A. PROTOCOL TITLE

1. Full Title
Immune Regulation and Costimulation in Natural History and Therapeutic Outcome of Chronic Hepatitis B

2. Brief Title
HBV Immunology Treatment Study

B. STUDY SPONSORSHIP

1. Funding Sponsor
National Institute of Diabetes and Digestive and Kidney Diseases

2. Primary Sponsor
Principal Investigator

C. PROTOCOL ABSTRACT

Hepatitis B virus (HBV) is largely a non-cytopathic virus. Therefore, liver disease pathogenesis and viral clearance in HBV infection is believed to be immune-mediated. At the same time, HBV persists with impaired antiviral immune effector responses that are potentially regulated by multiple immune pathways, including the CD28 costimulatory receptors and immune regulatory T cells. **We hypothesize that the balance between immune regulatory and effector responses in HBV-infected participants defines the natural history and treatment outcome.** The following Aims will examine if:

Aim 1. Therapeutic HBV suppression will enhance antiviral immune effector responses and reduce immune inhibitory factors in participants with chronic hepatitis B. We will also examine if antiviral therapy has a durable effect in host immune phenotype and define the immunological effect of interferon-alpha (IFNα) **therapy in chronic HBV participants.**

Aim 2. Antiviral immune effector and regulatory responses before, during and/or after therapy can predict long term therapeutic response.

To address these Aims, we are conducting an Immunology Study that is ancillary to the NIDDK-sponsored Hepatitis B Research Network (HBRN) Clinical Trials including 'Combination Therapy of Peginterferon Alpha-2a and Tenofovir versus Tenofovir Monotherapy in HBeAg-positive and HBeAg-negative Chronic Hepatitis B' and 'Combination Entecavir and Peginterferon Alpha-2a Therapy in HBeAg-Positive Immune-Tolerant Adults with Chronic Hepatitis B'.

D. OBJECTIVES

We hypothesize that the balance between immune regulatory and effector responses in HBV-infected participants defines the level of viremia, liver inflammation and treatment outcome. The following Aims will examine if:

Aim 1. Therapeutic HBV suppression will enhance antiviral immune effector responses and reduce immune inhibitory factors in participants with chronic hepatitis B (CHB). We will also examine if antiviral therapy has a durable effect in host immune phenotype and define the immunological effect of IFNα therapy in chronic HBV participants.

Aim 2. Antiviral immune effector and regulatory responses before, during and/or after therapy can

predict long term therapeutic response.
In these Aims, we will examine the frequency and phenotype of T cell, NK and dendritic cell subsets in HBV-infected participants before, during and after therapeutic HBV suppression, to determine if therapeutic HBV suppression leads to increased immune effector function with reduced regulatory T cell frequencies and immune inhibitory receptor expression. **We will further examine if the addition of IFNα further accentuates this immune augmentation and if baseline antiviral immune effector and regulatory responses (and their durability after treatment cessation) predict the kinetics of therapeutic virus suppression.**

E. BACKGROUND

The natural history of CHB includes phases of immune tolerance (high HBV viremia, low ALT), immune active state (fluctuating HBV viremia and active hepatitis) and immune control (virus suppression without liver disease) (1, 2). The underlying mechanisms for these phases of chronic HBV infection are not fully defined. While HBV is not readily sensed by the innate IFNα/β system in acute infection (3), it is suppressed by innate immune components including IFNα, toll-like receptor (TLR), NK or NKT cells (4, 5). In participants, acute HBV infection is resolved with a vigorous and broad HBV-specific effector T cell response whereas HBV persists with dysfunctional antiviral T cells that cannot control viremia and may promote liver damage. A dual role of HBV-specific T cells in liver injury as well as non-cytopathic cytokine-mediated virus control has been shown in HBV transgenic mice.

