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Optimization of culture conditions for HBV-specific T cell expansion in vitro from chronically infected patients

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Abstract

Background Hepatitis B virus (HBV) clearance depends on an effective adaptive immune response, especially HBV-specific T cell-mediated cellular immunity; however, it is difficult to produce enough HBV-specific T cells effectively.

Results In this work, we investigated the proportions of stimulated cells, serum, and culture media as the three primary factors to determine the most effective procedure and applied it to HLA-A2 (+) people. In parallel, we also examined the correlation between clinical parameters and HBV-specific immunity. Concerning amplification efficiency, 4×10^5 cells stimulation was superior to 2×10^6 cells stimulation, AIM-V medium outperformed 1640 medium, and fetal bovine serum (FBS) exceeded human AB serum under comparable conditions. As expected, this procedure is also suitable for developing HBV-specific CD8+T cells in HLA-A2(+) individuals. Expanded HBV-specific T cell responses decreased with treatment time and were negatively correlated with HBV DNA and HBsAg. Furthermore, the number of HBV-specific IFN-γ+SFCs was strongly correlated with the ALT level and negatively correlated with the absolute lymphocyte count and the ALB concentration.

Conclusions We confirm that stimulating 4×10^5 PBMCs in AIM-V medium supplemented with 10% FBS is the best approach and that HBeAg, HBsAg, and ALB are independent predictors of HBV-specific T-cell responses.

Keywords CD8+T cells, Expansion, Hepatitis B virus

Introduction

Hepatitis B virus (HBV) infection continues to be a significant global public health concern due to its association with end-stage liver diseases, including cirrhosis and hepatocellular carcinoma (HCC). According to statistical data, approximately 316 million individuals were estimated to be infected with chronic HBV globally in 2019, leading to approximately 555,000 deaths associated with HBV-related diseases [1]. Despite the widespread use of antiviral drugs such as nucleos(t)ide analogs (NAs) and peginterferon-alpha (PegIFNa), complete treatment remains difficult because covalently closed circular DNA (cccDNA) cannot be eliminated from the liver [2, 3].

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The eventual outcome of HBV infection is determined by how viral replication and host antiviral immunity interact. Adaptive immune responses are the central mechanisms for the resolution of virus infections, leading to the activation of virus-specific B and T cell functions. The central role of the HBV-specific immune response in the control of HBV infection is now well recognized: HBV-specific B cells generate antibodies [4, 5], while HBV-specific CD8+T cells destroy infected hepatocytes [6–8]. As predominant effector cells, HBV-specific CD8+T cells can be divided into two types—HLA class I-restricted and HLA class I-unrestricted—with the former classified as envelope (183–191, 335–343), core (18–27), HBx (52–60, 115–123) or polymerase (455–463, 575–583) based on epitope distinctions [9–11]. Interestingly, the infection stage and the targeted epitope have an impact on the phenotype, function, and frequency of HLA-restricted HBV-specific T cells. The majority of specific T-cell responses against multiple epitopes can be detected in acute infections, and PD-1 expression decreases with the control of HBV infection. Targeted core18-27 corresponded to the highest levels of expression of inhibitory molecules (PD-1, 2B4, and CD160), followed by envelope proteins, and the polymerase response showed the lowest levels [12]. In contrast to acute infection, a significant barrier to chronic infection is the low frequency of HBV-specific CD8+T cells in the blood, which forces most research to rely on evaluating these cells after enrichment or expansion in vitro [13–15]. Additionally, the efficiency of HBV-specific T cell expansion varies among antigenic epitopes, with core18-27 having a greater expansion factor than pol455-463 [10].

Most research on the in vitro expansion of HBV-specific T cells has been carried out in combination with RPMI-1640 medium supplemented with fetal bovine serum (FBS) [16, 17], human AB serum [18, 19], AIM-V medium supplemented with human AB serum [20–22], or DMEM supplemented with FBS [23]. Furthermore, different theories exist in the aforementioned literature regarding the concentration of serum (2% [24], 5% [22], 8% [18], or 10% [16]), supplemental cytokines (IL-2 [16], IL-2+IL-7 [25], or IL-2+IL-7+IL-15 [22]), and the proportion of stimulated cells (20% (4×10^5) [20, 21] or 100% (2×10^6) [18, 19]).

