Heterogeneous T cell response patterns within established clinical phases of Hepatitis B infection revealed through the rapid functional characterization of HBV-specific T cells in whole blood

Short title: Simple HBV-specific T-cell assay

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Abstract

Background & Aims: Virus-specific T cells are crucial for controlling viral replication and disease progression in chronic hepatitis B (CHB). Nevertheless, due to the complexity of their analysis, CHB management relies exclusively on virological and biochemical markers. We aimed to develop a point-of-care immunological test, the HBV-cytokine release assay (HBV-CRA), designed to measure multiple cytokines in whole blood after HBV-peptide pool stimulation.

Methods: We first assessed the assay's sensitivity by spiking whole blood with engineered HBV-specific T cells. Next, the performance of HBV-CRA was compared to ex vivo IFN-γ ELISpot assays in terms of sensitivity and consistency. We then applied the assay in a cross-sectional study of 235 CHB patients and longitudinally in acute HBV patients during HBsAg sero-clearance.

Results: The HBV-CRA detected cytokine secretion in response to peptide pools in approximately 80% of CHB patients. A profile of high IL-2 and IFN-γ after Core peptide stimulation was significantly associated with HBsAg clearance in acute HBV. The assay also revealed variability in cytokine levels and responses to different HBV proteins, even within the same clinical phase of CHB. Unsupervised clustering identified distinct HBV-specific immune patterns that did not segregate by clinical and virological categories. Notably, the assay revealed that NUC treatment impacted the functional profile of HBV-specific T cell responses.

Conclusions: The HBV-CRA is a rapid, easy-to-use assay that detects immune profiles linked to HBsAg clearance and distinguishes CHB patients based on antiviral T cell function. This capability to stratify patients within similar clinical phases offers a novel biomarker for host-viral interactions and could guide the selection of novel immunotherapies.

Key words: Hepatitis B virus, CHB, T cells, cytokines, liver disease, biomarker

Introduction:

Functional and quantitative defects of HBV-specific T cells are regarded as the primary reason for the inability of patients with chronic HBV infection (CHB) to control viral replication and achieve HBsAg seroconversion[1]. Research data indicate that a high frequency of HBV-specific CD8+ T cells in the bloodstream—observed only in acute but not chronic HBV infection—can lead to HBsAg loss and a 90% reduction in HBV-DNA levels[2].

Furthermore, while acute HBV patients possess a population of HBV-specific Th1-like CD4 T cells, in CHB patients, HBV-specific CD4+ T cell dysfunction is commonly observed[3,4]. These impairments are likely responsible for the failure of HBs-specific B cells to fully mature and produce anti-HBs antibodies[5,6], which are efficiently produced in acute HBV patients.

Importantly, while the profile of successful anti-viral immunity is similar among acute HBV patients, the adaptive immune defects of CHB patients can vary widely. Circulating CD4 and CD8 T cells specific for different HBV proteins are present at low frequencies (<0.1% of T cells)[7–10] in CHB patients but display heterogeneity in the expression level of exhaustion markers, not restricted to PD-1, but also TIM-3, Lag-3, and CD160[10–14]. Functionally, HBV-specific T cells in CHB patients show defects in their proliferation and production of cytokines[4,8,12,15]. These defects are heterogenous[16], are not always associated with the expression level of inhibitory receptors[14], and moreover, they are not only caused by classical T cell exhaustion mechanisms due to persistent antigenic stimulation[17]. They are different in relation to the HBV antigen specificity[10,11,16,18], or to their level of interaction with liver endothelial cells[19] and to their “priming history” since HBV-specific T cells induced by hepatocytes or by dendritic cells possess different functional profiles[20]. Furthermore, even though a better functional profile of HBV-specific T cells can be observed in CHB patients with very low viral load[11,14,18], or in CHB patients of younger age[21], the intricate spectrum of HBV-specific T cell immunity cannot be fully understood through the standard virological

tests (such as quantitative HBsAg and HBeAg positivity or negativity) and clinical assessments (serum ALT levels) commonly used in the management of CHB patients.

Only advanced immunological methods are currently able to define the complex nature of the HBV-specific T cells in CHB patients[22], but these methods are not easily translated into clinical practice for large clinical trials and, furthermore, can only be conducted in highly specialized laboratories.

The aim of this work was to develop a simple point-of-care immunological test that can define both the functional profile of HBV-specific T cells of acute HBV patients during the dynamic phase of HBV control and the complex heterogeneity of HBV-specific T cells in CHB patients. We modified a method that we developed to rapidly measure the frequency and function of SARS-CoV-2 specific T cells[23,24]. We designed HBV peptide pools covering HBV proteins of the different HBV genotypes and used them directly in the whole blood of acute HBV and CHB patients to stimulate the release of cytokines. After overnight incubation, different cytokines (IFN-γ, IL-2, IL-10, granzyme B, TNF-α, IL-4, IL-5) were measured. We report here the ability of this assay to rapidly gauge the presence of HBV-specific T cells directly in a small volume of whole blood and to depict their heterogeneous secretion of cytokines.

Material and Methods:

Study approval: Clinical assessment and blood sampling were performed at The Royal London Hospital, Barts Health NHS Trust, Singapore General Hospital and Policlinico of Milan in accordance with the Declaration of Helsinki. Written informed consent was obtained, and the study was approved by the Institutional Review Boards (refs: 17/LO/0266, 2022/2732, ID-4670).

Blood samples: 260 individuals were included in this study: HBV-vaccinated subjects (n=16), treatment-naïve CHB patients (n=99), NUC-suppressed CHB patients (n=75), HBsAg-negative CHB patients (n=61), and patients with acute resolving HBV infection (n=9). A subset of NUC-suppressed patients (n=10) was sampled monthly over three time points. Acute HBV patients were tested 1-4 times during viral clearance. Clinical and virological parameters are summarized in Tables S1, S2 and S3.

Peptide pools: A library of 838 overlapping 15-mer peptides (10-aa overlap) was used to identify HBV-specific T cells. These peptides were combined into six pan-genotype pools covering all HBV proteins from genotypes A, B, C, and D and sourced from T Cell Diagnostics. SARS-CoV-2-Spike peptide pool was generated as described before[23].

Ex-vivo ELISpot assays: PBMCs were isolated by Ficoll gradient and directly seeded at 400,000 cells per well onto anti-IFN-γ (1-D1K, Mabtech; 5 µg/mL) pre-coated 96-well plates. Cells were stimulated for 18h with peptide pools (2 µg/mL), DMSO, or PHA controls. Plates were developed using biotinylated IFN-γ detection antibody (7-B6-1, Mabtech; 1:2000), Streptavidin-AP (Mabtech), and KPL BCIP/NBT substrate (SeraCare). Spot-forming cells (SFC) were quantified with ImmunoSpot, and positive responses were calculated by subtracting 2x SFC of unstimulated wells from peptide-stimulated wells. All controls met quality criteria, with <5 SFC in negative controls and positive PHA controls.

HBV-Env$_{183-91}$ TCR T cells: HLA-A2-restricted HBV-Env$_{183-91}$-specific T cells were generated by electroporating mRNA encoding the TCR Vα and Vβ chains into 10-day expanded T cells from an HLA-A2+ subject[25]. HBV-Env$_{183-91}$-TCR expressing T cells were quantified via dextramer staining and diluted into 320 µl of whole blood from the same HLA-A2+ subject. The blood aliquots containing graded Env$_{183-91}$-TCR+ T cell numbers were pulsed with the

corresponding HBV-Env-$_{183\text{-}91}$ peptide (2 µg/mL). After overnight incubation, plasma IL-2, IFN-γ, granzyme B, and IL-10 concentrations were measured.

Whole blood HBV-CRA: On the same day of collection, whole blood drawn into heparinized tubes (320 µL blood per peptide pool) was mixed with 80 µL RPMI and stimulated with HBV peptide pools (2 µg/mL) or DMSO control. After 15 hours of culture at 37ºC, the culture supernatant (plasma) was collected and stored at -80ºC for cytokine quantification.

Cytokine quantification and analysis: Plasma cytokines (IFN-γ, IL-2, TNF-α, IL-4, IL-5, IL-10, and granzyme B) were measured using an Ella machine (ProteinSimple) per the manufacturer's instructions. Cytokine levels in DMSO controls were 2x subtracted from peptide-stimulated samples. Dimensionality reduction was performed using UMAP[26] and Phenograph[27] with nearest neighbors = 200, min_dist = 0.99, k = 30. UMAP results were converted to .fcs files and analyzed in FlowJo to generate cytokine secretion heatmaps.

Statistical analysis: All tests are stated in the figure legends. P-values (all two-tailed): <0.05 = *; <0.01 = **; <0.001 = ***; <0.0001 = ****.

