Drexel University

Annual Progress Report: 2009 Nonformula Grant

Reporting Period

July 1, 2013 – May 31, 2014

Nonformula Grant Overview

The Drexel University received $2,769,497 in nonformula funds for the grant award period June 1, 2010 through May 31, 2014. Accomplishments for the reporting period are described below.

Research Project: Project Title and Purpose

Therapeutic DNA vaccine for the Prevention of Hepatitis C Virus-associated Cancer - The main object 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.

Duration of Project

6/1/2010 - 5/31/2014

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.

Principal Investigator

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Other Participating Researchers (updated)

Michele A. Kutzler, PhD, Seth L. Welles, PhD, Scott Baliban (PhD candidate), Brian Latimer (MD, PhD candidate), Ebony Gary (PhD candidate), Mariana Bernui, PhD (postdoctoral fellow) – employed by Drexel University
David B. Weiner, PhD, Daniel Choo, PhD, Bernadette Ferraro, PhD, Michael J. Merva – employed by University of Pennsylvania
Sakkar A. Eva, PhD, Abdel A. Bior, PhD, undergraduate students (Jessica Laroda, Chandra Gadsen, Monica Effi, Ryan Hoffman, Fatima Jackson) – employed by Cheyney University
Niranjan Y. Sardesai, PhD, Amir S. Khan, PhD – employed by Inovio Biomedical Corporation

Expected Research Outcomes and Benefits

With more than 170 million individuals currently infected, hepatitis C virus (HCV) infection is a global pandemic, effecting 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

Progress for Specific Aim #1: We 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 mice; nonhuman primates). In the past budget year we completed the preclinical nonhuman primate study analysis (Human Vaccines & Immunotherapeutics HV0011R 2014, Volume 10 Issue 8). In this published study, we aimed to maximize vaccine recipients' opportunity to induce a broad T cell response with a novel antigenic sequence, multi-antigen vaccine strategy. We generated DNA plasmids encoding *consensus* sequences of HCV genotypes 1a and 1b non-structural proteins NS3/4a, NS4b, NS5a, and NS5b. Rhesus macaques were used to study the immunogenicity of these constructs. Four animals were immunized 3 times, 6 weeks apart, at a dose of 1.0mg per antigen construct, as an intramuscular injection followed by in vivo electroporation, which greatly increases DNA uptake by local cells. Immune responses were measured 2 weeks post-immunization regimen in immunized rhesus macaques and showed a broad response to multiple HCV nonstructural antigens, with up to 4680 spot-forming units per million peripheral blood mononuclear cells (PBMCs) as measured by Interferon-γ ELISpot. In addition, multiparametric flow cytometry detected HCV-specific CD4+ and CD8+ T cell responses by intracellular cytokine staining and detected HCV-specific CD107a+/GrzB+ CD8+ T cells indicating an antigen specific cytolytic response 2 weeks PIR compared with baseline measurements. At the final study time point, 6 weeks PIR, HCV-specific CD45RA- memory-like T cells remained detectable in peripheral blood. Data presented in this manuscript support the notion that vaccine immunogenicity studies using a macaque model can be used to depict key anti-HCV nonstructural antigenic cellular immune responses and support the development of DNA-based prophylactic HCV vaccines.
Immunization with all 4 antigenic HCV DNA plasmids induces strong and broad HCV-specific T cell response in both the spleen and liver. While data presented in Human Vaccines & Immunotherapeutics publication indicate strong and broad systemic IFNγ responses elicited by peripheral IM+EP immunization, it is possible that HCV-specific T cells might become dysfunctional once trafficked to the immunosuppressive milieu of the liver upon HCV infection. HCV-specific T cells must remain functional in the liver for our HCV vaccine to be efficacious. Therefore, in the past budget year we sought to demonstrate that HCV-specific T cells primed in the periphery by vaccination will remain functional, in terms of cytokine production and CTL killing, once they have trafficked to the liver. A table detailing immunization groups used for all experiments is shown in Figure 1A, and the immunization timeline schematic illustrating that mice were immunized intramuscularly, followed by *in vivo* electroporation (E.P.), three times, separated by three weeks is shown in Figure 1B. Immune responses were assayed 7 days