In this study, we aimed to investigate the optimal in vitro expansion protocol for HBV-specific T cells by controlling three factors: serum, medium, and the proportion of stimulated cells. Furthermore, by examining the correlation between the number of expanded specific T cells and virological indicators, we sought to reveal the significance of clinical indicators indicative of the HBV-specific immune response and to provide favorable methodological support for subsequent functional remodeling studies.

Methods and materials

Study cohort

One hundred thirty-three patients with chronic HBV (cHBV) infection were recruited at the Department of Clinical Laboratory, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China. Table 1 displays a summary of their fundamental data. Age, sex, and past medication use were verified when the subjects were initially enrolled in the study.

Table 1 Characteristics of patients with chronic HBV infection enrolled in the study

	CHB patients (*n* = 133)	
Clinical data	**Treatment-naive group (*n* = 54)**	**Antiviral treatment group (*n* = 79)**
Gender (male/female)	26/28	59/20
Age (years)	41(21–60)	38(27–73)
ALT (U/L)	40.5(9-365)	51(12–239)
AST (U/L)	32(11–375)	37(17–400)
γ-GGT (U/L)	28(9-338)	38(10–596)
TP (g/L)	77.6(64.8–85.2)	76.5(63.5–89.1)
ALB (g/L)	46.6(40.5–55.4)	46.1(33.3–54.9)
GLB (g/L)	29.7(15.8–41.8)	30.2(18-44.3)
WBC (10^9/L)	5.5(2.5–8.1)	5.1(2.4–9.5)
NEUT (10^9/L)	3.1(0.9–5.4)	2.8(0.7–5.9)
LYM (10^9/L)	1.7(0.9–3.1)	1.8(0.7–3.6)
HBeAg (+/-)	16	26/53
HBsAg (IU/ml)	1802.7(3.6-106135.0)	553.8(0-63472.5)
HBV DNA(< 2 Log10 IU/ml)	15	66
HBcAb (IU/ml)	13140.3(580-88324)	1665.0(118-89246)

All numerical data were expressed in median (range)

ALT, alanine aminotransaminase; AST, aspartat aminotransferase; γ-GGT, γ-glutamyltransferase; TP, Total protein; ALB, albumin; GLB, Globulin; WBC, white blood cell; NEUT, neutrophil; LYM, lymphocyte; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcAb, hepatitis B core antibody; HBV, hepatitis B virus; CHB, chronic hepatitis B

HBV peptide library

A library of 313 fifteen-mer peptides overlapping by 10 amino acids was used to identify HBV-specific T cells from HLA-A2- patients. The peptides covered the whole proteome of HBV genotype B (GenBank AF121243) and were purchased from Mimotopes. For HLA-A2+patients, peptides covering the HLA-A2-restricted epitopes of the HBV core (core 18–27: FLPSDFFPSV) were purchased from Sangon Biotech for HBV-specific T-cell culture. Detailed peptide library information is listed in Supplementary Table 1.

PBMC isolation and in vitro expansion of HBV-specific T cells

Peripheral blood mononuclear cells (PBMCs) were extracted from heparinized blood using Histopaque-1077 (Sigma-Oldridge, USA) density centrifugation at 400 × g for 30 min. The middle PBMC layer was carefully aspirated and washed twice with 4 mL of phosphate-buffered saline (PBS). Then, the generation of T-cell lines was performed as previously described in detail [21, 24]. Briefly, 2×10^6 PBMCs were stimulated with peptides and anti-CD28 (BD, USA), or 4×10^5 PBMCs were cocultured in medium with the remaining cells after being pulsed with 10 μg/ml of a panel of fifteen-mer peptides and anti-CD28 for 1 h at 37 °C. According to the group in which they are positioned, different mixes of medium and serum are used. The medium was refreshed with 20 U/ml IL-2 on days 3 and 7 of culture, and the cells were analyzed after a total of 10 days of culture. The specific reagents used are listed in Supplementary Table 2.

