Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Thomas Jefferson University

2. **Reporting Period (start and end date of grant award period):** 06/01/10-05/31/14

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Carol Prem

4. **Grant Contact Person's Telephone Number:** 215-955-1407

5. **Grant SAP Number:** 4100051723

6. **Project Number and Title of Research Project:** Therapeutic Vaccine Bridging the Gap in Racial Disparities in Colorectal Cancer

7. **Start and End Date of Research Project:** 06/01/10-05/31/14

8. **Name of Principal Investigator for the Research Project:** Scott A. Waldman, MD, PhD, FCP, FAHA

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 4,286,813.74

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Waldman	Principal Investigator	10% Yr 1; 9% Yr 2 - 3, 10% Yr 4	$ 100,621.58
Alexeev	Co-Investigator	5% Yr 1 - 2; 9% Yr 3 - 4	22,320.88
Eisenlohr	Co-Investigator	10% Yr 1 - 2; 4% Yr 3 - 4	67,147.42
Hyslop	Co-Investigator	10% Yr 1; 9% Yr 2, 3, 4	54,459.55
Mastrangelo	Co-Investigator	5% Yr 3 - 4	7,297.29
Myers	Co-Investigator	15% Yr 1, 2, 3, 4	149,865.20
Rappaport	Co-Investigator	10% Yr 1 - 2, 9% Yr 3	52,170.62
Sato	Co-Investigator	10% Yr 1; 9% Yr 2, 3, 4	59,707.98
Andrel	Statistical Analyst	10% Yr 2, 3, 4	22,579.99
Burgh	Clinical Research Assistant	12% Yr 1; 0% Yr 2	55,089.00
Croker	Statistical Analyst	24% Yr 1	14,345.76
Faust	Administrator	5% Yr 1; 4% Yr 2, 3, 4	17,300.10
Haaf	Clinical Nurse Coordinator	23% Yr 1; 20% Yr 2, 40% Yr 3, 50% Yr 4	90,592.11
Hagerty	Statistical Analyst	9% Yr 1; 36% Yr 2, 3, 4	27,807.56
Haslam	Clinical Research Assistant	15% Yr 4	5,037.25
Keintz	Development Coordinator	19% Yr 1	16,045.56
Laudadio	Clinical Research Nurse/Project Mgr	10% Yr 1, 2, 3; 17% Yr 4	41,680.73
Leis	Med Tech	15% Yr 4	9,064.11
Lin	Post Doc	50% Yr 4	8,963.36
Magee	Doctoral Student	90% Yr 1, 2, 3; 66% Yr 4	13,504.34
Oppong	Post Doc	15% Yr 4	8,362.62
Pallotto	CRU Director	25% Yr 4	23,719.90
Pequignot	Statistical Analyst	6% Yr 2, 22% Yr 3 - 4	52,413.72
Petrich	Nurse Clinical Specialist	30% Yr 1, 66% Yr 2, 100% yr 3 - 4	227,989.65
Puchalski	Clinical Research Nurse	15% Yr 4	3,555.61
Pullaro	Clinical Res Coordinator	15% Yr 4	7,659.52

Quinn	Clinical Research Assistant	30% Yr 2, 3, 4	27,594.12
Sims	Grants Manager	5% Yr 1; 4% Yr 2, 3, 4	15,138.27
Snook	Post Doctoral Fellow	94% Yr 1, 2; 58% Yr 3; 50% Yr 4	175,570.36
Sohon	Clinical Res Specialist	100% Yr 4	37,584.98
Soleiman	Admin Education Director	15% Yr 4	33,868.42
Swan	Clinical Research Assistant	10% Yr 1; 30% Yr 2	21,997.16
Thomas	Clinical Research Coordinator	50% Yr 4	8,568.18
Ueland	Nursing Clinical Specialist	4% Yr 1	2,445.72
Vizza	RN	15% Yr 4	9,380.53
Xiang	Doctoral Student	100% Yr 2, 3, 4	73,841.59

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
CTL Analyzer	At the time we had moved forward in the final phase of this project in order to analyze our data/samples in a timely/efficient manner the analyzer was necessary.	$81,400.00

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Chemokine-enhanced vaccination and T cell engineering in melanoma-bearing hosts	☒NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	June 2011	$250,000 (annual direct)	not funded
Occult lymph node metastases and racial disparities in rectal cancer outcomes	☒NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	June 2011	$250,000 (annual direct)	not funded

GUCY2C-targeted adoptive T cell therapy to treat metastatic colorectal cancer	☐NIH ☐ Other federal (specify:_____ _____) ☒ Nonfederal source (specify: _PhRMA_____)	Sept 2013	$100,000	not funded
GUCY2C-specific tolerance in colon cancer patients	☐NIH ☐ Other federal (specify:_____ _____) ☒ Nonfederal source (specify: _PhRMA_____)	Feb 2014	$100,000	$100,000
GUCY2C-targeted adoptive T cell therapy for metastatic colon cancer	☐NIH ☐ Other federal (specify:_____ _____) ☒Nonfederal source (specify: _AACR_____)	Feb 2014	$67,500	not funded
GUCY2C-specific CD4+ T cell tolerance mechanisms and outcomes in tumor immunity	☐NIH ☐ Other federal (specify:_____ _____) ☒ Nonfederal source (specify: _Margaret Q. Landenberger Research Foundation__)	April 2014	$100,000 (annual)	$100,000
Elimination of Adoptive Cell Therapy (ACT) Toxicity by Hypoxic Regulation of Antigen Receptors	☐NIH ☐ Other federal (specify:_____ _____) ☒ Nonfederal source (specify: WW Smith____)	June 2014	$125,000	pending
GUCY2C-specific CD4+ T cell tolerance mechanisms and outcomes	☒NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	June 2014	$232,500	pending

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

A. for widely Mapping GCC class I and II epitopes mediating CD8 and CD4 T cell responses in humans; directly leverages the clinical trial in Aim 1 of this proposal.
B. Mechanisms of tolerance induction to GCC by systemic metastases; directly expands studies outlined in Aim 2 of this proposal.
C. Mechanisms of CD4 tolerance to endogenous gut antigens; directly expands studies outlined in Aim 2 of this proposal.
D. Utility of GCC as a predictive marker of therapeutic responses in African American and Caucasian colon cancer patients; directly expands one focus of the clinical trial in Aim 1 of this proposal.
E. Utility of GCC as a predictive marker of therapeutic responses in African American and Caucasian rectal cancer patients; directly expands one focus of the clinical trial in Aim 1 of this proposal.
F. Commercialization of GCC as a prognostic and predictive marker in African American and Caucasian colon and rectal cancer patients; directly expands one focus of the clinical trial in Aim 1 of this proposal.
G. GCC-targeted chimeric T cell receptors (T bodies) to eliminate established tumors in mouse models of metastatic colorectal cancer; directly expands the approaches utilized in the clinical trial in Aim 1 to develop treatments metastatic disease.

12. Future of Research Project. What are the future plans for this research project?

<u>AIM</u> 1. SAFETY AND IMMUNE EFFICACY OF ADENOVIRAL (AV) GCC IN CAUCASIAN AND AFRICAN AMERICAN pN0 COLON CANCER PATIENTS WITH DEFINED OCCULT TUMOR BURDEN (TRANSLATIONAL AIM).

Full immunomonitoring studies will be carried out on all subjects to include antibody responses to GCC and the Ad5 vector and T-cell responses to GCC, PADRE and Ad5 at 30, 90 and 180 days post vaccination. Full assay validation including those for sample processing, ELISA (antibody; Figure 2) and ELISpot (T cell; Figure 3) were performed prior to analyzing samples from vaccinated subjects. Full immunomonitoring assessment is ongoing for the 3 subjects that have completed all follow-up visits and will be conducted for the remaining subjects following their final visit. In the context of a successful outcome from the current trial, future phase II and III clinical trials will employ Ad5-hGCC-PADRE meeting RCA guidelines, to ensure that subjects in future studies receive a consistent product.

AIM 3. MECHANISMS SHAPING RESPONSES TO CANCER MUCOSA ANTIGENS THAT INFORM CLINICAL STRATEGIES (MECHANISTIC AIM).

SUBAIM 3.A. TOLERANCE MECHANISMS TO SELF-ANTIGENS IN INTESTINE AND METASTATIC COLORECTAL CANCER.

C57BL/6-derived embryonic stem (ES) cells were transfected with the ROSA-stop^{flox}-Tac targeting construct and ~500 single neomycin-resistant clones were picked, expanded, frozen and tested for correct targeting using PCR (Figure 5). Production of chimeric mice from 3 targeted ES cell clones was attempted, however, no chimeric mice were produced. Before, other targeted ES clones could be injected in a second attempt, the Thomas Jefferson University (TJU) Transgenic Core was closed and production at TJU was terminated. Future work will utilize extramural transgenic facilities to produce the transgenic mice utilizing ES clones generated here. Future studies will employ that mouse model to explore endogenous tolerance mechanisms. Also, future studies are planned to determine the mechanism by which Trp2-specific CD8$^+$ T cells mediate MDSC polarization, the consequences of polarization (is beneficial or more suppressive?) and if this novel mechanism could be a therapeutic target for immunotherapeutic intervention in cancer.

SUBAIM 3.B. REGULATORY T CELL (TREG)-DEPENDENT TOLERANCE AND GCC RESPONSES.

We have successfully identified T cell receptors (TCRs) specific for the two dominant GCC-derived CD4$^+$ T cell epitopes recognized in GCC$^{-/-}$ (non-tolerant) mice. Generation of transgenic mice expressing these TCRs is ongoing and these mice will be very important tools to explore the mechanisms and consequences of GCC-specific CD4$^+$ T cell tolerance. Future experiments will employ the newly generated W11 and W14 TCR transgenic mice to define the fate of GCC-specific CD4$^+$ T cells in GCC$^{+/+}$ mice, the contribution of Treg development to GCC-specific tolerance, and the ability of GCC-specific Tregs to inhibit vaccine-induced antitumor immunity. Those results can be directly translated to next-generation vaccine approaches in patients, leveraging safety and efficacy data of Ad5-hGCC-PADRE produced in Aim 1.

SUBAIM 3.C. CHEMOKINE-POTENTIATED HETEROLOGOUS VACCINE STRATEGIES THAT AMPLIFY GCC- TARGETED RESPONSES.

We developed a GCC-targeted DNA vaccine to supplement GCC-targeted adenoviral vaccination to treat metastatic colorectal cancer. DNA plasmid vaccine expressing GCC elicited CD8$^+$ T cell responses, but those were of lower frequency than Ad5-GCC, resulting in poor protection from GCC-expressing metastatic colorectal cancer. However, the combination of GCC DNA and Ad5 in a prime-boost strategy, not only increased the quantity of GCC-specific CD8$^+$ T cells, but also produced CD8$^+$ T cell responses with higher TCR avidity. That prime-boost strategy also protected mice with colorectal cancer metastases better than all other regimens tested. Employing an Ad5-GCC$_{254-262}$ construct that minimizes TCR avidity confirmed the importance of TCR avidity in antitumor efficacy of GCC-specific responses to vaccination. Indeed, comparing survival benefit to T cell quantity, T cell cytokine multifunctionality and TCR avidity across all tested vaccination combinations revealed only TCR avidity predicting tumor outcomes. Thus, our studies revealed the previously unrecognized mechanism of functional T-cell avidity enhancement mediating synergy of DNA-Ad5 prime boost vaccinations. Those results will direct vaccine design for future GCC vaccine combinations in patients. Moreover, these results suggest that the magnitude and multifunctional cytokine profiles of cancer vaccine-induced T cell responses are poor predictors of outcomes following prime-boost immunizations. Rather, functional TCR avidity correlates with outcomes and will be explored as a biomarker of cancer vaccine efficacy.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	6			
Female	11			
Unknown				
Total	**17**			

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic	3			
Non-Hispanic	14			
Unknown				
Total	**17**			

	Undergraduate	Masters	Pre-doc	Post-doc
White	3			
Black	13			
Asian	1			
Other				
Unknown				
Total	**17**			

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes__X _____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

As a result of this funding, we recruited a new faculty member (Adam Snook, PhD) with expertise in cancer immunology and vaccinology to lead a program in vaccine development against GI malignancies, including colorectal, pancreatic, gastric, and esophageal cancers. This program has already attracted funding in the form of peer-reviewed grants, licensed technology to commercial entities for clinical development, and produced new intellectual property.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

Aims 1, 2 and 4 of the research project involved important collaborations with institutions through Pennsylvania. Aims 1 and 2 tested the safety and immunological activity of the Ad5-hGCC-PADRE vaccine and identified barriers underlying racial disparities in cancer vaccine trial participation. University of Pittsburgh Medical Center (UPMC), Einstein Medical Center in Philadelphia, Fox Chase Cancer Center in Philadelphia and Virtua healthcare system in New Jersey were all important collaborators in recruiting patients and performing the research. In addition, Aim 4 sought to prepare trainees from under-represented minorities for careers in biomedicine and Cheyney, Lincoln, and St. Joseph's Universities were vital collaborators in identifying trainees and overseeing the training program.

16(B) Did the research project result in commercial development of any research products?

Yes____X_____ No_____

If yes, please describe commercial development activities that resulted from the research project:

This program developed new intellectual property, in the form of patents, that has been the focus of licensing opportunities with regional and international biotechnology companies. Indeed, the vaccine project was licensed by Targeted Diagnostics & Therapeutics, Inc., a regional company based in Exton, PA. The technology involving T cells re-directed by chimeric antigen receptors was licensed to Millennium Pharmaceuticals (A Takeda Company).

16(C) Did the research lead to new involvement with the community?

Yes___X_____ No___ _____

If yes, please describe involvement with community groups that resulted from the research project:

The program developed new outreach programs with the community with respect to understanding racial and cultural barriers to enrolling in cancer clinical trials.

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project goals, objectives and specific aims

This ***Center of Excellence in Cancer Immunotherapy*** will develop a vaccine for secondary prevention of disease recurrence to reduce stage-specific racial disparities in colorectal cancer. There is an unmet need for improved therapeutics in colorectal cancer, the 3rd leading cause of cancer and 2nd leading cause of cancer mortality worldwide. In Pennsylvania, colorectal cancer incidence and mortality rates are higher than those expected in the nation with ~15,000 cases treated each year associated with a total in-patient annual cost of >$200M. Mortality reflects metastatic disease: ~50% of patients

present with clinically apparent metastases, while ~30% present with occult metastases. Moreover, there is a disparity in outcomes in early stage (stage I and II; lymph node negative; pN0) African Americans, who exhibit ~40% excess mortality compared to Caucasians, reflecting occult metastases. Reductions in disparities have been hampered by the absence of prognostic markers that identify patients at risk and the paucity of therapies that prevent metastatic disease.

This proposal advances an emerging paradigm in colorectal cancer detection and eradication, employing GCC as a ***prognostic marker*** and ***immunological target***. GCC is a protein whose expression is normally restricted to intestinal epithelial cells, but universally over-expressed by metastatic colorectal tumors. GCC is a marker of occult metastases in lymph nodes, which disproportionately burdens African American patients. Also, GCC is the index example of a new class of vaccine targets, cancer mucosa antigens, whose expression normally is restricted to mucosae, but extends to the immunologically naive systemic compartment upon metastasis of mucosal tumors. These antigens exploit the immunological separation of tissue compartments, in which systemic immune responses rarely extend to mucosae. Advantages of these antigens include systemic immunoreactivity profiles supporting durable antitumor immunity, and the limited immune cross talk between compartments that restricts autoimmunity. Viral vectors expressing GCC induce antigen-specific immune responses that prevent parenchymal metastases without autoimmunity.

This ***Center*** will translate advances in molecular diagnostics and immunotherapy into new vaccines that bridge racial disparities in colorectal cancer. Goals include (1) advancing GCC into clinical trials in African Americans and Caucasians, (2) defining barriers and culturally-sensitive solutions to improve participation in cancer vaccines trials, (3) mechanism-based optimization of cancer mucosa antigens as vaccine targets, and (4) developing the next generation of investigators from under-represented minorities in biomedicine. These goals will be accomplished through: (1) the ***Translational Research Program***, which will define the safety and immunological efficacy of a GCC-based vaccine in pN0 African American and Caucasian colon cancer patients variably burdened by occult metastases identified by GCC; (2) the ***Health Services Research Program***, which will identify barriers that prevent African Americans and Caucasians from participating in vaccine trials, and design strategies to increase informed participation; (3) the ***Biomedical Research Program***, which will define mechanisms shaping responses to cancer mucosa antigens that inform clinical strategies to maximize antitumor efficacy; and (4) the ***Training Program***, a collaboration of regional universities to recruit new scholars from under-represented minorities for careers in biomedicine. This program will define the:

AIM 1. SAFETY AND IMMUNE EFFICACY OF ADENOVIRAL (AV) GCC IN CAUCASIAN AND AFRICAN AMERICAN pN0 COLON CANCER PATIENTS WITH DEFINED OCCULT TUMOR BURDEN (TRANSLATIONAL AIM). There is an unmet need for immunotherapeutics that bridge racial disparities and protect against recurrence in patients with occult metastases. This phase I study explores the safety and immune efficacy of a vaccine employing AV-GCC in African American and Caucasian pN0 colon cancer patients variably burdened by occult metastases quantified by GCC RT-PCR. The hypothesis suggests that AV-GCC will induce immune responses in patients

with without autoimmunity. Further, AV-GCC will produce responses in both African American and Caucasian patients. Moreover, this vaccine will induce responses in patients with and without occult metastases.

AIM 2. BARRIERS UNDERLYING RACIAL DISPARITIES IN PARTICIPATION IN CANCER VACCINE TRIALS (HEALTH SERVICES RESEARCH AIM). With respect to participation in cancer vaccine trials, processes underlying decision-making, racial differences in those processes, and methods to facilitate informed decisions have not been defined. We will design education materials, revise survey data collection instruments, and adapt decision counseling materials related to the present cancer vaccine trial. We will pre-test contact and survey procedures with patients relative to a scenario that presents the hypothetical opportunity to participate in the trial. These materials and procedures will be used to pilot-test education and decision counseling interventions with patients eligible for that trial. These studies will identify barriers to participation in the trial, develop interventions that are sensitive to race and literacy, and refine interventions that facilitate informed decision making about enrollment in diverse populations. Also, they will characterize factors that influence enrollment and identify demographic, knowledge, and preference-related predictors of actual enrollment in the trial.

AIM 3. MECHANISMS SHAPING RESPONSES TO CANCER MUCOSA ANTIGENS THAT INFORM CLINICAL STRATEGIES (MECHANISTIC AIM). The utility of cancer vaccines will reflect an understanding of mechanisms modulating immune responses that can be exploited to maximize efficacy. Studies here model important immunologic attributes that mediate tolerance and predict inhibition to vaccine therapy (antigen exposure and tumor burden; Aim 3.A), explore strategies to overcome that resistance (Tregs; Aim 3.B), and identify receptor-dependent approaches to maximize those paradigms (adjuvanation; Aim 3.C), to optimize future clinical trials of AV-GCC.

SUBAIM 3.A. TOLERANCE MECHANISMS TO SELF-ANTIGENS IN INTESTINE AND METASTATIC COLORECTAL CANCER. Cancer vaccines will have their greatest impact on preventing metastases in patients with occult metastases. However, the impact of <u>endogenous</u> tolerance and tolerance <u>induced</u> by metastases on immunotherapeutic responses remains undefined. Studies here employ an *in vivo* model in which tissue, duration, and quantity of target antigen expression is precisely controlled. The working hypothesis suggests that endogenous tolerance opposes the immunotherapeutic efficacy of cancer mucosa antigens. A correlative hypothesis suggests that there is a threshold of exposure to antigen, reflecting duration and quantity, in metastatic sites inducing tolerance that attenuates immunological responses. Here, results with mice will be compared to quantitative relationships between immune responses to AV-GCC and occult tumor burden in pN0 patients in Aim 1.

SUBAIM 3.B. REGULATORY T CELL (TREG)-DEPENDENT TOLERANCE AND GCC RESPONSES. Tregs and their product, IL-10, are key mediators of tolerance opposing immune responses to cancer vaccines. Studies will explore the contribution of Tregs to tolerance barriers opposing immune responses to GCC, and their abrogation by cyclophosphamide and IL-10 immunoadhesin to reduce Treg-dependent immunoinhibition.

SUBAIM 3.C. CHEMOKINE-POTENTIATED HETEROLOGOUS VACCINE STRATEGIES THAT AMPLIFY GCC- TARGETED RESPONSES. While

heterologous prime-boost strategies involving 3 GCC-based viruses maximize antitumor protection, vaccine paradigms requiring 3 different viruses represent a regulatory hurdle to clinical translation. Here we explore a DNA-based GCC vaccine strategy to supplement virus-based GCC approaches that overcome endogenous tolerance mechanisms. These studies will optimize chemokine recruitment of APCs and effector cells to DNA vaccination sites, and CD40 ligand (CD40L)-dependent maturation of APCs, their antigen presentation and T cell activation to maximize immunotherapeutic responses to DNA-based GCC constructs that compliment AV-GCC in prime-boost regimens.