As to the mechanism of T cell dysfunction in HBV persistence, there is increasing evidence that antiviral effector T cells are functionally suppressed or 'tolerized' during chronic viral infections by one or more immune inhibitory pathways. These pathways include CD28 family costimulatory receptors such as PD-1 (programmed death-1) and CTLA-4 (cytotoxic T lymphocyte antigen-4) that are induced on effector T cells as well as regulatory T cells (FoxP3+ Tregs and IL10+ Tr1 cells) (6-9). While therapeutic response is linked to an underlying immune reactivity, the basis for this association is not clear. Furthermore, the mechanisms for immune tolerance in chronic viral infection are not fully defined. In recent years, there has been increasing evidence that antiviral effector T cells are actively suppressed or 'tolerized' by one or more immune inhibitory pathways induced by active viremia during chronic viral infections. These pathways include the various subsets of immune regulatory T cells (FoxP3+ Tregs and IL10+ Tr1 cells) as well as T cell expression of costimulatory receptors such as PD-1 (programmed death-1) and CTLA-4 (cytotoxic T lymphocyte antigen-4) (6-9). Such immune tolerance mechanisms and their interplay with antiviral immune effector responses may also be relevant in HBV persistence and therapeutic response. In fact, prior studies have shown that antiviral immune effector T cell responses were enhanced in CHB participants during lamivudine therapy (10, 11). However, antiviral immune effector responses were lost with recurrent viremia due to lamivudine-resistance (12), suggesting their downregulation by virus-induced immune tolerance mechanisms. We propose that prolonged therapeutic virus suppression will more effectively sustain the underlying antiviral immune effector responses while suppressing the inhibitory pathways, thereby tipping the balance towards long term therapeutic response. In **Aim 1**, we will capture such immunological events during and after therapy.

F. CHARACTERISTICS OF THE STUDY POPULATION

1. Target Population

We will recruit 3 groups of participants with CHB (HBeAg-positive immune active, HBeAg-negative immune active and immune tolerant) that are also being enrolled into HBRN clinical trials including: 1) 'Combination Therapy of Peginterferon Alpha-2a and Tenofovir versus Tenofovir Monotherapy in HBeAg-positive and HBeAg-negative Chronic Hepatitis B'; 2) 'Combination Entecavir and Peginterferon Alpha-2a Therapy in HBeAg-Positive Immune-Tolerant Adults with Chronic Hepatitis B'.
Sample Collection: Immunology blood will be drawn at 6 time points over a 5 year period for Immune Active participants and at 2 time points over a 2 year period for Immune Tolerant participants as shown:
Immune Active Trial: 3 tablespoons of immunology blood (45mL) at 6 time points:

1. Before therapy
2. 24 weeks or 6 months from starting therapy
3. 48 weeks or 1 year from starting therapy

4. 192 weeks or 4 years from starting therapy (at or around the time of repeat liver biopsy and conclusion of therapy
5. 6 months after stopping therapy
6. 12 months after stopping therapy

Immune Tolerant Trial: Immunology blood at 2 time points
1. Before therapy (27mL in 3 lavender top 10mL tubes)
2. 72 weeks after enrollment which is also 24 weeks after treatment cessation (45mL in 5 lavender top 10mL tubes)

Whenever possible, blood for the immunology study will be drawn at the same time as other required laboratory tests. Isolated PBMC will be used for Tier 1 assays. Tier 2 and functional assays will be performed later for select cases, based on initial results and PBMC availability.

2. Accrual

We will recruit 50 HBeAg-positive and 50 HBeAg-negative participants with Immune Active chronic hepatitis B that are randomized into the HBRN Clinical Trial for combined Peginterferon Alpha-2a + Tenofovir therapy or Tenofovir monotherapy. In addition, we will recruit 25 HBeAg+ Immune Tolerant participants in HBRN Clinical Trial for combined Entecavir and Peginterferon Alpha-2a therapy.

Patient Groups	**N**	**Therapy**
HBeAg-positive CHB	25	Peginterferon Alpha-2a /Tenofovir x 24 weeks, followed by Tenofovir x 168 weeks
HBeAg-positive CHB	25	Tenofovir x 192 weeks
HBeAg-negative CHB	25	Peginterferon Alpha-2a /Tenofovir x 24 weeks, followed by Tenofovir x 168 weeks
HBeAg-negative CHB	25	Tenofovir x 192 weeks
Immune Tolerant	25	Entecavir x 48 weeks followed by Peginterferon Alpha-2a at 9-48 weeks

As controls, we will recruit **20 participants with resolved HBV infection (**anti-HBc IgG-positive and HBsAg-negative persons with normal ALT activity and no active liver disease (e.g. due to hepatitis C, autoimmune or fatty liver disease) and **20 seronegative healthy persons** (anti-HBc-negative, HBsAg-negative, normal ALT activity, no active liver disease such as hepatitis C, autoimmune hepatitis or fatty liver disease). Since many persons in North America have received HBV vaccine that results in anti-HBs-positivity, we will recruit anti-HBs-positive as well as anti-HBs-negative persons. Efforts will be made to identify subjects matched for age range, gender and ethnicity as the HBV-infected patient groups.

