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:** The Trustees of the University of Pennsylvania

2. **Reporting Period (start and end date of grant award period):** 6/1/10-5/31/14

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

4. **Grant Contact Person's Telephone Number:** 215-662-7153

5. **Grant SAP Number:** 4100051725

5. **Project Number and Title of Research Project:** Novel Adjuvants for Cancer Vaccine Immunotherapy

6. **Start and End Date of Research Project:** 6/1/10-5/31/14

7. **Name of Principal Investigator for the Research Project:** Carl H. June, M.D.

8. **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:

   $ 4,621,644.30

   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
June	PI-Professor	7% Yr1; 7% Yr2; 11% Yr3; 14% Y4	$105,324.38
Zhao	Co-Investigator	32% Yr1; 33% Yr2; 16% Yr3; 22% Yr4	$158,370
Antzis	Research Specialist	33% Yr2; 11% Yr3	$10,414
Frigault	Research Specialist	90% Yr2	$19,256
Gilmore	Clinical Research Coordinator/Data Manager	28% Yr1; 40% Yr2; 5% Yr3	$43,820
Guedan Carrio	Post-Doc Researcher	100% Yr1; 3%Yr 3; 100% Yr4	$89,003.83
McGettigan	Research Specialist	46% Yr1; 50% Yr2; 8% Yr3	$44,836
Paulos	Post-Doc Researcher	62% Yr1	$25,040
Veloso	Regulatory Affairs	13% Yr1; 20% Yr2; 12% Yr3; 19% Yr4	$94,393
Vonderheide	Investigator	5%-Yr 1,2,4; 4%-Yr 3	$44,932
Borghaei	Co- Investigator Associate Professor	5% Yr 1-3; 13% Yr 4	$63,516
Kalos	Co-Investigator	9%-Yr 1; 10%-Yr 2; 8% Yr 3; 23% Yr 4	$84,537
Lacey	Co-Investigator	4% Yr 4	$5,129
Tchou	Clinical PI	11% Yr 2; 16.2% Yr 3; 14.8% Yr 4	$124,201
Anderson	Regulatory	9% Yr 4	$8,708
Mikheeva	Research Specialist	13% Yr 1; 15% Yr 2-3; 3% Yr 4	$34,286
Gupta	Research Specialist	8% Yr 3	$3,191
Chen	Research Specialist	6% Yr4	$4,388
Trosko	Research Specialist	1% Yr 2	$29
Bagnyukova	Post Doc Associate	16% Yr 4	$11,654
Liu	Research Associate	44% Yr 4	$29,087
Adams	Associate Professor	15% Yr 1-3; 25% Yr 4	$108,601
Robinson	Assistant Professor	15% Yr 1-3; 36% Yr4	$104,895
D'Souza	Research Specialist	46% Yr3; 73% Yr 4	$60,678
Shaller	Scientific Associate	46% Yr 4	$42,597
Shchaveleva	Scientific Technician II	50% Yr1; 20% Yr2; 49% Yr 4	$78,701
Simmons	Scientific Associate	75% Yr1; 69% Yr2; 50% Yr3; 47%Yr 4	$215,888
Kromer	Scientific Technician I	26% Yr 3	$11,693
Miller	Post Doc Associate	36% Yr 2; 6% Yr 3	$23,176
Railkar	Post Doc Associate	27% Yr 4	$16,028

Coukos	Investigator	3.5% Yr 1-4	$26,558
Facciabene	Co-Investigator	31% Yr 1-4	$91,155
De Sanctis	Visiting Student	83.5% Yr 1-4	$33,400
Ugel	Post Doc Associate	5.1% Yr 1-4	$3,552
Jung	MGR Admin& Finance	7.2% Yr 1-4	$17,145
Pierini	Visiting Student	93.9% Yr 1-4	$31,347
Rafail	Post Doc Associate	55.5% Yr 1-4	$102,027
Peng	Specialist	6.2 % Yr 1-4	$11,632
Aqui	PI	11% Yr1-4	$61,472
Tsao	Sr. Research Investigator	4% Yr3; 30% Yr4	$27,244
Nyarko	Research Fellow	100% Yr3	$5,000
Chew	Scientific Project Director	22% Yr1	$11,085.37
Haines	Research Specialist	23% Yr1; 88% Yr2; 58% Yr3; 10% Yr4	$134,127.92
Lee	Post-Doc Researcher	100% Yr1; 33% Yr2	$22,015.00
Leight	Administrative Officer	23% Yr1; 25% Yr2; 24% Yr 3; 10% Yr4	$53,795.12
Plesa	Scientific Project Director	18% Yr2; 11% Yr3; 11% Yr4	$46,076.02
Carroll	Director	8% Yr1	$4,995.19
Liu, R	Research Specialist	28% Yr1; 31% Yr2; 60% Yr3	$37,575.16
Leferovich	Research Specialist	17% Yr1; 19% Yr2	$24,276.63
Ebanks	Research Assistant	100% Yr2; 100% Yr3; 100% Yr4	$15,250
Wahome	Research Assistant	100%-Yr4	$1,500
Ihejirika	Research Assistant	100%-Yr4	$3,000

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
Facciponte	Post Doc Associate	100%
Baskerville	Associate Professor and Chair	5%
Chickwem	Co-Director	5%

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
None		

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__X____ No_____

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

Specific Aim 1. For last two years, part of a grant from the Breast Cancer Research Foundation (to Dr. Vonderheide, total award $250,000/year) has helped support this project. Specific Aim 3. NCI The Ruth L. Kirschstein NRSA Institutional Research Training Grant (T32) supported Dr. Facciponte effort.

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:
Vaccine to prevent breast cancer	☐NIH ☐ Other federal (specify:____) X Nonfederal source (specify: Breast Cancer Research Foundation)	June 2012 June 2013 June 2014	$$250K $250K $250K	$250K $250K pending
Development of Anti-Vasculature vaccine for BRCA-positive patients	☐NIH ☐ Other federal (specify:_____ _____) X Nonfederal source (specify: BASSER Research Center For BRCA)	Aug 2013	$200K	$150K
Ways and Means to improve the immune biology of anti-vasculature vaccine	XNIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	Dec 2013	$1.250K	Not funded

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:
Specific Aim 1. BCRF pending application mentioned above.
Specific Aim 2. The PIs are reviewing the results of the combined efforts on the project and are exploring new avenues for funding ongoing research.

Specific Aim 3. The NIH RO1 application mentioned above will be resubmitted in the future cycle of submission, considering the encouraging results obtained in the mouse models; the goal is to fund the clinical development of the tem1-TT DNA vaccine.
Specific Aim 4. The PI is exploring opportunities for other fund sources to continue the future plans for this project.

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

Specific Aim 1. We will enroll three additional metastatic breast cancer patients on the clinical trial of c-Met RNA CAR T cell (intratumoral injection) and complete the clinical assessments such as toxicity and response. We will complete the immune assessments on all six patients including evaluation of the post-treatment tumor biopsies for evidence of c-Met expression and tumor cytotoxicity. We will also examine for persistence of the infused cells. If biological activity is demonstrated in the absence of toxicity, we will work to enroll a second cohort of triple negative breast cancer patients. This cohort is described in the original protocol and will be allowed per protocol pending completion of evaluation of the first six patients treated with the same investigational cell product.

Specific Aim 2. The team is planning to continue the research project.

Specific Aim 3. In collaboration with the Basser Research Center for BRCA we are planning to conduct a standard first in human phase I dose escalation study to determine safety and tolerability, clinical and immunogenic response of TEM1-TT DNA vaccine in BRCA positive recurrent ovarian cancer patients who are platinum sensitive after completing secondary treatment with platinum-based therapy and are in remission. A 3+3 design will be employed whereby the following doses will be tested: 0.3 mg/dose, 1.5mg/dose and a 3 mg/dose. Four injections will be given in each cohort on Day 0, week 4, week 12, and week 24. Patients will receive a dose of cyclophosphamide infusion of 200 mg/m^2 the day before each vaccination. Cyclophosphamide has been shown to augment anti-tumor immune response in patients via downregulation of regulatory T-cells. Patients who have no evidence of disease will continue receiving maintenance vaccine every month until progression. A total of 15-18 patients will be enrolled, 3 in each cohort followed by an additional 6 patients allocated to the maximally tolerated dose. The primary objective of this study is to determine the safety and feasibility of TEM1-TT while the secondary objectives are immune and clinical responses. Immune response will be measured by interferon gamma response while the clinical response will be assessed looking at PFS at 6 months and 12 months respectively and by RECIST criteria. The strategy for clinical development of the vaccine will move in line with previous trials with DNA cancer vaccines. The steps include: a) manufacture GLP vectors for toxicology, and GMP vectors for a human trial; b) perform toxicology studies in small and large animals; c) submit an IND application; d) initiate a phase I study.

Specific Aim 4. The observation that CAR T cells bearing ICOS signaling domains have enhanced survival after adoptive transfer provides the rationale to test this concept in human trials in the setting of solid tumors and non-lymphoid hematologic malignancies, where improved persistence of CAR T cells remains as an unmet medical need. The results of this

research are analyzed for the feasibility of advancing into a clinical trial testing Th17 polarized CAR redirected T cells in cancer patients.

Specific Aim 5. The collaboration with Lincoln University allowed development of a research project studying the expression profile of genes and proteins in brain cancer cell lines that could give clues to immunotherapies for cancer of the brain; this research program involves and train undergraduate students in the translational research field. The data collected thus far have generated further questions and warrant us to repeat experiments and extend the study. We plan to do Western blot analyses of 4-HNE and 8-hydroxyguanosine protein adducts in the glioma cell lines. We need to complete gene oxidative stress expression studies and treat gliomas with pro-oxidants. Furthermore, we have purchased glioma profile and cancer drug targets arrays to study biomarkers of glioma cells and targets for anticancer therapies; we plan to run these arrays this summer. In the long term, we plan to study how treatment with antioxidants and pro-oxidants affects tumor growth as well.

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	4	1		
Female	5	1	1	
Unknown				9
Total	**9**	**2**	**1**	**9**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic	1			
Non-Hispanic	8	2	1	
Unknown				9
Total	**9**	**2**	**1**	**9**

	Undergraduate	Masters	Pre-doc	Post-doc
White	2	2		
Black	6		1	
Asian				
Other	1			
Unknown				9
Total	**9**	**2**	**1**	**9**

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

Yes__X_____ No_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:
Stefano Ugel, PhD, University of Padua
Francesco De Sanctis, Student, University of Perugia
Stefano Peirini, Student, University of Perugia

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.

This grant funding has improved access to state of the art cancer research for patients in Pennsylvania. CAR T cell therapy for cancer is an emerging therapeutic technology in which a patient's own immune cells are genetically modified to fight their cancer. The clinical trial under the Specific Aim 1 is a first in human testing of cMet CAR T cells. Additionally, the promising preclinical data set generated under Specific Aim 4 is considered for clinical testing in which a fundamental question as to the phenotype of T cells mediating the anti-tumor effects can be addressed. Knowledge gained from this research and future trials will impact cancer therapy development in the United States. The grant has supported a large collaborative effort to advance the clinical development of cutting-edge approaches to harness the immune system to fight cancer at UPENN institution. This new CAR technology has changed the dynamic of the collaborative efforts between pharmaceutical companies and academic institutions, and provides new opportunities for commercialization of anti-cancer therapies.

The addition of new investigators especially for Specific Aim 3 has contributed to the completion of the aim/project milestones earlier than planned and allowed advancement into regulatory path. The capacity and quality of this research has improved allowing advancement of a new investigational agent into clinical testing.

In addition to the clinical trial proposed in the initial grant application, this grant has allowed for extensive testing of additional potential clinical candidates in the cancer immunology field. The increased research capacity and quality gained has allowed for two additional projects to advance from the bench research to the clinical research.

The basic research at the Lincoln University has reached a higher standard through the Minority Core funded by this grant. The project is significant because it exposed undergraduate students to an interesting and challenging line of research. The basic research performed at the Lincoln University with undergraduate student trained in translational medicine through this program continues and will help generate more hypotheses on the role of ROS in therapeutic targets for gliobastomas.

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:
On August 2012, the University of Pennsylvania and Novartis announced a research and licensing agreement aimed at expanding the use of personalized T cell therapy using CARs for cancer. Based on this agreement, Novartis will have an exclusive global licensing to the technologies used in ongoing trial as well as future CAR-based therapies developed through the collaboration. As our project 1 proposes testing of cMet specific CAR technology for breast cancer patients, the outcomes of this research may fall under the stipulations of this agreement. The Novartis-UPENN alliance is expected to generate substantial new employment opportunities in the Commonwealth, thereby achieving one of the goals of the PA DOH program through technology transfer.

Additionally, this grant initiated a broad collaboration between FCCC and UPENN scientists and physicians, and Lincoln University faculty and students focused on developing novel immunotherapeutics for cancer and have exposed undergraduate student to laboratory experience in the field of translational research.

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

Yes_____ No__X_____

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

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:

As a result of this project, FCCC faculty presented lectures at Lincoln University on the topics of antibody development & therapy, biotechnology and novel therapeutics and at Allegheny College on development of biopharmaceuticals.

UPENN faculty presented lectures at the Lincoln University as part of the Minority Core program of this grant. The talks, given by all the Project PI's as well as other biomedical researchers, ranged in topics from novel cancer therapies, to pharmacology, to biomarkers of schizophrenia and autism. The seminar series has been very successful, as evidenced by the fact that Lincoln has been able to attract additional outside (non-Penn) speakers.

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.

Specific Aim 1. The first proposed aim/project was designed to test the clinical and immunological capability of novel bispecific T cell engaging reagents to redirect the force of polyclonal T cell populations against tumor cells in patients with advanced lung, ovarian, and breast cancer for a vaccine effect. A clinical protocol was developed to test safety of this reagent and the regulatory path forward was strategized. Our initial efforts were to develop the CD326/CD3 bispecific antibody construct from Micromet for this purpose; however, further results from the phase I study of this reagent in Europe became available and demonstrated poor clinical efficacy and importantly, it raised questions of liver toxicity. Although considerable effort was expended in collaboration with the company to develop alternative dose and schedule scenarios to mitigate these concerns, intellectual property issues became highly complex and jeopardized the scientific goals of this project such that the collaboration with the company were terminated.

In the interim, we became highly interested in the tumor target cMet as the focus of this project. Like CD326, cMet is overexpressed on the surface of solid tumor malignancies, but unlike CD326, a role for cMet as a receptor tyrosine kinase in tumor growth and development is clearly established, increasing its value as a potential immune target. Thus, on the second year of the grant, the project was changed with a newly proposed clinical study testing safety and feasibility of cMet redirected autologous T cells administered in patients with breast cancer. This new study proposed to use a cMet-specific chimeric antigen receptor (CAR) with transient expression on T cells for an increased safety design of the clinical study. The cMet CAR construct is comprised of an anti-cMet single chain Fv (scFv) fused to a hinge, transmembrane domain, and 41-BB and CD3zeta signaling domains. The transient expression of this cMet CAR on autologous T cells is achieved by using the mRNA electroporation technology, a manufacturing step developed in collaboration with MaxCyte® (http://www.maxcyte.com) using a closed, high scale, GPM validated mRNA Flow Transfection System. A GMP process for manufacturing of cMet CAR T cells was developed, and the cMet CAR mRNA ancillary reagent was manufactured.

In support for the correlative studies of this clinical trial, we evaluated the feasibility of applying the Quantigene platform to formalin-fixed and paraffin embedded (FFPE) samples as a novel technology allowing for the multiplexed quantification of mRNA expression in an attempt to identify the panel of antigens expressed on tumor cells as part of our larger programmatic effort to develop patient specific targeted therapeutics. The Quantigene platform was adapted to include detection of the cMet antigen as a direct applicability to the proposed clinical trial. This technology was used to evaluate cMet expression in multiple primary breast tumors in order to confirm the high level of cMet expression in these tumor types and establish the foundation for qualifying this assay for clinical use in the proposed trial. The Quantigene assay platform (Panomics) is a Luminex XMAP bead based assay that uses branched DNA (bDNA) technology. The bDNA assay is a sandwich nucleic acid hybridization method that uses bDNA molecules to amplify signal from captured target RNA. In bDNA technology RNA is measured directly from the sample source, without RNA purification or enzymatic manipulation, thereby avoiding inefficiencies and variability introduced by or errors inherent to these processes.

We have generated preclinical data indicating the safety profile, specificity of target recognition, and anti-tumor efficacy of cMet CAR redirected T cells, data supporting the development of the clinical protocol and the IND application. A clinical protocol was designed and it received the

NIH OBA RAC approval on December 2011. The IND application was submitted and the FDA allowed opening the IND on March 2012. The study went through several revisions by the UPENN regulatory committees which leaded to a modified study design and addition of a new patient population for an increased safety design. The study was officially opened on April 2013.

The clinical study is designed to test safety and feasibility of the cMet CAR mRNA eletroporated T cells by intratumoral (IT) injection in breast cancer subjects (primary objective). The study schema is shown in Figure 1.1. Secondary objectives include assessment of cMet directed responses in resected tumor tissue, immunogenicity and bioactivity of cMet CAR T cells, and the effect of local treatment on progression of metastatic disease at other sites.

This is an open-label, single-arm Phase I clinical trial of autologous cMet redirected T cells administered intratumorally (IT) in patients with cMet positive breast cancer in a two steps design to account for two separate but related study populations. Between 15 and 24 (depending on toxicity) patients in four cohorts (Table 1.1) will be treated at the University of Pennsylvania. Patients who have confirmed cMet expression and meet all the other inclusion criteria are allowed to enroll in the study. Details of the study including the inclusion/exclusion criteria are posted on the www.clinicaltrials.gov site NCT01837602.

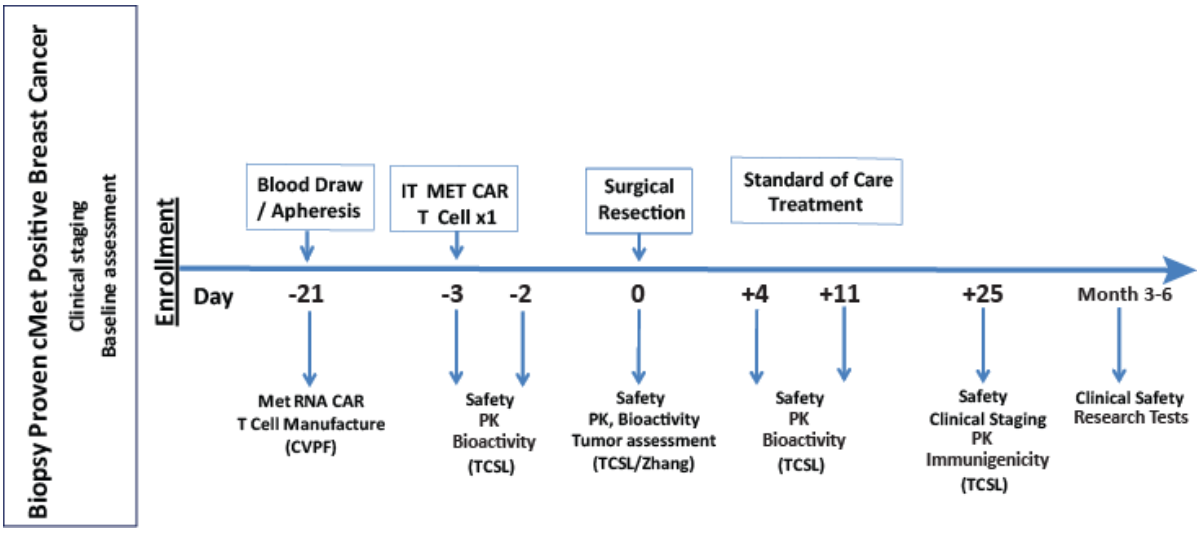


Figure 1.1 Study schema

During Step 1 (Cohort 1), six subjects with metastatic breast cancer and accessible metastatic deposits will be treated with cMet CAR T cells by IT injection in one of the metastatic deposits; first 3 subjects will be given a low cell dose of $3x10^7$ cells and the next 3 subjects will be treated with a high cells dose of $3x10^8$ cells. Step 2 subjects (Cohorts 2, 3, 4) will be newly diagnosed, operable, triple negative breast cancer (TNBC) subjects who will receive a single IT injection of cMet CAR T cells. Three doses will be tested with 3 patients per each of 3 cohorts. In the event of a single dose limiting toxicity (DLT0 in a cohort, 3 additional patients will be treated to ensure the rate of DLT is $\leq$1/6 patients. Three days after cMet CAR T cell injection (Day 0 in study schema, Figure 1), subjects will undergo resection of the metastatic (Cohort 1) or primary (Cohort 2, 3, 4) tumor treated and follow standard of care procedures. Cohort 1 patients will

undergo tumor staging before treatment and at 28 days after treatment (Day +25 on the protocol) or later. Treated subjects will be followed for 25 days after surgery (28 days after IT injection) and at 3-6 months or until metastatic breast cancer patients begin new therapy, whichever comes first. Patients will then be on follow-up for progression free survival for up to two years.

