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14. ABSTRACT

GT103 is an anti-complement factor H (CFH) mAb that kills tumor cells by complement dependent cytotoxicity (CDC), and inhibits the growth of tumors while stimulating an immune response. Our overall project objectives are to determine how GT103 causes tumor growth inhibition in mice and to define a cancer patient population who will best respond to GT103 as a cancer treatment. Our Specific Aims are: 1. Investigate the in vivo impacts of GT103 treatment on innate immune pathways; 2. Investigate antigen presentation and adaptive immunity in GT103 cancer immunotherapy; 3. Define a cancer patient population most amenable to GT103 treatment. In this first year of the grant, we have focused on Aims 1 and 2. We found that GT103 induces CDC by both the alternative and classical complement pathways, which suggests that GT103 does not only block CFH but may form immune complexes that could engage immune cells. In CMT167 subcutaneous lung tumor-bearing C57BL/6 mice, GT103 reduces the number of intratumoral immunosuppressive T regulatory cells and myeloid-derived suppressor cells and increases the number of intratumoral immune-modulatory dendritic cells over control. We discovered that multiple gene expression pathways were upregulated in the tumors of GT103 treated tumor bearing mice, including those for B-cell receptor signaling, regulation of cell-cell adhesion, leukocyte chemotaxis, regulation of Interleukin-1β production, leukocyte migration, regulation of IFNγ production, regulation of cell activation, and cell chemotaxis. In addition, we found that in the B16F10 melanoma model, GT103 and anti-PD-1 acted synergistically to suppress tumor growth. Future work will focus on integrating these findings with a focus on B cell- and T cell-mediated mechanisms of adaptive immunity, as well as beginning to study human correlates to our findings in mice.

15. SUBJECT TERMS
immunotherapy; non-small cell lung cancer; complement

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TABLE OF CONTENTS

1. INTRODUCTION: We previously reported that patients with non-recurrent stage I non-small cell lung cancer (NSCLC) had a significantly higher incidence of an antibody to complement factor H (CFH) than those with late-stage NSCLC, and had no side-effects attributable to the antibody (1). CFH is a regulatory protein that protects host cells, including tumor cells, from attack and destruction by the alternative pathway of complement dependent cytotoxicity (CDC). Since complement is a bridge between innate and adaptive immunity, we proposed that administering a tumor-specific CFH antibody would activate the complement system, cause CDC of tumor cells, and produce a robust adaptive immune response for long-term anti-tumor activity (2). Starting from human B cells from NSCLC patients expressing the antibody, we cloned a human anti-CFH mAb, GT103, that recognizes the same epitope recognized by the patient antibodies (3). GT103 triggers CDC of lung tumor cells, and inhibits tumor growth with lymphocytic infiltration of tumors suggestive of an adaptive immune response. GT103 has recently entered a Phase Ib clinical trial for NSCLC. The purpose of this grant is to more precisely define the immune response induced by this anti-CFH mAb. With this understanding, we can better interpret clinical results, design co-therapies with other agents, and develop mechanistic and predictive biomarkers of efficacy. The Specific Aims of the proposal are 1. Investigate the *in vivo* impacts of CFH mAb treatment on innate immune pathways; 2. Investigate the role of cellular immunity in CFH mAb cancer immunotherapy; and 3. Define a cancer patient population most amenable to treatment with a CFH mAb.

1. Amornsiripanitch N, Hong S, Campa MJ, Frank MM, Gottlin EB, Patz EF, Jr. Complement factor H autoantibodies are associated with early stage NSCLC. Clin Cancer Res. 2010;16(12):3226-31. Epub 2010/06/03. doi: 10.1158/1078-0432.CCR-10-0321. PubMed PMID: 20515868; PMCID: PMC2891404.

2. Bushey RT, Moody MA, Nicely NL, Haynes BF, Alam SM, Keir ST, Bentley RC, Roy Choudhury K, Gottlin EB, Campa MJ, Liao HX, Patz EF, Jr. A Therapeutic Antibody for Cancer, Derived from Single Human B Cells. Cell Rep. 2016;15(7):1505-13. doi: 10.1016/j.celrep.2016.04.038. PubMed PMID: 27160908; PMCID: PMC4871760.

3. Campa MJ, Gottlin EB, Bushey RT, Patz EF, Jr. Complement Factor H Antibodies from Lung Cancer Patients Induce Complement-Dependent Lysis of Tumor Cells, Suggesting a Novel Immunotherapeutic Strategy. Cancer Immunol Res. 2015;3(12):1325-32. Epub 2015/07/29. doi: 10.1158/2326-6066.CIR-15-0122. PubMed PMID: 26216416.

2. KEYWORDS: immunotherapy; non-small cell lung cancer; complement

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Milestone month/year are in parentheses.

Specific Aim 1: Investigate the *in vivo* impacts on CFH mAb treatment on innate immune pathways.

Major Task 1: Sub-Aim 1A. Determine the relevant complement activation pathway for CFH mAb action. (02/20) Completed as of 09/19.

Major Task 2: Sub-Aim 1B. Determine the ability of the CFH mAb to activate innate immunity locally and systemically. (11/19) In progress.

Major Task 3: Sub-Aim 1C. Investigate modulation of innate cellular immune response by the CFH mAb. (02/20) Completed as of 12/19 in the CMT167 lung tumor model.

Major Task 4: Sub-Aim 1D. Determine levels of tumor leukocytes, systemic innate immune status of lung cancer patients with or without CFH Abs. (10/20) At Duke, we continued to identify/collect the appropriate specimens in order to address Sub-Aims 1D and 2C. The Durham VA IRB has recently approved Dr. Shofer's protocol, but collection of specimens is currently on hold due to Covid-19.

Specific Aim 2: Investigate the role of cellular immunity in CFH mAb cancer immunotherapy.

Major Task 5: Sub-Aim 2A. Determine whether CFH mAb inhibition of tumor growth *in vivo* requires T lymphocytes. (12/20) Partially completed–we are will be continuing our investigation of T cells in Year 2.

Major Task 6: Sub-Aim 2B. Determine whether CFH mAb treatment induces lasting anti-tumor T cell immunity *in vivo*. (09/21) Not addressed during this period; scheduled for Year 2.

Major Task 7: Sub-Aim 2C. Characterize T lymphocyte population in tumor tissues of NSCLC patients with or without CFH antibodies. (11/20) Not addressed during this period.

