RESEARCH PROTOCOL

PROJECT TITLE:

The impact of Bepirovirsen on host HBV specific immune response in regard to HBsAg decline and its durability

SPONSOR/COLLABORATOR**:**

- **Sponsor – Chang Gung Memorial Hospital, Linkou**
- **Collaborator -- GlaxoSmithKline Research & Development Ltd**

ESTIMATED STUDY START DATE**:** July 13, 2025
ESTIMATED STUDY END DATE (analysis complete)**:** October 29, 2027

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3. ABSTRACT

The persistence of HBV infection results from an impaired host immune response that fails to clear the virus. HBV-specific T cells play a crucial role in the clearance of HBsAg. Bepirovirsen (Bepi) is an antisense oligonucleotide (ASO) that targets all messenger RNAs of the hepatitis B virus (HBV) and helps to reduce viral protein levels. After 24 weeks of weekly treatment with 300 mg of Bepirovirsen, followed by a 24-week follow-up period, about 10% of participants achieved HBsAg levels below 0.05 IU/mL, which is considered a functional cure. In this project, we aim to investigate changes in host immune phenotypes, particularly focusing on the numbers and functionality of HBV-specific T cells, using samples collected from participants enrolled in Bepirovirsen-containing trials. By comparing peripheral blood mononuclear cells (PBMCs) collected before the investigational product (IP) administration, at 7 weeks of Bepirovirsen treatment, at 24 weeks of Bepirovirsen treatment (end of IP), and 24 weeks after discontinuation of Bepirovirsen (end of follow-up), we will examine the host immune profiles and HBV-specific T cells in participants with and without a rapid reduction in HBsAg during the IP and placebo administration. This project may disclose the changes of the host antiviral immune response during Bepi administration and understood the possible immune pathogenesis explaining the durability after Bepi treatment.

Word count: 213

4. KEYWORDS: Dextramer staining, Functional cure, HBsAg rapid decline, HBV specific T cells, single cell RNA seq

5. CONTENT

5.1 Background

HBV specific T cells and the chronicity of HBV infection

HBV-specific T cells are vital for controlling the virus,(1, 2) but their numbers are limited in chronic HBV infection.(3, 4) Exhaustion of HBV-specific CD8+ T cells, particularly those expressing LAG3+Tim+PD-1+, contributes to persistent infection.(5) A distinct population of liver-resident CD8+ T cells with an activated immune profile are also present during liver damage.(6) Non-HBV-specific CD8+ T cells, including innate-like bystander CD8+ T cells (coined as CD38+HLA-DR+CD8+T cells), may play a role in hepatotoxic liver damage in HBV infection, associated with IL12/18 or IL15,(6, 7) similar to their role in chronic hepatitis C that found in our lab.(8).

The novel approaches for HBV specific immune response

It used to be hard to obtained HBV specific T cell information from ex vivo studies and most of the known HBV immunological information of human subjects derived from in vitro expansion.(3, 9) Recently, the utility of tetramer-staining method enables the better detection of HBV specific T cell response.(10) The intracellular cytokine staining and highly multiplexed combinatorial peptide-major histocompatibility complex (pMHC) tetramer strategy allows the detection of rare antigen-specific T cell directly ex vivo.(10, 11) Other novel investigation tools include single immune cell approaches, including Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITEseq), mass cytometry (Cytof) and/or 30 color flow cytometry (FACSymphony)(12, 13), which enable the investigation of immune mechanism of human diseases.(14, 15) CITEseq helps to understand the phenotype or function of different immune cell population via tis high throughput of huge amount of mRNA information. Symphony, the multicolor flow cytometry, help to investigate the protein expression of the immune cells, understanding the cellular immune response.(14, 16) Integrating these novel tools may help us to overcome the obstacle in the past and understand more of how antiviral immune response changes during treatment.