AIM 4. PREPARATION OF TRAINEES FROM UNDER-REPRESENTED MINORITIES FOR CAREERS IN BIOMEDICINE (TRAINING AIM). This aim seeks to capitalize on the success of durable programs created by ***Center*** investigators that have been national models for the recruitment and retention of individuals from under-represented minorities in careers in biomedicine. Specifically, this aim will provide training opportunities for students from under-represented minorities from Cheyney, Lincoln, St. Joseph's and Thomas Jefferson Universities through a summer internship program. This program will include a didactic component and a research component in which trainees are matched with mentors to participate in research projects through data collection, data analysis, presentation at local and national forums, and authorship on publications.

FINAL PROGRESS REPORT

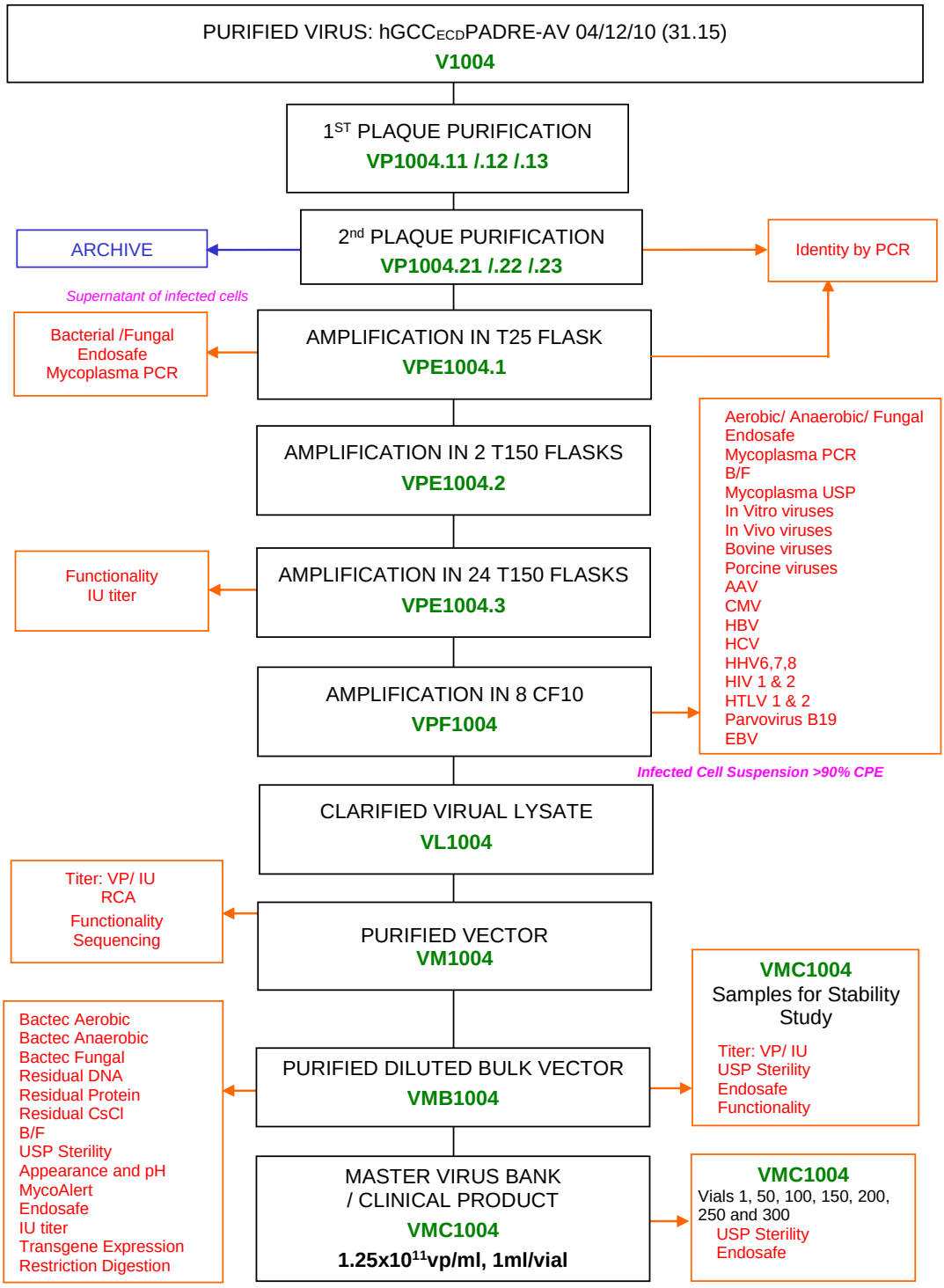
AIM 1. SAFETY AND IMMUNE EFFICACY OF ADENOVIRAL (AV) GCC IN CAUCASIAN AND AFRICAN AMERICAN pN0 COLON CANCER PATIENTS WITH DEFINED OCCULT TUMOR BURDEN (TRANSLATIONAL AIM).

There is an unmet need for immunotherapeutics that bridge racial disparities and protect against recurrence in patients with occult metastases. Aim 1 explored the safety and immune efficacy of a vaccine employing adenoviral GCC in African American and Caucasian pN0 colon cancer patients variably burdened by occult metastases quantified by GCC RT-PCR. Aim 1 consisted of 4 phases: 1) Vaccine Design, Manufacturing and Testing, 2) Preclinical Pharmacology and Toxicology 3) Regulatory Approval and 4) Clinical Testing.

1) Vaccine Design, Manufacturing and Testing

We developed a recombinant viral vector constructed from adenovirus type 5 (Ad5) that has been modified to carry the GCC gene. GCC represents a model cancer mucosa antigen, with expression normally confined to intestinal mucosa, but uniformly over-expressed in metastatic colorectal tumors. Ad5-delivered GCC-based vaccines induce antigen-specific $CD8^+$ T cell and antibody responses in wild type mice. These immune responses target colorectal cancer metastases in lung and liver in mouse models of prophylaxis and therapy. Importantly, immunization with GCC-based vaccines produces memory responses that provide durable protection against metastases in mice, modeling vaccination in pN0 patients with minimum residual disease. Moreover, GCC vaccination provides therapeutic efficacy in the absence of autoimmunity. Hence, we developed Ad5-hGCC-PADRE as an immunotherapeutic tumor vaccine targeting metastatic cancer cells over-expressing GCC in humans. Ad5 was selected because of its potency to induce antigen-specific immune responses in animal models and humans and because of its long and impressive safety record. PADRE is a $CD4^+$ T cell epitope that is active in most humans and is included in the vaccine because preclinical mouse studies demonstrated that inclusion of helper epitopes is critical to maximize $CD8^+$ T cell, antibody, immunological memory and antitumor responses.

Ad5-hGCC-PADRE DNA was generated at Thomas Jefferson University and transfected into HEK293 cells, which contain the E1 genes needed for viral replication. Viable Ad5-hGCC-PADRE virus was formed and collected and tested for the presence of hGCC-PADRE DNA, sequenced for hGCC-PADRE cassette accuracy and tested for its ability to produce hGCC-PADRE protein upon infecting cells *in vitro*. The vector was then produced under current Good Manufacturing Practices (cGMP) conditions appropriate to Phase I products, at Baylor College of Medicine, Center for Cell and Gene Therapy (CAGT) Vector Production Facility, a Class 10,000 manufacturing environment that operates in compliance with cGMP as applicable to products intended for Phase 1/2 clinical trials. An overview of the manufacturing process is shown below.


PURIFIED VIRUS: hGCCECDPADRE-AV 04/12/10 (31.15)
V1004
1ST PLAQUE PURIFICATION
VP1004.11 /.12 /.13
2nd PLAQUE PURIFICATION
VP1004.21 /.22 /.23
ARCHIVE
Identity by PCR
Supernatant of infected cells
Bacterial /Fungal
Endosafe
Mycoplasma PCR
AMPLIFICATION IN T25 FLASK
VPE1004.1
AMPLIFICATION IN 2 T150 FLASKS
VPE1004.2
Aerobic/ Anaerobic/ Fungal
Endosafe
Mycoplasma PCR
B/F
Mycoplasma USP
In Vitro viruses
In Vivo viruses
Bovine viruses
Porcine viruses
AAV
CMV
HBV
HCV
HHV6,7,8
HIV 1 & 2
HTLV 1 & 2
Parvovirus B19
EBV
Functionality
IU titer
AMPLIFICATION IN 24 T150 FLASKS
VPE1004.3
AMPLIFICATION IN 8 CF10
VPF1004
Infected Cell Suspension >90% CPE
CLARIFIED VIRUAL LYSATE
VL1004
Titer: VP/ IU
RCA
Functionality
Sequencing
PURIFIED VECTOR
VM1004
VMC1004
Samples for Stability Study
Titer: VP/ IU
USP Sterility
Endosafe
Functionality
Bactec Aerobic
Bactec Anaerobic
Bactec Fungal
Residual DNA
Residual Protein
Residual CsCl
B/F
USP Sterility
Appearance and pH
MycoAlert
Endosafe
IU titer
Transgene Expression
Restriction Digestion
PURIFIED DILUTED BULK VECTOR
VMB1004
MASTER VIRUS BANK
/ CLINICAL PRODUCT
VMC1004
1.25x10^11vp/ml, 1ml/vial
VMC1004
Vials 1, 50, 100, 150, 200, 250 and 300
USP Sterility
Endosafe

Vector Testing and Qualification

The final product of the manufacturing process was Ad5-hGCC-PADRE Master Virus Bank (MVB). The MVB was tested for quality control and a portion was vialed and labeled for clinical testing. Testing and qualification of the MVB and the clinical lot of Ad5-hGCC-PADRE vector is presented in Table 1 below.

Table 1
Testing performed on Ad5-hGCC-PADRE Master Virus Bank V1004

Test	Method	Specification	Harvest (Cell suspension)	Purified Bulk	MVB vial	Vialed Final Product
Aerobic/ Anaerobic/ Fungal	Bactec	Negative	PASS	PASS		
Sterility	USP	No Growth		PASS	PASS	PASS
Bacteriostasis / Fungistasis	Immersion USP/EP	Pass	PASS	PASS		
Mycoplasma	USP	Negative	PASS			
	PCR	Negative	PASS	PASS		
Mycoplasmastasis	Harmonized USP/EP/PTC/JP	Negative	PASS			
Endotoxin	Endosafe	<5.0EU/ml	PASS	PASS	PASS	PASS
Identity	Sequencing	As Expected		PASS		
Titer	Infectious Unit by CPE	Report result		PASS		
	VP by Spectrophotometer	Report result		PASS		
Activity/Potency	In Vitro Transgene Expression	Report result		PASS		
Residual Host DNA	Taqman PCR	Report result		PASS		
Residual Host Protein	Immunoenzymetric assay	Report result		PASS		
Residual CsCl	ICPMS (mass spectrophotometer)	<0.5mg/ml		PASS		
In vitro viral	3 indicator cell lines, 28 day culture	Negative	PASS			
In vivo viral	Inoculation of adult mice, suckling mice, embryonated hen eggs	Negative	PASS			
RCA	PCR + CPE confirmation	< 1 RCA/3x10e10 VP		FAIL#		
Bovine Virus	9 CFR	Negative	PASS			
Porcine Virus	Modified 9 CFR	Negative	PASS			
EBV	PCR	None Detected	PASS			
AAV	PCR	None Detected	PASS			
Parvovirus B19	PCR	None Detected	PASS			
CMV	PCR	None Detected	PASS			
HBV	PCR	None Detected	PASS			
HCV (RNA)	RT-PCR	None Detected	PASS			
HHV 6, 7, 8	PCR	None Detected	PASS			
HIV 1&2	PCR	None Detected	PASS			
HTLV 1&2	PCR	None Detected	PASS			
Appearance	Visual	Report result		PASS		
pH	pH meter	Report result		PASS		

Replication-Competent Adenovirus (RCA)

While Ad5-hGCC-PADRE passed all other quality control tests, RCA levels in the Ad5-hGCC-PADRE MVB/clinical product lot yielded 9/10 flasks positive when seeded with $3x10^9$ vp/flask by CPE and PCR, exceeding recommended levels. The GMP manufacturing process was repeated to produce a second lot of virus, however it also was RCA positive. Sequence analysis revealed no obvious cause for RCA development in our vector (i.e. an RCA "hot spot" within hGCC-PADRE), so a different Ad5 backbone possessing larger E1 and E3 deletions (pAdeno-X) was employed to reduce the risk of RCA during a third attempt at GMP manufacturing. Seed virus was then made with the hGCC-PADRE-pAdeno-X construct, but that seed material was also RCA positive, so full-scale GMP manufacturing was not performed. Thus, three attempts to produce RCA-free Ad5-hGCC-PADRE were unsuccessful.

Therefore, we contacted Michael Havert, at FDA, Center for Biologics, Evaluation and Research (CBER) Office of Cellular, Tissue and Gene Therapies (OCTGT) regarding Ad5-hGCC-PADRE. After reviewing the data and discussing it with his colleagues, he suggested that we move forward with the Investigational New Drug (IND) application employing the initial Master Virus Bank of Ad5-hGCC-PADRE that produced 9/10 positive flasks. Safety issues associated with this level of RCA are minimal. Indeed, wild type replication-competent Ad5 is largely benign in humans. There is substantial data documenting the benign nature of Ad5 as a natural human pathogen, as a vaccine, and as an anti-cancer agent. Ad5 infections in juveniles are endemic but benign, and most are sub-clinical. The most common symptoms of an Ad5 infection are flu-like, and include cough, gastroenteritis, conjunctivitis, cystitis, and rarely pneumonia. In addition, fully replicating oral adenoviral vaccines were given to thousands of military recruits in the 1960s without adverse effects. Various other examples of experimental infection of human volunteers or non-human primates with live non-attenuated natural or recombinant replication-competent adenoviruses have proven safe, despite evidence of viral replication *in vivo*. The only human patients known to be at risk from replicating adenoviruses are severely immunosuppressed patients. Subject inclusion and exclusion criteria in the proposed clinical trial of Ad5-hGCC-PADRE are designed to ensure that subjects in the study are not immunocompromised. Moreover, an intramuscular route of administration minimizes systemic exposure to Ad5-hGCC-PADRE, compared to an IV route. In the context of these data and preclinical safety data below, safety of RCA+ Ad5-hGCC-PADRE is not a significant concern. Mechanisms that produce RCA in Ad5-hGCC-PADRE and potential solutions are being explored. In the context of a successful outcome from the current study, future phase II and III clinical trials will employ Ad5-hGCC-PADRE meeting RCA guidelines, to ensure that subjects in future studies receive a consistent product.

2) Preclinical Pharmacology and Toxicology

Having identified the vaccine candidate as Ad5-hGCC-PADRE, preclinical studies were conducted under GLP conditions to define the pharmacology, pharmacokinetics (biodistribution) and toxicology in mice. However, examination of the sequence homology between murine and human GCC revealed only 69% identity. Thus, investigating Ad5-hGCC-PADRE in mice does not sufficiently model the human situation. Immune responses produced with Ad5-hGCC-PADRE in mice would likely target regions of dissimilarity between mouse and human GCC, producing immune responses that could not recognize mouse GCC. Thus, for studies of safety and efficacy in mice, mouse GCC-expressing vector (Ad5-mGCC-PADRE) was employed. This vector is limited by tolerance mechanisms operating against the self-antigen mouse GCC, and

produces immune responses that are quantitatively and qualitatively representative of those expected in human subjects immunized with Ad5-hGCC-PADRE. Similarly, because xenoantigen-specific responses are unlikely to cross-react with native antigen, Ad5-mGCC-PADRE, but not Ad5-hGCC-PADRE, is capable of producing immune responses that could recognize mouse GCC in intestine and produce autoimmune pathology. Therefore, only immunization of mice with Ad5-mGCC-PADRE is representative of the autoimmunity risk that could be seen in subjects immunized with Ad5-hGCC-PADRE. This topic was discussed during pre-PreIND and PreIND meetings with the FDA and it was agreed that preclinical studies with Ad5-mGCC-PADRE, not Ad5-hGCC-PADRE, would be most informative. Thus, Ad5-mGCC-PADRE was used in preclinical pharmacology, pharmacokinetics (biodistribution) and toxicology studies, producing results that are representative of those that could be expected when immunizing subjects with Ad5-hGCC-PADRE.

The Ad5-mGCC-PADRE test vector used for those GLP preclinical studies was produced under GLP conditions at the Baylor College of Medicine in the CAGT Vector Development Lab (VDL). The Ad5-mGCC-PADRE plasmid was produced in the same Ad5/CMV/V5 backbone at Thomas Jefferson University that was employed in Ad5-hGCC-PADRE. The plasmid was then transferred to the Baylor College of Medicine for GLP manufacturing. Importantly, the GLP and GMP laboratories use identical manufacturing procedures: both amplified virus in HEK293 cells, both employ CsCl ultracentrifugation to purify the virus and both formulate into a glycerol-based storage buffer. Moreover, the GMP laboratory provided an aliquot of the HEK293 master cell bank for production of the Ad5-mGCC-PADRE to ensure that it was produced in the same cells as the Ad5-hGCC-PADRE. After completing Ad5-mGCC-PADRE manufacturing, it was tested for RCA (negative), sterility (negative), mycoplasma (negative), and host cell DNA contamination (negative), before employing it in preclinical studies.

Pharmacology

Two large GLP studies were performed by WuXi AppTec, Inc to support or IND application. The first (study number 151047) was a maximum tolerated dose study and it will be described in greater detail in the Toxicology section below. The second study (number 151048) was a large 90-day study examining the immunogenicity, biodistribution and toxicity of Ad5-mGCC-PADRE. Each arm of that study (immunogenicity, biodistribution and toxicity) employed separate, but identically treated, animals reflecting the different processing requirements for the assays performed in each arm. The design of study 151048 is summarized below in Table 2. For clarity, each arm (immunogenicity, biodistribution and toxicity) is described within the respective sections below (Pharmacology, Pharmacokinetics and Toxicology).

Table 2
90-Day Toxicity, Immunogenicity and Biodistribution Study Design (Study 151048)

ARTICLE	DOSAGE	NUMBER OF ANIMALS (MALE/FEMALE)	IN-LIFE DURATION (DAYS) 14	30 ± 1	90 ± 1	SAMPLE COLLECTION AT TERMINATION
Control	0	Toxicity: 15M/15F	5M/5F	5M/5F	5M/5F	Blood for hematology, clinical chemistry; Tissues for histology.
		Immunogenicity: 15M/15F	5M/5F	5M/5F	5M/5F	Blood for ELISA, Spleen for ELISpot
		Biodistribution: 15M/15F	5M/5F	5M/5F	5M/5F	Blood for PCR, Tissues for PCR.
Ad5-mGCC-PADRE	10^{10} vp	Toxicity: 15M/15F	5M/5F	5M/5F	5M/5F	Blood for hematology, clinical chemistry; Tissues for histology.
		Immunogenicity: 15M/15F	5M/5F	5M/5F	5M/5F	Blood for ELISA, Spleen for cell ELISpot
Ad5-mGCC-PADRE	10^{11}vp	Toxicity: 15M/15F	5M/5F	5M/5F	5M/5F	Blood for hematology, clinical chemistry; Tissues for histology.
		Immunogenicity: 15M/15F	5M/5F	5M/5F	5M/5F	Blood for ELISA, Spleen for ELISpot
		Biodistribution: 15M/15 F	5M/5F	5M/5F	5M/5F	Blood for PCR, Tissues for PCR.

In vivo Ad5-mGCC-PADRE immunogenicity in C57BL/6 mice (WuXi Apptec Study No. 151048)

The purpose of these assessments was to examine both the humoral and T cell responses to the GCC and PADRE epitopes in the context of a 90-day GLP safety study in immunocompetent C57BL/6 mice. Ad5-mGCC-PADRE was administered to C57BL/6 mice once i.m. on Day 0 at 0 vp (viral particles), 10^{10} vp, and 10^{11} vp. All doses were prepared by dilution in buffer including the control group, which received buffer only. Animals were terminated on day 14, day 29 or day 90. Blood was collected, processed into plasma and stored for antibody analysis by ELISA. Spleens were collected, processed into a single-cell suspension and cryopreserved for T cell analysis by ELISpot.

Antibody Responses

mGCC-specific antibody responses were quantified at a fixed dilution (1/20) of plasma and compared to a standard curve of mouse monoclonal antibody specific for GCC (MS20). The limit of quantification for the assay was 20 ng/mL MS20 equivalents, reflecting the 1:20 dilution of samples and the lowest standard curve quantity of 1 ng/mL. All standard curves produced an R^2 value >0.99 confirming the accuracy of curve fitting. Anti-GCC antibody concentrations were interpolated from duplicate measurements and were undetectable in the 0 vp group, ranged from undetectable to 4287.7 ng/mL in the 10^{10} vp group and were undetectable to 5358.1 ng/mL in the 10^{11} vp group. Across all time points and both genders, 0/30 control (0 vp) mice exhibited GCC-specific antibodies above the limit of quantification. In comparison, GCC-specific antibody responses were detected in both the 10^{10} vp and 10^{11} vp Ad5-mGCC-PADRE-immunized groups, producing 70% and 86.7% overall response rates, respectively. There were highly

significant differences in response magnitude based on dose, sex and time. The magnitude of GCC-specific responses also increased with dose. Furthermore, responses increased from day 14 to day 29, and remained at approximately day 29 levels on day 90. In general, responses were also greater in females than males. Results are summarized in Table 3 below.