Specific Aim 3: Define a cancer patient population most amenable to therapeutic intervention with a CFH antibody.

Major Task 8. Perform assays for eight variables. (05/21)

Major Task 9. Establish statistical correlation of assay variables and outcome. (07/21)

Neither Major Task in Specific Aim 3 was addressed during this period.

Specific Aim 4: Determine if CFH-containing exosomes can inhibit metastasis.

This is a new Specific Aim added June 2020, with DOD approval.

Major Task 10. Perform exosome-mediated metastasis experiments in the CMT167 (WT vs CFH knockout)-C57BL/6 model. Scheduled for Year 2.

What was accomplished under these goals?

1) Major Activities

In the first quarter, we explored whether the alternative or classical complement pathways is the major contributor towards GT103-mediated CDC, using *in vitro* techniques.

In the second quarter, using the syngeneic CMT167-C57BL/6 subcutaneous lung tumor model, we identified cell types involved in the innate and adaptive immune responses after treatment of tumor-bearing mice with GT103. The most notable findings were decreased levels of T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs) and increased levels of dendritic cells (DCs) in the tumors of GT103-treated mice.

In the third quarter, using RNA-seq and gene ontology profiling of tumors, we identified gene expression pathways associated with tumor growth inhibition, notably B-cell receptor signaling, leukocyte migration and chemotaxis pathways, cell-cell adhesion, and cytokine production. We also obtained preliminary evidence that GT103 and anti-PD-1 act synergistically to inhibit tumor growth in the syngeneic B16F10-OVA-C57BL/6 model.

In the fourth quarter, Duke University research was shut down in April and May due to Covid-19. However, we were able to validate the genes identified by RNA-seq as upregulated by GT103 using real time PCR; we have explored the role of B cells in GT103-mediated growth inhibition; and we demonstrated no synergy between GT103 and anti-PD-1 in the CMT167-C57BL/6 subcutaneous lung tumor model.

2) Specific Objectives

Specific Aim 1 is to investigate the *in vivo* impacts on anti-CFH antibody treatment on innate immune pathways.

This aim is divided into an investigation of the complement pathways impacted by the antibody, and an exploration of the innate immune response to the antibody, both within the tumor and systemically. The innate immune response to GT103 vs. control in tumor bearing mice will be compared to that of NSCLC patients who have vs. do not have an anti-CFH autoantibody.

Specific Aim 2 is to investigate the role of cellular immunity in CFH mAb cancer immunotherapy. The focus of this aim is adaptive immunity.

Questions to be addressed are whether GT103 inhibition of tumor growth *in vivo* requires T lymphocytes, and whether CFH mAb treatment induces lasting anti-tumor T cell immunity *in vivo*. Because we discovered that genes involved in B lymphocyte activation are differentially expressed in tumors of mice treated with GT103, we are also focusing on the role of B lymphocytes in tumor growth inhibition. A parallel goal is to characterize the T lymphocyte population in tumor tissues of NSCLC patients with or without CFH antibodies.

Specific Aim 3 is to define a cancer patient population most amenable to therapeutic intervention with a CFH antibody by exploring biomarkers that correlate with the presence of the CFH autoantibody and outcome.

3) Significant Results

Specific Aim 1: Investigate the *in vivo* impacts on CFH mAb treatment on innate immune pathways.

Major Task 1: Sub-Aim 1A. Determine the relevant complement activation pathway for CFH mAb action. (Site 1, Dr. Patz)

Understanding the complement activation pathway that results from GT103 binding to its target is important for understanding the downstream immunological consequences of this event. Both the alternative and classical pathways result in CDC of cells and release of anaphylatoxins. If the classical pathway is the predominant pathway for CDC, this would also indicate that GT103 is capable of forming immune complexes with its target. Immune complexes, in addition to binding C1q to trigger the classical pathway, also activate immune cells, such as DCs, macrophages, and B lymphocytes through Fc-Fc receptor interaction.

Although *in vivo* experiments were originally planned, two *in vitro* experiments were performed that adequately addressed this issue. The first experiment uses a GT103 Fab construct. Lacking the Fc fragment, the Fab construct would only be able to carry out the blocking function of the CFH mAb, initiating the alternative pathway. The presence of the Fc fragment is necessary in order to bind C1q, initiating the classical pathway. Therefore, if CDC induced by the Fab is equivalent to that induced by the full mAb, the classical pathway is irrelevant to the mechanism of the antibody. If CDC is reduced in extent, or not induced by the Fab, we would conclude that the classical pathway plays a major role in the mechanism of the antibody.

To test these hypotheses, A549 cells were mixed with normal human serum and either full length GT103, the Fab construct, or IgG, and CDC was measured. **Figure 1** shows the results of this experiment. CDC in the presence of the Fab was reduced almost to the level of the negative control IgG. This result indicates that the classical pathway makes a strong contribution to the cytotoxicity triggered by GT103.

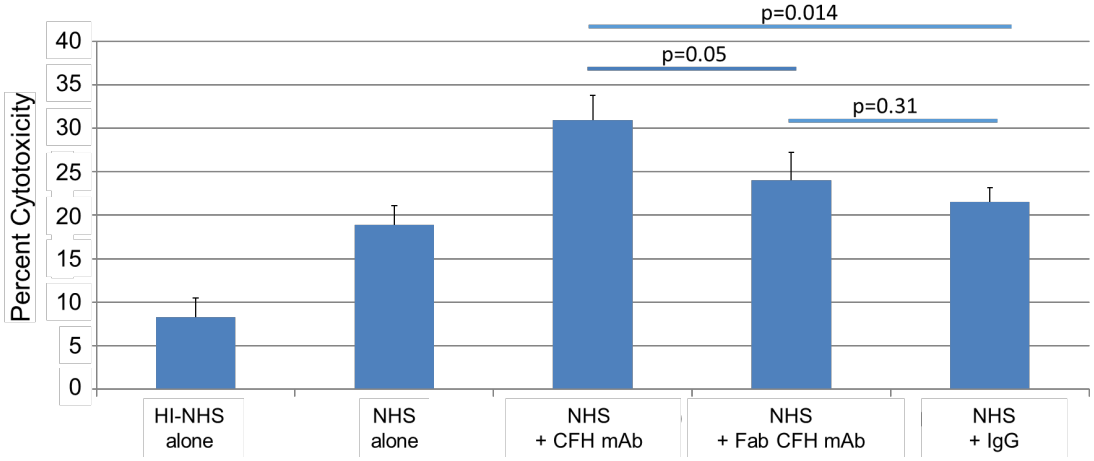


Figure 1. CDC in the presence of full length vs. Fab CFH mAb. A549 human lung cancer cells were incubated with normal human serum (NHS) as a source of complement alone or with GT103 (CFH mAb), Fab CFH mAb, or IgG. Heat inactivated (HI) NHS served as a negative control. After 24 hrs, lysis was measured by lactate dehydrogenase (LDH) release and expressed as percent cytotoxicity. The experiment was performed in triplicate and performed twice with similar results.