Results:

Sensitivity of the whole blood HBV-cytokine release assay (HBV-CRA)

During the COVID-19 pandemic, we demonstrated that whole blood stimulation with peptide pools covering SARS-CoV-2 proteins can detect SARS-CoV-2-specific T cells with more accuracy than other assays (ELISpot, AIM assay)[23]. However, while vaccine- or infection-induced SARS-CoV-2-specific T cells often comprise around 0.1% of total T cells, circulating HBV-specific T cells in CHB patients rarely reach this level. This different magnitude is depicted in Figure 1A, where PBMCs of COVID-19-vaccinated CHB patients were tested in parallel for T cells specific to SARS-CoV-2 Spike and the various HBV-proteins. While the ex vivo IFN-γ ELISpot assay clearly detected SARS-CoV-2-specific T cells, HBV-specific T cells were rarely found. To assess whether the whole blood HBV-CRA can achieve a sensitivity threshold suitable for detecting the low frequency of HBV-specific T cells, we diluted graded numbers of T cells with known specificity into whole blood. Therefore, we engineered a T cell receptor specific for the HLA-A2-restricted HBV-Env$_{183-91}$ epitope into T cells of an HLA-A2+ subject. HBV-Env$_{183-91}$-TCR expressing T cells were quantified by dextramer staining and diluted into the whole blood from the same subject (Figure 1B). After overnight culture with the corresponding HBV-Env-$_{183-91}$ peptide, plasma cytokines were measured. Figure 1C shows that IFN-γ and granzyme B levels >10 pg/mL were detected when 50-100 Env$_{183-91}$-TCR+ T cells (~0.01-0.02% of total T cells) were diluted in blood, matching the frequency of circulating HBV-specific T cells in most CHB patients. IL-2 was first detected at ~100 cells/320 µL of blood, but levels >10 pg/mL were observed only at higher T cell numbers (~1000), in line with the effector profile of these engineered TCR-T cells.

HBV-CRA in CHB patients and healthy controls: cytokine selection

After confirming that the sensitivity of the whole blood CRA aligns with the known frequency of HBV-specific T cells in CHB patients, we designed six peptide pools of overlapping 15-mer peptides (10-aa overlap) covering the Core (73 peptides), S (188 peptides), PreS (35 peptides), Polymerase (split into Pol-1 with 230 peptides and Pol-2 with 231 peptides) and X (83 peptides) proteins of HBV genotypes A, B, C, and D. These were organized into individual pools (Figure 2A). To assess HBV-peptide-induced cytokine secretion, each peptide pool was added to fresh whole blood and incubated overnight. Supernatants were then analyzed for Th1 cytokines (IFN-γ, IL-2, TNF-α), Th2 cytokines (IL-5, IL-4), granzyme B, and IL-10 (Figure 2B).

We initially tested samples from 10 virally suppressed CHB patients on NUC treatment and 16 healthy HBV-vaccinated individuals. The clinical and virological profiles of CHB patients (6 anti-HBe+, 4 HBeAg+, varying HBsAg levels) and the details of the vaccinated subjects are in Table S1.

Cytokine concentrations in blood cultures of CHB patients and vaccinated individuals stimulated with each peptide pool are shown in Figure 2C. Positive responses were defined as cytokine levels exceeding 10 pg/mL after subtracting twice the background levels. Frequencies of positive responses are summarized in Figure 2D. Most CHB patients showed IFN-γ and IL-2 secretion in response to multiple peptide pools, though cytokine concentrations varied widely, indicating heterogeneity in immune responses. Surprisingly, the S peptide pool elicited stronger IFN-γ (6/10) and IL-2 (7/10) responses in CHB patients than others. In contrast, the Core pool, often considered the most T cell-immunogenic HBV protein, induced IFN-γ/IL-2 secretion in fewer patients (4/10). The Pol-2 pool, covering the C-terminal polymerase region, stimulated IFN-γ/IL-2 secretion in more than half of the patients, while Pol-1 (N-terminal polymerase) and X pools triggered responses in just one patient each. The PreS peptide pool elicited no responses.

The cytokine response profile in vaccinated individuals differed markedly. Only the S peptide pool consistently induced IFN-γ/IL-2 secretion in most individuals, while PreS, Core, X, and Pol-1 pools did not. The Pol-2 pool induced responses in 3 of 16 individuals, confirming previous reports of polymerase-specific T cells detected via MHC pentamers in some vaccinated individuals[14,28], likely due to high HBV circulation in Asia.

Granzyme B secretion was predominantly detected with the S peptide pool in both CHB patients and vaccinated individuals. However, while granzyme B secretion always co-occurred with IFN-γ/IL-2 in vaccinated individuals, the was not always the case in CHB patients (e.g., C and Pol-2 pools).

Th2 cytokines (IL-4, IL-5) were undetectable in both CHB patients and vaccinated individuals. TNF-α and IL-10 secretion were frequently observed, particularly in vaccinated individuals, often independently of IFN-γ/IL-2. This suggests they may be driven by monocyte activation rather than T cell activity, and thus, may not provide reliable information about HBV-specific T cell function.

Enhanced sensitivity and reproducibility of HBV-CRA in longitudinal monitoring of HBV-specific T cell function in NUC-suppressed CHB patients compared to ELISpot

To evaluate whether the HBV-CRA detects reproducible patterns of HBV-peptide pool-induced cytokine secretion over time, we collected blood samples from 10 virally suppressed CHB patients on NUCs at four-week intervals, performing the assay at three time points. Concurrently, we isolated PBMCs to conduct fresh ex vivo ELISpot assays (Figure 3A).

HBV peptide-induced IFN-γ secretion showed a consistent response pattern across time points, with minor quantitative variations but stable T cell dominance in individual patients (Figure 3B). In contrast, the IFN-γ ELISpot assay exhibited greater variability, with low responses at one time point often not replicating in subsequent tests, leading to inconsistent immunodominance patterns (Figure 3C).

Correlation analysis between HBV-CRA and ex vivo ELISpot revealed that IFN-γ levels above 5pg/mL were often detected by HBV-CRA without corresponding spots in ELISpot assays (Figure 3D). However, a significant correlation was found when ELISpot results exceeded 10 SFC per million PBMCs, indicating higher sensitivity of the HBV-CRA.

To further evaluate the consistency of the HBV-CRA, we correlated cytokine levels at time points T1, T2 and T3 (Figure 3E). IFN-γ and IL-2 levels were highly consistent across all time points. Granzyme B levels also showed strong consistency between T1 and T2, though distinct responses emerged at T3, yet maintaining a significant correlation overall. TNF-α secretion was frequently detected but showed substantial variability over time, while IL-10 remained low but consistent across adjacent time points (T1 vs. T2 and T2 vs. T3). IL-5 remained consistently low or undetectable. In contrast, correlation analysis of IFN-γ ELISpot results over time confirmed its variability, with a strong correlation only between T1 and T2.

HBV-CRA captures dynamic kinetics of HBV-specific T cells in acute HBV infection.

Building on the reproducibility of the HBV-CRA in NUC-treated CHB patients, we evaluated its ability to capture dynamic changes in HBV-specific T cell responses during acute resolving HBV infection. Unlike NUC-suppressed CHB patients, acute HBV patients are characterized by a well-documented expansion and contraction of HBV-specific CD8+ and CD4+ T cells with a Th1 cytokine profile[29–31].

We longitudinally followed nine patients with acute HBV, performing the HBV-CRA at 1-4 different time points (Table S2). Among these, three patients were sampled from the acute phase (HBsAg+, HBV-DNA+, elevated ALT) through to HBsAg seroconversion and ALT normalization (Figure 4, upper graphs). Cytokine secretion in response to HBV peptide pools was measured at each time point, with Core, S and Pol-2 pools stimulating responses in Patient #6 and Core, S and X in Patients #4 and #8.

The HBV-CRA effectively tracked dynamic HBV-specific T cell fluctuations associated with viral clearance and HBsAg negativity. Th1 cytokines (IL-2 and IFN-γ) and granzyme B were consistently detected, while Th2 cytokines, TNF-α and IL-10 were minimal. IL-2 and IFN-γ showed coordinated kinetics, while granzyme B followed a distinct trajectory, suggesting differential regulation.

These results demonstrate the HBV-CRA's ability to quantify and characterize HBV-specific T cell responses during acute infection.

Marked heterogeneity in the HBV-specific T cell secretome among CHB patients

We then applied the HBV-CRA to a large cohort of CHB patients spanning all disease phases (Figure 5A). This cohort included treatment-naïve (n=99) and NUC virally suppressed CHB patients (n=75), as well as 61 HBsAg-negative patients, comprising those who had either spontaneously resolved CHB infection (n=24) or achieved HBsAg negativity following NUC therapy (functional cure; n=37). Additionally, longitudinal samples (1-3 time points, all HBV-DNA+, n=20) from 9 patients with acute HBV were included in the analysis.

