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:** Drexel University

2. **Reporting Period (start and end date of grant award period):** 6/1/2010 - 5/31/2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Noreen Robertson, DMD

4. **Grant Contact Person's Telephone Number:** (215) 895-6471

5. **Grant SAP Number:** 4100051718

6. **Project Number and Title of Research Project:** Therapeutic DNA vaccine for the Prevention of Hepatitis C Virus-associated Cancer

7. **Start and End Date of Research Project:** 6/1/2010 - 5/31/2014

8. **Name of Principal Investigator for the Research Project:** Jeffrey M. Jacobson, MD

9. **Research Project Expenses**.

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

   $ $2,815,858

   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
Drexel University College of Medicine			
Jeffrey Jacobson,MD	PI	25%	$189,122.95
Michele Kutzler, PhD	Co-investigator	25%	$122,736.48
Seth Welles, PhD	Biostatistics	10%	$17,355
Albert Sylvester	Technician and Masters student	40%	$23,250.93
Scott Baliban	Graduate Research Fellow (PhD)	100%	$89,260.50
Viraj Sanghvi	Graduate Research Fellow (PhD)	75%	$8,180
Jennifer Gyamfi	Technician and Masters student	75%	$38,499.63
Shannon Criniti	Administrator	20%	$37,736.26
Mariana Bernui	Postdoc Fellow	30%	$23,903.59
Brian Latimer	Graduate Research Fellow (MD PhD)	100%	$20,250.00
Toporovski, Roberta	Graduate Research Fellow (PhD)	100%	$2,250.00
Natalie Torres	Technician and undergraduate	10%	$1,040.00
INOVIO			
Niranjan Y. Sardesai, Ph.D.	Chief Operating Officer - Project Director	8%	$19,281
Amir Khan, Ph.D.	Director, Product Development - Investigator	8%	$9,790
Mark Bagarazzi, M.D.	Chief Medical Officer - Clinical Investigator	2%	$4,211
Jessica Lee	VP, Clinical Operations	3%	$333
Matthew Morrow, Ph.D.	Immunology Lead	0.25%	$383
Kate Broderick, Ph.D.	Associate Director R&D	0.25%	$3,365
University of Pennsylvania			
David Weiner, PhD	P.I.	2%	$3,934
Natalie Hutnick	Postdoctoral fellow	3%	$1,252
Colleen Lucke	technician	50%	$14,641
Atman Parikh	technician	44%	$2,090
Kendra Talbott	technician	8%	$1,160
Devin Myles	technician	70%	$23,330
Daniel Choo	Postdoctoral fellow	100%	$37,740

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
Drexel University College of Medicine		
Ebony Gary	Graduate Research Fellow (PhD)	50%
Cynthia Edisuryia	Medical Student	10%
Getrude Makurumidze	Summer research UG from Bryn Mawr	100%
Cheyney University of PA	**Position Title**	**% of Effort on Project**
Saber Aloui	Summer research student in Felix Kim Lab	100%
Deylan Moore	Summer research student	100%
Lennan Boyd	Summer research student	100%
Devon Moore	Summer research student in Sonia Martin Lab	100%
Ashley Benjamin	Summer research student in Ramesh Raghupathi Lab	100%
Ryan Hoffman	Summer research student in Barry Waterhouse Lab	100%
Monica Effi	Summer research student in Carroll Artlett Lab	100%
Chandra Gadsen	Summer research student	100%
Fatima Jackson	Summer research student in David Weiner lab	100%
Jessica Laroda	Summer research student in Chris Sell Lab	100%
Dr. Abdel Bior	Assistant Professor, Cheyney University of PA	100%
INOVIO		
Steve Kemmerrer	VP, Engineering	2%
Brian Waterman	Project Engineer	3%
Usman Khan	Quality Manager	2%
Catherine Ngo	Controller	2%
University of Pennsylvania		
Michael Merva	Lab Manager	2%

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
BD LSRF Fortessa special	Research carried out as described in our	$131,866.57

Ordering system (total cost $284,013). Drexel provided $152,146.43 towards the purchase of this instrument. CURE funds were used to pay $131,866.57.	CURE Grant required the phenotyping of cell subsets and functional analysis of T cells in response to our HCV DNA Vaccine and the BD Fortessa instrument was used primarily for these studies. The instrument was integral for the work and 1 paper has been published and 1 paper is under review using data from these experiments. Latimer, B., R. Toporovski, J. Yan, P. Pankhong, M. P. Morrow, A. S. Khan, N. Y. Sardesai, S. L. Welles, J. M. Jacobson, D. B. Weiner, and M.A. Kutzler. 2014. Strong HCV NS3/4a, NS4b, NS5a, NS5b-specific cellular immune responses induced in Rhesus macaques by a novel HCV genotype 1a/1b consensus DNA vaccine. Hum Vaccin Immunother. 2014 Jun 20;10(8). Under review at JCI: Brian P. Latimer, Ebony N. Gary, Scott M. Baliban, Jian Yan, Niranjan Y. Sardesai, Linda B. Couto, Jeffrey M. Jacobson, David B. Weiner, and Michele A. Kutzler. "A synthetic HCV genotype 1a/1b consensus DNA vaccine elicits functional, long-lived, memory T cells in heparo".	

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

Yes_____ No____X_____

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

11. Leveraging of Additional Funds

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

Yes_____ No_____X_____

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:
None	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

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:

1. National Cancer Institute: Etiology, Prevention, and Treatment of Hepatocellular Carcinoma (P01) PAR-08-245.

2. National Cancer Institute: (R43/R44) Small Business Innovation Research (SBIR) Grant - Phase I, Phase II, and Fast-Track PA-11-096.

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

This collaborative research project will continue to involve a number of leading scientists in the field of hepatitis research and vaccine development and includes a number of collaborating organizations from academia and industry. The team involved in this study will continue to share infrastructure, resources and expertise with the common goal of testing the DNA based vaccine for the prevention of Hepatitis C Virus (HCV)-associated cancer in Phase I therapeutic and prophylactic trials, as well as mentoring future research scientists in highly translational bench research. Findings published from work carried out in this grant show that T cell effector function is preserved within the liver and that selective recruitment of antigen-specific T cells to the liver may play a previously unappreciated role in the process of immune surveillance, which were tested in immunogenicity studies and challenge studies using both mouse and primate studies. In addition to the published preclinical studies, we have identified other funding sources and collaborators to aid in supporting our clinical trials to test vaccine in human cohorts (in collaboration with GeneOne Life Sciences in Seoul, Korea, study 1: PI; Sang Hoon Ahn, M.D, Ph.D.
- Yonsei University Severance Hospital and Jeong Heo, M.D, Ph.D. - Pusan National University Hospital; Recruiting: Phase I Trial to Evaluate the Safety, Tolerability and Immunogenicity of VGX-6150 for Second-line Therapy of Chronic Hepatitis C Infection (VGX-6150-01, ClinicalTrials.gov Identifier: NCT02027116). The second trial that will take place will be funded by NCI Division of Cancer Prevention as Dr. Jeff Jacobson and his Clinical Trial Unit has been added as an approved partner to the Phase 0/I/II Cancer Prevention Clinical Trials Program (Consortia for Early Phase Prevention Trials).

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	3	1
Female	8	1	3	2
Unknown	0	0	0	0
Total	**12**	**2**	**6**	**3**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic	1	0	1	1
Non-Hispanic	2	2	5	2
Unknown	0	0	0	0

Total	**3**	**2**	**6**	**3**

	Undergraduate	Masters	Pre-doc	Post-doc
White	1	1	5	2
Black	11	1	1	0
Asian	0	0	0	1
Other	0	0	0	0
Unknown	0	0	0	0
Total	**12**	**2**	**6**	**3**

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

Yes_____ No_____X_____

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

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

Yes____X_____ No_____

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

Although not directly related, the research into HCV vaccines pre-clinical development has parallels with exploration of multi-antigen formulations for other target pathogens. We have developed a mouse hepatocyte model for assessing T-cell localization to the liver. This model is presently being tested for use with a multi-component formulation of malaria and hepatitis B antigens.

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:

The collaborative nature of the research in this proposal provides an ideal model for an inter-academic-industry partnership. At Drexel University College of Medicine (DUCOM), funding through this award has strengthened our ability to carryout next step experiments and collaborate with University of Pennsylvania (UPENN). In addition, funding through this

award has supported graduate student stipends so that the PI can mentor and involve graduate students in this research project. In addition, a unique training opportunity was funded to continue to train minority students and faculty from Cheyney at DUCOM and UPENN. The funding provides a means to pay for resources including research consumables, reagents, animal per diems, etc. so that the collaborative research group can be productive and meet proposal research goals. At Inovio, the partial research funding provided helps us offset some of the costs associated with the pre-clinical development of interventions for a hard to treat disease that is otherwise underserved. In this regard, the collaboration that includes top-notch universities and academic collaborators at Drexel University and the University of Pennsylvania has increased the capacity to conduct such pre-clinical research at Inovio.

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

Yes_____X____ No_____

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

The collaborative nature of the research in this proposal provides an ideal model for an academic-industry partnership. At Inovio, the partial research funding provided helps us offset some of the costs associated with the pre-clinical development of interventions for a hard to treat disease that is otherwise underserved. Inovio and GeneOne Life Science collaborators on the grant will be testing the HCV DNA vaccine in Phase I clinical trials with the hopes of commercial development: (study 1: PI; Sang Hoon Ahn, M.D, Ph.D. - Yonsei University Severance Hospital and Jeong Heo, M.D, Ph.D. - Pusan National University Hospital; Recruiting: Phase I Trial to Evaluate the Safety, Tolerability and Immunogenicity of VGX-6150 for Second-line Therapy of Chronic Hepatitis C Infection (VGX-6150-01, ClinicalTrials.gov Identifier: NCT02027116). The second trial that will take place will be funded by NCI Division of Cancer Prevention as Dr. Jeff Jacobson and his Clinical Trial Unit has been added as an approved partner to the Phase 0/I/II Cancer Prevention Clinical Trials Program (Consortia for Early Phase Prevention Trials).

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:

We anticipate that we will be recruiting high risk individuals in Philadelphia and elsewhere for enrollment into the phase 1 safety trial of the HCV DNA vaccine in the trial funded by NCI Division of Cancer Prevention (Dr. Jeff Jacobson and his Clinical Trial Unit has been added as an approved partner to the Phase 0/I/II Cancer Prevention Clinical Trials Program (Consortia for Early Phase Prevention Trials).

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

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

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

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

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

Project Goals, Objectives and Aims as Presented in the Strategic Plan

Purpose

The main objective of this project is to test a next generation DNA vaccine strategy for the prevention of hepatitis C virus-associated cancer using preclinical testing to demonstrate immunogenicity and toxicology/safety studies leading to development of a strong platform for clinical testing. In addition, this research consortium will provide a structured mentoring program with students from a collaborating minority institution for the development of future research scientists in highly translational bench research.

Research Project Overview

Specific Aim #1 will test the hypothesis that *consensus* antigenic plasmids NS3/4A, NS4B, NS5A, and NS5B from diverse genotype 1a and 1b sequences will exhibit immunogenicity *in vivo* using small animal models (C57Bl6 and HLA transgenic mice). In addition, experiments will be carried out to optimize immunological assay conditions for HCV responses, including ELISpot, polyfunctional flow and CFSE T cell proliferation. Secondly, we will utilize a NHP model to test immunogenicity of the constructs in a larger animal model. The cross-reactivity of this approach will be explored. Finally, our collaborative group will work to determine toxicity, biodistribution and integration studies for HCV antigens using rabbits.

Specific Aim #2 consists of the *Education and Training Component* that will serve as a mechanism for providing education and training through hands-on research experiences and lecture workshops for faculty, undergraduate and graduate students at Cheyney and Drexel Universities. Throughout the course of the work and critical to the research will be the development and application of data generated throughout the project. There are two primary goals of the application with regard to education and training: (1) to encourage undergraduate and graduate students to pursue careers in biomedical research and (2) to provide a framework for faculty and students to collaborate on research related to the development, engineering, immunogenicity, and protective effects of the DNA vaccine platform.

To achieve this goal, the *Education and Training Component* will offer a 10-week summer internship program at Drexel University for Cheyney University students. By working closely with the faculty in the different components of the project, the students will be provided with valuable research experiences. Students will gain knowledge and skills on how to formulate research questions, develop a scientific methodology, analyze data and present research findings at a conference or through publications. As part of the summer internship program, students will conduct research projects under the supervision and mentorship of program faculty.

Expected Research Outcomes and Benefits

With more than 170 million individuals currently infected, hepatitis C virus (HCV) infection is a global pandemic, affecting approximately 3% of the entire world's population. In the United States chronic hepatitis C infection accounts for approximately one-third of all cases of hepatocellular carcinoma. While many important advances have been made in regard to immunotherapies for HCV, the field continues to be hindered by a general paucity of knowledge concerning how the virus not only interacts with the host immune system but more specifically what type of immune responses are critical for control and clearance of the virus. Understanding the immune correlates of protection against the virus is critical for designing vaccine strategies to combat infection. The role of antibodies in HCV infection is still not completely understood, with research being further compromised due to a lack of a widely accessible method of culturing HCV *in vitro*. Additionally, without a small animal model of HCV infection, elucidation of the specific cellular immune responses responsible for control of the virus has been slow, with the majority of what is known about the immune correlates of protection being gathered from chronically infected individuals. While chimpanzee models of infection have been instrumental in understanding the immune responses early in infection, unlike humans, these animals develop only mild clinical sequelae. Further complicating the situation is the mounting evidence supporting the idea that HCV viral proteins are able to modulate immune responses,

which may in part explain the propensity of the virus to persist. Therefore, success in the development of novel immunotherapies to combat HCV is of the utmost importance. Ultimately, by improving the treatment of HCV infection, we would also have an impact on reducing the incidence of HCV-related hepatocellular carcinoma, a difficult-to-treat cancer with a poor prognosis**.**

Summary of Research Completed Over the Entire Grant Term

SPECIFIC AIM 1

State Fiscal Year	Milestones for Specific Aim #1
6/1/10 - 6/30/11	Obtain recombinant DNA biosafety and animal protocol approval. Submission will occur prior to start of grant budget year. Manufacturing DNA constructs. Begin small animal dosing and immunogenicity studies in C56Bl6 parental strains.
7/1/11 - 6/30/12	Preclinical small animal studies, batch testing, immunogenicity of combined antigenic constructs, Coordinate technology training with Cheyney
7/1/12 - 6/30/13	Begin toxicology, biodistribution, integration study, Purchase NHP, begin NHP study. Use of HLA trangenics and parental strains to measure breadth of epitopes.
7/1/13 - 5/31/14	Complete toxicology, biodistribution, integration studies. Finish NHP study. Data collection and analysis, coordinate technology training with Cheyney

Our research team achieved DNA biosafety and animal protocol approval in the first month of the grant period. Our research team manufactured DNA constructs and began animal studies including dosing and immunogenicity as planned and achieved milestones. These data are shown in studies 1 through 12 below in the small animal (mouse strains) as planned. Summary of these studies are described here. The Nonhuman Primate (NHP) studies were carried out and milestones achieved. The experiments that describe outcomes from these milestones (NHP studies) are shown in Study 13-18 below. With regard to the milestone to begin toxicology, biodistribution, and integration studies, our collaborative research group completed these studies and have begun to enroll in a clinical study 1: PI; Sang Hoon Ahn, M.D, Ph.D. - Yonsei University Severance Hospital and Jeong Heo, M.D, Ph.D. - Pusan National University Hospital; Recruiting: Phase I Trial to Evaluate the Safety, Tolerability and Immunogenicity of VGX-6150 for Second-line Therapy of Chronic Hepatitis C Infection (VGX-6150-01).

Demonstration of immunogenicity of NS3/4A, NS4B and NS5A plasmids in a mouse model
Immunization induced potent NS3/4A-, NS4B- and NS5A-specific cellular immune responses in the periphery

Study #1. Immunization with the NS3/4A plasmid induced HCV-specific IFN-γ^+ and CD107$^+$ cytotoxic T cells in the periphery.

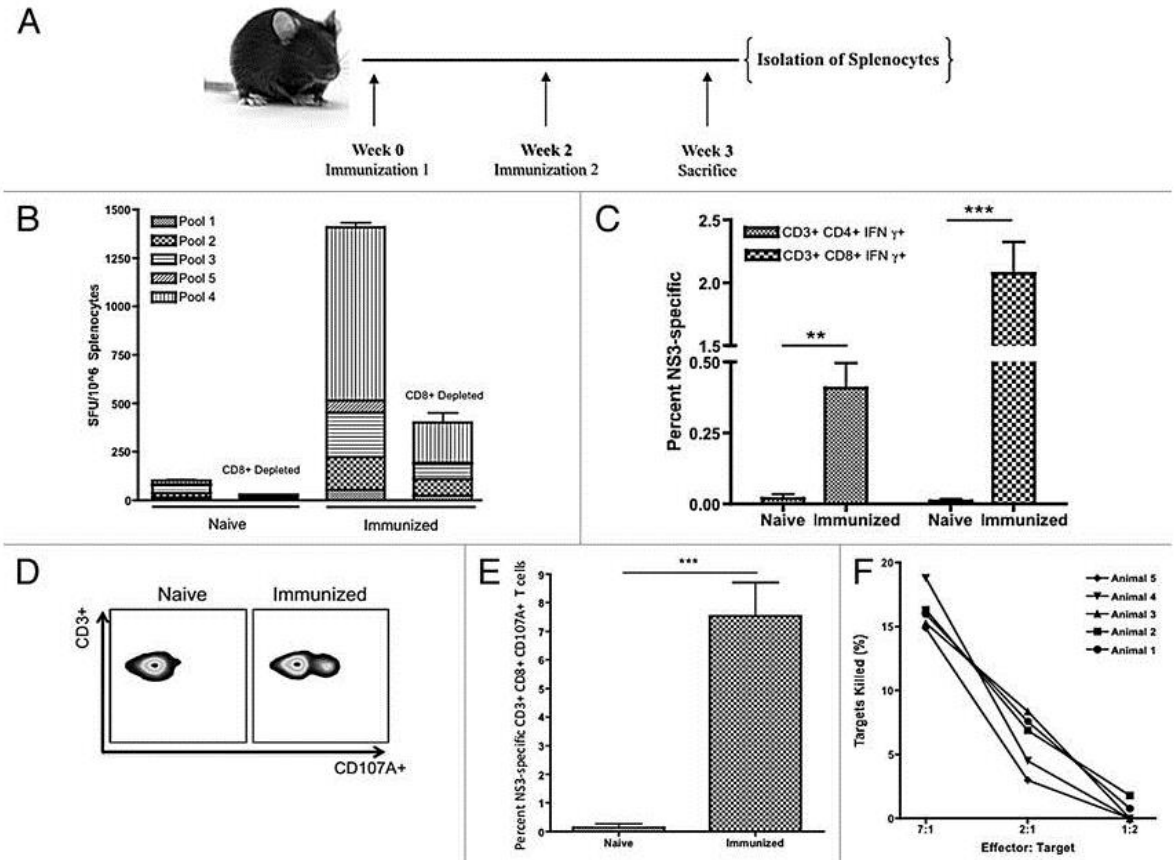
Given that both cytotoxic and IFN-γ+ HCV-specific T cells have been correlated with clearance of acute infection, we focused primarily on these responses when evaluating the immunogenicity

of the NS3/4A plasmid. Female 8-week-old C57BL/6 mice were divided into two groups: the native group and immunized group. The mice in the immunized group received 12.5 µg of the NS3/4A DNA by intramuscular injection (IM) into the quadriceps followed by electroporation (EP) using the CELLECTRA® adaptive constant current device (Inovio Pharmaceuticals Inc.). The device was configured to deliver two 0.1 Amp pulses of 52ms pulse width spaced apart by a 1 second delay. The mice received 2 immunizations, 2 weeks apart. One week after the last immunization, mice were sacrificed and the spleens were harvested (**Figure 1A**).