Table 3
GCC-specific antibody responses–Effect of time, dose and gender in C57BL/6 Mice (Study 151048)

Test/Control	Day	Male			Female		
		N	% responders	Mean antibody levels +/- SD	N	% responders	Mean antibody levels +/- SD
Control (0 vp)	14	5	0	-	5	0	-
	29	5	0	-	5	0	-
	90	5	0	-	5	0	-
10^{10} vp	14	5	0	-	5	60	65.9 +/- 56.9
	29	5	100	147.1 +/- 178.0	5	80	1372.5 +/- 1946.2
	90	5	80	254.08 +/- 66.9	5	100	381.5 +/- 256.7
10^{11} vp	14	5	80	145.9 +/- 174.2	5	80	551.7 +/- 209.8
	29	5	100	1817.9 +/- 891.7	5	100	1539.9 +/- 1757.3
	90	5	60	813.2 +/- 780.7	5	100	1730.3 +/- 2047.2

Rounded to nearest 1 decimal. The limit of quantification is 20. For the purposes of determining the mean, non-responders were considered 0.

T Cell Responses

Viable cell yields were sufficient to test responses specific for GCC, PADRE and the positive and negative controls in 78/90 samples. Thus, 12/90 samples were tested against only select antigens. No control (0 vp) animals produced GCC-specific or PADRE-specific responses at any time point or dose or in either sex. GCC-specific responses were observed in some 10^{10} vp and most 10^{11} vp animals, producing 16.7% and 53.3% overall response rates, respectively. The magnitude of responses increased over time ($P=0.011$), and with dose ($P<0.001$) and were unaffected by sex ($P>0.05$). GCC-specific T cell responses are summarized in below.

Table 4
GCC-specific T cell responses – time, dose and sex comparisons in C57BL/6 Mice
(Study 151048)

Test/Control	Day	Male			Female		
		N	% responders	Mean T cell levels +/- SD	N	% responders	Mean T cell levels +/- SD
Control (0 vp)	14	5	0	0 +/- 0	5	0	0.3 +/- 0.5
	29	5	0	0.4 +/- 0.5	5	0	1.0 +/- 1.1
	90	5	0	0.5 +/- 1.0	5	0	0.8 +/- 1.2
10^{10} vp	14	5	0	0.3 +/- 0.6	5	20	2.6 +/- 5.3
	29	5	40	18.5 +/- 25.4	5	0	1.1 +/- 1.6
	90	5	40	30.5 +/- 54.3	5	0	0.1 +/- .1
10^{11} vp	14	5	40	9.9 +/- 16.0	5	40	18 +/- 24.2
	29	5	60	104.1 +/- 117.5	5	40	46 +/- 68.7
	90	5	60	37.5 +/- 46.5	5	80	79.1 +/- 84.3

Numbers indicate spots/10^6 splenocytes. Responder is >5 spots and >4x above background. Rounded to nearest 1 decimal.

PADRE-specific responses were observed in most 10^{10} vp and 10^{11} vp animals, producing 63.3% and 89.5% overall response rates, respectively. The dose of Ad5-mGCC-PADRE had a significant impact on the magnitude of responses (P<0.001). However, time impacted responses only marginally (P=0.072), and responses were unaffected by mouse gender (P=0.67). PADRE-specific T cell responses are summarized below in Table 5 below.

Table 5
PADRE-specific T cell responses – time, dose and sex comparisons in C57BL/6 Mice
(Study 151048)

Test/Control	Day	Male			Female		
		N	% responders	Mean T cell levels +/- SD	N	% responders	Mean T cell levels +/- SD
Control (0 vp)	14	4	0	0.3 +/- 0.5	5	0	1.0 +/- 1.1
	29	5	0	0.6 +/- 0.4	5	0	0.4 +/- 0.5
	90	5	0	0.9 +/- 1.1	5	0	0.3 +/- 0.4
10^{10} vp	14	5	60	18.0 +/- 16.1	5	80	27.9 +/- 30.3
	29	5	40	9.5 +/- 10.7	5	80	31.9 +/- 18.3
	90	5	80	20.7 +/- 11.9	5	40	10.0 +/- 6.1
10^{11} vp	14	5	100	64.0 +/- 46.6	4	100	55.8 +/- 43.5
	29	2	100	18.0 +/- 14.1	5	100	33.1 +/- 8.7
	90	0	NA	NA	3	33.3	10.7 +/- 12.5

Numbers indicate spots/10^6 splenocytes. Responder is >5 spots and >4x above background. Rounded to nearest 1 decimal. NA = not tested due to insufficient sample.

Relationship Between Antibody and T Cell Responses

Comparison of the three immune responses measured within in each mouse revealed that GCC-specific T cell responses were a slightly better predictor of GCC-specific antibody responses

than PADRE-specific T cell responses. Comparing antibody response rates in GCC-specific T cell responders and non-responders revealed that 90.5% of T cell responders were also antibody responders, however, 40.6% of T cell non-responders were also antibody responders. In contrast 77.8% of PADRE responders were also antibody responders, while 23.8% of T cell non-responders were also antibody responders. Thus, neither measure was an accurate predictor. Examination of response magnitudes revealed a positive correlation between magnitude of antibody responses and GCC-specific T cell responses ($R^2 = 0.22$; $P<0.0001$) and between antibody responses and PADRE-specific T cell responses ($R^2 = 0.20$; $P<0.0001$), but not between GCC and PADRE –specific T cell responses ($R^2 = 0.02$; $P = 0.17$).

Pharmacokinetics

A 90-Day Biodistribution Assessment of Ad5-mGCC-PADRE in Mice (WuXi Apptec Study No. 151048)

The tissue biodistribution of Ad5-mGCC-PADRE was evaluated in groups of male and female C57BL/6 mice in context of the larger GLP safety, immunology and biodistribution study (study 151048). For the biodistribution arm of this study, Ad5-mGCC-PADRE was administered to groups of 5 C57BL/6 mice per sex by intramuscular injection once on Day 0 and at a fixed dose of 10^{11} vp. A vehicle control group was also included. All doses were prepared by dilution in formulation buffer including the vehicle control, which received formulation buffer only. Groups of mice were sacrificed on day 14, day 29 or day 90. Blood and tissues were collected for quantification of biodistribution using a validated qPCR method.

DNA was isolated from the following tissues: blood, injection site, ovaries, bone marrow, kidneys, small intestine, brain, large intestine, spleen, draining lymph node, liver, stomach, heart, lungs and testes. qPCR was performed on the samples employing a validated assay to quantify Ad5-mGCC-PADRE DNA copy number. The limit of quantification for the validated assay was ≤ 10 copies of Ad5-mGCC-PADRE linearized plasmid DNA standard. Further, assay validation demonstrated that >25 copies of standard DNA can be detected in DNA isolated from mouse tissues post-spiked with 50 copies of standard Ad5-mGCC-PADRE DNA.

The percentage of tissue samples having detectable vector is reported in **Table 6**. Overall, vector DNA was detectable in each tissue type of at least one animal receiving a fixed dose of 10^{11} vp of Ad5-mGCC-PADRE. At Day 14, vector was detected at the injection site, bone marrow, spleen and liver of all animals. Vector was detected in the lymph node, kidneys, lungs, blood and heart of at least 50% of the animals. Copy number was greatest at the injection site, followed by the lymph nodes and bone marrow. Copy number was greater than 10,000 at the injection site, bone marrow and lymph node in at least 50% of the animals.

At Day 29, vector levels were present at decreased frequency in the bone marrow, lymph node, kidneys, lungs, blood, and heart of animals receiving the test article dose of 1011 vp. At this time point, vector remained detectable at the injection site, spleen and liver of all animals. High copy number, defined as > 10,000 copies, remained at the injection site and in the bone marrow and decreased in the lymph node.

At Day 90, vector DNA was still detectable in each tissue type except the testes of at least one animal. The overall pattern of expression, however, was decreasing in frequency and magnitude. The frequencies of high copy numbers at the injection site and bone marrow remained essentially unchanged. By Day 90, vector remained at > 10,000 copies at the injection site (8/10 animals),

bone marrow (5/10 animals), lymph nodes (1/10 animals), large intestine (1/10 animals) and small intestine (1/10 animals).

There was no clear trend between male and female mice. Gender differences were evaluated based on high copy number. The vector biodistribution was similar among male and female animals when evaluating tissues consistently having high copy numbers (> 10,000). Values at the injection site, lymph nodes and bone marrow were similar among male and female animals. Blood, stomach, brain, kidney, lungs, heart and testes/ovaries consistently expressed < 10,000 copies in both male and female animals at all time points. In the remaining tissues, no consistent patterns related to animal gender were identified. Low frequency responses (1/5 animals, 20%) were scattered among the remaining tissues over all time points.

Table 6
Percentage of Ad5-mGCC-PADRE positive tissues

Tissue [N=10, except ovaries and testes (n=5)]	Overall percentage			Percentage > 10,000 copies		
	Day 14	Day 29	Day 90	Day 14	Day 29	Day 90
Injection site	100%	100%	100%	80%	90%	80%
Bone marrow	100%	70%	100%	50%	40%	50%
Draining lymph node	70%	50%	50%	70%	40%	10%
Large intestine	30%	50%	30%	0%	10%	10%
Small intestine	30%	30%	40%	0%	10%	10%
Spleen	100%	100%	80%	10%	10%	0%
Liver	100%	100%	90%	0%	10%	0%
Kidneys	70%	40%	50%	0%	0%	0%
Lungs	70%	40%	20%	0%	0%	0%
Blood	50%	20%	20%	0%	0%	0%
Heart	50%	10%	20%	0%	0%	0%
Stomach	20%	30%	10%	0%	0%	0%
Brain	10%	0%	20%	0%	0%	0%
Ovaries	0%	20%	20%	0%	0%	0%
Testes	40%	40%	0%	0%	0%	0%
% indicates percentage positive mice per tissue for each tissue type						

Toxicology

An initial maximum tolerated dose (MTD) study (WuXi AppTec Study Number 151047) was performed to establish the maximum deliverable/tolerable dose in C57BL/6 mice. Subsequent to this study, a 90-day GLP safety, biodistribution and immunology study was performed (WuXi AppTec Study Number 15148), and the toxicology results are presented here.

Acute Maximum Tolerated Dose Study in Mice - Single IM Injection (WuXi Study Number 151047)

Ad5-mGCC-PADRE was administered to C57BL/6 mice (5 mice/sex/group) by the intramuscular route once on day 0 at 1.7x1010 vp/kg, 4.25x1011 vp/kg, and at a fixed dose of 1011 vp (~4x1012 vp/kg at mean group weight of 24 g/mouse). A vehicle control group was also included, and all doses were prepared by dilution in formulation buffer. In-life toxicological

endpoints included: clinical observations, body weights feed consumption, hematology, and clinical chemistry. Mice were sacrificed on day 14, and gross pathology was evaluated.

All animals survived to study termination and appeared normal throughout the study. There were no toxicologically significant changes or effects on body weight, feed consumption, hematology, clinical chemistry or necropsy findings. There were no meaningful toxicological findings for hematology and clinical chemistry.

90-Day Single-Dose Intramuscular Toxicity Assessment in Mice (WuxI Study number 151048)

The purpose of GLP study number 151048 was to examine the safety, biodistribution and immunological effects of Ad5-mGCC-PADRE for up to 90 days post-treatment following a single i.m. dose in immunocompetent C57BL/6 mice. The immunology and biodistribution results of this study are presented above and the toxicology results are presented here.

Ad5-mGCC-PADRE was administered to C57BL/6 mice i.m. once on Day 0 at 1010 vp and 1011 vp. A control group was also included and received formulation buffer only. Animals were terminated on day 14, day 29 or day 90. Blood was collected for hematology and clinical chemistry assessment prior to necropsy. Tissues were collected for histology. The 90-day toxicity study was conducted in conjunction with Immunogenicity and Biodistribution studies described above. Thus, some analyses (such as mortality and clinical observations, body weights, etc.) were performed on animals in the immunogenicity, biodistribution and toxicology arms to increase the number of mice analyzed. Table 7 indicates the toxicological endpoints measure in each arm of the study.

Table 7
Toxicology Endpoints Measured in Study 151048 Arms

	Study Number 151048 Arm		
Toxicology Endpoint Measured (checked)	**Immunogenicity**	**Biodistribution**	**Toxicology**
Mortality/Moribundity	✔	✔	✔
Clinical Observations	✔	✔	✔
Body Weights	✔	✔	✔
Feed Consumption			✔
Hematology			✔
Clinical Chemistry			✔
Necropsy		✔	✔
Organ Weighs			✔
Histopathology			✔ (10^{11} vp/ animal dose and controls only)

Clinical observations for all animals prior to dosing, within 5 minutes post-dose, approximately 1-hour post-dose and approximately 3 hours post-dose were normal. Two hundred thirty-five of two hundred forty animals (97.9%) survived to study termination across the immunogenicity, biodistribution and toxicology arms of the study. One vehicle control mouse experienced lethargy with labored respiration on day 20 and early death on day 21. Four animals were humanely euthanized early due to ulcerative dermatitis (1 mouse at 0 vp, 1 mouse at 10^{10} vp and 2 mice 10^{11} vp). Spare animals were available to replace the five animals that did not survive until the scheduled termination date.

The most frequent abnormal clinical observation was alopecia and/or idiopathic ulcerative dermatitis, which were observed in sixteen mice (4 of which were humanely euthanized) spread across the safety, biodistribution and immunogenicity studies (5 mice at 0 vp, 2 mice at 10^{10} vp and 9 mice at 10^{11} vp). Other abnormal clinical observations included a cloudy eye (1 mouse at 10^{11} vp, day 77 through day 90). Mortality and clinical observations were not affected by test article treatments.

No treatment-related or toxicologically significant differences in body weight, body weight change, hematology or clinical chemistry were found among treatment groups.

Necropsy was performed on the animals in the biodistribution and toxicology arms of study 151048, but not the immunogenicity arm due to animal processing requirements for immune evaluation. With the exception of four animals (of the 150 total animals in the biodistribution and toxicology arms), tissues appeared macroscopically normal. Minor abnormalities, not related to the vector, were found in the other 4 animals. No treatment-related or toxicologically significant effects on organ weights were found.

Histopathology was performed on the vehicle and high-dose treated mice (0 vp or 10^{11} vp cohorts). Tissues from the lower dose (10^{10} vp) cohort were archived for future analysis if warranted. No specific patterns of treatment-related findings were present in the internal organs of mice treated with 0 vp or 10^{11} vp of Ad5-mGCC-PADRE.

Summary of Preclinical Pharmacology and Toxicology

Intramuscularly-administered Ad5-mGCC-PADRE at a dose of 10^{11} vp/animal (~4.5×10^{12} vp/kg) distributed primarily to the injection site, draining lymph nodes, liver, spleen and bone marrow. Levels in the injection site and barrow remained relatively constant during the 90-day evaluation period. Despite that biodistribution pattern, the anticipated antibody and T cell immune responses were observed, there were no treatment-related toxicities or adverse responses to the vector observed and the vector was well-tolerated at doses up to 10^{11} vp (4.5×10^{12} vp/kg). This dose is 3,600 fold higher than the proposed first-in-man human dose.

Based on the available data and lack of any significant toxicity in mice, these data supported the initiation of the proposed first-in man clinical study with the human clinical product Ad5-hGCC-PADRE and were included in applications to obtain regulatory approval for clinical testing.

3) Regulatory Approval

Regulatory approval of the vaccine focused on two parallel tracks: federal and institutional. Because our vaccine involves administration of recombinant DNA to humans, review by the Recombinant DNA Advisory Committee (RAC) of the NIH, Office of Biotechnology Activities was required. Our protocol was unanimously approved by the RAC at their quarterly meeting June 11th, 2013. Subsequently, institutional approvals (Clinical Cancer Research Review Committee; Institutional Biosafety Committee; and the Institutional Review Board) were obtained. The Investigational New Drug application was submitted to the FDA on October 16, 2013 and approved November 10, 2013.

4) Clinical Studies

The study proposed a phase I multi-institution study to assess the safety and immunogenicity of Ad5-hGCC-PADRE in Stage I and II colon cancer subjects.

Annually, approximately 51,370 Americans die of CRC, accounting for approximately 9 percent of all cancer deaths. While "curative" surgery removes all detectable tumor and is most successful in early-stage disease, occult metastases result in relapse. Recurrence rates range from ~10% for disease limited to mucosa (stage I) to >50% for tumors metastatic to lymph nodes (stage III). Standard treatment for patients with colon cancer is surgical resection of the primary tumor and regional lymph nodes for localized disease. The most important indicator of outcome following resection of colon cancer is the pathologic stage at presentation. The benefits of adjuvant chemotherapy are controversial for stage II (node-negative) disease while there is a clear benefit in stage III (node-positive) disease, with an approximate 30 percent reduction in disease recurrence and a 22 to 32 percent reduction in mortality. Thus, there is significant medical need for therapies to prevent relapse in early stage (I and II) colon cancer patients. GCC is a tumor-associated antigen expressed by nearly all colorectal (CRC) specimens, regardless of anatomical location or grade. The GCC-expressing adenovirus vaccine Ad5-GCC-PADRE induces GCC-specific immune responses in mice, producing antitumor immunity. Importantly, in addition to efficacy, Ad5-GCC-PADRE is very safe, producing no observable adverse effects in animal studies. Thus, Ad5-hGCC-PADRE is poised to meet the unmet need for therapies to prevent relapse in early stage colon cancer patients.

A Phase I clinical trial was designed to assess the safety and immunological activity of Ad5-hGCC-PADRE in early-stage colon cancer patients. The study objectives and design are as follows:

The Study Objectives

Primary Objectives

a) To determine the safety, tolerability and toxicity of Ad5-hGCC-PADRE

b) To determine whether Ad5-hGCC-PADRE induces an antibody response to GCC at 1 month following vaccination employing a validated ELISA assay.

Secondary Objectives

a) To determine whether Ad5-hGCC-PADRE induces a T cell response to GCC at 1 month following vaccination

b) To determine whether Ad5-hGCC-PADRE induces antibody and/or T cell responses to GCC that persist at 3 months and 6 months following vaccination

c) To determine whether development of antibody and/or T cell responses to GCC are related to:

- occult metastases quantified by GCC qRT-PCR
- race
- time to recurrence or disease-free survival

Table 8
Study Design

Visit	**Visit 1**	**Visit 2**			**Visit 3**	**Visit 4**	**Visit 5**
Day	**D28 ~ D7**	**D1**	**D3±1**	**D8±1**	**D30±3**	**D90±10**	**D180±10**
Consent	X						
Pathology Release Form	X						
Demographic Data	X						
Medical History	X						
Physical Exam (Including Weight)	X				X	X	X
Vital Signs	X	X			X	X	X
Safety Labs	X				X	X	X
Performance Status	X				X	X	X
HIV	X						
Pregnancy Test	X						
CEA	X						X
ANA	X				X	X	X
Immunomonitoring Blood		X			X	X	X
Ad5-hGCC-PADRE Injection		X					
Telephone Safety Assessment			X	X			
Concomitant Medication	X	X	X	X	X	X	X
Assess for Adverse Events	X	X	X	X	X	X	X

Clinical trial enrollment initiated immediately after FDA approval and the first subject was vaccinated on November 20, 2013. Currently, a total of 10 subjects have been vaccinated (Figure 1) and ***zero*** adverse reactions have occurred. Each subject will return to Thomas Jefferson University for 3 follow-up visits at 30, 90 and 180 days after vaccination. Each visit includes clinical assessment by physical exam and blood work and includes collection of blood samples to monitor vaccine activity (immunomonitoring). The study design included two primary endpoints: safety and immune activity and the study will be stopped if the vaccine is unexpectedly toxic or unexpectedly inactive. For the purposes of this clinical trial, immune activity is defined as GCC-specific antibody immune responses at 30-days post vaccination. If 1 or more of the first 10 subjects produce an antibody response at 30 days, enrollment will continue and 12 additional (22 total) subjects will be enrolled. Day 30 samples were collected from subject #10 on July 3, 2014 and assessment of day 30 antibody responses for the 10 enrolled subjects is ongoing.