ELISPOT assay

Enzyme-linked immunosorbent spot (ELISPOT) assays (Mabtech, Sweden) were performed as previously described in detail [26]. Briefly, T cells were incubated overnight with a panel of HBV peptides (5 μg/ml) after 10 days of in vitro expansion. A 100 μl dose of biotinylated detection monoclonal antibody (7B6-1) was applied after five rounds of washing with phosphate-buffered saline. After 2 h at room temperature, the plate was washed twice, 100 μl of streptavidin ALP was added to each well, and the plate was incubated for another 1 h at room temperature in the dark. After washing the plate five times, the ready-to-use substrate solution (BCIP/NBT-plus) was filtered through a 0.45 μm filter, and 100 μl/well was added. They develop until distinct spots emerge. The color development was stopped by washing extensively in distilled water. The cells were counted on an ELISPOT reader (AID, ELR08 IFL, Germany) according to the manufacturer's instructions.

Flow cytometry

The staining of 1×10^6 PBMCs was started with a Zombie Aqua™ Fixable Viability Kit (Biolegend, USA) for 15 min at room temperature in the dark. Then, PE-Dextramer (HLA-A*0201-FLPSDFFPSV) (Immudex, Denmark) was added to the cell sample, which was vortexed briefly according to the manufacturer's instructions. Next, the cells were stained for 30 min at 4 °C with PerCP-Cy5.5-conjugated anti-CD3 (clone UCHT1, Biolegend, USA) and FITC-conjugated anti-CD8 (clone G42-8, BD, USA) antibodies. The cell samples were washed once and then analyzed using a flow cytometer (CytoFLEX, Beckman Coulter, USA).

Statistical analysis

Statistical analyses were performed in SPSS 22.0 (IBM) as indicated in the following legends: Wilcoxon's paired t test and Spearman's rank correlation. All tests were 2-tailed. Differences were considered significant at the level of $p<0.05$.

Results

Schematic diagram of a multivariate combinatorial scheme for the in vitro expansion of HBV-specific CD8+T cells

We examined three primary parameters when designing the protocol: the fraction of stimulated cells, the kind of serum, and the type of medium. As shown in Fig. 1, PBMCs were isolated from heparin-anticoagulated peripheral blood according to the manufacturer's instructions and were then separated into two groups: (A) 2×10^6 PBMCs was stimulated, and (B) 4×10^5 PBMCs were stimulated with 10 μg/ml of both peptides. Serum and medium were divided into four groups based on permutations: α) AIM-V+human AB serum, β) AIM-V+FBS, γ) RPMI 1640+human AB serum, and δ) RPMI 1640+FBS. The serum content was 10% in all patients. On days 3, 6, and 9, half-volume fluid changes and IL-2 supplementation to 20 IU/ml were conducted, followed by quantification via ELISPOT or flow cytometry on day 10.

Comparison of outcomes from different expansion protocols and a representative schematic of the ELISPOT

In the first set of experiments, we investigated which of the two protocols—4×10^5 cells stimulation or 4×10^5 cells prestimulation—was most effective based on the combination of RPMI-1640 medium and human AB serum. Notably, 4×10^5 cells prestimulation had a significantly greater effect than the other treatments (Fig. 2A). After deciding on the 4×10^5 cells prestimulation, we contrasted the impact of RPMI-1640 medium and AIM-V medium using human AB serum. Surprisingly, we detected considerably greater amplification in AIM-V medium than in RPMI-1640 medium (Fig. 2B). Therefore, we were able to identify more appropriate media genres