following the final immunization c. We performed ELISpot (Figure 1C), intracellular staining assays (ICS Figure 1D) and acquired cell phenotype data by flow cytometry. Splenocytes and liver lymphocytes (LL) 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, as well as intracellular cytokines IFNγ, tumor necrosis factor-alpha (TNFα), and interleukin-2 (IL-2). 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 peptide-induced cytokine production. Statistically significant frequencies of cytokine-producing lymphocytes were detectable in both the Th1 and CTL compartments, within both the spleen and liver of each cytokine analyzed, when compared to either control group ($p<0.05$; Figure 1D). Importantly, IFNy+ CTL are correlated with acute resolution in natural HCV infection, and essential for HCV clearance in the chimpanzee model. Immunization induced IFNy+ CTL frequencies of 7.57% [6.78-8.35; 95% CI] and 4.09% [1.54-6.64] in the spleen and liver, respectively. Additionally, TNFα+ CTL frequencies of 7.41% [5.33-9.48] and 5.03% [4.27-5.78] and IL-2+ CTL frequencies of 0.51% [0.40-0.63] and 0.80%[-0.14-1.73] were detectable in the spleen and liver, respectively (Figure 1D). Whereas, CTL frequencies within either the pVAX of EnvC groups ranged from undetectable to 0.19% [0.08-0.29] in the spleen and liver for all cytokines studied. These data illustrate that immunization induces HCV-specific T cell responses of substantial magnitude and broad reactivity to regions spanning all four HCV antigens in both the spleen and liver. *In heparo generation of NS3/4A-expressing hepatocytes.* Although we have demonstrated that HCV-specific T cells can be stimulated to perform effector functions ex vivo, 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 HCV 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. 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. *Rapid memory recall responses clear antigen from the liver.* 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 (pVAX and EnvC) animals (Figure 2A). While control animals maintained robust expression of HCV NS3/4A-HA by IF staining of frozen liver sections, few antigen-positive (Anti-HA red) hepatocytes remained in HCV-immunized animals (Figure 2B). Most of the remaining antigen-positive hepatocytes observed in HCV-immunized animals displayed abnormal nuclear morphology as *ring-like* due to DNA condensation, implying apoptosis. When antigen expression was measured by mean fluorescence intensity (MFI), HCV-immunized animals

exhibited significantly less MFI than either pVAX or EnvC groups (Figure 2E). 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 3F). Clearance of antigen-expressing hepatocytes also coincided with a robust IFNγ response, as measure by 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 2D). The memory T cell recall response observed was composed of both HCV-specific CTL and Th1 cells, as demonstrated by ICS (Figure 2G). 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 either 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 2C; 2F). These data demonstrate that immunization elicits long-lived functional memory T cells *in heparo*, able to rapidly kill HCV antigen-expressing hepatocytes. *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, and subsequent associated sequelae. Because HCV-associated disease is largely a result of chronic liver inflammation, it is important that the inflammatory response subsides following an immunological insult. 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 by day 30. Liver sections from HCV-immunized mice closely resembled those of pVAX and EnvC groups (Figure 3A). Additionally, TUNEL-positive nuclei were undetectable in any immunization group (Figure 3C). In contrast, when frozen sections were stained for NS3/4A-HA expression, control animals continued to exhibit robust antigen expression, while antigen (anti-HA Red) was completely unobservable in sections of HCV-immunized mice (Figure 3B). 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 3D). Resolution was also apparent by serum ALT activity of HCV-immunized mice, which was indistinguishable from control animals (Figure 3F). Finally, in the interest of long-lived protection, we enumerated the number of HCV-specific central memory 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 3E). 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.

Progress for Specific Aim #2: This component serves 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. June 2013 through August of 2013 was the third and final summer session of the Education and Training Component in which 6 Cheyney University students (Chandra Gadsen, Fatima Jackson, Jessica Laroda, Monica Effi, and Ryan Hoffman) 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) on 8/17/2013, at Drexel Univ. College of Medicine's 2013 Discovery Day Research Symposium to be held 10/4/2013, and at the Alliance for Minority Participation sponsored by NSF in 10/2013. As shown in Table 1, 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.