Bepirovirsen and HBsAg seroclearance

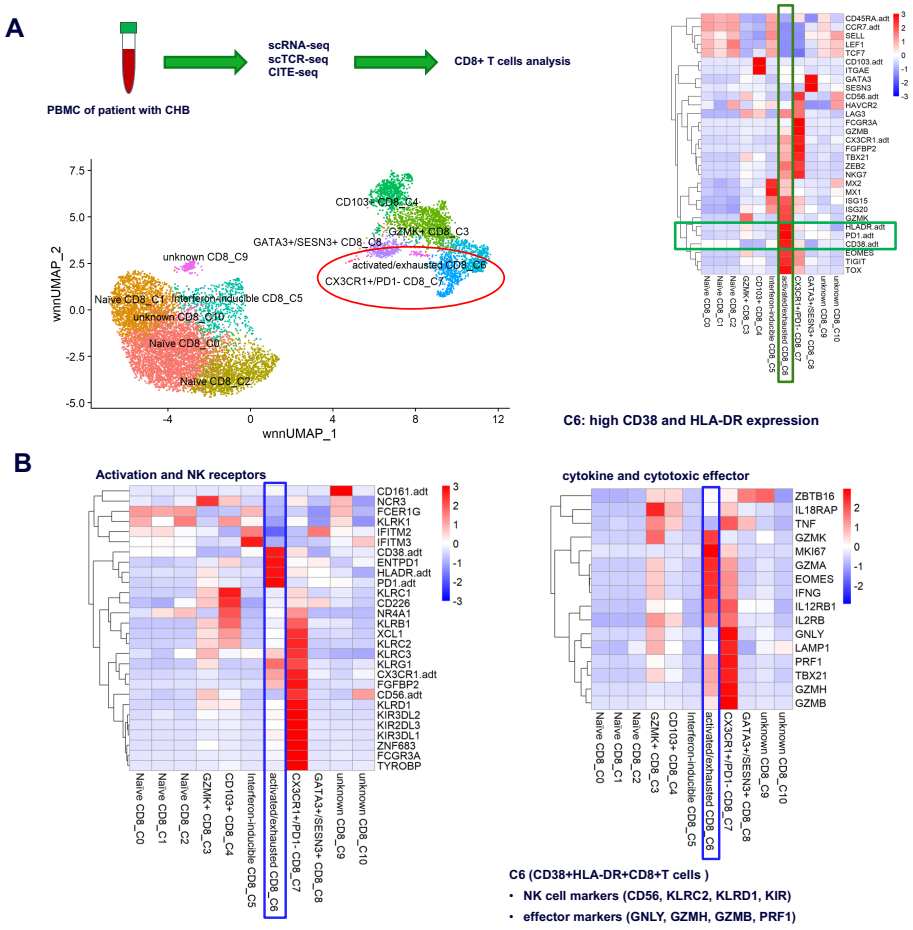
Bepirovirsen, an antisense oligonucleotide (ASO) against hepatitis B virus, rapidly reduces HBsAg levels. In a phase 2b trial (B-Clear study), about 30% achieved HBsAg loss after 24 weeks of Bepirovirsen treatment at a dose of 300 mg per week, but sustained loss occurred in only ~10% post- treatment .(17, 18) ALT elevation, even >10-20 times ULN, was observed in more than one-third of subjects during the first half of treatment.(17, 18) Apart from the hepatitis flare observed in natural

history or off-nucleos(t)ide analuge relapse which the decline in HBsAg occurs after the hepatitis flare,(19-21) the ALT elevation during Bepirovirsen treatment was preceded by a greater rapid decline in HBsAg (velocity: > 1 $\log_{10}$ IU/year).(17, 18)

The host immunity which possibly be responsible for HBsAg reduction

It has been reported that TLR8 activation by Bepirovirsen's immunostimulatory activity is linked to the early HBsAg reduction.(22) In a study using GS-9688, a TLR8 agonist, it enhanced immune cell activation and boosted interferon-gamma expression in HBV-specific CD8+ T cells,(23) possibly due to IL12 and IL18 from activated NK cells.(23-26) Our unpublished data shown CD38+HLA-DR+CD8+T cells, identified by unsupervised clustering by scRNAseq analysis (Figure 1A), which resemble innate-like CD8+T cells with NK receptor expression and effector function molecules (Figure 1B).