Table 1.1 Cell dosing and Number of Subjects per Cohort

Cohort	# of Subjects	Dose
1	3	$3x10^7$ cells
	3	$3x10^8$ cells
2	3	$3x10^7$ cells
3	3	$1x10^8$ cells
4	3	$3x10^8$ cells

At the time of this reporting, 3 subjects in the Cohort 1 were treated. Safety analysis indicates no overt toxicity related to IT injection of cMet CAR T cells. To date, no DLT was noted allowing progression of the study to the next cMet CAR dose level.

The presence of cMet CAR T cells in tumor samples and peripheral blood was determined using a quantitative RT-PCR assay specific for a sequence of the cMet CAR mRNA construct. The cMet specific signal was detected in tumor biopsies, but not in any of the peripheral blood samples collected up to day 28 post-treatment (day 25 on the protocol).

The excised tumor was analyzed for evidence of cMet directed responses. Preliminary immune-histochemistry (IHC) analysis indicates occurrence of tumor necrosis along the needle track associated with the presence of lymphocytes and macrophages.

From subjects 001, 003, and 004 we had tumor biopsy; from subject 004, we also have two lymph nodes. From subject 003, we had access to FFPE; from this same patient part of tumor biopsy was used to generate single cell suspension, and we had normal tissue for comparison of gene expression profiles.

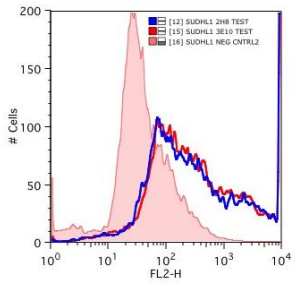
Gene expression in the tumor biopsies has been investigated with Quantigene assay. We used a 57-plex panel to interrogate 57 genes in total RNA extracted from biopsy samples, from five tumor cell lines, and from mixed normal donor PBMC, and from normal donor bone marrow. The 57 genes included the cMet gene, genes encoding T cell and other leukocyte markers, cytokines, chemokines, and various receptors, and transcriptional factors. Together this data set allowed us to asses a) tumor antigen gene expression, b) infiltration of the tumor tissues by various leukocyte subsets, including T cell subsets, and c) assess the quality of the T cell infiltrate, i.e. whether they belonged to Tregs, T helper 1 or other subsets, and their activation status, indicated by the expression of the various cytokine and chemokine genes plus activation markers such as CD69. The transcriptional levels of the genes are normalized by reference to the PPIB housekeeping gene. The results suggest that the breast tumor biopsy samples from patients 001, 003 and 004, and normal breast tissue from patient 003 contain cMet transcripts, as do all five tumor cell lines. The control PBMC and bone marrow RNA samples did not have a cMet

signal. In line with the IHC analysis, the tumor tissue that was treated 3 days prior with cMet CAR T cells showed infiltration with T cells, whereas the normal breast tissue did not contain CD3, CD4, CD8, and other T cell-associated transcripts. Also absent from normal breast were CD20 (B cell marker) and CD11c/ITGAX (dendritic cells); however, most of the breast and lymph node tumor biopsy samples showed these T cell, B cell and DC cell markers, suggesting their infiltration by these immune cells following cMet CAR T cell injection. Macrophages, melanocyte and platelet markers were present in all the biopsy samples. The panel included several genes encoding cytokines and chemokines including MIG, IP10, IL-2, IL-6, GMCSF, and TNF-α, most of which were seen in the biopsy samples and activated PBMC, but not in tumor cell lines. Since these cytokines and chemokines are generally only expressed by activated immune cells, we interpret this as a sign of immune activation. Several cytokine and chemokine receptors were also expressed in the biopsies. Finally, the biopsies from patients 001 and 003, but not the lymph node biopsies from patient 004 showed high expression of the GATA-3 gene. GATA-3 is a transcription factor that regulates luminal epithelial cell differentiation in the mammary gland. GATA-3 has been shown to promote the secretion of IL-4, IL-5, and IL-13 from Th2 cells, and induces the differentiation of Th0 cells towards Th2 while suppressing their differentiation towards Th1 cells. GATA-3 is mutated in a subset of breast cancers, and has been shown to be required for luminal A breast cancer (Kouros-Mehr Hosein, Kim JW, Bechis SK, Werb Z "GATA-3 and the regulation of the mammary luminal cell fate" April 2008 Curr. Opin. Cell Biol. 20 (2): 164–70)

This summary reveals that the milestones established for the Specific Aim 1 of this grant has been accomplished, with the exception of completing the trial's subjects' accrual. This delay is justified by the complete change of project toward the end of grant Year 1 which reset the milestones timeline. Leveraging additional funding sources, this clinical trial is progressing to testing safety of a 10-fold higher dose of cMet CAR T cells in an effort to establish proof-of -concept for using the cMet CAR T cells in the treatment of cMet expressing tumors.

In an effort to identify tumor antigens expression in breast cancer that could be exploited as targets for adoptive immunotherapy with CAR redirected T cell, we have reported the prospect of mesothelin as a second breast cancer surface target for engineered T cell (Tchou J, Wang LC, Selven B, Zhang H, Conejo-Garcia J, Borghaei H, Kalos M, Vonderheide RH, Albelda SM, June CH, Zhang PJ "Mesothelin, a novel immunotherapy target for triple negative breast cancer" Breast Can Res Treat, 2012; 133:799-804). Screening 99 primary breast cancer samples by immunohistochemistry analysis confirmed that mesothelin is over expressed in the majority of TNBC (67%) but only rarely (<5%) in other breast cancers. A significantly higher anti-tumor cytotoxicity by such T cells compared to control was observed *in vitro*, suggesting for the first time that mesothelin has promise as a novel immunotherapy target for TNBC for which effective targeted therapy is lacking to date.

Figure 2.1. FACS analysis of anti-ICOS antibodies using the ICOS "+" SUDHL1 cell line.



Specific Aim 2. The goals for this aim/project as proposed in the grant application are as follows.

1. To develop second generation BiTEs (bispecific scFvs or BiTEs) capable of engaging and stimulating T cells at the surface of tumor cells. Anti-human ICOS (a T cell stimulatory receptor) single-chain Fv molecules will be

isolated from combinatorial phage display libraries and will be employed in the generation of anti-EpCAM/anti-ICOS BiTEs. The ability of these BiTEs to selectively stimulate T cells will be determined in vitro.

2. To develop second generation BiTEs that selectively block T cell inhibitory receptors when the cells are bound to tumor cells. Anti-human CTLA-4 and PD-1 single-chain Fv molecules will be isolated and employed in the generation of anti-EpCAM/anti-CTLA-4/PD-1 BiTEs. The ability of these BiTEs to selectively stimulate T cells will be determined in vitro.

3. To validate the ability of the ability of the pro-stimulatory and anti-inhibitory BiTE molecules to promote anti-tumor effects and stimulate memory immune responses in NSG mice. The ability of combinations of BiTEs targeting EpCAM with one arm and CD3, ICOS or CTLA-4 or PD-1 with the second arm to effectively treat established tumors and mount a memory immune response will be assessed.

We were successful in cloning and expressing the PD1 target receptor as an Fc fusion (methods are described below). Attempts to similarly produce CTLA-4 and ICOS fusion proteins repeatedly failed. However, during the term of the project the targets became commercially available and were purchased. Phage display selections were carried out to isolate single chain antibodies (scFvs) that engage these targets. Selection results for anti-PD1 antibodies and anti-ICOS antibodies are provided in Table 2.1. The clinical success of Ipilimumab was reported during the period of these studies. Based upon this, the project's external reviewers at the Interim Review meeting directed us to employ the variable domains from established commercial antibodies (Table 2.1) rather than continuing to focus on developing higher risk new anti-CTLA-4 MAbs for the generation of bispecific reagents.

Table 2.1 Antibodies isolated/developed by the project team

Target	Phage display	Published seq
ICOS	4	-
PD1	2	2
CTLA-4	-	1
MET	1	1

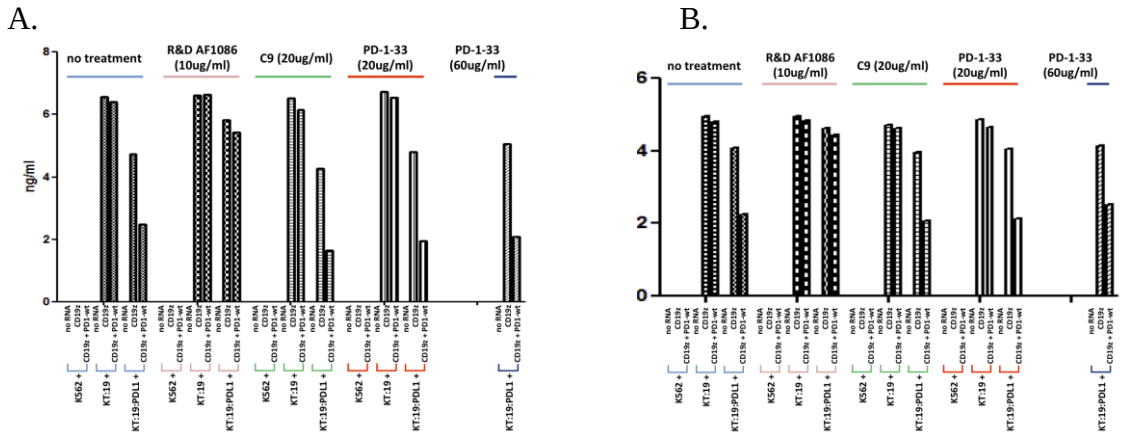
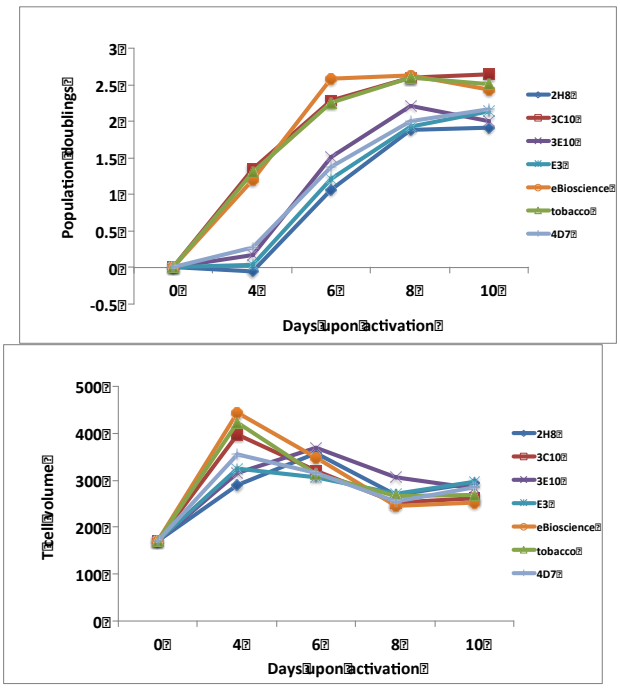


Figure 2.2. Impact of anti-PD1 antibodies on receptor function. The ability of anti-PD1 antibodies (C9 and PD-1-33) to block receptor function were measured using induction of IFNgamma production (A), IL2 production (B), cellular proliferation (not shown) as biomarkers of activity. A human PD-1 antibody from R&D (AF1086) was used as a positive control for blocking PD1 signaling. Lack of IFNgamma production and induction of proliferation suggests the antibodies are not effectively blocking PD1 function.

Antibodies were expressed from E. coli as scFv molecules and where possible were assayed for specificity against cells expressing the target antigens. An example of demonstration of anti-ICOS specificity is provided in Figure 2.1. ScFvs were then sent to UPENN to determine their ability to modulate T cell activity. The anti-PD1 antibodies did not exhibit the ability to block T cell function in the assay systems employed (Figure 2.2).

However, we believe that these negative results were due to the assay systems employed rather than the intrinsic activity of the antibodies, particularly as the PD1-33 (anti-PD1) antibody was derived from an antibody with known anti-PD1 activity. In contrast, one anti-ICOS antibody isolated from our phage libraries exhibited the ability to stimulate T cell proliferation (Figure 2.3). The June lab had problems with the CTLA-4 assay and is currently in the process of resolving those issues. As

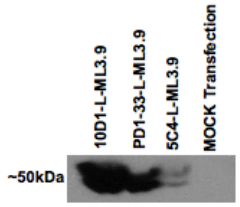
Figure 2.3. Receptor function of anti-ICOS antibodies. The ability of generated anti-ICOS antibodies (2H8, 3C10, 3E10, 4D7), positive control anti-ICOS antibodies (eBioscience, JMAB 52 [tobacco]), and a negative control antibody (E3) to stimulate receptor function were measured using population doubling and T cell volume.



stated above, these are antibodies with known in vivo function; however, it is not always possible to easily demonstrate in vitro function. This led to our decision to initiate construction of bispecific antibodies based upon the above inhibitory anti-PD1 and CTLA-4 scFvs.

BiTEs were initially planned employing anti-MET scFvs as tumor-targeting vehicles. Phage display selections were carried out to isolate anti-MET scFvs (Table 2.1). Due to a number of considerations including feedback from our External Advisors at the Interim Review Meeting regarding new concerns about MET as a cancer target and the potential of anti-MET antibodies to initiate tumor cell proliferation the decision was made to target the validated HER2 tumor associated antigen instead. BiTEs were generated targeting HER2 using the ML3.9 antibody in combination with the CTLA-4 or PD1 inhibitory T cell receptors. PD1-33/ML3.9, 5C4/ML3.9 and 10D1/ML3.9 bs-scFvs were expressed (Figure 2.4) and purified (data not shown) from HEK293 cells. These three bs-scFvs have been sent to UPENN for functional analysis. A new bispecific format was developed in order to incorporate the antibody's functional Fc domain. This format, which is described in Figure 2.5, designed to provide better pharmacokinetics/bioavailability than current BiTEs that require administration by continuous infusion.

Figure 2.4. Expression of 2nd generation BiTEs. Western blot of media from pools of cells transfected with anti-PD1/anti-HER2 and anti-CTLA4/anti-HER2 BiTEs constructed from clinically validated anti-CTLA4 and anti-PD1 mAbs demonstrating expression of the 2nd generation BiTEs.



Methods:

Target fusion protein expression: The genes encoding each of the three target receptors' extracellular domains (ECDs) were synthesized and cloned into pSec/Fc vectors to create in-frame fusion proteins with a human IgG1 Fc. The expressed fusion proteins were designed to contain an optimized thrombin cleavage site for liberation of the target ECDs and a 6X-HIS tag at the C-terminus of the Fc domain for purification and detection. HEK-293T cells were transiently transfected with vectors containing each ECD gene and the resulting fusion proteins were isolated and purified. PD-1 exhibited excellent production from transiently transfected cells with approximately 3.5 mg of protein recovered from 100 ml of media (Figure 2.6).

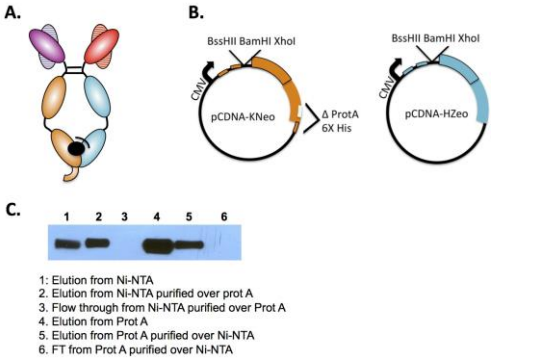
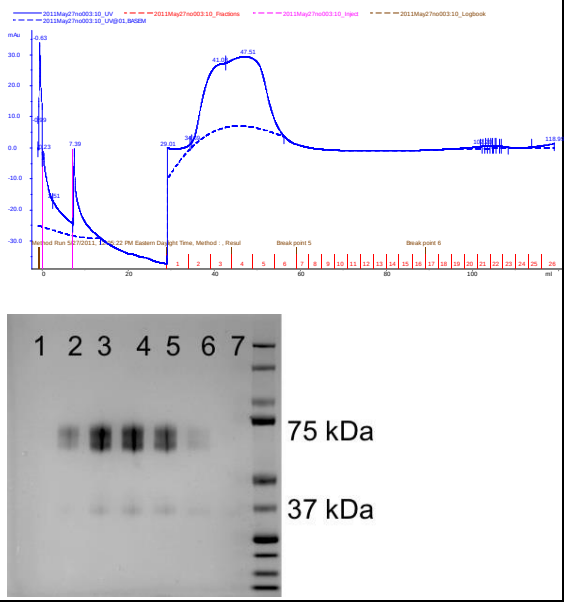


Figure 2.5. Schematic and Vector Map of new bispecific antibody construct. A depiction of our new bispecific antibody format (A), vector maps of the light and heavy chain domains (B) and elution profiles of test molecules verifying the behavior of the expression system are presented.

Table 2.2. Anti-PD1 selection output			
ROUND	LIBRARY STOCK (input phage)	#SELECTED PHAGE (clones)	AMPLIFIED PHAGE (pfu/mL)
1	$1x10^{17}$	$1x10^{5}$	$6x10^{15}$
2	$6x10^{15}$	$1.2x10^{7}$	$1x10^{16}$
3	$1x10^{16}$	$4.5x10^{8}$	$2.7x10^{15}$
4	$2.7x10^{15}$	$1.2x10^{8}$	----

Figure 2.6. Expression and purification of PD1-Fc.



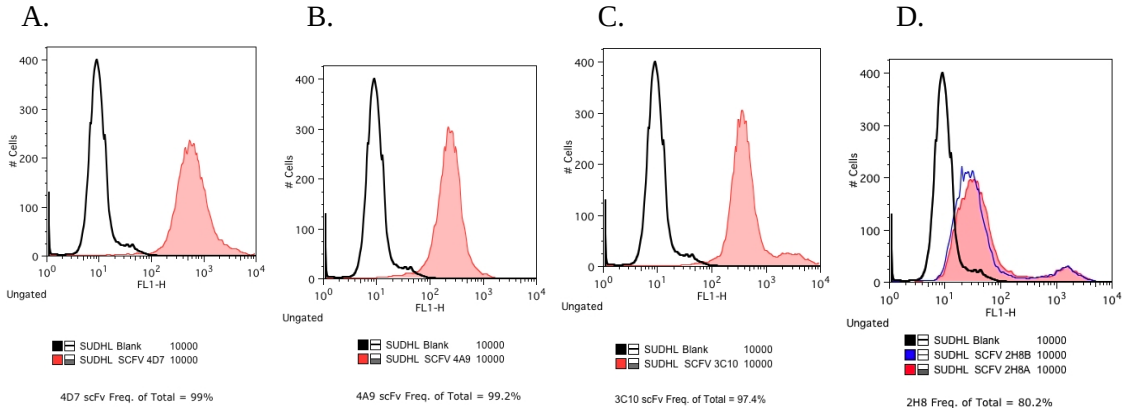


Figure 2.7. Flow cytometry analysis of anti-ICOS scFv. Anti-ICOS scFv (shaded area) exhibit binding to ICOS-positive SUDHL cells as compared to secondary antibody alone (open area). (A) = 4D7, (B) = 4A9, (C) = 3C10, and (D) = 2H8

Phage display selections: Briefly, $1x10^{17}$ pfu of the Nissim scFv combinatorial phage display library (Nissim et al EMBO J 1994) was depleted against Immunotubes coated with 100 µg/mL human IgG1 to reduce Fc-specific clones in the input phage. The depleted library was then used to select binders against immobilized ICOS-Fc and PD-1/Fc. Eluted phage were used to infect TG1 bacteria and enriched phage libraries were generated by infection with M13K07 helper phage for iterative rounds of panning. After the 3rd round, clones were analyzed by ELISA for binding to PD1 or ICOS and human IgG1 Fc. An example of output is provided in Table 2.2. ELISA and FACS analysis were employed to verify antigen binding. An example of FACS analysis is provided in Figure 2.7.