In the second experiment, we performed CDC experiments in the presence of normal human serum depleted for different complement factors. Factor B is cleaved to Bb which joins with C3b to form the alternative pathway convertase. If the alternative pathway is triggered by GT103, CDC would be impaired in Factor B-depleted serum. C1q initiates the classical pathway by binding to the Fc region of antibodies in immune complexes. As a consequence, C4 is cleaved to C4a which joins with C2a to form the classical pathway convertase. If the classical pathway is triggered by GT103, CDC would be impaired in C1q- or C4-depleted sera.

Figure 2 shows the result of this experiment. Although there was a small reduction in CDC of lung tumor cells in the presence of Factor B-depleted serum (10%), indicating some contribution of the alternative pathway to the GT103-mediated CDC, there were larger reductions in CDC with both C1q- and C4-depleted sera (33% and 49%, respectively). This result, along with the result of the first experiment, indicates that the classical pathway plays the major role in effecting CDC mediated by GT103.

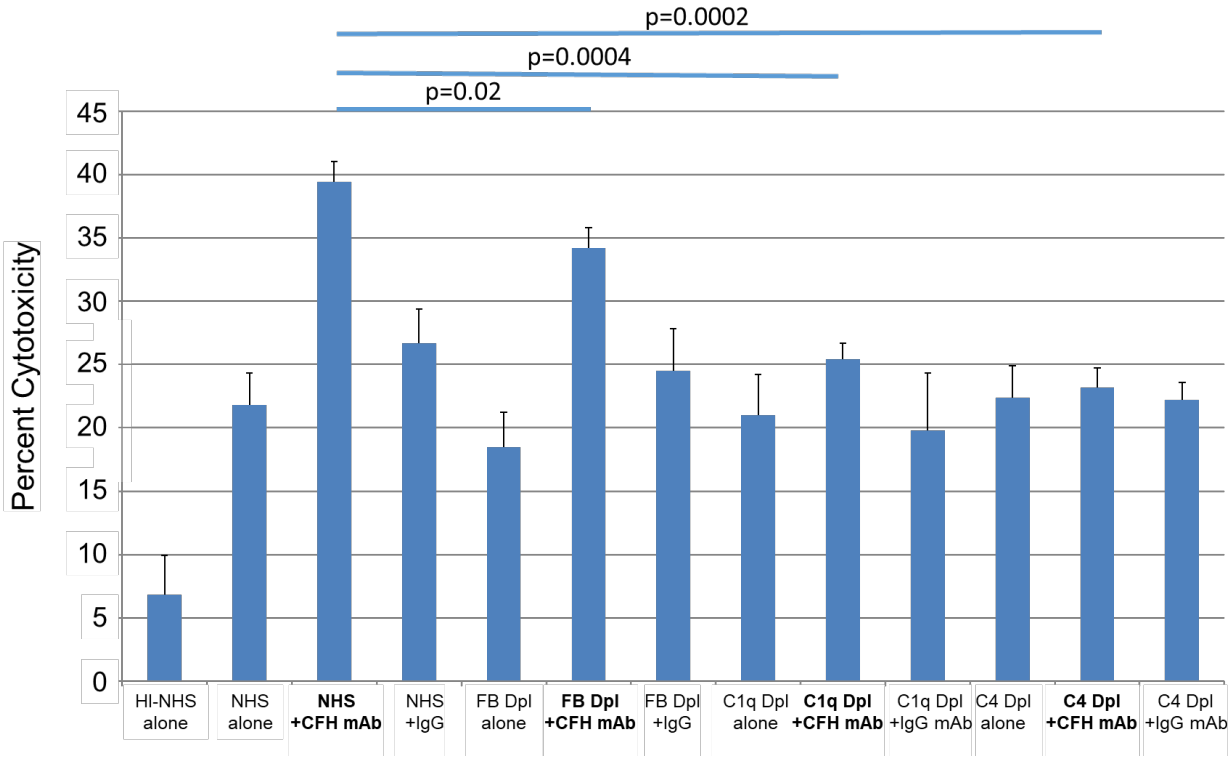


Figure 2. CDC in the presence of normal or complement depleted sera. A549 human lung cancer cells were incubated with GT103 (CFH mAb) or IgG negative control, plus intact NHS, or Factor B (FB), C1q, or C4-depleted (Dpl) serum. After 24 hrs, lysis was measured by LDH release and expressed as percent cytotoxicity. The experiment was performed in triplicate and performed twice with similar results.

Major Task 2: Sub-Aim 1B. Determine the ability of the CFH mAb to activate innate immunity locally and systemically. (Site 2, Dr. He) Tumors and sera of CMT167 tumor-bearing, GT103 or PBS-treated mice were used to explore the ability of GT103 to modulate changes in *both* innate and adaptive immunity. Six to eight week old C57BL/6 mice were implanted subcutaneously with 1.0×10^5 CMT167 cells in the right flank. One week after inoculation, mice were randomly grouped with 6 mice per group and treated for 3 weeks with vehicle (twice weekly) or GT103 (200 µg/mouse IP twice weekly). Tumors were measured bidimensionally every other day and tumor volume was calculated using the formula V = (smallest diameter)^2 x the largest diameter /2. After the mice were sacrificed, tumors were excised, weighed and portion of the tissue was stored in RNAlater (ThermoFisher) for cytokine expression analysis by RNA sequencing (RNA-seq). Another portion of the excised tumors was used to prepare single cell suspensions for flow cytometry. GT103 significantly inhibited tumor growth (p=0.008) (**Figure 3**); tumor weights were lower in the GT103-treated mice but this did not reach statistical significance (**Figure 4**). (Note that tumor weight reflects not only tumor cells but tumor cells and stromal cells combined.)