First, cytokine secretion levels (IFN-γ, IL-2, granzyme B, IL-5) in response to each HBV peptide pool were analyzed across all samples, excluding TNF-α and IL-10 due to their likely monocyte-driven production. We observed considerable heterogeneity in cytokine secretion levels within all patient groups (Figure 5B), with the S, Core, and Pol-2 pools eliciting the strongest median responses across all groups (Figure 5B, 5C, S1A). Acute HBV patients had the highest median IFN-γ secretion, while HBV-vaccinated subjects had peak IL-2 responses to the S pool, followed by Core and S-specific responses in acute HBV patients. Granzyme B secretion was greatest in response to the S pool across all groups. Interestingly, treatment-naïve patients and those who spontaneously resolved CHB exhibited substantially higher granzyme B levels than the other groups, suggesting that NUC therapy alters the functional profile of HBV-specific T cells (Figure 5B, 5C S1A, B).

The variability in cytokine secretion among CHB patients within the same clinical group precluded the identification of significant differences between groups. In contrast, acute HBV patients had significantly stronger IFN-γ and IL-2 responses to the Core pool, highlighting distinct HBV-specific T cell functions in patients actively clearing the virus (Figure 5D).

Identification of CHB patients with similar HBV-specific T cell profiles

To identify CHB patients with similar HBV-specific immune responses—based on quantity and combination of secreted cytokines in response to HBV peptide pools—we applied the UMAP algorithm to group samples based on cytokine secretion profiles (IFN-γ, IL-2, granzyme B, IL-5; Figure 6A). The UMAP showed substantial overlap between IFN-γ and IL-2 secretion but also distinguished samples with high IFN-γ but low IL-2 secretion, or vice versa (Figure 6B). IL-5 secretion was minimal and did not influence clustering, while granzyme B secretion formed a distinct cluster. Within this cluster, samples were arranged from low to high granzyme B levels, with some overlapping IFN-γ/IL-2 profiles and others showing exclusive granzyme B secretion (Figure 6B).

Figure 6C visualizes the HBV-specific cytokine secretion profiles induced by the six peptide pools in CHB patients of different disease categories and compares them with healthy vaccinated controls and acute HBV patients. As expected, in vaccinated controls, all S-peptide pool stimulated samples clustered in regions with medium-to-high IFN-γ, IL-2, and granzyme B, while samples stimulated with other pools clustered in the regions with low-to-negative cytokine levels. In contrast, CHB patients of all disease categories exhibited heterogeneous clusters across all peptide pools, with some clustering together (i.e., Core in HBeAg-negative hepatitis) and others forming multiple distinct (i.e., S and Core in HBeAg-positive infection, S in HBsAg-negative resolved CHB) or widely dispersed clusters (i.e., responses to all peptide pools in HBeAg-negative infection), depicting the marked heterogeneity of HBV-specific T cell responses even within the same disease category.

As expected from the analysis in Figure 5, in acute HBV patients, Core peptide pool responses clustered in regions with the highest levels of IFN-γ and IL-2, distributed along a gradient of granzyme B secretion ranging from high to none. Moreover, UMAP visualization also revealed differences between treatment-naïve and NUC-treated CHB patients (Figure 6D). In treatment-naïve patients, 92% of responses involved granzyme B, either alone (38%) or in combination with IFN-γ/IL-2 (54%). In contrast, NUC-suppressed patients had more responses secreting only IFN-γ/IL-2 (27%), a pattern even more pronounced in patients with HBsAg loss. Among spontaneously resolved CHB cases, only 4.6% of responses lacked granzyme B, compared to 47.6% in NUC-treated HBsAg-negative patients.

Next, to identify and subgroup CHB patients based on their HBV-specific T cell cytokine release profile, we combined the dimensional reduction method UMAP with PhenoGraph, an unsupervised clustering algorithm. With the k-nearest neighbors parameter of 30, PhenoGraph identified 13 distinct subgroups of cytokine release profiles (Figure 7A).
Cluster 1 (C1) and C2 comprise samples with high IFN-γ and IL-2 but no or only low granzyme B secretion, respectively. C3 includes samples secreting high levels of IFN-γ, IL-2, and granzyme B. C4 contains samples with medium levels of all three cytokines, while C5 and C6 contain samples with medium to high granzyme B combined with low IFN-γ or IL-2, respectively. C7 comprises samples exclusively secreting granzyme B. C8-C13 include samples with very low or no cytokine secretion (Figure 7B and 7C).

Next, we analyzed the distribution of samples from different groups across the 13 cytokine clusters (Figure 7D). This revealed substantial diversity in T cell responses within the same clinical category. Acute HBV patients predominantly fell into clusters C1–C3, characterized by high IFN-γ and IL-2 secretion, with varying granzyme B levels: no (C1), low (C2), or high (C3). In contrast, treatment-naïve CHB patients and those with resolved CHB (spontaneous HBsAg loss) were mostly in C3-C7, marked by high granzyme B secretion, either with (C3-C4) or without (C5-C7) IFN-γ and IL-2. Conversely, NUC-suppressed patients and those who

achieved functional cure (HBsAg loss after NUCs) showed more responses in C1 and C2, characterized by IFN-γ and IL-2 with no or low granzyme B.

This subgrouping approach allowed us to visualize individual patient response patterns to HBV peptide pools (Figure 7E). While some individuals showed similar cytokine profiles to the six HBV peptide pools, all falling within the same cluster, others exhibited distinct responses to each pool.

Discussion

Here, we developed a point-of-care assay, HBV-CRA, to provide a quantitative and functional profile of T cell immunity against HBV proteins using just ~2.5 mL of whole blood (320 µL per peptide pool for S, Pre-S1/S2, Core, Polymerase, and X), which is compatible with high-throughput processing. The assay is an adaptation of a method that we previously employed to measure SARS-CoV-2-specific T cells[23,24], including in low-resource settings such as rural areas of Kenya[32].

HBV-CRA simplifies testing by using fresh whole blood, eliminating the need for PBMC isolation, which is impractical in many facilities. The assay involves two steps: (1) overnight blood stimulation with peptide pools and supernatant collection, and (2) cytokine quantification. This modular design allows the cytokine analysis, which requires specialized equipment, to be performed in a separate laboratory (see ref 30), ensuring standardization and reproducibility.

The HBV-CRA's sensitivity (0.01-0.02% HBV-specific T cells among total T cells) is well-suited to detect the low frequency of HBV-specific T cells typically present in the circulatory compartment of CHB patients, which is notably lower than for viruses like SARS-CoV-2.

Pulsing whole blood of CHB patients with HBV peptide pools triggered cytokine secretion (primarily IFN-γ, IL-2, and granzyme B) to at least one pool in ~80% of cases, providing a quantitative and qualitative profile of circulating HBV-specific T cells. The HBV-CRA delivered reproducible results in NUC-suppressed patients over three months and exceeded the sensitivity of ex vivo ELISpot assays. In acute HBV, the assay effectively captured the dynamic changes of HBV-specific T cell responses[29,30] despite suppression of T cell function by arginase[33]. These findings support the assay's utility in monitoring fluctuations of T cell

responses that could occur after the initiation or termination of different treatment strategies in CHB patients or during hepatic flares.

The HBV-CRA not only offers superior sensitivity but also simplifies the assessment of functional HBV-specific T cell heterogeneity in CHB patients. Typically, this analysis would require advanced techniques such as HLA-tetramer staining of specific HBV-specific CD8 T cells, intracellular cytokine staining[14], or single-cell transcriptomic profiling. Instead, by simultaneously measuring multiple cytokines and combining their concentrations with a multidimensional reduction algorithm, we can derive a specific secretome profile for all HBV proteins present in each acute HBV and CHB patient.

We noted that among patients within each clinical category, various secretome profiles could be detected and that specific secretome profiles did not segregate into any specific clinical category of HBV infection. These findings might be caused by the differential mechanisms of HBV-specific T cell defects observed in CHB patients[1,17].

Nevertheless, some clear trends were also evident. Patients with acute HBV analyzed during the phase of HBV clearance (while still HBV-DNA+) showed high IL-2 and IFN-γ secretion in response to S and Core peptide pools, accompanied by relatively low granzyme B. However, this profile was not exclusive to acute HBV patients. Similar S and Core-specific secretome patterns (high IFN-γ and IL-2 with low or absent granzyme B) were also observed in some treatment naïve and NUC-treated CHB patients. It will be interesting to prospectively investigate whether CHB patients exhibiting this “HBV clearance-associated” profile respond better to specific treatments or maintain HBV control after NUC discontinuation. The potential protective efficacy of balanced IFN-γ and IL-2 co-production by HBV-specific T cells is supported by a recent study analyzing IL-2/IFN-γ secretion in CHB patients. Using ex vivo FluoroSpot assays, the authors identified IL-2 as a potential biomarker for viral control and response to checkpoint inhibitors[34].

