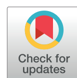

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Immunological biomarker discovery in cure regimens for chronic hepatitis B virus infection†

Adam J. Gehring[1,2,*], Patricia Mendez[3], Kirsten Richter[4], Hildegund Ertl[5], Eric F. Donaldson[6], Poonam Mishra[6], Mala Maini[7], Andre Boonstra[8], Georg Lauer[9], An de Creus[10], Kathleen Whitaker[11], Sara Ferrando Martinez[12,13], Jessica Weber[14], Emily Gainor[14], Veronica Miller[14]

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Summary

There have been unprecedented advances in the identification of new treatment targets for chronic hepatitis B that are being developed with the goal of achieving functional cure in patients who would otherwise require 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, HBc 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. Standardising immunological assays and data obtained from clinical trials will identify biomarkers that can be deployed in the clinic, independently of specialised immunology laboratories.

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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 standardisation of assays across diverse laboratories and collaboration among laboratory experts, immunologists, drug developers, regulators and the HBV research community to validate them for clinical research. A critical first step is to select, integrate and harmonise assays to monitor immune responses, potential immune-mediated toxicity, and 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 immunomodulators 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 regarding the incorporation of immunological assays into 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 standardise these techniques and biomarkers across diverse laboratories so that they will have prognostic or diagnostic value and can be used for the stratification of trial participants, to determine the effectiveness of novel HBV therapies and to 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

[1]Toronto Centre for Liver Disease, Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario, Canada; [2]Department of Immunology, University of Toronto, Toronto, Ontario, Canada; [3]Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, USA; [4]F. Hoffmann-La Roche, Roche Innovation Center Basel, Grenzacher Strasse 124, CH-4070 Basel, Switzerland; [5]The Wistar Institute, Philadelphia, PA 19104, USA; [6]Division of Antivirals, Center for Drug Evaluation and Research, US Food and Drug Administration, USA; [7]Division of Infection and Immunity, University College London, London, UK; [8]Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, The Netherlands; [9]Division of Gastroenterology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA; [10]Janssen Pharmaceuticals, Beerse, Belgium; [11]Division of Microbiology Devices Office of In Vitro Diagnostics and

Radiological Health Center for Devices and Radiological Health, US Food and Drug Administration, USA; [12]Microbial Sciences, Biopharmaceuticals R&D, AstraZeneca, Gaithersburg, US; [13]NeoImmuneTech, LLC 2400 Research Blvd, Suite 250 Rockville, MD 20850, USA; [14]Forum for Collaborative Research, University of California, Berkeley, USA

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* Corresponding author. Address: Princess Margaret Cancer Research Tower, Room 10-356, 101 College St, Toronto, ON M5G 1L7, Canada; Tel.: +1 (416) 634-7095.

E-mail address: adam.gehring@uhnresearch.ca (A.J. Gehring).

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Key point

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.

viral 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 patients with CHB.[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 patients with CHB, may lack key elements not easily measured in patients with acute or resolved HBV infection. These elements can only be defined by deploying standardised phenotypic and functional analyses, as outlined below, to validate the mechanism of action for immune-targeting drugs and evaluate the behaviour of the immune system in the setting of new therapies. Therapeutic interventions that impact immune responses provide the opportunity to characterise 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 programmes include: does reduction in viral antigen and viral load mediated by a direct-acting antiviral (DAA), such as small-interfering RNA (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 of immunotherapies, characterising the kinetics of the immune response and immunological biomarkers during and off treatment will provide meaningful information on the relationship between viral antigen reduction and immune responses. This information will help us predict clinical outcomes after discontinuation of therapy, inform combination strategies and guide patient selection and safety monitoring by improving our understanding of liver inflammation and damage.