Figure 1. CD38+HLA-DR+CD8+T cells represented as innate-like T cells in chronic hepatitis B patients. (A) By unsupervised clustering using scRNAseq analysis, the CD38+HLA-DR+CD8+T cells represented as activated/exhausted CD8+T cell cluster; (B) these CD38+HLA-DR+CD8+T cells cluster showed innate behavior with NK receptor and effector function molecules.



Additionally, CD38+HLA-DR+CD8+T cell is significantly correlated with serum ALT level in patients with chronic hepatitis B but decreases after NA treatment (Figure 2). In addition, TLR8 is present in monocytes but challenging to detect in CD8+ T cells from PBMC via single-cell RNA sequencing (Figure 3).

Figure 2. Percentage of innate CD8+T cells is highly correlated with serum ALT levels but decreased after NA treatment.

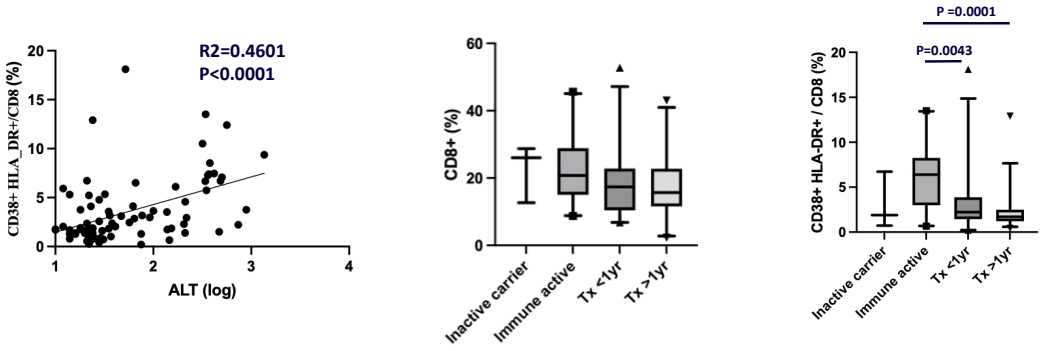
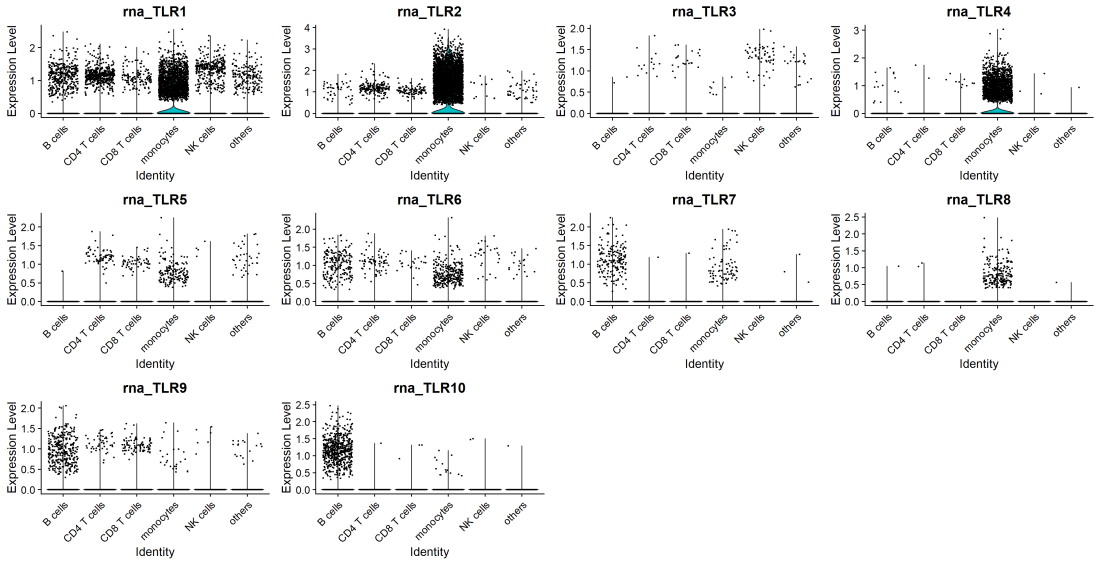