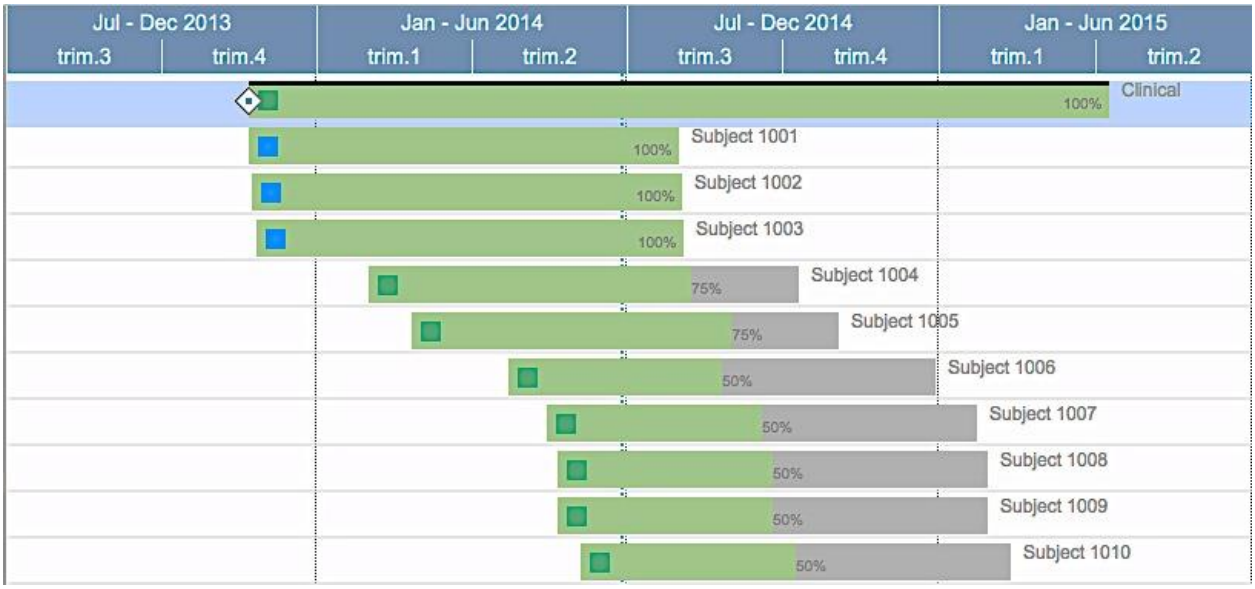


Figure 1. Progress on follow-up for the phase I trial. Ten subjects have been vaccinated. Three subjects have completed all visits (30, 90 and 180 days post-vaccination). Remaining subjects have completed 1-2 visits each**.**

Preclinical studies suggest that T cell responses to GCC and to the PADRE component of the vaccine are critical to the antitumor activity of the vaccine. Thus, full immunomonitoring studies will be carried out on all subjects to include antibody responses to GCC and the Ad5 vector and T-cell responses to GCC, PADRE and Ad5 at 30, 90 and 180 days post vaccination. Full assay validation including those for sample processing, ELISA (antibody; Figure 2) and ELISpot (T cell; Figure 3) were performed prior to analyzing samples from vaccinated subjects. Full immunomonitoring assessment is ongoing for the 3 subjects that have completed all follow-up visits and will be conducted for the remaining subjects following their final visit.

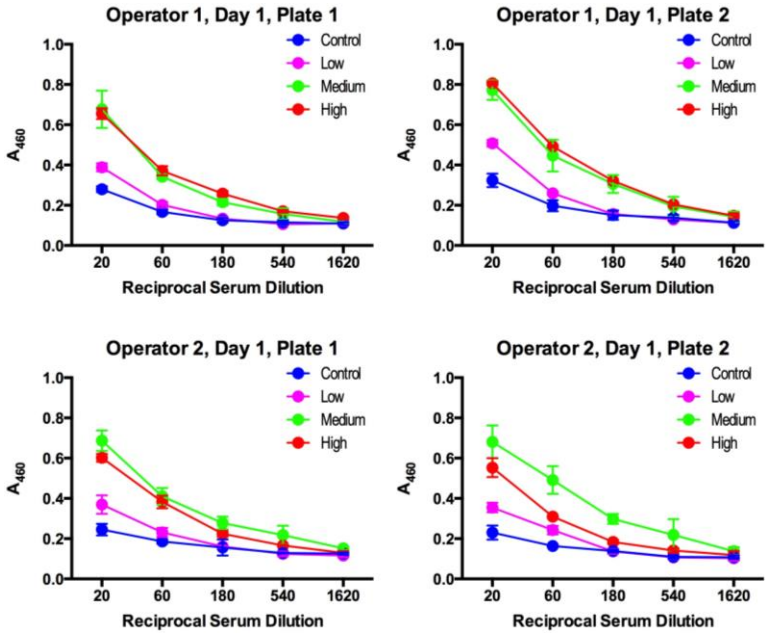


Figure 2. ELISA Assay Development and Validation. GCC-specific immune responses have never been measured in humans so a novel hGCC ELISA was developed and validated. A total of 23 development/validation experiments were carried out. A representative validation study is shown here to determine the inter-operator, inter-plate, inter-day reproducibility. Negative control serum was spiked with GCC monoclonal antibody at high, medium and low concentrations corresponding to the magnitude of responses observed in preclinical mouse experiments. Overall, a robust, sensitive and reliable assay was validated for assessing antibody responses to hGCC in human serum samples.

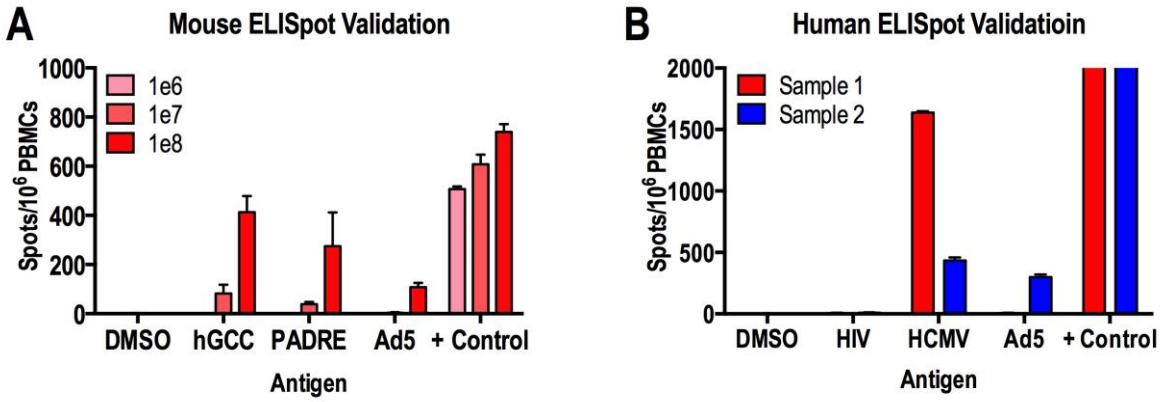


Figure 3. ELISpot Assay Validation A) A library of peptides spanning hGCC was synthesized. Mice were vaccinated with $1x10^6$ to $1x10^8$ IFU of Ad5-hGCC-PADRE and reactivity to the hGCC peptide library was confirmed. The hGCC peptide library will be used to quantify patient T-cell responses. **B**) Human ELISpot assay was validated on two human samples employing HIV and HCMV as positive and negative antigens, respectively. Ad5 responses were also confirmed. Overall, a robust, sensitive and reliable assay was validated for assessing T-cell responses to hGCC in human samples.

AIM 2. BARRIERS UNDERLYING RACIAL DISPARITIES IN PARTICIPATION IN CANCER VACCINE TRIALS (HEALTH SERVICES RESEARCH AIM).

Phase 1: Generation of the Decision Aid

Literature Review. A review of the literature identified factors positively associated with patient participation in clinical research, including white race, education, trust in study personnel, knowing others who have participated in research, and social support for participation. Factors negatively associated include lack of knowledge about the study, concerns about being part of an experiment, restrictive eligibility criteria, onerous participation requirements and costs, limited accessibility, fear of side effects, and fatalism. Decision aids have been reported to help patients make informed decisions about complex health care choices, such as participating in a vaccine trial. The most effective decision aids provide easy to understand information, facilitate values and preference clarification, and involve interaction with a trusted health professional.

Surveys of the Research Team and Patients. A draft informational booklet on a hypothetical CRC vaccine trial was distributed to 14 research team members to assess whether it clearly explained the purpose of the trial, contained accurate content, was easy to read, presented both pros and cons associated with trial participation, explained important keywords and concepts, covered appropriate topics, and included sufficient information to allow informed decision making about participation. Thirteen respondents from all three participating institutions provided feedback. Respondents recommended inclusion of more information about trial procedures and requirements for trial participation. The booklet was revised in accordance with these recommendations. The revised booklet was reviewed by colon cancer patients at all study sites. Surveys were completed by 20 patients, completing overall recruitment goals for Phase 1. Survey data revealed that older African American and Caucasian colorectal cancer patients found information in the booklet challenging to read and comprehend. In response to this feedback, and suggestions from the Interim Performance Review, we identified educational consultants (The Clear Language Group, see *Advisory Committee* below) that guided revision of the booklet, to add subheadings and to lower the reading level of the booklet from an 11th grade to an 8th grade reading level.

Decision Counseling Program. We completed development of an online decision counseling program, and created a draft Decision Counseling Training Manual. The manual includes the following sections: a research project overview, decision counseling background (purpose, theory, history, terms and examples), training specifics (roles, program components, how to conduct a session), and demonstration/evaluation of expected knowledge and competencies.

Advisory Committee. We convened a community advisory committee to guide participant recruitment and participation. J. DeBoy, PhD, Professor, Lincoln University, is the chair, , and current members include J. Ohene-Frempong, MS, Principal & Founding Member, The Clear Language Group, and S.B. Thomas, PhD, Director, Maryland Center for Health Equity. We are adding 2 colon cancer patients.

Phase 2 Survey: Testing the Decision Aid

Forty-nine patients completed the revised Phase 2 survey questionnaire.

Analysis Methods. Initially, frequency distributions of survey variables were generated, and univariable analyses were performed to assess the associations between independent variables (demographic background, knowledge, and attitudes) to the outcome variable (interest in trial

participation). Initially, outcome variable values was defined as "Yes", "Unsure," and "No" categories. For univariable analyses, independent variables were cross-tabulated with the outcome variable, which was dichotomized as "Yes" versus "Unsure or No" due to the small number of "No" responses (n=5). Fisher's Exact Tests were computed to assess statistical significance of the associations with outcome. Candidate variables that had a p-value of 0.20 or less, indicating that they might be significantly associated with the outcome, were included as predictors in a multivariable logistic regression model. After each model was estimated, the predictor with the highest p-value was removed and the model re-estimated until all of the remaining predictors were significantly associated with participation at the p=0.05 level. The demographic items (i.e., age, race, and gender) were included regardless of significance level. Logistic regression models were computed using SAS version 9.2.

A.2. Results. All participants reported that that the vaccine informational booklet was easy to read and described the purpose of the vaccine well. Most participants agreed that the eligibility criteria related to trial participation were explained well (82%), vaccine safety concerns were adequately addressed (84%), and sources of further information were clearly presented (84%). Almost all respondents (90%) reported that they would recommend the booklet to others.

As shown in Table 1, the distribution of background characteristics among Phase 2 survey respondents was as follows: < 65 years of age (57%), female (63%), white (53%), > high school education (53%), and married (49%). In terms of knowledge about the vaccine trial, 76% of survey respondents answered at least 9 out of 10 knowledge questions correctly. Perceptions related to colon cancer and the vaccine trial were distributed as follows: susceptible to colon cancer recurrence (47%), worries and concerns related to colon cancer recurrence (73%), worries and concerns about the vaccine trial (47%), salience and coherence related to trial participation (94%), response efficacy related to trial participation (84%), social support for trial participation (71%), belief that trial participation would be expensive (6%), belief that trial participation would require a lot of time and effort (12%), believe that trial participation would benefit others (96%), fatalistic attitude related to participation (31%), religiosity (44%), and mistrust of researchers (4%). In terms of outcome, 20 (41%) survey respondents reported that they were interested in participating in the vaccine trial, while 29 (59%) respondents indicated that they were either unsure about or were not interested in participating.

Univariable analysis results indicate that the following variables were associated with interest in trial participation at $p < 0.20$: age (p=0.152), marital status (p=0.080), education (p=0.152), knowledge (p=0.007), study worries and concerns (0.002), and trial participation response efficacy (p=0.015). The results of multivariable analyses (Table 2) show that factors significantly associated with interest in vaccine trial participation were: being male (OR=11.29, 95% CI: 1.71, 74.07), being African American (OR=5.24, 95% CI: 1.05, 26.02), and having a low level of worries and concerns about trial participation (OR=2.51, 95% CI: 2.51, 85.28).

Phase 3 –Decision Support for Participation in Colon Cancer Vaccine Trial:

The research team adapted a novel online decision support intervention (DSI) known as the Decision Counseling Program© (DCP) for use in facilitating patient participation in the vaccine trial. The DCP is a vehicle to deliver informational content to patients about available options related to any designated health decision. Here, we introduced the vaccine trial informational booklet as a decision aid. The DCP also is a tool that can be used to guide patients through a values elicitation and personal preference clarification exercise related to available options.

The research team configured the DCP for use in clarifying whether patients preferred to join the vaccine trial (Option 1) or not to join the trial (Option 2). Briefly, a trained decision counselor meets a patient, and uses the online DCP to review pertinent informational content and completes a decision counseling session. At the conclusion of the session, the decision counselor employs the DCP to produces a 1-page summary report, which displays the patient's preferred option (Option 1 or 2), how strongly the patient feels about the preferred option, and what factors are influencing preference.

In addition to adapting the DCP for use in the context of the vaccine trial, the research team trained 4 decision counselors to use the DCP. A representative of each study site – Jefferson, Pittsburgh, Fox Chase and Albert Einstein - underwent a 2-day training course. The training included a DCP overview, introduction to helping skills, DCP background and theory, case presentations, role playing, discussions and feedback. Refresher training was also offered to reinforce skills.

In terms of patient recruitment to the vaccine trial, patients potentially eligible for the trial were initially identified by clinicians and from electronic medical records. Potential participants were sent an invitation letter and the study educational booklet. This mailing also included instructions for opting out of further contact related to the project. Patients who did not opt out received a call from the designated decision counselor to review the informational booklet and discuss interest in joining the vaccine trial. In some cases, research staff met potential participants at the time of a clinic visit and reviewed these materials. In all sessions, a research staff member accessed the online DCP in order to complete a decision counseling session and determine whether the patient preferred to participate in the trial. Patients were also asked if they were willing to speak with a trial coordinator, irrespective of their expressed preference related to trial participation. Patient contact information for those who affirmed their willingness to speak with a coordinator was sent to trial personnel.

Patient Identification and Decision Counseling. A total of 70 potential participants were identified, and 56 (80%) were successful contacted. These contacts resulted in the following resolutions related to the trial: 19 completed a decision counseling session, 8 were found to be ineligible for the trial, and 29 declined participation in decision counseling and the trial.

Endpoint Survey Administration and Data Analysis. Research staff members attempted to contact the 19 patients who completed a decision counseling session in order to administer an endpoint survey, and were successful in completing the survey with 16 patients. Three patients were lost to follow-up. Frequency distributions of survey variables were generated for the respondents, and univariable analyses were performed to assess the associations between independent variables (i.e., demographic background, knowledge about the vaccine trial, and vaccine trial preference score) and the outcome variable (i.e., actual trial participation).

Results. Table 3 shows that of the 19 individuals who completed a decision counseling session, 14 preferred to join the vaccine trial, 3 were undecided, and 2 preferred not to join the trial. All individuals who completed a decision counseling session agreed to receive a follow-up call from the vaccine trial coordinator to discuss participation further. The trial coordinator succeeded in contacting 15 patients, but could not reach 4 patients. Overall, 16 individuals both completed a decision counseling session and an endpoint survey.

As shown in Table 4, background characteristics of 16 individuals with complete endpoint survey responses and decision counseling session results were as follows: < 65 years of age

(38%), white (81%), female (50%), > high school education (75%), and married (44%). In terms of knowledge about the vaccine trial, 75% of these individuals answered at least 9 out of 10 knowledge questions correctly. We also found that 75% of individuals preferred to participate in the vaccine trial.

Univariable analyses were conducted to determine if patient sociodemographic factors, knowledge about the trial, and trial participation preference were associated with actual participation in the vaccine trial. We determined that 75% of patients with a preference score that favored participation actually participated in the trial; while only 25% of those who a score indicating they were unsure about or did not favor participation did so. Thus, preference score was positively associated (p=0.118) with actual participation. The small number of individuals in this part of the study serves as a limitation to statistical inference.

All 16 of individuals with complete endpoint survey and decision counseling data reported on the survey that that the vaccine informational booklet was easy to read. Most participants also agreed that the booklet described the purpose of the vaccine sufficiently (94%), eligibility criteria related to trial participation were explained well (94%), vaccine safety concerns were adequately addressed (100%), and sources of further information were clearly presented (88%). Finally, 94% of respondents said that they would recommend the booklet to others.

Table 1: Univariable Analysis of Factors Associated with Interest in Trial Participation (n=49)

		Overall		Interested		Not Interested or Undecided		
Item/Scale	value	frequency	percent	frequency	percent	frequency	percent	p-value
Age								0.240
	< 65	28	(57)	9	(32)	19	(68)	
	≥ 65	21	(43)	11	(52)	10	(48)	
Gender								0.139
	male	18	(37)	10	(56)	8	(44)	
	female	31	(63)	10	(32)	21	(68)	
Race								0.155
	White	26	(53)	8	(31)	18	(69)	
	Black	23	(47)	12	(52)	11	(48)	
Marital status								0.080
	Married	23	(49)	13	(57)	10	(43)	
	Living alone	24	(51)	7	(29)	17	(71)	
	(missing)	2		0		2		
Education								0.155
	≤HS	23	(47)	12	(52)	11	(48)	
	>HS	26	(53)	8	(31)	18	(69)	
Knowledge (# correct)								0.008
	0-8	1	(24)	9	(75)	3	(2)	
	9-10	37	(76)	11	(30)	26	(70)	

Table 1: Univariable Analysis of Factors Associated with Interest in Trial Participation (n=49) (continued)

		Overall		Interested		Not Interested or Undecided		
Item/Scale	value	frequency	percent	frequency	percent	frequency	percent	p-value
Salience & Coherence								0.260
	<3	3	(6)	0	0	3	(100)	
	≥3	46	(94)	20	(43)	26	(57)	
Study Worries								0.003
	<3	26	(53)	16	(62)	10	(38)	
	≥3	23	(47)	4	(17)	19	(83)	
Worries/Concerns								1.000
	<3	13	(27)	5	(38)	8	(62)	
	≥3	36	(73)	15	(42)	21	(58)	
Susceptiblity								0.155
	<3	26	(53)	8	(31)	18	(69)	
	≥3	23	(47)	12	(52)	11	(48)	
Response Efficacy								0.015
	<3	8	(16)	0	0	8	(100)	
	≥3	41	(84)	20	(49)	21	(51)	
Social Support & Influence								0.345
	<3	14	(29)	4	(29)	10	(71)	
	≥3	35	(71)	16	(46)	19	54)	
Not expensive								1.000
	<3	3	(6)	1	(33)	2	(67)	
	≥3	46	(94)	19	(41)	27	(59)	
Time & effort OK								0.379
	<3	6	(12)	1	(17)	5	(83)	
	≥3	43	(88)	19	(44)	24	(56)	
Altruism								0.507
	<3	2	(4)	0	0	2	(100)	
	≥3	47	(96)	20	(43)	27	(57)	
Religiosity								0.770
	<3	27	(56)	10	(37)	17	(63)	
	≥3	21	(44)	9	(43)	12	(57)	
	(missing)	1		1		0		
Fatalism								0.218
	<3	33	(69)	11	(33)	22	(67)	
	≥3	15	(31)	8	(53)	7	(47)	
	(missing)	1		1		0		
Mistrust								1.000
	<3	47	(96)	19	(40)	28	(0)	
	≥3	2	(4)	1	(50)	1	(50)	

Table 2: Multivariable Analysis of Factors Associated with Interest in Trial Participation (n=49)

Variable	Values	OR	95% CI Lower	95% CI Upper	p-value
Gender	Female	ref			
	Male	11.29	1.72	74.07	0.0115
Race	White	ref			
	Black	5.24	1.05	26.02	0.0430
Worries/Concerns	≥3	ref			
	<3	14.62	2.51	85.28	0.0029

Table 3: Decision Counseling Vaccine Trial Participation Preference Scores (n=19)

Preference Score Range		Frequency	Percent
Prefer Not to Join the Trial			
0.000-0.332	Overwhelmingly	0	0
0.333-0.356	Very much	0	0
0.357-0.383	Much	1	5
0.384-0.416	Somewhat	0	0
0.417-0.454	A little	1	5
Undecided about Joining the Trial			
0.455-0.544	No preference	3	16
Prefer to Join the Trial			
0.545-0.582	A little	1	5
0.583-0.615	Somewhat	0	0
0.616-0.643	Much	2	11
0.644-0.666	Very much	1	5
0.667-1.000	Overwhelmingly	10	53

Table 4: Univariable Analysis of Factors Associated with Trial Participation (n=16)

		Overall		Participated		Did Not Participate		
Item/Scale	value	frequency	percent	frequency	percent	frequency	percent	p-value
Age								0.307
	< 65	6	(38)	5	(83)	1	(17)	
	≥ 65	10	(62)	5	(50)	5	(50)	
Gender								1.000
	male	8	(50)	5	(62)	3	(38)	
	female	8	(50)	5	(62)	3	(38)	
Race								1.000
	White	13	(81)	8	(62)	5	(38)	
	Black	3	(19)	2	(67)	1	(33)	
Marital status								1.000
	Married	7	(44)	4	(57)	3	(43)	
	Living alone	9	(56)	6	(67)	3	(33)	
Education								1.000
	≤HS	4	(25)	3	(75)	1	(25)	
	>HS	12	(75)	7	(58)	5	(42)	
Knowledge (# correct)								0.604
	0-8	4	(25)	2	(50)	2	(50)	
	9-10	12	(75)	8	(67)	4	(33)	
Trial participation preference								0.118
	No/Undecided	4	(25)	1	(25)	3	(75)	
	Yes	12	(75)	9	(75)	3	(25)	

AIM 3. MECHANISMS SHAPING RESPONSES TO CANCER MUCOSA ANTIGENS THAT INFORM CLINICAL STRATEGIES (MECHANISTIC AIM).