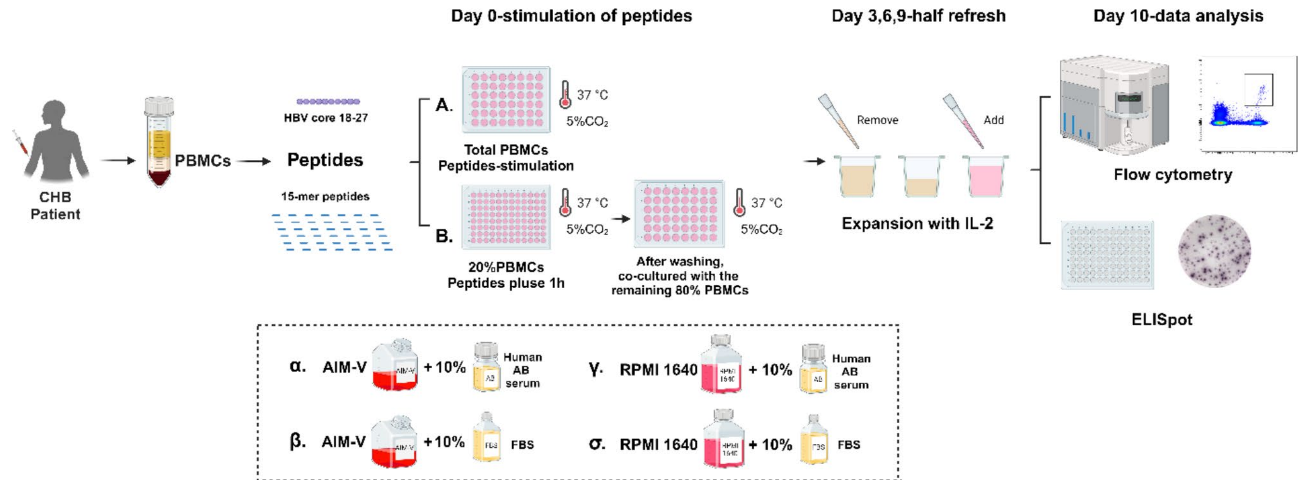

Fig. 1 A schematic illustration of HBV-specific T lymphocytes produced in vitro. Created in BioRender [27]

Fig. 2 Differences in the amplification efficiency of various culture techniques. **(A)** Comparison of B + γ (20%, 1640 medium, human AB serum) and A + γ (total, 1640 medium, human AB serum) from 10 CHB patients. **(B)** Comparison of B + γ (20%, 1640 medium, human AB serum) and B + α (20%, AIM-V medium, human AB serum) from 10 CHB patients. **(C)** Comparison of B + β (20%, AIM-V medium, FBS) and B + α (20%, AIM-V medium, human AB serum) from 10 CHB patients. CHB, chronic hepatitis B; FBS, fetal bovine serum. $P^{**} < 0.01$

and stimulation strategies. Next, we discovered that FBS was more favorable for specific T-cell expansion than human AB serum under comparable medium conditions (Fig. 2C). Therefore, we established the optimal in vitro expansion protocol of 4×10^5 cells prestimulation for 1 h in combination with AIM-V and FBS.

The combination of AIM-V and FBS is also suitable for the expansion of HBc-18-27-specific T cells

After determining that the combination of 4×10^5 PBMCs, AIM-V medium and human AB serum is best for the development of HBV-specific T cells, we aimed to investigate whether these conditions are applicable to the culture of HBc-18-27-specific T cells. Next, we selected four HLA-A2+patients for in vitro expansion experiments, which revealed that HBc-18-27-specific T cells could be generated successfully (Fig. 3A-D). These findings indicate that HBc-18-27-specific T cells can be successfully expanded using AIM-V combined with 10% FBS, with 4×10^5 cells stimulated first.

