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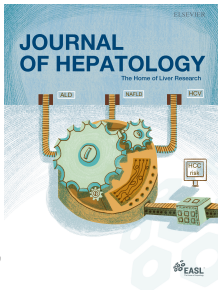

Immunological Biomarker Discovery in Cure Regimens for Chronic Hepatitis B Virus Infection

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PII: S0168-8278(22)00127-1

DOI: https://doi.org/10.1016/j.jhep.2022.02.020

Reference: JHEPAT 8622

To appear in: *Journal of Hepatology*

Received Date: 1 June 2021

Revised Date: 26 January 2022

Accepted Date: 16 February 2022

Please cite this article as: Gehring AJ, Mendez P, Richter K, Ertl H, Donaldson EF, Mishra P, Maini M, Boonstra A, Lauer G, de Creus A, Whitaker K, Martinez SF, Weber J, Gainor E, Miller V, Immunological Biomarker Discovery in Cure Regimens for Chronic Hepatitis B Virus Infection, *Journal of Hepatology* (2022), doi: https://doi.org/10.1016/j.jhep.2022.02.020.

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Key Points:

- Current evidence suggests that some level of immune reconstitution will be required for functional cure of chronic hepatitis B with both novel immune-targeting and direct acting antiviral therapies.

- Phenotypic and functional assays to characterize changes in the HBV-specific immune responses during and off treatment will be necessary to understand the relationship between viral antigen reduction and immune responses, predict clinical outcomes after discontinuation of therapy, inform combination strategies and improve our understanding of liver damage.

- The extent of immunological analyses should be carefully assessed with new experimental therapies that may not have logically predicted impacts on HBV-directed immunity and tested in ancillary studies in late Phase 1 or early Phase 2 clinical studies.

- In addition to validating the mechanism of action (MoA) for immunotherapies, the behavior of the immune response and immunological biomarkers during and off treatment will provide meaningful information to inform patient selection for clinical trials and safety monitoring related to combination therapy and liver inflammation.

- Standardization of assays across diverse laboratories is a challenge and subsequent validation for their use in clinical research will require collaboration among laboratory experts, immunologists, drug developers, regulators and the HBV research community.

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This publication is from a working group, which is part of the HBV Forum

Authors' Contributions: Adam Gehring contributed to all sections, abstract, background, functional analysis, conclusion and revisions. Patricia Mendez contributed to all sections, background, phenotypic analysis, conclusion and revisions. Kirsten Richter contributed to functional analysis. Hildegund Ertl contributed to phenotypic analysis. Eric F. Donaldson contributed to regulatory perspectives. Poonam Mishra contributed to regulatory perspectives. Mala Maini contributed to phenotypic analysis. Andre Boonstra contributed to phenotypic analysis. Georg Lauer contributed to phenotypic analysis. An de Creus contributed to functional analysis. Kathleen Whitaker contributed to regulatory perspectives. Sara Ferrando Martinez contributed to the background and phenotypic analysis. Jessica Weber contributed to images. Emily Gainor contributed to technical and critical revision. Veronica Miller contributed to the background and conclusion.

Financial Support: This work was supported by the Forum for Collaborative Research.

Abstract:

There have been unprecedented advances in identifying new targets for chronic hepatitis B therapy to achieve functional cure in patients who would otherwise face lifelong nucleoside analogue treatment. Many of the new investigational therapies either directly target the immune system or are anticipated to impact immunity indirectly through modulation of the viral lifecycle and antigen production. While new viral biomarkers (HBV RNA, HBcAg, small, middle, large HBs isoforms) are proceeding through validation steps in clinical studies, immunological biomarkers are non-existent outside of clinical assays for antibodies to HBs, and HBe. To develop clinically applicable immunological biomarkers to measure mechanisms of action, inform logical combination strategies, and guide clinical management for use and discontinuation of immune-targeting drugs, immune assays must be incorporated into Phase I/II clinical trials. This paper will discuss the importance of sample collection, the assays available for immunological analyses, their advantages/disadvantages and suggestions for their implementation in clinical trials. Careful consideration must be given to ensure appropriate immunological studies are included as a primary component of the trial with deeper immunological analysis provided by ancillary studies. Standardizing immunological assays and data obtained from clinical trials will identify biomarkers that can be deployed in the clinic, independent of specialized immunology laboratories.

Key words: Hepatitis B, clinical trial, immunology, drug development, therapy, viral hepatitis

Electronic Word Count: Seminar Article – 9272 (abstract, article & references)

Figures:
1. PBMC isolation figure
2. Radial figure with different assays and amount of blood required for each represented as tubes
3. Assay suggestions based on:
    a. Monitoring in clinical studies or
    b. Research-based investigation into mechanism

Tables:
1. List of assays, targets, strengths & limitations (paired with Figure 1)
2. Recommended assays for different classes of drugs

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This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

INTRODUCTION

As an increasing number of therapeutic approaches involving the immune system are being investigated, individually or in combination treatments, the need for "fit for purpose" immunologic assays and data is urgent. Meeting this challenge requires standardization of assays across diverse laboratories and collaboration among laboratory experts, immunologists, drug developers, regulators and the HBV research community for validation to allow them to be used in clinical research. A critical first step is to select, integrate and harmonize assays for monitoring immune responses, potential immune-mediated toxicity, and confirmation of target engagement in clinical trials. The Immune Monitoring Working Group of the HBV Forum, a project of the Forum for Collaborative Research, provides a neutral and independent setting to explore the current status and future directions of approaches to monitor immune modulators in the setting of novel therapies being tested for finite treatment of Chronic Hepatitis B (CHB).

The purpose of this paper is to provide recommendations for both clinical trial sponsors and immunologists for the incorporation of immunological assays in the clinical research setting where available biospecimens do not always meet expectations for the breadth and depth of analysis. The long-term goal is to standardize these techniques and biomarkers across diverse laboratories to be qualified so that they will have prognostic or diagnostic value and can serve for

stratification of trial participants, inform on the effectiveness of novel HBV therapies and guide potential combination therapeutic approaches.

GENERAL BACKGROUND

Functional HBV cure can only be achieved through the elimination or silencing of the HBV replication template, covalently closed circular DNA (cccDNA) in infected hepatocytes[1]. The immune system naturally achieves this via the coordinated action of innate and adaptive immune cells. HBV-specific CD4 T cells, CD8 T cells and B cells are critical for resolution of acute infection[2-4], providing the rationale for the induction of an effective, broad anti-HBV specific response as a therapeutic strategy to promote eradication of the virus in chronically infected patients. However, lack of complete virus clearance exposes the immune system to persistently high levels of viral antigen and liver inflammation, that over decades causes liver injury, leading to fibrosis and cirrhosis[5] and dysregulation of immune function. Chronic exposure to viral antigens drives progressive impairment of functional HBV-specific T and B cells in terms of both quantity and quality[6-13]. This presents the major obstacle for effective therapeutic immune restoration and a major reason that measuring immunity *ex vivo* to develop biomarkers that can influence patient stratification or predict outcomes of novel HBV therapies is so challenging.

A picture of what is considered a successful HBV-specific immune response emerges from extensive research comparing the immune response in patients that resolve acute infection to those of CHB patients[6, 14, 15]. However, the requirements for effective immune control may differ significantly after decades of chronic infection. Our understanding of effective immunity, or the metric for optimal restoration of immunity in CHB patients, may lack key elements not easily measured in patients with acute or resolved HBV infection. These elements can only be defined

by deploying standardized phenotypic and functional analyses, as outlined below, to validate the mechanism of action for immune targeting drugs and evaluate the behavior of the immune system in the setting of new therapies. Therapeutic interventions that impact immune responses provide the opportunity to characterize the key immunological mechanisms responsible for cccDNA clearance, identify crucial prognostic/predictive biomarkers and inform the development of future immunotherapy strategies.