Figure 3. The amount of TLR family among monocytes, B cells, CD4 T cells, CD8 T cells, and NK cells;



Therefore, adding Bepirovirsin to CHB patients on long-term NA treatment with suppressed HBV DNA to suppress HBsAg production may also activate innate-like T cells through IL12/15/18 produced by myelocytes through TLR8 activation at the same time, potentially causing liver inflammation. These immune response, driven by innate T cells, might offer a significant opportunity for rejuvenation of HBV-specific

T cells.

It's crucial to investigate Bepirovirsen's impact on the host immune system, especially its effects on HBV-specific and innate-like T cell responses during rapid HBsAg decline, TCR changes, and the phenotype of HBV-specific T cells at discontinuation to understand why only a fraction sustain HBsAg loss post-treatment.

5.2 RESEARCH QUESTIONS: Could the stimulation effect on TLR8 by Bepirovirsen help to rejuvenate the HBV-specific T cells?

We hypothesize that Bepirovirsen, in addition to inhibit HBs synthesis with a decline in HBsAg levels, may also activate myeloid cells through TLR-8 with subsequent activation of innate T cells by IL12/18 and IL15, that in turn may lead to ALT elevation, facilitating the restoration of HBV-specific T cells. These mechanisms may also account for the durability of off-therapy effects.

5.3 PRIMARY OBJECTIVES: **To explore the changes of HBV specific T cell phenotype during the administration of Bepirovirsen and its association with HBsAg decline and durability.**

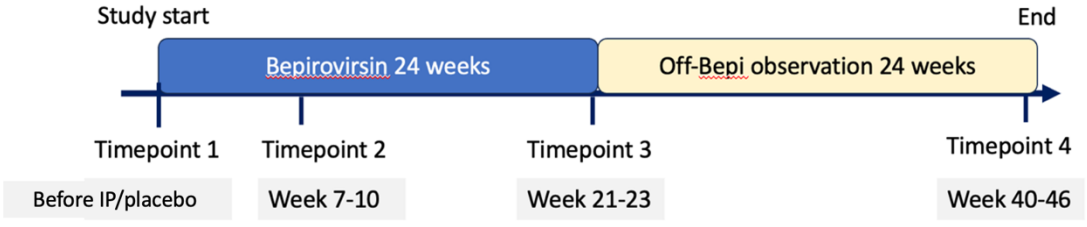
5.4 SECONDARY OBJECTIVES

1. **To investigate the changes of innate-like CD8+ T cells and their association with ALT elevation during Bepirovirsen**
2. **To investigate the phenotype changes of monocytes during the Bepirovirsen administration**
3. **To investigate the plasma cytokine patterns, especially the IL12/15/18, during Bepirovirsen treatment.**

5.5 STUDY DESIGN AND INVESTIGATION

5.5.1 STUDY POPULATION:

Single-center, retrospective observational (cohort) sub-study of the B-Well Phase 2b RCT (1:3 randomization, Bepirovirsen vs. placebo).



Target enrolment/sample size: 20 (10 for cross-sectional comparison, another 10 for longitudinal assessment)

5.5.2 INCLUSION CRITERIA

. CHB patients who enrolled in the Bepirovirsen-based clinical trials (e.g. B-well)
. Patients who had signed their consent for the possible sub-study of B-well.

