HBRN Ancillary Study Protocol
Embedded into the Adult Cohort Study
Version 3.1 05/05/2015

Application for Review of Human Research: IRB/REB Protocol Summary

***Principal Investigator*: _____**

A. PROTOCOL TITLE

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

2. Brief Title
HBV Immune Regulation and Pathogenesis

B. STUDY SPONSORSHIP

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

2. Primary Sponsor
Principal Investigator: Kyong-Mi Chang

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. **In this study, we hypothesize that the balance between immune regulatory and effector responses in HBV-infected patients defines the level of viremia, liver inflammation and treatment outcome.** The following Aims will examine if:

Aim 1: The clinical and virological status of chronic HBV infection is defined by distinct patterns of immune effector and regulatory responses.

Aim 2: Clinical flares of chronic hepatitis B reflect altered balance between immune regulatory and effector responses.

To address these Aims, we are conducting an Immunology Study that is ancillary to the NIDDK-sponsored Hepatitis B Research Network (HBRN) Study Cohort Study (entitled 'Observational Study of Persons with Hepatitis B Virus Infection in North America').

D. OBJECTIVES

1. Overall Objectives
We hypothesize that the balance between immune regulatory and effector responses in HBV-infected patients defines the level of viremia, liver inflammation and treatment outcome. The following Aims will examine the following 2 Aims:

Aim 1: The clinical and virological status of chronic HBV infection is defined by distinct patterns of immune effector and regulatory responses: We propose that one or more immune regulatory (e.g. FoxP3+ Tregs, HBV-specific IL10+ Tr1) and co-stimulatory pathways (PD1/CTLA4) are induced during chronic hepatitis B that define the extent of immune tolerance vs activation with associated disease activity and viremia.

Towards this end, the immune effector (T, NK, DC phenotype and function) and regulatory (FoxP3+ Tregs, IL10+ Tr1, PD1/CTLA4, DC PD-L1 expression) responses relative to serum HBV DNA, ALT, HBeAg, HBsAg and liver histology will be examined in a cross-sectional manner in patients with chronic HBV and control groups as discussed below.

Aim 2: Clinical hepatitis flares during chronic hepatitis B reflect altered balance between immune regulatory and effector responses. Ideally, it is desirable to monitor patients closely before, during and after a flare to understand the immunological mechanisms of clinical flares in chronic hepatitis B and downstream events that define a 'successful' or an 'abortive' flare. However, this requires a large-scale approach that is impractical and costly (i.e. in order to identify a few patients with true flares, we need to monitor many). Therefore, we chose to identify patients experiencing hepatitis flares and study their immune response longitudinally thereafter.

E. BACKGROUND

The natural history of chronic hepatitis B include 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 IFN□□□system in acute infection (3), it is suppressed by innate immune components including IFN□ toll-like receptor (TLR), NK or NKT cells (4, 5). In patients, 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). In **Aim 1**, immune regulatory and effector responses will be compared in a cross-sectional manner in chronic HBV patients at immune tolerant, immune active and immune control phases, testing the hypothesis that **the clinical and virological status of chronic HBV infection is defined by distinct patterns of immune effector and regulatory responses.** Since HBeAg is believed to be an immune tolerogen, we will also compare the immune regulatory pathways in HBeAg+ and HBeAg- patients.

ALT flares can occur before HBeAg seroconversion and disease remission, either spontaneously or with antiviral therapy. On the other hand, abortive ALT flares without seroconversion may contribute to liver injury. ALT flares also occur with HBV reactivation in inactive carriers, typically with immunosuppression (10). While HBV-specific T cells have been implicated in HBV flares (11, 12), the interplay between immune regulatory and effector responses has not been examined. ALT flares in HBeAg- patients are associated with precore mutation (13), although their immunological significance is not known. **In Aim 2, we will examine if clinical flares during chronic hepatitis B reflect altered balance between immune regulatory and effector responses.**

F. CHARACTERISTICS OF THE STUDY POPULATION

1. Target Population

We will identify and recruit HBV-infected subjects and control groups as defined below. Patients with chronic hepatitis B will be recruited from the HBRN Cohort Study.