Key questions for HBV cure programs include: does reduction in viral antigen and viral load mediated by a direct acting antiviral (DAA), such as siRNA or antisense oligonucleotide (ASO), impact the functional status of HBV-specific immunity? Can we identify patterns to discriminate between antiviral activity vs. drug hepatotoxicity? Can immunological biomarkers be used to guide potential combination therapies (concomitant vs sequential) and treatment durations? In addition to validating the mechanism of action (MoA) for immunotherapies, characterizing the kinetics of the immune response and immunological biomarkers during and off treatment will provide meaningful information to understand the relationship between viral antigen reduction and immune responses, predict clinical outcomes after discontinuation of therapy, inform combination strategies and improve our understanding of liver inflammation to inform patient selection and safety monitoring related to liver damage.

STRATEGY FOR IMMUNOLOGICAL ANALYSIS

A minimum amount of immunological information should be considered for every study whether the MoA of a drug candidate is immune targeting or not. Advances in technology and innovation provides the opportunity for additional in-depth analyses of HBV-specific immunity in focused translational sub-studies where objective reduction in viral biomarkers, liver damage and

functional cure are observed. Sub-studies allow strategic implementation of large volume blood collection and liver sampling to confirm mechanisms of action and define immune biomarkers that correlate with the antiviral response. In Table 1 we provide guidance for the minimum amount of immunological data required to assess responses and recommended a suite of assays to achieve the detailed analysis required to identify immune mechanisms associated with monotherapy, combination therapy and functional cure.

The following sections outline the utility of these assays, their application, benefits, and limitations. We feel this effort will provide a better understanding of immune responses across various novel HBV therapies. These recommendations reflect the state-of-the-art and will be revised as more data become available.

SAMPLE COLLECTION AND QUALITY FOR IMMUNOLOGICAL ASSAYS

Central to the generation of reliable immunological data is the collection and cryopreservation of high-quality biospecimens, commonly peripheral blood mononuclear cells (PBMC). Numerous variables impact the standardization of sample collection and processing (Table 2) including blood collection tubes, time to processing (fresh or after overnight shipping), the approach to density gradient separation, washing method and buffer, cell counting, freezing medium or freezing apparatus, liquid nitrogen storage and shipping of cryopreserved samples. The issues are familiar to the clinical research community, yet high quality PBMC collection continues to be a significant obstacle in clinical trials.

An overview of PBMC isolation is provided in Figure 1 and Table 2. Although all methods are roughly comparable, each has its benefits and downsides. Variation is mostly driven by donor-to-donor variation and inter-operator variability. In addition to the processing approach, the time

from collection to sample processing affects cellular quality and should be less than 8 hours from blood collection. Good cryopreservation is critical and should be consistent across all sites, which argues against using heat-inactivated (HI) fetal bovine serum (FBS). Specific lots can be used but import restrictions on FBS in different countries prevent some sites from using specified lots. This means that different sites may use different lots of FBS, differentially impacting noise in immunological assays. We recommend the use of 8.5ml ACD tubes for PBMC collection and cryopreservation in synthetic FBS or serum-free freezing medium to minimize lot to lot variability.

Serum and plasma are essential for viral and clinical biomarkers. Rapid processing of serum/plasma and storage at -80°C[16], with minimal freeze/thaw cycles is important to maintain biological activity of immune components.

EX VIVO VS. IN VITRO ANALYSIS

Long-term in vitro culture assays (10 – 14d) for T and B cells have been instrumental in defining the differences in magnitude of the immune responses between patients who resolve acute infection vs. those who have chronic infection[6, 14, 15, 17]. Robust, in vitro T cell expansion after nucleoside analogue withdrawal correlated with better viral control and lack of ALT elevation in CHB patients[18]. Improved T cell expansion was also demonstrated after therapeutic vaccination or starting treatment with nucleoside analogues but have not had significant impact on viral parameters[19-23]. Therefore, in vitro T cell expansion has a role in understanding the immune response during therapeutic interventions but will alter of cell phenotype and function, which will require validation using ex vivo assays.

As a primary approach, clinical studies should strive for *ex vivo* measurement of immune phenotype and function to obtain the most accurate assessment of therapeutic impact on HBV-

specific immunity. Fresh samples may be advantageous when investigating particular cell types or function, such as investigation of neutrophils and myeloid-derived suppressor cells, which do not efficiently survive the freeze/thaw process, and IFN-α production from plasmacytoid dendritic cells, which is severely impaired upon cryopreservation. For longitudinal analysis, cryopreserved samples from all time points should be run in a single experiment to avoid technical variability where the cell type and assay allows. To minimize variability in operators and equipment, immunological assays should be performed within centralized labs until robust biomarkers can be established. A repository of HBV research protocols can be found under ICE-HBV Protocols Database at https://ice-hbv.org/protocol/.

IMMUNOLOGICAL ASSAYS FOR HBV-SPECIFIC IMMUNITY

HBV-specific immunity: HBV-specific T cell Functionality

HBV-specific T cell magnitude and functionality are key distinguishing features between resolved and chronic hepatitis B and represent the basic information to be obtained in clinical studies. However, the low frequency and reduced function of HBV-specific T cells in CHB patients make them challenging to detect ex vivo using conventional assays[6, 15]. Strategies are emerging to improve detection of HBV-specific T cells using ELISpot assays and intracellular cytokine staining that can be applied to blood volumes consistent with clinical trials but sample quality is essential (see above).

Antigen selection

Virtually all assays to measure HBV-specific immune function require re-stimulation in culture. This can be a short stimulation, such as 5h for intracellular cytokine staining (ICS) or overnight stimulation such as those used in ELISpot assays. Standardized reagents for T and B cell stimulation are not available. Synthetic peptides, either representing exact epitopes or overlapping across viral proteins, provide a reproducible source of antigen-specific stimulation that works for both CD4 and CD8 T cells. Overlapping peptide libraries, consisting of peptides with a length of 15 – 18 amino acids, can be synthesized in large quantities and display a reasonable degree of genotype cross-reactivity due to conservation of the HBV genome at the amino acid level[24]. In addition to reproducibility, peptide libraries are stable and can be tailored to cover multiple HBV genotypes for multi-national clinical trials where patient ethnicity and genotypes will vary. A key advantage of overlapping HBV peptides for ICS or ELISpot studies is that they can detect responses in all patients and cover the full breadth of the response within a patient. In addition to covering the entire HBV proteome, peptides can be designed to cover specific targets, such as those included in vaccines. Long synthetic peptides, with a length of 40+ amino acids, have also been used to stimulate HBV-specific T cells, particularly when testing for vaccine-induced responses.

Use of recombinant antigens to study HBV-specific T cell immunity is discouraged. Recombinant antigens fail to efficiently stimulate T cells in the absence of professional antigen presentation and suffer from purity issues[25, 26], which can lead to non-specific immune activation and increase background in immune assays. Alternatively, recombinant antigens have been labelled with fluorescent dyes to successfully measure HBV-specific B cells (discussed below) and therefore have use in the correct scenarios[7, 13, 27].