5.5.3 EXCLUSION CRITERIA

. Patients with Hgb level < 10mg/dL

Planned Order of Experiment Execution:

In order not to disturb the original B-Well unblind schedule, we'll start with the 10 subjects (Cross-sectional part) first. We'll do the experiment on subjects whose HBsAg decline > 0.5 log during the IP/placebo injection, whom presumed to be on IP since the HBsAg kinetics be reported constant under long-term Nuc treatment. This portion may take about 6-9 months, before the unblind date, to finish the experiment, analysis and discussion. After the unblind procedure as originally B-well planned, the experiment on rest of the 10 cross-sectional subjects as well as longitudinal sampling subjects will be performed and analyzed.

5.5.4 EVALUATION OF HOST IMMUNITY

Evaluation of HBV-specific T cells

1. **Multimer approach:**
   For patients with HLA-A*11:01 or HLA-A*02:01, the tetramer will be used to identify the HBV specific T cells ex vivo. The phenotype of these HBV-specific T cells will be evaluated in two approaches. One is to evaluate the phenotype by multi-color flow cytometry (Symphony). Another is to evaluate the transcriptomic profiles by scRNAseq analysis. Finally, their effector functions will be evaluated after peptide stimulation. In case of too few HBV-specific T cells in some of the patients at some time point, in-vitro expansion of HBV-specific T cells will be done.
2. **Cytokine-synthesis pattern after peptide-pools stimulation**
   For the patients other than HLA-A*11:01/HLA-A*02:01, we will use the peptide pool stimulation and then by cytokine secretion assay to identify the HBV specific T cells ex vivo. After identifying these HBV specific T cells, the two approaches mentioned above (multicolor flow cytometry and scRNAseq analysis) will be chosen as well. In case of too few HBV-specific T cells in some

of the patients at some time point, in-vitro expansion of HBV-specific T cells will be done.

Evaluation of innate T cells

Based on the evidence shown in rationale section, we will then choose the CD38+HLA-DR+T cells as the marker of innate like bystander CD8+T cells. The phenotype of these innate T cells will be evaluated by multicolor flow cytometry and transcriptomic profiles by scRNAseq. In addition, their effector functions will be evaluated after cytokine stimulation (mainly IL12/18 and IL15)

Evaluation of monocytes in PBMCs

The monocytes in PBMCs will be one of the representative myeloid cells to evaluate the effects of Bepirovirsen on the myeloid cells, especially the effects through TLR8. The phenotype of these monocytes will be evaluated by multicolor flow cytometry and transcriptomic profiles by scRNAseq. Their function will be evaluated as well after simulation with TLRs agonists.

Plasma cytokine array evaluation

The immune effect of Bepirovirsen is believed through TLR8. Therefore, the plasma cytokine array will be performed with the quantitative cytokine array (BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokine Cytometric Bead Array (CBA) - I Kit) to evaluate and confirm the activation of TLR8 and their association with the changes of phenotype of CD8+T cell.

5.6 EXPECTED STUDY PROGRESS:

Year 1. **To investigate the changes of phenotype of innate-like CD8+ T cells monocyte and HBV specific T cells during Bepirovirsen, and their association with the ALT elevation during treatment**

In the first year, we'll perform the experiment using PBMC collected from Bepi-based trials. We'll apply for unblind of the subjects who had enrolled in this part of the projects.

HLA genotyping will be performed first. Those with HLA-A 11:01 genotype will undergo multimer approach for HBV specific T cell assessment, followed by CITE-seq/single cell RNA seq. Those with HLA-A non 11:01 will perform cytokine stimulation approach for HBV specific T cell assessment. Symphony (multicolor flow cytometry) will be performed the same times across different timepoints. Longitudinal changes of the numbers and phenotypes of these immune cells will be compared between (A). Bepi-experienced group vs. placebo group; (B). Among Bepi-experienced patients with vs. without HBsAg rapid reduction; (C). Among Bepi-

experienced patients with vs. without HBsAg loss.

Year 2. To investigate the serum cytokine patterns, including the IL12/15/18, during Bepirovirsen treatment.