The cellular immune responses induced by the NS3/4A plasmid were first measured by IFN-γ ELISpot. Briefly, Splenocytes were stimulated with five pools of 15 mer peptides over lapping by 8 amino acids and spanning the length of consensus NS3/NS4A. A total of 101 HCV consensus NS3/NS4A peptides were synthesized, resuspended in DMSO and pooled into five different pools at a concentration of 2µg/ml/peptide. 200,000 splenocytes were plated in a 96 well IFN-γ capture antibody (R&D Systems) coated plate and stimulated overnight in the presence of a specific peptide pool at 37˚C in 5% CO_2. Cells were washed and plates were incubated overnight with biotinylated anti-mouse IFN-γ detection antibody (R&D Systems). Streptavidin-alkaline phosphatase and 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt and nitro blue tetrazolium chloride were subsequently used to develop spots. Spots were counted using an automated ELISPOT reader (CTL Limited). Results were adjusted and graphed as the

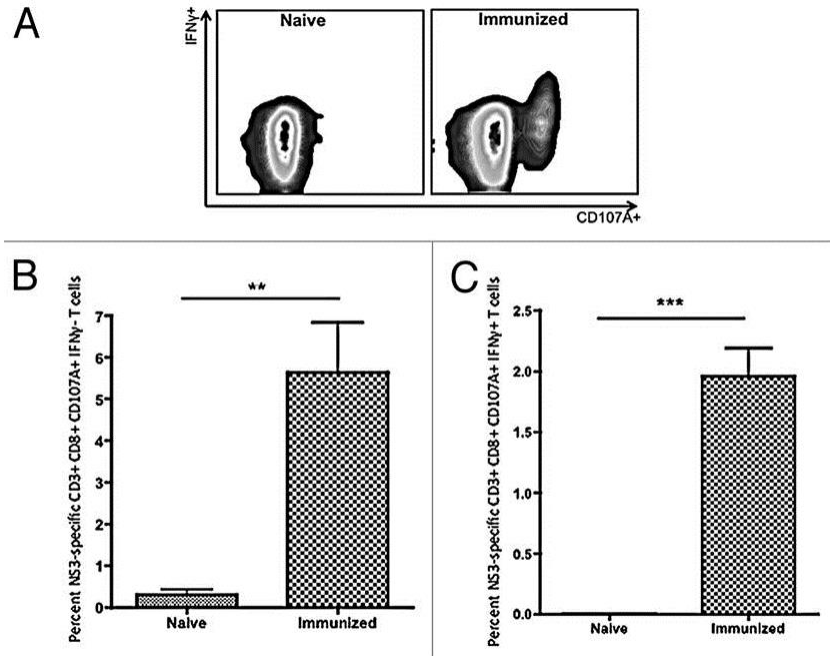
average number of spot forming units (SFU) per 1×10^6 splenocytes. As shown in **Figure 1B**, HCV NS3-specific IFN-γ T cell responses averaged approximately 1400 SFU per million splenocytes. Moreover, in order to determine whether CD8+ T-cells were responsible for the IFN-γ secretion detected in the immunized mice, CD8 lymphocytes were depleted from splenocytes using Dynabeads Mouse CD8 (Lyt2) (Life Technologies), and IFN-γ ELISpot was performed as described above after $CD8^+$ depletion. The results indicated that CD8+ T cells accounting for approximately 70 percent of the response (**Figure 1B**), supporting that the vaccine-induced IFN-γ production is mediated mainly by CD8+ T-cells.
Intracellular cytokine staining (ICS) assays were performed to further characterize the responses induced by the NS3/4A plasmid. Briefly, splenocytes were stimulated with 100 µl of either: (1) 2 µg/ml pConNS3/NS4A pooled peptides (2) 1 µg/ml Staphylococcus enterotoxin B (positive

Figure 1: Immunization with the NS3/4A plasmid induced HCV NS3-specific IFN-γ+ and $CD107A^+$ cytotoxic T-cell responses in the periphery. Immunization with the NS3/4A plasmid induced HCV NS3-specific IFN-γ+ and $CD107A^+$ cytotoxic T-cell responses in the periphery. (A) Immunization scheme, n = 5. (B) IFN-γ ELISpot. HCV NS3-specific responses were reported as the average number of spot forming units (SFU) per million splenocytes. (C) Average percent HCV NS3-specific $CD4^+$ and $CD8^+$ IFN-γ^+ T cells. (D) Representative CD107A staining. (E) Average percent HCV NS3-specific CD107A expression for each group. (F) Percent HCV NS3-specific granzyme B mediated killing as normalized to the number of NS3-specific effector CD8+ T cells. Significance was determined by Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.0005; NS, not significant).



control; Sigma-Aldrich) or (3) 0.1% dimethyl sulfoxide (negative control) all diluted in complete media supplemented with GolgiStop, GolgiPlug (BD Bioscience) and anti-CD107A. After 5 h of stimulation at 37°C, splenocytes were washed and stained for viability, then stained extracellularly for the surface markers; anti-CD4, CD8, CCR5 and CXCR3 for 30 min at 4°C. Cells were then permeabilized, washed and stained intracellularly with anti-IFNγ and CD3 for 45 min at 4°C. Specific function was reported as the percent function of the peptide stimulated group minus the percent function of the 0.1% dimethyl sulfoxide stimulated group (negative control) for each animal. As indicated in **Figure 1C**, the percentage of CD4+ IFN-γ+ and CD8+ IFN-γ+ T cells was 0.4% ± 0.09% and 2.1% ± 0.25%, respectively. In addition, CD107A expression was used as a phenotypic marker for HCV NS3-specific CD8+ cytotoxic T cells. Immunized mice expressed a large percentage of HCV NS3-specific CD107A with an average of 7.5% ±1.25% of CD8+ T cells staining positive (**Figure 1D and Figure 1E**). To confirm NS3-specific cytoxicity, a functional flow cytometric-based assay was employed to measure the amount of active granzyme B delivered to target cells. Target cells were pulsed with HCV NS3 antigens and co-incubated with effector cells that had been stimulated for 12 hours with HCV NS3 peptides. A dose dependent decrease in the percentage of targets killed was observed with an average of 16 percent HCV NS3-specific killing seen at the maximum effector to target ratio of 7:1 (**Figure 1F**).

Figure 2: Flow cytometric analysis of HCV NS3-specific $CD8^+$ T cells for CD107A and IFN-γ expression in the periphery. (A) Representative HCV NS3-specific $CD8^+$ T cell staining for CD107A and IFNγ. (B) Average percentage of HCV NS3-specific $CD8^+CD107A^+$ $IFN\gamma^-$ T cells. (C) Average percentage of HCV NS3-specific $CD8^+CD107A^+$ $IFN\gamma^+$ T cells. Significance was determined by Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.0005; NS, not significant). n = 5.



Further analysis revealed that all HCV NS3-specific CD8+ T cells stained positive for CD107A. However, when analyzed for IFN-γ expression, HCV NS3-specific CD8+ T cells fell into one of two major populations, either CD8+ CD107A+ IFN-γ- or CD8+ CD107A+ IFN-γ+ (**Figure 2A**). The percentage of HCV NS3-specific CD8+ CD107A+ IFN-γ- and CD8+ CD107A+ IFN-γ+ T cells averaged 5.6% ± 1.2% and 2.0% ± 0.23%, respectively (**Figure 2B and 2C**).

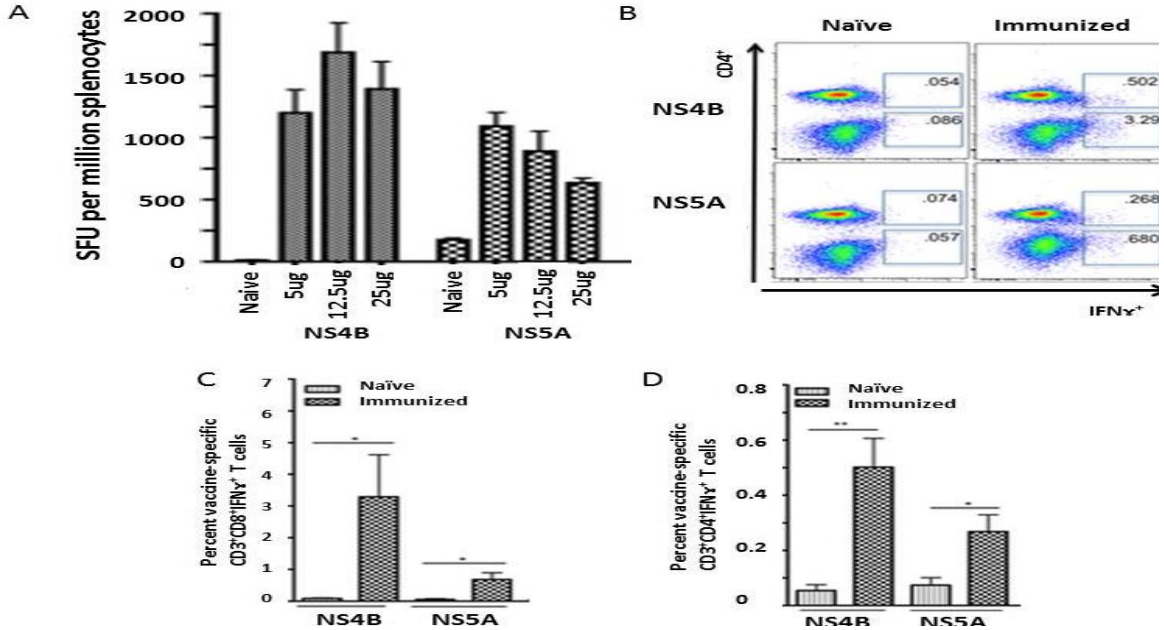
Study #2. Immunization with the NS4B and NS5A plasmids induced HCV-specific $CD8^+$ and $CD4^+$ T cells in the periphery.

The cellular immunogenicity of NS4B and NS5A constructs was determined by IFN-γ ELISpot assay. Animals were separated into three dosing groups for each construct with five animals per group. The animals were immunized intramuscularly with 5 µg, 12.5 µg or 25 µg of NS4B or NS5A plasmid, respectively, followed by electroporation as described above. The animals received two immunizations, two weeks apart and were sacrificed one week following the second immunization. Two sets of 15 mer peptides overlapping by 8 amino acids spanning the entire length of either NS4B or NS5A were utilized to stimulate the splenocytes, and the IFN-γ ELISpot assay was performed as described above. The results showed both NS4B and NS5A plasmids were strongly immunogenic (**Figure 3A**). The optimum dose for the NS4B plasmid vaccine was 12.5 µg (1687 $\pm$ 237 SFU/10^6 splenocytes), and the optimum dose for the NS5A plasmid vaccine was 5 µg (1091 $\pm$ 111 SFU/106 splenocytes), although the differences between doses were small.

Once the dosing was determined, a more detailed analysis of the cellular immune responses was performed. Dosing for the plasmids in all subsequent experiments was as follows: NS4B, 12.5 µg and NS5A, 5 µg. In order to determine the relative contributions of CD8+ and CD4+ T cell responses for each construct, splenocytes were intracellularly stained for IFN- γ and visualized with flow cytometry (**Figure 3B)**. The results of intracellular cytokine staining correlated well with results of the IFN-γ ELISpot assays. The majority of the IFN-γ responses to NS4B and NS5A were produced by CD8+ T cells, although CD4+ T cells specific for each plasmid were also identified. The average percentage of IFN-γ+ CD8+ T cells for the NS4B and NS5A plasmids were 3.29% ± 1.33% and 0.68% ± 0.22%, respectively (**Figure 3C)**. The average percentage of IFN-γ+ CD4+ T cells for NS4B and NS5A were 0.50% ± 0.11% and 0.27% ± 0.06%, respectively (**Figure 3D**).

Figure 3: Immunization with the NS4B and NS5A plasmids induced HCV-specific $CD8^+$ and $CD4^+$ T cells in the periphery. Animals (n = 5 per group) were immunized with 5 µg, 12.5 µg or 25 µg of the NS4B and NS5A plasmids, respectively. Animals received a total of two intramuscular immunizations, two weeks apart followed by electroporation. Animals were sacrificed one week following the last immunization after which splenocytes were isolated and analyzed. The response of each animal to each dose was determined with IFN-γ ELISpot assays. Splenocytes were isolated and individually analyzed for NS4B- or NS5A-specific T cell responses. (B, C, D) Splenocytes were intracellularly stained for IFN-γ and analyzed with flow cytometry. (C) Representative animal from each group. The values shown are the averaged response of five individual animals from each group. (D) and (E) graphical representation of percent HCV-specific IFN-γ+ T cell responses from isolated splenocytes. Values are reported as the average percent $\pm$ SE of the (D) $CD8^+$ IFN-γ+ or (E) $CD4^+$ IFN-γ^+ T cell responses of each animal (n = 5) from each group. Significance was determined by Student's t test (*$p<0.05$, **$p<0.005$ and ***$p<0.0005$).

Figure 3. Immunogenicity of the NS4B and NS5A plasmids in the periphery. (A) IFN-γ ELISpot dose response.



Immunization induced potent NS3/4A-, NS4B- and NS5A-specific T cell-based intrahepatic immunity

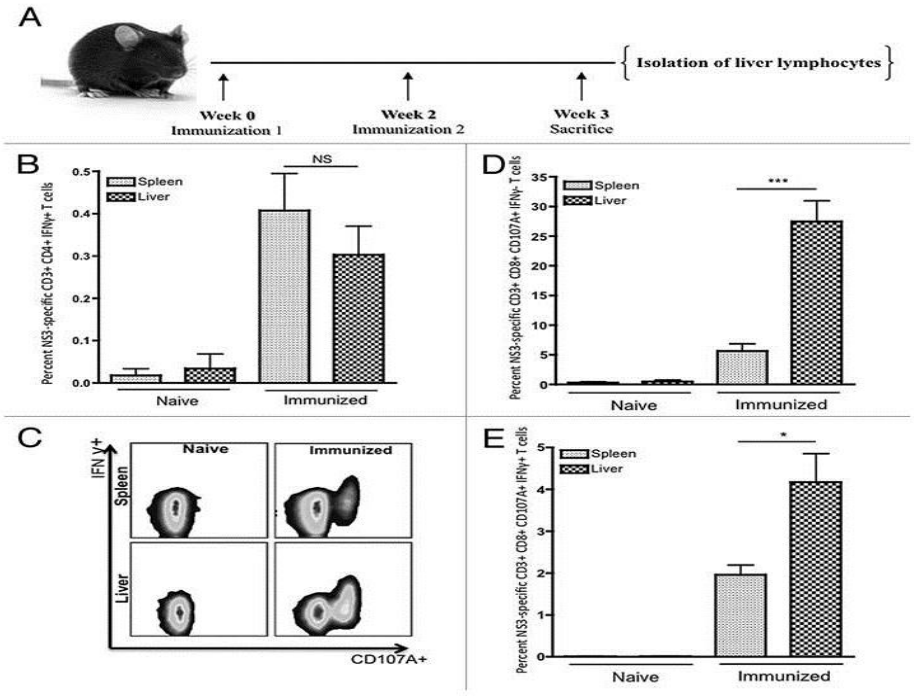
Given the importance of intrahepatic T cell responses in the clearance of HCV infection, once we had determined that our plasmid vaccines were able to induce strong T cell responses in the periphery, we wanted to determine whether these responses could be detected within the liver. Specifically, we were interested in determining whether HCV-specific T cells induced through peripheral immunization could migrate into and remain functional within the immunotolerant liver.

Study #3. The vaccine-induced HCV NS3-specific CD8+ T cells were enriched in the liver as compared with the periphery.

In order to detect the vaccine-induced NS3-specific CD8 and CD4 T cells in the liver, the liver lymphocytes were isolated from either naïve group or immunized group (n=5) and flow cytometric analysis was performed (**Figure 4A)**. Briefly, each liver was perfused with PBS and individually pulverized using a Stomacher machine (Seward). The resulting mixture was pelleted and resuspended in 10 ml ACK lysis buffer (Bioscience) for 5 min to clear the RBCs. The reaction was stopped with 10 ml R10 and pelleted. The pellet was resuspended in 10 ml of room temperature 40% isotonic percoll and centrifuged 1,200 rpm for 10 min in order to pellet the lymphocytes. The pelleted lymphocytes were resuspended in complete R10 media for the flow analysis.

The results showed that both HCV NS3-specific CD4+ and CD8+ IFN-γ+ T cells could be detected within the liver. Out of the CD4+ T cells isolated from the liver an average of 0.26% ± 0.09% expressed IFN-γ (**Figure 4B**). Likewise, both populations of HCV NS3-specific CD8+ T cells identified previously in the spleen were readily detected within the liver (**Figure 4C**). The percentage of HCV NS3-specific CD8+ CD107A+ IFN-γ- and CD8+ CD107A+ IFN-γ+ T cells within the resting liver were 27.5% ± 3.5% and 4.2% ± 0.68%, respectively (**Figure 4D and 4E**). Both populations of HCV NS3-specific CD8+ T cells were significantly enriched within the liver. Percentages of HCV NS3-specific CD8+ CD107A+ IFN-γ- and CD8+ CD107A+ IFN-γ+ T cells were approximately 5 and 2 fold higher in the liver as compared to the spleen, respectively.