In addition, the analysis of the HBV secretome profiles revealed an interesting trend of reduced granzyme B production by HBV-specific T cells associated with NUC treatment. A profile characterized by IFN-γ and IL-2 secretion without granzyme B was predominantly observed in CHB patients undergoing NUC treatment or those who became HBsAg-negative after treatment. Conversely, granzyme B secretion, either alone or in combination with IL-2/IFN-γ, was more frequently detected in treatment-naïve CHB patients, including those who spontaneously lost HBsAg.

We are particularly intrigued by the clear distinction between individuals who clear HBsAg spontaneously versus those who become HBsAg negative following NUC treatment. The reduced granzyme B production in NUC-treated patients might be related to the immunomodulatory effects of nucleotide analogs, which have been shown to induce Y3-IFN in the gut[35] and alter IL-10/IL-12 secretion by LPS-activated monocytes[36]. However, the persistence of this profile, even after treatment cessation, remains puzzling and warrants further analysis to clarify the effects of nucleotide analogs on immune responses.

On the other hand, these findings highlight the utility of our HBV-CRA in uncovering previously unobserved differences in HBV-specific immune responses induced by NUC treatment.

Approximately 10 years ago, Dammermann et al. reported a similar whole-blood CRA for assessing HBV-specific T cell responses[37]. While both assays share a similar approach, our method requires only 320 µL of blood per stimulation (vs. 1 mL), a shorter stimulation time (16 hours vs. 24 hours), and a lower peptide concentration (2 µg/mL vs. 5 µg/mL). Both assays detected IFN-γ and IL-2 in the whole blood of CHB patients after stimulation with peptide pools covering S and Core antigens. However, their study did not compare the HBV-CRA's sensitivity to classical methods, nor did it highlight how broad cytokine analysis can reveal the heterogeneity of HBV-specific T cells in CHB patients rather than merely indicating their presence or absence.

At this juncture, we chose to analyze the following cytokines: IFN-γ, IL-2, granzyme B, and a classical Th2 cytokine, IL-5. IFN-γ and IL-2 are Th1 cytokines important for inhibiting HBV replication[38] and maintaining HBV-specific CD8 T cell fitness[39], respectively. Granzyme B defines T cells with high lytic ability, and HBV-specific CD8+ T cells with low cytokine production but positive for granzyme and perforin were observed in CHB patients[14]. Although IL-5 was undetectable in the patients tested here, we included a Th2 cytokine due to its potential production in specific patient populations, particularly in non-urbanized areas[40]. In addition, we already have experimental evidence that therapeutic vaccine-induced HBV-specific T cells can produce Th2 cytokines in some CHB patients[41].

We initially included IL-10 and TNF-α in our secretome characterization due to their potential roles in HBV-specific T cell functionality. IL-10, though commonly associated with immune regulation, has been linked to highly functional T cell responses[24] and the promotion of CD8 T cell maturation in HBV transgenic mice[42]. TNF-α was selected for its ability to synergize with IFN-γ to induce noncytolytic HBV-DNA reduction in infected hepatocytes[43], and reports of TNF-α-producing CD4 T cells[44]. However, our data from CHB patients and healthy vaccinated subjects showed that IL-10 and TNF-α were detected regardless of IFN-γ and IL-2 secretion and exhibited fluctuating levels over time. This suggests that these cytokines may primarily reflect inflammatory states of myeloid cells[45] rather than HBV-specific T cell functionality. Consequently, we excluded them from the HBV secretome, although we cannot exclude that they may define HBV-specific T cell functionality in certain contexts. Future studies should explore other cytokines, such as IL-17 and IL-21, for a more comprehensive understanding of HBV-specific T cell responses.

The HBV-CRA has some limitations: it measures only circulating HBV-specific T cells and, similar to ELISpot assay, cannot distinguish whether cytokines originate from CD4+ helper or CD8+ cytotoxic T cells. It is also unable to detect anergic or functionally exhausted HBV-specific T cells. Furthermore, the use of 15-mer peptides for stimulation may underestimate the frequency of CD8 T cells[46].

Despite these limitations, our study demonstrates the feasibility of using a simple, minimally invasive technique to obtain both quantitative and qualitative measurements of HBV-specific T cells. This assay requires minimal blood manipulation, making it well-suited for point-of-care clinical settings. By providing detailed virus-specific T cell data, the HBV-CRA can enhance the understanding and management of CHB, as we recently demonstrated in CHB patients receiving a combination of siRNA and therapeutic vaccination.

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Figure legends:

Figure 1: Sensitivity of the whole blood HBV-cytokine release assay

(**A**) Isolated PBMCs from six CHB patients were stimulated with seven 15-mer overlapping peptide pools covering the HBV proteome (Core, Pre-S, S, Pol1, Pol2, X) and the SARS-CoV-2-Spike protein. IFN-γ-spot forming cells (SFC) were detected and quantified by ELISpot assay. (**B**) T cells from an HLA-A2+ individual were electroporated with mRNA encoding the Vα and Vβ chains of a TCR specific for the MHC-I/peptide complex presenting the HBV-Env$_{183\text{-}91}$ epitope. HBV-Env$_{183\text{-}91}$-TCR+ T cells were quantified via dextramer staining, and known quantities were spiked into fresh whole blood from the same subject to create a dilution series reaching from 10 to 8,000 HBV-specific T cells per 320 μL of blood. The blood samples were then stimulated with the Env$_{183\text{-}91}$ peptide or corresponding DMSO controls and cultured overnight at 37°C. Cytokines in the collected supernatants were quantified using the automated ELISA platform ELLA. (**C**) The levels of IFN-γ, IL-2, granzyme B, and IL-10 induced by peptide stimulation in blood samples containing the specified numbers of HBV-Env$_{183\text{-}91}$-TCR+ T cells are shown.

Figure 2: HBV-peptide induced cytokine release assay: CHB patients versus HBV vaccinated subjects

(**A**) Schematic of the HBV pan-genotype (A-D) peptide pool design. (**B**) Schematic of the whole-blood HBV-CRA. (**C**) Fresh whole blood from NUC-suppressed CHB patients (n=10) and healthy-vaccinated subjects (n=16) was stimulated overnight with the different HBV-peptide pools. Secreted cytokine concentrations are shown (red = CHB; blue = healthy-vaccinated). (**D**) The percentage of individuals with a peptide-induced cytokine secretion >10 pg/mL is shown (red = CHB; blue = healthy-vaccinated).

Figure 3: Consistency of the HBV-CRA and comparison with ELISpot assays

(**A**) NUC-treated CHB patients (n=10) were followed longitudinally, and their HBV-specific T-cell profiles were analyzed monthly with the HBV-CRA and ex-vivo ELISpot assay. (**B**) HBV-CRA: IFN-γ -secretion levels in response to the indicated peptide pools at T1 (Day 1), T2 (Day 30), and T3 (Day 60) (left). The percentage contribution of the single peptide pools to the total HBV-specific IFN-γ response is shown for individual patients across the three time points. (**C**) ex-vivo ELISpot: IFN-γ-SFU in response to the indicated peptide pools at T1 (Day 1), T2 (Day 30), and T3 (Day 60) (left). The percentage contribution of the single peptide pools to the total HBV-specific ELISpot response is shown for individual patients across the three time points. (**D**) Log-transformed IFN-γ-secretion levels from the HBV-CRA in response to individual peptide pools across all time points are plotted against the corresponding log-transformed IFN-γ-SFUs from the ex vivo ELISpot assays. (**E**) Secreted cytokine levels (IFN-

γ, IL-2, granzyme B, TNF-α, IL-10, IL-5) and (**F**) SFUs measured at different time points were correlated to monitor the consistency of the assays.

Figure 4: Fluctuation of the HBV-CRA in patients with acute HBV infection

Three patients diagnosed with acute HBV infection were followed longitudinally until HBsAg seroconversion and HBV-DNA negativity. Their HBV-DNA, HBsAg, and ALT levels are shown over time (upper panels). Blood samples were tested with the HBV-CRA at the indicated days post-hospital admission. Cytokine secretion levels (pg/mL) are shown in response to the indicated HBV-peptide pools.

Figure 5: Marked heterogeneity in the HBV-specific T cell secretome among CHB patients

(**A**) Healthy vaccinated, treatment-naïve CHB, NUC-treated CHB, resolved CHB, and acute HBV patients were analyzed with the HBV-CRA. n=number of subjects; #=number of samples. (**B**) Secretion levels (pg/mL) of IFN-γ, IL-2, granzyme B (GrzB), and IL-5 in response to the indicated HBV-peptide pools are shown for the various patient groups. Each dot represents one patient sample; bar represents median. (**C**) Heatmap showing the median secretion levels (pg/mL) of IFN-γ, IL-2, and granzyme B (GrzB) in response to the six HBV-peptide pools for each patient group. (**D**) IFN-γ, IL-2, and granzyme B (GrzB) secretion levels in response to the HBV-Core peptide pool are shown for the various patient groups. Each dot represents one patient sample; bar represents median. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test.