ELISpot & FluoroSpot

The magnitude and functionality of HBV-specific T cell responses can be assessed using ELISpot and Fluorospot assays. The production and release of an effector molecule (for example interferon gamma) [IFN-γ] is measured using a plate-based, antibody capture-detection method. This method provides high sensitivity because it captures cytokines produced from individual cells and amplifies the signal enzymatically or fluorescently to generate spots of varying size and density that can be read automatically by specific analyzers. Compared to ICS, HBV-HLA-multimer staining or ELISA, ELISpot provides orders of magnitude higher sensitivity, important for ex vivo analysis. In addition to effectively measuring the magnitude of HBV-specific T cell immunity, ELISpot assays are the preferred method to investigate breadth or diversity of the T cell response, particularly after in vitro expansion using small peptide pools or peptide matrices[15]. FluoroSpot assays offer similar sensitivity to ELISpot assays and allow multiplex cytokine analysis for detection and measurement of multiple cytokines (up to 4) co-produced by the same cell[28].

Compared to ICS, ELISpot/Fluorospot are less labor intensive, and less variable. Fewer reagents are needed and the assay analyzes immobilized cytokines on a plate rather than analysis of cells, as in flow cytometry. Although robotics to automate ELIspot plate development and data capture are available, manual aspects of handling cells for the assay cannot be avoided[29]. Cell preparation introduces the most variability as peptide stimulation can involve complicated peptide mixtures and accounting for vehicle toxicity issues, particularly if the concentration of DMSO is high. Accurate cell counting is imperative to accurately compare between time points. The tradeoff for speed and sensitivity of the ELISpot/Fluorospot assay is that the data returned are not as comprehensive as those obtained from ICS or HLA-multimers. No phenotypic data on the T cell

response are recovered, thus making it impossible to identify the cell type responsible for cytokine production or their differentiation status. Changes in T cell magnitude and functionality between pre- and post- treatment (longitudinal sampling post-treatment) with ELISpot/Fluorospot may help to select patients and timepoints to further analyze HBV-specific responses in greater depth.

The high sensitivity, moderate labor and low complexity of the assay and data acquisition make the ELISpot/Fluorospot assay preferable for initial assessment of T cell functionality and magnitude in Phase 1 & 2 clinical trials compared to ICS. The ELISpot/Fluorospot assay should be run for new investigational therapies: therapeutic vaccination, checkpoint blockade, innate immunomodulation, antigen reduction (siRNA, ASO, STOPs, NAPs) and antigen modulation (CAMs). The added benefit of the multi-cytokine fluorospot assay is the opportunity to quantify changes in T cell functionality, such as the potential for improved IL-2 production after checkpoint blockade or vaccination. Data from ex vivo ELIspot/Fluorospot assays can be used to define sampling windows for detailed analysis described below.

Intracellular cytokine staining

ICS can investigate cytokine production from HBV-specific T cells, innate-like T lymphocytes such as mucosal-associated invariant T (MAIT) cells and γδ T cells as well as natural killer (NK) cells. This method relies completely on multi-parametric flow cytometry or mass cytometry to measure cytokine production. It has the advantage of extracting subset-specific data, allowing functional interrogation of different T cell populations, based on surface marker or transcription factor expression. The ICS approach has proven particularly effective for detecting HBV-specific T cell responses following *in vitro* expansion, but this alters the phenotype and the functional profile of the cells, at least partially. Direct ex vivo ICS analysis of HBV-specific T

cells is feasible[30], but similar to HLA multimer studies, it requires large amounts of PBMC in order to detect significant antigen-specific populations. The assays are more difficult for CD4 compared to CD8 responses. The information gained in such assays is valuable as it can establish polyfunctionality (production of several cytokines by the same T cell), in combination with phenotype on the single cell level, which is currently not feasible by ELISpot.

While data obtained from ICS provide a deep picture of T cell functionality, the assay is relatively insensitive, more labor intensive than ELIspot and highly subject to end user experience and capability. The relatively low sensitivity of ICS makes it less effective for ex vivo analysis unless paired with pre-enrichment strategies and/or using large PBMC samples only available in intensified ancillary studies. The staining procedure for ICS has numerous washes/staining steps and requires combining multiple antibodies at the correct dilutions. Some reagent variability can be minimized by manufacturer premixed aliquots but, ultimately, multiple steps that introduce variability and cell loss remain. Furthermore, acquisition and analysis of data is user dependent. High parametric stains to detect cytokine production from multiple subsets introduces technical complexity of compensation that impacts both fluorescent- and mass-based cytometry techniques.

ICS is most effectively deployed in ancillary studies and less attractive as the initial strategy to monitor HBV-specific T cell responses in larger Phase 2 cohorts because of its labor intensity and variability. Using ICS in settings of therapeutic vaccination can quantify the specific CD4/CD8 T cell responses to vaccination and alternations in functionality induced by any adjuvant properties. For strategies such as checkpoint inhibitor therapy, receptor occupancy and HBV-specific CD4/CD8 T cell functionality may be measured simultaneously to assess the response to each HBV antigen, which could inform vaccine combination strategies. Identifying labs with proven experience in flow/mass cytometry and ICS will minimize laborious implementation and

allows for standardization of ICS staining panels and analysis pipelines, providing the depth of information that assays such as ELISpot cannot. These labs are likely to have the added benefit of performing the phenotypic analysis described below.

HBV-specific immunity: HBV-specific T cell phenotyping

Because HBV-specific T cells comprise a tiny fraction of the total T cell compartment in CHB patients (typically <1% in the blood), phenotypic analyses using flow or mass cytometry to detect changes in the overall composition of peripheral blood mononuclear cells fails to provide insight into T cell responses to HBV antigens. Therefore, MHC class I- or II-specific multimers are critical for analysis of HBV-specific T cells. Studies using HBV-specific CD8 MHC multimers directly ex vivo have elucidated differences between patients that cleared an acute infection and those that progressed to chronic infections. Because there are limited HLA-multimer reagents available, and these experiments require significant blood volumes, studies have had to focus on a limited selection of epitopes. Even with this limitation, these studies demonstrated that HBV-specific CD8 T cells are phenotypically heterogeneous, even within patients[9, 12, 31].

The low frequency of HBV-specific CD8 T cells in PBMC limits reliable detection and the challenge is even greater for HBV-specific CD4 T-cells, where the frequencies are typically lower than CD8 T cells. Enrichment strategies (e.g., magnetic bead-based enrichment of HLA multimer-specific T cells) greatly enhance characterization of responses but requires large numbers of PBMC (often 30 ml or more) necessitating careful planning of experiments and patient sampling. Large volume collections can be planned before and after therapy through leukapheresis but this also limits the number of patients available for investigation. Detailed analysis of few, well characterized patients can be highly informative but may miss wider

complexity of CHB patients due to heterogeneity of the response. Therefore, key features should be validated in follow-up studies where broader analysis is performed. Additional limitations for the analysis of antigen-specific T cells stem from the requirement to know the patient's HLA alleles and match available HLA-multimer reagents. Most studies have been limited to HLA-A2 and to a lesser degree to A11 and A24, limiting T cell analysis in Asian populations[32, 33]. A bigger library of HBV-specific epitopes restricted by different HLA alleles and assays incorporating the additional specificities would increase our ability to study whole patient populations and compare T cell phenotypes targeting a broader repertoire of epitopes[34]. Promising approaches to incorporate more specificities per assay include the use of multiplexed[35] or DNA barcoded[36] HLA multimers, but these reagents require further development for deployment in HBV infection[34].