2. Accrual

Patient Groups will be recruited with their phenotypes as defined in HBRN Cohort Study and with additional control groups as shown below. Patients with chronic hepatitis B will be recruited from subjects that are enrolled into the HBRN Cohort Study, as defined in the Cohort Study Protocol:

a. Immune tolerant: Presence of HBsAg and HBeAg and normal ALT levels on two occasions or more at least 6 months apart. HBV DNA levels of greater than 1,000,000 IU/mL.

b. HBeAg-positive chronic hepatitis B: Definite: Presence of HBsAg and HBeAg and abnormal serum ALT levels (at least twice the ULN) on two occasions or more at least 6 months apart. HBV DNA levels of greater than 10,000 IU/mL. **Probable:** Presence of HBsAg and HBeAg and HBV DNA greater than 10,000 IU/mL, but ALT levels between 1-2 times the ULN.

c. HBeAg-negative chronic hepatitis B: Definite: Presence of HBsAg without HBeAg but with abnormal serum ALT levels (at least twice the ULN) on two occasions or more at least 6 months apart. HBV DNA levels of greater than or equal to 1,000 IU/mL. **Probable:** Presence of HBsAg without HBeAg and HBV DNA greater than or equal to 1,000 IU/mL, but ALT levels between 1-2 times the ULN.

d. Inactive carrier: Definite: Presence of HBsAg without HBeAg and normal ALT levels on two occasions or at least 6 months apart. HBV DNA levels of less than 1,000 IU/mL. **Probable:** Presence of HBsAg without HBeAg and HBV DNA between 1,000-10,000 IU/mL, but ALT levels normal.

e. Acute Hepatitis B: Presence of HBsAg and IgM anti-HBc with serum ALT values greater than 300 IU/L and absence of known history of HBsAg positivity. Probable acute hepatitis B is when all above criteria are met except serum ALT is less than or equal to 300 IU/L or if there is any suspicion of chronic disease.

f. Chronic hepatitis B with ALT Flare: An ALT flare is defined as serum ALT greater than or equal to 10 times the upper limit of normal which corresponds to 300 IU/L in males or 200 IU/L in females. Thus, HBsAg-positive patients with an ALT flare will be enrolled into this group. It is anticipated that some patients enrolled into this group may be undergoing acute hepatitis B. A definitive diagnosis will be made over time based on their clinical course and laboratory parameters. Patients deemed to have acute hepatitis B will be re-assigned to acute hepatitis B cohort whereas patients with a flare of chronic hepatitis B (e.g. with known prior chronic hepatitis B) will remain in this group.

Patient Groups	N	HBs Ag	Anti-HBs	Anti-HBc	HBe Ag	ALT	HBV DNA (IU/ml)	Comments
Immune Tolerant	50	+	NA	NA	+	Normal	>1,000,000	
HBeAg+ CHB	50	+	NA	NA	+	Elevated*	>10,000	
HBeAg- CHB	50	+	NA	NA	-	Elevated*	≥1,000	
Inactive Carrier	50	+	NA	NA	-	Normal	<1000**	
CHB with ALT Flare	50	+	NA	NA	+/-	>10X ULN which is >300 in males or >200 in females		Needs clarification between chronic flare vs acute hepatitis B based on clinical course
Acute Hep B	50	+	NA	IgM+	+/-	>300		No known prior HBsAg positivity

* > 2 times ULN = definite, 1-2 times ULN = probable (ULN=30 IU/L for males, 20 IU/L for females)
** HBV DNA 1,000-10,000 IU/mL with normal ALT and no HBeAg-probable

g. Control group with resolved HBV infection: We will define resolved hepatitis B based on anti-HBc-positive status in HBsAg-negative persons with normal ALT activity and without ongoing liver disease (e.g. due to hepatitis C, autoimmune or fatty liver disease). Efforts will be made to identify subjects matched for age range, gender and ethnicity as the HBV-infected patient groups.

h. Seronegative controls: We will recruit control subjects that are negative for HBsAg and anti-HBc without a prior history of acute or chronic hepatitis B. 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	HBV DNA (IU/ml)	Comments
Resolved Hep B	20	-	+/-	+	NA	Normal		HBV DNA negativity to be confirmed later as a batch, if not available at recruitment
Seronegative control	30	-	+/-	-	NA	Normal		

Based on the foregoing, a total of 300 study subjects will be enrolled for **Aim 1** from all sites involved in the Immunology Study with a preference given to patients undergoing liver biopsy during the study, patients infected with HBV genotypes A, B, C or D (since the existing HBV peptide pools are derived from genotypes A-D) and those likely to enter the HBRN treatment protocol (for prospective analysis via a separate protocol to be submitted in the future).

From our site, we anticipate enrolling up to ____ patients over the course of the study through our clinics.

