Title: Effects of Persistent Innate Immune Activation on Vaccine Efficacy

NCT Number: NCT02429583

Last Approval Date: 12/08/2018

11.0 Objectives and Rationale

11.1 * Overview

Briefly state the ***purpose of this study***. Give enough background and rationale to provide both scientists and lay members of the IRB and ACCTS with the basis for exposing human participants to the risks involved.

Vaccines have been responsible for preventing millions of deaths and extending the average human lifespan. A better understanding of the factors that contribute to vaccine success should provide a useful blueprint for improving current vaccines and developing new vaccines against prevalent and emerging pathogens. Essential in the innate immune control of viral infections, type I interferons are traditionally described as immunostimulatory cytokines. However, emerging evidence suggests a paradoxical role for type I interferons in suppressing certain immune functions. Chronic infection by hepatitis C virus (HCV) causes constitutive interferon stimulation. Although not considered immunocompromised, chronic HCV patients demonstrate high failure rates to vaccination against hepatitis B virus (HBV). We hypothesize that this poor response is due to immunomodulatory effects of persistent interferon activation by chronic

HCV infection. We have conceived a clinical study to test this hypothesis, in which we will perform a comprehensive examination of the HBV vaccine response in patients chronically infected with HCV. We will recruit chronic HCV patients and healthy controls for HBV vaccination, measure their innate immune status by interferon-stimulated gene expression in peripheral blood, and determine its association to HBV vaccine response. We will then characterize those immune mechanisms compromised in chronic HCV by assessing the HBV-specific adaptive immune response, and the innate inflammatory response to vaccine antigen and adjuvant. These studies will enhance our understanding of the immunomodulatory effects of chronic viral infection, establish determinants of effective vaccine responses, and help guide vaccination strategies for HCV patients and other individuals with chronic inflammatory disease.

11.2 * Engaging Stakeholders: Describe any plans to engage other stakeholders (Scientists, practitioners, patients, advocacy groups, etc.) for hypothesis generation, or feasibility purposes.

This study was developed with input from clinicians and basic research scientists in the Center for the Study of Hepatitis C Virus, a cooperative endeavor of the Rockefeller University, Weill Cornell Medical College and New York –Presbyterian Hospital. Biostatisticians in the Rockefeller University Center provided additional guidance regarding statistical considerations and study design for Clinical and Translational Science.

11.3 * Hypothesis

Describe the ***research hypothesis*** in a single sentence.

We hypothesize that poor response to HBV vaccination in chronic HCV patients is associated with elevated expression of interferon-stimulated genes (ISGs) in peripheral blood mononuclear cells (PBMCs).

11.4 * Aim(s)

Indicate how you will ***address the hypothesis*** (e.g., to compare groups, to estimate a parameter, to ascertain feasibility). Since the sample size determination is usually based on the primary aim only, the primary aim should be sufficient to justify the study.

Aim 1: What is the relationship of persistent innate immune signaling to HBV vaccine non-response?
In order to assess the effects of persistent IFN signaling on an adaptive immune response *in vivo*, we will administer the HBV vaccine series to patients chronically infected with HCV pre-treatment and healthy volunteers. Vaccine response will be assessed by serum anti-HBsAg antibody titers. We will measure baseline and post-vaccination serum cytokines and total PBMC ISG expression signatures, and analyze their relationship to HBV vaccine response

Aim 2: What aspects of the adaptive immune response to HBV vaccination are compromised in chronic HCV infection?
Response to HBV vaccination is typically assessed by HBsAg antibody titers measured after completion of the vaccine series. A more detailed characterization of the adaptive immune response (or lack there of) to HBV vaccination in the context of chronic HCV is a necessary prerequisite to better understand the underlying mechanisms. Using PBMC samples collected from a subset of patients (Aim 1), we will assess the frequency and function of HBsAg-specific B cells by ELISPOT assay. We will also compare the immunophenotype of HBsAg-specific $CD4^+$ T cells from vaccine responders and non-responders by *ex vivo* stimulation and multiparametric flow cytometry analysis for activation markers and cytokine production with particular focus on $CD4^+$ T cell subsets important for humoral immunity.