All issues raised above are even more evident for the CD4 T cell responses, which are severely understudied and for which we lack the very basic information of defined viral epitopes. Emerging data indicates that HLA-multimers are effective for the detection of HBV-specific CD4 T cell responses in different stages of HBV infection[37, 38]. Preliminary observations indicate that more HBV-specific CD4 T cells are detectable in patients with functional cure[30]. A better understanding of the role of CD4 T cells for HBV control and during HBV therapy should be a high priority, as sustained viral control mediated by either CD8 T cells or antibodies usually requires a functional and long-lived CD4 response. A focused effort to have widely available multimer library for the detection of both CD4 and CD8 T cell responses targeting HBV in diverse patient populations would be a major facilitator for improved T cell immunology studies.

Despite the challenges associated with *ex vivo* phenotypic analysis of HBV-specific T cell immunity, the value of these data cannot be overstated given their importance in HBV control. The

resolution provided by phenotyping epitope-specific T cells allows for correlations of antigen-specific cellular phenotypes with therapeutic intervention. Understanding which inhibitory and/or activation receptors correlate with objective antiviral responses could improve checkpoint inhibitor therapies or refine patient selection by identifying stages of CHB most likely to respond to specific treatments. This resolution is likely to be highly valuable with antigen reduction strategies, which address the hypothesis that reduction in HBsAg can restore T cell functionality. Similarly, understanding how HBV-specific T cell phenotypes change with vaccination and how this relates to magnitude and functionality when a decline in viral biomarkers is observed will be highly informative for combination strategies. Due to the complexity, *ex vivo* T cell analysis often requires collaboration with specialized laboratories and dedication by the sponsor to ensure that large volumes of blood can be collected from a cohort of patients. This is more amenable through site-specific ancillary or sub-studies. For T cell targeted therapies, *ex vivo* phenotypic analysis will likely be critical to acquire a better understanding of the evolution of HBV-specific T cell responses required for HBV cure.

HBV-specific immunity: HBV-specific B cell phenotyping

Insight into the phenotype and function of B cells that specifically target HBV antigens is limited but there is evidence that B cells play a role in ongoing control of HBV in resolved and chronic infection, based on viral reactivation caused by the B cell-depleting drugs such as Rituximab[39]. HBV-specific B cells might be of importance to predict treatment success as B cells can become compromised in CHB[7, 10, 13].

Similar to virus-specific T cells, frequencies of HBV-specific B cells are very low (generally less than 0.5% of total B cells), requiring relatively large blood volumes for

phenotyping. HBV-specific B cells can be identified through fluorochrome-labeled HBsAg and HBcAg that bind to their respective B cell receptors[7]. Staining protocols make use of HBV antigens labeled to one or two different fluorochromes; staining with two different fluorochromes improves the specificity of the signal. These fluorochrome labeled "baits" are not yet commercially available. Therefore, comparison and standardization of reagents will be paramount once these reagents become widely accessible.

Detailed phenotyping of specific and global B cells to determine their frequency, and their memory and functional status may provide a better understanding of the fluctuations in viral parameters seen during the clinical phases of chronic HBV infection. Their analysis is highly relevant for examining potential therapeutic strategies aimed at boosting B cell functionality, for example the binding of anti-PD1 antibodies to PD-1 that has recently been reported to be upregulated on HBsAg-specific B cells[7, 13].

HBV-specific immunity: HBV-specific B cell functionality

Protocols for measuring antibody secreting B cells specific for HBsAg and HBcAg have been published but are not as standardized as functional assays for T cells[7, 27, 39]. The assays require a short, non-specific, memory B cell expansion followed by detection on either HBV antigen coated wells or with Ig-specific capture antibodies and biotinylated antigens. They are not yet widely used but combined with fluorescent HBV antigen baits described above provide the tools to investigate both the phenotypic and functional profile of HBV-specific B cells.

Serum/Plasma analysis

Serum and plasma are the easiest clinical samples to obtain and are essential for monitoring viral markers to assess antiviral responses. Serum has been useful to measure the MoA of innate immunomodulators through detection of cytokines predicted to be induced by these drugs[40]. The analysis of serum cytokines and immunological effector molecules has yet to predict antiviral responses but has been used to profile different stages of chronic hepatitis B and characterize the inflammatory profile of different type of liver damage[41, 42]. Despite being peripheral measurements, serum assays provide insight into potential intrahepatic immune activation, which can guide in-depth investigation of HBV-specific immunity outlined above and help define the timing for potential intrahepatic samples described below. It is important to remember that peripheral cytokines will likely not represent the full spectrum of immune markers produced in the liver or concentrations achieved within the microenvironment. However, with technologies now able to measure over 1,000 analytes in the serum, the power of this analysis is increasing beyond the measurement of conventional cytokines, providing real opportunities to identify peripheral immune biomarkers associated with viral control and liver damage. Given that serum analysis is the least invasive approach, analysis of serum for immunological biomarkers should remain a standard of future clinical trials.

Functionality of innate-like T cells and innate cells

Relatively little is known about the role of innate lymphocytes such as NK cells, γδ T cells, mucosal associated invariant T (MAIT) cells, and innate lymphoid cells (ILCs) in the control and pathogenesis of chronic hepatitis B. The cytokine profiles induced by pattern recognition receptors can stimulate the production of type I interferons (IFN) or IL-12 and IL-18, which in turn activate MAIT cells, γδ T cells and NK cells, to produce IFN-γ[40, 43]. However, innate

immunomodulators have yet to show significant antiviral efficacy through cytokine production alone.

Of the innate lymphocytes, NK cells have been studied the most. NK cells can display altered cytokine production[44] contribute to pathogenesis through production of effector molecules that induce hepatocyte apoptosis[45] and potentially regulate the HBV-specific T cell response[46]. However, their antiviral activity remains unclear. NK cells can serve are sentinels for the MoA of immune drugs, particularly type I interferons (IFN), where their activation status has been linked with objective responses[47]. Their activation profile can be measured using flow cytometry panels focused on TNF-related apoptosis inducing ligand (TRAIL) up-regulation on CD56hi NK cells. Type I IFNs, and likely other inflammation-inducing drugs that activate NK cells also serve to protect antigen-specific T cells from NK-mediated killing[48, 49], likely limiting the negative impact of this mechanism on immunotherapies. The cytotoxic activity of NK cells can be measured using target cell lines in vitro. Ex vivo cytotoxic activity has been associated with liver damage in CHB patients and, therefore, NK cell activation may serve as a biomarker for liver damage.

With the current knowledge, measuring the function of γδ T cells, MAIT cells or ILCs is not justified in clinical trials. Monitoring the phenotype of NK cells in treatments that are associated with type I IFNs or may induce liver damage could provide insight and serve as a biomarker. However, assessing the role of NK cell-mediated killing of HBV-specific T cells in the context of a clinical trial is unlikely to be practical. This depth of analysis may be carried out in a sub-study with drugs known to induce type I IFNs but the value of this has yet to be determined.

INTRAHEPATIC SAMPLING

To identify the immunological parameters associated with HBV control, we promote the use of intrahepatic sampling to analyze liver-infiltrating cells. This is an important step in understanding HBV-specific immunity at the site of infection but note that intrahepatic sampling does slow enrollment rates into clinical studies. Thus, liver sampling is primarily amenable to site-specific studies or investigator-initiated research studies. The objectives for liver sampling should be clearly defined and justified based on MoA or immunological signals identified in less invasive approaches described above to make the most efficient use of the low cell numbers obtained from liver sampling approaches.