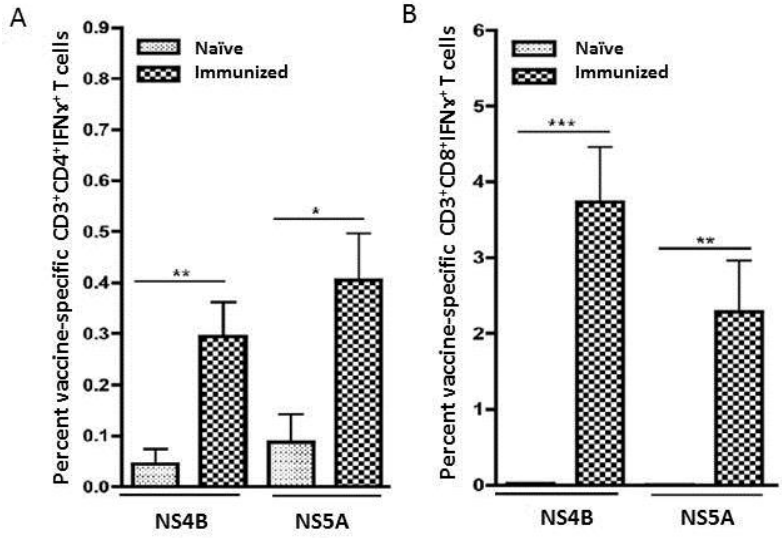
Figure 4: Flow cytometric analysis of HCV NS3-specific T-cell responses within the liver as compared with the spleen. (A) Immunization scheme, n = 5. (B) Average percent HCV NS3-specific CD4+IFNγ+ T cells. (C) Representative CD107A and IFNγ staining for HCV NS3-specific CD8+ T cells. (D) Average percentage of HCV NS3-specific $CD8^{+}CD107A^{+}$ $IFN\gamma^{-}$ T cells. (E) Average percentage HCV NS3-specific $CD8^{+}CD107A^{+}$ $IFN\gamma^{+}$ T cells. Significance was determined by Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.0005; NS, not significant).



Study #4. Vaccination with the NS4B and NS5A plasmids induced potent NS4B- and NS5A-specfic $CD4^+$ and $CD8^+$ T cells in the liver.

Mice were immunized as described in Study#2. Liver lymphocytes were isolated from each animal either in the naïve group or immunized groups and HCV-specific T cells were identified by IFN-γ expression and flow cytometry. Briefly, livers were individually pulverized using a Stomacher machine, strained and treated 5 min with 10 ml ACK lysis buffer (Bioscience). The mixture was pelleted and the hepatocytes were separated from the lymphocytes through the use of a 35% percoll (GE Healthcare) gradient. The pelleted lymphocytes were resuspended in complete media. Interestingly, HCV-specific T cells were identified in the livers of all immunized mice (**Figure 5)**. Strong $CD8^+$ T cell responses were detected within the livers of mice immunized with the NS4B or NS5A plasmid. The $CD4^+$ T cell responses for NS4B and NS5A were 0.29% $\pm$ 0.07% and 0.41% $\pm$ 0.09%, respectively (**Figure 5A)**. The $CD8^+$ T cell responses for NS4B and NS5A were 3.73% $\pm$ 0.73% and 2.28% $\pm$ 0.68%, respectively (**Figure 5B)**.

Figure 5. Flow cytometric analysis of the percentage of HCV-specific IFN-γ^+ T cell responses from isolated liver lymphocytes. Values are reported as the average percent $\pm$ SE of HCV-specific (A) $CD4^+$ IFN-γ^+ or (B) $CD8^+$ IFN- γ^+ T cell responses of each animal (n = 5) from each group. Significance was determined by Student's t test (*p<0.05, **p<0.005 and ***p<0.0005).



Liver expression of HCV antigens resulted in the activation of intrahepatic HCV-specific T cells and clearance of transfected hepatocytes.

While it had been shown that peripheral immunization could induce functional HCV-specific T cell based immunity within the resting liver, an effective vaccine strategy must also be able to induce HCV-specific T cells with the ability to become activated and remain functional following HCV antigen expression within the liver. However, observations of the apoptotic fate of selectively recruited T cells to the liver has led to theories that the liver may function as either

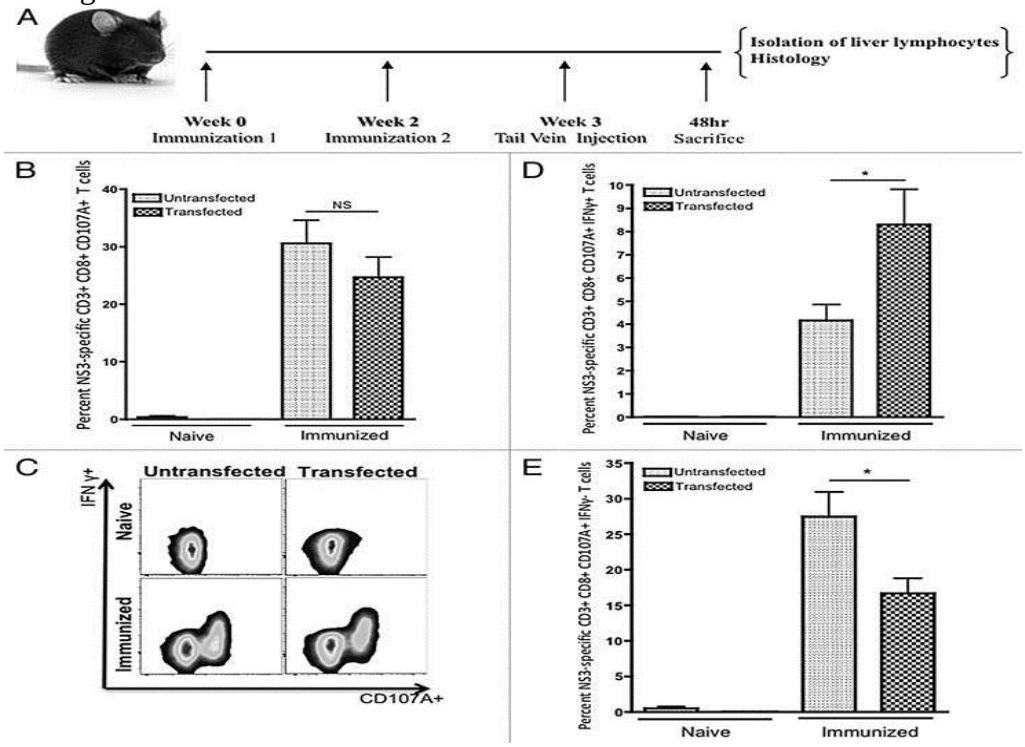
a ‘graveyard’ or ‘killing field’ for activated CD8+ T cells, which is thought to be one possible mechanism by which the liver may be able to induce tolerance.

Study #5. Liver expression of HCV NS3 resulted in the activation of intrahepatic HCV NS3-specific $CD8^+$ T cells.

Given these observations, first we were curious as to the fate of the vaccine-induced NS3-specific CD8+ T cells following activation by expression of their cognate antigen within the liver. In order to test this, we took advantage of a previously reported liver transfection method where tail vein injection of naked DNA was used to selectively transfect hepatocytes resulting in foreign protein expression in the liver. Briefly, in order to induce liver expression of HCV NS3 plasmid, animals in native or immunized group (n=5) each received a tail vein injection of 100 μg of the NS3/4A plasmid diluted in 300 μl PBS and were sacrificed 48 h following injection (**Figure 6A**). When the percentages of intrahepatic HCV NS3-specific $CD8^+$ $CD107A^+$ T cells were compared before and after liver transfection, no significant difference was seen. This finding suggested that the population of HCV NS3-specific CD8+ T cells observed prior to injection was the same observed following transfection (**Figure 6B**). Intrahepatic lymphocytes were then analyzed for expression of both IFNγ and CD107A. A large shift toward a gain of function was observed in the population of HCV NS3-specific $CD8^+$ T cells isolated from immunized animals following liver transfection (**Figure 6C**). An approximately 2-fold increase in the percentage of HCV NS3-specific $CD8^+CD107A^+$ IFNγ+ T cells was observed which coincided with an approximately 2-fold decrease of HCV NS3-specific $CD8^+CD107A^+IFN\gamma^-$ T cells. The percentages of HCV NS3-specific $CD8^+CD107A^+IFN\gamma^+$ and $CD8^+CD107A^+IFN\gamma^-$ were 7.8% ± 1.5% and 16.7% ± 2.1%, respectively (**Figure 6D and 6E**). Therefore, liver expression of HCV NS3 antigen resulted in activation of intrahepatic HCV NS3-specific CD8+ T cells as evidenced by the enhanced expression of $IFN\gamma^+$.

Figure 6. Flow cytometric analysis of intrahepatic HCV NS3-specific T-cell responses following HCV NS3 transfection within the liver. (A) Immunization and injection scheme, n = 5. Animals were given a tail vein injection of 100 μg of NS3/4A. After 48 h, animals were sacrificed and liver lymphocytes were individually analyzed. (B) Average percentage of HCV NS3-specific $CD8^+CD107A^+$ T cells detected within the untransfected vs. the transfected liver. (C) Representative CD107A and IFNγ staining of HCV NS3-specific $CD8^+$ T cells before and after liver transfection. (D) Average percentage of HCV NS3-specific $CD8^+CD107A^+IFN\gamma^+$ T cells detected within the untransfected vs. the transfected liver. (E) Average percentage of HCV NS3-specific $CD8^+CD107A^+IFN\gamma^-$ T cells detected within the untransfected vs. the transfected liver. Significance was determined by Student’s t-test (*$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$; NS, not significant).

Figure 6. Flow cytometric analysis of intrahepatic HCV NS3-specific T-cell responses following HCV NS3 transfection within the liver.



Study #6. Liver expression of HCV NS4B and NS5A resulted in the activation of intrahepatic HCV NS4B- and NS5A-specific $CD8^+$ T cells.

Next, we sought to determine whether liver-specific expression of either NS4B or NS5A proteins could also activate the vaccine-induced HCV NS4B- or NS5A-specific T cells detected within the liver. In order to induce liver expression of NS4B or NS5A, the hepatocytes of native or immunized mice were transfected by administering a tail vein injection with 100 µg of NS4B or NS5A plasmids diluted in 300 µl of PBS. The livers were allowed to transfect for 48 hours, after which they were harvested and the liver lymphocytes were isolated. The splenocytes were also isolated for flow analysis. The vaccine-induced HCV-specific T cells were identified by IFN-γ secretion detected through intracellular cytokine staining and flow cytometry. Following the tail vein injections, a massive increase in the percentage of both $CD4^+$ and $CD8^+$ HCV-specific T cells was seen in all immunized groups as compared to both the spleen and the untransfected liver (**Figure 7A)**. The percentage of $CD4^+$ HCV-specific T cells was 2.27% $\pm$ 0.70% and 2.55% $\pm$ 0.70% for mice immunized with the NS4B and NS5A plasmids, respectively **(Figure 7B and 7C)**. The percentage of $CD8^+$ HCV-specific T cells was 9.46% $\pm$ 1.53% and 6.98% $\pm$ 0.48% for mice immunized with the NS4B and NS5A plasmids, respectively **(Figure 7D and 7E)**. The largest fold increase, as determined by the percentage of HCV-specific IFN-γ^+ T cells in the liver before and after tail vein injection, was seen with the $CD4^+$ T cell response. The fold increase in the intrahepatic $CD4^+$ T cell response in NS4B and NS5A immunized mice was approximately 8 and 6 fold, respectively. While the $CD8^+$ T cell response remained the

dominant response in the liver both before and after tail vein injection, a slightly smaller fold increase was seen with the $CD8^+$ T cells response as compared to the $CD4^+$ T cells response. The fold increase in the intrahepatic CD8+ T cell response in both NS4B and NS5A immunized mice was approximately 3 fold.

Vaccine-induced HCV NS3-, NS4B- or NS5A-specific cytotoxic T cells rapidly clear HCV NS3, NS4B or NS5A-expressing hepatocytes.

Study #7. Vaccine-induced intrahepatic HCV NS3-specific cytotoxic T-cell responses within the liver clear HCV NS3-transfected hepatocytes.

Figure 7. Flow cytometric analysis of the percentage of HCV-specific IFN-γ^+ T cell responses from isolated lymphocytes from the spleen, untransfected liver and transfected liver. Lymphocytes from each animal (n = 5) were isolated and individually analyzed for NS4B- and NS5A-specific T cell responses. The isolated lymphocytes were intracellularly stained for IFN-γ and analyzed with flow cytometry. (A) Representative animal from each group. The values shown are the averaged response of five individual animals. (B) and (C) Graphical representation comparing the average percentage of HCV-specific $CD4^+$ IFN-γ^+ T cell responses in the liver before and after transfection. Values are reported as the average percent $\pm$ SE of $CD4^+$ IFN-γ^+ T cell responses to NS4B (B) or NS5A (C). (D) and (E) Graphical representation comparing the average percentage of HCV-specific $CD8^+$ IFN-γ^+ T cell responses in the liver before and after transfection. Values are reported as the average percent $\pm$ SE of $CD8^+$IFN-γ^+ T cell responses to NS4B (D) or NS5A (E). Significance was determined by Student's t test (*p<0.05, **p<0.005 and ***p<0.0005).

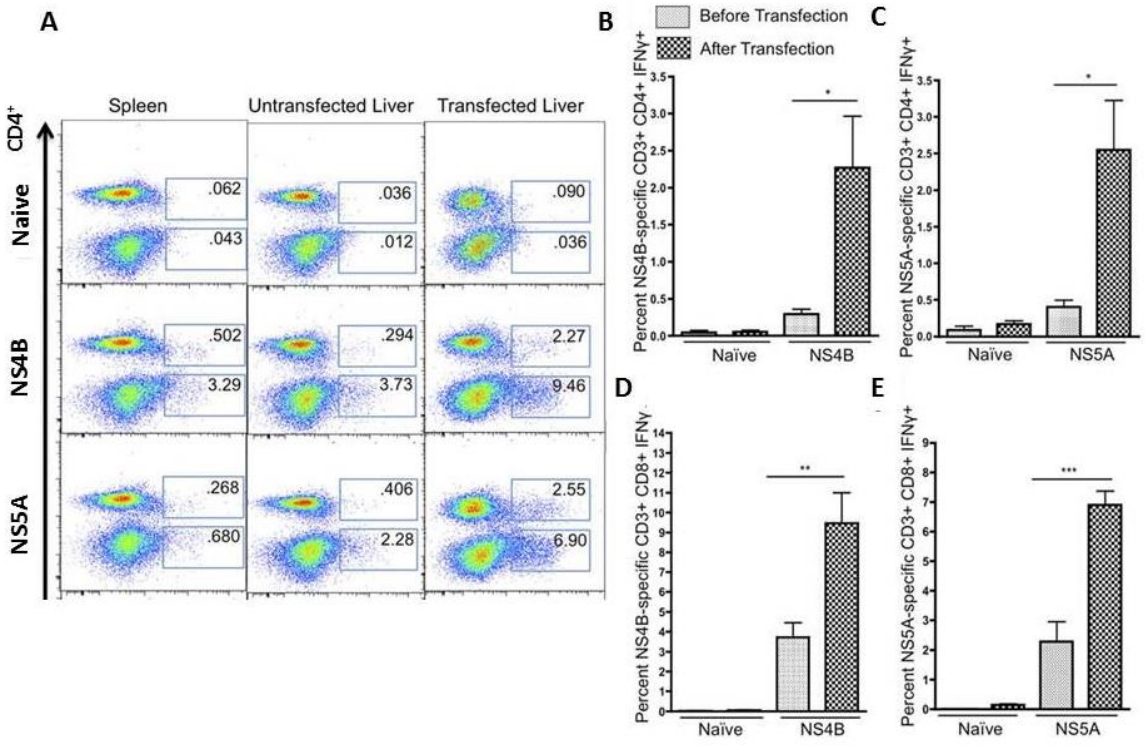
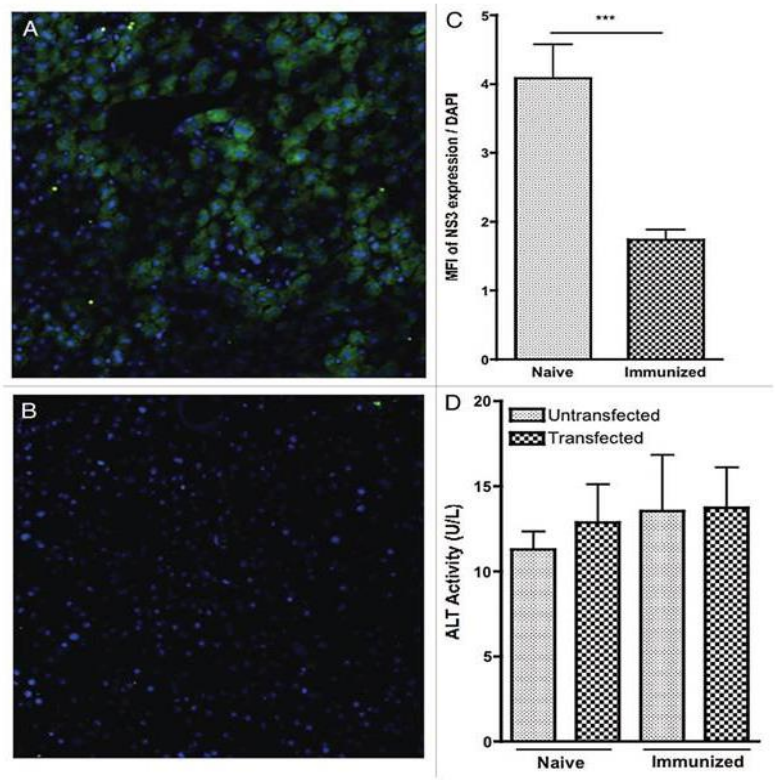


Figure 8. Intrahepatic HCV NS3-specific cytotoxic T-cell responses within the liver following HCV NS3 transfection. Intrahepatic HCV NS3-specific cytotoxic T-cell responses within the liver following HCV NS3 transfection. (A and B) Representative liver confocal images of hepatocyte expression of HCV NS3 in the (A) naïve group (B) immunized group. Expression of HCV NS3 was detected with an anti-HA antibody (green). Nuclei were stained with DAPI (blue). (C) Average MFI ratio of expression of HCV NS3 as normalized to DAPI. For each group, four images were captured for each animal (n = 4, one animal from each group was dropped due to insufficient sample quality). MFI values for HCV NS3 were calculated and normalized to the MFI value for DAPI for each image. (D) Average ALT activity before and after liver transfection of each group. Significance was determined by Student's t-test (*p < 0.05, **p < 0.005 and ***p < 0.0005).