Bispecific antibody generation: Initial plans called for expression of the BiTE molecules in E. coli. However, as discussed above, the presence of endotoxin associated with E. coli production led to inadvertent activation of T cells. Therefore, we needed to develop a mammalian expression system. A pSECTag2-based expression vector for production of BiTE molecules was generated essentially as previously described by us in Robinson et al, Br. J Cancer, 99:1415-25, 2008.

These results were presented at the IBC Antibody engineering meeting in December 2012 (Jimson D'Souza, Heidi Simmons, Tatiana Pazina, Carl June, Gregory P. Adams, Matthew K. Robinson "Engineering bs-scFv based immune modulatory antibodies").

Specific Aim 3. The goals for this aim/project as proposed in the grant application are as follows.

1. Optimize TEM1 vaccination. We will optimize DNA constructs, conditions of electroporation, and schedule of DNA prime with adenovirus boost vaccination. We will examine the contribution of cellular and humeral immunity and dissect the role of CD4 and CD8 cells as well as additional immune cell populations. Finally, we will identify the precise cellular targets and the immunogenic epitopes of tem1 responsible for tumor rejection.
2. Combine tem1 vaccination with pharmacologic therapy inhibiting angiogenesis. Because tem1 vaccines are expected to cause severe tumor ischemia - and will depend on rebound angiogenesis for recovery, we will test the role of blocking VEGF in combination with vaccine therapy.
3. Develop combinatorial vaccine therapy targeting tem1 and EpCAM. In mouse models of solid tumors we will test the efficacy of targeting the tumor vasculature with tem1 vaccine and the tumor cells with tumor antigen vaccines comprised of dendritic cells pulsed with whole tumor lysate or the epithelial antigen EpCAM.

We were successful in completing all aims of this project, more specifically, the development of the DNA vaccine targeting tumor endothelial marker1 (tem1). The work was published on the Journal of Clinical investigation in April 2014. We were also successful in generating new fusion constructs or implementing *mir* technology; the results indicate that none of the generated constructs resulted in a significant increase of the immunogenicity of tem1-TT construct. As an alternative to increase the immunogenicity, we are implementing different priming boosting strategies using the identified tem1 epitope in combination with DNA plasmid or adenoviral vector based vaccine.

DNA vaccination with tem1-TT breaks tolerance to tem1. Consistent with previous work in the human and the mouse, we found that tem1 is overexpressed in actively growing tumors of different background or histology (TC1, CT26 and LLC) and is expressed at very low to undetectable levels in mouse normal tissues (data not shown) as measured by qRT-PCR (Figure 3.1A). In the hyper-vascularized CT26 mouse colon carcinoma model, tem1 mRNA was found to localize with, or in close juxtaposition to, CD31 mRNA (Figure 3.1B), suggesting that tem1 is expressed by endothelial cells and/or pericytes. These findings support the notion that tem1 is an overexpressed vasculature-associated antigen in CT26 colon tumors, which can serve as a target for anti-tumor vascular therapy. Utilizing pcDNA3.1 as a DNA plasmid vector, the *tem1* gene

sequence was fused to the first domain (865–1120) of the C-fragment of the tetanus toxin sequence (TT) at its COOH. Expression levels of both sequences were measured by qRT-PCR following transient transfection of CHO cells (Figure 3.1C).

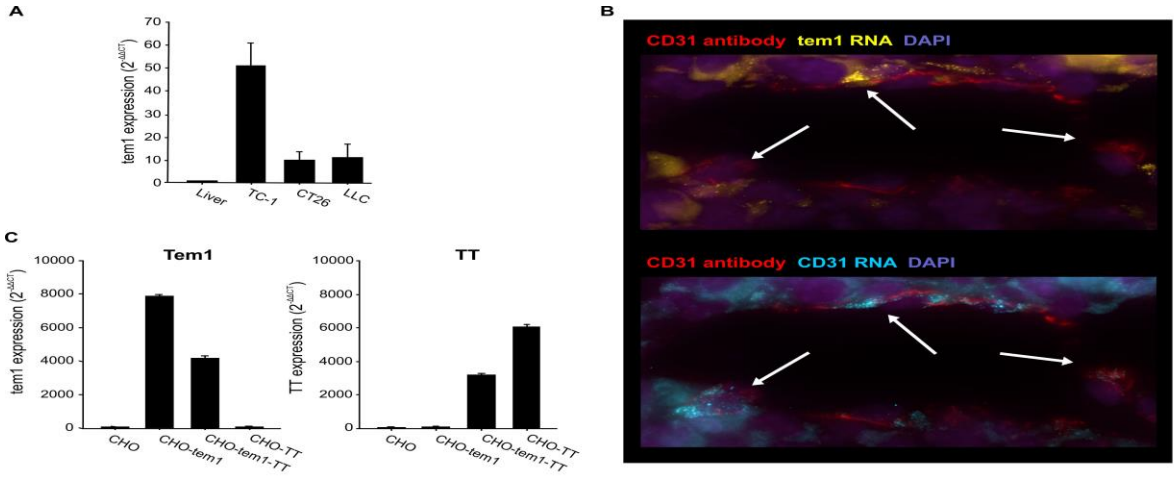


Figure_1

Figure 3.1. Physiological and plasmid DNA-induced tem1 expression. A) Tem1 is expressed in tumors in vivo. Quantitative RT-PCR was performed with a tem1 probe. An 18S probe was used as an endogenous control and samples were normalized with mouse liver. Post qRT-PCR calculations to analyze relative gene expression were performed by the comparative CT method (2−ΔΔCT method). Error bars denote SD (*n*=5). B) Tem1 RNA co-localizes with CD31 positive cells. The images show CT26 tumor sections subjected to immunofluorescence with a CD31 antibody (red) and single-molecule RNA fluorescent in situ hybridization (FISH) for tem1 (yellow, top) or CD31 (cyan, bottom). The bright spots in the RNA FISH channels correspond to individual transcript molecules. DAPI staining for cell nuclei is in purple. Magnification 100X. The images shown are 85 micrometers wide. C) Expression of the tem1-TT DNA plasmid. A fusion tem1-TT construct was generated by fusing codon-optimized tem1 cDNA (nucleotides 1–2297; amino acids 1–765) with the cDNA of the N-terminal domain of the fragment C of tetanus toxin (TT, 819 bp; 273 amino acids). Expression of tem1 or TT following transfection of the tem1-TT construct was tested with CHO cells and assessed by qRT-PCR. Error bars denote SD (*n*=3).

This fusion construct, in the dose of intramuscular injection of 50ug/mouse, was used to vaccinate healthy mice at one week intervals for a total of three immunizations. Splenocytes harvested from vaccinated mice were stimulated with a tem1 peptide library consisting of 15-mers that overlap by 10 aa. The tem1 library encompassing the entire protein sequence was divided into four pools: Pool A contains sequences that span aa 1 to 197, pool B aa 187 to 377, pool C aa 367 to 557, and pool D aa 547 to 765. This peptide-based approach facilitates the detection of $CD8^+$ and $CD4^+$ T cells that are specific for tem1 sequences in the context of MHC-I and MHC-II respectively. IFN-γ ELISpot and intracellular staining (ICS) indicated that the

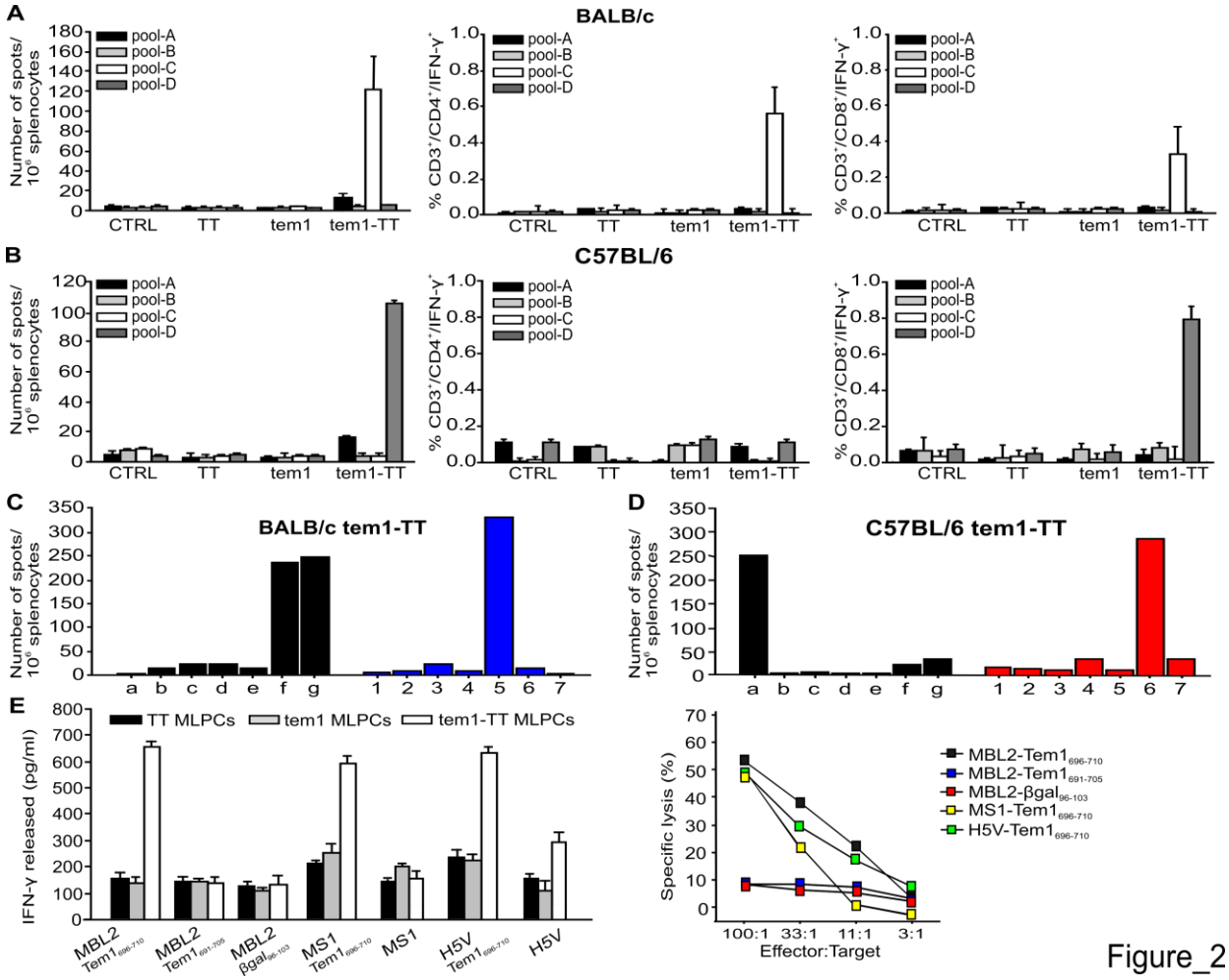


Figure 3.2. Characterization of tem1 immunodominant epitopes. Specific peptide pools restricted in H-2^d (A) and H-2^b (B) haplotypes. Splenocytes ($1x10^6$) from BALB/c (A) or C57BL/6 (B) mice vaccinated with tem-TT were cultured with the four tem1 peptide pools and tested by ELISpot (left panels) and ICS for IFN-γ secretion (middle panels). (A) Splenocytes from tem1-TT vaccinated BALB/c mice recognize Pool C (left panel); Pool C induces $CD3^+/CD4^+$ (center panel) and $CD3^+/CD8^+$ (right panel) T cell responses. (B) Splenocytes from tem1-TT-vaccinated C57BL/6 mice recognize Pool D. By ICS, there is a specific $CD3^+/CD8^+$ (right panel) response. Data are mean ± SD (*n*=5 mice/group) from one of three experiments. C), D) Characterization of tem1-derived peptides specific for H-2^d (C) and H-2^b (D) haplotypes. Peptides from Pool C (C) or pool D (D) were divided into seven mini-pools of seven 15-mer peptides, so each individual peptide was present in two mini-pools and could be identified through an experimental matrix design. (C) Reactivity to Pool C is present in mini-pools *f*, *g* and *5*. (D) Reactivity to Pool D is present in mini-pools *a* and *6*. Data is shown from one of three independent experiments. E) $Tem1_{696-710}$ induces IFN-γ secretion and cytotoxic effector activity. Splenocytes from mice vaccinated with TT, tem1 or tem1-TT vaccine was used in peptide-stimulated cultures and tested in an IFN-γ secretion ELISA (left panel) and CTL assay (right panel).

majority of $CD8^+$ and $CD4^+$ T cell epitopes in BALB/c mice were contained within Pool C (Figure 3.2A). Spleen-derived $CD8^+$ T cells from vaccinated C57BL/6 mice exhibited reactivity against tem1 pool D, whereas $CD4^+$ T cell responses were not detected (Figure 3.2B). As hypothesized, only the tem1-TT DNA fusion vaccine was able to break tolerance to tem1; specific responses were not detected in mice vaccinated with DNA encoding tem1 or TT adjuvant alone, in either mouse background strain. To characterize the tem1 sequences recognized by $CD4^+$ and $CD8^+$ T cells generated by the tem1-TT vaccine, Pools C and D were deconvoluted using a matrix scheme. For the BALB/c strain, Pool C was further divided into mini-pools. Tem1-specific reactivity was found against the mini-pools *f*, *g* and *5* as shown in Figure 3.2C. IFN-γ ICS analysis revealed that mini-pools *g* and *5*, corresponding to $Tem1_{516-530}$ (ITSATHPARSPPYQP), induced a stronger $CD8^+$ T cell response compared to mini-pools *f* and *5*, corresponding to $tem1_{511-525}$ (GHKPGITSATHPARS). Both $tem1_{516-530}$ and $tem1_{511-525}$ peptides stimulated a $CD4^+$ T-cell response (data not shown). We therefore decided to use the $tem1_{516-530}$ peptide for subsequent monitoring of the immune response in BALB/c mice. Using the same approach to further divide Pool D into mini pools for testing the C57BL/6 background, a specific tem1 response was detected for mini-pools *a* and *6*, corresponding to the $tem1_{696-710}$ peptide (GQSQRDDRWLLVALL*)* (Figure 3.2D). Tem1-TT exclusively primes and expands antigen-specific $CD8^+$ T cells that kill endothelial cells in the context of MHC-I/$tem1_{696-710}$ peptide but not control peptides. Using IFN-γ ICS, this sequence was found to specifically activate $CD8^+$ T cells, indicating that peptide $tem1_{696-710}$ contains the immunodominant $CD8^+$ T cell epitope for C57BL/6 mice (data not shown).

To confirm that the $Tem1_{696-710}$ peptide mediates target cell recognition and killing by CTL, splenocytes from vaccinated C57BL/6 mice were harvested and tested in a mixed leucocyte peptide-stimulated culture (MLPC). MBL2 leukemia cells were used as the stimulatory cells. MLPCs were pulsed with $Tem1_{696-710}$ peptide or control peptides for five days and tested for IFN-γ secretion by ELISA and in a ^{51}Cr cytotoxicity CTL assay. IFN-γ ELISA revealed that tem1-TT vaccine selectively generates antigen-specific T cells that recognize the $Tem1_{696-710}$ peptide but not control peptides $Tem1_{691-705}$ or βgal_{96-103} (Figure 3.2E, left panel). Splenocytes from vaccinated C57BL/6 mice were also able to recognize unpulsed or $Tem1_{696-710}$ -pulsed H5V endothelial cells (EC) that express endogenous tem1 protein, whereas MS1 EC cells that do not express tem1, required tem1 peptide pulsing to stimulate splenocytes from tem1-TT vaccinated mice (Figure 3.2E, left panel). In CTL assays, splenocytes from tem1-TT vaccinated mice efficiently lysed all three target cell types when they were pulsed with tem1 peptide (Figure 3.2E, right panel). Together these data suggest that tem1-TT can break tolerance and elicit functional $CD8^+$ T cells that are specific for tem1 epitopes. Furthermore, endothelial cells can naturally process the endogenous tem1 antigen and are able to present the cognate tem1 peptide in an MHC I-restricted fashion.

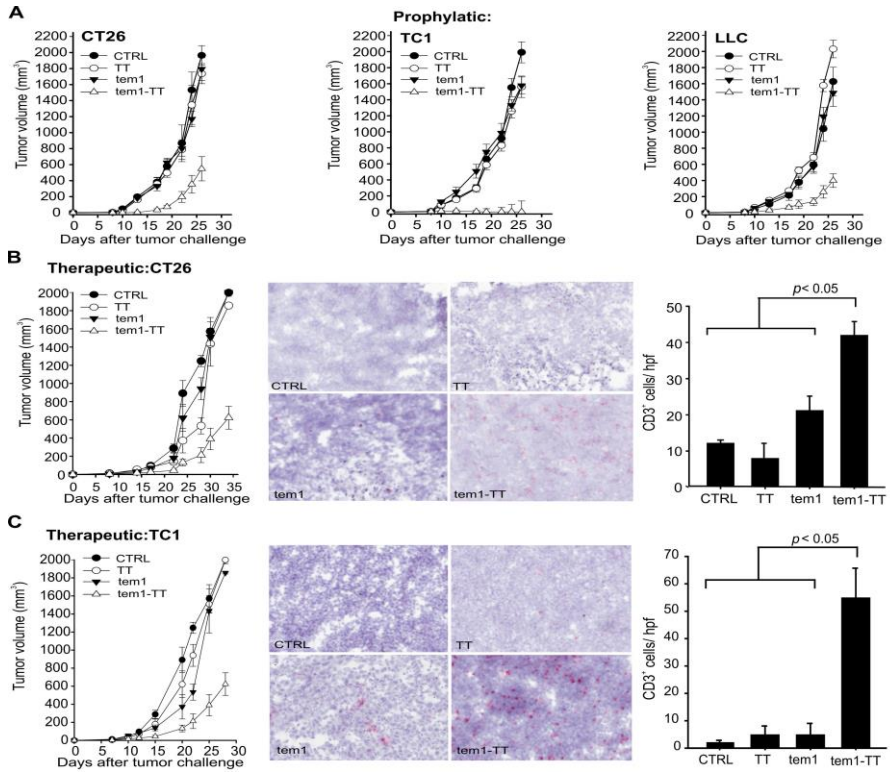


Figure 3.3. Tem1-TT vaccination controls tumor progression. A) Tumor control in a prophylactic setting. BALB/c and C57BL/6 mice were immunized three times with tem1-, TT- or tem1-TT vaccines then injected (s.c.) with $1x10^5$ CT26 (left), TC1 (middle) or LLC (right) cells. Data points are mean ± SD (*n* = 10 mice/group) from one of 5 experiments. All *p* values are pairwise multiple comparison procedures (Tukey test): in CT26 model, tem1-TT *vs* tem1, $p<0.05$; tem1-TT *vs* TT, $p<0.05$, in TC1 model tem1-TT *vs* tem1, $p<0.05$; tem1-TT *vs* TT, $p<0.05$, in LLC model tem1-TT *vs* tem1, $p<0.05$; tem1-TT *vs* TT, $p<0.05$. B) Inhibition of CT26 (B) and TC1 (C) tumor progression and induction of $CD3^+$ T-cell infiltration in a therapeutic setting. BALB/c mice were injected (s.c.) with $1x10^5$ CT26 cells (B) and C57BL/6 mice were injected with $1x10^5$ TC1 cells (C). Mice were immunized three days, one week and two weeks later with tem1-, TT- or tem1-TT vaccine and euthanized 33 tumor sections are shown in the middle (magnification 20X) and quantified in the right panel. Data are represented as mean ± SD of a representative experiment (*n* = 5 mice/group).