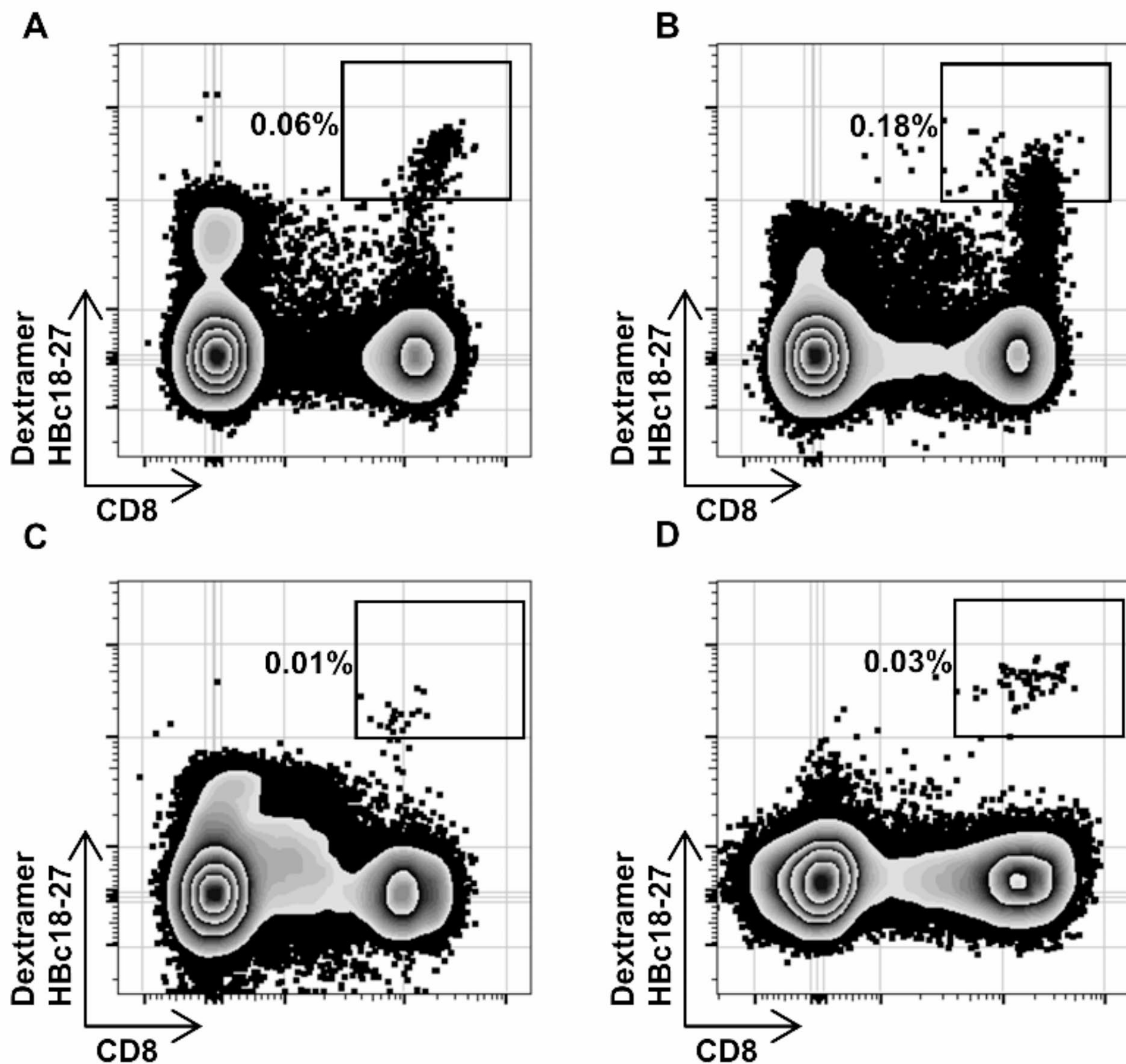

Fig. 3 Schematic representation of the detection of HBc-18-27-specific CD8+T cells. Results of four patients **(A-D)** with chronic hepatitis B using in vitro amplification tests

The amount of HBV-specific IFN-γ + SFCs following expansion is associated with laboratory indicators

Here, we collected laboratory data and clinical information on all the included patients and investigated the associations between them and the number of specific T cells. First, there was an increase in HBsAg (Fig. 4A) and HBV DNA (Fig. 4B) levels along with a decrease in specific T-cell numbers. In terms of therapeutic efficacy, we discovered that the number of HBV-specific T cells decreased with prolonged treatment (Fig. 4C), accompanied by the normalization of ALT (Fig. 4D). Interestingly, the lymphocyte (LYM) percentage and the serum ALB concentration (Fig. 4F) were significantly negatively correlated with specific T-cell counts. In addition, the factors significantly related to the number of HBV-specific T cells were ALB, HBeAg and HBsAg (Table 2). In summary, clinical characteristics are valuable for predicting the effect of HBV-specific T-cell expansion.