Control subjects will be recruited from participating clinical centers and the Immunology Center.

Additional Controls	**N**	**HBs Ag**	**Anti-HBs**	**Anti-HBc**	**HBe Ag**	**ALT**	**Serum HBV DNA**
Resolved Hep B	20	-	+/-	+	NA	Normal	HBV DNA negativity to be confirmed later as a batch, if not available at recruitment
Seronegative control	20	-	+/-	-	NA	Normal	

Sample Size Considerations: Based on the foregoing, a total of 125 study subjects starting HBRN clinical trials will be enrolled into the Immunology Study with additional 40 control subjects. A sample size of 50 will provide sufficient power (at least 80%) to detect differences of 20% in mean %FoxP3+ Tregs, 20% PD1/CTLA4 expression on T cells or 2 fold cytokine responses—for example, between 50 HBeAg+ vs 50 HBeAg- participants or 50 participants on combination therapy with Peginterferon Alpha-2a and tenofovir vs 50

participants on tenofovir alone for cross-sectional comparison. Paired comparison of immune responses between different time points (e.g. before vs. after therapy) within each subject may require less sample size (e.g. 25 within each group). A larger sample size is not pursued for practical reasons.

3. Key Inclusion Criteria

We will recruit subjects capable of giving informed consent with clinical, serological and virological characteristics as described in section 2. Study subject selection will not be restricted to any specific age, sex or ethnic background, except as defined in Key Exclusion Criteria below. For the 125 study subjects starting HBRN clinical trials, inclusion criteria for the HBRN Clinical Trials need to be met.

4. Key Exclusion Criteria

We will exclude children under 18 years of age, pregnant women, prisoners or persons unable to provide informed consent. Further exclusion criteria includes: Anemia with Hgb<10 or Hct<30 and active medical conditions such as congestive heart failure or chronic lung disease requiring oxygen, active coronary artery disease with unstable angina, sepsis and renal failure. Participants with significant medical conditions, autoimmune disease or immunosuppression will be excluded. The 125 study subjects starting HBRN clinical trials are also subject to exclusion criteria for the HBRN Clinical Trials.

5. Vulnerable Populations

Vulnerable populations such as children below age 18, pregnant women, fetuses/neonates and prisoners will not be included in this research study.

6. Populations vulnerable to undue influence or coercion

We will recruit subjects capable of giving informed consent. Subjects will be told that their participation is voluntary and that they are free to leave the study at any time.

G. STUDY DESIGN

1. Phase

Not applicable

2. Design

Groups will be identified and recruited for blood draw by the PI, co-investigators or their research staff with clinical phenotypes as defined in the HBRN Cohort Study protocol and additional control groups as defined above in **Section F**. As described below, this study involves blood draws (3 tablespoons or 45mL as described below) in HBV-infected participants before, during and/or after therapy relative to clinical, virological and therapeutic outcomes, as an Ancillary Study to the HBRN.

3. STUDY DESIGN, EXPERIMENTAL GROUPS:

Aim 1. To examine if therapeutic HBV suppression will enhance antiviral immune effector responses and reduce immune inhibitory factors in participants with chronic hepatitis B. In this Aim, we will examine the frequency and phenotype of T cell, NK and dendritic cell subsets in HBV-infected participants before, during and after therapeutic HBV suppression, to determine if therapeutic HBV suppression leads to increased immune effector function with reduced regulatory T cell frequencies and immune inhibitory receptor expression. **We will also examine if antiviral therapy has a durable effect in host immune phenotype and define the immunological effect of Peginterferon Alpha-2a therapy in chronic HBV participants.**

Aim 2. Host immune effector and regulatory responses before, during and/or after therapy can predict long term therapeutic response. In this Aim, we will compare the frequency and phenotype of T cell, NK and dendritic cell subsets in HBV-infected participants relative to therapeutic response to determine if they correlate with long term virus suppression, HBeAg loss and/or HBsAg loss.

Patient Groups include:

1. 25 HBeAg+ Immune Active CHB participants treated with tenofovir DF 300 mg once daily for 192 weeks.
2. 25 HBeAg- Immune Active CHB participants treated with tenofovir DF 300 mg once daily for 192 weeks.
3. 25 HBeAg+ Immune Active CHB participants treated with Peginterferon Alpha-2a 180 µg once weekly for 24 weeks in combination with tenofovir DF 300 mg once daily for 192 weeks.
4. 25 HBeAg- Immune Active CHB participants treated with Peginterferon Alpha-2a 180 µg once weekly for 24 weeks in combination with tenofovir DF 300 mg once daily for 192 weeks.
5. 25 HBeAg+ Immune Tolerant CHB participants receiving entecavir 0.5 mg daily orally for 48 weeks plus Peginterferon Alpha-2a 180 µg sq weekly during weeks 9-48 of treatment.
6. The participant groups and regimen are as defined in the Treatment Protocols.