Tem1-TT DNA vaccine suppresses tumor growth and induces $CD3^+$ T cell tumor infiltration. To evaluate the ability of tem1-TT vaccination to control tumor growth in vivo, we tested both prophylactic and therapeutic vaccine approaches using CT26, TC1 and LLC tumor models. Mice were vaccinated 3 times (i.m.) at weekly intervals, followed by tumor challenge (prophylactic) or tumor cells inoculated first and then 3-5 days later, followed by 3 weekly vaccinations (therapeutic). The prophylactic treatment with tem1-TT vaccine induced significant tumor protection compared to the tem1 or the TT single constructs in all three tumor models, CT26 (Figure 3.3A, left panel), TC1 (Figure 3.3A, middle panel) and LLC (Figure 3.3A, right panel). These results reinforce the notion that the fusion construct tem1-TT vaccine is essential to break tem1 tolerance

The prophylactic treatment with tem1-TT vaccine induced significant tumor protection compared to the tem1 or the TT single constructs in all three tumor models, CT26 (Figure 3.3A, left panel), TC1 (Figure 3.3A, middle panel) and LLC (Figure 3.3A, right panel). These results reinforce the notion that the fusion construct tem1-TT vaccine is essential to break tem1 tolerance and generate protective immunity. Therapeutic tem1-TT vaccination conferred significant tumor protection in comparison with single construct controls in CT26 (Figure 3.3B, left panel) and TC1 tumor-bearing mice (Figure 3.3C, left panel). Analysis of the $CD3^+$ T cell tumor infiltration by immunohistochemistry revealed a consistent, heavy infiltration of $CD3^+$ T cells within CT26 (42 ±5 $CD3^+$ cells/high powered field (hpf): tem1-TT *vs*. TT; $p<0.05$) (Figure 3.3B, right panel) and TC1 tumors (54 ±11 $CD3^+$ cells/hpf: tem1-TT *vs*. TT; $p<0.05$) (Figure 3.3C, right panel) after therapeutic vaccination with tem1-TT compared with single construct or control saline. Cumulatively, these data indicate that the tem1-TT vaccine induces significant T cell infiltration within tumors and exerts potent anti-tumor activity.

To demonstrate that the anti-tumor response by tem1-TT vaccine was indeed immune mediated and to determine if the immune response is cellular or humoral in nature, we performed an adoptive transfer of $CD3^+$ T cells or sera from tem1-TT immunized mice into TC-1 tumor-bearing mice (Figure 3.4). Splenocytes from tem1-TT immunized mice were enriched for $CD3^+$ cells and inoculated into naïve mice. In simultaneous experiments, the serum from immunized mice was also transferred to naïve mice. Mice were then sublethally irradiated and challenged with TC1 tumor. The anti-tumor response mediated by tem1-TT vaccine is based on the induction of a $CD3^+$ T cell response, while serum has no therapeutic efficacy. Therefore, although antibodies may be generated by the tem1-TT vaccine, our adoptive transfer experiments rule out any humoral contribution to anti-tumor efficacy.

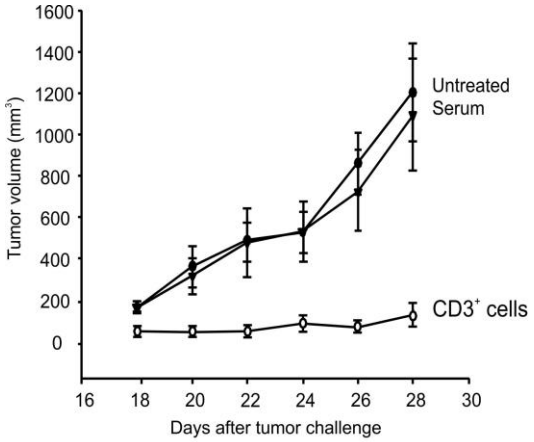


Figure 3.4. Therapeutic efficacy of $CD3^+$ T cells by adoptive cell transfer (ACT) from tem1-TT vaccinated mice into TC1-tumor bearing mice. C57BL/6 were vaccinated three times, after a week from the last vaccination mice were sacrificed and splenocytes used to magnetically isolate the lymphocyte population ($CD3^+$ T cells). Isolated lymphocytes or serum were injected into separate TC1 tumor-bearing mice (challenged the day before the ACT), sub-lethally irradiated (500 rads) three days before the ACT, i.v. or i.p., respectively. Log Rank $p<0.05$.

Tem1-TT vaccination inhibits tumor vascularization. To determine whether tem1-TT vaccination controls tumor progression by targeting the tumor vasculature, we utilized common readouts for measuring the effects on the vasculature but also measured the functional dynamics of the vasculature in the hypervascularized CT26 model. To ensure that tumors of the same size were studied, mice that were immunized with tem1-TT were sacrificed 10 days later than mice immunized with TT vaccine and available tumors were harvested. Tumors of similar volume

(~200 mm^3) from both groups were assessed for tumor hemoglobin levels by ELISA, as a readout for blood perfusion. Tumors from mice vaccinated with tem1-TT had markedly decreased hemoglobin levels compared to tumors from TT vaccinated mice, suggesting reduced red blood cell perfusion in these tumors (Figure 3.5A). We then assessed the effects of tem1-TT vaccination on the functional vasculature by measuring tumor vessel blood perfusion using ultrasound for each treatment group when tumors reached a volume of ~600 mm^3. Gas-filled microbubbles were injected i.v. and act as a contrast agent as the bubbles flow through the tumor blood vessels. Regions of perfusion in the tumors were visualized by ultrasound pulsing and contrast-enhanced Doppler imaging, which enabled us to determine the area of the tumor that was perfused (as a percentage of total tumor area) as well as the red blood cell flux (rate of blood flow) per unit area of the tissue (color-weighted fractional area. Contrast-enhanced images (post-perfusion) showed a marked reduction in tumor perfusion (colored regions) in the tem1-TT-treated group compared to the TT-treated control group (Figure 3.5B, left panels). Both the reduction in area of perfusion (p=0.008) and blood flux (p=0.010) in tem1-TT vaccinated tumor were statistically significant (Figure 3.5B), suggesting that tem1-TT vaccination disrupts the functional vasculature within the CT26 tumor.

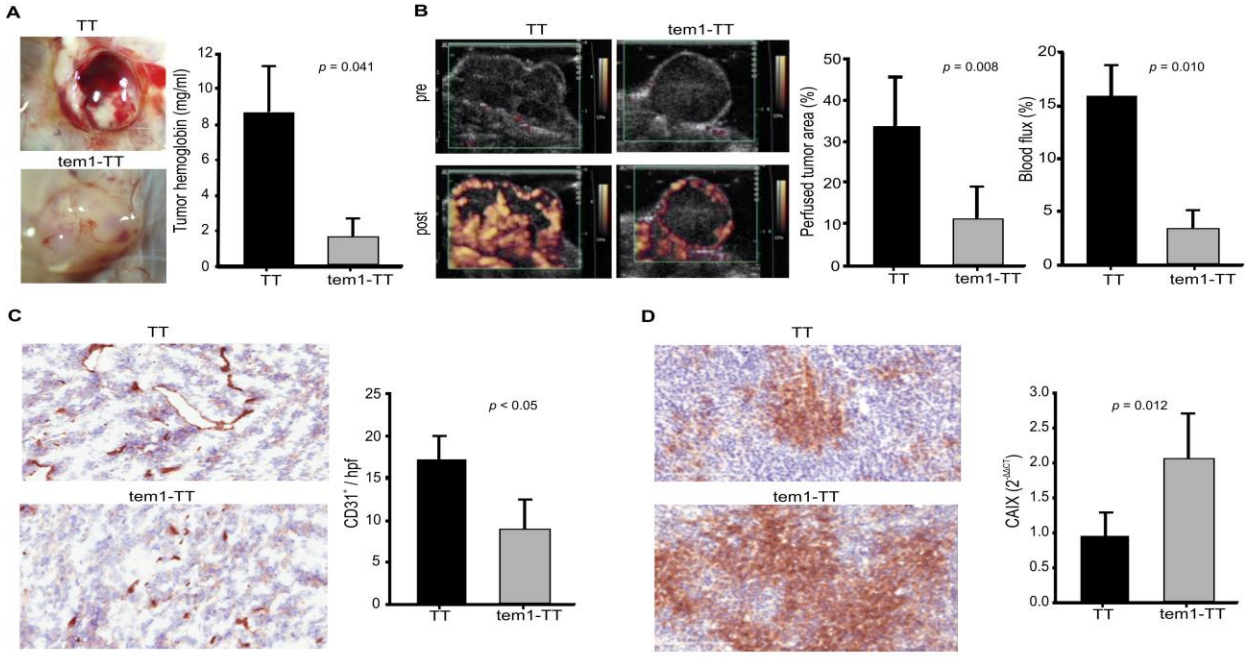


Figure 3.5. Tem1-TT vaccination inhibits CT26 tumor vascularization. A) Tem1-TT vaccine reduces tumor hemoglobin content. Tumors at ~200 mm^3 were excised from TT- or tem1-TT vaccinated mice and inspected grossly. Tumors from tem1-TT vaccinated mice appeared pale relative to control tumors. Reduced hemoglobin levels are observed in tumors from tem1-TT vaccinated mice by ELISA. B) Tem1-TT vaccine reduces tumor vascularity. Tumors from mice vaccinated with either TT- or tem1-TT vaccine were analyzed by Doppler ultrasound. Perfused tumor area and real blood flux are shown as measured and calculated by Doppler image analysis. C) Tem1-TT vaccine reduces CD31 expression in tumor mass. CT26 tumor from tem-1-TT immunized mice have a significant decrease of CD31 expression compared to TT-vaccination D) Tem1-TT vaccine increases carbonic anhydrase expression. Carbonic anhydrase (CAIX) expression was visualized by immunohistochemistry (magnification 20X) and also independently quantified by qRT-PCR in tumors from mice vaccinated with either TT- or tem1-TT vaccine.

We next assessed the apoptotic index of CT26 tumors by TUNEL assay. In Figure 3.6A, we observed an increase in cellular apoptosis in CT26 tumors from tem1-TT immunized mice as compared to tumors from TT immunized mice (p=0.014). We also observed co-localization of these apoptotic cells with the CD31 antigen as shown in Figure 3.6B, indicative of apoptotic endothelial cells of the vasculature. Lastly, we also observed a reduction of cells positive for Ki-67, a marker for proliferating cells, within tumors from the tem1-TT immunized group as compared to the TT-immunized group, indicating that tem1-TT vaccine lowers the proliferative capacity of the tumor, a likely consequence of disruption of the tumor vasculature (not shown). Next, the microvasculature density (MVD) of the tumors was measured by immunohistochemistry staining of CD31. CT26 tumors from tem1-TT treated mice displayed reduced MVD compared to TT treated tumors (Figure 3.5C, right panel). In addition to fewer $CD31^+$ endothelial cells per high powered field of view, structural disorganization of the vasculature was noted: tem1-TT treated tumor vessels appear flattened with less luminal space, suggesting collapse of the vessels as well as difficulty in the assembly of the new vasculature (Figure 3.5C, left panels). Lastly, we tested whether reduced tumor perfusion in tem1-TT-vaccinated mice was associated with an increase in tumor-associated hypoxia. Using immunohistochemistry, an increase in carbonic anhydrase IX (CAIX), a cellular biomarker of hypoxia, was observed in tumors of tem1-TT-treated mice relative to control TT vaccinated mice (Figure 3.5D, left panels). Analysis by qRT-PCR also showed an increase in CAIX message levels in tumors from tem1-TT treated mice relative to controls (Figure 3,5D, right panel). These data suggest an association between reduced tumor blood perfusion and hypoxia in CT26 tumors from tem1-TT vaccinated mice.

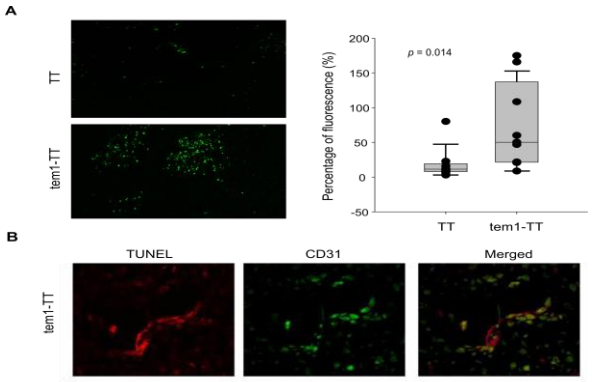


Figure 3.6. Tem1-TT vaccine induces specific ablation of tumor vasculature. A) Apoptotic status of CT26 tumors was assessed by TUNEL immunofluorescence in TT and tem1-TT treated mice **B)** CD31 and TUNEL double-staining of CT26 tumor from tem1-TT-immunized animals. Images identifies CD31 positive endothelial cells (red) and TUNEL positive apoptotic cells (green) within CT26 tumor excised from tem1-TT treated cells were detected in areas surrounding endothelial cells in CT26 tumor from TT-vaccinated mice (data not shown).

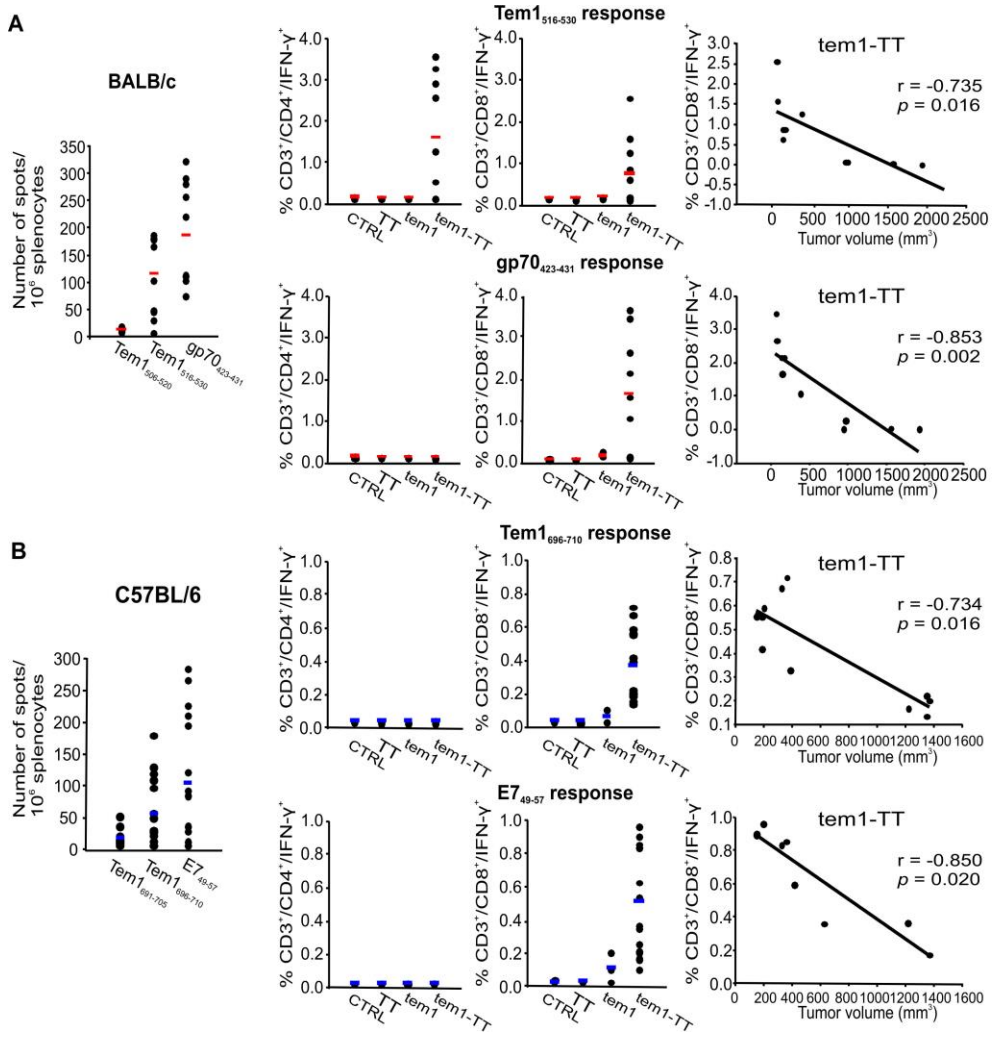


Figure 3.7. Tem1-TT vaccination induces secondary tumor cell-specific cytotoxic T cell responses in tumor bearing mice. A) Tem1-TT-vaccinated splenocytes mount specific responses against the immunodominant peptide of tem1 ($Tem1_{516-530}$) and the AH1 antigen but not control peptide ($Tem1_{506-520}$) as measured by ELISpot assay (left panel). Tem1-TT vaccine induces $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cell responses against tem1 antigen (middle panels). The $CD3^+/CD8^+$ T cell response from tem1-TT vaccinated mice correlates with suppression of tumor growth (upper right panel). The ICS assay confirms that tem1-TT immunization induces an AH1-specific $CD3^+/CD8^+$ T cells response (lower middle panels) that also correlate with tumor volume (lower right panel). B) Splenocytes from mice vaccinated with tem1-TT recognize $Tem1_{696-710}$ peptide and the E7 antigen but not control peptide ($Tem1_{691-705}$) (left panel). Only tem1-TT vaccination was able to induce both a tem1 (upper middle panels) and E7 (lower middle panels) $CD3^+/CD8^+$ T cell response, and both of these responses correlated with tumor volume (right panels). Correlation analysis was performed by Spearman's rank correlation.

Tem1-TT vaccine induces T cell responses against tumor-associated antigens as well as tem1 antigen. Next, we tested whether immunization against tem1 by tem1-TT vaccine resulted in 'epitope spreading', an immunological phenomenon in which immunotherapy or chemotherapy kills tumor cells and induces cross-presentation of tumor-associated antigens followed by cross-

priming. Tumor antigen-specific vaccines can also induce epitope spreading, inducing specificity against antigens unrelated to the original vaccine formulation. We hypothesized that the anti-vascular effects described above would provide a rich source of dead or dying tumor/stromal cells capable of supporting a corollary cross-priming event that would generate immune responses to antigens other than tem1. To this end, we utilized the immunodominant AH1 ($gp70_{423-431}$) peptide in the immunological assays. AH1 is the MHC-I associated epitope for CT26 tumor, and as such, AH1-specific $CD8^+$ T cells can cure mice of established CT26 tumor. AH1 peptide is derived from an endogenous retroviral gene product, gp70, expressed by CT26 cells.