Table 1. 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	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 2012-University of Pittsburgh Intramural Research Training Aw (Post-baccalaureate research prog for minority students) 2014- Selected from competitiv applicant pool and now enrolled Drexel Pathway to Medicine prog
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 2013- Selected from competitiv applicant pool and now enrolled Drexel Pathway to Medicine prog
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 Received *best undergraduate researcher* at Cheyney University senior awards 2013	Expected graduation December 2
Ashley Benjamin	2012	Cheyney University of Pennsylvania	Ramesh Raghupathi, PhD (DUCOM, Neurobiology and Anatomy)		Expected graduation December 2
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 2
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 2
Chandra Gadson	2013	Cheyney University of Pennsylvania	Michele Kutzler, PhD DUCOM Medicine)	*Third place oral presentation prize @ Alliance for Minority Participation sponsored by NSF *Selected for summer research SUNY; 2014	Expected graduation December 2
Fatima Jackson	2013	Cheyney University of Pennsylvania	David Weiner, PhD (UPENN Pathology)		Expected graduation May 201
Jessica Laroda	2013	Cheyney University of Pennsylvania	Cristian Sell, PhD (DUCOM Pathology)		Expected graduation May 201
Gertrude Makurumidze	2013	Bryn Mawr	Michele Kutzler, PhD (DUCOM Medicine)	Awarded Bryn Mawr Katherine Hepburn Summer fellowship *selected for summer research UPENN; CHOP 2014	Expected graduation May 201
Dr. Abdel Bior	2011, 2012, 2013	Assistant Professor, Cheyney University of Pennsylvania	Michele Kutzler, PhD (DUCOM Medicine)		