Statistical analysis: 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 IFN□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 size of 50 for each chronic HBV patient groups was based on sample size calculations as follows:

First, to examine **%FoxP3+ Tregs and PD1/CTLA4 expression on CD8 T cells will be larger in eAg+ immune tolerant group compared to eAg+/- immune active group,** our sample size of 50 for each group will ensure enough power (at least 80%) to detect a 20% increase in the mean percentage of FoxP3+ Tregs or PD1/CTLA4+ CD8 T cells in immune tolerant group compared to immune active groups at 5% level of significance. For example, an increase in mean FoxP3+ Treg frequency from 2.8% to 3.4% represents a 20% increase.

Second, to determine if HBV-specific IL10 response will be larger in immune tolerant group compared to immune active group or if HBe or core-specific IL10 response will be larger in HBeAg+ than HBeAg- chronic HBV patients, sample size of 50 for each group will allow sufficient power (at least 80%) to discern a 2 fold increase or decrease in HBV-specific IL10 response by IL10+ spot forming units (SFU) in Elispot Assay. For example, HBe or core-specific IL10+ Tr1 response may be two fold greater in eAg+ than eAg- patients (e.g. 400 SFU vs 200 SFU). By contrast, their response to HBsAg may be similar (e.g. 400 SFU vs 400 SFU).

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, with the exception that children under 18 years of age will be excluded.

4. Key Exclusion Criteria

We will exclude children under 18 years of age, 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. Patients with significant medical conditions, autoimmune disease or immunosuppression will be excluded.

5. Vulnerable Populations

Vulnerable populations such as children, 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.

7. Subject Recruitment

Study subjects will be identified directly by the Principal Investigator and healthcare providers at _____.

G. STUDY DESIGN

Patient Groups will be identified and recruited for blood draw by the PI, co-investigators and their research staff with clinical phenotypes as defined in the HBRN Cohort Study and additional control groups as defined above in **Section F**. As described below, this study involves blood draws (30-50ml or 2-4 tablespoons as described below) to compare immune responses in HBV-infected patients relative to their clinical, virological, demographic and histological parameters, as an Ancillary Study to HBRN Network Cohort Study.

Aim 1: The clinical and virological status of chronic HBV infection is defined by distinct patterns of immune effector and regulatory responses: We propose that one or more immune regulatory (e.g. FoxP3+ Tregs, HBV-specific IL10+ Tr1) and co-stimulatory pathways (PD1/CTLA4) are induced during chronic hepatitis B that define the extent of immune tolerance vs activation with associated disease activity and viremia. Towards this end, the immune effector (T, NK, DC phenotype and function) and regulatory (FoxP3+ Tregs, IL10+ Tr1, PD1/CTLA4, DC PD-L1 expression) responses relative to serum HBV DNA, ALT, HBeAg, HBsAg and liver histology will be examined in a cross-sectional manner in patients with chronic HBV and control groups as discussed below.

Patient Groups include Immune Tolerant, HBeAg+ CHB, HBeAg- CHB, Inactive Carrier, Resolved Hep B and Seronegative controls with phenotypes as defined in the HBRN Cohort Study with additional controls (defined in Section F2).

Study subjects will be enrolled from select sites involved in the Immunology Study with preference given to patients undergoing liver biopsy during the study, patients infected with HBV genotypes A, B, C or D (since the existing HBV peptide pools are derived from genotypes A-D) and those likely to enter the HBRN treatment protocol (for prospective analysis via a separate protocol to be submitted in the future).

HBV patients enrolled into the Cohort Study will be evaluated for their clinical phenotype. Subjects that qualify for the patient groups will be recruited into the Immunology Study during the next follow up visit (12 weeks according to the Cohort Study Protocol), however patients may be approached at 24 weeks or later if they appear to be eligible and were not approached at week 12).

Hypotheses: The following individual hypotheses can be tested by Tier 1 immune assays (see below) that will define the immunological characteristics for the 4 phenotypes or phases of chronic HBV infection:

1. %FoxP3+ Tregs and PD1/CTLA4 expression on CD8 T cells will be larger in eAg+ immune tolerant group compared to eAg+/- immune active group.
2. HBV-specific IL10 response will be larger in immune tolerant group compared to immune active group.
3. HBe or core-specific IL10 response will be larger in HBeAg+ than HBeAg- chronic HBV patients.
4. NK function (cytokines, CD107a mobilization) will be larger in immune than immune tolerant patients.

5. Dendritic cells from immune tolerant patients display increased PDL1 and reduced type I IFN compared to immune active patients or inactive carriers.