Splenocytes from CT26 tumor-bearing BALB/c mice vaccinated with tem1-TT exhibited a specific response against $Tem1_{516-530}$ but not against the $Tem1_{506-520}$ control peptide, as expected, but they also exhibited a vigorous response against the AH1 peptide as measured by IFN-γ ELISpot (Figure 3.7A, left panel). Tumor-bearing mice immunized with control TT vaccine did not respond to any of these peptides (data not shown). As measured by IFN-γ ICS, tem1-TT vaccine induced both $CD4^+$ and $CD8^+$ T cell responses against $Tem1_{516-530}$, while response to AH1 was restricted to $CD8^+$ cells, as expected (Figure 3.7A, middle panels). The frequency of the tem1 $CD8^+$ T cell response from tem1-TT vaccinated mice was inversely correlated with tumor volume (Figure 3.7A, right upper panel); the $CD4^+$ T cell response also inversely correlated with tumor volume (data not shown). A similar inverse correlation was found between the AH1-specific $CD8^+$ T cell response from tem1-TT vaccinated mice and tumor volume (Figure 3.7A, right lower panel). To further understand the potency of the anti-vascular tem1 response, and subsequent anti-tumor AH1 response, we performed adoptive T cell transfer from either naïve or CT26 tumor-bearing mice that were all tem1-TT immunized to recipient mice that were challenged with CT26 tumor (Figure 3.8). $CD3^+$ T cells from naïve tem1-TT immunized (A) and tumor-bearing tem1-TT immunized (B) donor mice

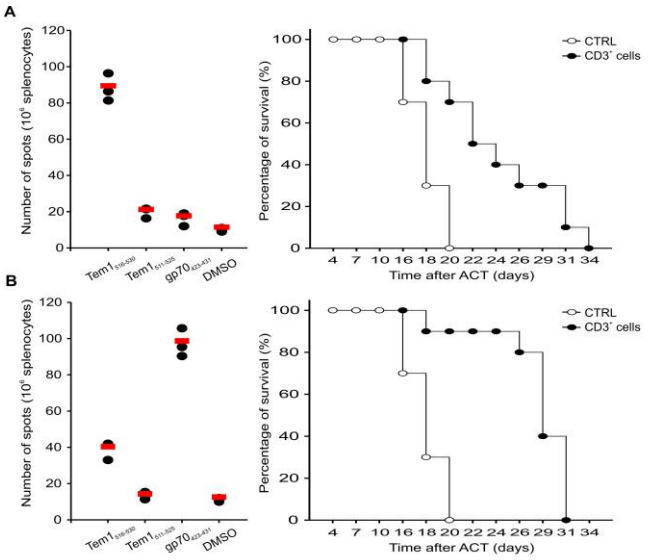


Figure 3.8. Adoptive Cell Transfer (ACT) of the lymphocyte population of tem1-TT vaccinated mice. A) BALB/c tumor-free mice were vaccinated three times**,** after a week from the last vaccination mice were sacrificed and splenocytes were tested for AH1-specific ELISpot assay (left panel). Isolated lymphocytes were injected i.v. in CT26 tumor-bearing mice (challenged the day before the ACT) sub-lethally irradiated (500 rads) three days before the ACT. The log Rank ($p<0.001$). B) CT26 tumor-bearing mice were vaccinated three times, after a week from the last vaccination, mice were sacrificed and splenocytes were tested for their ability to recognize the tem1-specific, AH1-specific and control peptide through ELISpot assay (left panel). Isolated lymphocytes were injected i.v. in CT26 tumor-bearing mice. log rank ($p<0.001$).

both significantly increased the survival of recipient mice injected with CT26. This result shows that the anti-tem1 immune response generated by tem1-TT in the non-tumor bearing donor mice (A), without concomitant cross-priming and epitope spreading as in (B), is sufficiently potent to control tumor growth that is comparable to $CD3^+$ cells adoptively transferred from tem1-TT immunized tumor-bearing mice (B). As expected, adoptively transferred $CD3^+$ cells from naive mice vaccinated with tem1-TT exhibited a response against $Tem1_{516-530}$ but not against AH1 as in (B). To confirm the epitope spreading phenomenon, we analyzed a second tumor model, TC1; a C57BL/6 epithelial lung tumor cell transformed by human papilloma virus (HPV) E6 and E7 viral oncogenes. E7 derived peptide ($E7_{49-57}$) is the immunodominant epitope that can cure mice of established tumors. Mice immunized with tem1-TT or control vaccine were challenged with TC-1, as described previously. Splenocytes from tem1-TT-vaccinated mice displayed immune responses against $Tem1_{696-710}$ peptide that were $CD8^+$ specific (Figure 3.5B, left and upper middle panels) but not against control peptide ($Tem1_{691-705}$), and against the $E7_{49-57}$ $CD8^+$ T cell-specific epitope (Figure 3.7B, lower middle panels). As we observed in the CT26 model, the frequency of both tem1 and E7 specific $CD8^+$ immune responses correlated with TC1 tumor control (Figure 3.7B, upper, lower right panels). Thus, effective tem1-TT vaccination elicited a T cell-mediated response against both the tumor vasculature antigen (tem1) and also against antigens expressed by the tumor cells themselves (gp70 and E7).

Tem1-TT vaccination does not affect wound healing or reproduction. Physiologic angiogenesis is critical for wound healing, and anti-angiogenic agents such as bevacizumab have been associated with important safety concerns including wound healing. We assessed whether wound repair was impaired by tem1-TT immunization in C57BL/6 mice by wounding animals on their backs after immunization. There was no significant difference in time to wound closure between tem1-TT and the control vaccinated groups (Figure 3.9A), despite the generation of robust anti-tem1 responses, as measured by IFN-γ ELISpot (Figure 3.9B) and IFN-γ ICS (Figure 3.9C). Reproductive organs are sites of de novo angiogenesis, with both the corpus luteum and placenta being heavily dependent on formation of new blood vessels. Anti-angiogenic compounds have been associated with defective luteogenesis and reproductive dysfunction, pregnancy loss and teratogenesis. Previous studies have reported tem1 expression in several tissues of the mouse embryo. Postnatally, although tem1 expression is down-regulated in most organs, some expression persists in the renal glomerulus and in the adult uterus, where the expression pattern varies with the stage of the estrous cycle. Immunological targeting of the vascular tem1 antigen could therefore have significant side effects on the reproductive system. To test whether tem1-TT immunization affects various aspects of mouse reproduction, female mice were immunized with tem1-TT or TT-vaccine and then mated with male mice.

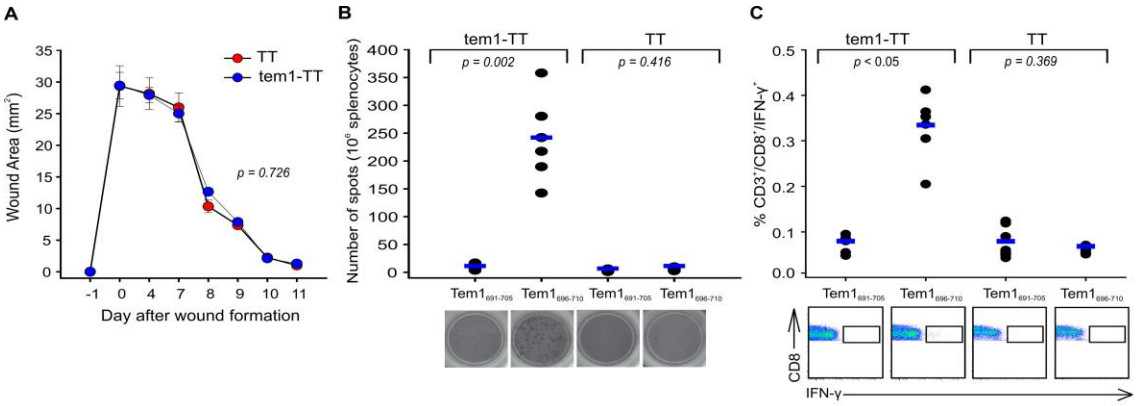


Figure 3.9. Wound healing is not delayed by tem1-TT immunization. A) Time course of wound healing. C57BL/6 mice were vaccinated five times with either tem1-TT vaccine or TT vaccine. Ten days after the final vaccination, two circular wounds were inflicted on the upper back of the mice. The wound area was measured every two days. Data are represented as mean ± SD of two experiments (n = 10 mice/group for each experiment). All *p* values are pairwise multiple comparison procedures (Tukey test): tem1-TT *vs* TT, p=0.726. B, C) Evaluation of the tem1-specific response. Twenty days after the punch biopsies, mice were sacrificed to check the immune response against tem1 antigen by IFN-γ ELISpot of bulk splenocytes ($1x10^6$) (B) and IFN-γ intracellular cytokine staining of bulk splenocytes (C). Statistical analysis for the indicated comparisons was performed with Student's t test.

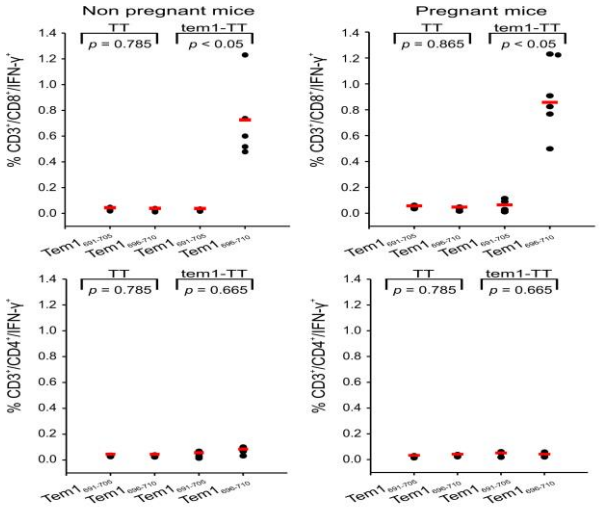


Figure 3.10. Tem1 specific immune responses are similar in immunized non-pregnant and pregnant mice. Forty-five days after the last immunization, both pregnant and non-pregnant mice were boosted with a single injection of tem1-TT or TT DNA. Two weeks later the mice were sacrificed to assess the immune response against tem1 antigen.

The pregnancy success rate, time to gestation, total litter size and pup weight at birth were quantified in tem1-TT immunized and control immunized groups (Figure 3.11A). Tem1-TT immunization had no effect on pregnancy rate (p=0.572), gestation time (p=0.698), number of pups (p=0.552), or pup mass at birth (p=0.409). Furthermore, there were no anatomical or histological abnormalities in embryos from tem1-TT-vaccinated or control groups during early gestation (embryonic age, day 10 or E10; Figure 3.11B, left 4 panels) or late gestation (E19; Figure 3.11B, right 4 panels). We also assessed the potential effects of tem1-TT immunization on various organs of vaccinated pregnant mice postpartum and found no abnormality in heart, lungs, liver, uterus, ovary or the placenta

(Figure 3.11C). Similarly, organs from non-pregnant mice, including the uterus and the renal glomeruli, had no abnormalities following tem1-TT immunization. To ensure that pregnancy did not dampen the effectiveness of tem1-TT vaccination, we measured tem1-specific T cell responses ($Tem1_{696-710}$) by IFN-γ ELISpot (Figure 3.11E) and ICS (Figure 3.10) in the same female mice that were impregnated, and gave birth to pups above and also in tem1-TT mice that did not become pregnant following vaccination.Tem1 responses were roughly equal between pregnant and non-pregnant mice that were tem1-TT immunized (Figure 3.11E). The ICS assay similarly shows that the tem1 response was roughly of equal frequency between pregnant and non-pregnant mice and $CD8^+$ specific as expected with this tem1 peptide (Figure 3.10).

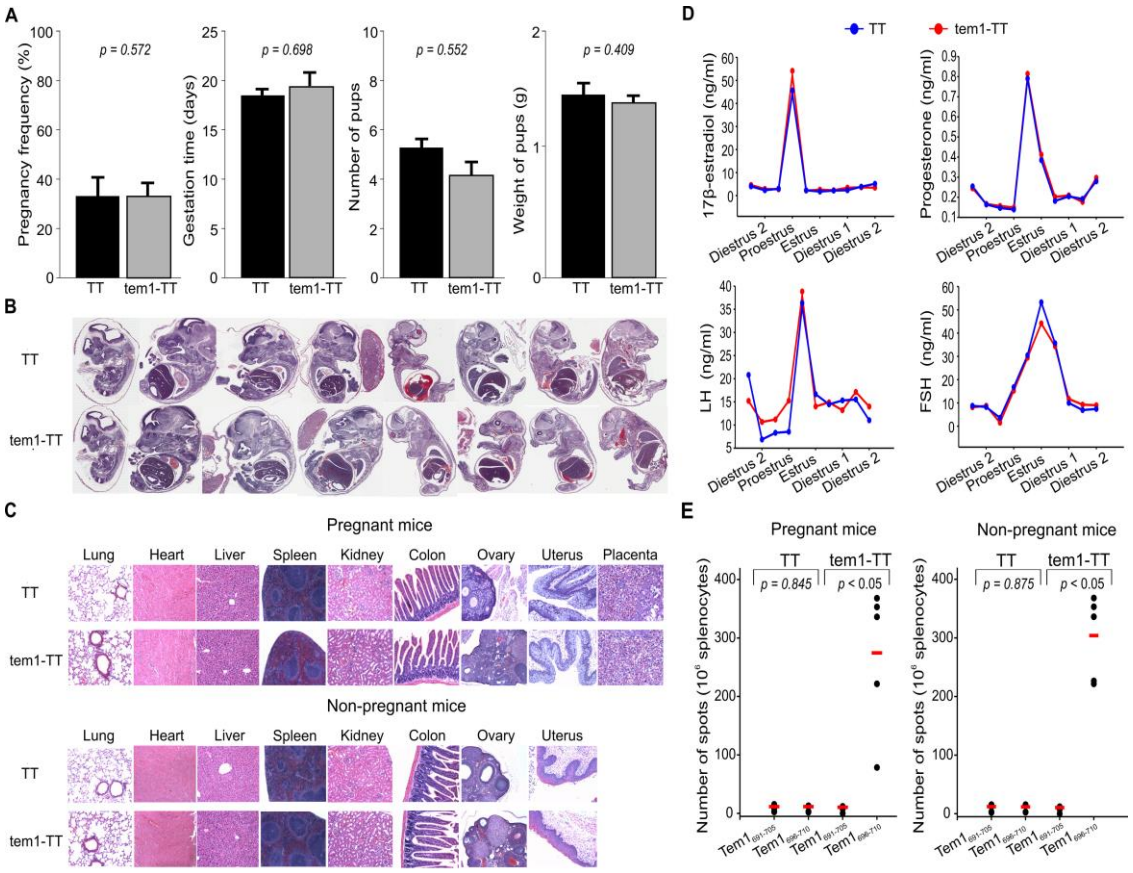


Figure 3.11. Tem1-TT vaccine does not affect reproduction. C57BL/6 female mice were vaccinated 5 times with either tem1-TT vaccine or TT vaccine followed by mating a week later**.** A) Tem1-TT does not alter reproduction. Vaccination has no impact on fertility, gestation time, numbers of pups or weight of pups (n=15 for each group). Data are representative of two independent experiments. Statistical analysis was performed with Student's t test. B) Tem1-TT vaccine does not affect embryonic development. H&E staining of embryos of female mice vaccinated with TT and tem1-TT. No abnormalities were detected in embryos in either group. C) Tem1-TT vaccination does not induce tissue pathology. Histological analysis of lung, heart, liver, spleen, kidney, colon, ovary, uterus and placenta from pregnant (postpartum) and non-pregnant mice (no placenta) revealed no pathological effects by either vaccine. Magnification is 10x except spleen and ovary which is 4x. D) Tem1-TT vaccination does not affect the estrus cycle. In a separate independent experiment, sera from immunized mice had synchronized estrus cycles and were evaluated for reproductive hormones. No fluctuations were noted. E) Tem1-specific T cell responses are unaltered by pregnancy. Splenocytes ($1x10^6$) of vaccinated pregnant and non-pregnant mice were tested against $Tem1_{696-710}$ peptide or control peptide ($Tem1_{691-705}$) using IFN-γ ELISpot. Forty-five days after the last immunization, mice were boosted with a single injection of tem1-TT or TT DNA, two weeks later the mice were sacrificed. There was significant recognition of $Tem1_{696-710}$ peptide by tem1-TT-vaccinated splenocytes from both pregnant and non-pregnant mice compared to TT-vaccinated splenocytes (n = 5).

Lastly, because anti-angiogenic agents may disrupt luteogenesis, we also tested the reproductive endocrine function following tem1-TT vaccination in a separate independent experiment in which mice were vaccinated only. Vaccinated mice then had their estrous cycle synchronized by medroxyprogesterone, and serum levels of 17β-estradiol (E2), progesterone (P4), luteinizing hormone (LH), and follicle stimulating hormone (FSH) were measured longitudinally (Figure 3.11D). Patterns of E2, P4, FSH and LH expression were similar between TT and tem1-TT vaccinated groups, therefore tem1-TT did not modulate hormone levels during estrus. Collectively, tem1-TT vaccination appears to have no untoward effects on physiological processes in mice that depend on *de novo* angiogenesis. In previous mouse studies, DNA vaccination with tumor-associated antigens AH1 and E7 induced $CD8^+$ dependent regression of established tumors that expressed those antigens. In the studies presented here, the fusion of the tem1 antigen to the TT adjuvant increased the immunogenicity of the target antigen and the efficacy of tumor control. It also generated robust CTL activity against tumor cell proper immunodominant epitopes such as AH1 and E7. This was not achieved by direct targeting of these antigens, but rather indirectly, by targeting the vasculature antigen tem1. Taken together our data suggest a model (Figure 3.12) whereby tem1-TT vaccine induces an expansion of tem1-specific T lymphocytes. These effector T cells target tem1-expressing cells in the tumor vasculature, including tumor-associated endothelial cells and/or pericytes. This immune attack impairs the tumor vasculature, with the increases of local tumor-associated hypoxia and likely inflammation-related mediators, resulting in apoptosis of local tumor cells. These apoptotic bodies are scavenged by macrophages and dendritic cells that process and present additional tumor-associated antigens. This secondary cross-priming event then expands tumor-specific cytotoxic $CD8^+$ T cells which work hand in hand with the anti-tem1 immune response to eradicate larger numbers of tumor cells, resulting in improved control of tumor growth. The anti-tem1 response and the anti-tumor immune responses against AH1 and E7 significantly correlated

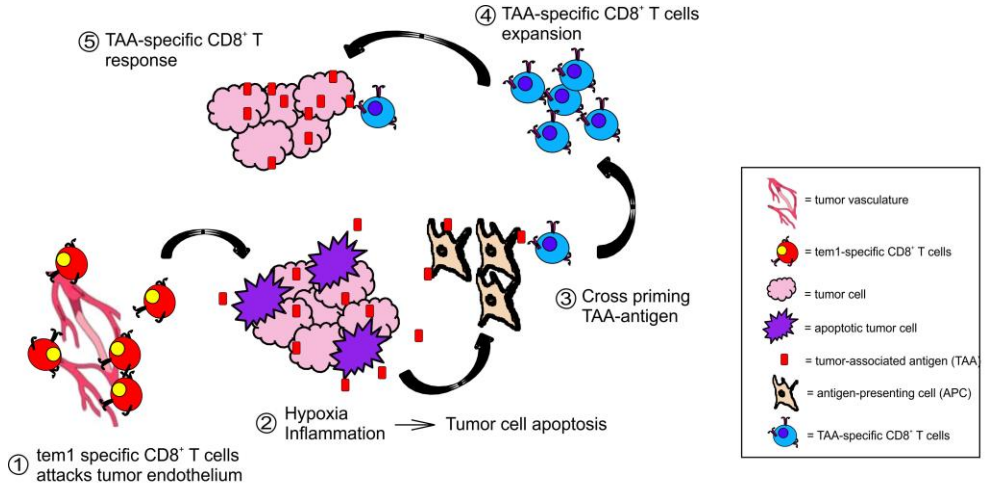


Figure 3.12. Proposed mechanism of action of Tem1-TT vaccination. 1. Tem1-TT vaccine induces expansion of tem1-specific $CD8^+$ T cells that target the CT26 tumor vasculature. These cytolytic effectors target and kill tem1-expressing cells, most likely tumor-associated endothelial cells and/or pericytes. **2.** This event results in reduction of the tumor vasculature, inducing inflammation and tumor hypoxia resulting in localized tumor cell death. **3.** Dead tumor cells are scavenged by antigen-presenting cells (APCs) which process relevant tumor antigens (e.g. AH1 antigen in CT26 tumor model). **4.** This crosspresentation event results in a secondary cross-priming event that expands tumor specific cytotoxic $CD8^+$ T cells to tumor cell derived epitopes. **5.** Tumor-specific cytotoxic $CD8^+$ T cells lyse and eliminate tumor cells, resulting in control of tumor growth.

with control of tumor growth. Further studies will address the kinetics of the tem1 and AH1 immune response. Our findings are consistent with the general paradigm of "epitope spreading" as a mechanism underlying superior immunotherapeutic outcomes and extends this concept to include T cell specificities against tumor-associated vasculature antigens.