Sample Collection: For **Immune Active participants**, approximately 3 tablespoons (45mL) of blood will be drawn in 5 lavender top 10mL EDTA tubes at 6 time points over 5 years. For **Immune Tolerant participants**, approximately 2 tablespoons (27mL) of blood in 3 lavender top 10-mL EDTA tubes will be drawn at baseline and 3 tablespoons (45mL) of blood will be drawn at 72 weeks(24 weeks after treatment cessation) in 5 lavender top 10mL EDTA tubes. Blood draw schedule is shown in **Table 1**. The blood volume calculations are based on realistic assessments of 8-9mL of blood per phlebotomy tube. Isolated PBMC will be used for Tier 1 assays. Tier 2 and functional assays will be performed in the future for select cases, based on initial results and PBMC availability as described in **Section 5** (Methods).

Table 1. Patient Groups and Blood Draw Schedule

	Immune Active						**Immune Tolerant**
Blood draw	**Tenofovir 192 weeks**		**Tenofovir 192 weeks +** Peginterferon Alpha-2a **24 weeks**			**Blood Draw**	**Entecavir 48 weeks +** Peginterferon Alpha-2a **9-48 week**
	HBeAg+ IA	**HBeAg- IA**	**HBeAg+ IA**	**HBeAg- IA**			**Immune Tolerant**
	N=25	**N=25**	**N=25**	**N=25**			**N=25**
1. Baseline	45mL	45mL	45mL	45mL		**1. Baseline**	27mL
2. Week 24	45mL	45mL	45mL	45mL		**2. Week 72[3]**	45mL
3. Week 48	45mL	45mL	45mL	45mL			
4. Week 192	45mL	45mL	45mL	45mL			
5. Week 216[1]	45mL	45mL	45mL	45mL			
6. Week 240[2]	45mL	45mL	45mL	45mL			

[1]24 weeks after end of treatment
[2]48 weeks after end of treatment
[3]24 weeks after end of treatment

Hypotheses: The following hypotheses can be tested in **Aims 1 and 2:**

Therapeutic HBV suppression results in enhanced antiviral effector T cell response and/or reduce immune regulatory and inhibitory pathways (FoxP3+ Tregs, IL10+ Tr1 response and PD-1/CTLA-4). This hypothesis can be tested in 4 groups of Immune Active participants, examining their immune phenotype and function at baseline (before therapy) to those measured at the time of therapeutic virus suppression (i.e. paired comparison of baseline vs first time point with undetectable HBV DNA). Immune parameters during various time points (during or after therapy) can be compared cross-sectionally between patient groups (HBeAg+ vs HBeAg-; tenofovir vs tenofovir + Peginterferon Alpha-2a) and correlated with concurrent levels of serum HBV DNA, HBV antigens (quantitative HBeAg, HBsAg) and ALT. If phenotypic changes are observed in immune

subsets (T, NK, DC) during therapeutic HBV suppression, their functional significance can be examined further in a subset of participants with sufficient PBMC via Tier 2 assays.

Peginterferon Alpha-2a with oral antiviral regimen promotes a greater and more durable enhancement in antiviral effector function and phenotype and/or reduction in immune inhibitory parameters compared to oral antiviral therapy alone. The immune modulatory effect of IFNα in this process can be examined at week 12, comparing participants with and without Peginterferon Alpha-2a added to their regimen. This can lead to cross-sectional comparison of 25 HBeAg+ Immune Active participants treated with combined Peginterferon Alpha-2a and tenofovir and 25 HBeAg+ Immune Active participants treated with tenofovir alone. Similar comparison will be made for HBeAg- participants. If appropriate, we can combine the results from HBeAg+ and HBeAg- participants for larger sample size. The durability of immunological changes occurring during and after antiviral therapy can be monitored thereafter (48 and 192 week time points during therapy; 24 and 48 week time points post-treatment cessation).