As we identify the immune regulatory or costimulatory pathways relevant in chronic HBV infection, the functional relevance of those pathways can be examined more directly by in vitro immune regulatory blockade in a subset of patients with sufficient PBMC (Tier 2 assays). Among patients with immune active disease, we can also examine the relationship between immune parameters and liver disease (ALT activity, histological activity, fibrosis).

Chronic hepatitis B is a dynamic condition with evolutions in clinical phenotype grouping and serological/virological status over time

To better define evolution in host immune response in persons with and without specific phenotype change (e.g., HBeAg seroconversion), we will study participants to include weeks 192 and 240 post-baseline.

Specifically, we can test the hypotheses that:

1) immune regulatory measures but not effector responses increase over time in chronic hepatitis B thereby worsening immune inhibition;

2) immune regulatory pathways are downregulated and antiviral effector functions improve after virus control +/- HBeAg or HBsAg seroconversion.

3) Individuals with/without HBeAg or HBsAg seroconversion have more sustained restoration of immune effector function and loss of regulatory responses.

The immune regulatory/activation measures include percentage, mean fluorescence intensity or function for:

-Adaptive immune cells: CD4, CD8, Tregs and antigen-specific T cells for their frequency, phenotype (PD1, CTLA-4, CD28, CTLA-4) and function (proliferation, cytokines, cytolytic potential, activation) with additional focused evaluation of B cells.

-Innate immune cells: NK cell subsets (e.g. CD56+CD16+/CD3-, CD56+CD16-/CD3- or CD56-CD16+/CD3-), NKT cells and $\gamma\delta$ T cells for frequency and phenotype (activating receptors NKG2D, NKG2C, NKG2D, NKp30 and NKp46; inhibitory receptors CD158a/b, CD94 and NKG2A); also dendritic cells (mDC and pDC) for frequency and expression of costimulatory ligands (e.g. CD80/86, PDL1/L2).

Sample Collection: 50ml (3.3 tbsp) blood will be drawn in five 10-ml lavender EDTA tubes preferably at 12 weeks and 24 weeks from enrollment for the Cohort Study. PBMC from baseline visit will be used for initial Tier 1 assays. Remaining PBMCs will be cryopreserved and used for one or more Tier 2 assays and further functional analysis based on initial assay results. The immune assays are described in **Section G. 5** (Methods).

If liver biopsy is performed at any times other than 12 or 24 weeks, 30ml (2 tbsp) blood will be drawn 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.

If a patient is not eligible at the week 12 visit (or blood could not be obtained at that visit), the patient could be enrolled at week 24 or later, as long as the patient has not started HBV therapy. For all participants, blood draws will occur at weeks 192 and 240, provided the participants did not receive antiviral therapy. (see below)

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 two additional time points over a 5 year period, for additional immune analyses.

Relevant time points during the Cohort Study for immunology study in Aim 1*:

Baseline:	Enrollment into the Cohort study followed by definition of patient phenotype Possible enrollment into the Immunology Study
Week 12**:	Enrollment into the Immunology Study 1st Blood draw (50ml for immunology)
Week 24:	2nd Blood draw (50ml for immunology) for those enrolled at week 12 Enrollment and first blood draw (50ml for immunology) for patients who are enrolled at this visit
Week 48:	2nd blood draw (50ml for immunology) for patients who are enrolled at week 24 visit
Week 192:	3rd blood draw (50 ml for immunology)
Week 240:	4th blood draw (50ml for immunology)

*HBRN participants who were not initially enrolled into the Immunology Study during the 1st year will be enrolled at any time during the Cohort Study for at least one blood draw, provided that they remain eligible.

**If liver biopsy is performed at times other than weeks 12 or 24, patients will be enrolled into the Immunology Study with 30ml of blood drawn for immunology study at the visit for liver biopsy.

Aim 2: Spontaneous clinical hepatitis flares during chronic hepatitis B reflect altered balance between immune regulatory and effector responses. Spontaneous flares will be identified among subjects enrolled into the HBRN Cohort Study for prospective monitoring. Ideally, it is desirable to monitor patients closely before, during and after a flare to understand the immunological mechanisms of clinical flares in chronic hepatitis B and downstream events that define a 'successful' or an 'abortive' flare. However, this requires a large-scale approach that is impractical and costly (i.e. in order to identify a few patients with true flares, we need to monitor many). Therefore, we chose to identify patients experiencing hepatitis flares and study their immune response longitudinally thereafter.

Patient Groups: We will recruit 50 HBeAg+/- CHB patients with ALT flare as defined in Section F.2 according to the Cohort Study. Findings from these subjects will be compared to those in patient groups from Aim 1.