In the early half of the second year, we'll investigate the cytokine expression patterns including IL12/15/18 (known to expand innate CD8+T cells), proinflammatory and anti-inflammatory cytokinesw during Bepirovirsen treatment and the differences across the three different comparison settings: (A). Bepi-experienced group vs. placebo group; (B). Among Bepi-experienced patients with vs. without HBsAg rapid reduction; (C). Among Bepi-experienced patients with vs. without HBsAg loss.
The latter half of the second year, we'll complete the analysis and start the drafting of the manuscript.

5.7 STUDY ENDPOINTS

5.7.1 PRIMARY ENDPOINTS

The changes of HBV-specific CD8 T cell phenotype during and after Bepirovirsin treatment and their relationship to the durability of HBsAg loss during follow-up.

5.7.2 SECONDARY ENDPOINTS

The changes of innate-like T cell phenotype during and after Bepirovirsin treatment and their relationship with ALT elevation during treatment and the off-therapy viral control

5.8 EXPERIMENTAL METHOD

HLA genotyping

Human leukocyte antigen(HLA) determination was performed by using PCR amplification on genomic DNA with HLAssure SE A Locus SBT Kit #50110(TBG Biotechnology Corp., New Taipei City, Taiwan). Genome DNA was extracted from whole blood by using PureLink™ Genomic DNA Mini Kit(Invitrogen) according to the manufacturer's instructions. Briefly, group specific and locus specific sequence-specific primer pairs are designed to selectively amplify target sequences that are specific to a single allele or group of alleles. This amplification method is based on the principle that only primers with completely matched sequences to the target sequences result in amplified products under controlled PCR conditions. Mismatched primers do not generate amplicons. Reactions with amplicons are purified and subjected to locus specific sequencing reactions that determine the exact DNA sequence of each sequenced allele. The HLAssureTM SE SBT Kit supplies a panel of primer mixes that is designed to provide amplify and sequence HLA alleles. A

sequence analysis software can be used to assist the determination of HLA types by analyzing the resulting sequencing reactions.

Generation of HLA-A*11:01 and HLA-A*02:01 dextramer

Major histocompatibility complex (MHC) class I epitope-specific dextramers were generated by conjugation of biotinylated peptide-MHC class I monomers (Biolegend) with PE-conjugated streptavidin. Peptide exchange was performed at 0.2 mg/ml of HLA-A*11:01 monomer (Biolegend) in 20ul PBS with 400μM of peptide of interest in a 96-well plate according to the manufacturer's instructions. We will validate the only reference of HBV specific peptide for HLA 11:01 CHB Asian patients according to Cheng et al Sci. Immunol. 2019(10) As for those whose HLA-A*02:01, the dextramer will generated according to the information provided Rivino L et al JCI 2018.(27)

The major HLA-A genotype in Taiwan is 11:01 (~ 50%). For those whose HLA-A genotype other than 11:01 or 02:01, peptide-pool stimulation and cytokine detection will be performed for HBV specific T cell detection.

Intracellular cytokine assay and staining

Intracellular staining. T cells directly ex vivo or expanded T cell lines will be stimulated overnight at 37°C with or without HBV peptide pools (5 μg/ml) (core pool, HBx pool, envelope pool, and polymerase pool, respectively) in the presence of 2 μg/ml brefeldin A (Sigma-Aldrich). As a positive control, cells will be stimulated with PMA (2 ng/ml) and ionomycin (1 μg/ml). Cells will be stained with the yellow LIVE/DEAD fixable dead cell stain kit (Invitrogen) and anti-CD3 V500 (clone UCHT1), anti-CD4 BV605 (clone SK3), and anti-CD8 APC CY7 (clone SK1) antibodies. Cells will be subsequently fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences — Pharmingen) and stained with anti–IFN-γ PE (clone 25723, R&D Systems), anti–TNF-α AF 700 (clone MAb11), and anti–IL-2 PerCPCY5.5 (clone MQ1-17H12) antibodies and analyzed on a BD-LSR II FACS Scan. Data were analyzed by FlowJo (Tree Star Inc.). Antibodies will be purchased from BD Biosciences — Pharmingen unless otherwise stated.