Strategy for immunological analysis

A minimum amount of immunological information should be considered for every study whether the mechanism of action of a drug candidate is immune targeting or not. Advances in technology and innovation provide 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 for 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 on the minimum amount of immunological data required to assess responses and recommend 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 (PBMCs). Numerous variables impact the standardisation 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 Fig. 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 foetal

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, RNA-seq/scRNA-seq
	Potential improvement of HBV-specific T and B cells	Advanced	Peripheral[*] and/or intrahepatic[^,#] immunophenotyping, T- and B-cell function	• Serum Proteomics • ELISpot/FluoroSpot • HBV-HLA multimers • Fluorescent antigen baits • RNA-seq/scRNA-seq
Therapeutic vaccines	Induction of HBV-specific T- and B-cell responses, reduction of circulating antigens, reduction of infected cells	Standard	Peripheral HBV-specific T- 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[^,$]	• HBV-multimers • Fluorescent antigen baits • HBV-specific cell sorting + 'omics
Immune checkpoints	Improvement in HBV-specific T- and B-cell responses	Standard	Target engagement[§] Peripheral HBV-specific T- and B-cell[¶] magnitude and function HBV-specific antibody responses	• Multi-cytokine FluoroSpot • Fluorescent antigen baits • B-cell ELISpot
		Advanced	Phenotypic analysis (peripheral & intrahepatic[^]) • Exhaustion • Activation Transcriptional changes in HBV-specific T and B cells • Peripheral & liver[^,$]	• 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- and 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

ASO, antisense oligonucleotide; CAMs, capsid assembly modulators; NAPs, nucleic acid polymers; RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA sequencing; siRNA, small-interfering RNA; STOPs, S-antigen transport-inhibiting oligonucleotide polymers.

[†]Standard: Easy implementation, standardised technique; Advanced: Specialised, limited to site-specific studies or investigator-initiated research studies.

[*]In general, analysis timepoints should occur at baseline, peak of response, EoT, and 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 reduction patterns. Phenotypic and functional analyses are highly recommended in clinical trials exploring HBV cure.

[¶]Recommend implementation despite no standardised 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 4–24 h post-dose and EoT, optional at antiviral or inflammatory events.

[$]Liver fine needle aspirate at baseline and EoT, optional at antiviral or inflammatory events.

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Table 2. Comparison of different methods for PBMC isolation.

	Manual density gradient	Frit barrier	Cell preparation tubes (CPTs)
Examples	Ficoll overlay	SepMate, LeucoSep, Accuspin	Sodium citrate, sodium heparin
Benefit	High PBMC yield and viability, lowest cost, accommodates 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*, longer processing time	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)

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

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 8.5 ml ACD tubes for PBMC collection and cryopreservation in synthetic FBS or serum-free freezing medium to minimise 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 the biological activity of immune components.

Ex vivo vs. in vitro analysis

Long-term *in vitro* culture assays (10–14 days) 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 alanine aminotransferase elevation in patients with CHB.[18] Improved T-cell expansion was also demonstrated after therapeutic vaccination or the start of treatment with nucleoside analogues but does not seem to have a significant impact on viral parameters.[19–23] Therefore, *in vitro* T-cell