Patients will be recruited for immunology blood draw according to the existing HBRN Cohort Study follow up schedule. In the Cohort Study, the follow-up intervals in patients diagnosed with ALT flare will be every 4 weeks unless ALT is greater than 1000 IU/L or total bilirubin is greater than 2.5 mg/dl in the absence of Gilbert's syndrome, then follow-up intervals will be every 2 weeks.

An ALT flare is considered to be resolved when the participant no longer meets the ALT definition of flare, ALT must drop to 300 IU/L or below in males or 200 IU/L or below in females. When the ALT flare is considered to be resolved, the follow-up will revert to the original follow-up schedule for the Cohort Study. The routine follow-up schedule for the Cohort Study is at weeks 12, 24 and 48 during the first year. Beyond week 48, follow-up evaluations will be conducted at 24 week intervals.

The following summarizes the relevant time points for immunology study in Aim 2:

Visit 1: Unscheduled follow-up visit within 1-2 weeks of initial ALT flare (50ml for immunology study)
Visit 2: Follow up visit at 4 weeks from initial flare (50ml for immunology study)
Visit 3: First follow up visit after the resolution of flare--either at 24 weeks per original follow-up interval or before initiating therapy, whichever occurs first (50ml for immunology study)

All subjects will further undergo blood draws for Immunology Study (3.3 tbsp. for immunology) at weeks 192 and 240 from enrollment (similar to Aim 1).

This will result in immune monitoring at two time points close to the initial ALT flare (1-2 weeks, then at 4 weeks from flare) and after resolution of flare or before therapy. In patients who did not resolve ALT flare and were initiated on therapy, we anticipate that these subjects will be recruited for immune monitoring after treatment initiation through a separate treatment protocol.

To enhance recruitment, all patients with flares will be recruited from sites recruiting for Immunology Study, even without a baseline PBMC collection. Of note, Toronto site estimated ~24 flares annually among HBeAg+ patients (with ~40% seroconversion). Based on this projection, we anticipate sufficient recruitment. However, if recruitment is insufficient within the first 2 years, we can expand the study to the entire network, if this meets with SC approval.

Successful outcome will be defined in the short term by ALT normalization and HBV DNA <2000 IU/L at the time of the visit and in the long term by HBeAg seroconversion (this outcome will be defined months thereafter).

Hypotheses: The following individual hypotheses can be tested:
1. Immune regulatory parameters (%FoxP3+ Tregs, T cell expression of PD1/CTLA4 and/or HBV-specific IL10+ Tr1 response) are lower during hepatitis flares compared to immune tolerant patients.
2. NK cells are more activated (NKG2D, CD69) and cytotolytic during flare than after resolution.
3. Immune effector responses during a hepatitis flare (HBV-specific T cell IFN□response, NK IFN□/TNF□response, DC IFN□**)** are greater in patients who achieve successful HBeAg seroconversion, compared to those with abortive flares.

Sample Size Calculations: We used similar sample size considerations as shown for Aim 1 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 flare vs and immune tolerant or inactive carrier patients.

Sample Collection: 50ml blood will be drawn in 10-ml lavender EDTA tubes according to the 3 visits as above. PBMCs will be used for initial Tier 1 assays. Remaining PBMCs will be cryopreserved and used for one or more Tier 2 assays and further functional analysis based on initial assay results—if sufficient PBMC remain.

3. 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 (18M 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 IFN□/IL10 response to HBV/Flu peptides and controls by Elispot (**7.4M PBMC**)
Antigen-specific IFNγ/IL10 response using overlapping HBV peptides (core, env and RT from geno A-D) with Flu, LPS, PHA and media controls.
c. T cell proliferation to HBV/Flu and controls by 3H Thymidine uptake (**6.6M PBMC**)

Tier 2 analyses in select patients (with sufficient cells) to define the mechanism of immune dysfunction.
a. HBV-specific T cell IFN□/TNF□response +/- PD1 and/or CTLA4 block (**32M PBMC**)

Intracellular IFN□and TNF□staining with HBV and Flu peptides +/- PD1 and/or CTLA4 blockade

b. HBV-specific T cell function +/- IL10R block (**16M PBMC**)
Intracellular IFN□and TNF□staining with HBV and Flu peptides +/- IL10R blockade

c. HBV tetramer+ T cell expansion (**32M**) +/- select blockade

d. DC cytokine production upon TLR 7/9 (CpG2216) and TLR4 (LPS) stimulation (**5M PBMC**)

e. NK CD107a mobilization and cytokine production with K562 +/- IL12/IL15 (**5M PBMC**)

f. Serum cytokine/chemokine analysis

g. B cell frequency and phenotype

Tier 3 analyses with comparison to Biomarker, Virology and other Ancillary Studies: Findings from Tier 1 and 2 assays will be compared to serum cytokines/chemokines (IFN□, TNF□, IL10, TGF□, IP-10), 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.