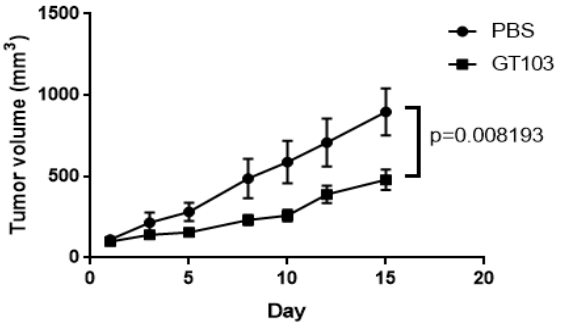


Figure 3. Effect of GT103 on the growth of syngeneic CMT167 tumors. Tumor growth was plotted as mean ± SEM for N=6 for each treatment group.

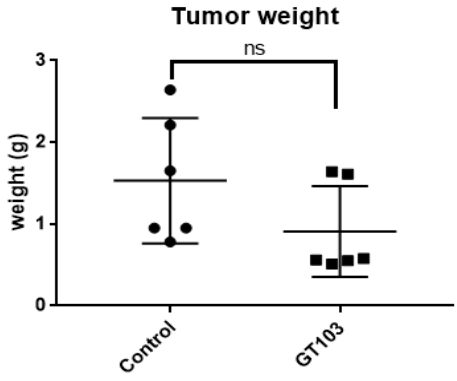


Figure 4. Final CMT167 tumor weights. Final weights of tumors excised from sacrificed mice in the experiment of Figure 3 are plotted.

Analysis of tumors. Gene ontology analysis of RNA-seq data highlighted the B-cell receptor signaling, regulation of cell-cell adhesion, leukocyte chemotaxis, regulation of Interleukin-1β production, leukocyte migration, regulation of IFNγ production, regulation of cell activation, and cell chemotaxis as pathways relevant to the intratumoral response to GT103. The most significant differentially up-regulated genes, are summarized in **Table 1**. Down-regulated genes were largely involved in metabolic processes.

All of the genes identified as up-regulated in GT103-treated tumors by RNA-seq were confirmed as differentially expressed by real time RT-PCR **(Figure 5)**.

Analysis of sera. Serum was used to probe mouse cytokine arrays (RayBiotech C1000 arrays C3 and C4). A receptor tyrosine kinase also on this array, Axl, was downregulated and 7 cytokines were upregulated as summarized in **Table 2**. Axl downregulation is significant as it functions in inhibition of Toll-like receptors on innate immune cells; therefore, this inhibition is expected to be relieved in GT103-treated mice.

Table 1. Genes that are significantly uprated in tumors after GT103 treatment

Ccl17	Important roles in Th2-type immune responses
Ffar2	Involved in the inflammatory response
Il2ra	T and B cell activation
Ctla4	Inhibitory receptor acting as a major negative regulator of T-cell responses.
Cebpg	B cell differentiation and immune response
Efnb3	T cell costimulation and cell-cell adhesion
Ccl22	Trafficking of activated/effector T-lymphocytes to inflammatory sites
Hsph1	Regulates MHC-I biosynthesis and NK-T cell activation
Slc39a10	B cell proliferation and BCR signaling
Src	Gene transcription, immune response, cell adhesion, cell cycle progression, apoptosis, migration, and transformation
Itga3	Cell matrix adhesion, migration and invasion

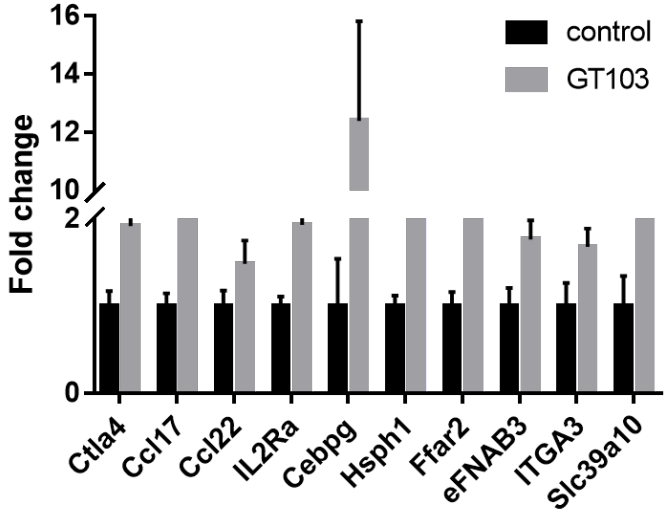


Figure 5. Real time RT-PCR of serum from CMT167 tumor-bearing C57BL/6 mice (N=6) treated with GT103 vs. PBS.

Table 2. Differentially expressed cytokines and related proteins in the sera of GT103- vs. PBS treated CMT167 tumor-bearing mice

Down-regulated	
Axl	Receptor tyrosine kinase involved in several cellular functions including growth, migration, aggregation and anti-inflammation in multiple cell types. Plays important role in inhibition of Toll-like receptor (TLR)-mediated innate immune response.
Up-regulated	
IFN-g	Produced by lymphocytes activated by specific antigens or mitogens, has important immunoregulatory functions. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the antiviral and antitumor effects of the type I interferons.
IGFBP5	IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture.
IL10	Major immune regulatory cytokine that acts on many cells of the immune system where it has profound anti-inflammatory functions, limiting excessive tissue disruption caused by inflammation.
IL17A	Involved in inducing stromal cells to produce proinflammatory and hematopoietic cytokines.
LIX	Participates in the recruitment of inflammatory cells by injured or infected tissue.
VCAM1	Functions in leukocyte-endothelial cell adhesion. Interacts with integrin alpha-4/beta-1 (ITGA4/ITGB1) on leukocytes, and mediates both adhesion and signal transduction. The VCAM1/ITGA4/ITGB1 interaction may play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation.
IGFBP2	Inhibits IGF-mediated growth.

