRODUCTS

Publications:

1. García-Hernándes ML, Uribe-Uribe NO, Espinosa-González R, Kast WM, Khader SA, Rangel-Moreno J. A Unique Cellular and Molecular Microenvironment is Present in Tertiary Lymphoid Organs of Patients with Spontaneous Prostate Cancer Regression. Front Immunology. 2017 17; 8:563.

Websites or other Internet Sites:

Nothing to report

Technologies or techniques:

Nothing to report

Inventions, patent applications, and/or licenses:

Nothing to report

Other products:

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals that have worked on the project:

Name	W. Martin Kast
Project role	Principal Investigator
eRA Commons	wmkast
Nearest person month worked	1
Contribution	Oversaw and provided guidance in project progression.
Funding Support	N/A

Name	Mikk Otsmaa

Project role	Graduate Research Assistant
eRA Commons	N/A
Nearest person month worked	4
Contribution	Design and clone bacterial construct and optimized E. coli expression and purification of LIGHT-VTP constructs.
Funding Support	N/A

Name	Parkash Gill
Project role	Co-I
eRA Commons	parkashg
Nearest person month worked	1
Contribution	Responsible for generating stable protein production cell lines and protein production of the LIGHT-DLL4 and LIGHT-EphB4 fusion proteins
Funding Support	N/A

Changes in active other support of the PIs or senior/key personnel since start of grant award:

Nothing to report

Other organizations that are involved:

Nothing to report

SPECIAL REPORTING REQUIREMENTS

Nothing to report

APPENDICES

References:

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