***4. Data Usage*:** The following data will be obtained HBRN/DCC at baseline and prospectively:

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

5. Study Duration

The duration of this study is 7 years (2008-2015).

H. DRUGS OR DEVICES

Not Applicable

I. STUDY PROCEDURES

1. Recruitment with Informed Consent

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

Initial Consent: Study subjects 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 the HBRN Cohort Study or because they belong to 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 HBV-infected subjects in Aim 1, 50ml blood will be drawn as follows:
-at 12 and 24 weeks from enrollment into the Cohort Study
-at 1-2 other visits if blood draw was missed at either 12 and/or 24 week blood draw
-follow-up blood draws at weeks 192 and 240 from enrollment.

***For subjects undergoing a liver biopsy at times other than week 12 or 24 visits**, 30ml blood will be drawn at the time of liver biopsy for screening flowcytometry analysis. This may occur before or after the 12

week visit depending on liver biopsy scheduling. Five additional slides will be cut from the biopsy to examine intrahepatic immune subsets and HBV gene expression by further ancillary mechanisms.

For subjects in Aim 2 who experience ALT flares, 50ml blood will be drawn at each visit as follows:
-Within 1-2 weeks of initial ALT flare;
-Follow up at 4 weeks from initial flare;
-First follow up visit after the resolution of flare--either at 24 weeks per original follow-up interval or before initiating therapy, whichever occurs first
-follow-up blood draws at weeks 192 and 240 from enrollment.

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 2 additional time points over a 5 year period, for additional immune analyses.

-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) 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
Data will be submitted to the Data Coordinating Center (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 (ex. 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

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 IFN□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 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 Hepatitis B Clinical Research Network Cohort Study will be coded to protect the patients' 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 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

A Data and Safety Monitoring Board (DSMB), with members appointed by the NIDDK, consists of individuals who are independent of the institutions and investigators participating in the HBRN and who have no financial ties to the outcome of the study. The ongoing review of the data by this independent committee assures the investigators and the NIDDK that the study can continue. The roster and charter of the DSMB members will be provided to investigators participating in the study for submission to their IRB/REB.

The DSMB charter will be developed by the NIDDK. The DSMB will review the study protocol and recommend recruitment initiation and continuation. It will monitor all aspects of the study (e.g., recruitment, protocol deviations, breeches of confidentiality, data quality, attrition, descriptive characteristics), and recommend protocol modifications, including early study termination. Reports will be prepared by the DCC. Tables showing study progress will be presented by clinical center and overall. These will include recruitment, protocol deviations, attrition, breeches of confidentiality, and data quality. The DCC will maintain a cumulative summary of breeches of confidentiality to be forwarded to the DSMB for their meetings via conference call or in person. Based on the data presented, the DSMB will recommend continuation or termination of the study. A summary of the DSMB findings will be forwarded to all investigators for submission to their respective IRB/REBs.

Who will monitor this study? Check all that apply.

☐ Principal Investigator
☐ Sponsor or contract research organization
☐ NCI sponsored cooperative group
☐ Cancer Center (if mandated by CTSMRC)
☐ Medical monitor
☐ Safety monitoring committee
☐ Data and safety monitoring board

Appendix 1.

Participating Centers:

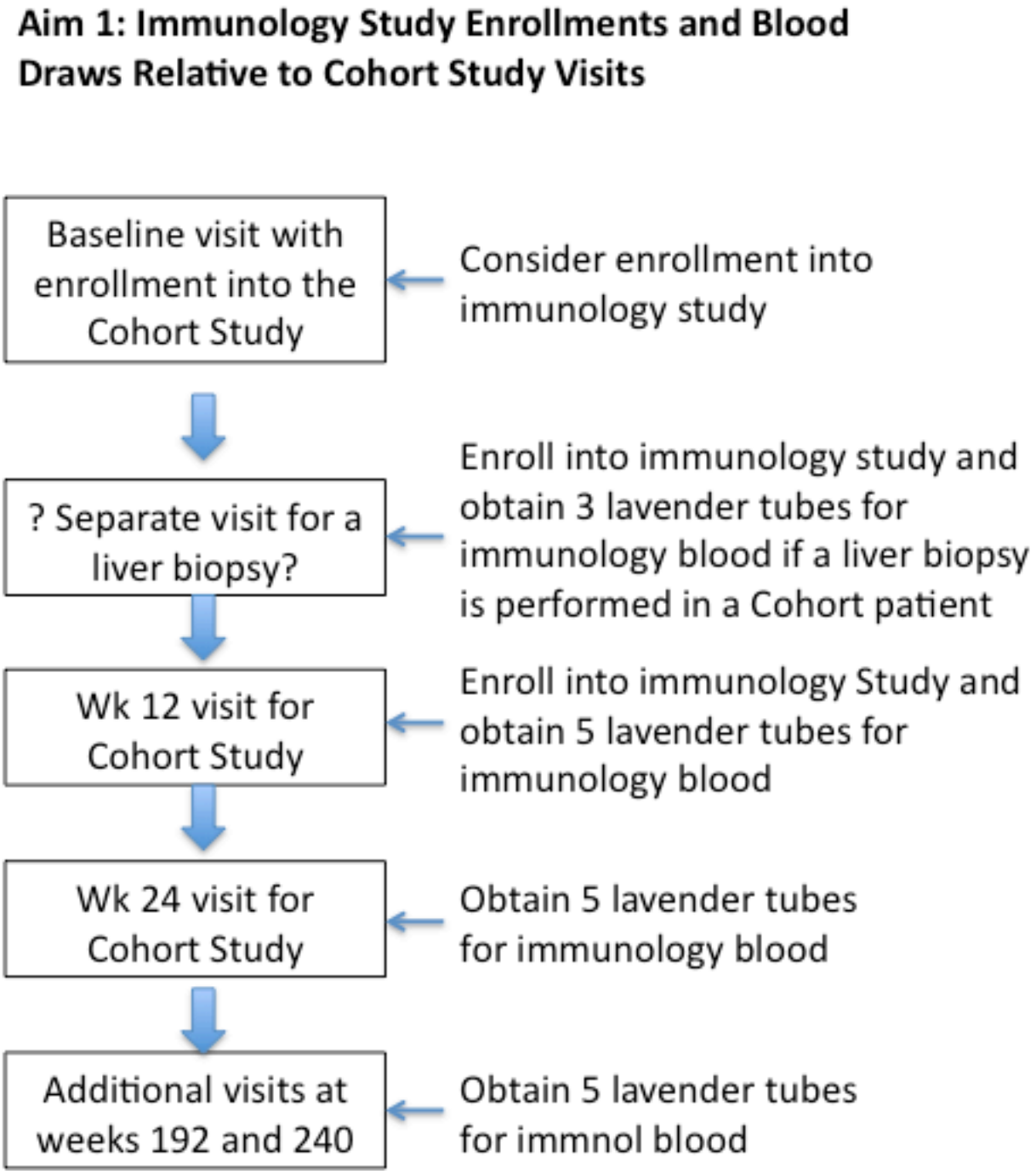
Philadelphia, PA: University of Pennsylvania School of Medicine (Immunology Center)
Pittsburgh, PA: University of Pittsburgh Graduate School of Public Health (DCC)

Boston, MA: Beth Israel Deaconess Medical Center, Massachusetts General Hospital
Minnesota: Mayo Clinic Rochester, University of Minnesota
North Carolina: University of North Carolina,
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

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. Hoofnagle JH. Reactivation of hepatitis B. Hepatology 2009;49:S156-165.
11. Tsai SL, Chen PJ, Lai MY, Yang PM, Sung JL, Huang JH, Hwang LH, et al. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen seroconversion. J Clin Invest 1992;89:87-96.
12. Liaw YF. Hepatitis flares and hepatitis B e antigen seroconversion: implication in anti-hepatitis B virus therapy. J Gastroenterol Hepatol 2003;18:246-252.
13. Kumar M, Chauhan R, Gupta N, Hissar S, Sakhuja P, Sarin SK. Spontaneous increases in alanine aminotransferase levels in asymptomatic chronic hepatitis B virus-infected patients. Gastroenterology 2009;136:1272-1280.