Phenotyping of lymphocytes from liver biopsies

Specific immune subsets are enriched within the liver, and may re-circulate through the peripheral blood, but to what extent the functional or transcription phenotypes of these subsets are different in the liver compared to the blood is not yet clear [50, 51] (Genshaft et al is unpublished data). We may miss an essential piece of the HBV-specific immune response using only blood for analysis. Phenotypic analysis of liver lymphocytes will likely provide a more accurate picture of disease- or treatment-induced immune cell changes in the microenvironment of a chronic infection[52, 53].

Liver biopsies can be justified in clinical trials for patients that meet inclusion/exclusion criteria rather than restricting them based on clinical need, such as patients with active hepatitis. However, frequent core biopsy sampling to monitor the intrahepatic effects of treatment strategies is not feasible. If only a single biopsy can be collected, the most informative time point is likely at the end of treatment (EoT) rather than at baseline. Differences between placebo and treatment groups are likely to be most evident at EoT. However, if longitudinal FNAs are possible, sampling

at baseline and EoT would be the minimum with additional FNAs taken when antiviral/inflammatory events are anticipated or during follow-up.

Isolation protocols need to be standardized to ensure adequate recovery of lymphocytes from liver biopsies; gentle mechanical disruption without enzymatic digestion steps is generally sufficient to obtain viable cell suspensions[54]. Liver fine-needle aspirate (FNA) collection is less standardized but allows for more frequent sampling. However, due to the nature of collection, peripheral blood contamination can be an issue if the needle penetrates a large vessel, requiring methods to control for contamination. Methods to quantitate the practical processing techniques and to assess the level of blood contamination to allow standardization of serial FNAs are being developed (e.g OPPT-FNA (Optimising Practical and Processing Techniques for FNA)[51, 53] (Genshaft et al is unpublished data). Furthermore, cryopreservation may be possible, but protocols are not yet standardized[55]. Currently, liver FNAs require rapid isolation and testing, which will be a challenge for multi-center clinical trials.

Longitudinal sampling of the liver using FNAs in clinical studies provides the power to measure dynamic changes in intrahepatic immunity. It has been used in chronic hepatitis C patients to assess the effect of standard of care treatment and novel antiviral compounds[56-58]. However, in CHB patients, only one study used longitudinal sampling to assess the impact of tenofovir treatment on NK cells[59]. Both core biopsies and liver fine needle aspirates (FNAs) have been used to detect HBV-specific T cell using HLA-multimers[53]. The advantage of using the intrahepatic samples for phenotypic analyses is that HBV-specific T cells are more frequent in the liver than blood and can often be detected without additional enrichment [60]. In addition to the advantage of repeated sampling using FNAs, the cells are collected as a suspension and do not require mechanical or enzymatic digestion, aiding analysis of viable hepatocytes in parallel with

leukocytes[52]. Multiple FNA passes can provide sufficient cell yields for some parallel analyses but assays should be prioritized according to the expected mechanism of action of drugs under investigation. Some specific examples where intrahepatic sampling is likely to be valuable is phenotypic analysis of T cells after checkpoint blockade, HBV-specific T cell recruitment after therapeutic vaccination, innate immunomodulation, or direct-acting antivirals (DAAs) that reduce HBV antigens.

Imaging to assess lymphocyte phenotypes in liver

Multiplexed imaging, such as imaging mass cytometry, for immunophenotyping of core needle liver biopsies might improve immune monitoring by providing not only frequency and phenotype but also spatial distribution/location of immune cell subsets within tissues[61]. Tissue dissociation is not required, and preservation methods are standardized for pathology laboratories. New multiparameter immunostaining platforms allow in situ analysis of cell types requiring more complex combinations of antibodies and in situ hybridization reagents. In addition, immunofluorescence can be combined with spatial genomics technologies to provide state-of-the-art resolution of the liver microenvironment. Panels of antibodies, fixation conditions, platforms and analysis strategies used need to be further validated and standardized for multicenter clinical studies but the standardized preservation of biopsies make them amenable to centralized processing and analysis.

GENERAL IMMUNE PROFILING

Phenotypic analysis by multiparameter flow or mass cytometry allows direct ex vivo analysis of a range of immune cell subsets in parallel with sophisticated characterization of their

features. In many cases, phenotypic analysis uses fewer cells than functional assays. This allows for multiple analyses to be performed on a single sample, providing rapid and broad assessment of immune status, measuring changes in immune composition or the activation/differentiation state of immune populations. Mass cytometry is a variation of flow cytometry, which uses antibodies labeled to heavy metal ions and time-of-flight mass spectrophotometry. There is less spillover as compared to fluorochromes because each metal has its defined mass rather than a fluorescent emission spectrum[10], which allows for simultaneous analysis of more parameters[14]. Mass cytometry provides increased resolution of cell phenotypes since more markers are simultaneously used.

The limitation of phenotypic analysis by either cytometric method is that changes in immunological signatures need to be robust to separate specific effects of the therapeutic intervention from patient heterogeneity. Also, broad changes in immunological phenotypes of total immune cell populations cannot be extrapolated to HBV-specific immunity, which is specifically affected by the persistent presence of HBV antigens. Proper panel design, validation, compensation, and gating strategies can also be highly user dependent and a source of variation in clinical studies if samples are acquired at multiple sites. Therefore, while general immune profiling with cytometry-based approaches is accessible, and high-resolution analysis is available, using limited samples from clinical trials should have a defined purpose because it provides limited insight into HBV-specific immunity. Some examples of this would be measuring a surface marker to gauge mechanism of action, monitoring leukopenia of specific cell types, phenotyping of innate immune cells or receptor occupancy of therapeutic antibodies. Otherwise, samples are better used for HBV-specific T or B cell analysis or functional assays.

SINGLE-CELL RNA SEQUENCING

Understanding the processes of functional cure will refine the development of immune biomarkers that are likely to be specific to either individual drugs or drug classes. This process will likely be accelerated as new "omics" technologies are incorporated into clinical trials with a focus on immunity. Already mentioned are large serum inflammatory panels that measure >1,000 analytes with minimal sample volume.

Single-cell RNA sequencing is rapidly becoming accessible for use in clinical studies but has so far been primarily restricted to the blood. This technology fits well with the liver FNA sampling approach, where few cells are available for analysis. Single-cell RNA sequencing provides a high-resolution snapshot of the intrahepatic immune response. When combined with longitudinal sampling, will likely identify dynamic changes in the transcriptional profile of multiple cell types simultaneously. In addition to monitoring immunological changes, it may be possible to compare the transcriptional profile between infected and uninfected hepatocytes[62]. This data is of particular interest where immunotherapies or DAAs are targeted to the liver such as innate immunomodulators, modulation of checkpoint inhibitors in the liver or HBV antigen reduction.

Thus far, HBV-specific T & B cells have not been readily detected in current high throughput single cell RNA sequencing techniques and require flow cytometry-based cell sorting into individual wells for single cell analysis. Combining single-cell RNA sequencing with HLA multimer DNA-barcoded libraries that can simultaneously test for responses targeting numerous HBV epitopes may help overcome this obstacle. However, HLA-multimer reagents and epitopes remain limited for HBV and the low frequency of HBV-specific T and B cells presents a numerical challenge of the current technologies. In addition, hepatocytes have been captured in current single

cell technologies, but these examples used digested liver tissue from biopsies or resections[63, 64]. It is currently unclear if the hepatocytes recovered from liver FNAs will be of sufficient quality to measure their transcriptional profiles using the single-cell RNA sequencing strategies.

Lastly, the development of spatial transcriptomics platforms can combine phenotypic and transcriptional data with localization within the liver tissue in core biopsy samples. These strategies allow for a systems immunology approach starting from the plasma and ending in individual cells in the patient liver.