Aim 3: Does chronic HCV infection modulate the innate immune response to HBV vaccination?
In order to assess if persistent IFN stimulation in chronic HCV infection impairs innate immune cell function and in turn, the immune response to HBV vaccination, we will isolate monocytes and dendritic cells in pre and post vaccination PBMC samples from subsets of healthy, HCV-infected vaccine responders, and HCV-infected vaccine non-responders. In *ex vivo* monocyte experiments, we will measure inflammasome dependent and independent responses to vaccine antigen, adjuvant (Alum), and related stimuli. Using small numbers of peripheral blood conventional dendritic cells, we will use low-input RNA-Seq to measure baseline levels and vaccine-induced changes in ISG signature and inflammasome components in order to determine those factors associated with effective vaccine response and those that are compromised in chronic HCV infection.

Aim 4 What risk factors associated with chronic HCV infection and its complication is linked to immune activation or modulation?
In order to evaluate the correlation between the factors associated with chronic HCV infection and persistent immune activation or modulation with HBV vaccine non-response, a detailed health questionnaire has been added to this study to increase the ability to correctly understand and interpret the predicted outcomes of the study.

11.5 * Primary Outcome(s)

Indicate which ***variable(s)*** will be assessed to judge the primary specific aim. Give measurement units, if applicable.

In order to determine the relationship of persistent innate immune signaling to HBV vaccine non-response, we will measure:

-**HBV vaccine response versus non-response status** (seroconversion, effective response defined as anti-HBsAg antibody titers 10 IU/L)

-**Serum biomarkers of innate immune activation** (Luminex assay for multiplex cytokine / chemokine panel)

-**Interferon-stimulated gene (ISG) expression in PBMCs** (RNA-Seq with analysis focus on curated ISG list defined in the Rice laboratory)

11.6 * Secondary Outcome(s)

Indicate which ***additional variable(s)*** will be assessed to judge the secondary outcome(s). Give measurement units, if applicable.

In order to determine which aspects of the adaptive immune response to HBV vaccination are compromised in chronic HCV infection (Aim 2), we will measure:

-**Serum anti-HBsAg antibody titers post-vaccination doses over time** (IU/L, and change in titers between time points)

-**Frequency and functional status of anti-HBsAg antibody-producing B cells post-vaccination doses over time** (ELISPOT assays)

-**Frequency and functional status of HBsAg-specific CD4+ "helper" T cells** (flow cytometry assays)

In order to determine if chronic HCV infection modulates the innate immune response to HBV vaccine antigen or adjuvant (Aim 3), we will measure:

-**Functional response of monocytes (isolated from patient PBMCs) stimulated ex vivo with vaccine antigen and/or adjuvant**

-**Gene expression profile of conventional dendritic cells (isolated from patient PBMCs) measured by RNA-Seq**

11.7 * Methods and Procedures

Please provide a description of the laboratory and clinical analyses and procedures that will be performed. Include the role of external collaborators and consultants when appropriate. Please refer to Help text for Guidance.

Aim 1: What is the relationship of persistent innate immune signaling to HBV vaccine non-response?

Given the risk of hepatic superinfection, HBV vaccination is recommended in all patients diagnosed with chronic HCV. With the support of Weill Cornell Medical College (WCMC) clinicians in the Center for the Study of Hepatitis C (a multidiscplinary center at The Rockefeller University and WCMC), and the clinicians from the Bellevue Hospital Hepatology/Infectious Disease clinic, we refer patients with chronic HCV without a history of HBV infection or vaccination, for which HBV vaccination is indicated. Patients identified at WCMC will be asked to call 1-800-RUCARES (1-800-782-2737) if they are interested in participating in this study. All research activities will be performed at the Rockefeller University outpatient clinic therefore, WCMC and Bellevue Hospital are not considered engaged institutions in this research. The hope is to also recruit chronic HCV patients identified through the Rockefeller research volunteer repository database.