The results of this project are published (Facciponte et al, Tumor endothelial marker 1–specific DNA vaccination targets tumor vasculature, 2014 in The Journal of Clinical Investigation).

Specific Aim 4. Significant progress has been achieved over the past few years demonstrating the potential for adoptive T cell transfer to treat cancer. One of the most promising approaches is the introduction of chimeric antigen receptors (CARs) to redirect T cell specificity with high affinity antibody-based recognition units. CARs are synthetic molecules containing three distinct modules: an extracellular target binding module, a transmembrane module that anchors the molecule into the cell membrane, and an intracellular signaling module that transmits activation signals. Over the past decade a new effector $CD4^+$ T helper cell subset that secretes IL-17 was discovered, and it has become clear that T_H17 cells represent an independent subset of T helper cells. T_H17 cells regulate host defense and exacerbate autoimmune diseases. Naturally arising endogenous T_H17 cells have been found in various human tumors; however their function in cancer immunity is unclear. When adoptively transferred into tumor-bearing mice, T_H17 cells have been found to be more potent at eradicating melanoma than T_H1 or non-polarized (T_H0) T cells. Importantly, T_H17 cells have considerable plasticity, and can acquire certain type 1 characteristics (such as IFN-γ production) depending on the inflammatory conditions. The ability of T_H17 cells to acquire T_H1 cell-like features appears to be a prerequisite for potent antitumor activity. One obstacle to the use of T_H17 cells for adoptive cell transfer is the identification of robust culture conditions that limit the inherent plasticity of this subset.

Two properties of CAR T cells that correlate with potency are the specific lymphocyte subsets that are infused and the signaling domains of the CAR. Pre-clinical studies indicate that cells with extensive proliferative capacity are more potent. In mice adoptive transfer experiments indicate that T_H17 cells have higher in vivo survival and self-renewal capacity than T_H1 polarized cells. We have previously shown that ICOS, but not CD28, is necessary for optimal expansion and function of human T_H17 cells. ICOS is constitutively expressed on T_H17 cells and anti-CD3/ICOS stimulation induced RORγt and T-bet expression in these cells, leading to increased secretion of IL-17A, IL-21, and IFN-γ compared to CD3/CD28 stimulation. This project allowed us to study the potency of T_H17 polarized CAR redirected T cells. CAR construct with various signaling domains were tested and we show that CARs bearing ICOS signaling domains have enhanced stability as T_H17/T_H1 cells and increased persistence after transfer into tumor bearing mice.

The CAR constructs used in this study contain the SS1 scFv that recognizes human mesothelin (Figure 4.1A). The scFv was fused to the TCR-ζ signal transduction domain with the CD28, 4-1BB or ICOS signaling domains in tandem. As negative control for signal transduction, we used a chimeric receptor containing a truncated form of the TCR-ζ intracellular domain (del ζ). $CD4^+$ T cells were activated with beads coated with anti-CD3 and anti-ICOS antibodies in the presence of T_H17 polarizing cytokines, transduced with lentiviral vectors encoding SS1-based chimeric receptors, and expanded. For all functional assays, T cell cultures were normalized for equivalent

CAR expression by addition of mock transduced T cells as required. The surface expression of all CAR constructs was similar (Figure 4.1B).

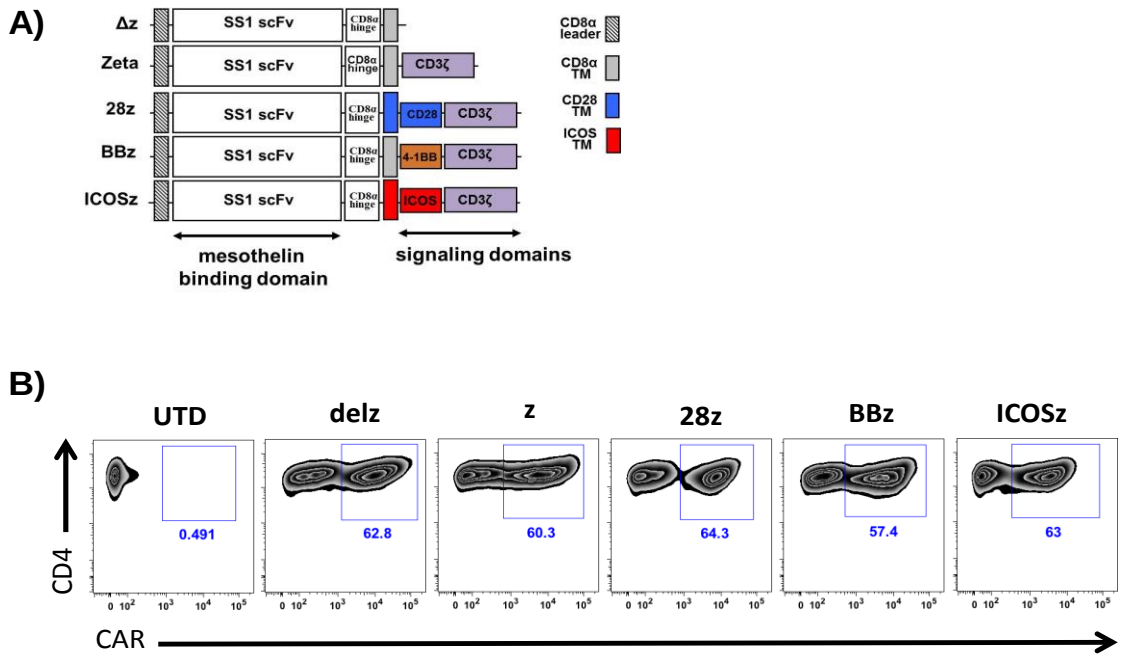


Figure 4.1. Redirection of T_H17 cells with SS1-CARs (A) Schematic representation of a panel of chimeric receptors that contain the SS1 single chain fragment and differ in the transmembrane and the intracellular domains. (**B**) Schematic of the experimental protocol. Peripheral blood $CD4^+$ T cells were stimulated with antibodies to CD3/ICOS beads and cultured under T_H17 polarizing conditions. Human IL-2 was added 3 days after activation. T cells were transduced with lentiviral vectors 24 hours following stimulation. Once T cells rested down, they were cryopreserved. For functional assays, T cells where thawed and stimulated with surrogate antigen in media without cytokine supplementation. (**C**) Surface expression of the SS1 scFv fusion proteins on human $CD4^+$ T cells at the time of functional evaluation.

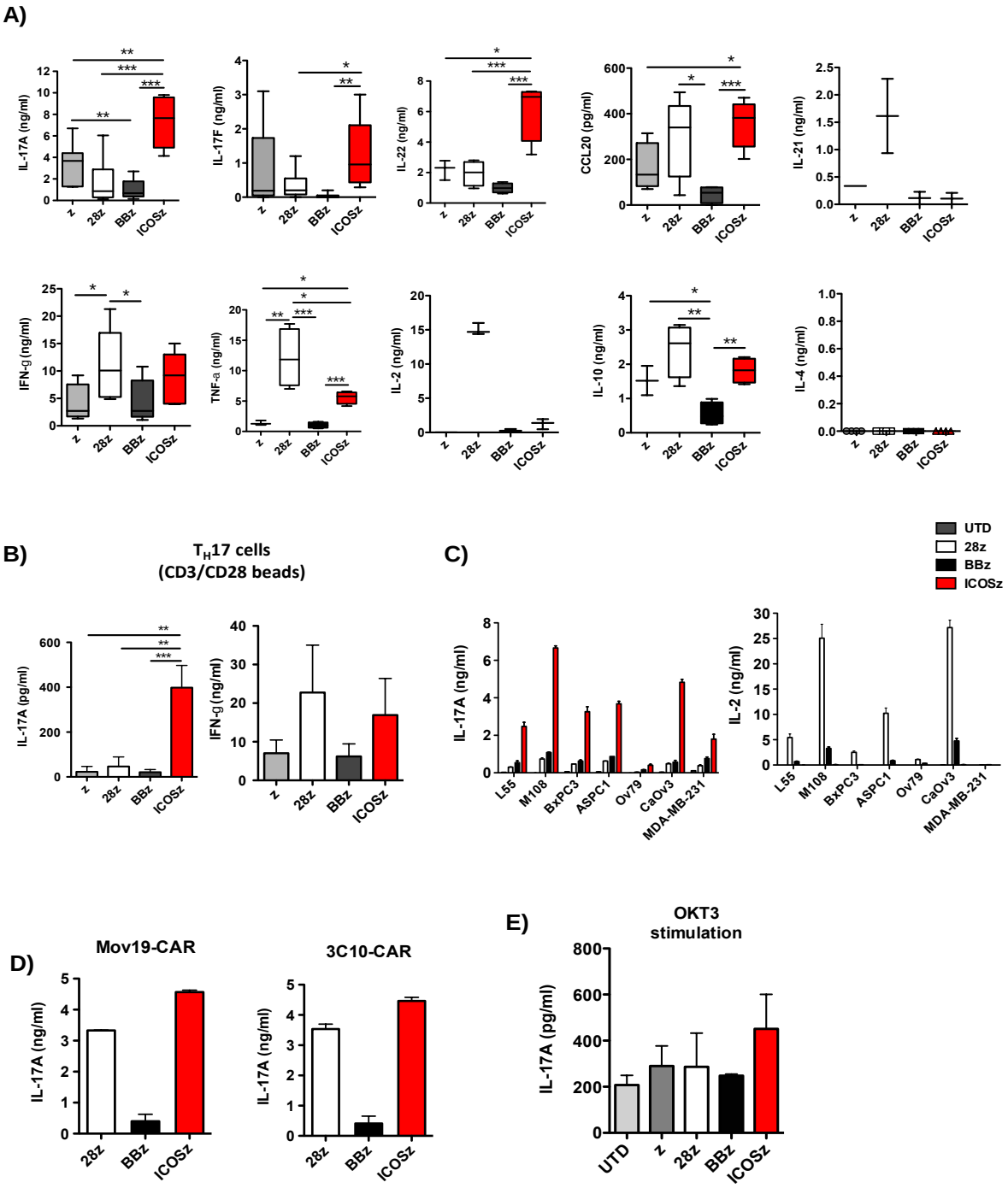
In vitro function of ICOS-based CAR cells. One of the functional hallmarks of T_H17 cells is the production of large amounts of IL-17A, IL-17F and IL-22. To test the cytokine response of the mesothelin redirected T cells, we co-cultured them with tumor cells that expressed mesothelin and quantified the concentrations of cytokines in the supernatants by ELISA (Figure 4.2A). We observed a significant enhancement of IL-17A, IL17-F and IL-22 secretion by the ICOSζ cells, consistent with a predominantly T_H17 phenotype. The secretion of IL-17 required surrogate antigen signaling because cells expressing a CAR with a truncated ζ chain did not secrete IL-17 (data not shown). Importantly, addition of the CD28 or the 4-1BB costimulatory domains to CD3ζ domain impaired expression of T_H17 cytokines, while addition of the ICOS domain significantly increased IL-17A production. In contrast, no differences in IL-17A secretion were observed when T cells were stimulated through their native TCR receptor (Figure 4.2B), confirming that differences observed in IL-17A secretion following antigen encounter require signaling through CAR costimulatory domains. The enhancement of IL-17 secretion following ligation of the ICOSζ CAR was consistently observed upon T cell exposure to cancer cells

derived from various tissues including lung, mesothelioma, pancreas, ovary and breast (Figure 4.2C), and when using ICOSζ–based CARs with distinct antigen specificities. In addition, ICOSζ CART cells cultured under T_H17 polarizing conditions but stimulated with anti-CD3/CD28 beads rather than anti-CD3/ICOS beads, showed enhanced IL-17A secretion when compared to ζalone, 28ζ and BBζ redirected T cells although the amount of IL-17A secreted was 20-fold lower (Figure 4.2D). These T_H17 cells expressed similar levels of IFN-γ compared to cells pre-expanded with anti-CD3/ICOS beads.

In contrast to the enhanced secretion of T_H17 cytokines by the ICOSζ cells, we found that only 28ζ CAR T cells secreted robust amounts of IL-2 after co-culture with mesothelin-expressing targets (Figures 4.2A and C). 28ζ cells secreted significantly more TNF-α and consistently more IL-21 (although significance was not reached due to donor variability) than cells transduced with the other CARs. This is consistent with studies showing that stimulation by natural ligand or agonistic antibodies to CD28 induces IL-2 and IL-21 secretion, while ligation of the endogenous ICOS molecule does not. Cells expressing either ICOSζ or 28ζ CARs secreted similar amounts of CCL20, IFN-γ and IL-10, while cells expressing the BBζ CAR had low level secretion of all cytokines. IL-4 secretion could not be detected in any of the conditions tested.

(figure 4.2 on following page)

Figure 4.2. T_H17 cells redirected with an ICOS-based CAR release high amounts of IL17-A, IL-17F and IL-22 but low amounts of IL-2. (A) T_H17 cells transduced with various CARs were cocultured with surrogate antigen, APC cells transduced with mesothelin (K562meso). Supernatants from several different healthy donors (n=4-9) were obtained 24 hours after coculture, and cytokine production was analyzed by ELISA. Box plots show median (line) and 25th–75th percentile (box). The end of the whiskers represents the minimum and the maximum of all of the data **(B)** Redirected T_H17 cells were stimulated with plate bound anti-CD3 (OKT3). IL-17A was analyzed by ELISA 24 hours after stimulation. Data represent the means ± SD for 2 different normal donors. **(C)** T_H17 cells were cultured with the indicated tumor cells expressing mesothelin. IL-17A and IL-2 production was analyzed by ELISA 24 hours upon antigen recognition. Error bars indicate standard deviation (SD) in duplicate samples. Results are representative of at least two different experiments. **(D)** $CD4^+$ T cells from different normal donors (n=3) were activated with anti-CD3/CD28 beads, cultured under T_H17 polarizing conditions, and redirected with the different CARs. After their primary expansion, $CD4^+$ T cells were cultured with K562meso cells. Secretion of IL17-A and INF-γ production was analyzed by ELISA 24 h upon antigen recognition. Error bars indicate standard deviation (SD). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.



Surface phenotype of ICOS-based CAR T cells. Human T_H17 cells can be identified by their surface expression of the lectin-like receptor CD161/NKRP1A, a marker of all human IL-17-producing T-cell subsets. Therefore, we measured the frequency of $CD161^+$ T cells after surrogate antigen recognition. We observed a progressive enrichment of $CD161^+$ cells in the ζ and ICOS ζ CAR cultures. In contrast, cells expressing CD161 were not enriched in the cultures

containing BB ζ or 28 ζ CAR T cells (Figure 4.3A). This enrichment was reproducible as it was observed in ICOS ζ CAR expressing T cells from 12 healthy donors (Figure 4.3B). In contrast to T_H17 cytokine production, no differences in CD161 expression were observed between cells redirected with ζ or ICOS ζ CARs (Figure 4.3C), indicating that signaling through 4-1BB and CD28 are detrimental for the expansion of $CD161^+$ cells, while ICOS is not necessary but not detrimental. Enrichment required initial exposure to T_H17 polarizing conditions, as T cells transduced with the panel of CARs under non-polarizing conditions did not exhibit subsequent enrichment for CD161 expressing cells when cultured with mesothelin-expressing tumor cells (Figure 4.3D).

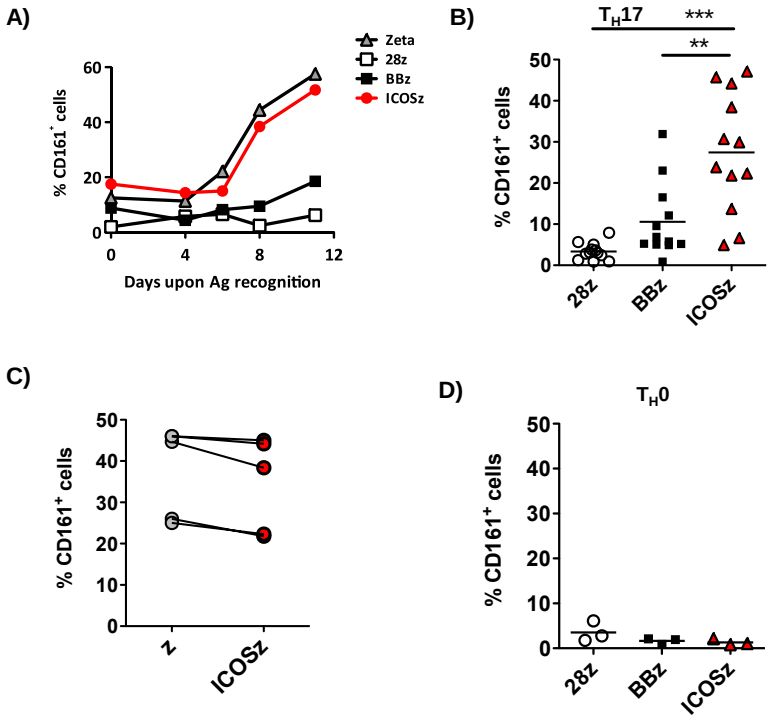


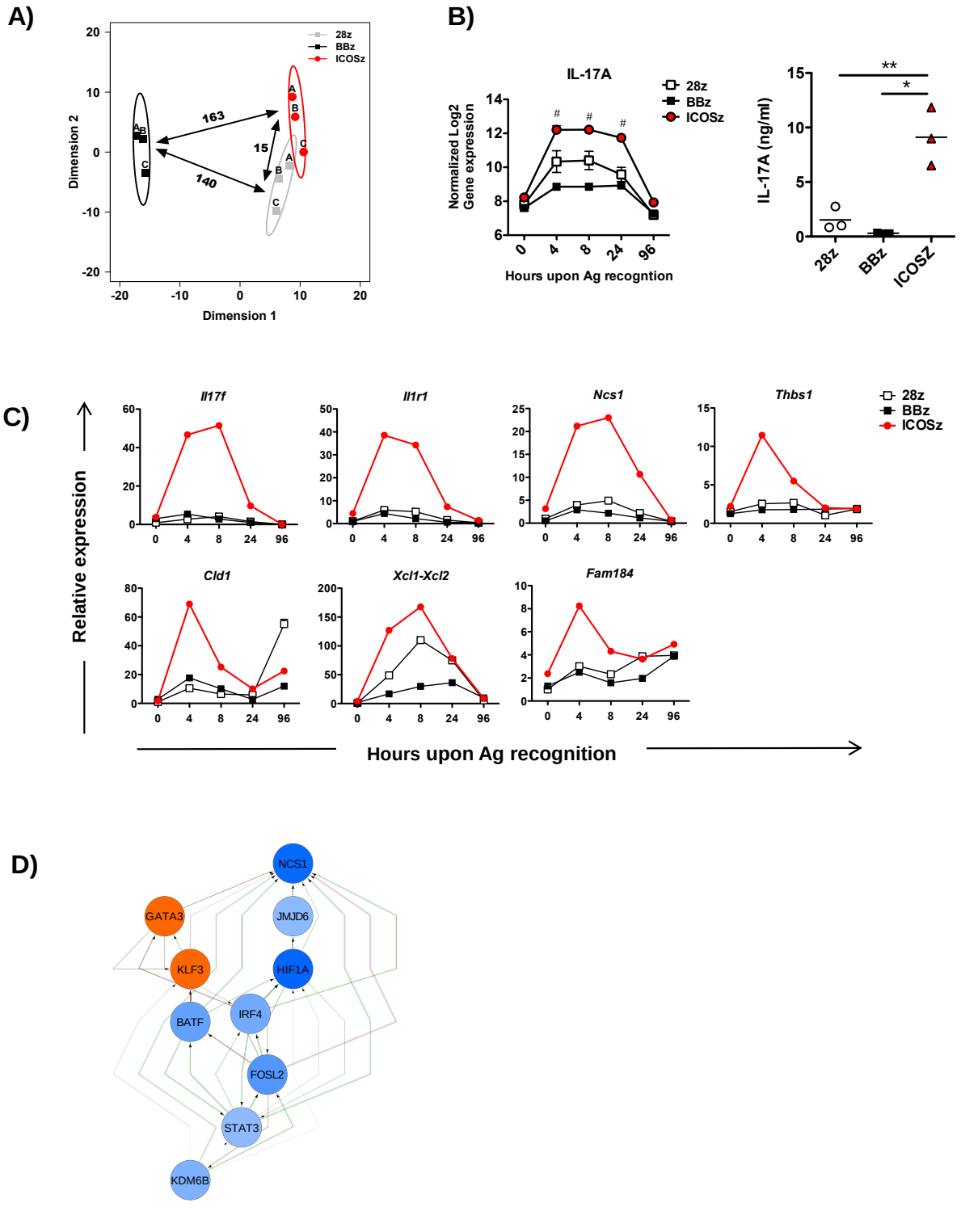
Figure 4.3. T_H17 cells redirected with ICOS endodomain have increased expression of CD161. Redirected T_H17 cells were cocultured with irradiated APC expressing mesothelin. (A) CD161 expression by CAR^+CD4^+ T cells in response to mesothelin-specific stimulation was analyzed by flow cytometry at indicated time points. The percentage of CAR^+ T_H17 (B, C) or T_H0 (D) cells expressing CD161 at day 8 after stimulation in several different normal donors is plotted. *p < 0.05, **p < 0.01 and ***p < 0.001.