Major Task 3: Sub-Aim 1C. Investigate modulation of innate cellular immune response by the CFH mAb. (Site 2, Dr. He)

Single cell suspensions from CMT167 tumor tissue from GT103-treated or control treated mice were prepared and analyzed for innate natural killer (NK) cells, NK-T cells, MDSCs, and DCs by flow cytometry. The flow cytometry data are presented below in **Major Task 5: Sub-Aim 2A**. In this experiment, there were no significant differences in NK and NK-T cells, but MDSCs were significantly decreased (p=0.026) and DCs were significantly increased (p=0.0022).

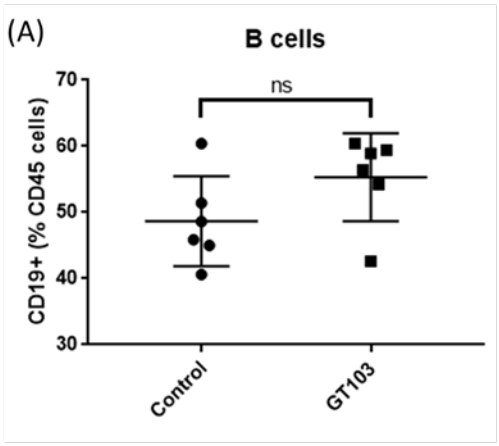
Major Task 4: Sub-Aim 1D. Determine levels of tumor leukocytes, systemic innate immune status of lung cancer patients with or without CFH Abs. (Site 1, Dr. Patz; Subaward site, Dr. Shofer)

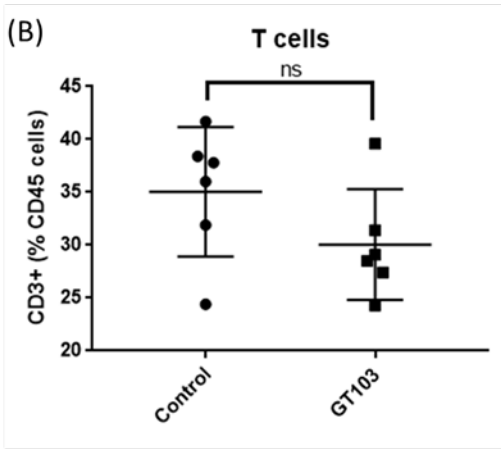
We are continuing to collect tissue for this Sub-Aim.

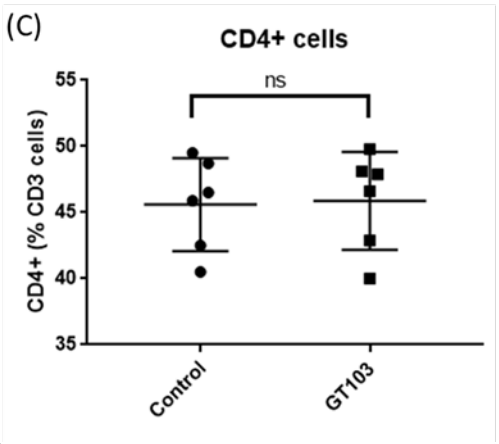
Specific Aim 2: Investigate the role of cellular immunity in CFH mAb cancer immunotherapy.

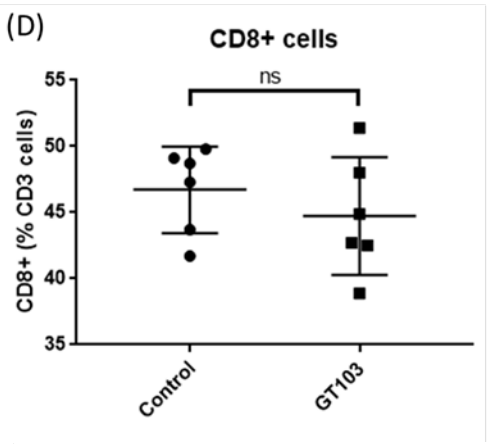
Major Task 5: Sub-Aim 2A. Determine whether CFH mAb inhibition of tumor growth *in vivo* requires T lymphocytes. (Site 2, Dr. He)

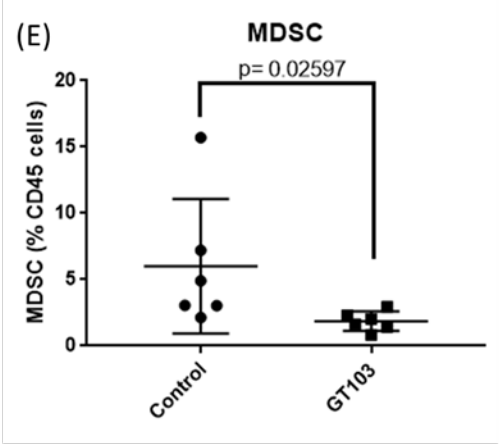
Immunophenotyping of tumors from GT103-treated vs. PBS-treated mice bearing CMT167 tumors is shown below. GT103 did not significantly change total B and T cell infiltration in tumors (**Fig 6A, B**) and no effect was seen on overall CD4+ and CD8+ subsets of T cells (**Fig 6C, D**). However, GT103 treatment inhibited trafficking of immunosuppressive leukocytes, MDSCs and Tregs, into the tumor microenvironment (**Fig 6E, F**) and led to an increase in dendritic cell infiltration (**Fig 6G**). It also led to increase in percentage of Perforin+ CD8 cells, total NK cells and NK-T cells, though the increases were not found to be significant (**Fig 6H- J**).