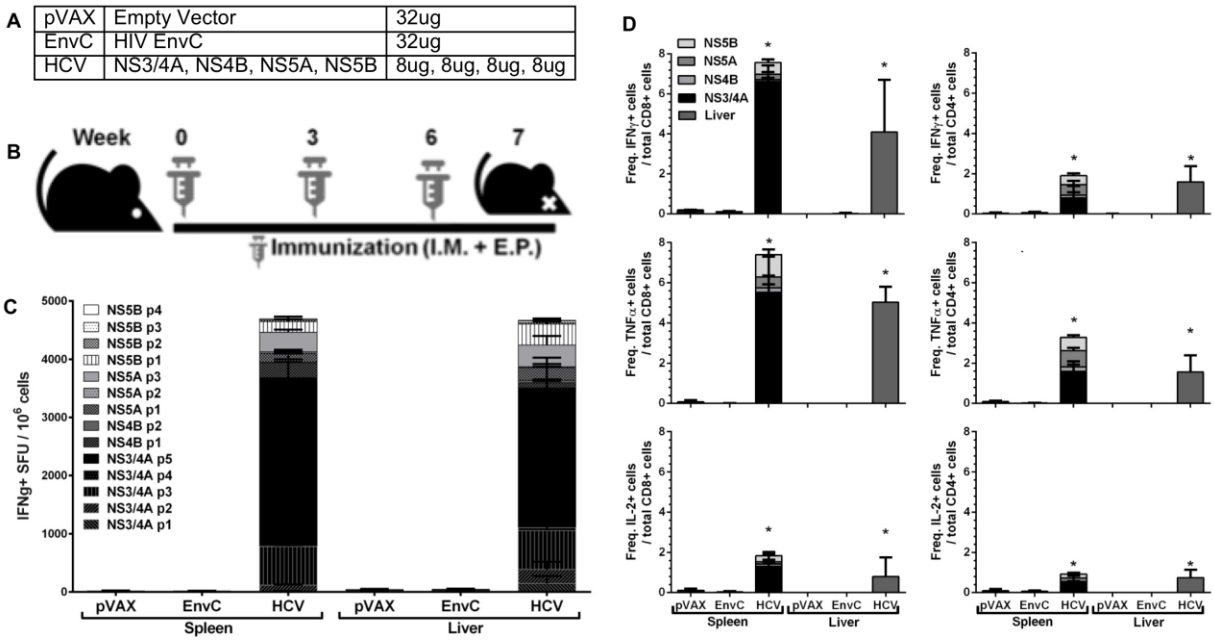

Figure 1. *Immunization elicits strong and broadly reactive HCV-specific CTL and Th1 responses* - A. Table detailing immunization groups used for all experiments. The HCV group received a combination of 8ug ConNS3/4A, 8ug ConNS4B, 8ug ConNS5A, and 8ug ConNS5B (32ug total DNA). Two control groups each received 32ug of their respective plasmids; pVAX, which is the empty backbone used in all other plasmids, and HIV-1 envelope C (EnvC), which served as an irrelevant antigen. B. 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 D. Stacked IFNγ ELIspot results from the spleen and liver depicting the magnitude of total responses, and the breadth of reactivity by HCV peptide pools. d. Frequencies of HCV-specific CD8+ (*left*) and CD4+ (*right*) isolated from the spleen and liver, defined by production of cytokines, IFNy (*top*), TNFα (*middle*), and IL-2 (*bottom*), in response to HCV peptide stimulation. Error bars represents standard deviation. * $p<0.05$.