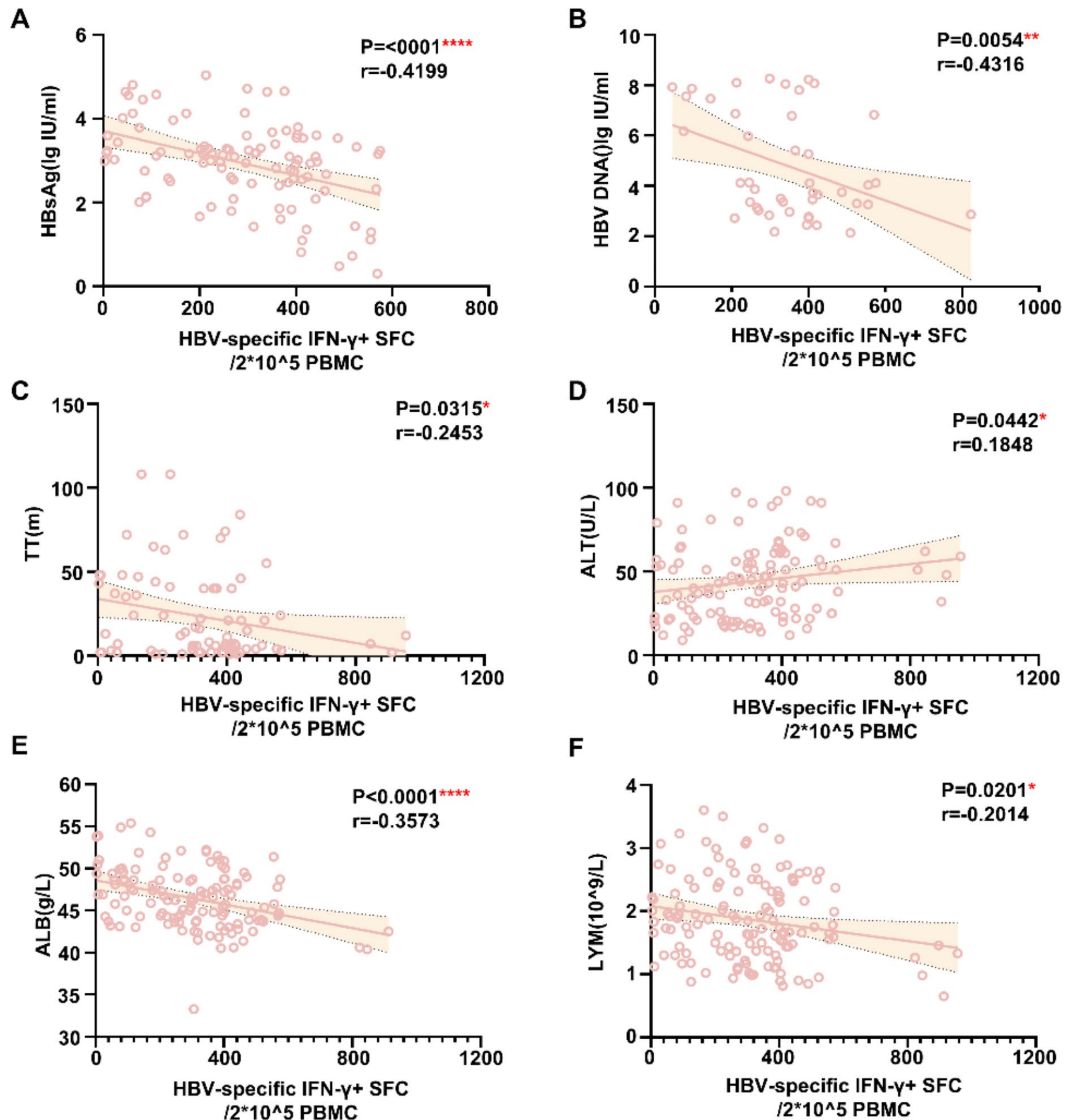

Fig. 4 Correlations between laboratory indicators and the number of HBV-specific T cells. Correlations between the levels of HBsAg **(A)** and HBV DNA **(B)**, TT **(C)**, the levels of ALT **(D)** and ALB **(E)**, the amount of LYM **(F)**, and the number of HBV-specific IFN-γ + SFCs measured by ELISpot. HBsAg, hepatitis B surface antigen; TT, treatment time; ALT, alanine aminotransferase; ALB, albumin; LYM, lymphocyte; SFC, spot-forming cell; ELISpot, enzyme-linked immunosorbent spot. *P** < 0.05, *P*** < 0.01, *P***** < 0.0001