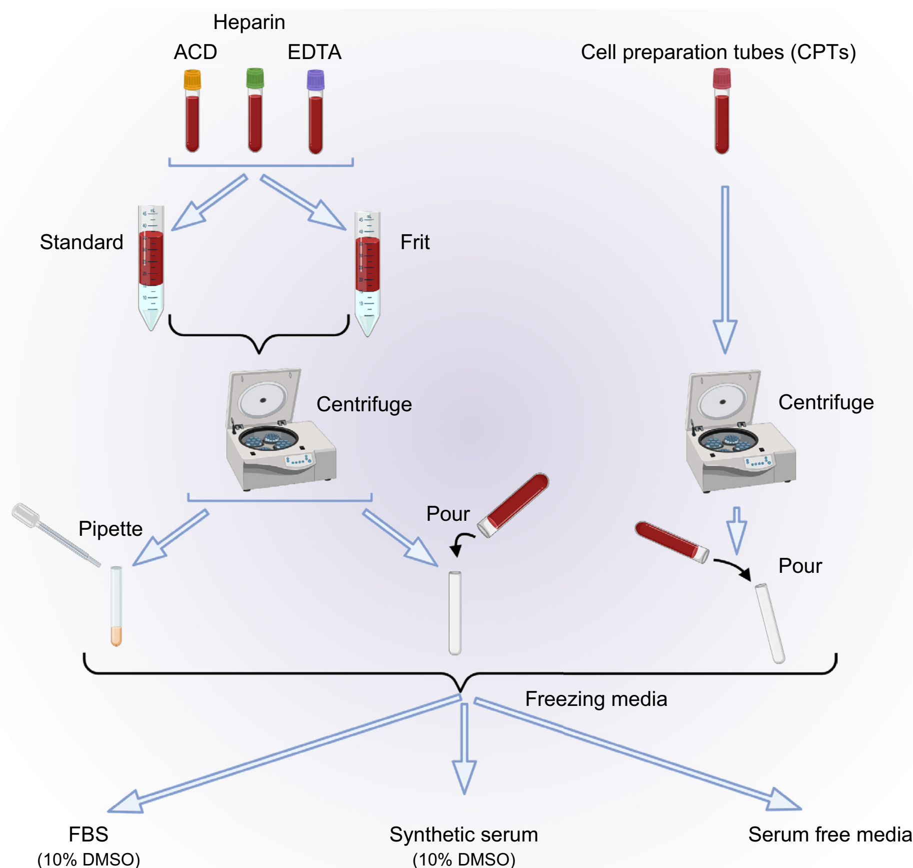

Fig. 1. Isolation of peripheral blood mononuclear cells from blood. Figure provides comparisons of different methods of isolating peripheral blood mononuclear cells from blood and the comparative steps taken as part of this process.

expansion will be important to uncover the role of the immune response during therapeutic interventions, but *in vitro* expansion will alter cell phenotype and function; thus, validation using *ex vivo* assays will be required.

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 functions, such as investigation of neutrophils and myeloid-derived suppressor cells, which do not efficiently survive the freeze/thaw process, and interferon (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 minimise variability in operators and equipment, immunological assays should be performed within centralised laboratories 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 required in clinical studies. However, the low frequency and reduced function of HBV-specific T cells in patients with CHB 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 (ICS) 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, *e.g.* 5 h for ICS, or overnight stimulation, *e.g.* for ELISpot assays. Standardised 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 synthesised 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 multinational 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 noise 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 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 analysers. Compared to ICS, HBV-HLA-multimer staining or ELISA, ELISpot provides orders of magnitude higher sensitivity, which is important for *ex vivo* analysis. In addition, ELISpot assays are the preferred method to investigate the breadth or diversity of the HBV-specific 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 enable 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 labour intensive, and less variable. Fewer reagents are needed and the assay analyses immobilised cytokines on a plate rather than analysing 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 must account for vehicle toxicity issues, particularly if the concentration of DMSO is high. Accurate cell counting is imperative to accurately compare

Key point

Phenotypic and functional assays to characterise 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.

between time points. The trade-off 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 analyse HBV-specific responses in greater depth.

The high sensitivity, moderate labour and low complexity of the assay and data acquisition make the ELISpot/Fluorospot assay preferable to ICS for the initial assessment of T-cell functionality and magnitude in phase I and II clinical trials. The ELISpot/Fluorospot assay should be run for new investigational therapies: therapeutic vaccination, checkpoint blockade, innate immunomodulation, antigen reduction (siRNA, ASO, S-antigen transport-inhibiting oligonucleotide polymers, nucleic acid polymers) and antigen modulation (capsid assembly modulators). 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 the detailed analyses described in the following sections.

Intracellular cytokine staining

ICS can be used to investigate cytokine production from HBV-specific T cells, innate-like T lymphocytes (such as mucosal-associated invariant T [MAIT] cells and $\gamma\delta$ T cells), as well as natural killer (NK) cells. This method relies completely on multiparametric flow cytometry or mass cytometry to measure cytokine production. It has the advantage of extracting subset-specific data, enabling 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 numbers of PBMCs in order to detect significant antigen-specific populations (Fig. 2). 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 labour 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 involves numerous washes/staining steps and requires the combination of multiple antibodies at the correct dilutions. Some reagent variability can be minimised 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, introduce 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 II cohorts because of its labour intensity and variability. Using ICS in settings of therapeutic vaccination can quantify the specific CD4/CD8 T-cell responses to vaccination and alterations 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 guide vaccine combination strategies. Identifying laboratories with proven experience in flow/mass cytometry and ICS will minimise laborious implementation and allows for standardisation of ICS staining panels and analysis pipelines, providing the depth of information that assays such as ELISpot cannot. Another benefit of such laboratories is that they will likely be able to perform the phenotypic analysis described in subsequent sections.