Exclusion criteria will include clinical, laboratory, or biopsy evidence of cirrhosis, detectable anti-HBsAg titers at baseline, and history of HIV infection. We will also recruit 30 healthy volunteers with no history of HCV, HBV infection, or HBV vaccination. Prior to initiating vaccination, we will collect comprehensive clinical data on all study participants, including age, gender, complete metabolic panel, complete blood counts, liver function tests, and uric acid levels. In addition, for the chronic HCV patients we will send blood for HCV viral load, HCV genotype, IFNL3/4 SNP genotype, and obtain a liver biopsy fibrosis score (if available - no biopsies will be performed as part of this study).

Patients will be vaccinated with the HBsAg vaccine (Recombivax HB; Merck & Co., Inc.) at standard dose (10 g) and standard schedule (three doses: 0, 1 month, 6 month). We will collect peripheral blood samples on a defined schedule, with time points at -5 to -3 weeks pre-vaccination, 0 weeks (vaccine dose #1), 3 days, 4 weeks (vaccine dose #2), 6 weeks, 24 weeks (vaccine dose #3), and 30 weeks. Volunteers will recive a telephone callor email from a member of the research team at week 26 to access their health status. The approximate total amount of research blood from 112 ml to 180 ml (~ 7½ to 12 tablespoons) will be obtained at certain study visits. In compliance with IRB guidelines and appropriate ethical practice, and ensuring sufficient material for all experiments proposed, the total amount of research blood collected will not exceed 550 ml over an 8-week period. All peripheral blood samples will be separated into components (PBMC, plasma), aliquoted and cryopreserved. We will measure HBsAg antibody titers at several time points throughout the study (weeks 6, 24, and 30), in order to track the antibody response and to monitor for a possible memory response, as would be present in a subject who incorrectly reported no history of HBV vaccination. In such a case, if a subject is found to have a dramatic increase in anti-HbSAg antibody titers (>40 IU/L) at the 4 week time point (following a single vaccine dose) characteristic of immune memory (indicating previous vaccination), additional history will be taken and that subject may be excluded from the remainder of the study.

Volunteers will be asked to undergo urine drug testing at study visits Screen I, Days 0, 28, and 168. Individuals who have a positive drug screen (except for Methadone use) at the Screen I visit will be considered screen failed. Individuals who have a negative drug screen at Screen I but have a positive drug screen at one of the subsequent drug testing visits, will remain in the study, continue to receive the remainder of the Hepatitis B vaccination series, and blood will be drawn for research purposes.

At screening visit I, the Hepatitis C volunteers will have an additional 8.5 ml of blood drawn for HCV viral load and genotype which will be sent to the commericial lab, LabCorp, for analysis.

Starting with the second screening visit, all participants will be asked to fast for approximately 8 hours prior to their clinic visit in an effort to minimize variability in gene expression.

The study visit window is as follows: for Days 0, and 3 visits there will be no visit window; for Weeks 4 and 6 there will be +/- 3 days visit window; Week 24 there will be a +/- 14 days window and for Week 30 there will be a +/- 7 days visit window.

There are no known additional risks of HBV vaccination in chronic HCV patients relative to healthy control patients.

We will **evaluate clinical response to HBV vaccination by measuring HBsAg antibody titers in serum collected at the 30 week time point** (6 weeks after completing vaccine series). Based on HBsAg titers, patients will be assigned to HBV vaccine responder status (seroconversion, defined as anti-HBsAg 10 IU/L) and HBV vaccine non-responder status (anti-HBsAg < 10 IU/L). Thus, we will use these data to divide our patient cohort into **three experimental groups**: 20 **chronic HCV^{+}/HBV vaccine non-responder patients (HCV-NR)**, 20 **chronic HCV^{+}/HBV vaccine responder patients (HCV-R)**, and 20 age (+/- 5 years) and gender matched **healthy/HBV vaccine responder patients (CTRL-R)**. We will also use these data to determine the response rate to HBV vaccination in our patient cohort, a value that has been reported as highly variable in different chronic HCV populations.