The External Scientific Advisory Committee re-prioritized the laboratory-based aims, focusing on *Chemokine-potentiated heterologous vaccine strategies that amplify GCC-targeted responses* (Aim 3.C) prior to examining T_{reg}-dependent tolerance and GCC responses (Aim 3.B). This strategy reflects the (1) importance of prime-boost strategies to induce maximum GCC-targeted anti-tumor immunity, (2) potential utility of DNA-based strategies to amplify responses to Ad5-hGCC-PADRE, and (3) the low regulatory bar for introducing DNA-based vaccines into patients, creating a unique opportunity to expand clinical studies in Aim 1. Thus, studies in Aim 3.C were initiated in 2011 while studies in Aim 3.B began in 2012.

SUBAIM 3.A. TOLERANCE MECHANISMS TO SELF-ANTIGENS IN INTESTINE AND METASTATIC COLORECTAL CANCER. Cancer vaccines will have their greatest impact on preventing metastases in patients with occult metastases. However, the impact of endogenous tolerance and tolerance induced by metastases on immunotherapeutic responses remains undefined. Here, we sought to generate an *in vivo* model in which tissue, duration, and quantity of target antigen expression is precisely controlled. The working hypothesis suggests that endogenous tolerance opposes the immunotherapeutic efficacy of cancer mucosa antigens. Moreover, we employed metastatic tumor models to examine tumor-induced tolerance to model and endogenous antigens, identifying a previously unrecognized contribution of antigen-specific $CD8^+$ T cells to MDSC homeostasis.

Results

Endogenous Tolerance - Transgenic Model

We developed a unique model antigen containing well-defined $CD4^+$ T, $CD8^+$ T and B cell epitopes imbedded into the hIL-2Rα (Tac) backbone. This Tac model antigen leverages the previous development of transgenic mice expressing T cell receptors (TCRs) specific to the SIINFEKL and $OVA_{323-339}$ epitopes (OT-I and OT-II) and peptide-MHC complex-specific antibodies. We produced a Tac-transgenic constructed targeting the Rosa26 locus (Figure 4). Tac was cloned into pRosa26-DEST vector via Gateway cloning to produce the Rosa-$stop^{flox}$-Tac targeting construct. This plasmid possesses a splice acceptor site, followed by the $stop^{flox}$ (floxed neomycin resistance expression cassette) and Tac cDNA, all of which are flanked by ROSA26 homologous sequence for homologous recombination into intron 1 of ROSA26. The ROSA26 locus was identified by gene-trap mutagenesis screening performed in mouse ES cells. The ubiquitous expression of ROSA26 in embryonic and adult tissues, together with the high frequency of gene-targeting events observed at this locus in murine ES cells has led to the establishment in the past 10 years of over 130 knock-in lines expressing successfully from the ROSA26 locus a variety of transgenes including reporters, site-specific recombinases and, recently, noncoding RNAs. Thus, it serves as an ideal locus for inducible Tac expression, driven by the endogenous ROSA26 promoter.

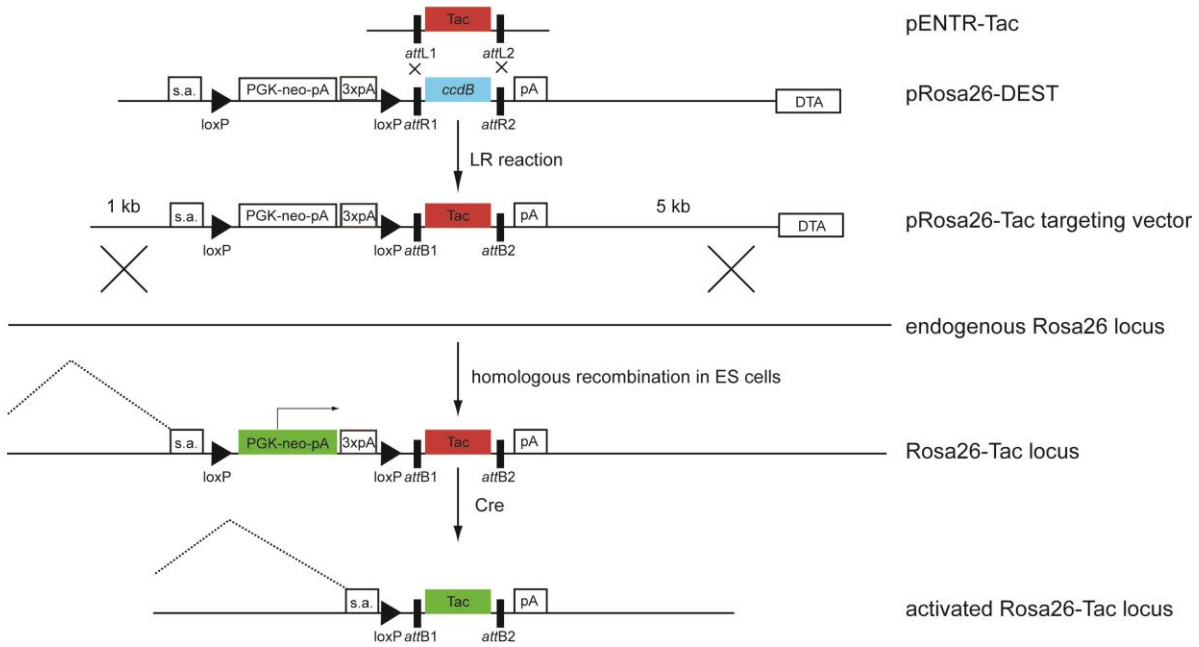


Figure 4. Design of the novel transgenic model. The model Tac antigen possessing B, $CD8^+$ T and $CD4^+$ T cell epitopes (HA Tag, $Ova_{257-264}$, and $Ova_{329-337}$, respectively) was cloned into the pRosa26 targeting vector. Homologous recombination will inserted Tac downstream of the endogenous ROSA26 promoter following a splice acceptor and floxed stop cassette. Tac expression is "off" (red) until Cre-mediated recombination excises the stop cassette, resulting in Tac expression (green).

C57BL/6-derived embryonic stem (ES) cells were transfected with the ROSA-$stop^{flox}$-Tac targeting construct and ~500 single neomycin-resistant clones were picked, expanded, frozen and tested for correct targeting using PCR (Figure 5). Production of chimeric mice from 3 targeted ES cell clones was attempted, however, no chimeric mice were produced. Before other targeted ES clones could be injected in a second attempt, the Thomas Jefferson University (TJU) Transgenic Core was closed and production at TJU was terminated. Future work will utilize extramural transgenic facilities to produce the transgenic mice utilizing ES clones generated here.

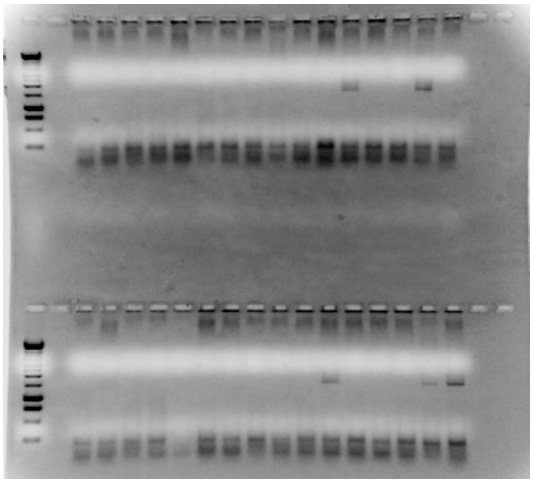


Figure 5. Rosa-Tac ES Screening. About 500 clones were screened following targeting with ROSA-$stop^{flox}$-Tac targeting construct by PCR. Here, 32 clones were screened, identifying 5 potential clones (band ~2000 bp). The PCR assay employed primers upstream of the targeting sequence and within the Tac transgene, identifying clones with productive 5' homologous recombination events.

Tumor-Induced Tolerance

We employed the Tac model antigen to explore tumor-induced tolerance reflecting its incorporation of defined $CD4^+$ and $CD8^+$ T and B cell epitopes (Figure 6A). Tac was cloned into the B16 melanoma cell line syngeneic with C57BL/6 mice compatible with the Tac T cell epitopes. Indeed, B16-Tac, but not parental B16, cells presented SIINFEKL and activated T cells collected from Tac immunized mice (Figure 6B). Also, immunization of C57BL/6 mice with adenovirus expressing Tac (Ad5-Tac) eliminated B16-Tac lung metastases (Figure 6C), curing mice of disease (Figure 6D) when administered ***before*** tumor challenge.

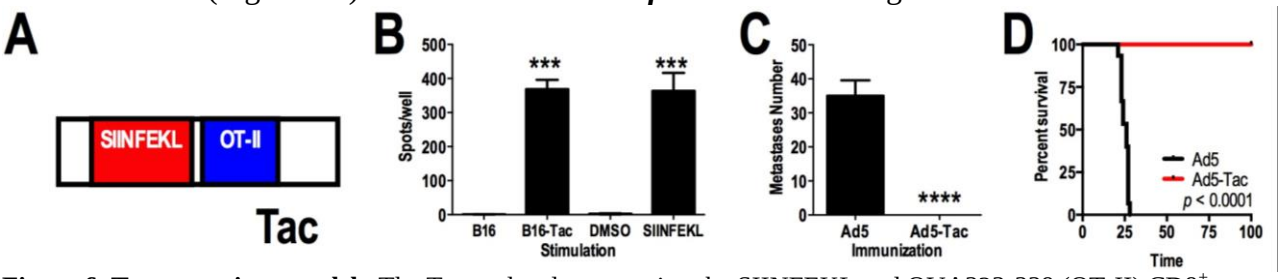


Figure 6. Tumor antigen model. The Tac molecule possessing the SIINFEKL and OVA323-339 (OT-II) $CD8^+$ and $CD4^+$ T cell epitopes, respectively (**A**) was inserted into B16 melanoma cells syngeneic with the C57BL/6 background ($H\text{-}2^b$) compatible with these $H\text{-}2^b$-restricted T cell epitopes. **B**) B16-Tac cells presented SIINFEKL epitope detected by activation of T cells obtained from mice immunized with SIINFEKL. Splenocytes pulsed with DMSO or SIINFEKL peptide served as controls. **C-D**) Immunization of mice with adenovirus expressing Tac (Ad5-Tac) cured mice with B16-Tac metastases in lungs, quantified by tumor enumeration (**C**) and survival (**D**). ****, Two-way ANOVA (**B**), T-test (**C**), Mantel-Cox Log-Rank Test (**D**).

To explore Tac-tolerance induced by growing B16-Tac tumors, Ad5-Tac was administered ***after*** establishing lung or subcutaneous tumors (Figure 7A). While not statistically significant, there was a trends towards reduced SIINFEKL-specific responses in mice with B16-Tac tumors compared to those with parental B16 tumors, suggesting possible antigen-specific tolerance to Tac (Figure 7A). To more closely monitor the fate of SIINFEKL-specific cells in this model, we adoptively transferred $CD45.1^+$ OT-I $CD8^+$ T cells (SIINFEKL TCR transgenic) to $CD45.2^+$ mice (Figure 7B). Mice were then challenged with B16 or B16-Tac cells IV to establish lung metastases and mice were immunized 7 days later. T cell fates (Figure 7C and D) and tumor burden (Figure 7E) were quantified 10 days after immunization. We observed an expected Ad5-Tac-induced expansion of OT-I cells, regardless of the established B16-Tac tumor (Figure 7C). Similarly, B16-Tac tumors and Ad5-Tac immunization each induced the acquisition of IFNγ effector function in OT-I cells (Figure 7D). Moreover, all B16-Tac tumors were eliminated, independent of Ad5-Tac immunization (Figure 7E). Collectively, these data suggest that established B16-Tac tumors do not induce antigen-specific tolerance in the form of deletion (Figure 7C) or anergy (Figure 7D).

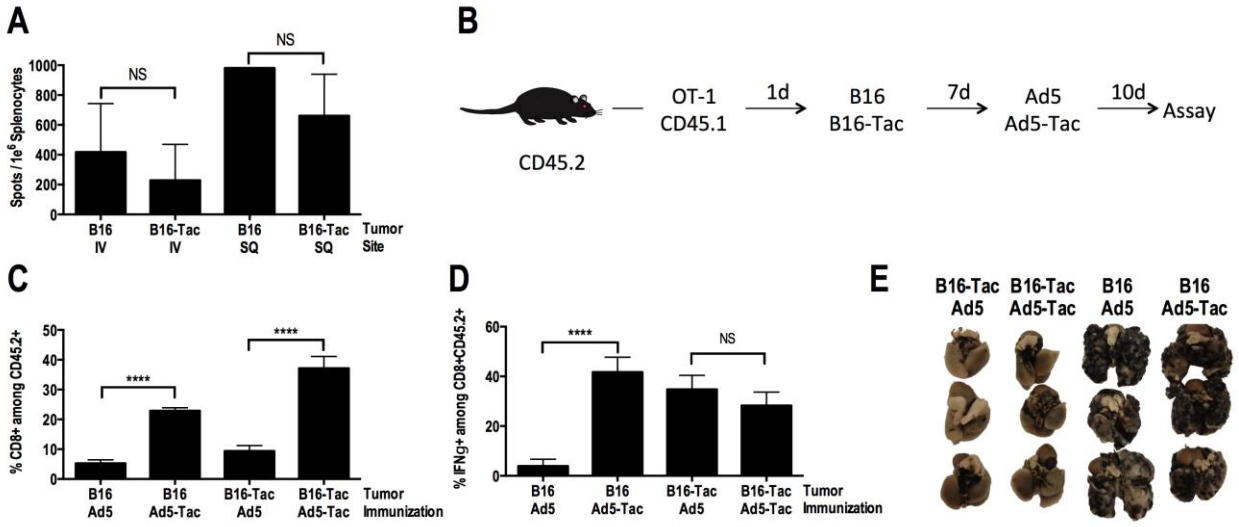


Figure 7. Tolerance to the model tumor antigen Tac. A) C57BL/6 mice were challenge intravenously (IV) or subcutaneously (SQ) with parental B16 (Tac-negative) or B16-Tac (Tac-expressing) cells to establish tumors in lung or subcutaneously. Mice were then immunized with Ad5-Tac and SIINFEKL-specific responses were quantified 10 days later. Though not statistically significant, there was a trends towards reduced responses (tolerance) in mice with Tac-expressing tumors. **B-E**) To explore possible deletion and/or anergy of SIINFEKL-specific cells, CD45.1$^+$ OT-I specific cells were transferred to CD45.2$^+$ C57BL/6 mice, followed by IV challenge with B16 or B16-Tac cells 1 day later and immunization with Ad5 or Ad5-Tac 8 days later. T cells and tumor immunity were analyzed 10 days after immunization. The percentage of OT-I CD8$^+$ T cells (**C**) and effector cytokine IFNγ expression (**D**) among OT-1 cells were quantified to measure deletion and anergy respectively. E) Transferred OT-1 cells eliminated B16-Tac, but not B16, tumors regardless of immunization. **** p<0.0001, One-way ANOVA.

Next, we examined tumor-induced tolerance to the endogenous melanocyte differentiation and B16 melanoma antigen tyrosinase-related protein 2 (Trp2). Because Trp2-deficient B16 melanoma cells are not available, we compared responses to Trp2 immunization with Ad5-Trp2 between mice with growing B16 tumors and those with no tumors (Figure 8). Ad5-Trp2 produced Trp2-specific CD8$^+$ T cell responses in normal (no tumor) mice and in those with established B16 tumors (Figure 8A). Interestingly, Trp2-specific responses in spleens were increased, rather than decreased, in mice with established B16 tumors (Figure 8A), similar to results obtained with Tac (Figure 7B-E). Similarly, tumor-draining (TDLN), but not non-draining (NLN), lymph nodes possessed increased Trp2-specific CD8$^+$ T cell responses in Ad5-Trp2 immunized mice with established B16 tumors (Figure 8B), suggesting results obtained in splenocytes were representative of those at the tumor. To confirm, we isolated tumor-infiltrating cells from tumors and analyzed them by FACS staining (Figure 8C-E). Tumors of mice immunized with Ad5-Trp2 possessed increased lymphocytes, reflecting primarily an increase in CD3$^+$CD8$^+$ T cells, but not CD3$^+$CD4$^+$ T cells or CD3$^+$CD4$^+$CD25$^+$ Tregs (Figure 8C), consistent with Ad5-Trp2-induced activation and expansion of Trp2-specific CD8$^+$ T cells and their infiltration into tumors and participation in ongoing antitumor responses. We also found a modest increase in CD11c$^+$ cells, but not CD11b$^+$ cells or myeloid-derived suppressor cells (MDSCs) in tumors (Figure 8D). However, when we examined MDSC subtypes (Granulocytic and Monocytic) based on their expression levels of CD11b, GR1 and Ly6C, we observed a dramatic shift in the relative contributions of these subtypes to the MDSC population (Figure 8E). Almost all tumor models demonstrated a preferential expansion of granulocytic subset of MDSC,[25] consistent with our results in Ad5-immunized mice. However, Ad5-Trp2 induced a shift towards monocytic MDSCs (Figure 8E). We do not yet know the mechanisms or

consequences of MDSC repolarization induced by Ad5-Trp2. Future studies are planned to determine the mechanism by which Trp2-specific CD8$^+$ T cells mediate MDSC polarization, the consequences of polarization (is beneficial or more suppressive?) and if this novel mechanism could be a therapeutic target for immunotherapeutic intervention in cancer.

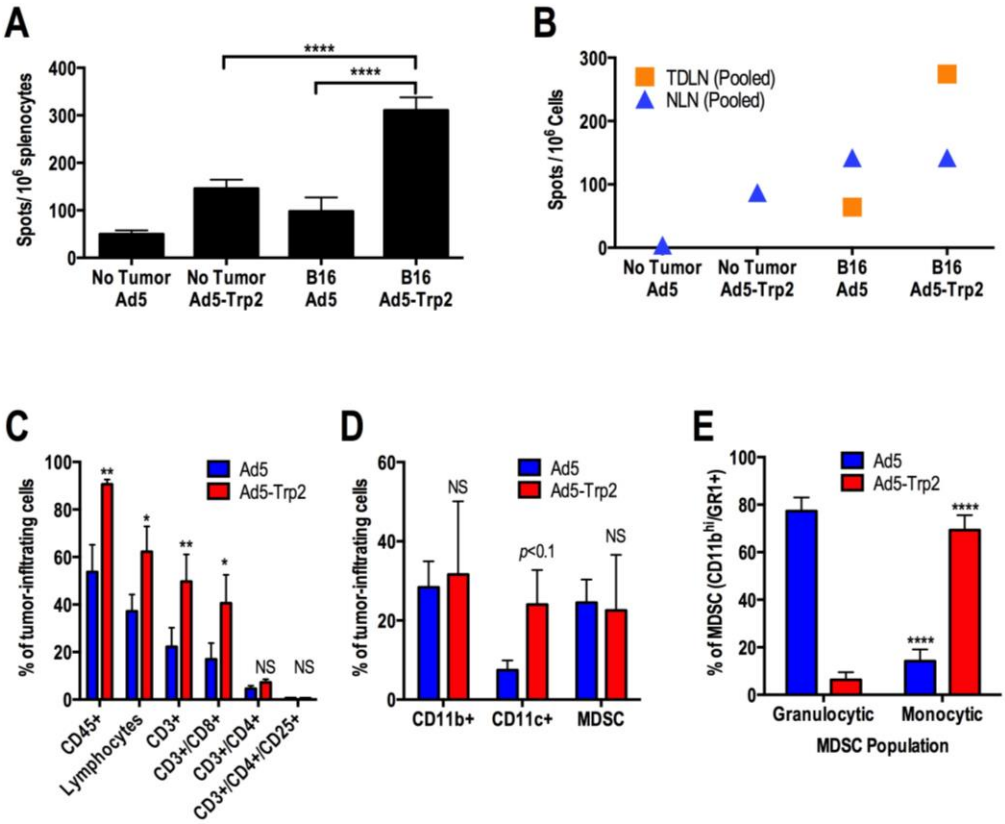


Figure 8. Tolerance to the endogenous tumor antigen Trp2. A-B) C57BL/6 mice were challenge subcutaneously with parental B16 cells to establish tumors subcutaneously. Mice were then immunized with Ad5 or Ad5-Trp2 seven days later and Trp2-specific CD8$^+$ T cell responses were quantified 10 days later in spleens of individual mice (**A**) or in tumor-draining (TDLN) and non-draining lymph nodes (NLN) (**B**). **C-E**) Tumors were collected from mice in **A-B**, dissociated into single-cell suspensions and FACS analysis of immune cell types was performed. **** $p<0.0001$, One-way ANOVA (**A**), Two-way ANOVA (**C-E**).