Regulatory Perspectives

Antiviral drugs developed to modulate innate and adaptive immune responses to chronic HBV infection are likely to target host factors and induce or repress immune biomarkers prior to having an impact on HBV replication or clearance of HBV-infected hepatocytes. Nonclinical pharmacology studies can be used to describe the specific mechanism of action of the drug and to demonstrate that immune modulation in cell culture and animal models of HBV infection results in antiviral activity. These studies can be used to demonstrate that HBV replication, as measured by HBV DNA, is reduced, or that the HBV cccDNA reservoir is reduced by assessing HBsAg loss or cccDNA levels directly, or both. In addition, given that these antiviral drugs may target host factors, it is important to assess the impact of polymorphisms in the target to determine any impact on activity. If proof-of-concept studies are performed with animal models, it is important to determine that the target of the drug is conserved, having similar affinity, between the animal species being assessed and the human target.

Clinical assessment during the development of immunomodulators is likely to be challenging, given that the greatest impact of these types of drugs will likely be a reduction in

infected cells resulting in depletion of the cccDNA reservoir. Complete depletion of the cccDNA reservoir to below the limit of detection may take a long time and will vary depending on the mechanism of action of the drug. Currently, the only endpoint sufficient to predict a sustained response off-treatment is HBsAg loss, the assessment of which may be complicated by HBsAg expressed from integrated HBV DNA[1]. In addition to host immune markers, clinical trial protocols may assess several exploratory HBV endpoints (HBcrAg, HBV RNA, etc.) early in the development program in an attempt to identify potential markers that correlate with the immunomodulatory activity and may predict response to antiviral therapy. The assays used to measure these HBV markers in clinical trials of new immune modulatory therapies need to be standardized and validated during the subsequent course of clinical development.

When developing assays for clinical assessment of patients undergoing treatment for HBV (or post-treatment), it is important to first define the intended use of the in vitro diagnostic. What is the analyte being measured, who will be tested (where, e.g., point of care, high complexity laboratory and when), what are the appropriate specimen types, and how will the results be used in patient management? Analytical studies in support of the diagnostic may vary according to the technology, the end user, quantitative or qualitative nature of the diagnostic, and what is being reported (individual analytes vs a composite score). The clinical validation of the assay also depends on the intended use. It is often advantageous for the developer of the in vitro diagnostic to partner with the drug manufacturer enabling access to specimens, patient demographics and outcomes. If the assay will be submitted to FDA for approval, then it is advisable to participate in the presubmission process for in vitro diagnostic devices[65].

Conclusion

In the beginning, we laid out example questions that, by incorporating immunology into clinical trials, we could potentially address and move the goal of HBV cure forward. To address these questions, we have put clear emphasis on the ex vivo measurement of HBV-specific immunity. Each assay provides an additional layer of information. The measure of HBV-specific T cell functionality and magnitude is a basic assessment of the immune response that could be altered by vaccination, innate immunomodulators, antigen reduction, or checkpoint blockade. However, therapies such as vaccination are likely to boost immunity in the majority of patients whereas an objective decline in viral biomarkers may occur in only a minority of patients. Understanding why only a fraction of patients respond falls on the next level of analyses, investigating the phenotypic and functional profiles of individual T and B cells and differences in intrahepatic immunity that result in viral decline to discriminate between responders and non-responder. With respect to DAAs, such as siRNA/ASO, removal of viral antigens from the circulation may not alter the total peripheral HBV-specific T cell response, as suggested by recent studies in CHB patients where HBsAg levels did not impact HBV-specific T cell frequency[10, 66]. However, phenotypic changes at the individual HBV-specific cell level may predict timing to add-on immune-stimulatory compounds to the combination therapy to maximize the immunological response. Any changes in immunological magnitude or phenotype can then be weighed against ALT elevations to define effective antiviral inflammatory responses. Liver sampling can then be used to further resolve immune responses at the site of infection and validate the antiviral effect. Using these complicated immunological experiments as a guide, serum analysis can be focused to specific time points, and on specific analyte classes, to define peripheral biomarkers

By measuring the magnitude and functionality of HBV-specific immune responses and obtaining a detailed phenotype of the HBV-specific T and B cells, we will be able to measure how novel single agent and combination therapies reshape immunity. Knowing these pathways will help distinguish between drug and immune related liver damage. In line with this understanding, current strategies for therapy withdrawal are related to viral biomarkers, which so far have not predicted which patients can safely stop therapy. One could anticipate that this information might be provided by immunological biomarkers.

The ultimate goal is to integrate and interpret a comprehensive dataset in patients that achieve functional cure on novel hepatitis B therapies to focus efforts on specific aspects of the immune system that were responsible. The initial effort to define these biomarkers are likely to require centralized analysis to standardize assays given the challenges of measuring HBV-specific immunity. These may be different for different stages of chronic hepatitis B but without making the effort to obtain these data, these biomarkers will remain elusive. Therefore, it will require a dedicated effort by sponsors to incorporate the assays described above into clinical trials and collaboration with research labs that have demonstrated expertise. These collaborative efforts between sponsors and researchers should extend beyond data generation, allowing access to trial immunological data for analysis and publication by non-industry scientists associated with the clinical studies. This will ensure different perspectives towards data analysis, which is much more likely to identify immunological biomarkers or mechanisms associated with HBV cure.

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Table 1 Phenotypic and Functional Analyses Guide

Class of Drug	Immunologic Effect(s)	Implementation†	Outputs*	Assays
Innate Immune Agonists	Myeloid Cell Activation	Standard	Cytokine profiles◊	Serum Cytokines, RNAseq/scRNAseq
	Potential improvement of HBV-specific T and B cells	Advanced	Peripheral* and/or intrahepatic^# immunophenotyping, T & B cell function	• Serum Proteomics • ELISpot/FluoroSpot • HBV-HLA-multimers • Fluorescent antigen baits • RNAseq/scRNAseq
Therapeutic Vaccines	Induction of HBV-specific T & B cell responses, reduction of circulating antigens, reduction of infected cells	Standard	Peripheral HBV-specific T cell and B cell¶ magnitude and function Clinical HBV antibody assays	• Multi-cytokine FluoroSpot • Fluorescent antigen baits • B cell ELISpot
		Advanced	Phenotypic analysis (peripheral & intrahepatic) • Exhaustion • Activation Intrahepatic trafficking^$	• HBV-multimers • Fluorescent antigen baits • HBV-specific cell sorting + 'omics
Immune checkpoints	Improvement in HBV-specific T and B cell responses	Standard	Target engagement§ Peripheral HBV-specific T cell and B cell¶ magnitude and function HBV-specific antibody responses	• Multi-cytokine FluoroSpot • Fluorescent antigen baits • B cell ELISpot
		Advanced	Phenotypic analysis (peripheral & intrahepatic^) • Exhaustion • Activation Transcriptional changes in HBV-specific T & B cells • Peripheral & liver^$	• HBV-HLA-multimers • Fluorescent Antigen Baits • Sorted HBV specific cells + 'omics • Intrahepatic HBV specific cells

ImmTAV	Polyclonal T cell activation	Standard	Phenotypic analysis of peripheral immune cell activation markers	High parameter cytometry

		Advanced	• Intrahepatic target engagement
			• Phenotypic analysis (peripheral & intrahepatic^)
			  ○ Exhaustion
			  ○ Activation
			• Transcriptional changes in T cells
			  ○ Peripheral & liver^#
				High parameter cytometry