First, we determined whether vaccine-induced response was able to clear HCV NS3 expressing hepatocytes. Briefly, mice were injected intravenously with 100 μg of NS3/4A plasmid in 2mL

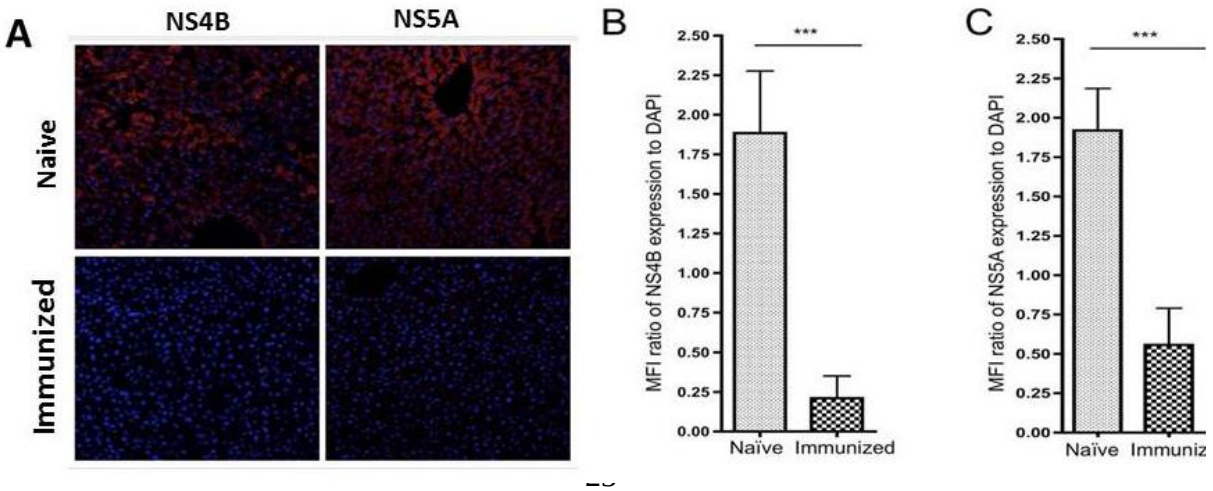
(about 10% volume of the mouse weight) of Ringers solution within a period of 7 seconds to transiently transfect the liver. A lobe of liver from each animal was stained with an anti-hemagglutinin (HA) monoclonal antibody for expression of NS3. Clearance was assessed by measuring the expression of NS3 in hepatocytes of immunized mice versus naive controls.

Representative confocal images for each group are shown in **Figure 8A and 8B**. Clearance of transfected hepatocytes for each group was quantified by the mean florescent intensity (MFI) of NS3 expression normalized to the number of cells present within each field as measured by the MFI of nuclear DAPI staining (**Figure 8C)**. Compared to the naïve controls, dramatic reductions in the number of NS3 expressing hepatocytes were seen in the immunized group. Additionally, this response was found to be highly specific and did not result in generalized liver damage as measured by serum alanine aminotransferase (ALT) activity (**Figure 8D)**.

Study #8. Vaccine-induced intrahepatic HCV NS4B- or NS5A-specific cytotoxic T-cell responses within the liver clear HCV NS4B- or NS5A-transfected hepatocytes.

Next, we determined whether vaccine-induced response was able to clear HCV NS4B or NS5A expressing hepatocytes. Briefly, mice were injected intravenously with 100 μg of NS4B or NS5A plasmid in 2mL (about 10% volume of the mouse weight) of Ringers solution within a period of 7 seconds to transiently transfect the liver. A lobe of liver from each animal was stained with an anti-hemagglutinin (HA) monoclonal antibody for expression of NS4B or NS5A. Clearance was assessed by measuring the expression of NS4B or NS5A in hepatocytes of immunized mice versus naive controls. Representative confocal images for each group are shown in **Figure 9A**. Clearance of transfected hepatocytes for each group was quantified by the mean florescent intensity (MFI) of NS4B or NS5A expression normalized to the number of cells

Figure 9. Clearance of NS4B or NS5A transfected hepatocytes. (A) Confocal microscopy. Representative confocal image of hepatocyte expression of NS4B or NS5A in each group. Expression of each construct was detected with an anti-HA antibody (red). Nuclei were stained with DAPI (blue). (B and C) Graph of MFI ratio of expression of NS4B (B) or NS5A (C) as normalized to DAPI. For each group, three images were captured for each animal (n = 5). MFI values for NS4B or NS5A (red) were calculated and normalized to the MFI value for DAPI (blue) for each image. The values shown are the averaged response ± SE of five individual animals from both the naive and immunized groups. Significance was determined by Student's t test (*p<0.05, **p<0.005 and ***p<0.0005).



present within each field as measured by the MFI of nuclear DAPI staining (**Figure 9B and 9C)**. Compared to the naive controls, dramatic reductions in the number of NS4B or NS5A expressing hepatocytes were seen in two immunized groups. Animals immunized with NS4B or NS5A had approximately 9 and 3 fold reductions in foreign antigen expression within the liver compared to naive controls. The amount of clearance observed in each immunization group correlated well with the HCV-specific CD8+ T cell response detected within the transfected livers.

Immunogenicity induced by co-immunization of NS3/4A, NS4B and NS5A plasmids in mice

Study #9. Co-immunization with the NS3/4A, NS4B and NS5A plasmids induced strong and broad HCV-specific T cell responses in the periphery and liver.

At present, the target of an HCV vaccine is to achieve immunological benchmarks observed in patients during acute resolution (AR) of HCV infection, namely, a rapid, strong, and broadly reactive T cell response against non-structural HCV gene products (Thimme R et al., Proc Natl Acad Sci U S A 2002 99:15661-8). Therefore, after demonstrating that vaccination with the NS3/4A, NS4B or NS5A plasmid alone elicited strong T cell responses in mice, we decided to combine all three plasmids into a single formulation to achieve the broadly-reactive T cell profile observed in AR patients. Female 8-week-old C57BL/6 mice were divided into two groups (n=4): the native group and immunized group. Animals received either 24 µg of pGX0001 (empty vector) or HCV (8ug, NS3/4A; 8ug, NS4B; 8ug, NS5A) I.M., immediately followed by E.P. (**Table 1**) as described earlier. Animals received a total of three immunizations, separated by three weeks. Seven days after the last immunization, mice were sacrificed and splenocytes and liver lymphocytes were isolated (**Figure 10A**).

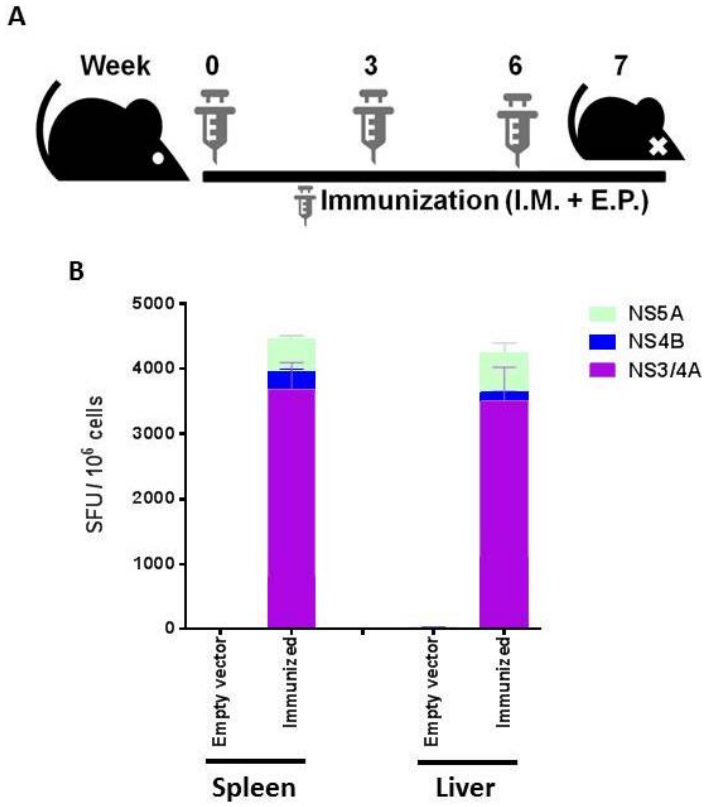
Table 1: DNA Plasmid Combination Experimental Groups

Mouse Group	Antigen	Dose
1	pGX0001(Empty vector)	24 µg
2	NS3/4A, NS4B, NS5A	8 µg, 8 µg, 8 µg

Immunogenicity of the HCV vaccine was first determined by IFNγ ELISpot assays as described earlier. While there was a dominant IFNγ response against the NS3/4A peptide pools in C57BL/6 mice, IFNγ secretion was detectable in response to all three plasmid vaccines in the spleen and liver, respectively. Broadly reactive responses across all antigens underscored the advantage of a multi-antigenic DNA vaccine strategy. At day 7 post 3rd immunization, the average antigen-specific IFNγ responses of the immunized animals totaled 4463.3 and 4243 SFU (spot-forming units)/per million cells by IFNγ ELISpot assay in the spleen and liver, respectively. Whereas IFNγ responses of the control group totaled 11.7 and 28.3 SFU/per million cells (**Figure 10B**) in the spleen and liver, respectively.

Figure 10. Co-immunization with the NS3/4A, NS4B and NS5A plasmids induced strong and broad HCV-specific T cell responses in the periphery and liver determined by ELISpot. (A) Immunization timeline schematic illustrating that mice were immunized intramuscularly, followed by *in vivo* electroporation (E.P.), three times, separated by three weeks. Immune responses were assayed 7 days following the final immunization (B) Stacked IFNγ ELISpot results from the spleen and liver depicting the magnitude of total responses, and the breadth of reactivity by HCV NS3/4A, NS4B and NS5A peptide pools.

Figure 10. Co-immunization with the NS3/4A, NS4B and NS5A plasmids induced strong and broad HCV-specific T cell responses in the periphery and liver determined by ELISpot.

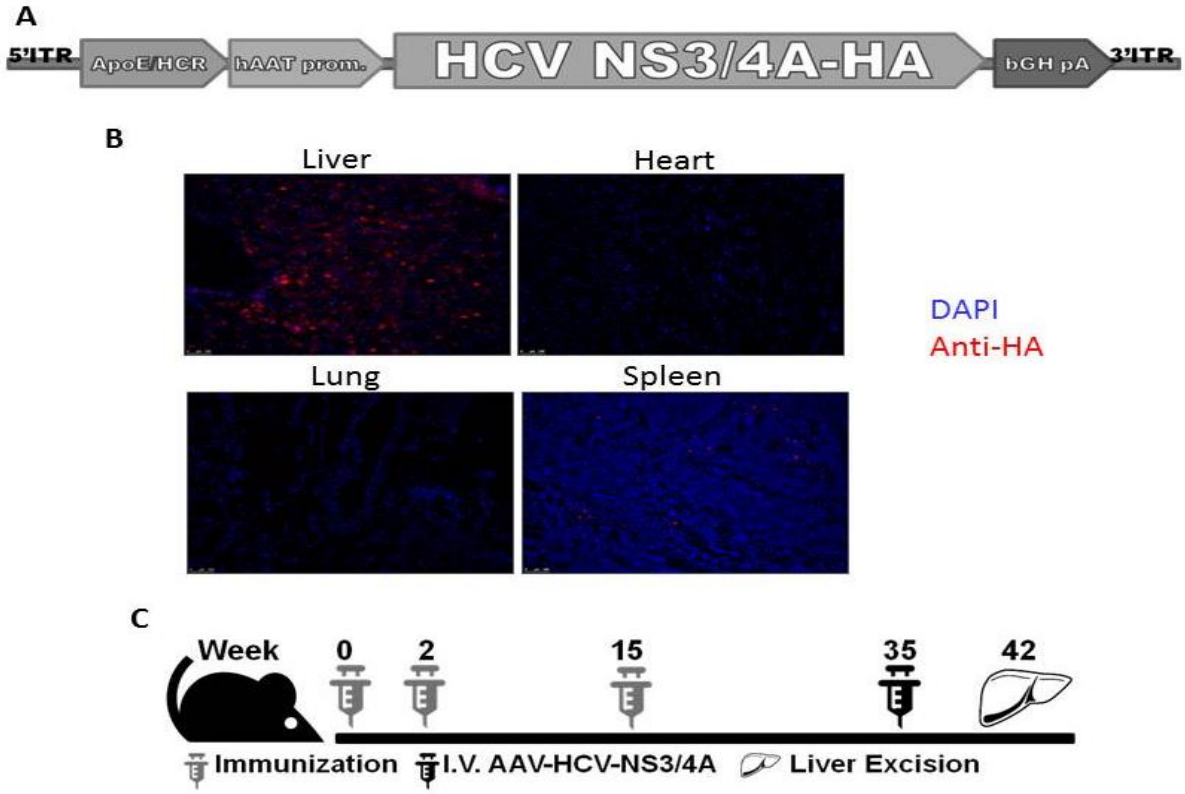


Rapid memory recall responses induced by co-immunization of NS3/4A, NS4B and NS5A plasmids cleared the HCV antigen-expressing hepatocytes

Study #10. *In heparo* generation of NS3/4A-expressing hepatocytes

After we demonstrated that vaccination with the combined HCV vaccine could elicit strong HCV-specific cellular immune responses, we aimed to further demonstrate T cell function in the presence of immunosuppressive regulatory mechanisms present in the liver. Due to the poor availability of affordable HCV animal models suitable for vaccine research, we opted to develop an uncomplicated and widely-available model to test T cell killing within the immunologically unique environment of the liver, which would also be applicable to fields of study beyond HCV. In order to generate HCV antigen-expressing hepatocytes, we chose a well-established gene therapy platform, which is poorly immunogenic, has been safely administered to animals and humans, and can chronically express transgene product in the liver. Animals were intravenously injected with a gene therapy adeno-associated viral vector (AAV) to deliver an HA-tagged NS3/4A expression cassette, under the control of the widely-used hepatocyte-specific promoter, human alpha-antitrypsin 1 (hAAT) promoter with upstream hepatocyte control region (HCR) to restrict antigen expression to only hepatocytes (**Figure 11A**). Tissue-specificity was tested in pGX0001 (empty vector)-immunized mice, I.V.-injected with $2x10^{11}$ vg AAV8-HCV-NS3/4A-HA in 300 µl PBS. Tissues were harvested 7 days following injection. Expression of HA-tagged NS3/4A antigen was detected by immunofluorescence staining for HA peptide of frozen tissue sections. As expected, robust antigen expression was observed in the liver, while antigen was completely undetectable in heart and lung tissue, and sparse HA^+ cells were rarely observed within the spleen (**Figure 11B**). With a means to generate target hepatocytes, we were able to measure HCV-specific memory T cell function by clearance of HCV antigen-expressing hepatocytes and concomitantly analyze HCV-specific memory T cell frequency and phenotype in mice immunized 20 weeks prior to AAV-HCV-NS3/4A-HA, which we refer to as an *in heparo* killing assay (**Figure 11C**).

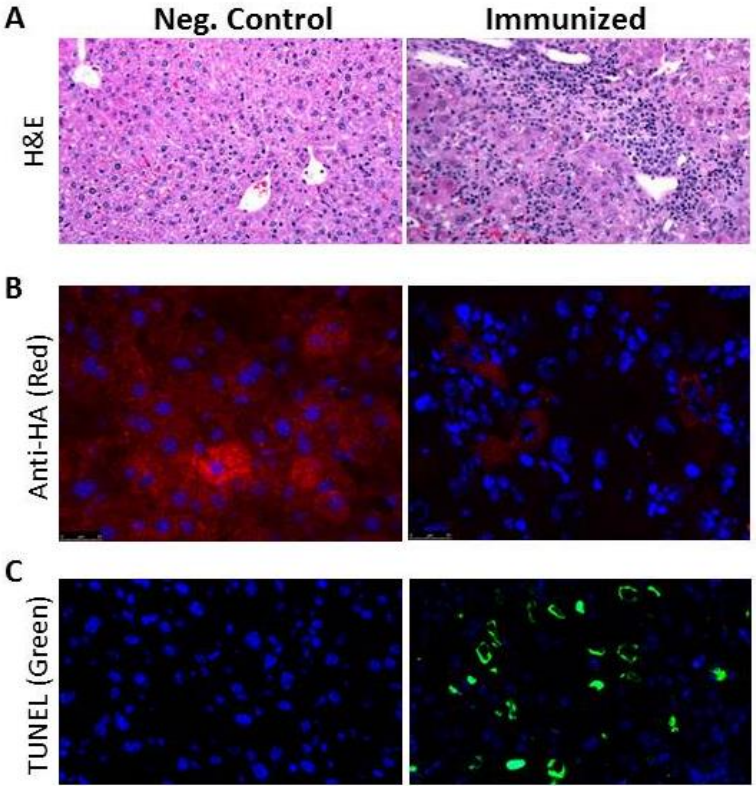
Figure 11. Generation of HCV antigen-expressing target hepatocytes (A) Schematic illustrating the AAV expression vector used to generate target hepatocytes. Antigen-matched HCV NS3/4A with C-terminal HA tag was expressed under the control of a hepatocyte specific promoter. (B) Robust liver-specific antigen expression. To confirm tissue-specificity, frozen sections of liver, spleen, lung, and heart were stained for HA-tagged protein expression and DAPI. (C) *In heparo* killing assay schedule. Thrice immunized mice were rested for 20 weeks before generation of target hepatocytes.