Host immune effector and regulatory responses before, during and/or after therapy can predict long term therapeutic response. This hypothesis will be tested by comparing the immune responses before, during and after therapy in long-term virological or serological responders and nonresponders. In 25 HBeAg+ Immune Active participants on Peginterferon Alpha-2a + tenofovir or tenofovir alone, we project 10 responders and 15 nonresponders based on a conservative estimate of 40% long term virological response. Combining 25 HBeAg+ and 25 HBeAg- Immune Active participants on similar therapeutic regimen, we can project 20 responders vs 30 nonresponders per therapeutic regimen. Similar comparison can be made for HBeAg seroconversion in 25 HBeAg+ particpants with Peginterferon Alpha-2a + tenofovir and 25 HBeAg+ participants on tenofovir alone.

HBsAg seroconversion is estimated at 5% for tenofovir monotherapy group and 15% for Peginterferon Alpha-2a + tenofovir combination therapy group. For all 100 Immune Active CHB (Chronic Hepititis B) participants combined, we can project 10 participants with HBsAg seroconversion (2-3 on Tenofovir alone, 7-8 on Peginterferon Alpha-2a + tenofovir combination). Although small, this sample size of 10 may provide meaningful and novel information about baseline, on-treatment and off-treatment immunological events that may be associated with HBsAg seroconversion. Furthermore, a larger sample size is not practical within the scope of our study.

Antiviral therapy can modulate a durable change in host immune phenotype in Immune Tolerant participants. In this Aim, we will examine if limited duration of antiviral therapy (with Peginterferon Alpha-2a and entecavir) can induce host immunity, by comparing immune phenotype before therapy and 1 year after treatment cessation. While the amount of blood available for immunological analyses is limited (due to blood draws needed for the clinical trial itself), this provides a novel opportunity to compare immune responses in treated and untreated control participants. Due to the limited blood draw, the initial study will be limited to multi-color flowcytometric phenotyping and limited functional analyses. The sample size is small for practical reasons, but will provide insights to substantial changes based on considerations discussed re: sample size calculations.

Sample Size Calculations: Individual considerations are described above for each hypothesis. In general, we used sample size considerations in which a sample size of 50 provided sufficient power (at least 80%) to detect differences of 20% in mean %FoxP3+ Tregs, 20% PD1/CTLA4 expression on T cells or 2 fold cytokine responses between 50 HBeAg+ vs 50 HBeAg- participants or 50 participants on combination therapy with Peginterferon Alpha-2a and tenofovir vs 50 participants on tenofovir alone for cross-sectional comparison. Paired comparison of immune responses between different time points (e.g. before vs after therapy) within each participant may require less sample size (e.g. 25 within each group). A larger sample size is not pursued for practical reasons.

4*. Immunological Assays*:

Immune analyses will include Tier 1 and 2 immunology assays and Tier 3 analyses involving comparison with measurements from other HBRN studies.

Tier 1 analyses (17M PBMC) performed in all subjects for Immune effector and regulatory responses.

a. Immune phenotype by screening FACS analysis (**2M PBMC**) PD-1, CTLA-4 and CD28 expression on T cells; HLA A2; %FoxP3+ Tregs, NK/DC frequency/phenotype
b. T cell IFNγ/IL10 response to HBV/Flu peptides and controls by Elispot (**7.4M PBMC**).
c. T cell proliferation to HBV/Flu and controls by 3H Thymidine uptake (**6.6M PBMC**)

Tier 2 analyses in select participants to define the mechanism of immune dysfunction.

a. DC cytokine production upon TLR 7/9 (CpG2216) and TLR4 (LPS) stimulation (**5M PBMC**)
b. NK CD107a mobilization and cytokine production with K562 +/- IL12/IL15 (**5M PBMC**)
c. HBV-specific T cell IFNγ/TNFα response +/- PD1 and/or CTLA4 block (**32M PBMC**)
d. Intracellular IFNγ and TNFα staining with HBV and Flu peptides +/- PD1 and/or CTLA4 blockade
e. HBV-specific T cell function +/- IL10R block (**16M PBMC**)
f. Intracellular IFNγ and TNFα staining with HBV and Flu peptides +/- IL10R blockade
g. HBV tetramer+ CD8 T cell expansion in HLA A2+ participants (**32M**) +/- select blockade
h. Serum cytokine/chemokine analyses (including IFNγ, TNFα, IL10, TGFβ, IP-10)

Tier 3 analyses with comparison to Biomarker, Virology and other Ancillary Studies: Findings from Tier 1 and 2 assays will be compared, quantitative serology (HBeAg, HBsAg) and HBV DNA sequence as well as intrahepatic HBV expression (HBcAg, cccDNA) and immunohistochemistry (FoxP3, PD-L1, T/NK) from concurrent Virology, Biomarker or other ancillary studies.