HBV-specific immunity: HBV-specific T-cell phenotyping

Because HBV-specific T cells comprise a tiny fraction of the total T-cell compartment in patients with CHB (typically <1% in the blood), phenotypic analyses using flow or mass cytometry to detect changes in the overall composition of PBMCs fail to provide insights into T-cell responses to HBV antigens. Therefore, MHC class I- or II-specific multimers are critical for the analysis of HBV-specific T cells. Studies using HBV-specific CD8 MHC multimers directly *ex vivo* have elucidated differences between patients who cleared an acute infection and those who 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

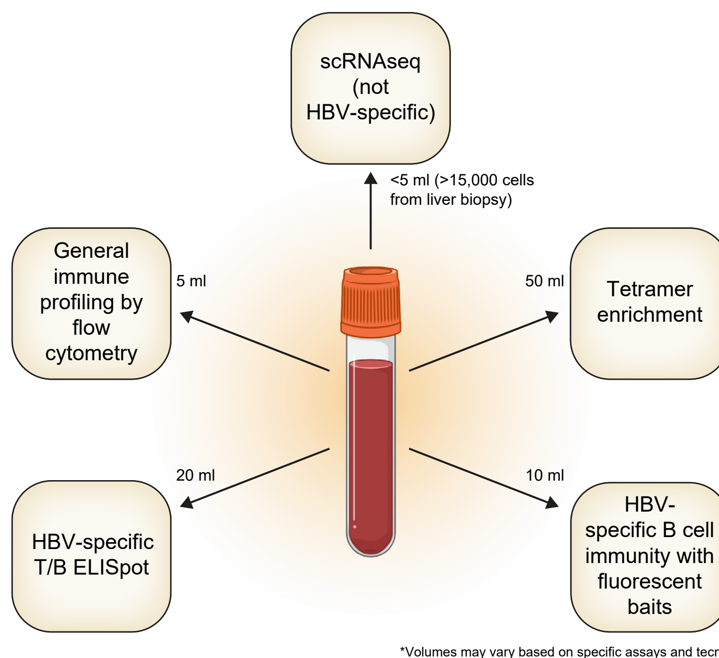

Fig. 2. Blood volumes required for different assays. Blood volumes contain a radial figure with the estimated amount of blood required for each type of assay.

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 PBMCs limits reliable detection and the challenge is even greater for HBV-specific CD4 T cells, which are typically present at a lower frequency than CD8 T cells. Enrichment strategies (*e.g.*, magnetic bead-based enrichment of HLA-multimer-specific T cells) greatly enhance characterisation of responses but require large numbers of PBMCs (often 30 ml or more) necessitating careful planning of experiments and patient sampling (Fig. 2). 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 a few well-characterised patients can be highly informative but may miss the wider complexity and heterogeneity present in patients with CHB. Therefore, key features should be validated in follow-up studies where broader analysis is performed. Additional limitations to 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 prior to their application in HBV infection.[34]

All of the aforementioned issues are even more evident for the measurement of CD4 T-cell responses, which are severely understudied and for which very basic information regarding defined viral epitopes is still lacking. 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 in 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 develop a widely available multimer library for the detection of both HBV-specific CD4 and CD8 T-cell responses in diverse patient populations would be a major facilitator of 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

Key point

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 I or early phase II clinical studies.