In order to assess the relationship of chronic viral immune activation and HBV vaccine response, we will measure markers of innate immune signaling in both serum and PBMCs collected prior to, and immediately following vaccine dose #1. We will quantify serum cytokines by Luminex microbead-based immunoassay using a multiplex cytokine/chemokine panel that includes markers of inflammation, IFN response and Th1/Th2 response (IFN-alpha2, IFN-gamma, CXCL10, IL-10, IL-12(p40), IL-12(p70), IL-1beta, IL-2, IL-4, IL-6, TNF, and FLT3L). In order to measure IFN signatures in PBMCs, we will use RNA-Seq with a focused analysis targeting ISGs, inflammation markers, and immune signaling components. We will quantify expression levels in relation to a reference standard of External RNA Control Consortium (ERCC) controls – a calibrated panel of defined RNA molecules at defined concentrations that will be "spiked in" to each library preparation to serve as a universal reference across samples [50].

Other Reseach Assessment

Fibrotest or Fibrosure is a blood test that combines the age and sex of the patient with a set of six parameters for the assessment of fibrosis in chronic liver disease. Serum from the patient is analyzed in a CLIA certified reference laboratory. The set of parameters analyzed includes alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, gamma-glutamyl transpeptidase (GGT), total bilirubin and ALT levels using an equation originally designed by Biopredictive. It has been validated for chronic hepatitis C with 85% sensitivity especially for the evaluation of the early stages of fibrosis.

Overall, it is significantly less invasive and expensive than the gold standard of liver biopsy.

References:
1.) Halfon et al, FibroTest-ActiTest as a non-invasive marker of liver brosis. Gastroenterol Clin Biol 2008; 32:22-38

2.) Poynard et al, Standardization of ROC curve areas for diagnostic evaluation of liver fibrosis markers based on prevalences of fibrosis stages. Clin Chem. 2007;53:1615-1622

A Fibroscan procedure will be performed on all Hepatitis C participants one time over the course of the study. Fibroscan or transient elastography is a non-invasive ultrasound of the liver. The transducer and probe of the ultrasound device measures the velocity of the shear wave as it passes through the liver (1, 2). This measure is converted to a liver stiffness measurement. It has been validated extensively in assessing hepatitis C virus infection associated liver fibrosis. It is significantly less invasive than a liver biopsy (1, 2). The combination of Fibroscan and serum blood test such as Fibrosure increases the sensitivity and specificity of identifying hepatic fibrosis in stages F2, F3 and F4 (2).

References:
1-Foucher et al., Diagnosis of cirrhosis by transient elastography (Fibroscan): a prospective study. Gut. 2006, Mar;55(3):403-8

2-Nguyen-Khac and Capron, Noninvasive diagnosis of liver fibrosis by ultrasonic transient elastography (Fibroscan). Eur J Gastroenterol Hepatol. 2006 Dec;18(12):1321-5

Aim 2: What aspects of the adaptive immune response to HBV vaccination are compromised in chronic HCV infection?

We will perform a systematic characterization of the adaptive immune response to HBV vaccination in the patient groups (HCV-NR, HCV-R, CTRL-R) identified and measured for IFN signature in Aim 1. As antibody response to HBV is typically assessed as a single endpoint, we will begin by measuring anti-HBsAg antibody titers (ELISA), along a full time course (0 weeks, 3 days, 4 weeks, 6 weeks, 24 weeks, and 30 weeks). We will direct our analyses both to absolute measures of antibody titers and changes over time, particularly changes immediately following each vaccine dose. In this manner, we aim to better define HBV non-response in chronic HCV as a quantitative (overall lower titers in HCV-R and HCV-NR groups compared to CTRL-R), qualitative (diminished induction of antibody production following vaccine doses 2 and 3 in HCV groups), and/or categorical (failed antibody response in HCV-NR, equivalent antibody response in HCV-R and CTRL-R) defect.