Aim 3.A Summary

We have generated a unique model antigen (Tac) possessing defined antibody, CD4$^+$ T cell and CD8$^+$ T cell epitopes to explore mechanisms of endogenous and tumor-induced tolerance. Generation of transgenic mice expressing the Tac antigen was not completed, however, we remain committed to this model and are actively exploring extramural transgenic facilities to complete the transgenesis processes initiated during these studies. Future studies will employ that mouse model to explore endogenous tolerance mechanisms. The Tac model is advantageous because it also has utility in exploring tumor-induced tolerance. We generated B16 melanoma cells expressing Tac and found that tumors induced a paradoxical expansion and activation of Tac-specific CD8$^+$ T cells, mediating tumor elimination in the presence of adequate CD8$^+$ T cell precursor frequency. Similarly, CD8$^+$ T cell responses specific to the endogenous melanoma antigen Trp2 were also increased in the presence of established B16 tumors. Interestingly, that enhancement produced a dramatic shift in MDSC subset frequencies from predominantly granulocytic (~80%) to predominantly monocytic (~70%). The mechanisms mediating this shift

and results of this shift are unknown. Future experiments will explore these mechanisms and determine if the shift produces a positive or negative effect on antitumor immunity, identifying a potential target for development of cancer immunotherapeutics.

SUBAIM 3.B. REGULATORY T CELL (TREG)-DEPENDENT TOLERANCE AND GCC RESPONSES. Tregs and their product, IL-10, are key mediators of tolerance opposing immune responses to cancer vaccines. Studies will explore the contribution of Tregs to tolerance barriers opposing immune responses to GCC, and their abrogation by cyclophosphamide and IL-10 immunoadhesin to reduce Treg-dependent immunoinhibition.

Results

T_{reg}-dependent tolerance and GCC responses (Aim 3.B) studies were initiated in 2012 and remain ongoing. In that context, we are producing transgenic mice expressing GCC-specific $CD4^+$ T cell receptors (TCRs), to define the fate of GCC-specific $CD4^+$ T cells in $GCC^{+/+}$ mice. To isolate TCRs, $GCC^{-/-}$ mice (not tolerant to GCC) were immunized with Ad5-GCC and T cells were fused with a thymoma cell line (BWZ) expressing an NFAT-induced LacZ reporter gene. Thus, TCR activation of hybrids results in NFAT-mediated LacZ expression, permitting simple quantification of TCR-mediated hybridoma activation. About 20,000 T cell hybrids generated from immunized $GCC^{-/-}$ mice were screen for GCC-specificity by stimulating them with a mixture of GCC-derived $CD4^+$ T cell epitopes $GCC_{125\text{-}139}$ and $GCC_{153\text{-}167}$ and quantifying LacZ expression (Figure 9). We previously identified $GCC_{125\text{-}139}$ and $GCC_{153\text{-}167}$ as the dominant epitopes in $GCC^{-/-}$ mice immunized with Ad5-GCC.[4] Two hybrids (W14 and W11) were identified.

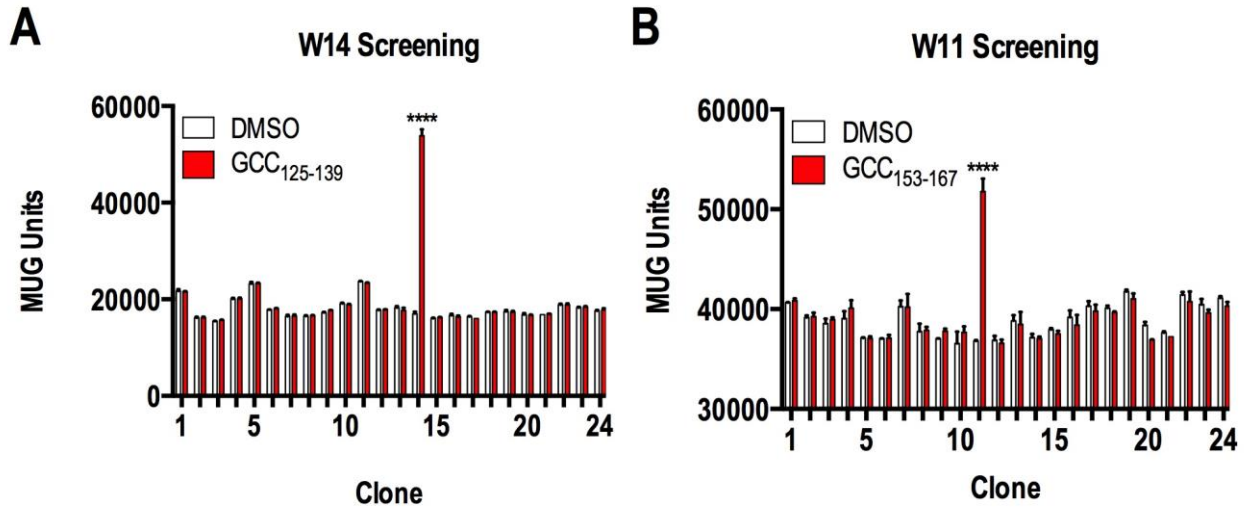


Figure 9. GCC-specific T cell hybridoma screening. $GCC^{-/-}$ mice were immunized with Ad5-GCC and splenocytes were collected 2 week later and stimulated ex vivo with GCC-derived, H-2^d-restricted epitope peptides and IL-2 for 1 week to enrich for GCC-specific $CD4^+$ T cells. Following stimulation, cultured cells were fused with BWZ cells stably-expressing NFAT-induced β-galactosidase. Productive fusion events were selected and cloned prior to screening by stimulation with DMSO or GCC peptide. Antigen-specific stimulation was measured by induction of β-galactosidase expression by fluorometric (MUG) assay identifying $GCC_{125\text{-}139}$ (**A**) and $GCC_{153\text{-}167}$ (**B**) specific T cell hybridomas W14 and W11, respectively. and D). Positive clones were isolated and expanded and their specificity was confirmed (B and E). The affinity of isolated hybridomas was compared to splenocytes assayed directly ex vivo from Ad5-GCC-immunized $GCC^{-/-}$ mice (C and F). **** $p<0.0001$, Two-way ANOVA.

To confirm the specificity of each T cell hybridomas, we stimulated them with cognate and control peptide (Figure 10A and B). Indeed, each hybridomas was activated by its cognate GCC epitope, but not by the other GCC epitope, confirming the specificity of each hybridomas. We further characterized the hybridomas by comparing their avidity for GCC epitope to that of $CD4^+$ T cells examined directly *ex vivo* from immunized $GCC^{-/-}$ mice (Figure 10C and D). The avidity of each hybridoma was comparable to those of T cells produced by Ad5-GCC immunization and measured directly *ex vivo*. Thus, these T hybridomas possess TCRs representative of those found in $GCC^{-/-}$ mice and are ideal model TCRs to examine endogenous tolerance mechanisms to GCC. TCR α and β chains were obtained from hybridomas by PCR, transgenic constructs were produced and transgenic mouse generation is ongoing.

Aim 3.B Summary

We have successfully identified T cell receptors (TCRs) specific for the two dominant GCC-derived $CD4^+$ T cell epitopes recognized in $GCC^{-/-}$ (non-tolerant) mice. Generation of transgenic mice expressing these TCRs is ongoing and these mice will be very important tools to explore the mechanisms and consequences of GCC-specific $CD4^+$ T cell tolerance. Future experiments will employ the newly generated W11 and W14 TCR transgenic mice to define the fate of GCC-specific $CD4^+$ T cells in $GCC^{+/+}$ mice, the contribution of Treg development to GCC-specific tolerance, and the ability of GCC-specific Tregs to inhibit vaccine-induced antitumor immunity. Those results can be directly translated to next-generation vaccine approaches in patients, leveraging safety and efficacy data of Ad5-hGCC-PADRE produced in Aim 1.

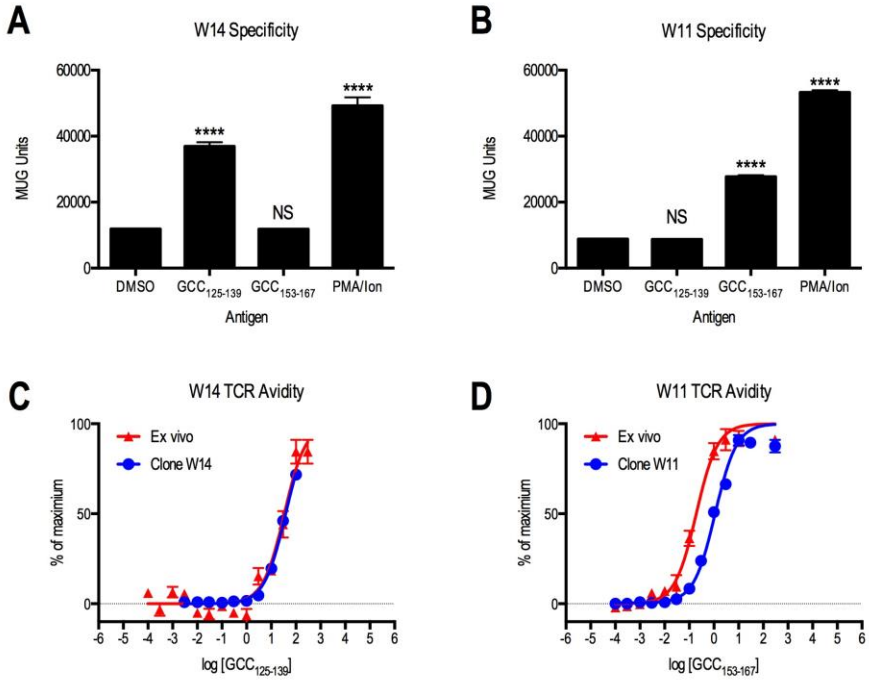


Figure 10. GCC-specific T cell hybridoma specificity and avidity. The W14 (**A**) and W11 (**B**) T cell hybridoma specificity was confirmed by stimulation each hybridoma with $GCC_{125-139}$, $GUCY2C_{153-167}$, or PMA+Ionomycin (PMA/Ion) positive control. Induction of NFAT-induced β-galactosidase expression was quantified by MUG assay to measure T cell activation. Each clone responded to its cognate epitope, but not the other GCC epitope, confirming the T cell hybridoma specificity. The W14 (**C**) and W11 (**D**) T cell hybridoma avidities were compared to those of T cells analyzed directly *ex vivo* from GCC-/- mice immunized with Ad5-GCC. The avidity of each clones was comparable to the endogenous responses to each GCC epitope. **** $p<0.0001$, One-way ANOVA vs. DMSO control.

SUBAIM 3.C. CHEMOKINE-POTENTIATED HETEROLOGOUS VACCINE STRATEGIES THAT AMPLIFY GCC- TARGETED RESPONSES. While heterologous prime-boost strategies involving 3 GCC-based viruses maximize antitumor protection, vaccine paradigms requiring 3 different viruses represent a regulatory hurdle to clinical translation. Here, we explored a DNA-based GCC vaccine strategy to supplement virus-based GCC approaches that overcome endogenous tolerance mechanisms. We identified a heterologous strategy of DNA vaccine priming and adenovirus-based vaccine boosting (D+A) that safely enhances immune responses and induces superior antitumor immunity, preventing disease progression. The optimal antitumor immune response induced by D+A vaccination resulted from a generation of GCC-specific T cells with high functional TCR avidity. In the context of enhanced immune responses, superior antitumor immunity, and absence of toxicity, the D+A vaccination strategy identified here is poised for clinical translation to prevent recurrent disease in early stage colorectal cancer patients. Moreover, mechanisms underlying synergy of DNA and viral vectors identified here may be applicable to other vaccine approaches for various malignancies.

Results

Route of administration effects transgene expression

DNA vaccines have been administered intramuscularly (IM) and intradermally (ID) to treat cancers. Though some data suggests route of injection influences the type of response (humoral vs cellular), a systematic evaluation of transgene expression and immunogenicity in the context of different routes of DNA vaccination remains unexplored. To visualize and quantify the protein expression level at different vaccination sites, we employed a plasmid expressing green fluorescent protein (GFP) administered IM or ID with electroporation (EP). IM administration of GFP plasmid produced more GFP expression than ID administration, in a time-dependent fashion (Figure 11A). Increased antigen expression following IM administration suggests this route may be favorable for DNA vaccine administration. Moreover, GFP plasmid administration in the absence of EP nearly eliminated antigen expression (Figure 11B), consistent with literature suggesting a 100-1000 fold increase in transgene expression with EP. Thus, transgene expression was optimized by IM, rather than ID, administration of DNA plasmid in the context of EP.

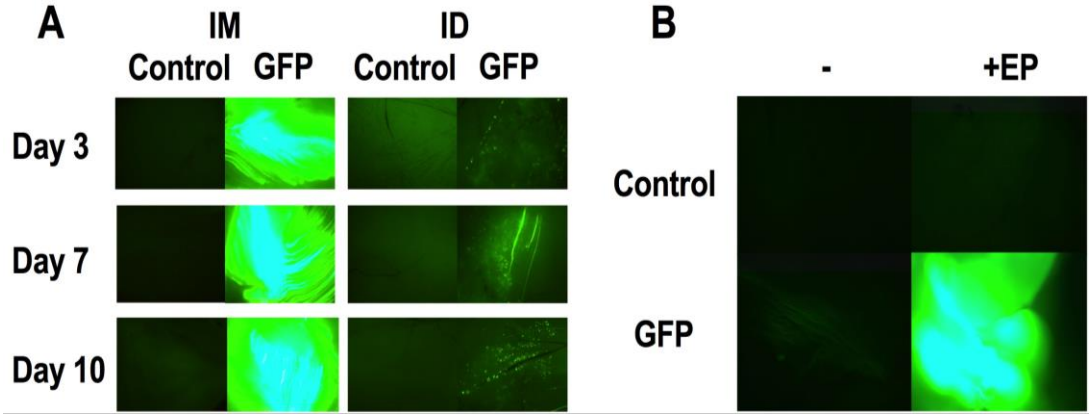


Figure 11. Route and Electroporation Enhance Transgene Expression. A) Time course of protein expression by electroporation of IM or ID administration of control or GFP-expression plasmid. GFP expression was imaged by fluorescence microscopy. **B**) EP enhanced the transfection rates of plasmids into muscle receiving control or GFP-expressing plasmids. Muscle tissues were collected on day 6 and GFP expression was imaged by fluorescence microscopy.

CCL20 and CCL21 expressed in situ recruit dendritic cells and T cells to the vaccine site

DNA vaccines have been largely limited by poor immunogenicity and various chemokines and adjuvants have been examined as mechanisms to improve DNA vaccine immunogenicity. Previously, we showed that plasmids expressing the chemokines CCL20 and CCL21 (pCCL20 and pCCL21, respectively) promote the efficacy of melanoma-specific DNA vaccines by recruiting immature dendritic cells and naïve T cells following intradermal administration. To extend that principle to muscular administration, a mixture of pCCL20 and pCCL21 was delivered IM with EP. Tissues were collected stained with H&E (Figure 12A) or anti-CD45 (Figure 12B) to reveal immune cell recruitment in chemokine plasmid-treated muscles.

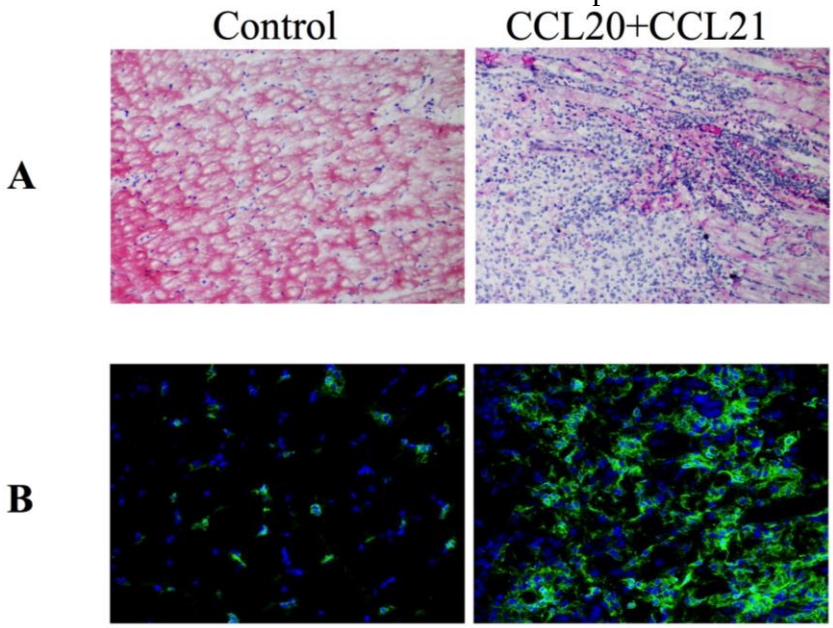


Figure 12. CCL20/CCL21-expressing plasmids enhance immune cell recruitment to muscle. A) H&E staining of muscle tissue collected 4 days after EP with control plasmid or CCL20 and CCL21 -expressing chemokine plasmids. **B**) CD45 immunofluorescence staining of the same muscle tissues collected in A. Slides were treated with anti-CD45 primary antibody and fluorescence-labeled secondary antibody (Green). DAPI (Blue) was used to stain nuclei.

Ad5-GCC is superior to DNA-GCC Vaccination

In the context of robust transgene expression following IM, rather than ID, DNA delivery (Figure 11), and robust chemokine-mediated immune cell recruitment in muscle (Figure 12), we compared the immunogenicity of IM and ID vaccinations administered with chemokine plasmid adjuvantation (Figure 13A). Intramuscular immunization with CCL20 and CCL21 plasmids and GCC plasmid produced GCC-specific $CD8^+$ T cell responses quantified by ELISpot that were ~15-fold greater than ID immunization (Figure 13A), consistent with greater transgene expression in muscle (Figure 11). However, adenoviral vector immunization with GCC (Ad5) produced GCC-specific $CD8^+$ T cell response that were ~13-fold greater than those produced by the optimal IM DNA vaccination (Figure 13B). Moreover Ad5 vaccination produced an 18-fold increase in median survival time of mice with GCC-expressing metastatic colorectal cancer compared to DNA vaccination (Figure 13C). Taken together, IM DNA vaccination with pCCL20/21 optimizes GCC-specific $CD8^+$ T cell responses, but those are not comparable to Ad5 vaccination.

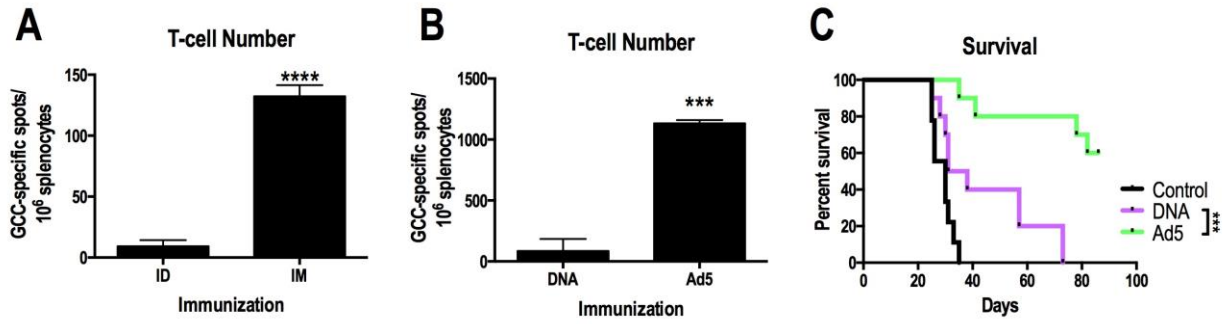


Figure 13. Adenoviral vaccination is superior to DNA vaccination for GCC. A) CCL20 and CCL21 plasmids were administered with EP on day 0 IM or ID, followed 12 days later by GCC plasmid with EP. GCC-specific T cell responses to the dominant $GCC_{254-262}$ epitope were measured by IFN-γ ELISpot 14 days after the last DNA administration. **B-C**) A comparison of the optimal DNA vaccination (IM identified in **A**) and Ad5-GCC vaccination revealed the superiority of Ad5 by GCC-specific ELISpot 14 days after immunization (**B**) and by antitumor immunity following intravenous challenge with GCC-expressing CT26 colorectal cancer cells and monitoring of survival (**C**). *** $p < 0.001$, **** $p < 0.0001$, One-way ANOVA (**A** and **B**) and Mantel-Cox Log-Rank Test (**C**).

Heterologous GUCY2C DNA-Ad5 prime-boost induces robust GUCY2C-specific immune responses and antitumor immunity.