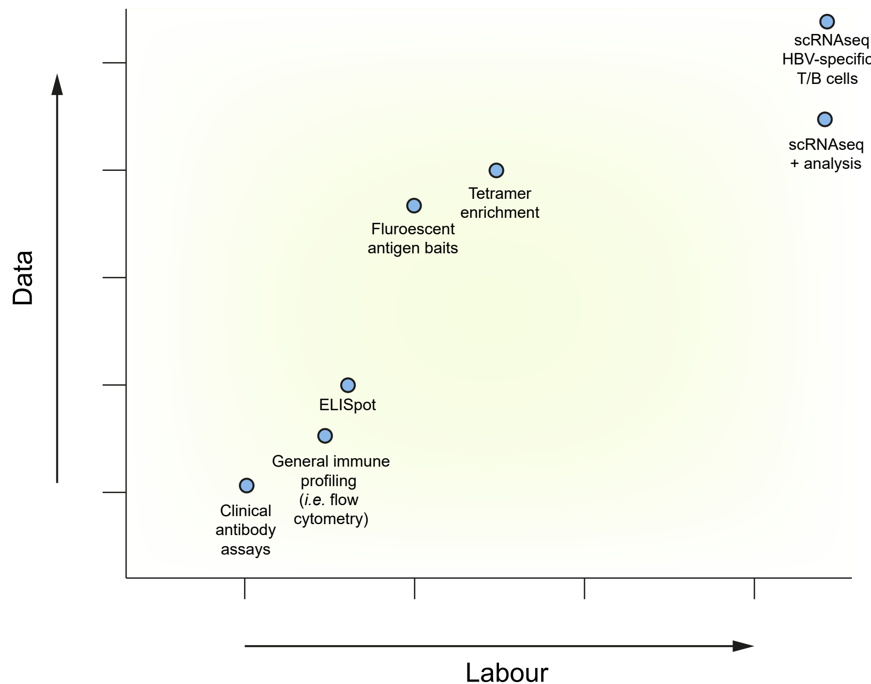

Fig. 3. Labour input *vs.* data output provides a comparison of the amount of labour needed to conduct each assay and the data produced by these efforts. Assay suggestions are based on monitoring in clinical studies or research-based investigations into mechanisms. scRNAseq, single-cell RNA-sequencing.

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. Due to its complexity, *ex vivo* T-cell analysis often requires collaboration with specialised 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 B cell-depleting drugs such as rituximab.[39] HBV-specific B cells might be of importance in predicting 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-labelled HBsAg and HBcAg that bind to their respective B-cell receptors.[7] Staining protocols make use of HBV antigens labelled to one or two different fluorochromes; staining with two different fluorochromes improves the specificity of the signal. These fluorochrome-labelled “baits” are not yet commercially available. Therefore, comparison and standardisation 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 improve our 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 PD1 that has recently been reported to be upregulated on HBsAg-specific B cells.[7,13]

HBV-specific immunity: HBV-specific B-cell unctionality

Protocols for measuring antibody-secreting B cells specific for HBsAg and HBcAg have been published but are not as standardised 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 the fluorescent HBV antigen baits described, 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 mechanism of action 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 characterise the inflammatory profile of different types 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 and help define the timing for potential intrahepatic sampling. 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 for 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, MAIT cells, and innate lymphoid cells in the control and pathogenesis of chronic hepatitis B. The cytokine profiles induced by pattern recognition receptors can stimulate the production of type I IFNs 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 as sentinels for the mechanism of action of immune drugs, particularly type I IFNs, where their activation status has been linked with objective responses.[47] Their activation profile can be measured using flow cytometry panels focused on TRAIL (TNF-related apoptosis inducing ligand) upregulation 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 patients with CHB and, therefore, NK cell activation may serve as a biomarker for liver damage.

With our current knowledge, measuring the function of γδ T cells, MAIT cells or innate lymphoid cells 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.

Key point

In addition to validating the mechanism of action for immunotherapies, the behaviour 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.