In order to assess the role of B cells in HBV vaccine non-response, we will evaluate the frequency and functional status of anti-HBsAg antibody-producing B cells at several time points following initial and subsequent vaccine doses. We will measure the frequency of anti-HBsAg IgM- and IgG- producing B cells by established ELISPOT assay [84,85] at 0, 4, 6, and 30 week time points.

Although $CD8^+$ cytotoxic T cells do not make a significant contribution to HBV vaccine response, functional $CD4^+$ T cells are required for a protective response [62-64]. In order to assess the role of $CD4^+$ T cells in HBV vaccine non-response, we will take several complementary approaches to evaluate different relevant functional cellular subsets. First, we will quantify $CD4^+$ T cell subsets at time points pre- and post- vaccination by flow cytometric immunophenotyping. Immunophenotyping analysis will focus on subset numbers and changes in the distribution of different subsets in response to vaccination. Next, we

will assess the frequency and cytokine profile of HBsAg-specific $CD4^+$ T cells, thereby determining the quality of the T cell response in different vaccine-response patient groups and subsequently relating it to ISG expression signatures (Aim 1). Using established peptide libraries [59,62]to bypass the need for antigen processing, we will stimulate PBMCs (HCV-NR, HCV-R, CTRL-R; time points 0, 4, 6, 24, and 30 weeks) with HBsAg peptides and supplemental co-stimulation via anti-CD28 and anti-CD49a antibodies. Activated, antigen-specific $CD4^+$ T cells will be identified via upregulation of CD154 combined with intracellular staining for cytokines (ICS) according to an established assay [86]. A panel of cytokine markers will be used to identify which, if any, $CD4^+$ T cell subsets are functionally impaired in HBV vaccination in the context of chronic HCV: T_H1 (IFN-gamma), T_H2 (IL-4), T_H17 (IL-17), and/or T_{FH} (IL-21).

Aim 3: Does chronic HCV infection modulate the innate immune response to HBV vaccine antigen or adjuvant?

In order to evaluate the effects of chronic HCV on innate immune signaling functions, we will perform a series of *ex vivo* stimulation experiments on monocytes isolated from the patient groups described above (HCV-NR, HCV-R, CTRL-R; Aims 1, 2). Monocytes are readily available in large numbers from PBMC samples and are an important cell type for inflammasome signaling *in vivo* [101]. We will characterize monocyte frequency and activation status in patient groups prior to and immediately following vaccination (time points 0 weeks and 3 day) by flow cytometry, assessing expression of CD14, CD16 (proinflammatory monocytes), PD-1 (upregulated by monocyte activation), PD-L1, TIM3, and HLA-DR [102-104]. Next, we will perform *ex vivo* stimulation experiments to evaluate potential differences in the capacity of monocytes from each patient group to mount a proinflammatory response to treatment with adjuvant or other stimuli, using a variety of assays including ELISA, Western Blot and qRT-PCR. Compared across groups and relative to IFN gene expression signatures (Aim 1), these results will identify those innate immune pathways impaired in chronic HCV and their possible association with HBV vaccine failure.