5. Data Usage

The following data will be obtained HBRN/DCC at baseline and prospectively:

1. Demographic: age, gender, ethnicity, alcohol use, autoimmune disease, immunosuppressive therapy.
2. Clinical: ALT, AST, total bilirubin, GGT, alkaline phosphatase, albumin, platelets, INR, Cr, ANA, AFP
3. Virology: HBV genotype, HBV DNA titer, sequence variation.
4. Serology: HBeAg, anti-HBe, HBsAg, anti-HBs, anti-HBc, quantitative HBeAg & HBsAg, anti-HCV, anti-HDV, anti-HIV.
5. Histology: Inflammation grading, fibrosis score, steatosis, HBV immunostaining for HBsAg and HBcAg.

6. Study Duration

The duration of the overall study is 5 years (2012-2015).

H. DRUGS OR DEVICES

Not Applicable

I. STUDY PROCEDURES

1. Recruitment with Informed Consent

Study participants will be identified and recruited by the site Principal Investigator, co-investigators or research staff from participants enrolled into a HBRN clinical trial with clinical phenotypes as defined in the HBRN Cohort Study protocol and additional control groups as defined above in **Section F**.

Initial Consent: Study participants will be fully informed of the reasons for the study, the extent and duration of their cooperation, the voluntary nature of their involvement, and their ability to drop out of the study at any time without prejudice. They will be told that:

They are being recruited because they are enrolled in a HBRN clinical trial or because they belong to a control group of persons (acute hepatitis B, resolved hepatitis B, HBV-uninfected seronegative control).

They will be told of blood draws for the Immunology Study as follows:
For **Immune Active participants**, approximately 3 tablespoons (45mL) of blood will be drawn in 5 lavender top 10-mL EDTA tubes at 6 time points over 5 years. For **Immune Tolerant participants**, approximately 2 tablespoons (27mL) of blood in 3 lavender top 10-mL EDTA tubes will be drawn at baseline and 3 tablespoons (45mL) of blood will be drawn at 72 week (24 weeks after treatment cessation) in 5 lavender top 10-mL EDTA tubes. Blood draw schedule is shown in **Table 1**. The blood volume calculations are based on realistic assessments of 8-9mL of blood per phlebotomy tube. Isolated PBMC will be used for Tier 1 assays. Tier 2 and functional assays will be performed in the future for select cases, based on initial results and PBMC availability as described in **Section 5** (Methods).

If liver biopsy is performed at any times, 30mL blood will be considered for immunology study at the time of liver biopsy for screening FACS analysis. Five additional slides will be cut from the biopsy to examine intrahepatic immune subsets (T, NK, Tregs) and HBV gene expression by immune histochemistry via further ancillary mechanism.

For control groups with naturally resolved HBV infection or healthy seronegative subjects without prior HBV infection, the blood draw will be limited to 3-4 tablespoons at initial recruitment with one additional time point over 5 year period, if additional immune analyses are needed for the study.

They will be told that immunological parameters defined in this study will be compared to clinical, virological, demographic and genetic parameters that are obtained from the Hepatitis B Clinical Trial Network Studies.

They will not derive any personal medical benefits from participation in the Immunology Study.

They will be reassured that this information will be kept confidential, accessible only to authorized persons within the research team except as may be required by law.

They will be provided with institutionally approved consent forms to read and sign after all questions have been answered to their satisfaction. They will receive a copy of the consent and contact telephone numbers for the principal investigator and the research coordinator.

They will/ will not be provided monetary compensation for participating in the Immunology Study.

Coded blood samples will be shipped overnight to the Immunology Center in Philadelphia (University of Pennsylvania & Philadelphia VA Medical Center) for further processing, immune analyses and storage.

2. Tissue Specimens
Blood samples will be processed for lymphocyte and plasma separation followed by immune analyses. Samples that remain will be coded, archived and stored in research freezers and liquid nitrogen tanks at the Immunology Center.