Transcriptional signature of T cells expressing ICOS-based CARs. The enhanced expression of CD161 together with the selective secretion of IL-17 suggests that the ICOSζ CAR can be used to maintain a T_H17 cell phenotype. To fully characterize the spectrum of Th17 related markers in our cells, we next used gene expression analysis to perform an unbiased characterization of the effects of the CAR signaling domains on T cell activation after antigen recognition. Redirected $CD4^+$ cells from three healthy donors were restimulated with surrogate antigen using immobilized recombinant mesothelin-Fc and gene expression levels determined prior to activation and at multiple time points after stimulation. Signaling through CARs induced the differential expression of 886 genes compared to resting T cells at 4 hours upon antigen recognition (false discovery rate [FDR] < 0.05, > 2-fold change [FC] in expression). Multidimensional scaling (MDS) analysis of these 886 genes revealed that T cells redirected with ICOSζ and 28ζ signaling domains had a closely related gene expression profile, with only 15 differentially expressed genes (Figure 4.4A). By contrast, BBζ T cells exhibited a distinct gene expression profile, with 163 and 140 differentially expressed genes when compared to

ICOSζ and 28ζ T cells, respectively. We identified only 2 genes that were markedly up regulated by CD28ζ-expressing T cells, compared to both ICOSζ and BBζ CAR T cells: IL-2 and IL-8. In contrast, we found 11 genes that were differentially up regulated in ICOSζ cells compared to both 28ζ and BBζ T cells. Of note, 5 of the 11 genes upregulated in the ICOSζ cells were T_H17-associated transcripts, such as *Il17a, Il17f, Ccl20, Il1r1* and *Klrb1* (which encodes CD161). As predicted by the cytokine secretion data, *Il17a* was the most differentially expressed transcript in ICOSζ-redirected cells, and was induced 16-fold (4h vs 0h) (Figure 4.4B). Other genes that were preferentially activated by ICOS include *Thbs1, Xcl2, Fam184a, Cldn1, and klrk1,* although their specific roles for T_H17 biology are currently unknown. Differences in gene expression observed by microarray analysis were validated independently using ELISA or RT-PCR analysis (Figure 4.4B-C).

The T_H17 developmental regulatory network in the mouse has been recently characterized, and it was shown that a gene's involvement in T_H17 function can be predicted using a network-based "KCRI" score. This score quantifies the extent to which each gene is regulated by five T_H17 core transcription factors (TFs): Stat3, Batf, Irf4, Maf, and Rorc. Using this network resource, we tested whether the six uncharacterized genes we identified were regulated by the five core T_H17 TFs. We found that *Ncs1* was significantly upregulated in T_H17 cells compared to T_H0 cells, and ranked 46 out of more than 22,000 tested genes for T_H17 function based on the "KCRI" scoring scheme, similar to well-established T_H17 genes including: *Il17a* (ranks in parentheses; 6), *Il23r* (7), *Il17f* (8), *Rorc* (11), *Il1r1* (19) *Ahr* (107) and *Ccl20* (143). Figure 4.4D shows the significant regulatory inputs for Ncs1 identified in the T_H17 differentiation network model.

Figure 4.4. Transcriptional signature of T cells expressing ICOS-based CARs. Redirected T_H17 cells from three different human normal donors were stimulated with immobilized recombinant mesothelin. Gene expression levels were determined on prior to stimulation (day 0) and 4, 8, 24 and 96 hours upon antigen recognition. **(A)** Multidimensional scaling analysis (MDS) analysis of 886 differentially expressed genes between the three sets of CAR T cells at 4 hours upon activation reveal three distinct clusters. The number of differentially expressed genes (FDR<0.05, FC>2) between a pair of CARs is indicated with pair-connecting arrows. **(B)** Relative log2 expression of *Il17a* at indicated time points upon antigen recognition. #FDR<0.05, FC>2 compared to both 28z and BBz. The cytokine levels of IL-17A were validated by ELISA. Error bars represent SEM (3 different normal donors). *$p < 0.05$, **$p < 0.01$ **(C)** Expression of genes that were found significantly upregulated in ICOSz T cells compared to both 28z and BBz T cells was validated in at least one of the three healthy donors by quantitative reverse transcription polymerase chain reaction (qRT-PCR) at indicated time points and shown as ratio versus β-actin. **(D)** We display the significant regulatory inputs for Ncs1 identified in the T_H17 differentiation network model. Blue and orange nodes depict the extent of T_H17 upregulation and downregulation respectively (compared to a T_H0 control). Edges connecting two genes are colored green for activation and red for repression. Edges emitting from IRF4, STAT3, BATF, MAF and Fosl2 are supported by both TF ChIP-seq and TF knockout RNA-seq and thus should be considered as validated. Edges emitted from other TFs were inferred from transcriptomic analysis of a large compendium of mouse immune cells and thus should be considered as likely but not validated.


A)
28z
BBz
ICOSz
Dimension 2
Dimension 1
163
15
140
B)
IL-17A
Normalized Log2 Gene expression
Hours upon Ag recogntion
IL-17A (ng/ml)
28z
BBz
ICOSZ
C)
Il17f
Il1r1
Ncs1
Thbs1
Cld1
Xcl1-Xcl2
Fam184
Relative expression
Hours upon Ag recognition
D)
NCS1
GATA3
JMJD6
KLF3
HIF1A
BATF
IRF4
FOSL2
STAT3
KDM6B

$CD4^+$ cells redirected with ICOSζ showed a T_H17 core molecular signature with a T_H1 bias. We next performed gene set enrichment analysis (GSEA) of the microarray data obtained using cells at 4 hours following antigen recognition (Figure 4.5A). We observed a nonrandom distribution of genes associated with T_H17 cells at the top of the ranked gene list for all groups. Cells expressing the ICOSζ CAR had the highest maximal enrichment score (ES) of 0.784, followed by 28 ζ and BB ζ, confirming that redirection of T_H17 with an ICOS ζ -based CAR can be used to sustain a T_H17 core molecular signature.

Because it has been suggested that T_H17 cells must acquire T_H1 cell-related features to eradicate tumors in mice, we next analyzed whether signaling through ICOS could induce the expression of T_H1-related genes, as well as T_H2- and T_{reg}-related genes. Heat map representation of log2-fold changes of T helper signature gene expression at 4 hours after stimulation relative to non-stimulated cells demonstrated that T_H17 cells redirected with ICOS ζ highly overexpressed T_H17-related genes, including *Rorc*, *Il22* and *Il26* (Figure 4.5B-C). Notably, they also overexpressed some T_H1-related genes, including *Ifnγ*, *Tnfα* and *Tbx21* (T-bet) (Figure 4.5B, D). Importantly, ICOS signaling in T_H17 cells did not induce the expression of T_{reg}- or T_H2-related genes, with the exception of *Il13* (Figure 4.5B). Also, contrary to cytokine and transcription factor expression, signaling through CAR did not induce significant changes in expression of most cell surface markers, including *Ccr6*, *Ccr4* and *Ccr10*. Collectively, these results suggest that ICOS-based CARs can sustain T_H17 function with a T_H1 bias.

Comparison of antitumor efficacy of T_H17/T_H1 polarized T cells expressing CD28, 4-1BB and ICOS based CARs. We next analyzed the antitumor activity of retargeted T_H17 cells. For this experiment, in addition to redirected T_H17 cells we also redirected T_C17 cells ($CD8^+$ T cells cultured under T_H17 polarizing conditions). Both T_H17 and T_C17 redirected cells efficiently lysed mesothelioma (M108) tumor cells, and no differences in cytotoxicity were observed between CAR signaling domains (Figure 4.6A). T_C17 cells secreted robust amounts of IFN-γ following antigen recognition, although IL-17 secretion was not detected. Next, the antitumor activity of mixed redirected T_H17 and T_C17 cells was analyzed in NSG mice bearing M108 tumors (Figure 4.6B-C). A potent antitumor effect was observed in all groups treated with mesothelin-specific CAR T cells. T cells redirected with BBζ showed a relatively slow tumor decline, with all mice showing significant tumor shrinkage after treatment. 28ζ and ICOSζ treatments showed a more rapid tumor decline, leading to tumor eradication in most mice (Figure 4.6B). Treated mice were sacrificed on day 51 after T cell injection as they developed xenogeneic graft versus host disease, and mice treated with the ICOSζ CAR T cells had the most severe GVHD. We have previously shown that this tumor does not regress following injection of non-gene modified allogeneic T cells.

Figure 4.5. T_H17 cells redirected with ICOS endodomain showed increased expression of T_H17/T_H1-related genes. (A) Gene set enrichment analysis (GSEA) for gene lists ranked by log2-fold change capturing expression patterns from T_H17 cells with three different CAR signaling domains. Shaded area around each line reflects the mean enrichment score plus and minus one standard deviation for each corresponding CAR from three donor replicates, respectively. The vertical red lines indicate where in the ranked list human orthologous of 74 literature-derived T_H17-related mouse genes were recovered. Blue line shows the performance of 10,000 randomly permuted gene sets in the ranked gene list from T_H17 cells with ICOS. **(B)** Heat map of log2-fold change in expression of T helper signature genes at 4 hours relative to 0 hours. (**C**,**D**) Expression of selected genes was measured by qRT-PCR and expressed relative to β-actin. The cytokine levels of IFN-γ were validated by ELISA. *$p < 0.05$, **$p < 0.01$.

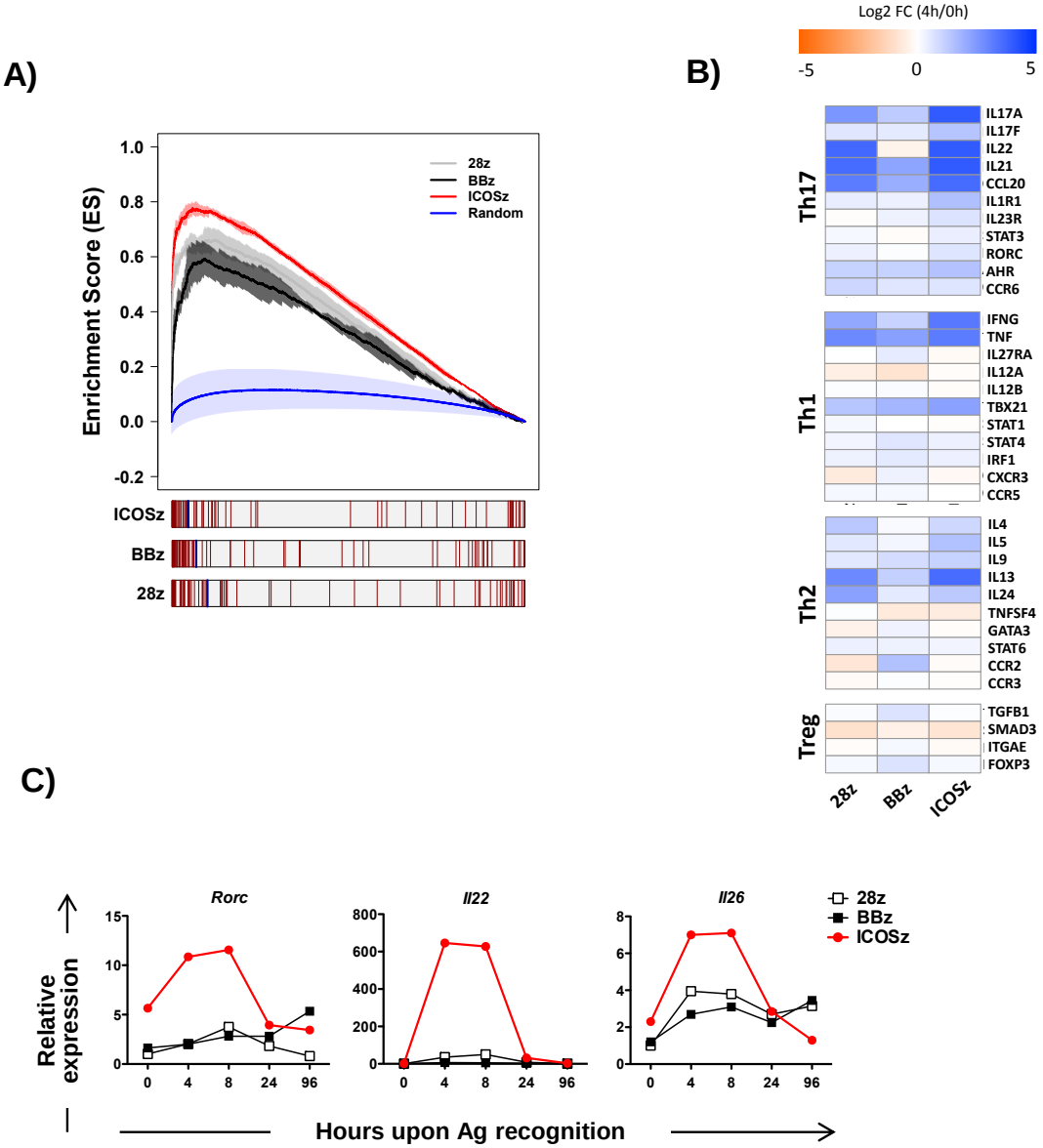
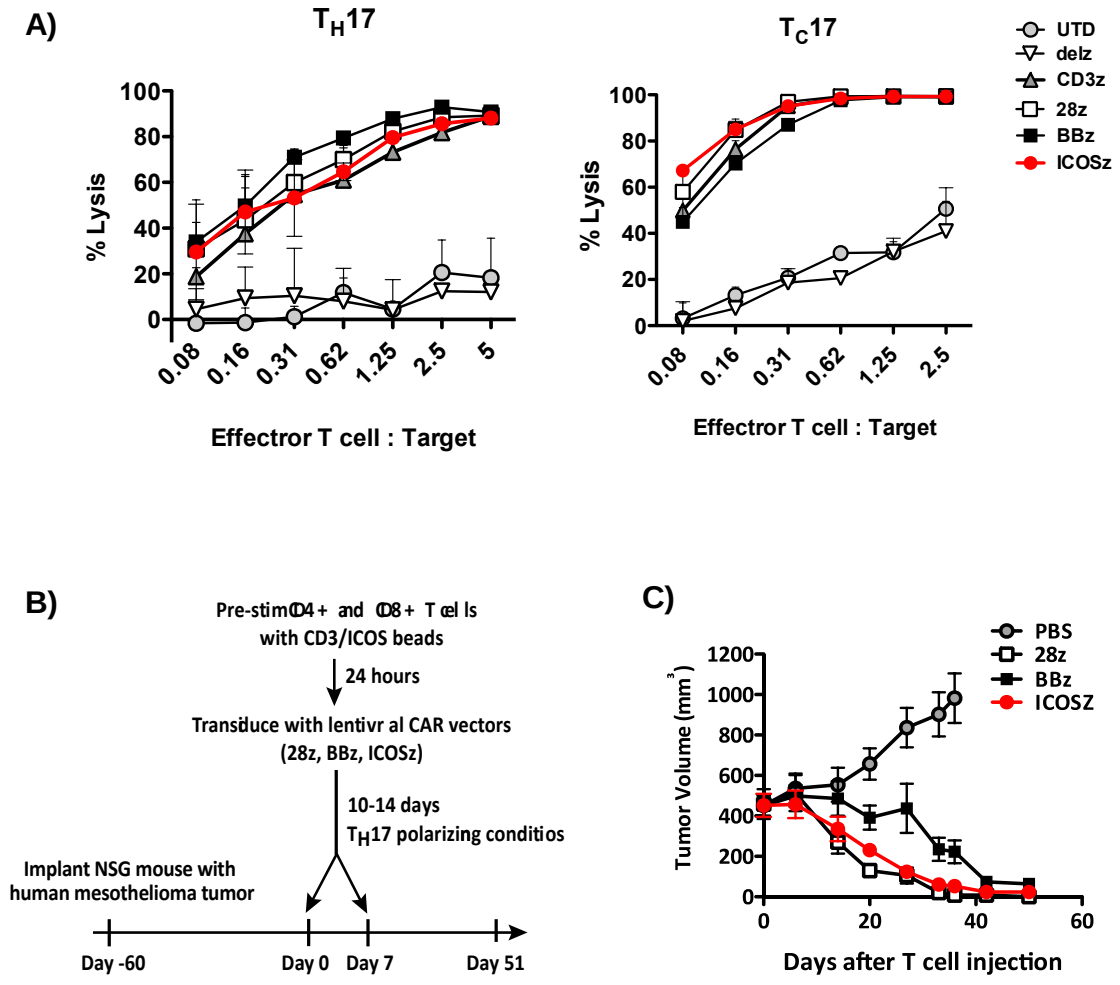

A)
Enrichment Score (ES)
28z
BBz
ICOSz
Random
ICOSz
BBz
28z
B)
Log2 FC (4h/0h)
-5
0
5
Th17
IL17A
IL17F
IL22
IL21
CCL20
IL1R1
IL23R
STAT3
RORC
AHR
CCR6
Th1
IFNG
TNF
IL27RA
IL12A
IL12B
TBX21
STAT1
STAT4
IRF1
CXCR3
CCR5
Th2
IL4
IL5
IL9
IL13
IL24
TNFSF4
GATA3
STAT6
CCR2
CCR3
Treg
TGFB1
SMAD3
ITGAE
FOXP3
28z
BBz
ICOSz
C)
Rorc
Il22
Il26
Relative expression
Hours upon Ag recognition
D)
NS
**
*
IFN-g (ng/ml)
28z
BBz
ICOSz
Tbx21
Relative expression
Hours upon Ag recognition

Figure 4.6. T_H17 /T_C17 cells redirected with mesothelin specific CARs eradicate advanced pre-established tumors. (A) T_C17 and T_H17 cells were cocultured with M108 target cells stained with CFSE for 18 hours at the indicated effector-target (E:T) ratios. Specific cytolysis was determined using a flow cytometry-based assay. Percent specific lysis was calculated as [experimental release (cpm) - spontaneous release (cpm)]/[maximal release (cpm) - spontaneous release (cpm)] × 100. Error bars indicate standard deviation (SD) in duplicate samples. Representative of two different experiments. (**B**) As shown schematically, human primary M108 tumors were established in the flanks of NSG mice. After 8 weeks, when the tumors reached a large volume of 500 mm^3, mice were treated with 2 intratumoral injections of 10 x 10^6 T_H17 and T_C17 cells (80% and 60% CAR+) or PBS on days 61 and 67. **(C)** Tumor volume was analyzed at indicated time points. Results are expressed as a mean tumor volume (+/- SD) with n=9 for all groups. Representative of two different experiments.