Cytokine stimulation

PBMCs were cultured at 3×10^6 cells per well in 6 well plate. Cytokine-stimulated cultures received 10 ng/ml recombinant human IL-12, 50 ng/ml recombinant human IL-18, 25 ng/ml recombinant human IL-15 and 50 U/ml recombinant human IL-2,

100ng/ml recombinant human IL-33, recombinant human TNF-α (R&D SYSTEM), 0.5 ng/ml LPS(Sigma-Aldrich) (28). Cells were cultured in RPMI-1640 with 10% FBS for 48 h. For positive control, add 50 ng/ml PMA and 500 ng/ml ionomycin mix in RPMI1640 with 10% FBS for 5 hours, then the addition of 10 μg/ml brefeldin A in the last two hours. Cells were collected and stained IFN-γ, TNF-α and degranulation markers CD107a in order to analysis the functional assay of the $CD38^{+}HLADR^{+}CD8^{+}$ and $CD38^{-}HLADR^{-}CD8^{+}$ T cells.

Multicolor-Flow cytometric analysis（Symphony）

It is a novel and powerful analytical tool to identify and analyze distinctive phenotypes in heterogeneous populations. The BD FACSymphony™ flow cytometer could further enable the simultaneous measurement of ~ 50 different characteristics of a single cell in addition to its inherent benefits of flow cytometry, which reduce the spillover between different fluorescence expression and improve the signal-to-noise ratio for detection of low protein amounts. The following antibodies were used for multi-parametric flow cytometry (as listed in the table). Analyses were performed using a Symphony (BD Biosciences). Data were evaluated with FlowJo (Treestar, USA).

Expansion of virus-specific CD8+ T cells and assessment of effector function

In patients who failed to be assessed HBV specific T cell response by ex vivo approach, in vitro expansion will be done for substitute analysis. PBMCs ($1\sim2\times10^{6}$) were stimulated with epitope-specific peptides (10 μg/mL) and anti-CD28 mAb (0.5 μg/mL, R&D) and expanded for 14 days in rIL2-containing (20 IU/mL, R&D) complete RPMI culture medium(29).

Cytokine secretion assay and flow cytemtry (CSA-Flow)

For the cytokine secretion assay, cells will be harvested and washed once in ice-cold MACS buffer (PBS/2 mM EDTA/5%BSA). Cells will be then resuspended in serum-free RPMI medium and labelled with capture antibodies for IFN-gamma (final dilution 1:10) or TNF-alpha (final dilution 1:10) (Miltenyi Biotec) for 10 min on ice. Cells will be further diluted in pre-warmed medium at 1×10^{5} cells/ml and be incubated while rotating (using a MACS rotor) at 37° C/5% CO2 for 45 min. Cells will be then placed

on ice for 10 min to terminate the secretion phase. Cells will be subsequently washed twice in ice-cold MACS buffer before labelling with detection antibodies for IFN-gamma(final dilution 1:10) or TNF (final dilution 1:40). Thereafter, the cytokine-secreting T cells were sorted on BD FACSAria (BD Biosciences). Data analysis was performed with FlowJo Software (TreeStar)

Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq)
Single cell suspension from the PBMCs or culture. Cells reaching > 80% will be harvested for sequencing. Cell hashing will be performed following manufacturer's instructions (TotalSeq™-A Cell Hashing and TotalSeq™-A Antibodies Protocol for Simultaneous Proteomics and Transcriptomics with 10X Single Cell 3' Reagent Kit v2; BioLegend, San Diego, CA, USA). Briefly, after cells counting and viability assessment, single cells suspension samples will be incubated 10 min on ice with Human TruStain FcX™ Fc Blocking reagent (BioLegend). Next, sample-specific TotalSeq A antibodies and suitable surface protein specific Antibody-Oligonucleotide Conjugates (Abseq BD® Ab) will be added with subsequent incubation on ice for 30 min. Thereafter, Cell suspensions are loaded into Chromium microfluidic chips with 30 v2 chemistry and used to generate single-cell gel bead emulsions (GEMs) using the Chromium controller (10X Genomics) as manufacturer instructions. In all cases, suspensions containing 10000 cells are loaded on the instrument with the expectation of collecting up to 7,000 GEMs containing single cells. GEM-RT is performed in a Thermal cycler and all subsequent steps to generate single-cell libraries are performed according to manufacturer recommendations. Libraries are sequenced at the Genomic medicine core lab on Nova Seq instrument (Illumina). Trimmed FASTQ files (26bp Cell barcode and UMI Read1, 8bp i7 index, and 100bp Read2), are generated using the Cell Ranger mkfastq command (a 10X Genomics wrapper around BCL2Fastq). Primary data analysis (alignment, filtering, and UMI counting) to determine gene transcript counts per cell (producing a gene-barcode matrix), quality control, clustering and statistical analysis are performed using Cell Ranger count (10X Genomics) and GRCh38 (human hg38) as genome assembly/annotation references. Outputs from multiple independent samples of single-cells are combined using Cell Ranger aggr (10X Genomics) based on mapped read counts to normalize sequencing depth and produce aggregated gene x cell barcode matrices and clustering models.

The Loupe Cell Browser v2.0.0 (10X Genomics) is used to visualize results

Single cell RNA sequencing data processing

Single-Cell RNA-seq. Single-cell suspensions were loaded onto a Chromium Single Cell Chip (10x Genomics) according to the manufacturer’ s instructions for co-encapsulation with barcoded Gel Beads at a target capture rate of ~5000 individual cells per sample. We barcoded captured mRNA was barcoded during cDNA synthesis and converted the barcoded cDNA into pooled single-cell RNA-seq libraries for Illumina sequencing using the Chromium Single Cell 3′ Solution (10x Genomics) according to the manufacturer’ s instructions. All samples for a given donor were processed simultaneously with the Chromium Controller (10x Genomics) and the resulting libraries were prepared in parallel in a single batch. We pooled all of the libraries for a given donor, each of which was barcoded with a unique Illumina sample index, for sequencing in a single Illumina flow cell. All of the libraries were sequenced with an 8-base index read, a 26-base read 1 containing cell-identifying barcodes and unique molecular identifiers (UMIs), and a 98-base read 2 containing transcript sequences on an Illumina HiSeq 4000.

5.9 STATISTICAL METHODS AND DATA ANALYSIS

The continuous variables were expressed as mean ± standard deviation values. Two-tailed Student's unpaired t-test or Mann-Whitney U-test was used to evaluate the difference between the two groups where appropriate. Differences between groups of categorical variables were analyzed using chi-square test. One-way analysis of variance with post Hoc comparison Fisher’s least significant difference was performed to evaluate the difference between multiple groups. Principle component analysis will be applied for the results from multicolor flow cytometry and single cell RNAseq. A P-value <0.05 was considered statistically significant. The statistical analyses were performed using the SAS 9.4 software

5.10 ETHICS AND DATA MANAGEMENT

IRB Status: Submitted for review (under review).

Confidentiality: Dual de-identification; coded keys in locked cabinet accessible only to PI, co-PI, and IRB.

Data Sharing: Aggregated results shared between GlaxoSmithKline Research & Development Ltd and hospital; no individual identifiers disclosed.

5.11 STUDY TIMELINE

Milestone	Target Date (Projected) (dd/month/year)
Final Protocol approved	13/June/2025
IEC / IRB submission	16/June/2025
IEC / IRB approval	07/July/2025
Initiation of experimental phase	14/July/2025
Completion of experimental phase.	10/July/2026
Initiation of validation phase	13/July/2026
Completion of validation phase	9/July/2027
Final HBS which was analyzed	6/Aug/2027
Database Freeze	3/Sep/2027
Final Report (or draft manuscript) delivered to GlaxoSmithKline Research & Development Ltd in accordance with the Data Reporting and Communication Clause	11/Oct/2027
Manuscript in accordance with the Publication Clause submitted for publication within 18 months in which all HBS was analyzed.	29/Oct/2027

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