3. Study Organization – Sites
Recruitment for the Immunology Ancillary Study will be conducted at clinical centers within the United States and Canada (listed in Appendix 1). Immune Analysis will be performed in Philadelphia at the Immunology Center based at the University of Pennsylvania and Philadelphia VA Medical Center. The HBRN Data Coordinating Center (DCC) located at the University of Pittsburgh will coordinate operations, develop and implement data and other systems, maintain the database and perform data analyses. This study will use a biospecimen and a genetic repository. A central virology lab, immunology lab, pathology lab, and one or more central testing labs will be utilized to perform tests and to store specimens identified in the protocol.

4. Data Management

De-identified immunology data from the immunology laboratory will be submitted to the Data Coordinating Center (DCC) as spreadsheets via emails. Data from clinical centers will be submitted to the DCC via a distributed web-based data entry system. Clinical center coordinators and other project personnel will be trained and certified to collect and enter data using established systems. Clinical centers will have the option to use any front end device (e.g. TabletPC, laptop, desktop) that provides access to the Internet.

DCC personnel will closely monitor clinical center adherence to study protocol and data collection practices for complete and accurate research data. Monitoring will be performed via established data management procedures with on-site monitoring visits conducted at designated intervals, or as needed, to facilitate the smooth conduct of the study. At the time of the on-site visit, DCC personnel will have access to all study and participant documents and to clinical center personnel. All participant and study documents will be kept confidential. Identifiers such as participant name and address will not be included on any data sent to the DCC.

DCC personnel meet weekly to discuss study status, recruitment, compliance, review data issues, clinical center participation, and other issues that arise during the course of the study.

5. Statistical Analysis

The statistical analysis will be supported through the Data Coordinating Center for the Hepatitis B Clinical Research Network. Patient subgroups will be examined using descriptive statistics. Continuous variables (e.g. %PD-1+, %CTLA-4+, or cytokine SFU/million) will be compared between patient subgroups using nonparametric tests (or Student's t-test for normally distributed data). Frequencies and contingency tables will be used for categorical variables (e.g. +/- increased IFNγ response to inhibitory blockade). Correlations between parameters will be tested by Spearman rank correlation. Stratification and multivariate analyses will examine the effect of potential confounding variables. Linear regression or Poisson regression analysis will be used to formally adjust for potential confounders.

Our sample sizes provide sufficient power to detect meaningful differences based on sample size calculations. For example, the sample size of 50 for each chronic hepatitis B group provides 80% power to detect a 20% increase in the mean percentage of FoxP3+ Tregs or PD1/CTLA4+ CD8 T cells or a 2 fold difference in HBV-specific cytokine response between patient groups.

6. Confidentiality

Clinical sites are responsible for the confidentiality of the data associated with participants in the HBRN in the same manner they are responsible for the confidentiality of any patient information within their sphere of responsibility. All forms used for the study data will be only identified by coded identifiers to maintain subject confidentiality. All records will be kept in locked file cabinets at the clinical centers with access limited to HBRN study staff. All study staff will identify participants by the participant identifier number generated at the clinical center. Clinical information will not be released without written permission of the participant, except as necessary for monitoring by the Institutional Review Board/Research Ethics Board (IRB/REB) or Data and Safety Monitoring Board (DSMB). Clinical information may be reviewed during site visits by the DCC and the NIDDK Project Officer. Participants grant permission to share research data with these entities in the consent document. Federal regulations govern the protection of participant's rights relative to data confidentiality and use of research data.

Consent procedures and forms, and the communication, transmission and storage of participant data will comply with individual site IRB/REB and NIH requirements for compliance with The Health Insurance Portability and Accountability Act (HIPAA)/ Personal Health Information Protection Act (PHIPA). The DCC will require that clinical centers provide documentation from the site IRB/REBs with the appropriate authorization or consent form.

7. Subject Privacy/Protected Health Information

All data and samples collected in the HBRN clinical trials will be coded to protect the participants' identity. As such, data and samples collected from subjects enrolled into the Ancillary Immunology Study will contain no health information/identifiers except for the date of sample collection.

J. RISK/BENEFIT ASSESSMENT

1. Potential Study Risks

Risks from the blood draw needed for this study are minor, including bruising, swelling, black and blue marks, fainting, and /or infection at the site. With repeated large volume blood draws, anemia can occur. There is also potential risk related to confidentiality since personal and medical information will be gathered in the course of the study. We will make every effort to minimize these risks. All adverse events will be reported to the responsible agencies as required by the Institutional Review Board/Research Ethics Board and/or Federal Agencies.

2. Potential Study Benefits

The results of this study will not benefit the subject directly. However, information gained from this study may be helpful in our understanding of HBV pathogenesis and therapy.