CAR T_H17 cells reprogramed with ICOS endodomains have long-lived persistence in vivo. Long-term persistence of adoptively transferred T cells correlates with antitumor effects in clinical trials. Because it has been shown that natural T_H17 cells are long lived *in vivo*, we next sought to assess the in vivo persistence of CAR-modified T_H17 and T_C17 cells in the peripheral blood of

treated mice (Figure 4.7A-B). The average number of $CD4^+$ T cells per µl of blood and per spleen at the time of sacrifice (day 51) was higher in mice treated with ICOSζ-based CARs, when compared with 28ζ and BBζ groups. Of note, T_H17 cells redirected with ICOS also showed a higher frequency of $CD161^+$ cells, correlating with in vitro results (Figure 4.7C). $CD8^+$ T cells redirected with BBζ persisted better, although differences were not significant (Figure 4.7A). To extend these results, we performed a similar experiment with NSG mice bearing non-small cell lung tumors. In order to confirm that T cell persistence was due to tumor recognition, and not to recognition of xenoantigens by the endogenous TCR of these cells, this experiment was done in duplicate using mice with or without tumors (empty mice). T_H17 cells redirected with ICOSζ showed an increased *in vivo* expansion and persistence that was significantly greater than 28ζ and BBζ groups. Importantly, ICOSζ T cell expansion was dependent on tumor antigen encounter, as empty mice showed significantly fewer ICOSζ cells compared to mice with tumors (Figure 4.7D). In summary, these results suggest that reprogramming $CD4^+$ T cells with an ICOS-based CAR signaling domain promotes T_H17 cell function and phenotype and enhances T cell persistence in tumor bearing mice.

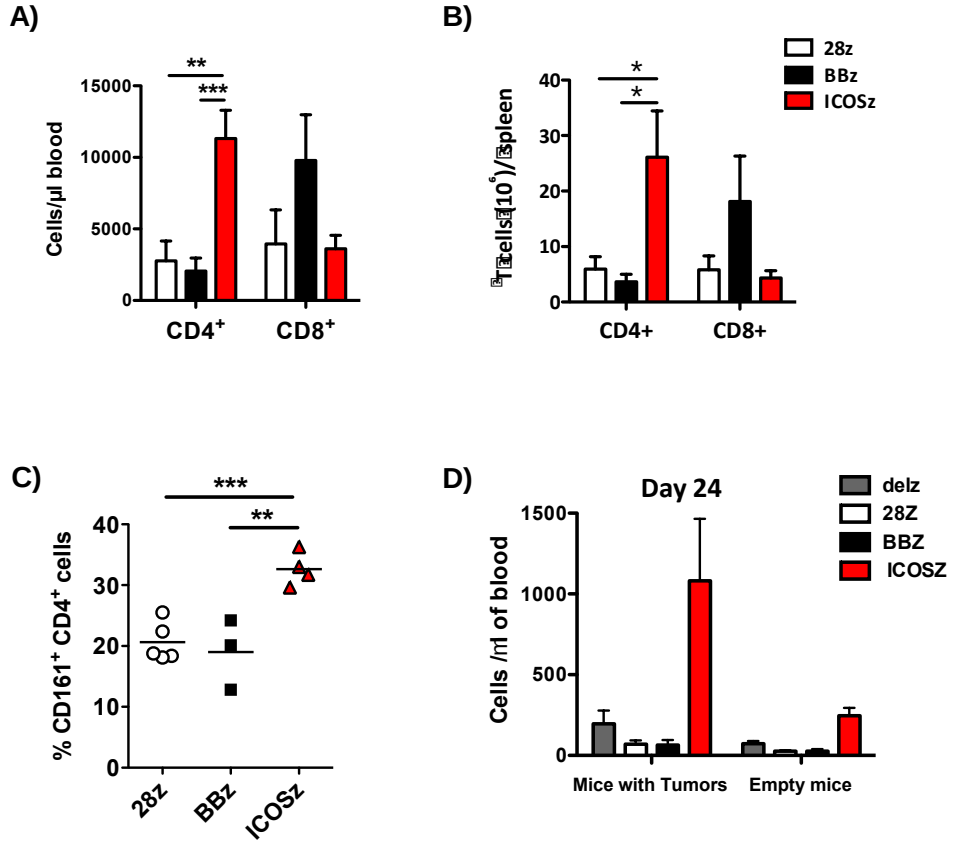


Figure 4.7. T_H17 cells programmed with an ICOS based CAR show enhanced persistence that is dependent on antigen encounter. (A) NSG mice from the experiment shown in figure 6 that were treated with intratumoral injections of redirected T_H17 /T_C17 cells were killed on day 51 after T cell infusion. Peripheral blood was quantified for the presence of human $CD4^+$ and $CD8^+$ T cells by a FACS Trucount assay. Results are expressed as a mean absolute T-cell count per µL of peripheral blood +/- SD (n=9 for all groups). Representative of two different experiments. (**B-C**) Redirected T cells were obtained from mouse spleens on day 51 after treatment. The number of $CD4^+$ and $CD8^+$ T cells (**B**) and the percentage of $CD161^+$ CAR^+ $CD4^+$ T cells (**C**) were analyzed by flow cytometry. Error bars represent SD (**D**) Human L55 non-small cell lung cancers were established in the flanks of NSG mice. After 3 weeks, when the tumors reached a volume of 150 mm^3, mice without tumors (empty mice) or with pre-established tumors were treated with 2 i.v. injections of 10 x 10^6 T_H17 /T_C17 cells . The concentration of $CD4^+$ T cells was determined in the blood of treated animals three weeks post-infusion. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Representative of one experiment.

In summary, we find that CAR T cells expressing an ICOS signaling domain in T_H17 cells are preferable to CAR T cells expressing CD28 or 4-1BB domains due to a stabilized T_H17 core molecular signature and an enhanced persistence of the CAR T cells in tumor bearing mice. CAR T cells with ICOS domains retain a "bipolar" bias towards a T_H1 and T_H17 signature, a plasticity that appears to be a prerequisite for potent antitumor activity in several preclinical models. Enhanced in vivo persistence is a desirable effect for long-term antitumor effects. Our studies suggest that the ex vivo manipulation of CAR T cells with an ICOS signaling domain can stabilize T_H17 function and promote CAR T cell persistence in mice bearing human tumor xenografts. This enhanced persistence required stimulation through the CAR receptor.

To our knowledge our findings are the first to demonstrate that the expression of selected CAR endodomains can program T cells for their subsequent differentiation fates and effector functions. The balance of Tregs to effector T cells is an important biomarker for the outcome of many immunotherapies, and the ability of ICOS to limit IL-2 secretion may be an important feature in improving this ratio. The observation that CAR T cells bearing ICOS signaling domains have enhanced survival after adoptive transfer provides the rationale to test this concept in human trials in the setting of solid tumors and non-lymphoid hematologic malignancies, where improved persistence of CAR T cells remains as an unmet medical need.

These results were published by Guedan et el (Sonia Guedan, Xi Chen, Aviv Madar, Carmine Carpenito, Shannon McGettigan, Matthew J. Frigault, John Scholler, Nathalie Scholler, Richard Bonneau, Carl H. June "ICOS---based chimeric antigen receptors program bipolar TH17/TH1 Cells" Blood 2014) and presented to the following meetings:

1. American Association of Immunologist annual meeting (2013).
   Communication type: Oral presentation
   Authors: Sonia Guedan, Carmine Carpenito, Aviv Madar, Xi Chen, Shannon McGettigan, Matthew Frigault, John Scholler, Nathalie Scholler, Richard Bonneau, Carl June.
   Title: Redirection of T_H17 cells with a car containing the ICOS costimulatory domain enhances function, antitumor activity and persistence of T_H17 cells.

2. American Society of Gene and Cell Therapy 15th annual meeting (2012).
   Communication type: Oral presentation
   Authors: Sonia Guedan, Carmine Carpenito, Shannon McGettigan, Matthew Frigault, John Scholler, Yangbing Zhao, Carl June.
   Title: Redirection of T_H17 cells with a car containing the ICOS costimulatory domain enhances function, antitumor activity and persistence of T_H17 cells.

<u>Specific Aim 5</u>. The specific aims of this training program in translational research are to:
1. Provide a basic foundation of the transdisciplinary research techniques appropriate for translational research and
2. Provide intensive, supervised research experiences with mentors in translational research at the undergraduate and graduate level.

The objectives of this training program are to:
1. Motivate and facilitate minority undergraduate and graduate students to pursue careers in clinical and translational research and
2. For graduate level training, increase the pool of investigators trained to conduct independent and collaborative translational cancer research.

To achieve these aims, we developed a training program to provide undergraduate and graduate trainees with an introduction and the basic tools necessary to pursue careers in biomedical research. In addition to didactic training, trainees complete a mentored research project in novel diagnostics and/or therapeutics.

A. Undergraduate Training Program

Our undergraduate efforts focused on expanding a pre-existing relationship with Lincoln University, a Historically Black University (HBCU), to provide selected undergraduate students with educational training and mentorship. To achieve this goal, we established both a Summer Undergraduate Internship and a monthly seminar series for undergraduate students at Lincoln University during the academic year.

Pre-existing infrastructure at UPENN through the Institute of Translational Medicine and Therapeutics (ITMAT) was utilized to assist in quickly establishing the summer internship program. We expanded on the ITMAT-sponsored summer internship program, which partners with five local colleges, including Lincoln University. The ITMAT-sponsored internship provides a substantial, mentored experience in translational and clinical approaches to understanding a disease and developing effective therapeutic modalities. The internship consists of research on a project of interest developed in consultation between the student and mentor. The project addresses a specific and meaningful question based on current insights relevant to the disease and therapy. The internship also consists of several hours each week devoted to coursework and a seminar series highlighting methodologies in translational research. Time is spent as well evaluating literature relevant to the specific research question. The 10-week internship culminates with a presentation describing the project in terms of the question asked, information obtained, and placement of the work within the larger context of clinical and translational research efforts.

In total, seven undergraduate students completed summer internships through this program (Table 5.1). All but one of these students were from Lincoln University (see table below). Each student presented their project at the CTSA Research Symposium, held at the conclusion of the internship.

Table 5.1 Summary of Undergraduate Students

Year	Student	School	Mentor
2010	Bianca Montalmont	Lincoln	Nicole Aqui
2011	Shauna Ebanks	Lincoln	Michael Kalos
2012	Anup Misra	Lincoln	Carl June
2012	Jhoneil Cooper	Lincoln	Daniel Rader
2012	Jodi-Ann Foster*	Lincoln	Steven Willi
2013	Danielle Mathis	Lincoln	Andrea Facciabene

2013	Connor Paez	Penn	Bruce Sachais
*Funded through ITMAT			

One of our most recent interns, Connor Paez, has continued to work with his mentor throughout the academic year, and will spend the summer in Dr. Sachais' laboratory.

Partnership with Lincoln University. We provided speakers for the seminar series at Lincoln during the 2011-2012, 2012-2013, and 2013-2014 academic years. The talks, given by all the Project PI's as well as other Penn biomedical researchers, ranged in topics from novel cancer therapies, to pharmacology, to biomarkers of schizophrenia and autism. The seminar series has been very successful, as evidenced by the fact that Lincoln has been able to attract additional outside (non-Penn) speakers. We plan to maintain our participation in the series during the next academic year.

One of our primary objectives was to establish collaborations that would allow Lincoln students to continue engaging in translational research while at Lincoln. As such, through this grant, we provided funding to Karen Baskerville, PhD, as Associate Professor and Chair of the Department of Biology at Lincoln. Her proposed project is outlined below.

The long-term goals of the proposed research are to study the expression profile of genes and proteins in brain cancer cell lines that could give clues to immunotherapies for cancer of the brain and to train undergraduate students in the research. Tumors in the brain are quite varied and difficult to treat. We hypothesize that the brain, particularly in response to aging, may be subjected to oxidative stress, compromising critical neuroprotective and immunoprotective mechanisms and, thus, contributes to brain cancer. The levels of reactive oxygen species (ROS) in brain tumor cells could give insight on the development of cancer therapies. There is, however, a paradox. High levels of ROS can lead to tumor development, but ROS can also lead to cell death, causing cancer cells to decrease. There is also contradictory evidence for the therapeutic use of antioxidants in cancer. Using glioma cell lines, the hypothesis will be tested with the following specific aims: 1) To determine the expression levels of oxidative stress markers in gliomas, the most common primary tumors in the brain; and 2) To elucidate clues for therapies for gliomas by treating brain cancer cell lines with antioxidants and pro-oxidants.

Undergraduate Student Involvement. Shauna Ebanks, a May 2014 graduate of Lincoln University, worked on the research project for the entire funding period. She cultured glioma and normal glial cell lines, isolated RNA from the samples for gene expression studies, and did Western blot analyses. Shauna worked on the parent grant as a research intern at the University of Pennsylvania in the summer of 2011. She presented her research at the annual science fair at Lincoln and won an award for her presentation. In addition to her research accomplishments, Shauna was the valedictorian of her graduating class and plans to go to medical school in the near future.

Two other students have worked on the project. Josephat Wahome worked on the project during the last year and a half of funding and contributed to the project by doing Western blots of oxidative stress markers. Patrick Ihejirika began working in the lab in the fall of 2013 and is completing the gene expression studies.

Studies and results generated at Lincoln University. For Aim 1, experiments have been done to determine the levels of ROS, SOD2, and other oxidative damage markers in rat glioma cell lines. A rat glioblastoma cell line (ATTC CRL-2433), a type of glioma cell, was studied and compared to a normal glial cell line. Using fluorescence microscopy and MitoSox (Life Technologies) as an indicator, high levels of superoxide, a ROS in mitochondria, were found in the glioma cells. These cancer cells also had high levels of 4-hydroxynonenal (4-HNE), a lipid peroxidation marker. There appeared to be lower levels of SOD2 in the glioma cells compared to normal cells. In Western blots, there was decreased protein expression of SOD2 in the gliobastoma cells. RNA has been isolated from cells to study gene expression profiles of oxidative stress markers.

For Aim 2, experiments have been done to determine the therapeutic effects of antioxidants on glioblastomas. Glioma cells treated with vitamen E, an antioxidant, appeared to have less superoxide and more SOD2 activity. Data have to be quantified, and experiments have to be repeated. We have not yet treated cells with pro-oxidants.

Significance of studies at Lincoln University. The significance of this research project is that it addresses elucidating mechanistic clues and therapies for a very aggressive form of cancer in the brain. The project will help us generate more hypotheses on the role of ROS in therapeutic targets for gliobastomas. The project is also significant because it exposed undergraduate students to an interesting and challenging line of research.

Plans for research developed at Lincoln University. The data collected thus far have generated further questions and warrant us to repeat experiments and extend the study. We plan to do Western blot analyses of 4-HNE and 8-hydroxyguanosine protein adducts in the glioma cell lines. We need to complete gene oxidative stress expression studies and treat gliomas with pro-oxidants. Furthermore, we have purchased glioma profile and cancer drug targets arrays to study biomarkers of glioma cells and targets for anticancer therapies; we plan to run these arrays this summer. In the long term, we plan to study how treatment with antioxidants and pro-oxidants affects tumor growth as well. None to date regarding data from the subaward, but we plan to submit a journal article by the end of 2014.

B. Graduate Fellowship

Ernestina Nyarko, a UPENN medical student, completed her independent study project with Dr. Jun Mao, focusing on: 1.) cancer survivor's perceptions of survivorship care from primary care physicians, and 2.) immunological markers in acupuncture as treatment of althralgia in breast cancer patients on aromatase inhibitor therapy. She is currently working on a manuscript for publication. Prior to starting her work with Dr. Mao, Ernestina took Clinical Trials and Translational Research (EP 815), also funded by this grant, which provided an overview of scientific and ethical research conduct. Ernestina graduated from the Perelman School of Medicine in May 2013, and is a resident in family practice at Carolinas Medical Center. She hopes to continue her interest in population studies, particularly women's health.

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

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?

___1__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?

___15__Number of subjects originally targeted to be included in the study
___3__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:
_____Males
__3___Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
__3___Not Latinos or Hispanics
_____Unknown

Race:

_____American Indian or Alaska Native
_____Asian
__1___Blacks or African American
_____Native Hawaiian or Other Pacific Islander
__2___White
_____Other, specify:_____
_____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.)
University of Pennsylvania, Philadelphia, USA

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):
Mesothelin, a novel immunotherapy target for triple negative breast cancer	Tchou J, Wang LC, Selven B, Zhang H, Conejo-Garcia J, Borghaei H, Kalos M, Vondeheide RH, Albelda SM, June CH, Zhang PJ.	Breast Cancer Research and Treatment	Feb 2012	☐Submitted ☐Accepted XPublished
Tumor endothelial marker 1–specific DNA vaccination targets tumor vasculature	Facciponte JG, Ugel S, De Sanctis F, Li C, Wang L, Nair G, Sehgal S, Raj A, Matthaiou E, Coukos G, Facciabene A.	The Journal of Clinical Investigation	Oct 2012 and Aug 2013	☐Submitted ☐Accepted XPublished
ICOS-based chimeric antigen receptors program bipolar T_H17/T_H1 cells	Guedan S, Chen X, Madar A, Carpenito C, McGettigan SE, Frigault MJ, Lee J, Posey AD Jr, Scholler J, Scholler N, Bonneau R, June CH.	Blood	Oct 2013	☐Submitted ☐Accepted XPublished

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:
Specific Aim 1. Upon enrollment, treatment, and analysis of six patients on this study, a report will be submitted to a peer-reviewed journal.

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

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.

None

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.")

a. Title of Invention: Tumor vascular marker-targeted vaccines: US20120035529-A1

b. Name of Inventor(s): Andrea Facciabene, George Coukos

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention): As from the results section 17.

d. 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: February 9, 2012

e. 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:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No X

If yes, how many licenses were granted?_____

g. 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:

a. Title of Invention: Switch Costimulatory Receptors

b. Name of Inventor(s): Zhao Y, June C

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

d. 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: July 29, 2011

e. 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:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No X

If yes, how many licenses were granted?_____

g. 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:

a. Title of Invention: RNA Engineered T Cells for the Treatment of Cancer

b. Name of Inventor(s): Zhao Y, June C

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

d. 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: Sept 16, 2011

e. 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:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No X

If yes, how many licenses were granted?_____

g. 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:

a. Title of Invention: Use of ICOS-Based CARs to Enhance Antitumor Activity and CAR Persistence

b. Name of Inventor(s): Guedan S, Xhao Y, Scholler J, June C

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention): As from the results section 17

d. 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: Feb 22, 2012

e. 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:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No X

If yes, how many licenses were granted?_____

g. 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.*