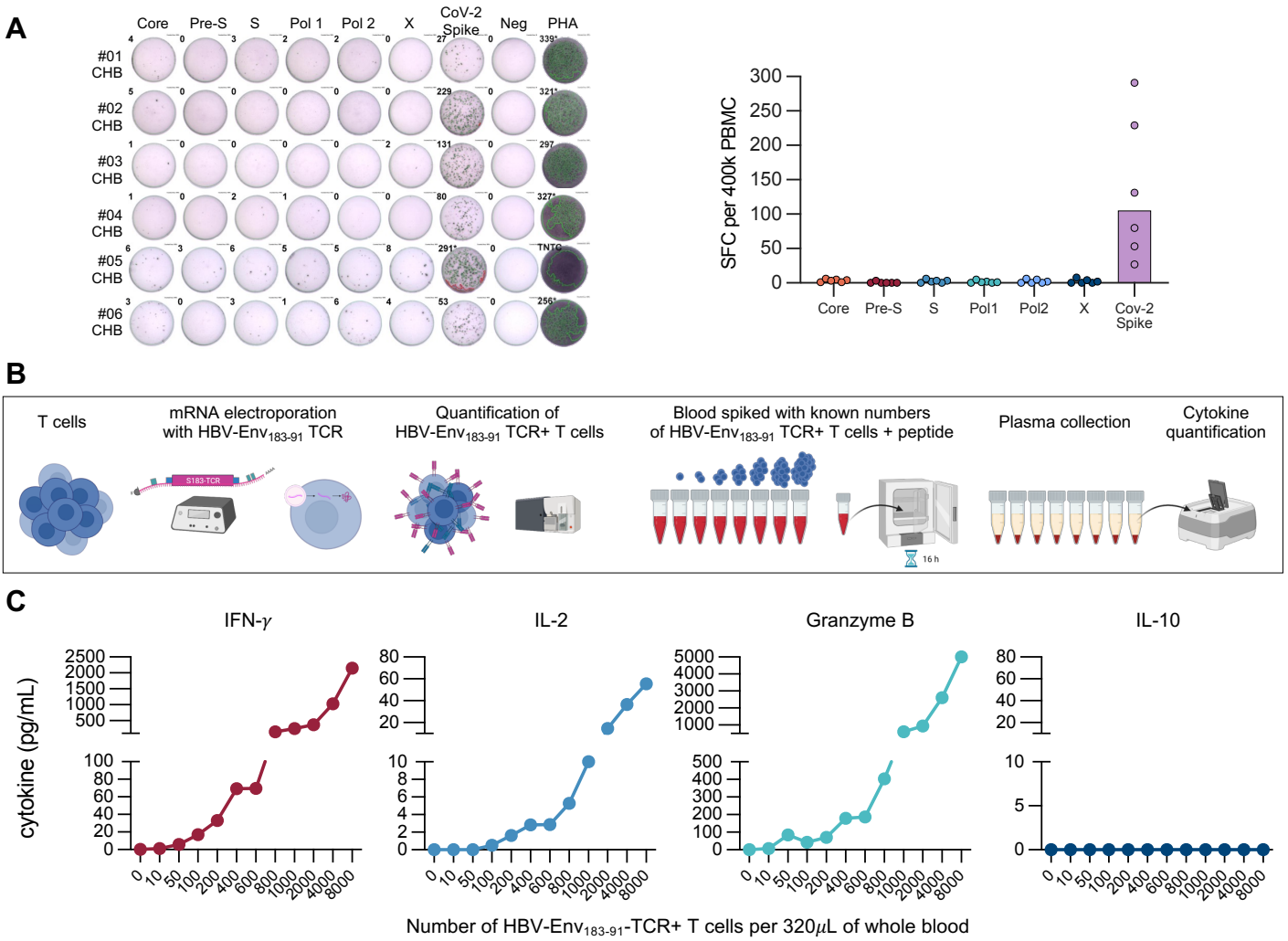
Figure 6: Visualization of HBV-specific T cell secretomes with UMAP

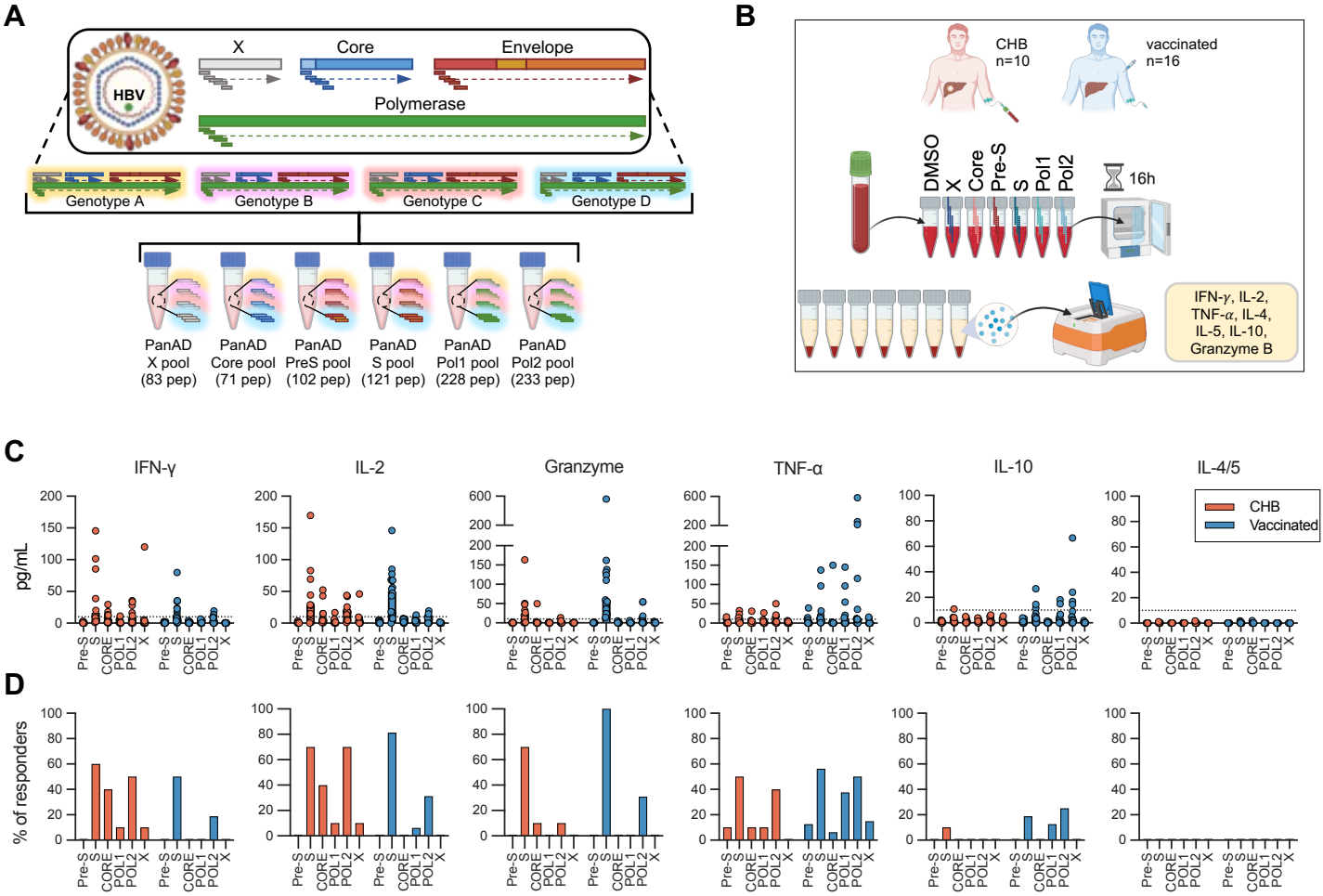
(**A**) Schematic of whole blood stimulation with HBV-peptide pools and analysis of the cytokine secretion profile (after 2x DMSO control subtraction) using dimensional reduction algorithm UMAP. (**B**) UMAP plots generated with all analyzed blood culture supernatants (n = 1626) with indicated cytokine secretion heatmaps. (**C**) Concatenated cytokine secretion profiles of peptide-pool stimulated whole blood from healthy vaccinated (yellow), different categories of treatment-naïve CHB (different shades of red), HBeAg+ and HBeAg- NUC treated CHB (light and dark blue), HBsAg- CHB (functional cure after NUC, light green; spontaneously resolved, dark green) and patients with acute HBV (HBsAg+, HBV-DNA+; grey) overlaid on the global UMAP plot of all analyzed samples (black dots; each dot corresponds to one culture supernatant). (**D**) Concatenated cytokine secretion profiles of all six peptide-pool stimulated whole blood samples from HBsAg+ treatment-naïve (n=99; red) and NUC-treated (n=75, blue) CHB patients, and resolved (HBsAg-) treatment-naïve (n=24; dark green) and previously NUC-treated (n=37; light green) CHB patients overlaid on the