3. Alternatives to Participation

There are no alternate procedures or treatments for subjects. They may elect not to take part in this study.

4. Participant withdrawal

If a participant chooses to withdraw, all data collected up to the point of withdrawal will remain in the study database, but no further data may be collected. The participant must submit a written request to withdraw or verbally to the clinical center personnel. This is consistent with HIPAA/PHIPA guidelines and regulations. A participant may also withdraw consent for use of data or stored specimens – in this case, any specimens collected from this subject will be destroyed and data deleted.

5. Data and Safety Monitoring

This is an ancillary immunology study for the NIDDK-sponsored HBRN Clinical Trials. The HBRN Clinical Trials will be monitored by a Data and Safety Monitoring Board (DSMB), with members appointed by the NIDDK and in the manner as defined in the Clinical Trial Protocol.

Appendix 1.

Participating Centers:
Philadelphia, PA: University of Pennsylvania School of Medicine (Immunology Center)
Pittsburgh, PA: University of Pittsburgh Graduate School of Public Health (DCC)

Boston, MA: Beth Israel Deaconess Medical Center, Massachusetts General Hospital
Minnesota: Mayo Clinic Rochester, University of Minnesota
San Francisco, CA: UCSF, California Pacific Medical Center
Texas: University of Texas Southwestern,
Toronto, Ontario, Canada: University of Toronto
Virginia: Virginia Commonwealth University
Washington: University of Washington Medical Center, Virginia Mason Medical Center

Appendix 2.

Table 1. Patient Groups and Blood Draw Schedule

	Immune Active						**Immune Tolerant**	
Blood draw	**Tenofovir 192 weeks**		**Tenofovir 192 weeks +** Peginterferon Alpha-2a **24 weeks**			**Blood Draw**	**Entecavir 48weeks +** Peginterferon Alpha-2a **9-48 weeks**	
	HBeAg+ IA	**HBeAg- IA**	**HBeAg+ IA**	**HBeAg- IA**			**Immune Tolerant**	
	N=25	**N=25**	**N=25**	**N=25**			**N=25**	
1. Baseline	45mL	45mL	45mL	45mL		**1. Baseline**	27mL	
2. Week 24	45mL	45mL	45mL	45mL		**2. Week 72[3]**	45mL	
3. Week 48	45mL	45mL	45mL	45mL				
4. Week 192	45mL	45mL	45mL	45mL				
5. Week 216[1]	45mL	45mL	45mL	45mL				
6. Week 240[2]	45mL	45mL	45mL	45mL				

[1]24 weeks after end of treatment
[2]48 weeks after end of treatment
[3]24 weeks after end of treatment

REFERENCES:

1. Yim HJ, Lok AS. Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. Hepatology 2006;43:S173-181.
2. Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. Hepatology 2007;45:1056-1075.
3. Wieland SF, Chisari FV. Stealth and cunning: hepatitis B and hepatitis C viruses. J Virol 2005;79:9369-9380.
4. Ferrari C, Missale G, Boni C, Urbani S. Immunopathogenesis of hepatitis B. J Hepatol 2003;39 Suppl 1:S36-42.
5. Guidotti LG, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. Annu Rev Pathol 2006;1:23-61.
6. Boni C, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, et al. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. J Virol 2007;81:4215-4225.
7. Isogawa M, Furuichi Y, Chisari FV. Oscillating CD8(+) T cell effector functions after antigen recognition in the liver. Immunity 2005;23:53-63.
8. Xu D, Fu J, Jin L, Zhang H, Zhou C, Zou Z, Zhao JM, et al. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol 2006;177:739-747.
9. Zhang Z, Zhang JY, Wherry EJ, Jin B, Xu B, Zou ZS, Zhang SY, et al. Dynamic programmed death 1 expression by virus-specific CD8 T cells correlates with the outcome of acute hepatitis B. Gastroenterology 2008;134:1938-1949, 1949 e1931-1933.
10. Boni C, Penna A, Ogg GS, Bertoletti A, Pilli M, Cavallo C, Cavalli A, et al. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. Hepatology 2001;33:963-971.
11. Boni C, Penna A, Bertoletti A, Lamonaca V, Rapti I, Missale G, Pilli M, et al. Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. J Hepatol 2003;39:595-605.
12. Mizukoshi E, Sidney J, Livingston B, Ghany M, Hoofnagle JH, Sette A, Rehermann B. Cellular immune responses to the hepatitis B virus polymerase. J Immunol 2004;173:5863-5871.