Study #11. Rapid memory recall responses cleared HCV antigen from the liver

To ensure complete contraction of HCV-specific terminal effector T cell populations, immunized animals were rested for 20 weeks before generation of HCV NS3/4A-expressing hepatocytes. By day 7 following AAV-HCV-NS3/4A-HA injection, dense perivascular and interstitial cellular infiltrates were apparent by H&E stained liver sections in HCV-immunized mice, whereas no appreciable changes were observed in control animals (**Figure 12A**). While control animals maintained robust expression of HCV NS3/4A-HA by IF staining of frozen liver sections, few antigen-positive hepatocytes remained in HCV-immunized animals **(Figure 12B)**. When antigen expression was measured by mean fluorescence intensity (MFI), HCV-immunized animals exhibited significantly less MFI than the control group (**Figure 12D**). Loss of HCV antigen in the liver coincided with a significant increase in serum ALT activity of 152 U/L [93-211; 95% CI | norm <40], which is a common clinical marker of liver damage (**Figure 12E**). Clearance of antigen-expressing hepatocytes also coincided with a robust IFNγ response, as measure by

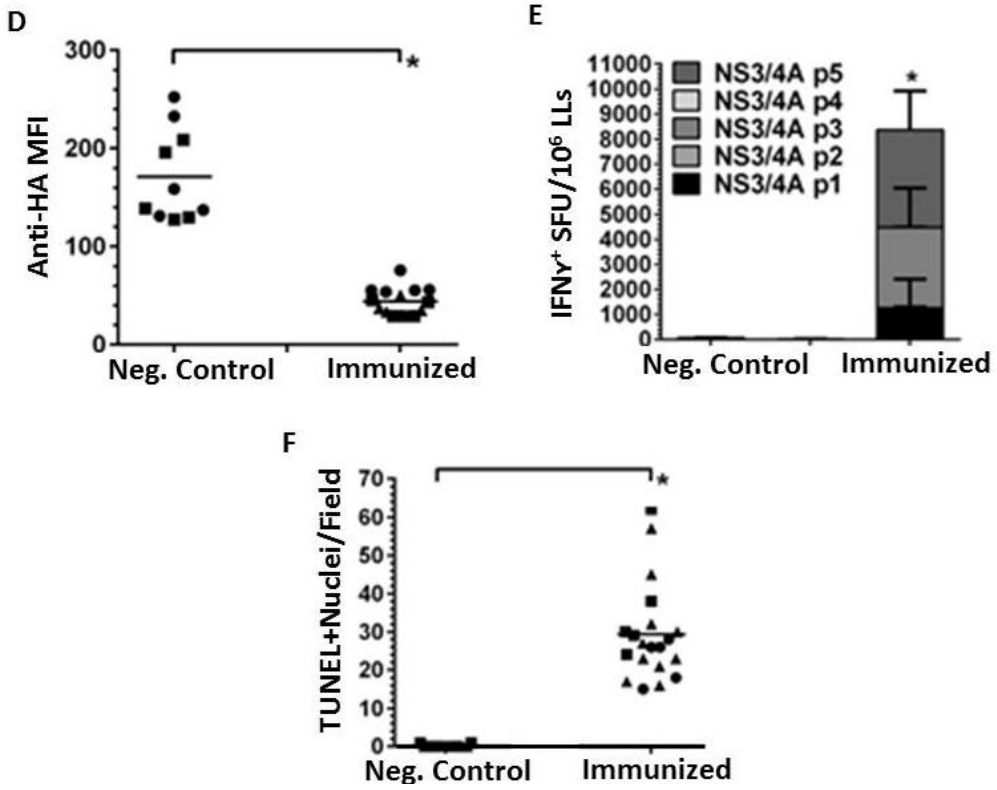
Figure 12 (A-C). HCV Antigen was cleared in the liver by day 7 following AAV-HCV-NS3/4A-HA injection. Mice (n=4), immunized and rested for 20 weeks, were administered $2x10^{11}$vg AAV-HCV-NS3/4A-HA. On day 7 post-AAV injection, perfused livers were excised to assess acute HCV-specific functional memory T cell responses. (A) Immunological consequences of HCV-specific T cell memory were observed by H&E. (B) Hepatocyte-specific expression of NS3/4A-HA was observed by IF staining for HA-tagged protein (red) and DAPI (blue). (C) Apoptotic hepatocytes were identified by TUNEL staining.



ELISpot, with broad reactivity across multiple NS3/4A peptide pools representing a significant total response of 9,450 [7,057-11,843; 95% CI] SFU (**Figure 12E**).

Finally, we aimed to investigate the cause of HCV-antigen clearance, to clarify if antigen-expressing hepatocytes were indeed, killed, or if antigen-expression was merely suppressed by the proinflammatory cytokine milieu. Frozen liver sections were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to enumerate apoptotic liver cells. As expected, there were no detectable apoptotic cells observed in the control group, whereas significant numbers of nuclei per high-power field (many with abnormal ring-like morphology) were observed in HCV-immunized mice, confirming loss of liver antigen expression by HCV-specific killing (**Figure 12F**). These data demonstrate that immunization elicits long-lived functional memory T cells *in heparo*, able to rapidly kill HCV antigen-expressing hepatocytes.

Figure 12 (D-F). HCV Antigen was cleared in the liver by day 7 following AAV-HCV-NS3/4A-HA injection. Mice (n=4), immunized and rested for 20 weeks, were administered $2x10^{11}$vg AAV-HCV-NS3/4A-HA. On day 7 post-AAV injection, perfused livers were excised to assess acute HCV-specific functional memory T cell responses. (D) Mean fluorescence intensity (MFI) of NS3/4A-HA protein IF staining. Bars represent grand means. (E) Quantification of HCV-specific IFNγ-secreting liver lymphocytes in response to antigen-matched peptide pool stimulation by ELIspot assay. (F) Quantification of TUNEL-positive nuclei per field. Bars represent grand means. Error bars represents standard deviation. * $p<0.05$.

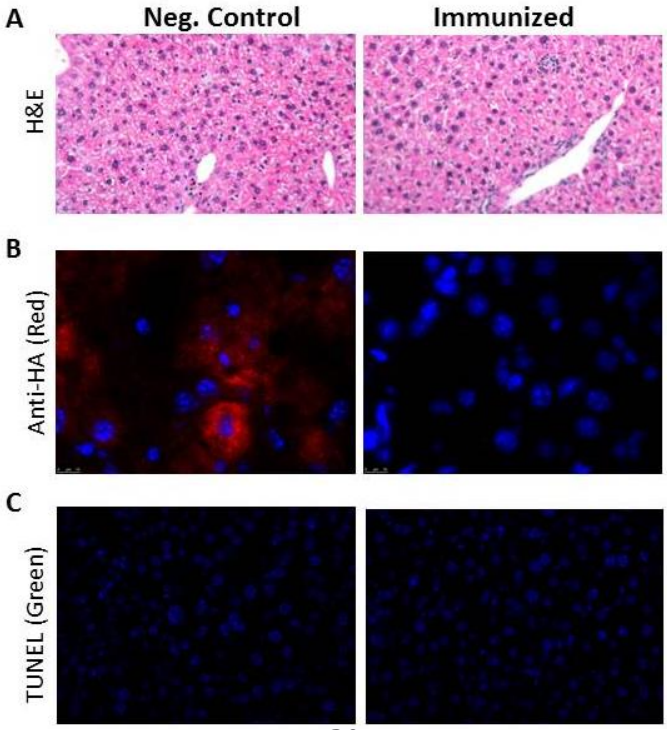


Study #12. Liver inflammation induced by HCV antigen is self-limiting

The aim of an HCV T cell vaccine is to rapidly clear infected cells to prevent chronic infection. Because HCV-associated disease is largely a result of chronic liver inflammation, it is important that the inflammatory response subsides following an immunological insult (Block TM et al., Oncogene 2003 22:5093-107).

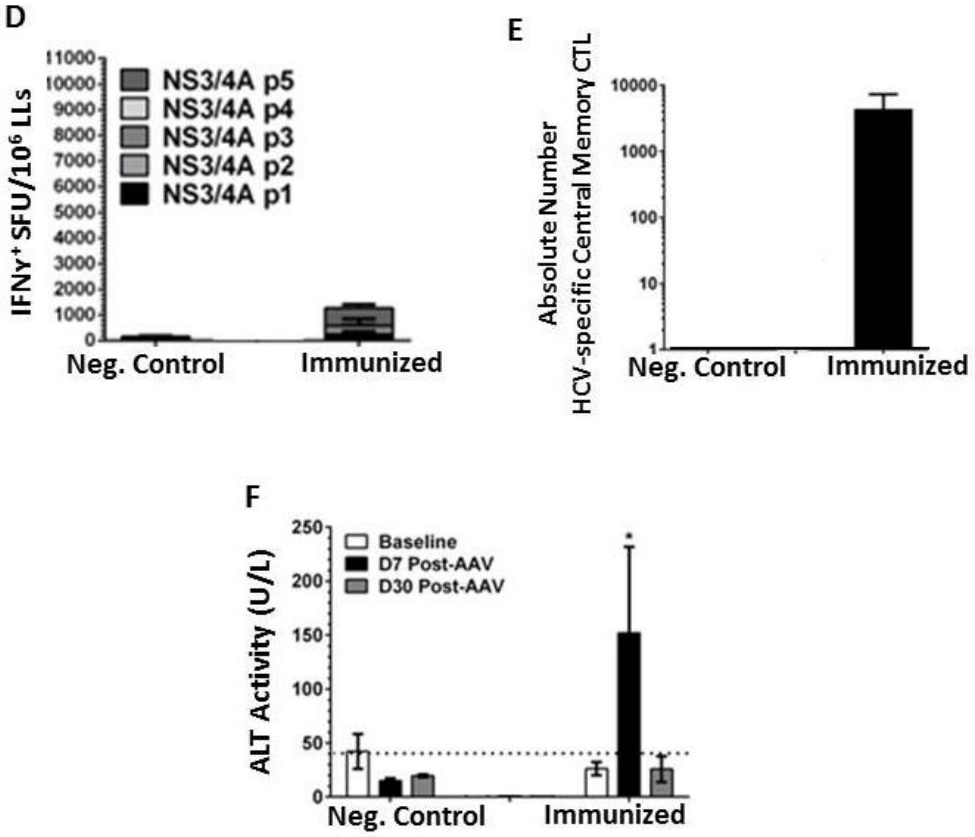
To assess resolution of inflammation, livers were excised from immunized and rested mice thirty days following AAV-HCV-NS3/4A-HA injection. By H&E, the cellular infiltrate apparent on day 7 were no longer observable. Liver sections from HCV-immunized mice closely resembled those of the negative control group (**Figure 13A**). In contrast, when frozen sections were stained for NS3/4A-HA expression, control animals continued to exhibit robust antigen expression, while antigen was completely unobservable in sections of HCV-immunized mice (**Figure 13B**). Additionally, TUNEL-positive nuclei were undetectable in any immunization group (**Figure 13C**). Resolution of inflammation by H&E coincided with a substantial decrease in HCV-specific IFNγ-secreting cells by ELIspot, although SFU counts remained marginally greater than control groups (**Figure 13D**). Resolution was also apparent by serum ALT activity of HCV-immunized mice, which was indistinguishable from control animals (**Figure 13E**). Finally, in the interest of long-lived protection, we enumerated the number of HCV-specific central memory

Figure 13 (A-C). Inflammation induced by HCV antigen resolved by day 30 following AAV-HCV-NS3/4A-HA injection. Mice, immunized and rested for 20 weeks, were administered $2x10^{11}$vg AAV-HCV-NS3/4A-HA. On day 30 post-AAV injection, perfused livers were excised to assess resolution of HCV-specific functional memory responses observed on day 7 post-AAV. (A) Resolution of inflammation was observed by H&E. (B) Hepatocyte-specific expression of HA-tagged NS3/4A persisted in control groups. (C) TUNEL staining of frozen liver sections, TUNEL+ (green) and DAPI (blue).



CTL still residing within the liver. We defined central memory CTL as CD3+/CD4-/CD8+ cells that produced one or more cytokines, IFNγ, TNFα, or IL-2, in response to HCV peptide stimulation, and highly expressed both CD44 & CD62L. Absolute numbers of HCV-specific memory CTL isolated from HCV-immunized livers were 100-fold greater than numbers isolated from control animals (**Figure 13F**). Together these data demonstrate that HCV-specific inflammation induced by a robust immunological insult in the liver is self-limiting, and resolves by day 30.

Figure 13 (D-F). Inflammation induced by HCV antigen resolved by day 30 following AAV-HCV-NS3/4A-HA injection. Mice, immunized and rested for 20 weeks, were administered $2x10^{11}$vg AAV-HCV-NS3/4A-HA. On day 30 post-AAV injection, perfused livers were excised to assess resolution of HCV-specific functional memory responses observed on day 7 post-AAV. (D) Contracted HCV-specific IFNγ-secreting liver lymphocytes assessed by ELIspot assay. (E) Absolute number of HCV-specific central memory CTL isolated from livers, defined as CD3+/CD8+/CD44hi/CD62Lhi and produced one or more cytokines (IFNγ, TNFα, IL-2) in response to HCV peptide stimulation. (F) Serum ALT activity determined at baseline prior to AAV injection, day 7 post-AAV injection during the memory T cell recall response, and day 30 post-AAV injection after resolution of inflammation.



Demonstration of immunogenicity of HCV NS3/4A, NS4B and NS5A plasmids in a Rhesus macaque model

While not a natural host for infection, the macaque has proven to be an important vaccination model for predicting successful human immune responses to multiple disease antigens because of the similarity of the macaque immune system to that of humans, as well as, the number of immune reagents available in order to dissect these responses. As a result, the immunogenicity of HCV NS3/4A, NS4B and NS5A (alone or combined) were assessed in a non-human primate model.

Immunization of NS3/4A plasmid induced strong NS3-specific multiepitope T cell responses in rhesus macaques mimicking human immune responses. Ten Chinese rhesus macaques were divided into two groups: the naïve group and immunized group. The plasmid was subsequently diluted in sterile water formulated with 1% (wt/wt) poly-L-glutamate sodium salt (MW = 10.5 kDa) (Sigma-Aldrich, St. Louis, MO). Each macaque in the immunized group was intramuscularly injected with 1 mg of NS3/4A plasmid diluted in a total volume of 0.75 ml. The site of injection was electroporated using the CELLECTRA adaptive constant current electroporation device (three pulses of 0.5 Amp constant current, 1 second apart and 52 ms in length). Each animal received a total of three immunizations, 4 weeks apart. Animals were bled once prior to immunization to test for preexisting NS3-specific T cell responses and were subsequently bled 2 weeks following each of the three immunizations. PBMCs were isolated using standard Ficoll-Hypaque density gradient centrifugation, resuspended in complete culture medium (RPMI 1640 with 2mM/L l-glutamine supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100µg/mL streptomycin, and 55 µmol/l β-mercaptoethanol).

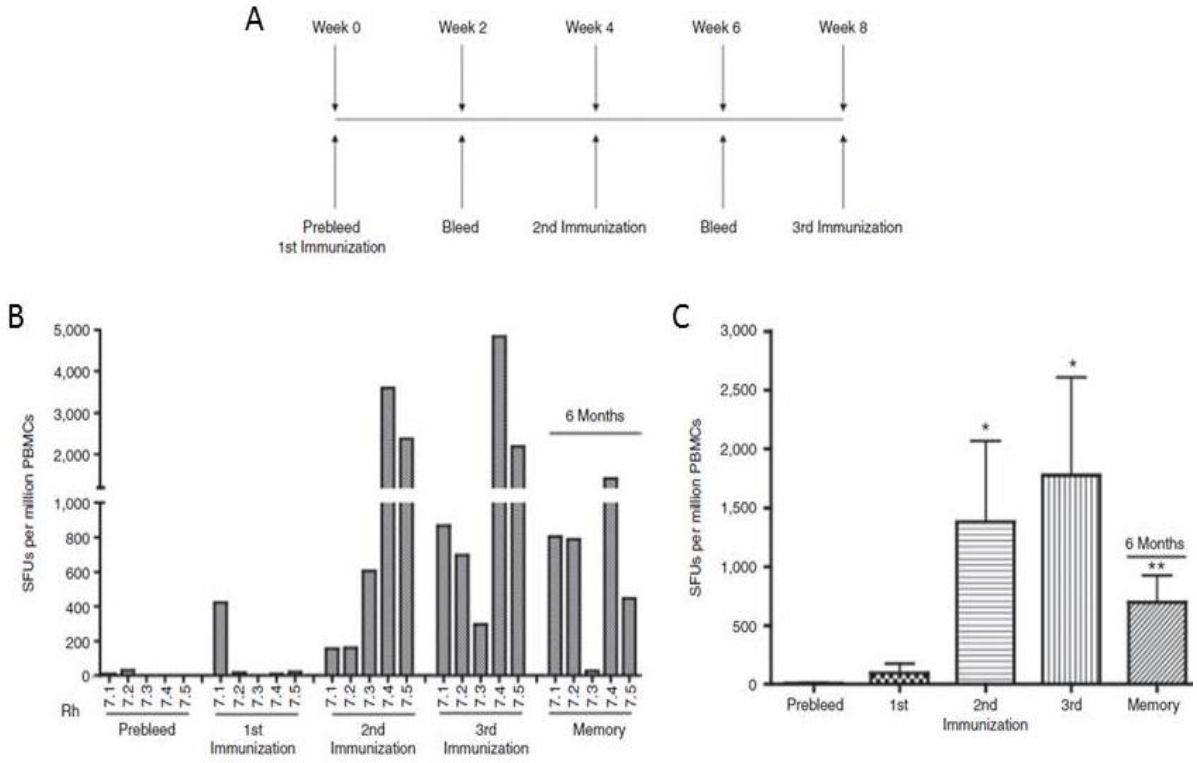
Study #13. Immunization of rhesus macaques with NS3/4A induces strong NS3-specific T cell responses measured by ELISpot.

Each rhesus macaque (n = 5) received a total of three intramuscular immunization of 1 mg of NS3/4A followed by electroporation. The three immunizations were administered 4 weeks apart with bleeds conducted 2 weeks following each immunization (**Figure 14A**). Given the importance of IFN-γ production by HCV-specific T cells in the resolution of acute infection, the initial responses to each immunization with NS3/4A plasmid were monitored through the use of quantitative IFN-γ enzyme-linked immunosorbent spot (ELISpot) assays. Anti-monkey IFN-γ capture and detection antibodies (MabTech, Sweden) were used. PBMCs were stimulated with a total of 101 HCV NS3 and NS4A 15-mer peptides (Invitrogen), overlapping by eight amino acids and spanning the entire length of the NS3/4A protein. These peptides were pooled into five different stimulation pools at a concentration of 2 µg/ml/peptide. The PBMCs were plated at a concentration of 200,000 cells per well. The average number of SFU was adjusted to 1×10^6 splenocytes for graphing purposes.

Analysis of peripheral blood mononuclear cells (PBMCs) from each animal conducted prior to immunization revealed no preexisting immune responses to pConNS3/NS4A, as detected by IFN-γ ELISpot assay. However, following the second immunization, detectable NS3- and NS4A-specific T cell responses were seen in all five animals, ranging from 157 to 3,602 spot forming units (SFU)/10^6 PBMCs (**Figure 14B**). Following the third immunization, the majority of the animals (three of five) showed an overall increase in total NS3- and NS4A-specific T cell responses, which ranged from 295 to 4,843 SFU/10^6 PBMCs (**Figure 14B**). The total T cell

response induced by NS3/4A averaged 1,381 ± 690 and 1,778 ± 830 SFU/10^6 PBMCs, for the second and third immunizations, respectively (**Figure 14C**). The duration of the T cell response to pConNS3/NS4A was also assessed by allowing the animals to rest for 6 months following the third immunization. NS3-specific responses to pConNS3/NS4A remained detectable in four of five animals, ranging from 447 to 1,414 SFU/10^6 PBMCs (**Figure 14B**). The average response among the four macaques was 864 ± 201 SFU/10^6 PBMCs (**Figure 14C**).

Figure 14. Induction of strong NS3/4A-specific cellular immune responses in NHP. (A) Immunization schedule. (B) IFN-γ ELISpot assay results for each individual animal. PBMCs were stimulated with a total of 101 HCV NS3 and NS4A 15-mer overlapping by either amino acids and spanning the entire length of the NS3/4A protein. (C) Average responses as measured by IFN-γ ELISpot assay. Values are represented as mean ± SEM, n=5 per group. Significance was determined by Student's *t*-test (* $P<0.05$, ** $P<0.005$ and *** $P<0.0005$.