Table 2 Univariate and multivariate analyses for HBV-specific T cells/2*10^5 PBMC in CHB patients

	HBV-specific T cells/2*10^5 PBMC (*n* = 133)			
	r	p	β	p
Gender (female)	0.013	0.855		
Age (years)	0.064	0.463		
ALT (U/L)	0.079	0.369		
AST (U/L)	0.080	0.359		
γ-GGT (U/L)	0.067	0.441		
TP (g/L)	-0.143	0.114		
ALB (g/L)	-0.357	**< 0.0001**	-0.348	**0.003**
GLB (g/L)	0.118	0.190		
WBC (10^9/L)	-0.185	**0.033**	0.081	0.859
NEUT (10^9/L)	-0.195	**0.025**	-0.230	0.540
LYM (10^9/L)	-0.201	**0.020**	-0.176	0.369
HBeAg-positive	-0.250	**0.004**	-0.395	**< 0.001**
HBsAg (IU/ml)	-0.420	**< 0.0001**	-0.408	**0.008**
HBV DNA > 100 (IU/ml)	0.070	0.424		
HBcAb (IU/ml)	-0.063	0.498		
Treatment-naive	0.683	0.036		
Treatment				
NAs	reference			
IFN	0.302	**0.007**	-0.183	0.268
Treatment-time (month)	-0.245	**0.032**	-0.056	0.664

Note: In two-variable correlation analysis, Spearman's rank correlation was used for two continuous variables, and Kendall's rank correlation was used if one of the variables was discontinuous; the r values represent the correlation coefficients. In multiple linear regression analysis, the stepwise regression was used to determine the variables in the regression equation; the β values represent the standardized regression coefficients

Discussion

We performed detailed culture and expansion experiments on HBV-specific T cells during chronic infection. Importantly, all investigations were carried out immediately ex vivo, eliminating the effects associated with frozen cells. We determined potential situations by varying the permutations of the three most significant impacting elements—an unprecedented methodological exploration.

Previous studies have reported that specific T cells are not completely absent from the circulation but are below the detection limit of standard direct ex vivo assays [12, 15]. Two approaches that aim to acquire and analyze certain T cells have steadily surfaced in recent years to address this difficulty, one of which is magnetic bead enrichment. Large numbers of specific cells were obtained by Schuch A et al. [10] and Hoogeveen R C et al. [12] using the magnetic bead method, which made it possible to study thousands of T cells specific to a virus, even from small populations of people who were chronically infected. However, this method tends to place a high demand on source samples, requiring hundreds or even thousands of milliliters of peripheral blood. Another method is in vitro amplification, which involves stimulating with a particular peptide and incubating the sample for several days to continuously and somewhat regularly increase the otherwise negligible level of specificity. This type of approach is commonly used to characterize immune responses and functions against specific T cells [28]. Obviously, the latter requires only a small initial cell volume, making relevant clinical research easier to undertake, although in vitro T-cell multiplication has been shown to cause dramatic alterations in their phenotype [12].

Many protocols have been refined recently for in vitro expansion, wherein the usage of IL-2 and cell density are essential. The effects of expansion on HBV-specific T cells revealed unanticipated differences depending on the number of stimulated cells. Typically, the total amount of starting cells for an expansion experiment falls between 1×10^6 and 3×10^6. Bertoletti A et al. [29] were the first to present the concept of prestimulating 20% of cells for 1 h, and only a few studies have since followed suit [21, 24, 30, 31]. We confirm that the 4×10^5 cells is, in fact, superior to the 2×10^6 cells stimulus. This finding suggested that the mechanism of initial stimulation by the antigenic peptide influences the intensity of specific T-cell proliferation, as mentioned in previous studies [32, 33]. A brief interaction with the antigen stimulates CD8+T-cell proliferation without the need for additional antigenic stimulation. Therefore, the question arises as to why the remaining T cells have equal opportunities for stimulation even though they are not triggered by the antigenic peptide. A reasonable hypothesis is that pulsed antigen-presenting cells (APCs) provide considerable stimulatory efficacy to T cells. Interestingly, peptide-pulsed dendritic

cells (DCs) have been employed to boost tumor-specific T-cell responses in melanoma patients through in vitro restimulation with efficacy comparable to that of peptides [34]. The use of 30–40% of PBMC for individual stimulation might be explored in various combinations in future studies; this would be a worthwhile project.