Antigen Modulation (siRNA, ASO, NAPs, STOPs)	Decrease in antigen-specific activation	Standard	Peripheral HBV-specific T & B cell¶ magnitude and function	• Multi-cytokine FluoroSpot • Fluorescent antigen baits • B cell ELISpot
	Potential re-invigoration of HBV-specific T and B cells	Advanced	Phenotypic analysis (peripheral & intrahepatic^) • Activation markers • Exhaustion markers	• HBV-HLA-multimers • Fluorescent antigen baits • Sorting Ag specific cells + 'omics
Replication Inhibitors (HBV entry inhibitors & CAMs)	Transient immune restoration	Standard	HBV-specific T cell magnitude and function	ELISpot/FluoroSpot

†Standard: Easy implementation, standardized technique; Advanced: Specialized, limited to site-specific studies or investigator-initiated research studies
**In general, analysis timepoints should occur at baseline, peak of response, EOT (end of treatment), and FU (follow up), as appropriate; Potential timepoints reflect the minimum time required to observe changes in the immune biomarkers and could be modified based on s-ag reductions patterns. Phenotypic and Functional Analyses are highly recommended in clinical trials exploring HBV Cure*

¶ *Recommend implementation despite no standardized assays*

◊ *Sampling should be considered 4-24 hours after dosing, as appropriate*

§ Sampling should occur at weeks 2-8 post treatment, as appropriate
^ Core liver biopsy at baseline and EOT for (minimum EOT)
#Liver Fine-needle aspirate at baseline and 4 – 24h post-dose and EoT, optional at antiviral or inflammatory events
$ Liver Fine-needle aspirate at baseline and EoT, optional at antiviral or inflammatory events
[A]Depending on dosing schedule: example 2nd dose, 4 weeks post, EOT, 24, 48 weeks post EOT; [timepoints depend on vaccine discovery and the schedule]
Abbreviations:

- *siRNA: small interfering RNA*
- *ASO: antisense oligonucleotide*
- *NAPs: nucleic acid polymers*
- *STOPs: S-antigen Transport-inhibiting Oligonucleotide Polymers*

Table 2: Comparison of different methods for PBMC isolation

	Manual Density Gradient	**Frit Barrier**	**CPT tubes**
Examples	Ficoll overlay	SepMate, LeucoSep, Accuspin	Sodium Citrate, Sodium heparin
Benefit	High PBMC yield and viability, Lowest cost, Accommodate a wide range of input blood volumes	Reduced operator variability, Reduced time to process	Whole blood directly collected into separation tubes, Reduced time to process, Reduced operator variability
Downside	Operator variability*, Higher processing time Low cost	Potential for operational [variability] difficulties*, Medium cost	Possible contamination of samples with erythrocytes, Expensive, Restricted in range of input blood volumes (size 4-8 mL)

mL= milliliter

*With proper training, the main source of variability is based on blood donors. However, there will be site to site variability.

Figure 1: Peripheral Blood Mononuclear Cell (PBMC) Blood Isolation

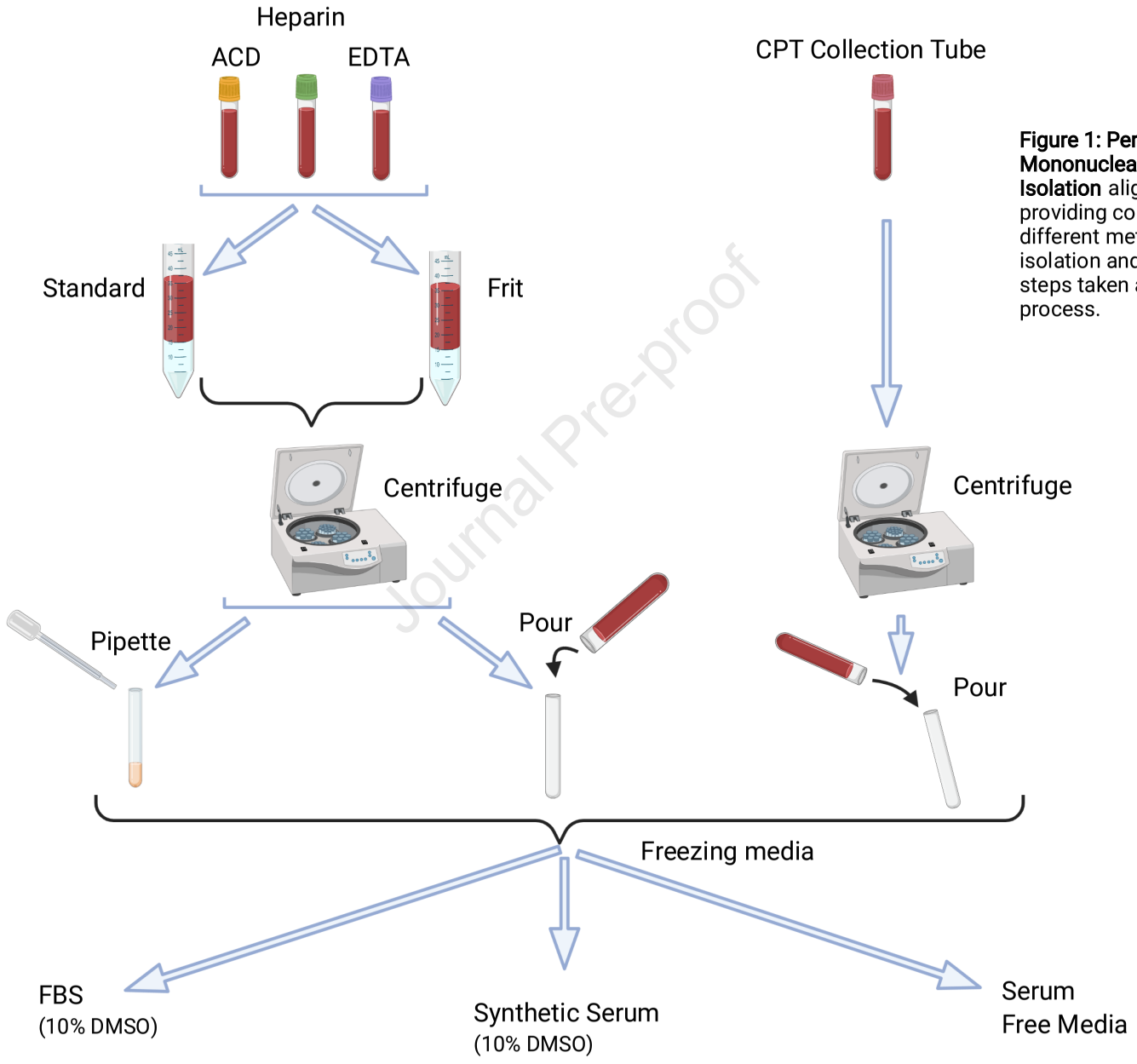
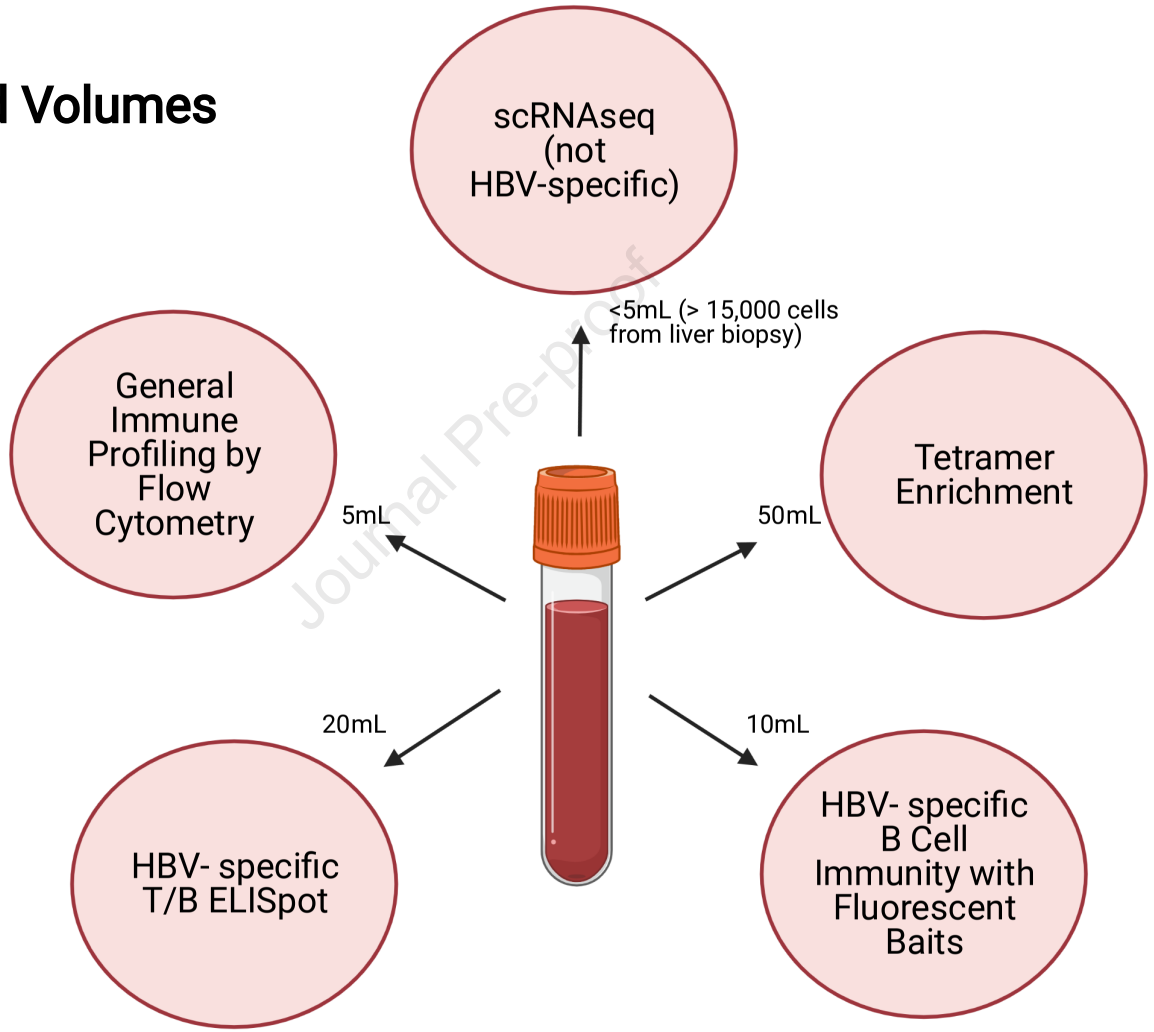