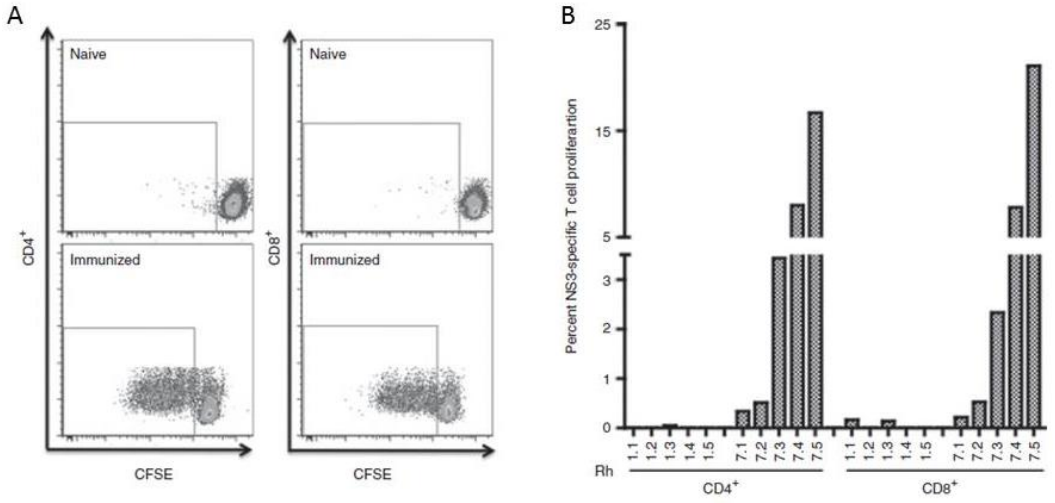


Study #14. Immunization of rhesus macaques with NS3/4A induced robust NS3-specific $CD4^+$ and $CD8^+$ T cell proliferation.

Numerous studies have found that an important correlate for both control of chronic HCV infection and clearance of acute infection is a strong and sustained anti-HCV $CD4^+$ T cell proliferative response, especially when directed against HCV NS3. Therefore, because of this correlation, we were interested in determining the proliferative capacity of NS3-specific T cells induced in response to immunization with NS3/4A plasmid. Animals were rested for ~2 months following the third immunization at which point the proliferative response of NS3-specific T cells were assessed through CFSE dilution assays. Briefly, PBMCs were pelleted and resuspended in 1 ml CFSE (Molecular Probes, Eugene, OR) diluted 1:2000 in phosphate-buffered saline. PBMCs were incubated at 37 °C for 10 minutes and pelleted. Cells were then resuspended in complete media at a concentration of 10^6 PBMCs/100 µl and plated in round bottom 96 well plates, stimulated with 100 µl complete media containing either 2 µg/ml NS3 peptides. Cell cultures were incubated for 5 consecutive days and then stained for flow cytometry analysis. PBMCs were first stained with a live/dead marker, and then stained with the following extracellular antibodies, CD3, CD4, and CD8 for 30 minutes at 4°C. PBMCs were washed twice with phosphate-buffered saline and fixed with 1% paraformaldehyde. Fifty thousand $CD3^+$ events were collected per sample.

NS3-specific proliferation was calculated as the total percentage of either $CD4^+$ or $CD8^+$ T cells to complete at least one round of cell division following a 5-day stimulation with NS3 peptides. Compared to the naive animals, immunized animals mounted robust NS3-specific proliferative responses, as seen with both $CD4^+$ and $CD8^+$ T cells (**Figure 15A**).

Figure 15. Immunization with NS3/4A plasmid induced robust $CD4^+$ and $CD8^+$ T cell responses in NHP (n=5). Proliferative responses were measured with CFSE dilution assay ~ 2 months following the last immunization. PBMCs were stimulated with HCV NS3 peptides, overlapping by either amino acids and spanning the entire length of the NS3 protein. (A) Representative dot plots are shown for both the native group and the NS3/4A immunized group. (B) Individual NS3-specific proliferative responses for each animal.



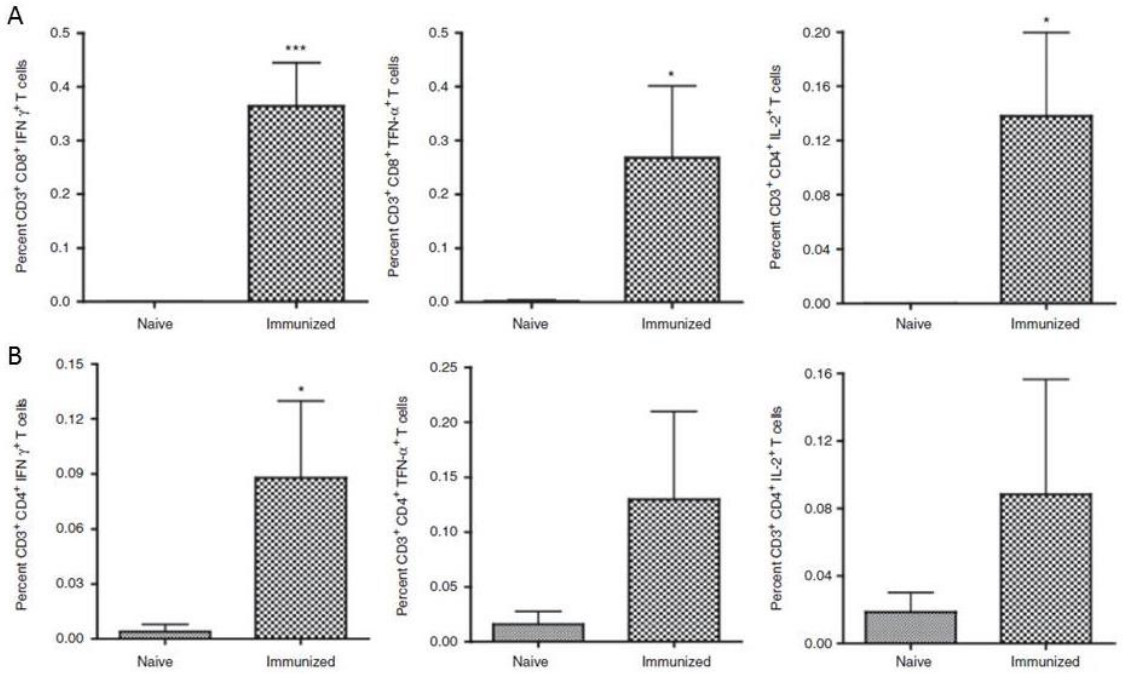
Total NS3-specific T cell proliferation averaged 5.8% ± 3% and 6.4% ± 3.9% for $CD4^+$ and $CD8^+$ T cells, respectively. **Figure 15B** shows the total percent $CD4^+$ and $CD8^+$ proliferation of each animal. The total percent NS3-specific proliferation did vary greatly among the five individual animals. Animals Rh7.1 and Rh7.2 showed only minimal proliferation, while animals Rh7.3, Rh7.4, and Rh7.5 showed robust NS3-specific proliferative responses.

Study #15. Immunization of rhesus macaques with NS3/4A induced polyfunctional T cell responses.

Polyfunctional T cells have been associated with the control of numerous viral infections. Studies of HCV-infected patients have suggested the importance of polyfunctional $CD8^+$ T cells in the effective control of HCV replication and have been suggested to be an important correlate for clearance of HCV in acutely infected patients. To test for these responses, animals were rested for approximately two months following the third immunization, at which point, the phenotypic and functional characteristics of immunization induced $CD4^+$and $CD8^+$ T cells were assessed through intracellular cytokine analysis by polychromatic flow cytometry. Briefly, PBMCs were resuspended in complete media at a concentration of 1×10^6 cells/100 µl and plated in a round bottom 96-well plate along with anti-CD107a. Cells were stimulated for 5 hours at 37 °C with 100 µl of either 2 µg/ml HCV NS3/NS4A peptides, 1 µg/ml Staphylococcus enterotoxin B (positive control; Sigma-Aldrich) or 0.1% dimethyl sulfoxide (negative control) diluted in complete media supplemented with GolgiStop and GolgiPlug (BD Biosciences, San Jose, CA). Following incubation, cells were washed and stained for viability and surface markers with anti-CD3, CD4, CD8, CD95, CD28, CD14, CD16, and CD20 for 30 minutes at 4 °C. Cells were permeabilized and washed using BD Cytofix/Cytoperm Solution Kit (BD Bioscience) and then stained intracellularly with anti-IFN-γ, IL-2, and TNF-α for 45 minutes at 4 °C. Following staining, cells were washed and then fixed with 1% paraformaldehyde and stored at 4 °C until analysis. The percent of specific function was calculated as the percent function of the peptide-stimulated group minus the percent function of the 0.1% dimethyl sulfoxide-stimulated group for each animal. The threshold for positive responses was set at 0.02%.

As shown in **Figure 16,** both $CD8^+$ T cells and $CD4^+$ T cells from immunized animals were able to produce IFN-γ, IL-2, and TNF-αin response to peptide stimulation as compared to naïve animals. Following stimulation, the $CD8^+$ T cell response for immunized animals averaged 0.36% ± 0.08%, 0.27% ± 0.13%, and 0.14% ± 0.06%, for IFN-γ, TNF-α, and IL-2 production, respectively (**Figure 16A**). The $CD4^+$ T cell response for immunized animals averaged 0.09% ± 0.04%, 0.13% ±0.8%, and 0.09% ± 0.07%, for IFN-γ, TNF-α, and IL-2 production following stimulation, respectively (**Figure 16B**).

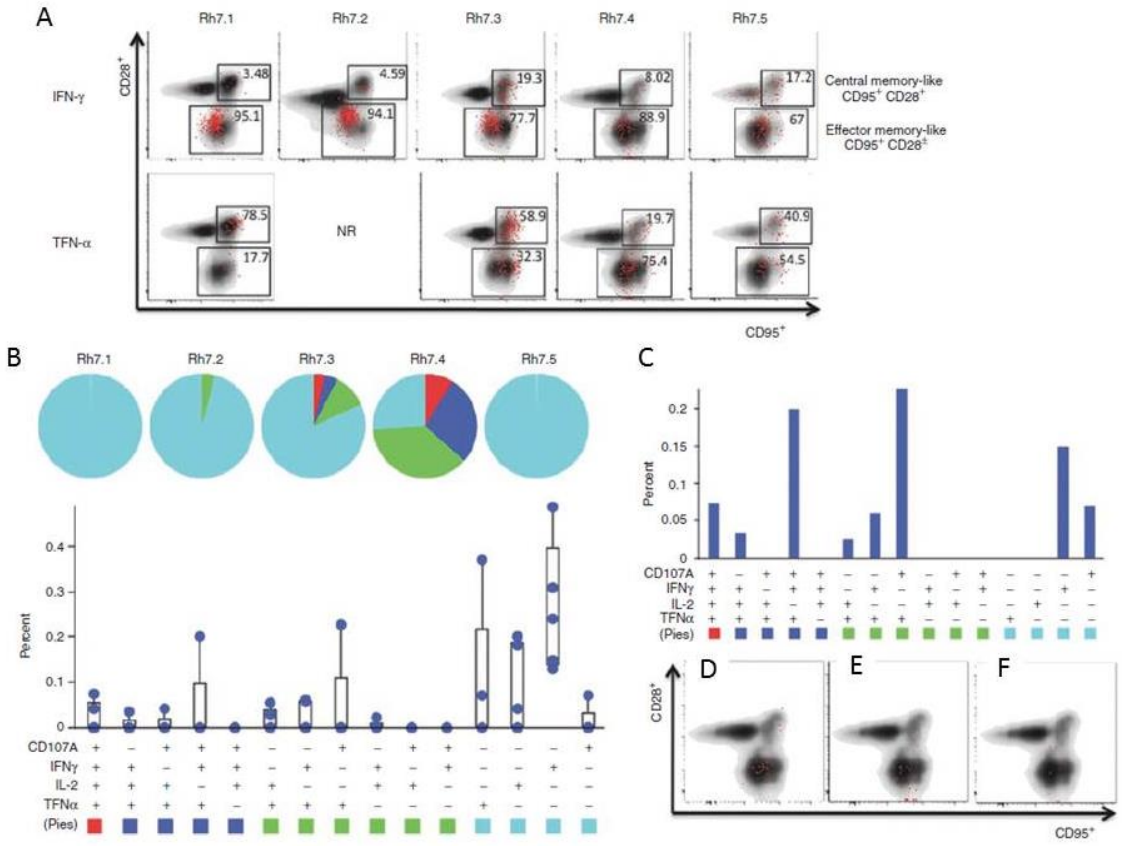
Figure 16. Immunization with NS3/4A plasmid induced NS3-specific T cells able to produce numerous cytokines in NHP. PBMCs were stimulated with HCV NS3 peptides, overlapping by eight amino acids and spanning the entire length of the NS3 protein. PBMCs were stained intracellularly for IFN-γ, IL-2, and TNF-α ~2 months following the last immunization. (**A**) Average NS3-specific CD8+ T cell responses for each group. (**b**) Average NS3-specific CD4+ T cell responses for each group. Values are represented as mean + SEM, n = 5 per group. Significance was determined by Student's t-test (*P <0.05, **P < 0.005, and ***P < 0.0005).



Next, we performed a detailed analysis of both NS3-specific $CD8^+$ T cell responses and NS3-specific $CD4^+$ T cell responses. For all five animals, the predominant cytokine produced by $CD8^+$ T cells in response to peptide stimulation was IFN-γ. TNF-α was also commonly expressed and was identified in four out of five animals. While an important correlation to progression of HCV infection to the chronic state is loss of effector function suggesting the importance of sustained effector function in viral control, an effective vaccine must also have the ability to induce potent memory responses in order to be protective long-term. Therefore, both $CD8^+$ IFN-γ^+ and $CD8^+$ TNF-α^+ responses were further characterized by their expression of both CD28 and CD95. $CD8^+$ IFN-γ^+ T cells were mostly $CD28^-$ $CD95^+$, which is suggestive of a more effector memory-like phenotype, while the majority of $CD8^+$ TNF-α^+ T cells were more evenly distributed between $CD28^-$ $CD95^+$ and $CD28^+$ $CD95^+$ populations, suggesting that these cells display both an effector memory-like and a central memory-like phenotype (**Figure 17A**). Polyfunctionality was analyzed based on four different functions, IFN-γ, TNF-α, IL-2, and CD107A. In **Figure 17B**, each pie chart represents the total NS3-specific $CD8^+$ T cell response

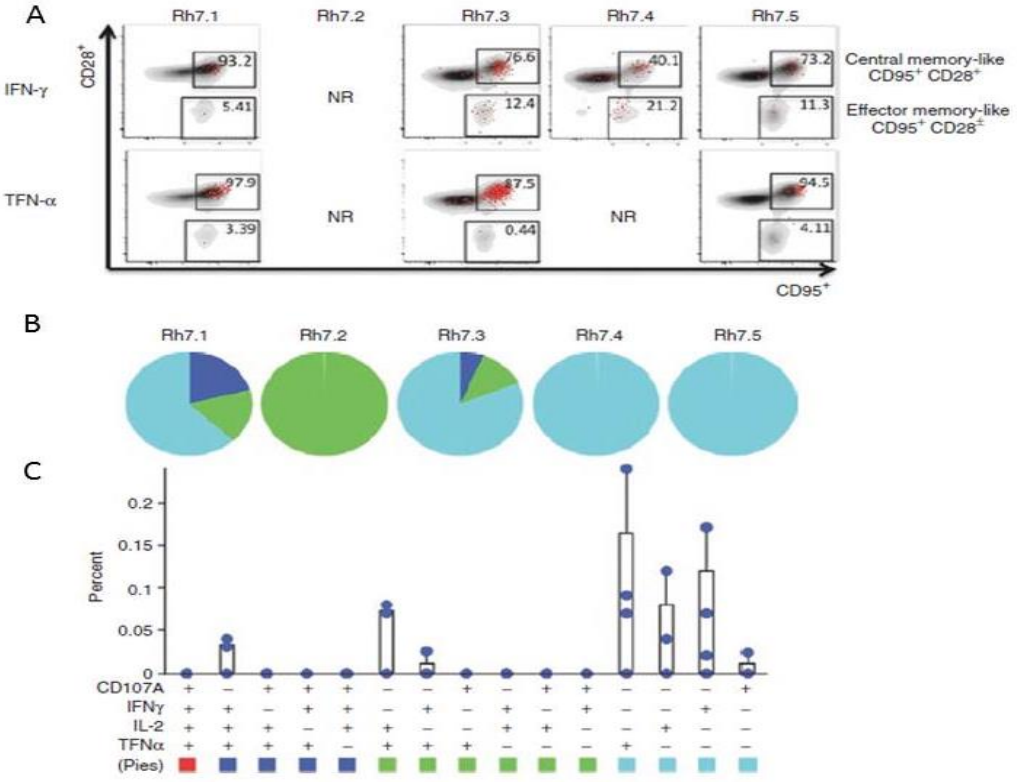
for each animal as graphed by function. Light blue, green, dark blue and red represent one, two, three and four functions, respectively. Three out of five immunized animals were able to mount $CD8^+$ T cells with two or more functions, while two animals were able to mount a vaccine specific $CD8^+$ T cells able to perform all four functions. The graph in **Figure 17B** represents the average $CD8^+$ T cell responses for all five animals. While some animals were able to mount a polyfunctional $CD8^+$ T cell response, the average $CD8^+$ T cell response among the five animals was predominantly a monofunctional IFN-γ or TNF-αresponse. Animal Rh7.4, however, was able to mount a much more diverse T cell response as compared with the other four animals; $CD8^+$ T cells able to produce numerous combinations of functions in response to immunization were detected. **Figure 17C** shows the individual NS3-specific $CD8^+$ T cell responses of Rh7.4. The three most dominant multi-functional responses were further characterized based on the markers CD28 and CD95, (**Figure 17D, E and F).** $CD8^+$ T cells able to express both TNF-α and CD107A (**Figure 17D**) and $CD8^+$ T cells able to express TNF-α, CD107A and IFN-γ (**Figure 17E**) were predominately $CD28^-$ $CD95^+$, suggesting that these two types of responses are mainly effector memory-like. On the other hand, $CD8^+$ T cells able to produce all four functions, TNF-α, CD107A, IL-2, and IFN-γ, fell into both $CD28^-$ $CD95^+$ and $CD28^+$ $CD95^+$ populations, suggesting that this type of response is both effector and central memory-like (**Figure 17F**).