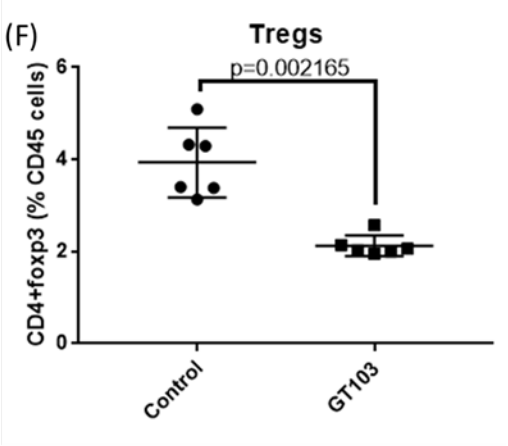

(A)
B cells
ns
CD19+ (% CD45 cells)
70
60
50
40
30
Control
GT103

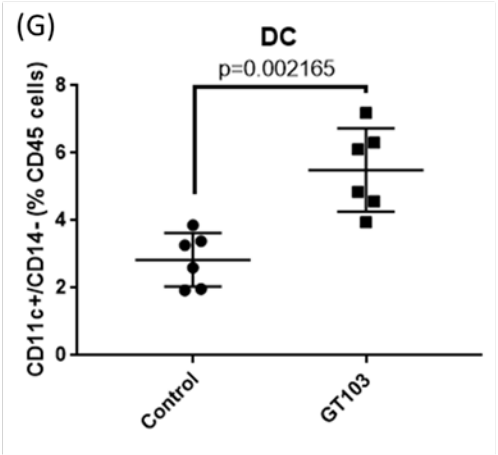

(B)
T cells
ns
CD3+ (% CD45 cells)
45
40
35
30
25
20
Control
GT103

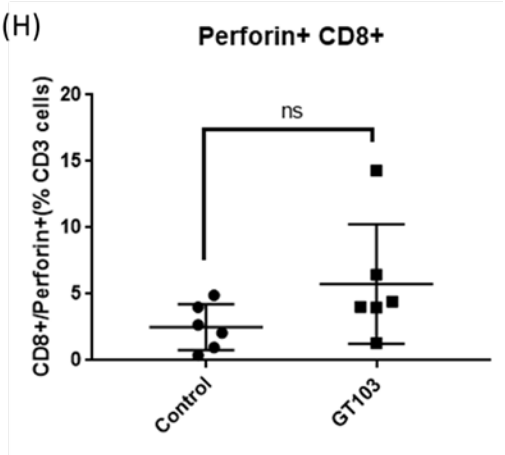

(C)
CD4+ cells
ns
CD4+ (% CD3 cells)
55
50
45
40
35
Control
GT103


(D)
CD8+ cells
ns
CD8+ (% CD3 cells)
55
50
45
40
35
Control
GT103


(E)
MDSC
p= 0.02597
MDSC (% CD45 cells)
20
15
10
5
0
Control
GT103


(F)
Tregs
p=0.002165
CD4+foxp3 (% CD45 cells)
6
4
2
0
Control
GT103


(G)
DC
p=0.002165
CD11c+/CD14- (% CD45 cells)
8
6
4
2
0
Control
GT103


(H)
Perforin+ CD8+
ns
CD8+/Perforin+(% CD3 cells)
20
15
10
5
0
Control
GT103

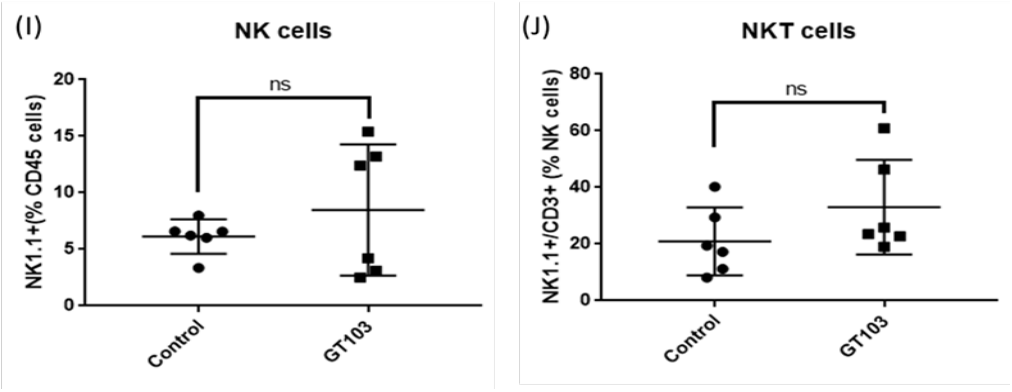


Figure 6. Immuno-phenotyping of tumor infiltrating lymphocytes in control (PBS)-and GT103-treated tumors

With respect to T lymphocytes, we observed that the most significant effect of GT103 was to decrease Tregs. Although we saw no significant increase in CD4+ and CD8+ subsets in the GT103-treated tumors, we intend to investigate whether there is an increase in *tumor antigen-specific* T cells in these tumors, and if so, will measure their effector functions.

Major Task 6: Sub-Aim 2B. Determine whether CFH mAb treatment induces lasting anti-tumor T cell immunity *in vivo*. Scheduled for Year 2.

Major Task 7: Sub-Aim 2C. Characterize T lymphocyte population in tumor tissues of NSCLC patients with or without CFH antibodies. We expect to continue to collect tissue for this Sub-Aim in Year 2. (Site 1, Dr. Patz; Subaward site, Dr. Shofer)

Additional experiments bearing on the role of cellular immunity in CFH mAb cancer immunotherapy

1. The requirement of B cells for efficacy of GT103. (Site 2, Dr. He) The gene ontology analysis referenced above indicated that genes involved in B cell receptor signaling were upregulated in tumors of GT103-treated mice. In order to further explore the relevance of this finding, we performed an experiment in which B cells were ablated or not, CMT167 tumors were initiated, and mice were treated with GT103 or isotype-matched control IgG2. If B cells are required for GT103 efficacy, then GT103-mediated growth inhibition will be lost when B cells are ablated with the anti-CD20 antibody. As shown in **Figure 7**, in the absence of B cells, GT103 efficacy is reduced, confirming that B cells are necessary for its activity.

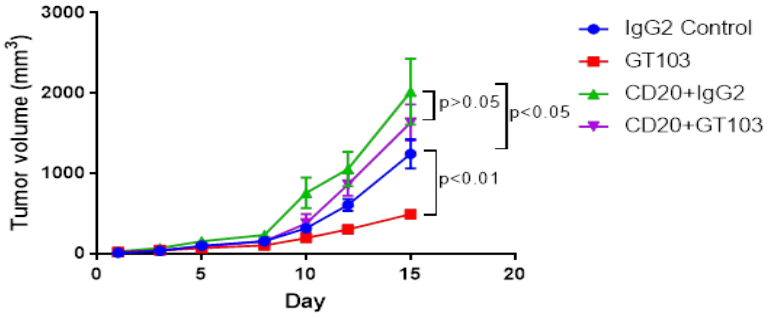


Figure 7. Inhibition of CMT167 tumor growth by GT103 requires B cells. Ten six to eight week old C57BL/6 mice were injected with anti-CD20 (Biolegend) (250 µg IV) three days prior to tumor inoculation to deplete B-cells. The 10 B-cell depleted and 10 nondepleted C57BL/6 mice were inoculated s.c. with 2.5 x 10^5 CMT167 cells. When tumors became palpable, mice in each group were dosed three times a week with control IgG2 (100 µg IP) or GT103 (200 µg IP). Tumor volume was plotted as mean ± SEM for n=3-5 for each treatment group.