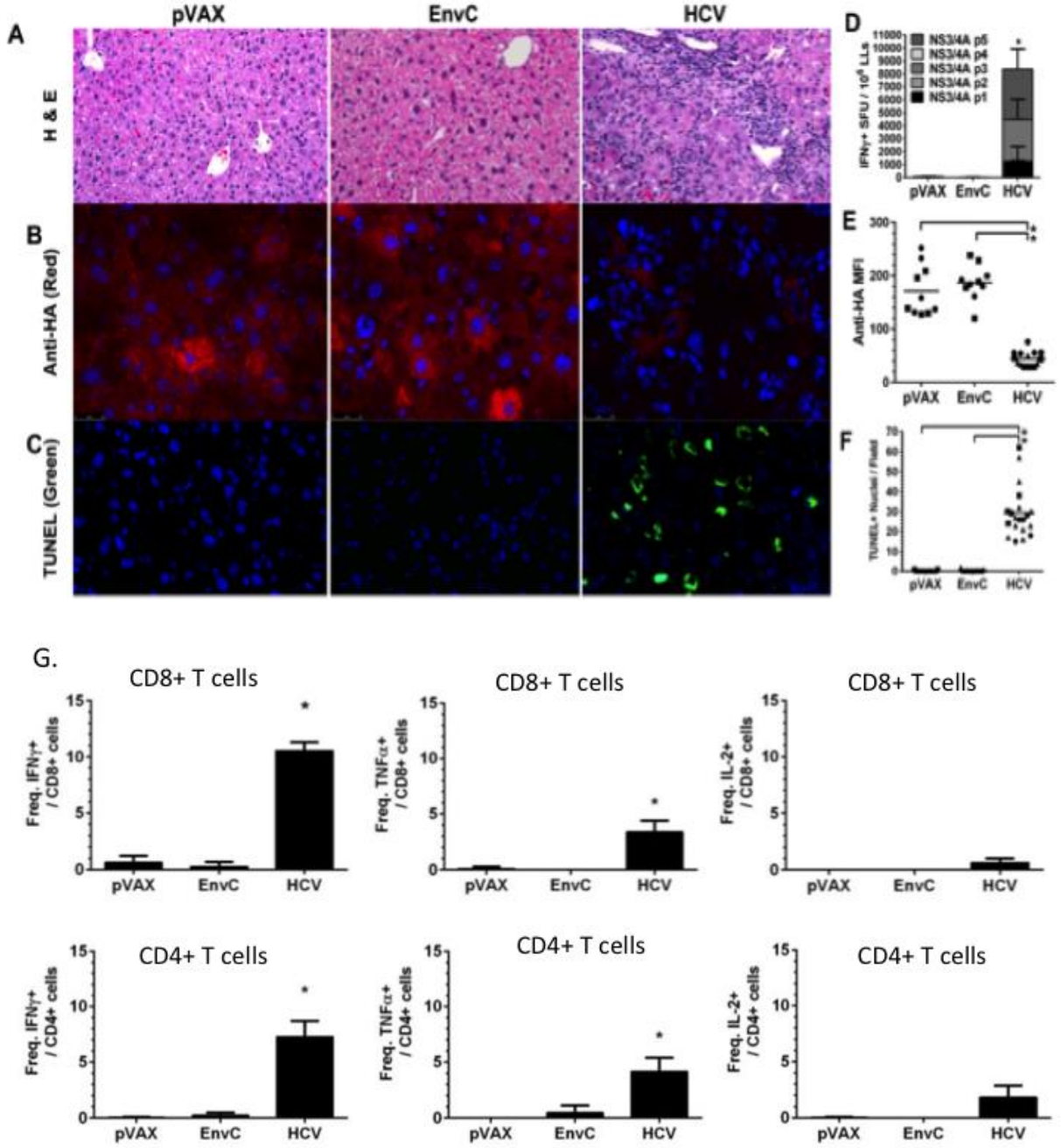

Figure 2. *HCV Antigen is cleared in the liver by day 7 following AAV-HCV-NS3/4A-HA injection* - Mice, immunized and rested for 20 weeks, were administered $2x10^{11}$vg AAV-HCV-NS3/4A-HA. On day 7 post-AAV injection, perfused livers were excised to assess acute HCV-specific functional memory T cell 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 stainining. d, Quantification of HCV-specific IFNγ-secreting liver lymphocytes in response to antigen-matched peptide pool stimulation by ELIspot assay. e, Mean fluorescence intensity (MFI) of NS3/4A-HA protein IF staining. Bars represent grand means. f, Quantification of TUNEL-positive nuclei per field. Bars represent grand means. g, Frequencies of HCV-specific CD8+ and CD4+ T cells, as measured by production of IFNγ, TNFα, or IL-2, in response to antigen-matched peptide pool stimulation. Error bars represents standard deviation. * $p<0.05$.

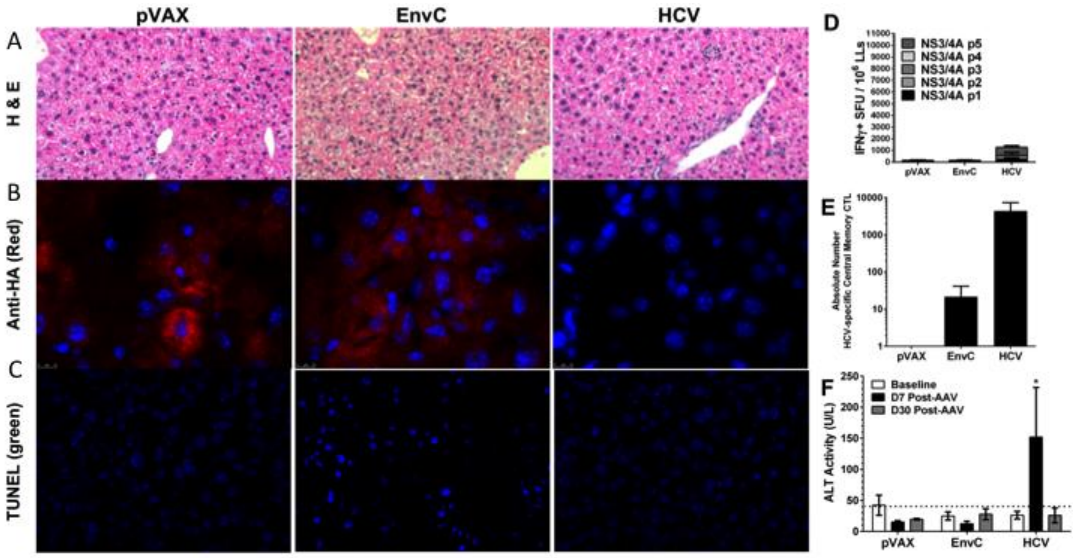

Figure 3. *Inflammation resolves by day 30 following AAV-HCV-NS3/4A-HA injection* - Mice, immunized and rested for 20 weeks, were administered $2x10^{11}$vg AAV-HCV-NS3/4A-HA. On day 30 post-AAV injection, perfused livers were excised to assess resolution of HCV-specific functional memory responses observed 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). 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.