Although monocytes are important sensors of inflammatory stimuli and produce many immune mediators in response to adjuvant, it is likely that dendritic cells are directly responsible for vaccine antigen presentation and T cell stimulation. In order to determine the DC contribution to HBV vaccine response in chronic HCV infection, we will focus our efforts on *bona fide* cDCs. As we have previously detected reduced numbers of cDCs in the peripheral blood of patients chronically infected with HCV[92], we will begin by using flow cytometry to enumerate the frequency (BDCA-1^+ and BDCA-3^+) and activation status (CD83, CD86, HLA-DR, PD-L1, TIM3) of cDCs in PBMC samples collected from the three patient groups subdivided in Aim 1 (HCV-NR, HCV-R, CTRL-R) at time points preceding and immediately following the initial HBV vaccine dose (0, and 3 days). These experiments will indicate whether there are any gross defects in cDC function during HBV vaccination of chronic HCV patients. However, potential alterations in cDCs are likely to be subtle and beyond the scope of these flow cytometry assays.Given the extremely low frequency (< 0.1%) of cDCs in peripheral blood, functional *ex vivo* experiments with large numbers of cells typically requires patient leukapheresis, which is not practical given our frequent sample schedule and study design. We plan to bypass this limitation and comprehensively assess the effects of chronic HCV infection and HBV vaccination on cDC by using low-input RNA-Seq. This methodology will allow us to test our hypotheses on cDC function regarding the effects of IFN signature and inflammatory signaling dysregulation on HBV vaccination with the additional advantages of providing robust transcriptome data to deeply explore (and perhaps settle) the question of cDC functional changes in chronic HCV infection. We will isolate BDCA-1^+ cDC (proposed as most effective in priming $CD4^+$ T cell responses [111,112]) by FACS from PBMC samples collected at various time points (baseline, and day 3) in the patient groups described (HCV-NR, HCV-R, CTRL-R, Aim 1). RNA-Seq libraries will be prepared by specialized protocol for low input concentrations [113].

Aim 4 What risk factors associated with chronic HCV infection and its complication is linked to immune activation or modulation?
In order to evaluate the correlation between the factors associated with chronic HCV infection and persistent immune activation or modulation with HBV vaccine non-response, a detailed health questionnaire has been added to this study to increase the ability to correctly understand and interpret the predicted outcomes of the study.

This study is designed to identify the differences in the immune response of individuals who achieve seroprotective anti-Hepatitis B surface antigen (anti-HBsAg) titers (>10 IU/L) versus non-responders. This study will be conducted in chronic Hepatitis C virus (HCV) infected and uninfected, healthy volunteers. The attached questionnaire has been added to increase the ability to correctly understand and interpret the predicted outcomes of this study.

Section A reviews the demographic information of the study population. Chronic HCV infection is associated with older age, male sex, homelessness and lower socioeconomic status[1]. Obtaining this baseline demographic information would allow us to identify our study population.

Section B of the questionnaire addresses alcohol and substance abuse. Heavy alcohol use in the setting of chronic HCV infection would increase liver injury and rapid progression into cirrhosis and hepatocellular cancer. Our questionnaire with the CAGE questions would assist in identifying individuals at risk of alcohol abuse. This enables us to offer counseling as an intervention during the visit.

Long-term opioid use decreases the proliferative capacity of macrophage progenitor cells and lymphocytes; this ultimately affects both the innate and adaptive immune response[2]. Increased prevalence of chronic Hepatitis C virus infection is strongly associated with injection drug use (IDU)[1]. IDUs have been shown to have an elevated baseline immune activation compared to non-IDUs[3]. However, recent meta-analysis in HBV vaccination studies did not show a significant association with seroprotection rates[4].

Section C addresses the medical history of the volunteers. Patients with history of major chronic HCV complications such as cirrhosis and hepatocellular cancer are automatically excluded from this study. However, other complications with further immunosuppression include; cryoglobulinemia (treated with systemic steroids or rituximab) and HCV-induced renal failure managed with hemodialysis. Failure to identify these conditions would distort interpretation of the data collected. Therefore, this questionnaire would aid in the identifying these patients.

Progressive liver fibrosis in chronic HCV infection is associated with persistent immune activation[5]. Section D would assist in identifying patients with previous analysis of hepatic fibrosis by invasive (liver biopsy) vs. non-invasive (Fibroscan) methods. Scoring of hepatic fibrosis would differentiate patients with minimal or no fibrosis from those with late stage fibrosis. These two groups have been shown to have different peripheral inflammatory markers[5]. Information regarding liver fibrosis would significantly increase the robustness of the data generated from the study.