Figure 17. Detailed analysis of vaccine-induced NS3-specific CD8$^+$ T cell responses. PBMCs were stimulated with HCV NS3 peptides, overlapping by eight amino acids and spanning the entire length of the NS3 protein. (**A**) Analysis of CD8+ IFN-γ$^+$ and CD8$^+$ TNF-α$^+$ T cell responses (red) based on CD28 and CD95 staining. (**B**) PBMCs were stained intracellularly for IFN-γ, TNF-α, IL-2, and CD107A and were analyzed for polyfunctionality. Pie charts represent the proportion of NS3-specific CD8$^+$ T cells for each animal that have one function (light blue), two functions (green), three functions (dark blue), and four functions (red). The graph is the average polyfunctional NS3-specific CD8$^+$ T cell response among all five animals. Values are represented as mean ± SEM, n = 5 per group. (**C**) Graph of the polyfunctional NS3-specific CD8$^+$ T cell response of Rh7.4. (**D**–**F**) The three largest NS3-specific polyfunctional CD8$^+$ T cell responses (red) of Rh7.4 were plotted based on CD28 and CD95 expression. (**D**) TNF-α$^+$ CD107A$^+$, (E) TNF-α$^+$ CD107A$^+$ IFN-γ$^+$, (**F**) TNF-α$^+$ CD107A$^+$ IL-2$^+$ IFN-γ$^+$.



Like the $CD8^+$ T cell response, the predominant cytokine responses for $CD4^+$ T cells were also IFN-γ and TNF-α. Four out of five animals mounted an NS3-specific $CD4^+$ IFN-γ^+ response, while three out of five mounted a $CD4^+$ TNF-α^+ response. Analysis of cell surface expression of both CD28 and CD95 revealed that the majority of $CD4^+$ IFN-γ T cells were $CD28^+$ $CD95^+$, suggesting the majority of these T cells displayed a more central memory-like phenotype (**Figure 18A**). However, animals Rh7.3, Rh7.4, and Rh7.5 showed a smaller, but a distinct population of $CD28^-$ $CD95^+$ $CD4^+$ IFN-γ^+ T cells, suggesting that these animals were also able to mount a $CD4^+$ IFN-γ^+ effector memory-like response, as well. Similarly, the majority of $CD4^+$ TNF-α T cells displayed central memory-like phenotype, falling mostly into the $CD95^+$ $CD28^-$ population (**Figure 18A**).

Figure 18. Detailed analysis of vaccine-induced NS3-specific $CD4^+$ T cell responses in NHP. PBMCs were stimulated with HCV NS3 peptides, overlapping by eight amino acids and spanning the entire length of the NS3 protein. (**A**) Analysis of $CD4^+$ IFN-γ^+ and $CD4^+$ TNF-α^+ T cell responses (red) based on CD28 and CD95 staining. (**B and C**) PBMCs were stained intracellularly for IFN-γ, TNF-α, and IL-2 and were analyzed for polyfunctionality. (**B**) Pie charts representing the proportion of NS3-specific CD4+ T cells for each animal that have one function (light blue), two functions (green), three functions (dark blue), and four functions (red). (**C**) Average polyfunctional response among all five animals (NR, no response). Values are represented as mean + SEM, *n* = 5 per group.

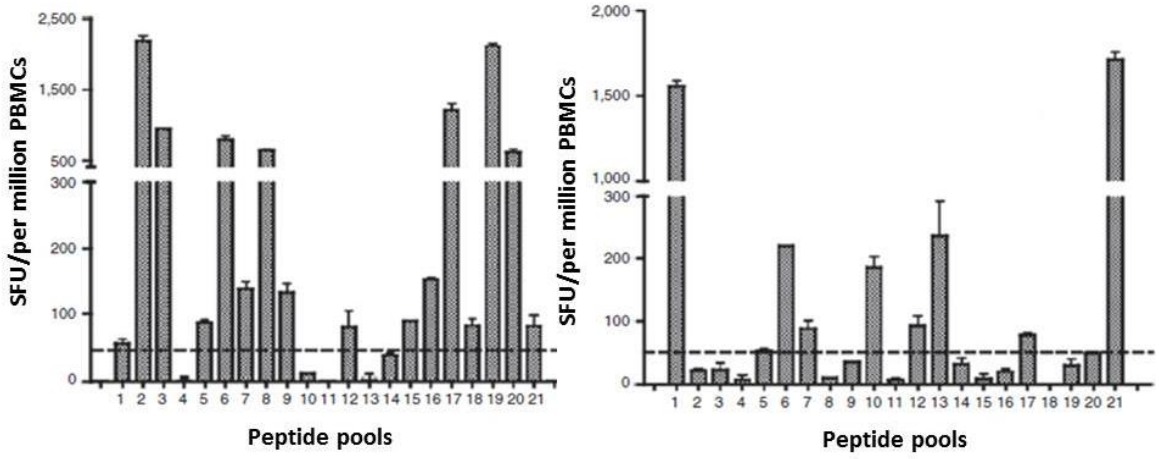


Finally, the polyfunctionality of the NS3-specific $CD4^+$ T cell response was analyzed. **Figure 18B** represents the total $CD4^+$ T cell response of each animal as graphed by function. Three out of five animals had $CD4^+$ T cells able to perform two or more functions, while two animals had $CD4^+$ T cells responses able to perform three functions; IFN-γ, IL-2, TNF-α. **Figure 18C** represents the average NS3-specific $CD4^+$ T cell response. As seen with the $CD8^+$ T cell responses, the average $CD4^+$ T cell response is predominantly monofunctional, with the highest average responses being TNF-α or IFN-γ.

Study #16. Immunization of rhesus macaques with NS3/4A induced broad T cell responses.

It is believed that a successful immune response against HCV includes a strong $CD8^+$ T cell response able to recognize and target multiple epitopes within the viral genome, while persistent infection is often marked by low frequencies of HCV-specific $CD8^+$ T cells able to recognize only a small number of epitopes. We were therefore interested in the breadth of the NS3-specific T cell response induced in the animals following immunization. Therefore, the T cell response of the two highest responders, Rh7.4 and Rh7.5, were mapped using a matrix epitope mapping technique and IFN-γ ELISpot assays. Briefly, 101 HCV NS3/4A 15-mer overlapping peptides spanning the length of the NS3/4A protein were pooled into 21 separate pools. Each individual peptide was represented in two pools of the 21 pools. These pools were then used to stimulate PBMCs in an IFN-γ ELISpot assay as described earlier. As seen in **Figure 19A and B**, Rh7.4 and Rh7.5 both mounted broad T cell responses in response to immunization and were able to recognize numerous peptides spanning the length of the HCV NS3 protein.

Figure 19. Matrix epitope mapping of Rh7.4 and Rh7.5 with IFN-γ ELISpot assays. PBMCs were stimulated with 21 separate pools of HCV NS3 and NS4A peptides. Each pool contained ~10 of the 101 HCV NS3 and NS4A 15-mer overlapping peptides spanning the length of pConNS3/NS4A. Each individual peptide was represented in two pools of the 21 pools. (**A**) Matrix epitope mapping of animal Rh7.4 (**B**) Matrix epitope mapping of animal Rh7.5.



Immunogenicity induced by co-immunization of NS3/4A, NS4B and NS5A plasmids in NHP
A broad primary immune response to nonstructural HCV antigens would be important host immune response needed for protection against HCV. Therefore, after we demonstrated that co-immunization with the NS3/4A, NS4B and NS5A plasmids induced strong and broad HCV-specific T cell responses in the periphery and liver in a murine model, in this study, we went forward to use a non-human primate model to test immunogenicity of our complete formulation of HCV antigens including NS3/4A, NS4B and NS5A.

Eight Female rhesus macaques (*Macaca mulatta*) of Indian origin (n = 4) were divided into two groups: the naïve group and immunized group. Each animal in the immunized group was immunized at weeks 0, 6, and 12 with 1.0 mg per plasmid construct of NS3/4A, NS4B and NS5A. DNA was formulated in sterile WFI and delivered via IM injection into the quadriceps muscle in a total volume of 0.75 mL per injection followed by in vivo electroporation (three pulses of 0.5 Amp constant current, 1 second apart and 52 ms in length) using the constant current CELLECTRA® device (Inovio Pharmaceuticals, Inc. Animals were bled 2 weeks previous to the first immunization, 2 weeks following each immunization, and additionally, 4 weeks following the final immunization. Ten or 20 mL of blood were collected in EDTA tubes, and PBMCs were isolated using standard Ficoll-Hypaque density gradient centrifugation, resuspended in complete culture medium (RPMI 1640 with 2mM/L l-glutamine supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, 100µg/mL streptomycin, and 55 µmol/l β-mercaptoethanol). Red blood cells (RBC) were lysed with ammonium chloride-potassium (ACK) lysis buffer (Cambrex BioScience).

Study #17. Immunization of rhesus macaques with NS3/4A, NS4B and NS5A induces strong and broad HCV-specific T cell responses measured by ELISpot.

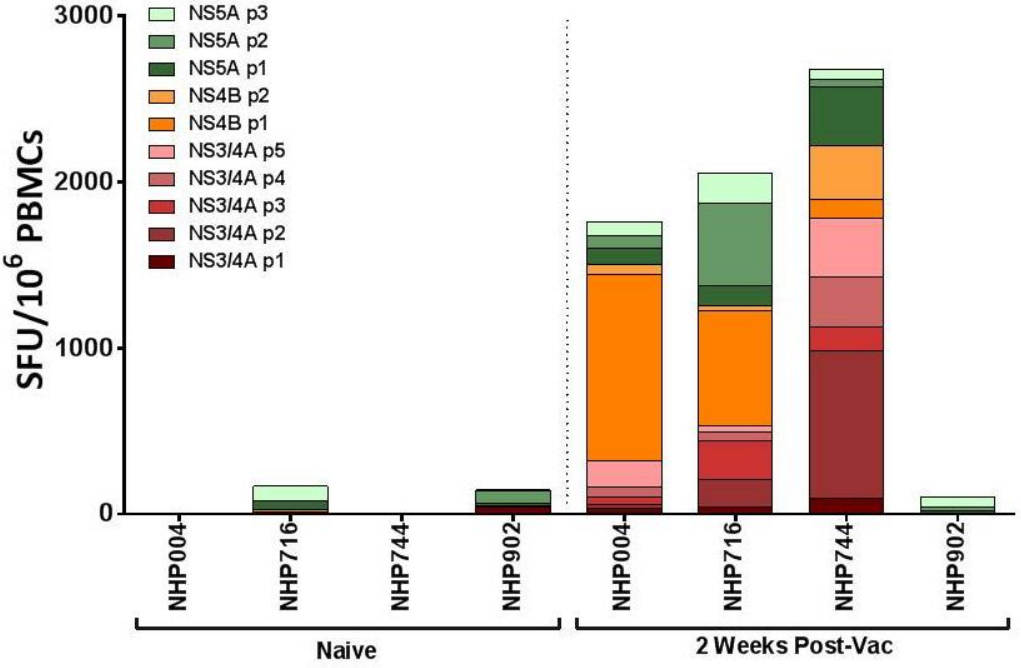
Immunogenicity was monitored through the use of IFNγ ELISpot assays as described earlier (**Figure 20**). While all constructs demonstrated immunogenicity in the context of vaccination, the immunodominant domains of each antigen varied among the outbred animals, which are consistent with what is seen in the genetically diverse human population, and highlights the importance of a multi-antigenic vaccine strategy. At 2 weeks post 3rd immunization, antigen-specific IFNγ responses were above background in 3/4 animals with total responses ranging from 103 to 2680 spot-forming units /10^6 PBMCs (SFU), and in one animal, NHP902, did not exceed its baseline response in the IFNγ ELISpot assay. Antigen-specific IFNγ reactivity against peptides of HCV NS3/4A, NS4B and NS5A were variable between animals and ranged from undetectable to 1783 SFU, 3 to 726 SFU and 100 to 796 SFU, respectively. These data illustrate that, in an outbred heterogeneous group of non-human primates, our HCV vaccine can induce PBMCs of substantial magnitude that are broadly reactive to regions spanning all three HCV vaccine components.

Study #18. HCV-specific effector CD4+ and CD8+ T lymphocytes are elicited by HCV immunization.

In order to further study the IFNγ responses induced by HCV immunization by identifying PBMC populations responsible for IFNγ production, as well as detect other effector cytokines

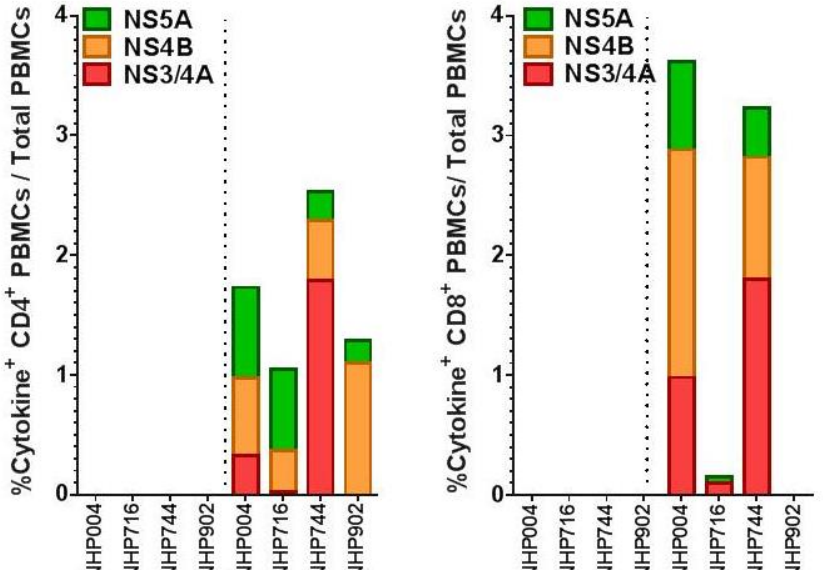
produced in response to antigen stimulation, we performed intracellular staining assays (ICS) as described earlier and acquired cell phenotype data by flow cytometry at 2 weeks post the last immunization. Briefly, PBMCs were stimulated ex vivo with antigen-matched peptides in the presence of Golgi transport inhibitors and immunofluorescently stained for T cell markers (CD3, CD4, and CD8), intracellular cytokines (IFNγ; tumor necrosis factor-α, TNFα; and interleukin-2, IL-2), and cytolytic markers (surface CD107a, LAMP-1; and granzyme B, GrzB). From acquired flow cytometry data, $CD3^+/CD4^-/CD8^+$ T cells (putative cytotoxic T lymphocyte; CTL) and $CD3^+/CD8^-/CD4^+$ T cells (Type 1 helper T lymphocytes; Th1) were analyzed for HCV-specific cytokine responses. T lymphocytes were included in the reported HCV-specific frequencies if they stained positive for one or more effector cytokines: IFNγ, which is an aforementioned correlate of acute resolution; TNFα, which is another important pro-inflammatory and antiviral effector cytokine; IL-2, which is necessary for the proliferation and survival of T cells), or exhibited a cytolytic phenotype ($CD107a^+/GrzB^+$, which indicates a cell is both producing the cytolytic enzyme GrzB and actively degranulating), in response to HCV peptide stimulation.

Figure 20. HCV-specific interferon-γ responses detectable in peripheral blood 2 weeks following the last immunization (n=4). Each animal in the immunized group was immunized at weeks 0, 6, and 12 with 1.0 mg per plasmid construct of NS3/4A, NS4B and NS5A. IFNγ ELIspot assays were performed on NHP PBMCs isolated at baseline and 2 weeks post the last immunization by stimulating cells overnight with antigen-matched overlapping linear peptide pools. HCV-specific IFNγ responses, as spot-forming units ($IFN\gamma^+$ cells)/million PBMCs, are represented as stacked responses, demonstrating specificity across multiple regions of multiple antigens for individual NHPs.



As shown in **Figure 21,** total cytokine responses were predominately due to HCV-specific CD8+ CTL. However, HCV-specific CD4+ Th1 cells were also detectable. NHP004 and NHP744 demonstrated Th1 and CTL reactivity to all 4 HCV antigens with total 1.73% and 2.53% Th1, and 3.62% and 3.23% CTL HCV-specific frequencies, respectively. Cytokine responses were detectable but limited to less than 4 antigens in NHP716 and NHP902 with total 1.05% and 1.29% Th1, and 0.15% and 0% CTL HCV-specific frequencies, respectively. Such variable T cell reactivity profiles further highlights the importance of a multi-antigenic vaccine in diverse populations. Taken together, these data illustrate that HCV immunization elicits both peripheral HCV-specific Th1 and CTL that react to one or more of the 4 HCV antigens within our HCV vaccine formulation.

Figure 21. Cytokine production of HCV-specific T cells following immunization. Cytokine production, by intracellular cytokine staining, following antigen-matched peptide stimulation revealed HCV-specific T cells in peripheral blood 2 weeks post last immunization, compared with baseline, by flow cytometric analysis. $CD3^+/CD4^+/CD8^-$ (Th1) or $CD3^+/CD8^+/CD4^-$ (CTL) cells were counted as cytokine-positive if they produced one or more of IFNγ, TNFα, or IL-2. Total cytokines responses are represented as stacked responses to demonstrate breadth across multiple antigens.



Summary of nonclinical immunogenicity experience with NS3/4A, NS4B and NS5A in murine and rhesus macaque models

Immunization with individual NS3/4A, NS4B and NS5A plasmids induced potent NS3/4A-, NS4B- and NS5A-specific cellular immune responses in the periphery and the liver as well. Liver expression of HCV antigens resulted in the activation of intrahepatic vaccine-induced HCV-specific cytotoxic T cells and rapid clearance of NS3, NS4B or NS5A-expressing

hepatocytes. Moreover, when combined as a single formulation, vaccination was able to elicit strong and broad HCV-specific T cell responses in the periphery and liver. The in *heparo* killing assay results indicated that rapid memory recall responses induced by co-immunization of NS3/4A, NS4B and NS5A plasmids cleared the HCV antigen-expressing hepatocytes. In addition, the immunogenicity studies in a more relevant non-human primate model showed that immunization with NS3/4A, NS4B and NS5A plasmids induced robust and broad T cell responses. The vaccine-induced T cells are polyfunctional, and exhibited strong proliferative capacity. Results of these assays investigating NS3/4A, NS4B and NS5A plasmids in mouse and Rhesus macaque models have been published [1-5].

1. Lang KA, Yan J, Draghia-Akli R, Khan A, Weiner DB. Strong HCV NS3- and NS4A-specific cellular immune responses induced in mice and Rhesus macaques by a novel HCV genotype 1a/1b consensus DNA vaccine. *Vaccine* 2008,**26**:6225-6231.
2. Lang Kuhs KA, Ginsberg AA, Yan J, Wiseman RW, Khan AS, Sardesai NY, *et al.* Hepatitis C virus NS3/NS4A DNA vaccine induces multiepitope T cell responses in rhesus macaques mimicking human immune responses [corrected]. *Mol Ther* 2012,**20**:669-678.
3. Lang Kuhs KA, Toporovski R, Ginsberg AA, Olsen AL, Shedlock DJ, Morrow MP, *et al.* Peripheral immunization induces functional intrahepatic hepatitis C specific immunity following selective retention of vaccine-specific CD8 T cells by the liver. *Hum Vaccin* 2011,**7**:1326-1335.
4. Lang Kuhs KA, Toporovski R, Yan J, Ginsberg AA, Shedlock DJ, Weiner DB. Induction of intrahepatic HCV NS4B, NS5A and NS5B-specific cellular immune responses following peripheral immunization. *PLoS One* 2012,**7**:e52165.
5. Latimer B, Toporovski R, Yan J, Pankhong P, Morrow MP, Khan AS, *et al.* Strong HCV NS3/4a, NS4b, NS5a, NS5b-specific cellular immune responses induced in Rhesus macaques by a novel HCV genotype 1a/1b consensus DNA vaccine. *Hum Vaccin Immunother* 2014,**10**.