2. Efficacy of GT103 in combination with anti-PD1. (Site 2, Dr. He) The therapeutic blockade of PD-L1 from binding to PD-1 utilizing immune checkpoint monoclonal antibodies is an established modality of treatment for stage III and IV NSCLC. The routine use of immunotherapy has resulted in the improvement of overall survival in numerous clinical trials for advanced NSCLC. However, it is still the case that many patients either don't

respond or acquire resistance to this therapy. Failure of immune checkpoint therapy can be attributed to insufficient numbers or function of anti-tumor T cells, or generation of T-cell memory. As our immunoprofiling experiment above showed that GT103 reduces the number of Tregs and MDSCs found in the tumor microenvironment, and these cell types suppress effector T cells, we decided to examine whether GT103 could augment anti-PD1 therapy.

Experiment 1. B16F10-OVA subcutaneous melanoma tumor growth with GT103 in combination with anti-PD-1

B16F10-OVA tumor-bearing C57BL/6 mice were treated with anti-PD1 and GT103, as monotherapy or in combination, and compared with vehicle control (**Figure 8**). While anti-PD-1 treatment alone did not inhibit tumor growth (previously known to be the case with this model), GT103 monotherapy led to tumor growth retardation. Throughout the majority of the time course, the combination of anti-PD-1 and GT103 led to slower tumor growth than was seen with either antibody alone.

Experiment 2. CMT167 subcutaneous lung tumor growth with GT103 in combination with anti-PD-1 therapy

CMT167 tumor-bearing C57BL/6 mice were treated with anti-PD-1 and GT103, as monotherapy or in combination, or with IgG2 control. In this model, no synergistic effect of anti-PD-1 and GT103 was observed, although the experiment had to be halted after only 15 days due to tumor necrosis (**Figure 9**).

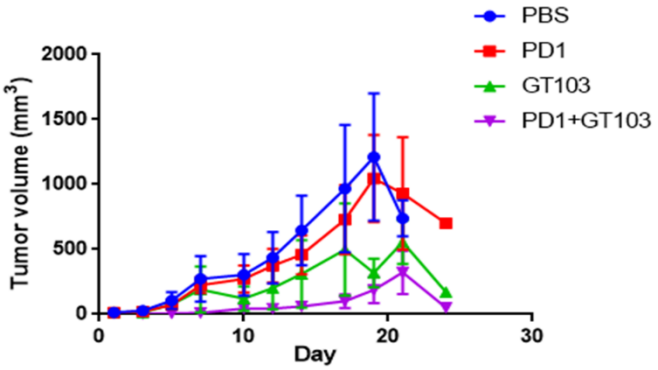


Figure 8. Effect of GT103, anti-PD-1, and combination on the growth of subcutaneous B16F10 melanoma tumors in C57BL/6 mice. Volume of subcutaneous tumors was plotted as mean ± SEM for N=5 for each treatment group.

Specific Aim 3: Define a cancer patient population most amenable to therapeutic intervention with a CFH antibody.

Major Task 8. Perform assays for eight variables.

Major Task 9. Establish statistical correlation of assay variables and outcome.

We are continuing to collect tissue for these two Major Tasks. (Site 1, Dr. Patz; Subaward site, Dr. Shofer)

Specific Aim 4: Determine if CFH-containing exosomes can inhibit metastasis.

Major Task 10. Perform exosome-mediated metastasis experiments in the CMT167 (WT vs CFH knockout)-C57BL/6 model. (Scheduled for Year 2)

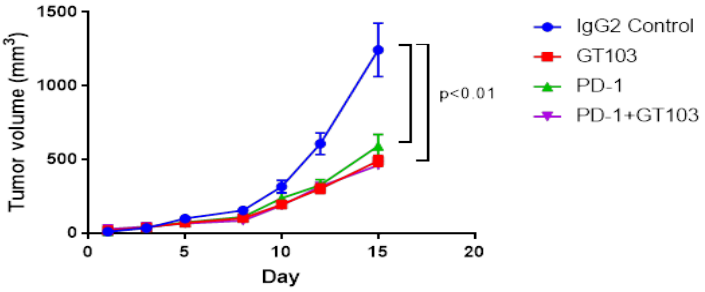


Figure 9. Effect of GT103, anti-PD-1, and combination on the growth of subcutaneous CMT169 lung tumors in C57BL/6 mice. Volume of subcutaneous tumors was plotted as mean ± SEM for N=10 for each treatment group.

4) Other Achievements

- **What opportunities for training and professional development has the project provided?**

Ruchi Saxena, PhD, a post-doctoral fellow, performed the mouse tumor growth experiments, and subsequent immunology and molecular biology experiments, at Site 2. Her mentor is Dr. You Wen He.

- **How were the results disseminated to communities of interest?**

Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We have four main goals for Year 2:

1) Complete the analysis of the how GT103 generates adaptive immunity against tumors.

Major Task 5: Sub-Aim 2A. Determine whether CFH mAb (GT103) inhibition of tumor growth in vivo requires T lymphocytes. This sub-aim contains two experiments: 1, determine the effector function of tumor infiltrating lymphocytes in GT103-treated tumors and 2, carry out depletion of CD4+, CD8+, NK, and NKT cells to identify which cell types are important for GT103 activity.

Major Task 6: Sub-Aim 2B. Determine whether CFH mAb (GT103) treatment induces lasting anti-tumor T cell immunity in vivo. This sub-aim contains three experiments: 1, determine whether treatment results in tumor infiltration of CD8+ anti-tumor T cells; 2, determine whether tumor cells killed by GT103 can vaccinate mice against a challenge with live tumor cells; 3, determine whether mice previously treated with GT103 which abolished their tumor can withstand a re-challenge with a higher dose of tumor cells.

Because gene expression analysis of GT103-treated tumors indicated B cell receptor signaling was an important part of GT103 action, we will continue to explore the role of B cells in tumor growth inhibition.

2) Determine if CFH-containing exosomes can promote metastasis.