Section E addresses other illness or vaccinations at the time of enrollment. This would aid in identifying potential confounders within the study. Overall, addition of this questionnaire would deepen the interpretation of data collected from this novel study.

1. Denniston et al., Ann Intern Med. 2014;160(5): 293-300
2. Roy and Loh, Neurochem Res. 1996;21(11): 1376-86
3. Deren et al., AIDS. 2012; NIDA Grant # P30DA011041-12S1
4. Kamath et al., Vaccine. 2014;32(2014) 2265-2274
5. Imbert-Bismut et al., The Lancet. 2001;357:1070-1074

The majority of patients in this study will be chronically infected with HCV. Chronic HCV infection is typically asymptomatic, but can be associated with fatigue and rarely with other symptoms. Though chronic HCV can progress to decompensated liver disease, this study excludes patients with cirrhosis and /or evidence of liver failure.

Any patient with chronic HCV who does not have a primary care provider will be referred to the WCMC Hepatitis C center and/or to other primary care providers depending upon their medical coverage and/or travel convenience.

11.8 * Data Analysis

Describe method(s) of data analysis. Include the role of external collaborators as appropriate.

For RNA-Seq analyses (Aim 1), in order to measure markers of innate immune signaling in PBMC, and measure those genes and pathways that respond to HBV vaccination, we will quantify expression levels in relation to a reference standard of External RNA Control Consortium (ERCC) controls – a calibrated panel of defined RNA molecules at defined concentrations that will be "spiked in" to each library preparation to serve as a universal reference across samples [50]. Assessing differences between groups (HCV-NR, HCV-R, CTRL-R) will require an analysis strategy capable of managing the high degree of interpatient variability typical in human gene expression experiments. As such, we will employ a linear modeling analysis for RNA-Seq data, as implemented in the *limma* and *voom* Bioconductor statistical packages [51,52]. In addition to advantages in managing interpatient variability [53], this analysis also allows for the facile implementation of gene set analysis [54], which will be used in the statistical comparison of the IFN signature across groups. The interferon signature for each sample will be quantified by a scoring system based on the scaled expression value (*limma* and *voom* results) for each of more than 350 ISGs drawn from a curated list defined in our laboratory (Rice) [55]. With these data, we will determine the

relationship of persistent innate immune stimulation (as measured by ISG induction) to HBV vaccine response in chronic HCV patients.

As demonstrated by other studies, we expect to observe a diminished response rate to HBV vaccination in patients chronically infected with HCV as compared to healthy volunteers. In the non-responder HCV patient group, we expect to detect evidence of innate immune signaling, as measured by ISG expression in PBMCs. Furthermore, we anticipate a discernable quantitative and/or qualitative relationship between IFN signature in PBMCs and response status to HBV vaccination. This effect will also likely correlate with serum levels of cytokines and chemokines such as IP-10, as measured by Luminex assay. Of note, levels of ISG induction may be related to HCV viral load and/or liver inflammation, clinical variables (measured with patient enrollment) that will also be considered in these analyses. If we do not detect an association between IFN signature and HBV vaccination, we expect that the data generated in these experiments will be of great utility in exploring other potential correlates of vaccine non-responsiveness. As an alternative to our targeted ISG strategy, we can expand our RNA-Seq approach to a more discovery-oriented, transcriptome-wide analysis directed towards identifying other genes or pathways related to vaccine response status. In addition, we can perform other targeted analyses on these datasets informed by the results of functional experiments in Aims 2 and 3.