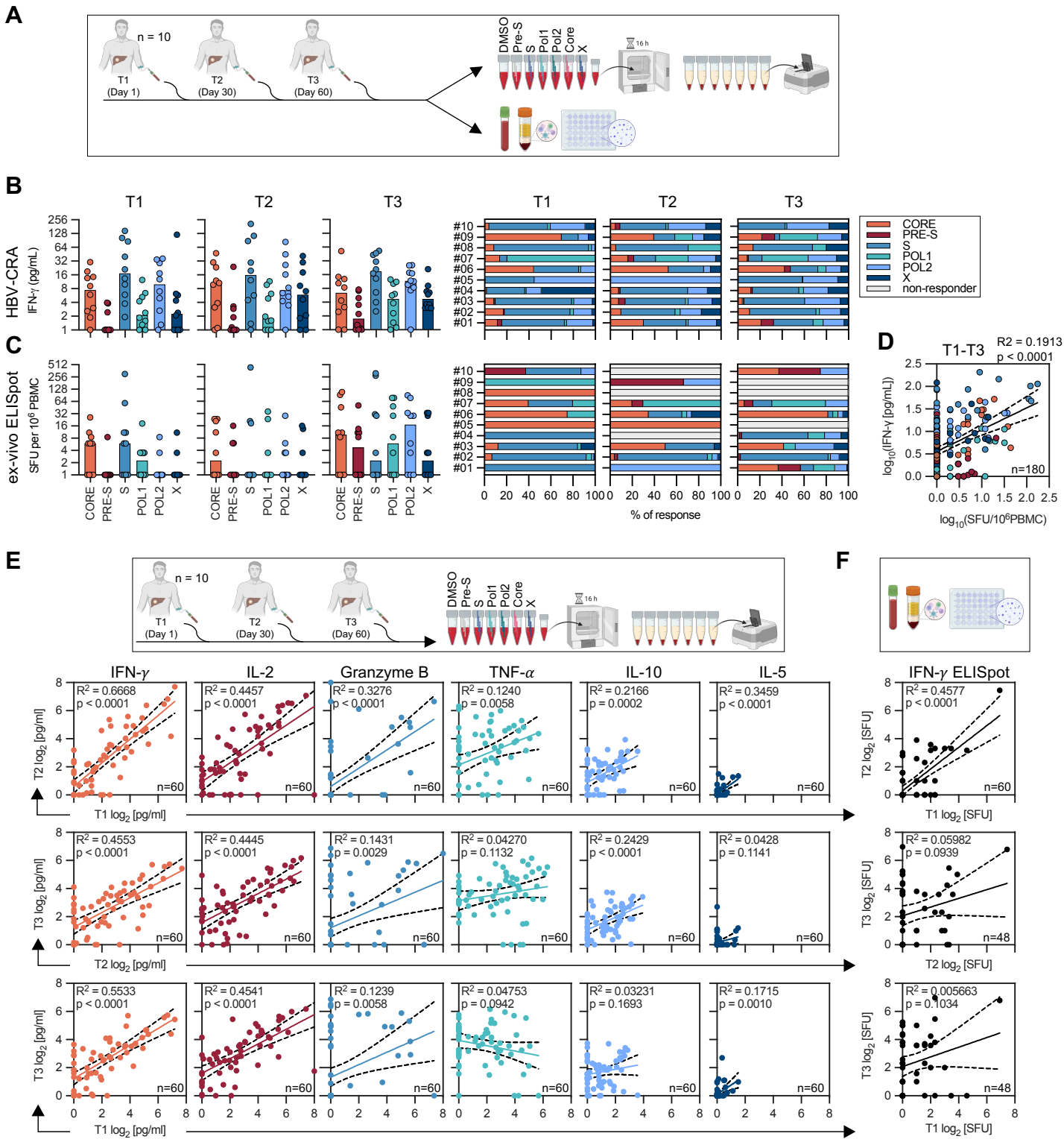
global UMAP plot of all analyzed samples (black dots). Bar graphs (right) show the percentage of peptide-stimulated blood cultures from treatment-naïve and NUC-treated CHB patients (upper panel: HBsAg+, lower panel: HBsAg-) with cytokine secretion levels >10pg/mL that contained either only IFN-γ and IL-2 (yellow), IFN-γ, IL-2, and granzyme B (orange), or only granzyme B (red). n = number of subjects; # = number of samples; t = number of tests.

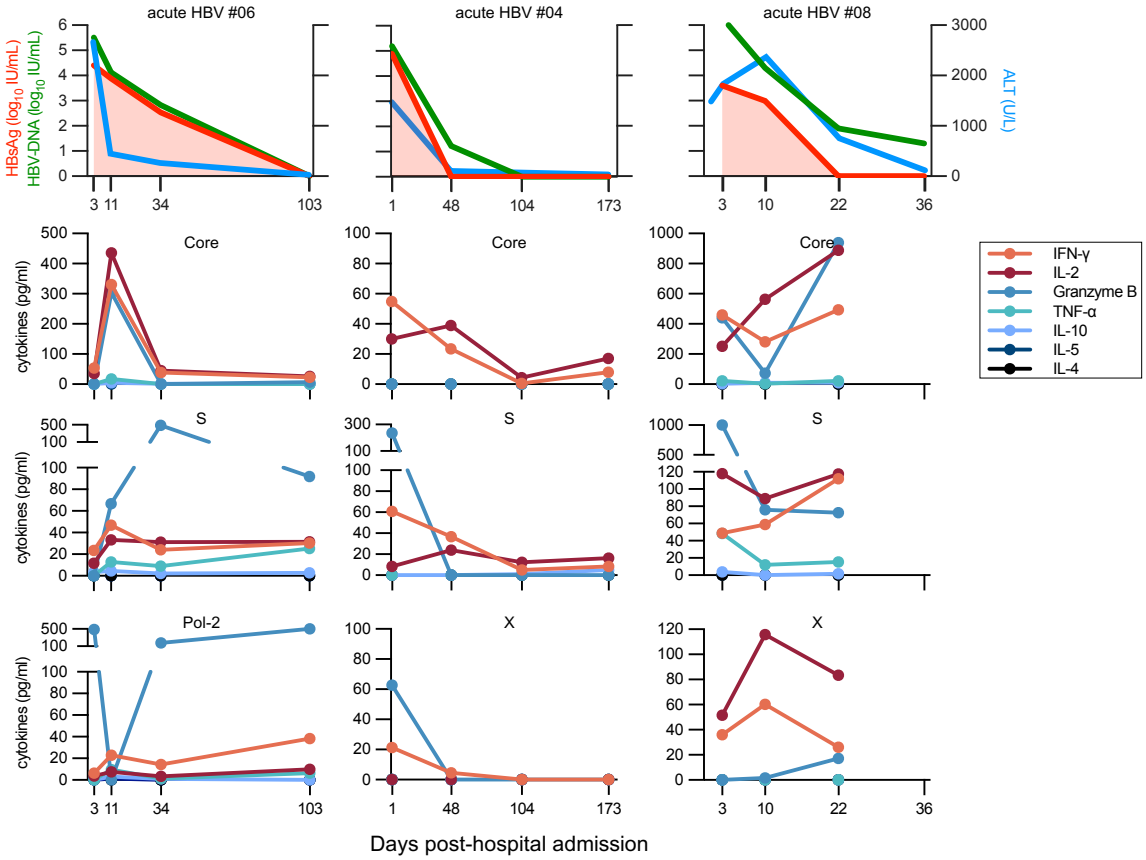
Figure 7: Clustering of samples with similar HBV-specific T cell cytokine secretion profiles with Phenograph