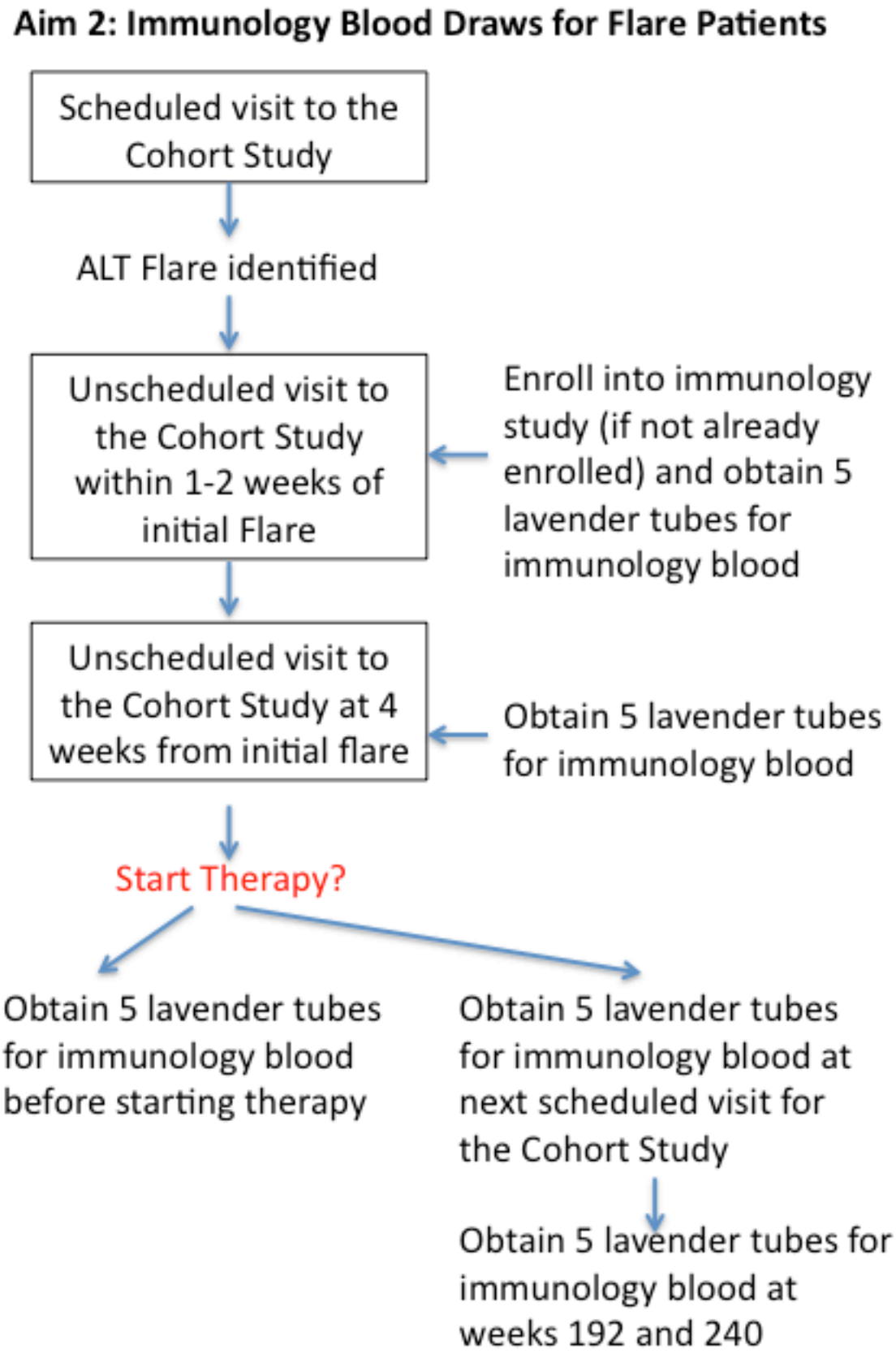
Appendix 2.



Immunology samples may be drawn at Week 24 and Week 48 or at other times in those instances when a patient is not enrolled into the Immunology Study in the first year but is eligible.

HBRN participants who were not enrolled into the Immunology Study during the 1st year will be enrolled at any time during the Cohort Study for at least one blood draw, provided that they remain eligible.

Appendix 3.


Aim 2: Immunology Blood Draws for Flare Patients
Scheduled visit to the Cohort Study
ALT Flare identified
Unscheduled visit to the Cohort Study within 1-2 weeks of initial Flare
Enroll into immunology study (if not already enrolled) and obtain 5 lavender tubes for immunology blood
Unscheduled visit to the Cohort Study at 4 weeks from initial flare
Obtain 5 lavender tubes for immunology blood
Start Therapy?
Obtain 5 lavender tubes for immunology blood before starting therapy
Obtain 5 lavender tubes for immunology blood at next scheduled visit for the Cohort Study
Obtain 5 lavender tubes for immunology blood at weeks 192 and 240