The following manuscript is in preparation:
A synthetic HCV genotype 1a/1b consensus DNA vaccine elicits functional, long-lived, memory T cells *in heparo*. Brian P. Latimer, Ebony N. Gary, Scott M. Baliban, Jian Yan, Niranjan Y. Sardesai, Linda B. Couto, Jeffrey M. Jacobson, David B. Weiner and Michele A. Kutzler

SPECIFIC AIM 2

State Fiscal Year	Milestones for Specific Aim #2
6/1/10 - 6/30/10	• Identification of three undergraduate students to enroll in first cycle of summer research internship program • Matching students to faculty mentors
7/1/10 - 6/30/11	• Begin collaboration between labs at DUCOM and Cheyney (exchange technology – immunological techniques, DNA vaccine development, small

	animal immunizations) • Summer internships, symposium presentations, publications • Internship program evaluation • Identification of three undergraduate students to enroll in second cycle of summer internship program • Match students to faculty mentors
7/1/11 - 6/30/12	• Continue collaboration between labs at DUCOM and Cheyney (exchange technology – immunological techniques, DNA vaccine development, small animal immunizations) • Summer internships, symposium presentations, publications • Internship program evaluation • Identification of three undergraduate students to enroll in third cycle of • summer internship program • Match students to faculty mentors
7/1/12 - 6/30/13	• Continue collaboration between labs at DUCOM and Cheyney (exchange technology – immunological techniques, DNA vaccine development, small animal immunizations) • Summer internships, symposium presentations, publications • Internship evaluation • Identification of three undergraduate students to enroll in fourth cycle of summer internship program • Match students to faculty mentors
7/1/13 - 5/31/14	• Continue collaboration between labs at DUCOM and Cheyney (exchange technology – immunological techniques, DNA vaccine development, small animal immunizations) • Summer internships, symposium presentations, publications • Internship program evaluation

Progress for Specific Aim #2: This component served as a mechanism for providing education and training through hands on research experiences and lecture workshops for faculty, undergraduate and graduate students at Cheyney and Drexel Universities. The time periods of June 2011 through August of 2013 were summer sessions for the Education and Training Component in which 10 Cheyney University students (listed in **Table 2)** took part in a 10-week summer internship program at Drexel University and the University of Pennsylvania. These students and Dr. Abdel Bior (Assistant Professor of Biology at Cheyney University) worked under the direction of faculty in the different components of the project and have completed hypothesis driven research in line with the aims of the grant. With great success, the students presented formal oral presentations based on their summer projects to DUCOM faculty and fellow summer students as part of the Summer Undergraduate Research Fellowship program (SURF), at Drexel Univ. College of Medicine's Discovery Day Research Symposium, and at the Alliance for Minority Participation sponsored by NSF. As shown in **Table 2,** minority students in our program have demonstrated high level of motivation and accomplishments and in general, we believe, have gained new knowledge and bench research skills, including responsible conduct in research, how to formulate research questions, develop and troubleshoot new scientific methodology, analyze data and present research findings.

Progress for Aim 2 Milestone Achievement.

- Identification of undergraduate students to enroll in cycles of summer research internship program

Matching students to faculty mentors under CURE grant faculty mentorship and direction, since 2010, 14 Cheyney University and 1 Bryn Mawr minority undergraduate students were matched with research mentors at DUCOM (including 4 in my lab) and UPENN. Each spring, CURE faculty would present research as part of the Cheyney University Department of Biology seminar series. This would give CURE faculty an opportunity to present the research and meet several students from Cheyney University, thus driving interest from Cheyney students in the match summer program. We continued to do these presentations each year for the summer research program. This was very important for the success to recruit students.

- Matching students to faculty mentors

Cheyney's Dr. Eva and Bior would collect formal applications for our summer research program from students (included transcript, personal career statement, and research resume). Dr. Kutzler would work with CURE faculty to read through the applications and match student research interest with our faculty at UPENN and DREXEL. Students would be notified of their acceptance in the program and they would be required to sign a "contract" that would state their commitment to a full time summer research program. At that time we would give the students information on the lab mentor match, student housing and expectations for the summer, as well as professionalism guidance.

- Continue collaboration between labs at DUCOM and Cheyney (exchange technology – immunological techniques, DNA vaccine development, small animal immunizations)

The students would begin the research program in June each year where students carryout summer "wet bench" hypothesis-driven research projects, present findings in both oral (DUCOM SURF) and poster presentations (Discovery Day and the Philadelphia Alliance for Minority Participation), receive training in responsible conduct of research, and other professional development seminars along side other DUCOM SURF students. The Cheyney students were matriculated into Drexel's formal SURF (summer undergraduate research program which usually totals about 20 summer students). This helped the students to build their network and the Cheyney students attended the responsible conduct of research seminars throughout the summer with these other SURF students. The students were required to attend monthly seminars at DUCOM that review responsible conduct in research (RCR Training) topics including; "Use of Humans in Biomedical Experimentation", "Scientific Record Keeping", "Responsible Conduct of Research, Scientific Integrity", "Use of Animals in Biomedical Experimentation", "Professionalism and Career Paths", and "How to Create and Deliver an Effective Poster Presentation". With regard to scientific methodology and technique development, the students worked closely in the lab with faculty mentor and students in the labs to learn new techniques, analyze data and think critically about the experiments they carried out.

- Summer internships, symposium presentations, publications

The research consortium provided a structured mentoring program with students from a collaborating minority institution for the development of future research scientists in highly translational bench research. Dr. Michele Kutzler oversees the summer minority undergraduate student training program in collaboration with Cheyney University of Pennsylvania that is a major component of a nonformula research grant award (July 1, 2010- June 30, 2014) funded by the PA Department of Health (PI: Jeff Jacobson). In addition, Dr. Kutzler's laboratory mentors a faculty member (Dr. Abdel Bior) from Cheyney University each summer. This component serves as a mechanism for providing education and training through hands-on research experiences and lecture workshops held at DUCOM for minority faculty and undergraduate students at Cheyney University of Pennsylvania. There are two primary goals of this component with regard to education and training: (1) to encourage minority undergraduate students to pursue careers in biomedical research and (2) to provide a framework for faculty and students to collaborate on research related to the development, engineering, immunogenicity, and protective effects of the DNA vaccine platform.

- Internship evaluation

Since 2010, students mentored in labs as part of this program have received honors for their research projects as noted in Table 2 below, and are now applying to medical and graduate schools, including one who was accepted into and participated in the University of Pittsburgh Intramural Research Training postbaccalaureate program for minority students, and two others who were accepted into and are attending Drexel's pathway to medical school program. At the end of the summer, students give specific, conference-styled presentations describing their research projects to an audience of Drexel Med faculty and graduate/medical students. The students meet daily/weekly with their mentors to prepare and review the content of their oral presentations. In addition, the students return in October to present their research at Discovery Day – the annual day of research at Drexel Med. By working closely with the faculty in the different components of the project, the students are provided with valuable research experiences. Students gain knowledge and skills on how to formulate research questions, develop a scientific methodology, analyze data and present research findings at a conference or through publications. As part of the summer internship program, students conduct research projects under the supervision and mentorship of program faculty. We also provided a survey to the students to get feedback on the summer research experience which was useful to improve the program each summer. Some of the improvements made were to include meetings during the summer just for the Cheyney students as they were spread over several labs, adding lessons in professionalism for the students (important as this was the first formal position for many students in which they had a "boss" and "professional colleagues", we added lectures on biotechniques to help improve didactic training component to lab work. The Summer Research Experience (SRE) evaluation rubric that was used to improve the program year to year is shown in Table 3 below.

Table 2. The *Education and Training Component*. This table summarizes to date the accomplishments and mentoring through this grant. This component serves as a mechanism for providing education and training through hands on research experiences and lecture workshops for minority faculty, undergraduate and graduate students at Cheyney, Bryn Mawr and Drexel Universities.

Name of undergraduate Student	SURF Program year	UG University	Faculty Mentor	Awards/Publications	Postgraduation position
Saber Aloui	2011	Cheyney University of Pennsylvania	Felix Kim, PhD (DUCOM Pharmacology)		Graduate May 2012
Deylan Moore	2011 2012	Cheyney University of Pennsylvania	2011-David Weiner, PhD (UPENN Pathology) 2012- Michele Kutzler, PhD (DUCOM Medicine)	Best UG researcher at Cheyney University	Graduate 2012 University of Pittsburgh Intramural Research Training Award (Post-baccalaureate research program for minority students)
Lennan Boyd	2011	Cheyney University of Pennsylvania	Michele Kutzler, PhD (DUCOM Medicine)	*First place poster prize @ Alliance for Minority Participation sponsored by NSF *Honorable mention poster prize @DUCOM Discovery Day 2011	Graduate 2012 Enrolled in Drexel Pathway to Medicine program
Devon Moore	2012	Cheyney University of Pennsylvania	Sonia Navas-Martin, PhD (DUCOM Micro/Immuno)	*Honorable Mention poster prize @ Alliance for Minority Participation sponsored by NSF	Expected graduation December 2013
Name of undergraduate Student	**SURF Program year**	**UG University**	**Faculty Mentor**	**Awards/Publications**	**Postgraduation position**
Ashley Benjamin	2012	Cheyney University of Pennsylvania	Ramesh Raghupathi, PhD (DUCOM, Neurobiology and Anatomy)		Expected graduation December 2013
Ryan Hoffman	2012 2013	Cheyney University of Pennsylvania	2012- Barry Waterhouse, PhD (DUCOM, Neurobiology and Anatomy) 2013- Ramesh Raghupathi, PhD (DUCOM, Neurobiology and Anatomy)		Expected graduation December 2013
Monica Effi	2012 2013	Cheyney University of Pennsylvania	2012- Shira Ninio, PhD (DUCOM Micro/Immuno) 2013- Carol Arlett, PhD (DUCOM Micro/Immuno)	*Second Place poster prize @ Alliance for Minority Participation sponsored by NSF Penn State Hershey research projects	Expected graduation December 2013
Chandra Gadsen	2013	Cheyney University of	Michele Kutzler, PhD DUCOM Medicine)		Expected graduation May 2013
Name of undergraduate Student	**SURF Program year**	**UG University**	**Faculty Mentor**	**Awards/Publications**	**Postgraduation position**
Fatima Jackson	2013	Cheyney University of Pennsylvania	David Weiner, PhD (UPENN Pathology)		Expected graduation May 2013
Jessica Laroda	2013	Cheyney University of Pennsylvania	Cristian Sell, PhD (DUCOM Pathology)		Expected graduation May 2013
Getrude Makurumidze	2013	Bryn Mawr	Michele Kutzler, PhD (DUCOM Medicine)	Bryn Mawr Katherine Hepburn Summer fellowship	Expected graduation May 2016

Table 3. Summer Research Experience (SRE) Evaluation Matrix

Questions	Data Source	Methods	Timeline
1. What effect is the SRE program having on the students with respect to student learning and increased student interest biomedical research?	• Research participants • Program Mentors	• Pre-program and post-program assessments • Performance-based assessments - final project presentations	• At student entry and at the end. of program • Week 1 • Week 10
2. How effective have the recruitment strategies been?	• Data on the student performance in lab work	• Compiling data • Observation • Evaluation of lab performance	• Once • Weekly • Weekly
3. How do the program participants evaluate each one of the program components? • the research experience • the workshops • information about career opportunities • training in data presentation	• SRE Students	• Observation • Interviews • Surveys • Student laboratory performance • Participation in Discovery Day	• Week 5 • Week 10 • Week 10
4. Have the participants developed an increased interest in biomedical research?	• Participating students • Program Mentors	• Survey	• Pre and Post project data • Week 1 • Week 10
5. How effective has the mentoring been?	• Participating students • Program Mentors	• Interview • Survey	• End of project • Week 9-10
6. What are the findings with respect to comparisons of SRE and Non-SRE students with respect to interest in pursuing biomedical research?	• SRE participants • Non-SRE participants	• Interview • Survey	• Follow-up data- beyond the program
7. What are the findings with respect to tracking students beyond graduation with the aim of determining the degree to which the research site experience has been a lasting influence?	• Participating students	• Web-based follow-up survey	• Yearly
8. Overall Program evaluation and obtain input for improvement	• Graduate student mentors • Faculty mentors	• Interviews • Focus group meetings	• After week 10 of the program

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?

___ ___Yes
___X___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?

_____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?

_____Number of subjects originally targeted to be included in the study
_____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
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics

_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____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.)

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
___N/A___ 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:	Date Submitted:	Publication Status (check appropriate box below):
1. Hepatitis C virus NS3/NS4A DNA vaccine induces multiepitope T cell responses in rhesus macaques mimicking human immune responses	Lang Kuhs, K. A., A. A. Ginsberg, J. Yan, R. W. Wiseman, A. S. Khan, N. Y. Sardesai, D. H. O'Connor, and D. B. Weiner.	The journal of the American Society of Gene Therapy 20: 669-678.	September 2011	☐Submitted ☐Accepted ☒ Published
2. Peripheral immunization induces functional intrahepatic hepatitis C specific immunity following selective retention of vaccine-specific CD8 T cells by the liver.	Lang Kuhs, K. A., R. Toporovski, A. A. Ginsberg, A. L. Olsen, D Shedlock, M. P. Morrow, J. Yan, R. G. Wells, and D. B. Weiner.	Human vaccines 7: 1326-1335.	December 2011	☐Submitted ☐Accepted ☒ Published
3. Induction of intrahepatic HCV NS4B, NS5A and NS5B-specific	Lang Kuhs, K. A., R. Toporovski, J. Yan, A. A. Ginsberg, D. J. Shedlock, and D. B. Weiner.	PloS one 7: e52165.	December 2012	☐Submitted ☐Accepted ☒ Published

cellular immune responses following peripheral immunization.				
4. Strong HCV NS3/4a, NS4b, NS5a, NS5b-specific cellular immune responses induced in Rhesus macaques by a novel HCV genotype 1a/1b consensus DNA vaccine.	Latimer, B., R. Toporovski, J. Yan, P. Pankhong, M. P. Morrow, A. S. Khan, N. Y. Sardesai, S. L. Welles, J. M. Jacobson, D. B. Weiner, and M. A. Kutzler.	Human Vaccines & Immunotherapeutics 2014; 10(8):20 - 19; PMID: 24949624; http://dx.doi.org/10.4161/hv.29590	April 2014	☐Submitted ☐Accepted ☒ Published
5. A synthetic HCV genotype 1a/1b consensus DNA vaccine elicits functional, long-lived, memory T cells *in heparo*.	Brian P. Latimer, Ebony N. Gary, Scott M. Baliban, Jian Yan, XiaoYang, Linda B. Couto, Katherine High, Niranjan Y. Sardesai, Jeffrey M. Jacobson, David B. Weiner and Michele A. Kutzler	Under review	November 2014	☒Submitted ☐Accepted ☐ Published
6. Development towards a prophylactic hepatitis C virus vaccine.	Brian Latimer and Michele Kutzler	Under review	August 2014	☒Submitted ☐Accepted ☐ Published

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

Yes___ _____ No_____X_____

If yes, please describe your plans:

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 thus far. Although outside the scope of this grant, we look forward to the clinical translation of the work in the clinical trials supported under this grant.

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.

Results of these assays investigating NS3/4A, NS4B and NS5A plasmids in mouse and Rhesus macaque models have been published [1-5] and the next step will be to test the vaccine plasmid formulation in the clinic for safety then efficacy.

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_____ No___X_____

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:

b. Name of Inventor(s):

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___ No____

If yes, indicate date patent was filed:

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____
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____

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____

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____X_____ No_____

If yes, please describe your plans:

In addition to the published preclinical studies, we will seek funding sources to aid in supporting our clinical trials to test vaccine in human cohorts (study 1: PI; Sang Hoon Ahn, M.D, Ph.D. - Yonsei University Severance Hospital and Jeong Heo, M.D, Ph.D. - Pusan National University Hospital; Recruiting: Phase I Trial to Evaluate the Safety, Tolerability and Immunogenicity of VGX-6150 for Second-line Therapy of Chronic Hepatitis C Infection (VGX-6150-01)). The second trial that will take place will be funded by NCI Division of Cancer Prevention as Dr. Jeff Jacobson and his Clinical Trial Unit has been added as an approved partner to the Phase 0/I/II Cancer Prevention Clinical Trials Program (Consortia for Early Phase Prevention Trials). If successful in clinical trials, the vaccine will be moved forward in clinical development with current partners (NIH and GeneOne Life Sciences) and potential future partners. The vaccine constructs being tested in this research award were previously reported in patent filings and licensed to Inovio Pharmaceuticals. Inovio has also filed several patent applications and has several issued patents covering the underlying electroporation based delivery technology being deployed in this research. Much of the current development work is being performed using underlying technologies brought to the partnership by the three research partners. However, we will be filing new patent applications as new data emerge.

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.*

All Key investigators biosketches were included in the original grant application. The collaborative research project involved a number of leading scientists in the field of hepatitis

research and vaccine development and includes a number of collaborating organizations from academia and industry. The team involved in this study shared infrastructure, resources and expertise with the common goal of developing a DNA based vaccine for the prevention of HCV-associated cancer, as well as mentoring future research scientists in highly translational bench research. Oversight of the research consortium was directed by Dr. Jeffrey M. Jacobson, the Chief of the Division of Infectious Diseases and HIV Medicine at the Drexel University College of Medicine in Philadelphia. The other participating institutions are the University of Pennsylvania and Cheyney University. Drs. Michele A. Kutzler (Project 1) of DUCOM and David Weiner (Project 2) of the University of Pennsylvania provided the immunology laboratory expertise and performed the immunologic assays for the preclinical studies outlined in aim 1. Inovio is the manufacturer of the vaccine and provider of the electroporation equipment. Dr. Niranjan Sardesai led the Inovio team on the program (Project 3). Dr. Seth Welles of the Drexel University School of Public Health provided the statistical input to the design of the study. Dr. Michele Kutzler coordinated and supervised the educational core research training component in aim 2. The educational core entails a collaborative effort between DUCOM and Cheyney University (PI: Sakkar Eva, Ph.D.) and includes training and collaborative research related to the development and testing of DNA vaccines.