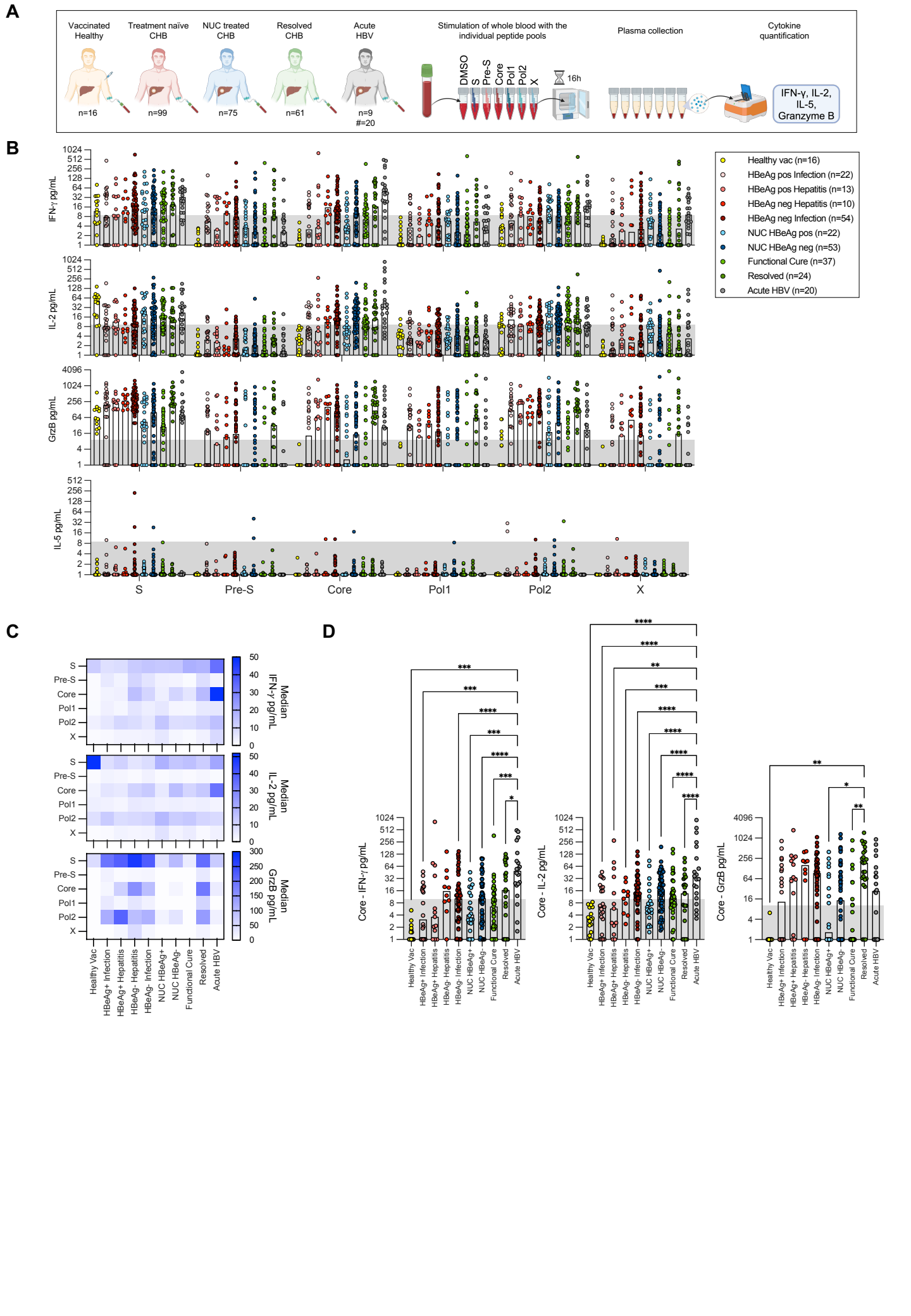
(**A**) UMAP plot with 13 color-coded PhenoGraph clusters. (**B**) Cytokine levels (IFN-γ, IL-2, and granzyme B) of all samples that fell into the PhenoGraph clusters 1 to 13 (each dot one sample; black line = median). (**C**) Heatmap of median cytokine concentrations of samples in clusters 1 to 13. (**D**) The portions of the 13 clusters identified by PhenoGraph in HBV-CRAs are shown as bar plots for each patient group in response to the six HBV-peptide pools. The colors of the 13 clusters correspond to those of the UMAP in panel (A). (**E**) Each line represents an individual, with the color code indicating the cluster to which their cytokine secretion profile in response to the HBV peptide pools was assigned.

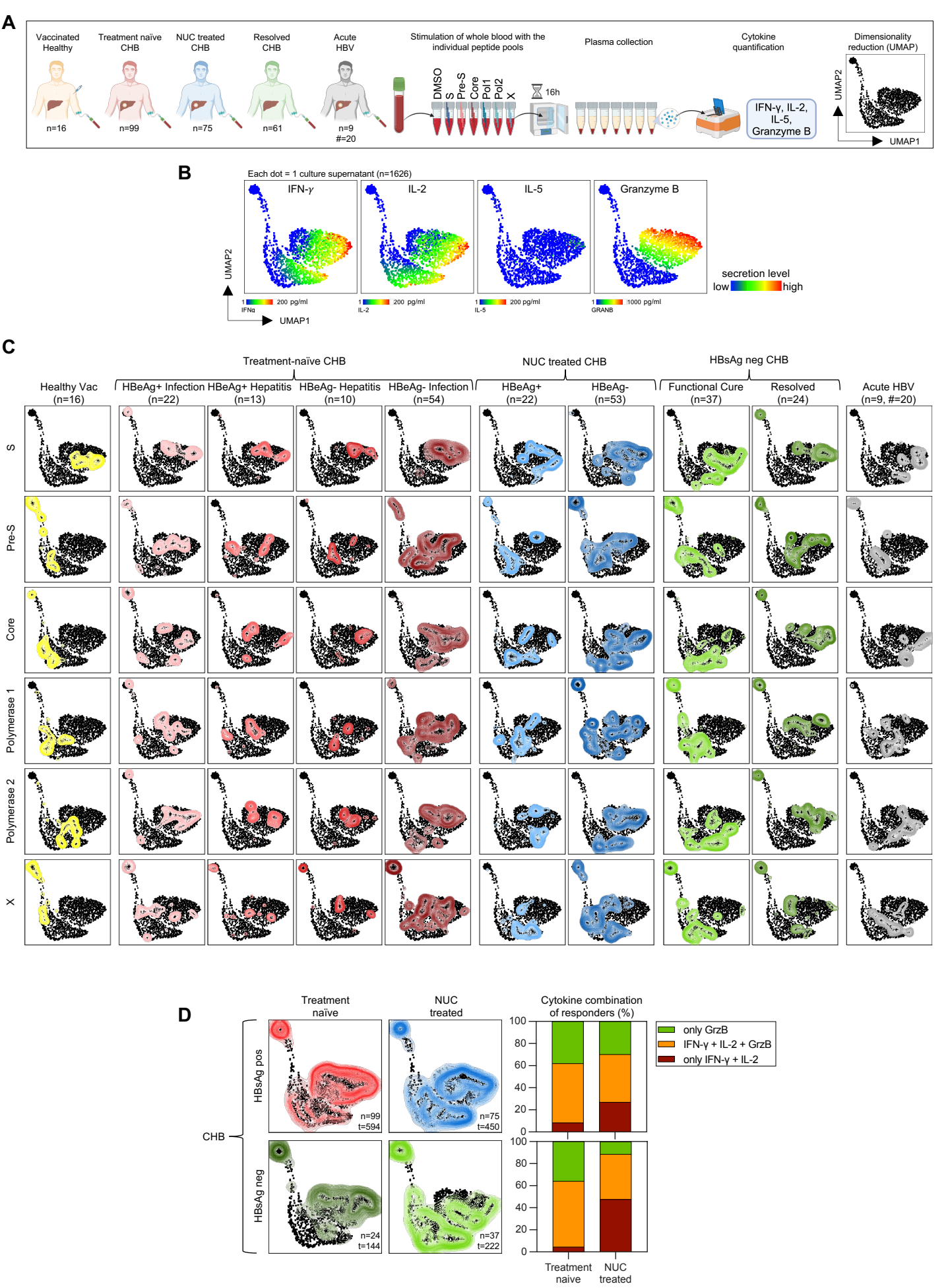

A
Core
Pre-S
S
Pol 1
Pol 2
X
CoV-2 Spike
Neg
PHA
#01 CHB
#02 CHB
#03 CHB
#04 CHB
#05 CHB
#06 CHB
SFC per 400k PBMC
Core
Pre-S
S
Pol1
Pol2
X
Cov-2 Spike
B
T cells
mRNA electroporation with HBV-Env183-91 TCR
Quantification of HBV-Env183-91 TCR+ T cells
Blood spiked with known numbers of HBV-Env183-91 TCR+ T cells + peptide
Plasma collection
Cytokine quantification
S183-TCR
16 h
C
IFN-γ
IL-2
Granzyme B
IL-10
cytokine (pg/mL)
Number of HBV-Env183-91-TCR+ T cells per 320μL of whole blood