While GCC DNA vaccination alone was poorly immunogenic, we hypothesized that it could be combined with Ad5-GCC in prime-boost regimens to produce antitumor immunity superior to either vaccine alone. To maximize GCC-specific antitumor responses, mice were immunized with different heterologous prime-boost strategies or Ad5 alone and challenged with GCC-expressing CT26 colorectal cancer cells to establish lung metastases (Figure 14A). While Ad5 followed by DNA vaccination (A+D) was more effective than Ad5 alone, DNA vaccination followed by Ad5 (D+A) was by far the most effective strategy, curing ~50% of mice, while no other vaccine produced more than 10% overall survival (Figure 14A). Surprisingly, quantification of T cell response to $GCC_{254-262}$ by ELISpot demonstrated that A+D produce equal T cell responses to Ad5 alone, and D+A was only ~2-fold higher than Ad5 alone (Figure 14B). Similarly, multifunctional cytokine responses (IFNγ, TNFα, MIP1α) were also similar between the vaccination regimens (Figure 14C). While the magnitude and effector cytokine responses were comparable between vaccine regimens, we hypothesized that differences in antitumor efficacy could reflect functional TCR avidity. TCR avidity correlates with antitumor immunity following adoptive T cell transfer with T cells expressing TCRs of varying avidities, suggesting that TCR avidity enhancement may underlie D+A efficacy. Indeed, A+D enhanced TCR avidity ~4-fold over Ad5 alone, while D+A enhanced avidity ~10-fold (Figure 14D). Interestingly, the avidity enhancement was specific to heterologous prime-boosts since Ad5+Ad5 homologous prime-boost produced identical TCR avidity to Ad5 alone (Figure 14D).

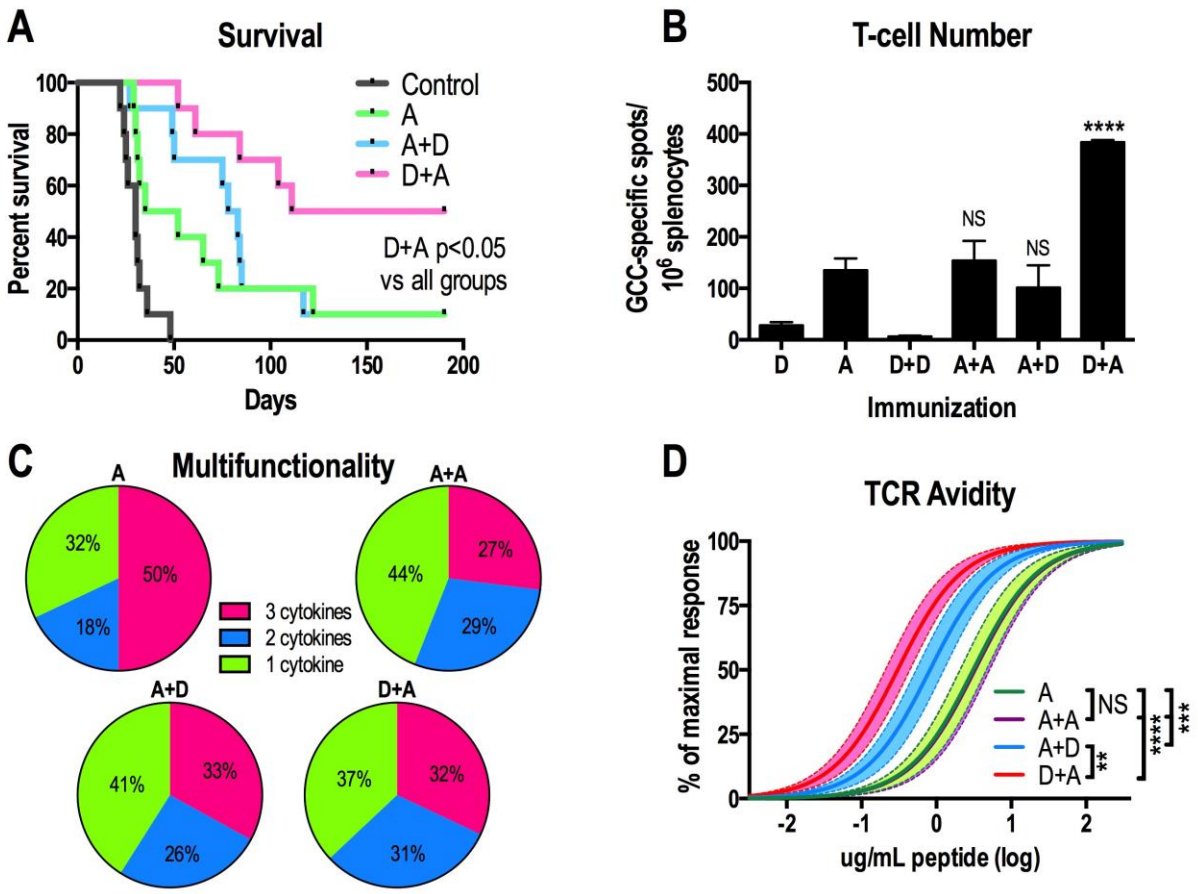


Figure 14. DNA-Ad5 Prime-Boost Vaccination Maximizes GCC-Specific Antitumor Immunity. A-D) Mice were immunized with control vaccine (Control), Ad5-GCC (A), DNA-GCC (D), or homologous and heterologous combinations of Ad5 and DNA (A+D, D+A). **A**) Following immunization, mice were challenged with GCC-expressing CT26 colorectal cancer cells to establish lung metastases and survival was monitored. **B-D**) T cells were collected from immunized mice 14 days after the final immunization and $GCC_{254-262}$-specific T-cell number (**B**), cytokine multifunctionality (**C**) and TCR avidity (**D**) were quantified by IFNγ ELISpot (**B**), IFNγ, TNFα, MIP1-α FACS (**C**) and IFNγ ELISpot (**D**), respectively. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Mantel-Cox Log-Rank Test (**A**), One-way ANOVA (**B**) and Sum-of-Squares F Test (**D**).

Functional TCR avidity enhancement mediates DNA-Ad5 antitumor synergy

While enhancement of TCR avidity, rather than T cell quantity or cytokine responses seem to correlate with antitumor efficacy, we wanted to experimentally manipulate TCR avidity, while preserving the magnitude and cytokine profile of GCC-specific T cell responses. To that end, we created an adenovirus expressing only the H-2^d class I epitope $GCC_{254-262}$ (Ad5-$GCC_{254-262}$). We have previously shown that viral vectors expressing only the minimal MHC class I epitope, maximize peptide-MHC densities, producing low-avidity T cell responses. Thus, Ad5-$GCC_{254-262}$ should produce low-avidity T cell responses. Indeed, the magnitude (Figure 15A) and cytokine profiles (Figure 15B) of $GCC_{254-262}$-specific responses were comparable following the optimized D+A immunization and Ad5-$GCC_{254-262}$ immunization, while Ad5-$GCC_{254-262}$ produced ~20-fold lower TCR avidity (Figure 15C). As expected, Ad5-$GCC_{254-262}$ produce almost no antitumor protection (~10 day increased median survival) while D+A cured 60% of mice (Figure 15D).

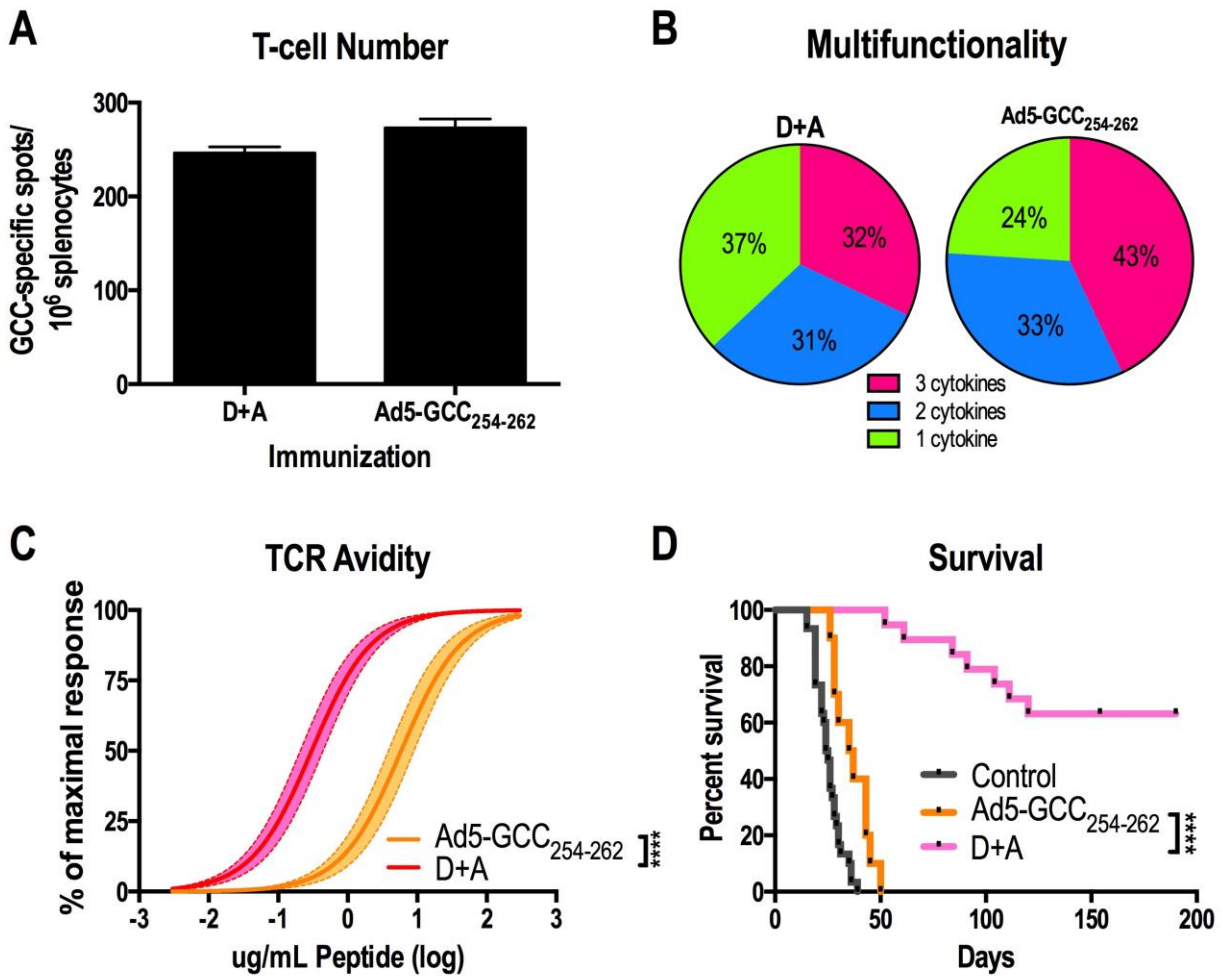


Figure 15. DNA-Ad5 Prime-Boost Synergy Reflects TCR Avidity Enhancement. A-D) Mice were immunized with control vaccine (Control), Ad5-GCC$_{254-262}$ or heterologous prime-boost with DNA-GCC followed by Ad5-GCC (D+A). **A-C**) T cells were collected from immunized mice and GCC-specific T-cell number (**A**), cytokine multifunctionality (**B**) and TCR avidity (**C**) were quantified as in **Figure 14**, confirming that Ad5-GCC$_{254-262}$ produces comparable quantity and cytokine profiles of GCC-specific T cells, but >20-fold lower TCR avidity. **D**) Following immunization, mice were challenged with GCC-expressing CT26 colorectal cancer cells to establish lung metastases and survival was monitored. **** $p < 0.0001$, Sum-of-Squares F Test (**E**) and Mantel-Cox Log-Rank Test (**D**)

Comparing survival benefit to T-cell quantity (p>0.05), T-cell cytokine multifunctionality (p>0.05) and T cell avidity (p<0.0001) across all tested vaccination combinations revealed only T cell avidity predicting tumor outcomes (Figure 16), revealing the previously unrecognized mechanism of functional T cell avidity enhancement mediating synergy of DNA-Ad5 prime boost vaccinations.

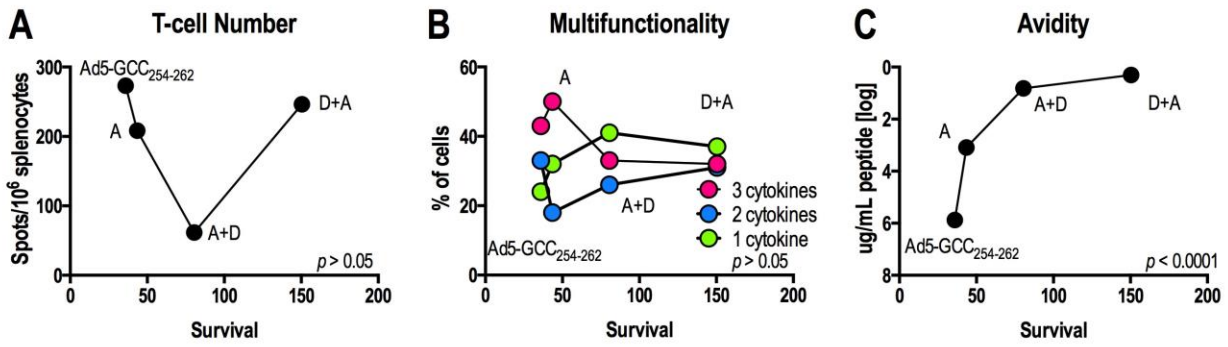


Figure 16. Functional TCR Avidity Predicts Antitumor Efficacy. A) T cell number, **B**) 1, 2, or 3 cytokine multifunctionality, and **C**) TCR avidity produced by the tested vaccine combinations were correlated with median survival outcomes (days) in mice with metastatic GCC-expressing colorectal cancer. One-way ANOVA post-test for linear trend.

GCC DNA-Ad5 prime-boost is safe.

Enhancing TCR avidity in adoptive T cell therapy for melanoma enhances antitumor immunity and autoimmunity, suggesting a strong relationship between efficacy and toxicity in T cell immunotherapy. However, immunologic compartmentalization between systemic and mucosal immune systems prevents autoimmunity in mice receiving Ad5-based GCC vaccines, suggesting that the increased TCR avidity and antitumor immunity observed with DNA+Ad5 prime-boost vaccination would ***not*** come at the expense of increased autoimmunity. Indeed, mice immunized with DNA-GCC or the heterologous GCC DNA-Ad5 prime-boost strategy were free of autoimmune toxicity (Table 9) in tissues associated with GCC expression (small and large intestines, cecum, salivary gland and hypothalamus) as well as tissues devoid of GCC (heart, lung, liver, kidneys and stomach). Thus DNA+Ad5 prime-boost immunization enhances TCR avidity, maximizing antitumor immunity, without concomitant autoimmunity.

Table 9
DNA and DNA+Ad5 Safety

	Tissue		
Tissue	**Control**	**DNA**	**DNA+Ad5**
Salivary Gland	0/5	0/5	0/5
Stomach	0/5	0/5	0/5
Small Intestine	0/5	0/5	0/5
Large Intestine	0/5	0/5	0/5
Cecum	0/5	0/5	0/5
Brain	0/5	0/5	0/5
Heart	0/5	0/5	0/5
Lung	0/5	0/5	0/5
Liver	0/5	0/5	0/5
Kidney	0/5	0/5	0/5
Fraction of animals experiencing histopathological evidence of toxicity			

Aim 3.C Summary

We developed a GCC-targeted DNA vaccine to supplement GCC-targeted adenoviral vaccination to treat metastatic colorectal cancer. DNA plasmid vaccine expressing GCC elicited $CD8^+$ T cell responses, but those were of lower frequency than Ad5-GCC, resulting in poor

protection from GCC-expressing metastatic colorectal cancer. However, the combination of GCC DNA and Ad5 in a prime-boost strategy, not only increased the quantity of GCC-specific $CD8^+$ T cells, but also produced $CD8^+$ T cell responses with higher TCR avidity. That prime-boost strategy also protected mice with colorectal cancer metastases better than all other regimens tested. Employing an Ad5-$GCC_{254-262}$ construct that minimizes TCR avidity confirmed the importance of TCR avidity in antitumor efficacy of GCC-specific responses to vaccination. Indeed, comparing survival benefit to T cell quantity, T cell cytokine multifunctionality and TCR avidity across all tested vaccination combinations revealed only TCR avidity predicting tumor outcomes. Thus, our studies revealed the previously unrecognized mechanism of functional T-cell avidity enhancement mediating synergy of DNA-Ad5 prime boost vaccinations. Those results will direct vaccine design for future GCC vaccine combinations in patients. Moreover, these results suggest that the magnitude and multifunctional cytokine profiles of cancer vaccine-induced T cell responses are poor predictors of outcomes following prime-boost immunizations. Rather, functional TCR avidity correlates with outcomes and should be explored as a biomarker of cancer vaccine efficacy. Moreover, strategies that specifically enhance TCR avidity may have the greatest efficacy in cancer vaccination.

AIM 4. PREPARATION OF TRAINEES FROM UNDER-REPRESENTED MINORITIES FOR CAREERS IN BIOMEDICINE (TRAINING AIM).

Trainees

Jefferson collaborated with a number of local universities to assist with the recruitment of underrepresent minority students for the Summer Research Internships in Cancer Immunotherapy which began June, 2010. The faculty liaisons would review applicants at their institutions and forward their recommended students to the Jefferson Faculty Selection Committee. The candidates were ranked based on their academic record, intellectual ability, personal qualities and potential for research, as well as, passion for science, and personal maturity. The Jefferson Selection Committee received approximately 20 applications per year for the five positions available. Most of the student applicants came from Cheyney, Lincoln and Saint Joseph's Universities although there were also applicants from Temple and Drexel. In addition to providing 4 positions for Under-Graduates, we also had one position each year for a senior accepted into a Graduate program or a first year Jefferson Medical College student. All of the students were under-represented minorities, out of the 17 participants (3 students participated for 2 years) 11 were female and 6 were male; 13 were Black, 3 Hispanic and 1 Asian. See Table

The trainees were each placed with a mentor who was involved with one of the program objectives. The research experience for the students provided laboratory activities on a project for 10 weeks. At the end of the program, the students were expected to prepare a research report and make a 30 minute presentation to program faculty and their peers. We also encouraged students to make an oral or poster presentation at their home institution's research day. Some students also worked with their mentor to develop an abstract for submission to a scientific meeting and a manuscript for submission to a refereed journal for publication. Students were also required to participate in the didactic program described below. Students were expected to take time outside of the regular work hours to read articles, text book chapters, and other materials related to the internship.

At the end of each summer program, trainees were asked to complete a program evaluation of their research experience and the didactic program. Students were also asked to complete online

evaluations to assess the impact of our program on their college performance and future career. The online assessment instruments were developed by David Lopatto, PhD at Grinnell College as part of his ongoing research on undergraduate education, student learning and attitudes with a grant supported by Howard Hughes Medical Institute. The use of these assessment tools were suggested by one of the members of the External Advisory Committee, Dr. David Usher at the University of Delaware. Students were also required to complete IRB training in basic human subjects and good clinical practice and provide a copy of their certification.

Didactic Program & Curriculum Development

The didactic program, originally designed by Dr. Elizabeth Rappaport with input from the Principal Investigator, other co-investigators and members of the Training Oversight Committee, provided 20 hours of lectures on research topics and cancer immunology. The program consisted of two 2-hour lectures per week by Jefferson faculty, on biomedical research topics including research ethics, laboratory safety, animals in research, and human subject usage. Research areas included cancer biology and immunology, health services research focused primarily on barriers to clinical trial participation, new methods for cancer diagnosis and treatment, clinical translational research, data management, analysis and biostatistics. The cancer topics included cancer genetics, fundamentals of immunology, hematopoiesis, pathobiology of cancer, tumor immunobiology and immunotherapy. In Years 2 and 3, in response to student and mentor feedback, students were provided with a copy of the textbook "How the Immune System Works" by Lauren M. Sompayrac to help the students with the immunology lectures. Students were given access to the Jefferson Medical library, where two didactic sessions were presented by librarians. These sessions focused on scientific inquiry including development of hypotheses, study design, data collection and management, data analysis and interpretation, and written and oral presentation of research results.

As suggested at the Interim Performance Review, the students were provided opportunities to meet and interact with minority faculty members and students at Jefferson as well as the Associate Dean of the Jefferson Medical College Office of Diversity and Minority Affairs. The Director of TJU Admissions met with each student individually to discuss medical school application process and other postgraduate education and training.

In Year 4 the students were provided the opportunity to participate with the Jefferson first year medical students in the Jefferson Medical College Summer Seminar Series which included topics on laboratory safety, animal care and use, research ethics and protection of human subjects in research, principles of clinical research and clinical trial design, patient decision-making about clinical trial participation, a review of cell biology, an introduction to cancer biology, and instruction from library staff on media literacy, use of Internet-based search tools, and reference management. This part of the program provided the students with an excellent opportunity to interact with the medical students and receive coaching on what they would need to do to be ready for careers in biomedicine.