AIM-V is primarily used for immunological studies and lymphocyte culture, providing a better environment for cell survival and functional maintenance than relatively simple RPMI 1640 medium [35], which is widely used for the culture and proliferation of a wide range of cell types. Under identical serum conditions, we discovered that the amplification efficacy of the AIM-V medium was superior to that of the 1640 medium. This conclusion is consistent with the findings of Sato K et al. [36], who reported that the multiplicity of amplification was significantly greater in AIM-V medium than in RPMI-1640 medium on day 12 of incubation. Next, we had to take into account the effect of serum factors because the total cell expansions in serum-free medium conditions were all significantly lower than those in AIM-V medium (supplemented with 5% human serum) [37]. Previous research has indicated that fewer T cells expand from human AB serum than from FBS [38]. Our findings additionally verified that the FBS-supplemented medium produced the greatest quantity of specific spots in an overwhelming manner. Interestingly, the accumulation of specific T cells was also observed in HLA-A2+patients under the same culture conditions.

It is challenging to identify the FBS element that produces better performance due to the complicated components of serum. Nevertheless, several scholars have analyzed by enzyme-linked immunoassay screening and concluded that the proliferative capacity of ASCs is related to serum platelet-derived growth factor (PDGFs) and transforming growth factor-beta (TGF-β) [39], the former being potent mitogens for ASCs [40], and the latter playing an important role in memory T-cell pool formation [41], whereas the concentrations of both tend to be at intermediate levels in FBS [39]. These findings imply that serum selection has a significant impact on HBV-specific T-cell expansion, even though the underlying mechanisms are not yet understood.

Our investigation demonstrated a negative correlation between sero-virological parameters and specific T-cell responses, which is consistent with prior findings [41–44], implying that increased specific T-cell immune responses contribute to viral suppression. The predictive effects of treatment duration and ALT on HBV-specific immunity were associated with the fact that prolonged interferon therapy results in a steady decrease in the number of HBV-specific T cells and stronger specific immunity in responders [45]. Furthermore, our findings suggest that the serum ALB concentration and the absolute lymphocyte count are inversely linked with HBV-specific T-cell immunity through an unknown mechanism. Overall, the strength of HBV-specific immunity is affected by both virologic and clinical biochemical markers. We regretfully cannot now distinguish between the specificity of HBV-specific T lymphocytes for distinct HBV antigens because the peptides employed in this work encompass the whole proteome of HBV genotype B. Future research on these findings will be interesting, though.

Conclusions

In conclusion, given the diversity of current culture methods, we included three major variables to examine the expansion of HBV-specific T cells under various culture protocols and successfully established an ideal expansion protocol that was also applied to HLA-A2+-specific T cells. Notably, the partial stimulation regimen with a 4×10^5 density of AIM-V medium supplemented with 10% FBS produced the best outcomes by a large margin.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12896-024-00908-8.

Supplementary Material 1

Acknowledgements

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Author contributions

Li Wang: Writing - Conceptualization, Methodology, Investigation, Data Curation, Original Draft, Visualization .Hongjiao Chen: Investigation, Data Curation. Yuanqi Yang: Methodology, Validation, Data Curation, Investigation. Ying Huang: Resources, Validation. Weixian Chen: Conceptualization, supervision, project administration. Di Mu: Conceptualization, Supervision, Funding acquisition, Writing - Review & editing, Resources.

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Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the World Medical Association Declaration of Helsinki, and all subjects provided written informed consent. This work was approved by the Ethics Committee of The Second Affiliated Hospital of Chongqing Medical University (2021 – 150).

Competing interests

The authors declare no competing interests.

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