For functional studies described in Aims 2 and 3, appropriate statistical tests will be tailored to different experimental assay systems. For each parameter (secondary endpoint) (e.g. upregulation of receptors or expression of a cytokine following stimulation), we will calculate the means and standard deviations (SD) of groups and identify outliers that will be excluded from the analysis. For longitudinal samples, the response to treatment over time will be compared between groups using repeated measures ANOVA and Bonferroni post test comparisons to detect statistical differences in parametric (normally distributed) values or log transformed values (cytokine/protein expression). For variables that depart from the normality assumption, the Kruskal-Wallis test followed by Dunns post test comparisons will be used instead. For IFNL SNP status analysis, the proportion of HCV-R will be compared across IFNL3/4 genotype groups (eg. IFNL3/IL-28B: C/C, C/T, or T/T; IFNL4: TT/TT, TT/ G, G/ G) using the Chi-square test for trends on an ordinal exposure variable. Correlations between different parameters will be examined using Spearman rank correlations. All p values will be two-tailed and values < 0.05 will be considered statistically significant.

For low-input RNA-Seq cDC experiments described in Aim 3, analysis will be performed using similar linear modeling methods described above (Aim 1). Analysis will be targeted to compare IFN signatures in cDCs pre and post vaccination as they relate to vaccine response status. Additional analysis will focus on the induction differences of immunostimulatory genes (antigen processing, cytokine, cell signaling) in response to HBV vaccination across patient groups. In addition to the analyses described here, these experiments will generate transcriptomic datasets describing the evolving cDC response to an Alum-adjuvanted vaccine. In future studies, these results can be compared to similar existing, publically available datasets measuring transcriptomic response to yellow fever vaccine [109], influenza vaccine [110], pneumococcus vaccine [110], poly(IC:LC) adjuvant [114]and MP59 adjuvant [115]. Such comparisons will be invaluable in expanding our understanding of the innate immune mechanisms of effective adjuvants and inform the design process for new and improved vaccines.

For all analyses, if RNA-Seq data will be deposited to public or restricted database repositories, we will remove all personally identifiable information possible, and deposit data in the NCBI dbGaP repository.

11.9 * Explain the rationale for the choice of statistical measures and the number of participants proposed for the study, including the power calculations when applicable.

We aim to recruit 100 patients with chronic HCV without a history of HBV infection or vaccination, for which HBV vaccination is indicated. Based on the response to vaccination (primary outcome, measured at study time point week 26), participants will be divided into 3 groups (HCV-R, HCV-NR and CTRL-R) as outlined above. Based on published data, we expect 50% to 80% of chronic HCV patients to respond to the vaccine. In an attempt to match age (+/-5 years) and gender, we will select 20 chronic HCV^+/HBV vaccine non-responder patients (HCV-NR) and 20 chronic HCV^+/HBV vaccine responder patients (HCV-R) for additional characterization.

We will also recruit 30 healthy volunteers with no history of HCV, HBV infection, or HBV vaccination. From this number, we expect a 90-95% vaccine response rate (27-29 subjects), and we will select 20 healthy /HBV vaccine responders (also based on antibody titers at week 26) for the CTRL-R group. [We may be able to include samples from our pilot study in this group].

We do not have sufficient preliminary data to calculate sample size but with a preliminary power calculation, assuming that the HCV-R will be similar to the CTRL-R group and our estimate of recruiting 20

patients in each group, our study will be powered at >90% to detect a difference at a two-sided 0.05 significance level, if the true difference between groups (HCV-NR and HCV-R) is 2 times the standard deviation.

11.10 * Will samples be coded?

(•) Yes ( ) No

If Yes, Please describe coding scheme consistent with GCP. If samples will not be coded, please provide justification for this proposed departure from GCP practice.

All research data will be coded using a unique identifier (e.g. CRI-0844-100 for Chronic HCV patients and CRI-0844-200 for healthy volunteers) assigned to each study participant upon study enrollment; study participants' names will not be associated with any reseach data. The personal computers used to store and analyze the research data used by the PI and his designees will be password protected. The data will be periodically backed-up using a back-up system provided by Information Technology Service at the Rockefeller University. These safe checks will ensure the risks to subjects' confidentiality are minimal.

If available, upload the Data and Sample Sharing Management Plan approved by RU IT.

Version	Title	Category	Expiration Date	Document Outcome	Checked Out	View Document
No Document(s) have been attached to this form.						