Program Administration

The Training Oversight Committee consisted of Program Liaisons from the three major contributing institutions, Dr. Steven Hughes at Cheyney University, Dr. Judith Thomas at Lincoln University and Dr. Catalina Arango at Saint Joseph's University, as well as, the Program Director Dr. Elizabeth Rappaport and Program Administrator. The Training Oversight Committee met regularly to review student recruitment and program elements. They developed

recruitment strategies and timelines, reviewed the evaluations of the Summer Internship Program and made appropriate changes in the program; looked for venues and opportunities for students to present their summer research projects and discussed tracking students post internships. The faculty participants from Cheyney, Lincoln and Saint Joseph's Universities expressed their regret that the program was ending and agreed that this was an excellent program which greatly benefitted their students.

	Summer Interns in Cancer Immunotherapy 2010 - 2014						
Trainee	**Year**	**Institution**	**Sex**	**Race**	**Mentor**	**Project Name**	**Outcomes as of June 30, 2014**
Ross,Shannel E	Year 1	JMC	F	Black	R. Myers, PhD	Factors affecting patients' decisions to participate in colorectal vaccine trial	
Nwafor,Ebelechukwu C	Year 1	Cheyney	M	Black	T. Hyslop, PhD	A comparison of analytical methods to assess change in tumor volume over time	Enrolled in PhD Program at Howard University
Fritzinger,Audrey J	Year 1 & 2	St. Joseph	F	Black	T. Sato, MD PhD & V. Alexeev, PhD	Year 1 & 2: IL-10 regulation of immune response to tumors	BS; worked at Phila. Public Health Management Corp; enrolled in Physician Associate Program at Yale Univ.
Gutierrez Jr,Baudelio	Year 1	Drexel	M	Hispanic	R. Myers, PhD	Factors affecting patients' decisions to participate in colorectal vaccine trial	
Lash,Elizabeth F	Year 1 & 2	St. Joseph	F	Black	L. Eisenlohr, DVM, PhD	Year 1 & 2: Using TAC as a model antigen to examine immunologic tolerance	BS, 2012; employed as Research Technician at TJU; applying for medical school
Diallo,Safiatou	Year 2	Lincoln	F	Black	V.Alexeev, PhD	In vivo T cell engineering	
Waters,Simone	Year 2	Cheyney	F	Black	S. Waldman, MD, PhD	Adoptive cell therapy using chimeric antigen receptors	
Goka,Selasie Q	Year 2	JMC	F	Black	R. Myers, PhD	Factors affecting patients' decisions to participate in colorectal vaccine trial	
Austin,Diamond S	Year 3	Lincoln	F	Black	M. Magee, PhD	A method to compare chimeric antigen receptor surface detection & increase its expression	
Ihejirika,Patrick Nzeribe	Year 3	Lincoln	M	Black	A. Snook, PhD	Creating a model antigen system to study the mechanisms of GCC-specific tolerance	BS, 2013, plans to apply for medical school
Sangster,Nathaniel J.	Year 3	Lincoln	M	Black/ Hispanic	V.Alexeev, PhD	Development of DNA vaccination approach for tumor immunotargeting	BS, 2013
Stevens,Evelyn M	Year 3	Lincoln	F	Black	T.Hyslop, PhD & R. Myers, PhD	Development of decision support intervention for recruitment to colon cancer	BS, 2012; Enrolled in MPH program at Temple University; Research Assistant at UPenn

						vaccine study	
Thelmo,Franklin L	Year 3 & 4	St. Joseph	M	Hispanic	S. Selvan, PhD & T.Sato, MD, PhD	Year 1:The production of an *in situ* dinitrophenyl (DNP) vaccine for B16F10 melanomas. Year 2: Unraveling the mechanism of 2,4-dinitrofluorobenzene-associated cell death.	BS, 2014, plans to apply for medical school
Walker,Kayla M	Year 4	St. Joseph	F	Black	R. Myers, PhD	Race and interest in genetic and environmental risk sssessment	Enrolled in BS, 2016
Smith-Parris,Raven	Year 4	Cheyney	F	Black	M. Magee, PhD	The conception of GCC-specific chimeric antigen receptors	
Ebanks,Shauna A	Year 4	Lincoln	F	Black	B. Xiang, PhD	Adoptive T cell therapy for metastatic colorectal cancer	BS, 2014
Khurana,Anmol R	Year 4	JMC	M	Asian	V.Alexeev, PhD	Prophylactic vs. therapeutic: DNA vaccine to metastatic colorectal cancer using molecular marker guanylyl cyclase-C	

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

__X___Yes
_____No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X___No – the study is ongoing

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

___12_ Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

__10-22 Number of subjects originally targeted to be included in the study
___10__ Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
__5___Males
__5___Females
__0___Unknown

Ethnicity:
__0___Latinos or Hispanics
__10__Not Latinos or Hispanics
__0___Unknown

Race:
__0____American Indian or Alaska Native
__0____Asian
__2____Blacks or African American
__0____Native Hawaiian or Other Pacific Islander
__8____White
__0____Other, specify:_____
__0____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

United States

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X___No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the

publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Occult tumor burden predicts disease recurrence in lymph node-negative colorectal cancer.	Hyslop, T., Schulz, S., Barkun, A., Weinberg, D and Waldman, S.A.	Clin Cancer Res	2011	☐Submitted ☐Accepted ☒Published
2. GUCY2C-targeted cancer immunotherapy: past, present and future.	Snook, A.E., Magee, M.S., and Waldman, S.A.	Immunol Res	2012	☐Submitted ☐Accepted ☒Published
3. Epitope-targeted cytotoxic T cells mediate lineage-specific antitumor efficacy induced by the cancer mucosa antigen GUCY2C.	Snook, A.E., Magee, M.S., Marszalowicz, G.P.; and Waldman, S.A.	Cancer Immunol Immunother	2012	☐Submitted ☐Accepted ☒Published
4.Analytic Lymph Node Number Establises Staging Accuracy by Occult Tumor Burden in Colorectal Cancer.	Hyslop, T., Weinberg, D., Schulz, S., Barkun, A. and Waldman, S.A.	Journal of Surgical Oncology	2012	☐Submitted ☐Accepted ☒Published
5. GUCY2C opposes systemic genotoxic tumorigenesis by suppressing AKT-	Lin, J.E., Snook, A.E., Li, P., Stoecker, B.A., Kim, G.W., Magee,	PLoS One	2012	☐Submitted ☐Accepted ☒Published

dependent intestinal barrier dysfunction.	M.S., Garcia, A.V.M., Hyslop, T., Schulz, S. and Waldman, S.A.			
6. Occult tumor burden contributes to racial disparities in stage-specific colorectal cancer outcomes.	Hyslop, T., Schulz, S., Weinberg, D., Barkun, A. and Waldman, S.A.	Cancer	2012	☐Submitted ☐Accepted ☒Published
7. Knowledge cycle transforms therapeutic innovation.	Waldman, S.A. and Terzic, A.	Clin Pharm Ther	2012	☐Submitted ☐Accepted ☒Published
8. GUCY2C molecular staging personalizing colorectal cancer management.	Gong, J., Schulz, S., Hyslop, T., and Waldman, S.A.	Biomarkers in Medicine	2012	☐Submitted ☐Accepted ☒Published
9.Translational medicine individualizes healthcare discovery, development and delivery.	Terzic, A.T. and Waldman, S.A.	Biomarkers in Medicine	2013	☐Submitted ☐Accepted ☒Published
10. Information hierarchies optimize patient-centered solutions.	Terzic, A.T. and Waldman, S.A.	Clin Pharm Ther	2013	☐Submitted ☐Accepted ☒Published
12. Molecular staging of node negative patients with colorectal cancer.	Hyslop, T. and Waldman, S.A.	J Cancer	2013	☐Submitted ☐Accepted ☒Published
13. Guanylyl cyclase C as a biomarker in colorectal cancer.	Hyslop, T. and Waldman, S.A.	Biomarkers in Medicine	2013	☐Submitted ☐Accepted ☒Published
14. Immunotherapeutic strategies to target prognostic and predictive markers of cancer.	Magee, M.S., Snook, A.E., Marszalowicz, G.P., and Waldman, S.A.	Biomarkers Medicine	2013	☐Submitted ☐Accepted ☒Published
15. Advances in cancer immunotherapy.	Snook, A.E. and Waldman, S.A.	Discovery Med	2013	☐Submitted ☐Accepted ☒Published

16. Mechanisms of weight regain following weight loss.	Blomain, E., Dirhan, D., Valentino, M., Kim, G., and Waldman, S.A.	ISRN Obesity	2013	☐Submitted ☐Accepted ☒Published
17. The adipose tissue production is increased in End Stage Renal Disease.	Martinez Cantarin, M.P., Waldman, S.A., Doria, C., Frank, A.M., Maley, W.R., Ramirez, C.B., Keith, S. and Falkner, B.	Kid Inter	2013	☐Submitted ☐Accepted ☒Published
18. GUCY2C at the intersection of obesity and colorectal cancer.	Kim, G., Lin, J.E., and Waldman, S.A.	Trends Endocrin Metab	2013	☐Submitted ☐Accepted ☒Published
19. New advances in models and strategies for developing anti-obesity drugs.	Kim, G., Lin, J.E., and Waldman, S.A.	Exp Opinion Drug Discovery	2013	☐Submitted ☐Accepted ☒Published
20. Colorectal cancer immunotherapy.	Xiang, B., Snook, A.E., Magee, M.S., and Waldman, S.A.	Discovery Med	2013	☐Submitted ☐Accepted ☒Published
21. Translating colorectal cancer prevention through the guanylyl cyclase C signaling axis.	Blomain, E.S., Lin, J.E., Kraft, C.L., Trela, U.T., Rock, J.M., Aing, A.S., Snook, A.E., and Waldman, S.A.	Expert Rev Clin Pharmacol	2013	☐Submitted ☐Accepted ☒Published
22. Intestinal GUCY2C Prevents TGF-B Secretion Coordinating Desmoplasia and Hyperproliferation in Colorectal Cancer.	Gibbons, A.V., Lin, J.E., Kim, G.W., Marszalowicz, G.P., Li, P., Stoecker, B.A., Blomain, E.S., Rattan, S., Snook, A.E, Schulz, S. and Waldman, S.A.	Can Res	2013	☐Submitted ☐Accepted ☒Published
23. Anti-obesity pharmacotherapy: New drugs and emerging targets.	Kim, G., Lin, J.E., Blomain, E.B., and Waldman, S.A.	Clin Pharm Ther	2013	☐Submitted ☐Accepted ☒Published
24. Immunotargeting and eradication of orthotopic melanoma	Igoucheva, O., Grazzinin. M., Pidich, A., Kemp,	Nature	2013	☐Submitted ☐Accepted ☒Published

using a chemokine-enhanced DVA vaccine.	D.M., Larijani, M., Farber, M., Lorton, J., Rodeck, U., Alexeev, V.			
25. Molecular insights provide the critical path to disease mitigation.	Waldman, S.A., and Terzic, A.	Clin Pharm Ther	2014	☐Submitted ☐Accepted ☒Published
26. Managing the innovation supply chain to maximize personalized medicine.	Waldman, S.A., and Terzic, A.	Clin Pharm Ther	2014	☐Submitted ☐Accepted ☒Published
27. Tumor radiation therapy creates therapeutic vaccine responses to the colorectal cancer antigen GUCY2C.	Witek, M., Magee, M.S., Xiang, B., and Waldman, S.A., Snook, A.E.	International Journal of Radiation Oncology, Biology, Physics	2014	☐Submitted ☐Accepted ☒Published
28. Selective antigen-specific CD4+ T cell, but not CD8+ T- or B-cell, tolerance corrupts cancer immunotherapy.	Snook, A.E., Magee, M.S., Schulz, S., and Waldman, S.A.	Eur J Immun	2014	☐Submitted ☐Accepted ☒Published
29. Gut-brain endocrine axes in weight regulation and obesity pharmacotherapy.	Merlino, D., Blomain, E.S., Aing, A.S., and Waldman, S.A.	J Clin Med	2014	☐Submitted ☒Accepted ☐Published
30. The Paracrine hormone for the GUCY2C tumor suppressor, guanylin, is universally lost in colorectal cancer.	Wilson, C., Lin, J. E., Li, P., Snook, A.E., Gong, J., Sato, T., Chengbao, L., Girondo, M.A., Rui, H., Hyslop, and Waldman, S.A.		2014	☒Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes__X_____ No_____

If yes, please describe your plans:

We will submit manuscripts on (1) novel ad5-GCC vaccine safety and efficacy in stage I and II colon cancer patients; (2) the efficacy of a culturally-sensitive decision counseling program to improve participation by under-represented minorities in cancer clinical trials; and (3) the utility of heterologous prime-boost vaccine strategies using adenoviral and DNA antigen expression systems to improve the efficacy of cancer vaccines.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None. The studies involved a first-in-man clinical trial of a new immunotherapeutic. The impact of this study on human health will be unknown for several years.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

These studies produced the new drug Ad5-hGCC-PADRE that may have utility in preventing colorectal cancer recurrence in patients. This project included the creation, GMP manufacturing, testing and clinical exploration of the Ad5-hGCC-PADRE vaccine in patients.

These studies also identified mechanisms underlying the synergy of DNA and Ad5 prime-boost vaccination regimens. Those results may be translated directly into clinical studies examining DNA-GCC-PADRE combined with Ad5-hGCC-PADRE, which may have superior efficacy than Ad5-hGCC-PADRE alone. Moreover, those findings may be applied to other tumor antigens, leading to vaccine approaches to enhance the activity and antitumor efficacy of treatments for various malignancies.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes__X_____ No_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

Title of Invention: Cell-Based Anti-Cancer Compositions With Reduced Toxicity And Methods Of Making And Using The Same

a. Name of Inventor(s): SA Waldman, AE Snook, M Magee

b. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

Colorectal cancer is the third most common cause of death from malignant disease in Western countries. Worldwide, it has been estimated there are at least half a million new cases of colorectal cancer each year. Current immunotherapeutic approaches to treat colorectal cancer remain poorly efficacious.

We have identified a novel class of vaccine targets for tumors arising from mucosae (aerodigestive, urogenital, breast, other), termed cancer mucosal antigens (CMAs). These antigens are normally expressed only in the mucosal compartment and their expression persists after mucosal cells undergo neoplastic transformation and become cancer cells. Moreover, these antigens continue to be expressed after these tumor cells metastasize. The theoretical advantage of these antigens includes (1) only partial tolerance in the systemic compartment, which is normally naïve to these antigens, permitting an effective systemic immune response to them which provides anti-metastatic tumor efficacy and (2) absence of cross compartmental immune responses, avoiding mucosal inflammation and autoimmunity. Indeed, the first cancer mucosal antigen, guanylyl cyclase C (GCC), has confirmed these theoretical advantages. GCC immunization induces a systemic immune response, demonstrating incomplete systemic tolerance to this mucosal antigen; that immune response demonstrates superior anti-metastatic tumor efficacy, effectively preventing colon cancer metastases to lung and liver in prophylactic and therapeutic models; and anti-tumor efficacy is produced in the complete absence of mucosal or systemic autoimmunity.

Immunization with GCC-expressing viral vectors (active immunotherapy) produced CD8+ T cell responses that effectively prevented and treated metastatic cancer in mice. Pre-clinical and clinical data in the literature have demonstrated that adoptive immunotherapy employing modified T cells also has therapeutic efficacy, especially against bulky metastatic disease. This invention constitutes the use of unmodified or modified CMA-directed T cells as therapeutic agents for cancer. Specifically, autologous CMA-specific T cells can be harvested from patients, cultured in vitro and re-infused to the patient to treat CMA-expressing tumors. Moreover, autologous T cells of undefined specificity can be harvested, modified in vitro to express either a) CMA-specific T cell receptor (TCR) or b) a modified TCR containing CMA-specific antibody domains fused to TCR domains. These modified TCRs are known as chimeric antigen receptors (CARs) or T-bodies. These engineered cells can be infused to patients to treat CMA-expressing tumors. CMA-directed T cells have the potential to effectively treat and prevent tumor metastases originating from mucosae, including aerodigestive, urogenital and breast.

c. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____X_____ No____

If yes, indicate date patent was filed: March 15, 2013

d. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___X
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

e. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes__X_____ No____

If yes, how many licenses were granted?_____1_____

f. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No___X

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No__X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Dr. Adam Snook, PhD.
A. Personal Statement
I am a junior faculty member at Thomas Jefferson University in the Jefferson Medical College, Department of Pharmacology and Experimental Therapeutics. My doctoral and post-doctoral training, as well as my early independent focus, has been in cancer immunology and the development of novel cancer immunotherapeutics. I identified guanylyl cyclase C (GUCY2C) as a novel target for immunotherapy in cancers of the gastrointestinal system (esophageal, stomach, colon and rectal cancers). I developed mouse models, vaccines and immunoassays required to explore GUCY2C immunobiology, demonstrating vaccine-induced immune responses and antitumor efficacy targeting GUCY2C. Importantly, I have

translated that discovery into an ongoing Phase I clinical trial examining the safety and immunogenicity of the GUCY2C vaccine, Ad5-GUCY2C in colon cancer patients. Beyond the clinical potential of Ad5-GUCY2C, I am interesting in exploring tolerance mechanisms underlying vaccine efficacy and safety. Additionally, I am developing innovative paradigms to treat patients with bulky tumor metastases using adoptive cell therapy (ACT). While a vaccine relies on a patient's immune system to create the antigen-specific immune response, ACT using engineered T cells offers unique advantages. Specifically, tolerance removes antigen-specific T cells during their maturation, limiting the number and the potency of T cells that escape tolerance and can be harnessed by vaccination. In contrast, T cells can be engineered and expanded ex vivo to produce large numbers of cells expressing receptors of high affinity, maximizing their potency. This technique has produced remarkable clinical successes in melanoma, neuroblastoma and leukemia. However, ACT approaches are commonly associated with life-threatening toxicities following antigen recognition in normal tissues. The research plan proposed in the present application will test the hypothesis that hypoxia-regulated antigen receptor expression will maximize antigen expression in T cells in tumors and minimize their expression in T cells in normal tissues. This approach should prevent ACT toxicity and permit its safe and effective use in metastatic cancer patients.

B. Positions and Honors Positions and Employment

2008-2010 Post-Doctoral Fellow, Department of Pharmacology and Experimental Therapeutics
Thomas Jefferson University, Philadelphia
2010-2013 Post-Doctoral Fellow, Microbiology & Immunology, Thomas Jefferson University,
Philadelphia
2013-present Research Instructor, Department of Pharmacology and Experimental Therapeutics Thomas Jefferson University, Philadelphia
Honors and Awards
1997 Henkel Corporation Trylon Chemicals Award,
1997-2001 Half-Tuition Academic Scholarship, University of the Sciences in Philadelphia
1998 Member, Alpha Lambda Delta, Freshman Honor Society
1999 Member, Alpha Chi, National Scholarship Society

2001 Alumni Association Commencement Award
2001 Ortho-McNeil Pharmaceutical/Janssen Pharmaceutical Award for Research Excellence 2001 Summa Cum Laude Scholastic Award
2001-2008 Measey Foundation Fellowship
2006 Sigma Xi Student Research Day Presentation Winner
2007 Jefferson College of Graduate Studies Alumni Travel Fellowship Award
Memberships in professional societies:
American Association for the Advancement of Science (AAAS) Society for Immunotherapy of Cancer (SITC)
The American Association of Immunologists (AAI)
The American Association for Cancer Research (AACR) Sigma Xi, Scientific Research Society
Rho Pi Phi, International Pharmaceutical Fraternity

Notable Speaking Invitations:
Tumor Immunology (An AACR Special Conference in Cancer Research), 2008, Platform Presentation Targeted Immunotherapeutics & Vaccine Summit, 2008, Invited Speaker
Jefferson Vaccine Center Meeting, 2008, Invited Speaker

C. Active Research Support

Pharmaceutical Research and Manufacturers of America Foundation (PhRMA)
(Snook, A.E. PI) 07/01/14-06/30/14
GUCY2C-specific tolerance in colon cancer patients
This project will define GUCY2C-specific tolerance mechanisms in colon cancer patients. This project will provide a critical path for future studies to clarify molecular and cellular mechanisms by which GUCY2C- specific CD4 T cells are selectively tolerant.

Margaret Q. Landenberger Research Foundation
(Snook, A.E. PI) 07/01/14-06/30/16
GUCY2C-specific CD4+ T cell tolerance mechanisms and outcomes in tumor immunity
This project will define GUCY2C-specific CD4+ T cell tolerance mechanisms in mice and the positive (antitumor immunity) and negative (autoimmunity) outcomes associated with reversing tolerance. This
project will provide a critical path for future studies to define new immunotherapeutics to maximize tumor
immunity, without autoimmunity.

NIH/NCI 1R01 CA170533 (Waldman, S.A., PI) 08/01/12-06/30/16
GUCY2C hormone signaling at the intersection of obesity and colorectal cancer (PQ1)
This project explores the relationship between obesity, cancer, and hormones regulating the intestinal tumor suppressor GUCY2C.
Role: Researcher