Figure 1: Peripheral Blood Mononuclear Cell (PBMC) Blood Isolation aligns with Table 1, providing comparisons of different methods of PBMC blood isolation and the comparative steps taken as part of this process.

Figure 2: Blood Volumes

Figure 2: Blood Volumes contains a radial figure with the estimated amount of blood required for each type of assay.



*Volumes may vary based on specific assays and tecnhology.

Figure 3: Labor Input vs. Data Output

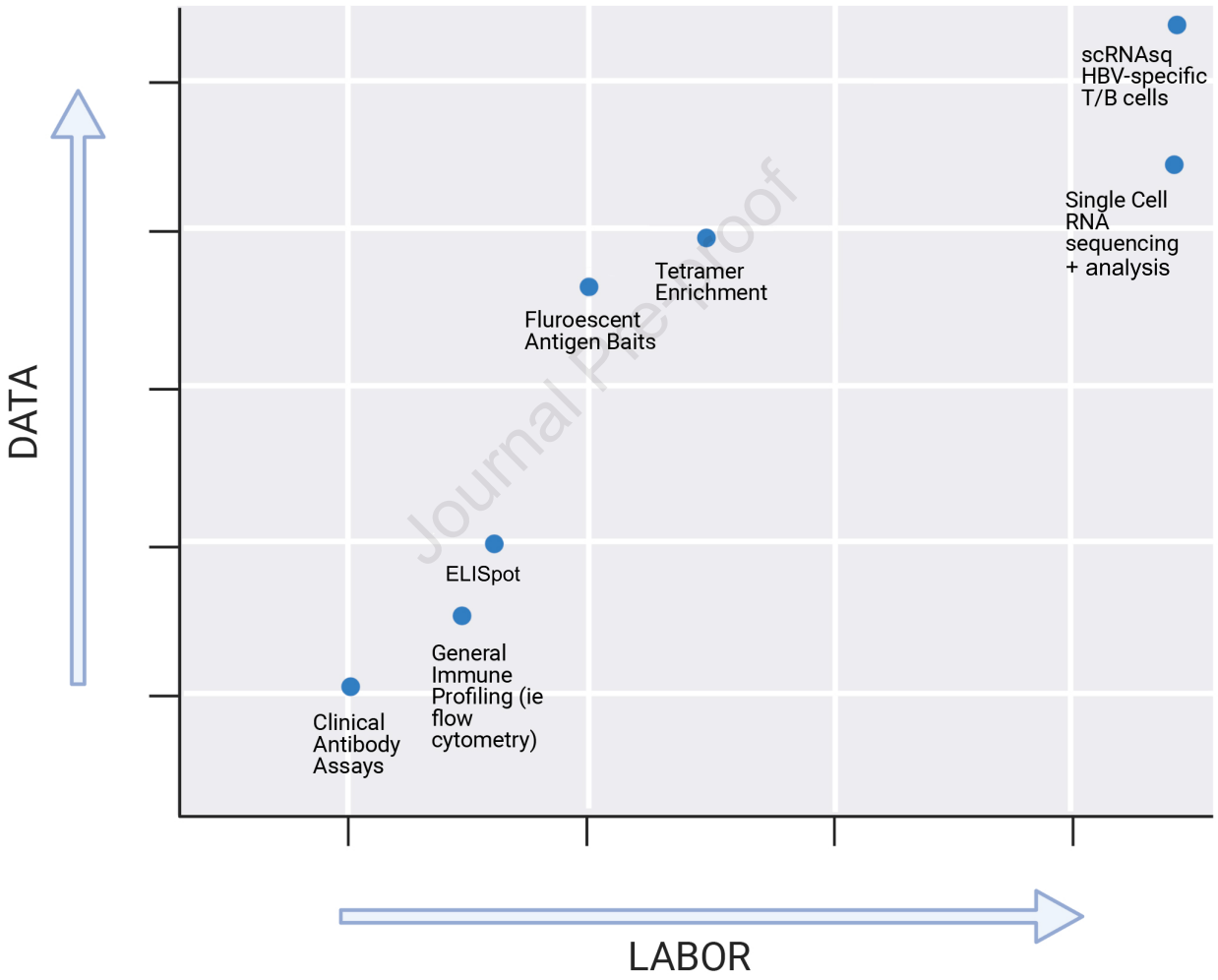


Figure 3: Labor Input vs. Data Output provides a comparison of the amount of labor needed to conduct each assay and the data produced by these efforts. Assay suggestions are based on monitoring in clinical studies or research-based investigations into mechanism.