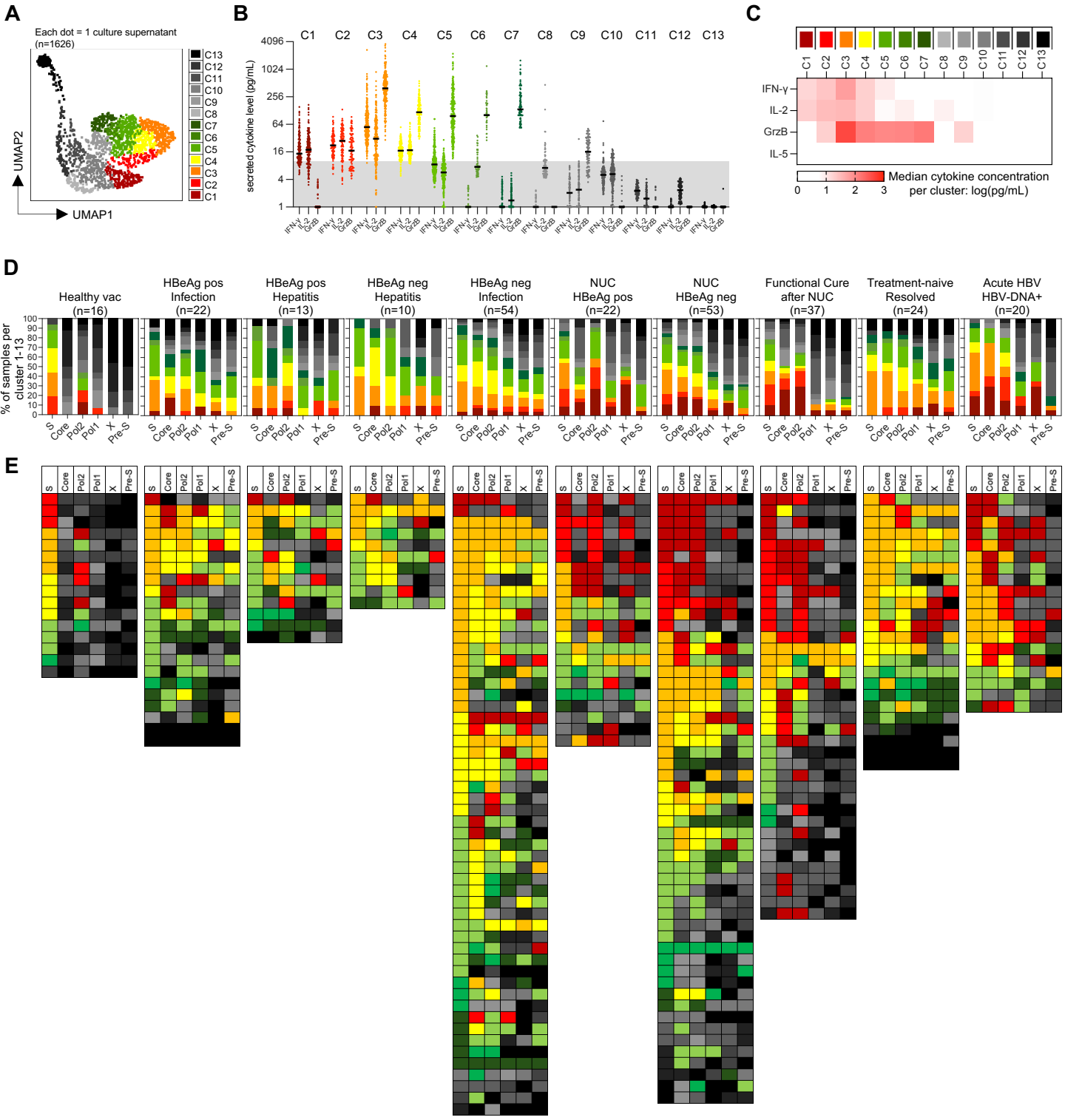

A
HBV
X
Core
Envelope
Polymerase
Genotype A
Genotype B
Genotype C
Genotype D
PanAD X pool (83 pep)
PanAD Core pool (71 pep)
PanAD PreS pool (102 pep)
PanAD S pool (121 pep)
PanAD Pol1 pool (228 pep)
PanAD Pol2 pool (233 pep)
B
CHB n=10
vaccinated n=16
DMSO
X
Core
Pre-S
S
Pol1
Pol2
16h
IFN-γ, IL-2, TNF-α, IL-4, IL-5, IL-10, Granzyme B
C
IFN-γ
IL-2
Granzyme
TNF-α
IL-10
IL-4/5
pg/mL
CHB
Vaccinated
D
% of responders
Pre-S
S
CORE
POL1
POL2
X


A
n = 10
T1 (Day 1)
T2 (Day 30)
T3 (Day 60)
DMSO
Pre-S
S
Pol1
Pol2
Core
X
16 h
B
T1
T2
T3
HBV-CRA
IFN-γ (pg/mL)
CORE
PRE-S
S
POL1
POL2
X
non-responder
C
ex-vivo ELISpot
SFU per 10^6 PBMC
% of response
D
T1-T3
R2 = 0.1913
p < 0.0001
log10(IFN-γ [pg/mL])
log10(SFU/10^6PBMC)
n=180
E
IFN-γ
IL-2
Granzyme B
TNF-α
IL-10
IL-5
IFN-γ ELISpot
T2 log2 [pg/ml]
T1 log2 [pg/ml]
T3 log2 [pg/ml]
T2 log2 [SFU]
T1 log2 [SFU]
T3 log2 [SFU]
n=60
n=48
F


acute HBV #06
acute HBV #04
acute HBV #08
HBsAg (log10 IU/mL)
HBV-DNA (log10 IU/mL)
ALT (U/L)
Core
S
Pol-2
X
cytokines (pg/ml)
IFN-γ
IL-2
Granzyme B
TNF-α
IL-10
IL-5
IL-4
Days post-hospital admission


A
Vaccinated Healthy
Treatment naïve CHB
NUC treated CHB
Resolved CHB
Acute HBV
n=16
n=99
n=75
n=61
n=9
#=20
Stimulation of whole blood with the individual peptide pools
DMSO
S
Pre-S
Core
Pol1
Pol2
X
16h
Plasma collection
Cytokine quantification
IFN-γ, IL-2, IL-5, Granzyme B
B
IFN-γ pg/mL
IL-2 pg/mL
GrzB pg/mL
IL-5 pg/mL
S
Pre-S
Core
Pol1
Pol2
X
Healthy vac (n=16)
HBeAg pos Infection (n=22)
HBeAg pos Hepatitis (n=13)
HBeAg neg Hepatitis (n=10)
HBeAg neg Infection (n=54)
NUC HBeAg pos (n=22)
NUC HBeAg neg (n=53)
Functional Cure (n=37)
Resolved (n=24)
Acute HBV (n=20)
C
Median IFN-γ pg/mL
Median IL-2 pg/mL
Median GrzB pg/mL
Healthy Vac
HBeAg+ Infection
HBeAg+ Hepatitis
HBeAg- Hepatitis
HBeAg- Infection
NUC HBeAg+
NUC HBeAg-
Functional Cure
Resolved
Acute HBV
D
Core - IFN-γ pg/mL
Core - IL-2 pg/mL
Core - GrzB pg/mL


A
Vaccinated Healthy
Treatment naïve CHB
NUC treated CHB
Resolved CHB
Acute HBV
n=16
n=99
n=75
n=61
n=9
#=20
Stimulation of whole blood with the individual peptide pools
DMSO
S
Pre-S
Core
Pol1
Pol2
X
16h
Plasma collection
Cytokine quantification
IFN-γ, IL-2, IL-5, Granzyme B
Dimensionality reduction (UMAP)
UMAP2
UMAP1
B
Each dot = 1 culture supernatant (n=1626)
IFN-γ
IL-2
IL-5
Granzyme B
UMAP2
UMAP1
secretion level
low
high
1 200 pg/ml
IFNg
1 200 pg/ml
IL-2
1 200 pg/ml
IL-5
1 1000 pg/ml
GRANB
C
Treatment-naïve CHB
NUC treated CHB
HBsAg neg CHB
Healthy Vac (n=16)
HBeAg+ Infection (n=22)
HBeAg+ Hepatitis (n=13)
HBeAg- Hepatitis (n=10)
HBeAg- Infection (n=54)
HBeAg+ (n=22)
HBeAg- (n=53)
Functional Cure (n=37)
Resolved (n=24)
Acute HBV (n=9, #=20)
S
Pre-S
Core
Polymerase 1
Polymerase 2
X
D
Treatment naïve
NUC treated
Cytokine combination of responders (%)
CHB
HBsAg pos
HBsAg neg
n=99
t=594
n=75
t=450
n=24
t=144
n=37
t=222
100
80
60
40
20
0
only GrzB
IFN-γ + IL-2 + GrzB
only IFN-γ + IL-2
Treatment naive
NUC treated


A
Each dot = 1 culture supernatant
(n=1626)
UMAP2
UMAP1
C13
C12
C11
C10
C9
C8
C7
C6
C5
C4
C3
C2
C1
B
secreted cytokine level (pg/mL)
4096
1024
256
64
16
4
1
IFN-γ
IL-2
GrzB
C
IFN-γ
IL-2
GrzB
IL-5
Median cytokine concentration
per cluster: log(pg/mL)
0
1
2
3
D
% of samples per cluster 1-13
Healthy vac (n=16)
HBeAg pos Infection (n=22)
HBeAg pos Hepatitis (n=13)
HBeAg neg Hepatitis (n=10)
HBeAg neg Infection (n=54)
NUC HBeAg pos (n=22)
NUC HBeAg neg (n=53)
Functional Cure after NUC (n=37)
Treatment-naive Resolved (n=24)
Acute HBV HBV-DNA+ (n=20)
S
Core
Pol2
Pol1
X
Pre-S
E