Intrahepatic sampling

To identify the immunological parameters associated with HBV control, we promote the use of intrahepatic sampling to analyse liver-infiltrating cells. This is an important step in understanding HBV-specific immunity at the site of infection, but it should be noted that intrahepatic sampling does slow enrolment 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 mechanism of action or immunological signals identified using the less invasive approaches described earlier 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 transcriptional 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 fine needle aspirates (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 standardised 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 FNA collection is less standardised 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 quantify and assess the level of blood contamination that allow for standardisation 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 standardised.[55] Currently, liver FNAs require rapid isolation and testing, which will be challenging to apply in multicentre 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 patients with chronic hepatitis C to assess the effect of standard of care treatment and novel antiviral compounds.[56–58] However, in patients with CHB, only one study used longitudinal sampling to assess the impact of tenofovir treatment on NK cells.[59] Both core biopsies and liver FNAs have been used to detect HBV-specific T cells using HLA multimers.[53] The advantage of using intrahepatic samples for phenotypic analyses is that HBV-specific T cells are more abundant 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 prioritised according to the expected mechanism of action of drugs under investigation. Some specific examples where intrahepatic sampling is likely to be valuable are phenotypic analysis of T cells after checkpoint blockade, HBV-specific T-cell recruitment after therapeutic vaccination, innate immunomodulation, or DAAs that reduce HBV antigens.

Imaging to assess lymphocyte phenotypes in the liver

Multiplexed imaging, such as imaging mass cytometry, for immunophenotyping of core needle liver biopsies might improve immune monitoring by revealing 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 standardised for pathology laboratories. New multiparameter immunostaining platforms allow *in situ* analysis of cell types requiring more complex combinations of antibodies and *in situ* hybridisation 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 need to be further validated and standardised for multicentre clinical studies but the standardised preservation of biopsies make them amenable to centralised processing and analysis.

General immune profiling

Phenotypic analysis by multiparameter flow or mass cytometry allows for direct *ex vivo* analysis of a range of immune cell subsets in parallel with sophisticated characterisation 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, enabling the rapid and broad assessment of immune status, including measuring changes in immune composition or the activation/differentiation state of immune populations. Mass cytometry is a variation of flow cytometry, which uses antibodies labelled to heavy metal ions and time-of-flight mass spectrophotometry. There is less spillover 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, it 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, such as innate immunomodulators or checkpoint inhibitors, or DAAs that reduce HBV antigens are targeted to the liver.

Thus far, HBV-specific T and 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 for 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 single-cell RNA sequencing strategies.

Lastly, the development of spatial transcriptomics platforms can combine phenotypic and transcriptional data with localisation within the liver tissue in core biopsy samples. These strategies allow for a systems immunology approach starting in 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. Non-clinical 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 and/or cccDNA levels directly. 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 programme in an attempt to identify potential markers that correlate with immunomodulatory activity and may predict response to antiviral therapy. The assays used to measure these HBV markers in clinical trials of new immunomodulatory therapies need to be standardised 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

Key point

Standardisation 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.

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 the FDA for approval, then it is advisable to participate in the pre-submission process for *in vitro* diagnostic devices.[65]

Conclusion

At the start of this seminar, we laid out example questions, with relevance to HBV cure, that could potentially be addressed by the inclusion of immunology in clinical trials. 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 but also complexity (Fig. 3). 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 most 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-responders. 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 patients with CHB and chronic hepatitis C, where viral antigen levels did not impact virus-specific T-cell profiles.[10,66,67] However, phenotypic changes at the individual HBV-specific cell level may predict the optimal timing to add immunostimulatory compounds to the combination therapy to maximise the immunological response. Any changes in immunological magnitude or phenotype can then be weighed against alanine aminotransferase 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 treatment 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 who achieve functional cure on novel hepatitis B therapies to focus efforts on the specific aspects of the immune system that were responsible. The initial effort to define these biomarkers will likely require centralised analysis to standardise 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 aforementioned assays into clinical trials and collaborate with research laboratories 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.

Abbreviations

ASO, antisense oligonucleotide; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; DAA, direct-acting antiviral; EoT, end of treatment; IFN, interferon; FBS, foetal bovine serum; FNA, fine needle aspirates; ICS, intracellular cytokine staining; MAIT, mucosal-associated invariant T; NK, natural killer; PBMCs, peripheral blood mononuclear cells; siRNA, small-interfering RNA.

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Conflict of interest

Please refer to the accompanying ICMJE disclosure forms for further details.

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.

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Supplementary data

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