Exosomes are ~30-150 nm extracellular vesicles of endocytic origin that mediate intracellular communication. In lung cancer, as in other types of cancer, exosomes facilitate metastasis by promoting formation of a premetastatic niche and inducing immunosuppression. Exosomal PD-L1 is a powerful contributor to the immunosuppression of T cells and higher expression of PD-L1 on exosomes is associated with poorer outcomes and less responsiveness to checkpoint inhibitors.

We have recently discovered that GT103 can bind exosomes from a wide array of tumor cell lines, and can induce both complement-dependent lysis and antibody-dependent phagocytosis, effectively destroying them. We hypothesize that GT103, operating through these mechanisms and reducing the overall PD-L1 burden, can inhibit metastasis. In order to address this hypothesis, we must first demonstrate that CFH on exosomes promote metastasis. We have modified the SOW to incorporate 2 experiments under a new Major Task 10:

Major Task 10. Perform exosome-mediated metastasis experiments in the CMT167 (WT vs CFH knockout)-C57BL/6 model.

Experiment 1: C57BL/6 mice will be inoculated on the flank with 5 x 10^5 wt CMT167 cells or with a CFH knockout cell line (CMT167 KO). After 21 days, mice will be euthanized and the lungs will be harvested and examined to determine the number of pulmonary metastases present. Expected result: If CFH plays a role in the formation of metastases, potentially through its presence in exosomes, we would see more metastases in the animals with wild type CMT167 tumors compared to those with the CMT167 KO tumors.

Experiment 2: If we observe the expected result in Experiment 1, we propose to test directly the contribution of CFH-containing exosomes in facilitating the formation of pulmonary metastases. This will be accomplished by intravenously injecting exosomes isolated from the wild type and KO CMT167 cell lines in mice bearing wt or KO tumors. Expected result: If exosomes containing CFH promote metastasis, exosomes from the wild type cell line should be able to stimulate metastasis of the KO cell line, which are otherwise unable to metastasize. Observance of the expected result would warrant further study to determine the ability of GT103 to suppress metastasis through direct action on exosomes.

3) Analyze innate and adaptive immune status of human tumor tissues as described in the following Major Tasks:

Major Task 4: Sub-Aim 1D. Determine levels of tumor leukocytes, systemic innate immune status of lung cancer patients with or without CFH Abs.

Major Task 7: Sub-Aim 2C. Characterize T lymphocyte population in tumor tissues of NSCLC patients with or without CFH antibodies

4) Begin Specific Aim 3. **Define a cancer patient population most amenable to therapeutic intervention with a CFH antibody.** This Specific Aim is divided into assays for 8 variables. The timeline assigns these assays to be divided between Years 2 and 3.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

GT103 is a novel anti-cancer agent whose mechanism we are still elucidating. The major findings of the project to date are that GT103 (1) reduces immunosuppressive cell types in the tumor microenvironment, (2) increases potentially immunostimulatory DCs, and (3) requires B cells for its function. If the reduction in Tregs and MDSCs in tumors treated with GT103 translates into higher effector T cell activity, then GT103 could constitute a new class of immunotherapy for NSCLC.

- **What was the impact on other disciplines?**

Nothing to Report

- **What was the impact on technology transfer?**

This research is being conducted in parallel with a Phase Ib clinical trial of GT103. The results from this proposal may help guide the direction of this trial, and may help select for patients who will respond to therapy.

- **What was the impact on society beyond science and technology?**

Nothing to Report

5. CHANGES/PROBLEMS: Changes in approach and reasons for change

1) Use of single cell line for mouse experiments: We proposed to use two mouse lung tumor models. So far, we have only used one, the CMT167 model because the other, LLC, has much lower CFH on the cell membrane. We may revisit using a second cell line in the future.

2) Change of approach in Specific Aim 1: The approach was changed from an *in vivo* to an *in vitro* one. This reduced the number of mice needed for the project, and we feel we adequately answered the basic question of whether GT103 triggers the alternative or classical complement pathway.

3) Change of approach in Specific Aim 2: The approach was made more comprehensive. Instead of performing biochemical assays and cytokine arrays in order to obtain evidence for innate and adaptive immune activation in GT103-treated tumors, we performed RNA-seq and gene ontology profiling to query a greater number of signaling molecules than originally planned.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Our laboratory experiments were delayed for most of Quarter 4 of Year 1 due to university-mandated Covid-19 closure.

Our human tumor tissue and serum collection was likewise delayed and Dr. Shofer, our collaborator at the VA, was waiting for IRB approval to begin collecting his share of the samples.

Laboratory work has now resumed and Dr. Shofer has obtained his IRB approval. However, the pace of human tissue collection may still be limited by institutional and local policies due to COVID-19.

- **Changes that had a significant impact on expenditures**

Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

- **Significant changes in use or care of human subjects**

Nothing to Report

- **Significant changes in use or care of vertebrate animals**

Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

Nothing to Report

6. PRODUCTS

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name	Edward F. Patz, Jr., MD	Michael J. Campa, PhD	You Wen He, MD, PhD	Ruchi Saxena, PhD
Project Role	Initiating PI	Collaborator	Partnering PI	Postdoctoral Fellow
Person Months	1.8	4.8	2.40	7.00
Contribution to Project	Supervising research	Tissue curation, carrying out in vitro experiments, supervising laboratory	Supervising research	Carrying out tumor growth, immunology, molecular biology experiments

James E. Herndon III, PhD, statistician, Latonia Strader, clinical coordinator, and Rex Bentley, MD, pathologist, had no effort in Year 1.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Title: A TriVac Platform to Enhance Antitumor-Specific Immunity
PI: You-Wen He
Time Commitment: 5% / 0.60 calendar months
Supporting Agency: Goldman Sachs Philanthropy Fund/Emerson Collective – P.O. Box 15203, Albany, NY 12212-5203
Grants Officer: Reed Jobs
Performance Period: 5/6/2019 - 5/5/2021
Level of Funding: $89,957
Project Goals: The goal of this project is to test the treatment efficacy of TriVac designs in different tumor models using TAAs and neoantigens.
Specific Aims: Aim 1: The two specific aims are straightforward with aim 1 to investigate the antitumor efficacy of TriVac in

mouse tumor models and in vitro priming of human T cells using TAAs and aim 2 to test whether TriVac can enhance neoantigen induced antitumor immunity.

- **Overlap:** None**What other organizations were involved as partners?**

  Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.*