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ORIGINAL ARTICLE

Dynamic changes of T cell receptor repertoires in patients with hepatitis B virus-related acute-on-chronic liver failure

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Abstract

Background and aims T cell-mediated immune injury plays a critical role in the pathogenesis of hepatitis B virus-related acute-on-chronic liver failure (HBV-ACLF). Given the high short-term mortality and crucial role of T cells in the disease progression, it is necessary to investigate the dynamics of T cell clones during HBV-ACLF. The aim of this study was to longitudinally investigate dynamic changes in the composition and perturbation of T cell receptor β (TCRβ) chain repertoires and to determine whether TCR repertoire characteristics were associated with HBV-ACLF patient outcomes.

Methods Peripheral blood mononuclear cells (PBMCs) were collected at two time points from 5 HBV-ACLF patients. Global $CD4^+$ and $CD8^+$ T cells were sorted using magnetic beads. TCRβ complementarity-determining region 3 was analyzed by unbiased high-throughput sequencing.

Results During HBV-ACLF, there was a significant decrease in the diversity of T cell repertoires and an increase in proportion of the most 100 abundant clonotypes of CD8 T cells but not CD4. Decreased CD8 repertoire diversity was positively correlated with the reduction of the Model for End-Stage Liver Disease (MELD) score.

Conclusions There was significant clonal expansion in CD8 but not in CD4 T cell repertoires in HBV-ACLF patients during disease progression. Patients with greater clonal expansions in CD8 T cell repertoires may have better outcomes. CD8 TCRβ repertoire diversity may serve as a potential predictive marker for disease outcome.

Keywords Hepatitis B virus · Acute-on-chronic liver failure · T cell receptor · High-throughput sequencing · Repertoire · Beta chain · Complementarity-determining region · Diversity · Clonotype · Clonal expansion

Introduction

Hepatitis B virus-related acute-on-chronic liver failure (HBV-ACLF) is the most common type of liver failure, with high 28-day mortality in the Asia–Pacific and African regions [1, 2]. It is characterized by rapid deterioration of liver function, altered mentation, and coagulopathy that subsequently progresses to multiple organ failure in individuals with pre-existing chronic hepatitis B (CHB) [2, 3]. Given the lack of options of therapies and prognosis models, there are pressing demands to investigate HBV-ACLF pathogenesis, which is still not fully understood. Both innate and adaptive immune dysfunctions have been reported in HBV-ACLF patients [4, 5]. In acute HBV infection, T cell responses play an important role in liver damage, whereas the immune function of T cells in HBV-ACLF patients remains controversial. Some studies have shown the significant decrease of $CD4^+$ and $CD8^+$ T cells, or increase of regulatory T cells (Tregs) frequencies in HBV-ACLF compared with CHB

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patients [6–9], while recent study indicated that patients with HBV-ACLF had higher levels of IL-17-producing $CD8^+$ T (Tc17) cells than patients with CHB, suggesting the proinflammatory role of Tc17 cells in the pathogenesis of HBV-ACLF [10]. All the studies analyzed T cell frequency by cell staining and flow cytometry. However, dynamic changes in cellular immunity, especially alterations in T cell components, have not been systematically investigated in patients with HBV-ACLF.

The majorities of CD4 and CD8 T cells in human are αβ T lymphocytes, which recognize the antigen peptides presented by the major histocompatibility complex (MHC) through T cell receptors (TCR) on the surface [11]. Each αβ TCR is composed of α and β chains. It is estimated that the theoretical diversity of αβ TCR could be up to 10^{18}, due to the combination of different variable (V), diversity (D), and joining (J) gene segments plus the non-template nucleotide additions and deletions at the V(D)J junctions, making TCRs capable of binding to the various peptide/MHC complexes [12]. There are three highly variable regions called complementary determining regions (CDRs) in each single chain. CDR3, which falls at the junction between V(D)J gene segments, is the most diverse and the main region that interacts with the peptide presented by the MHC.

Several studies have identified skewed TCR repertoires in peripheral CD4 [6] and CD8 T cells [13] in patients with chronic severe hepatitis B or HBV-ACLF through gene melting spectral pattern or CDR3 spectrum analysis, which only obtained limited CDR3 motifs and roughly described the degree of perturbation by CDR3 sequence length. Advances in next-generation sequencing (NGS) technologies now enable the routine analysis of millions of T cell receptors in a single experiment. TCR sequencing is a powerful technology to quantitatively track T cell clones in any given T cell population globally or by single cells. The diversity and components of clonotypes in T cell repertories can be assessed by the nucleotide CDR3 sequences.

However, no cross-sectional or longitudinal study has comprehensively evaluated the TCR repertoire of ACLF patients. In this study, we used NGS to investigate the dynamic changes in TCR repertoires of peripheral CD4 and CD8 T cells in HBV-ACLF patients during hospitalization. By analyzing massive numbers of TCR sequences, we were able to accurately monitor the clonal expansion and composition of T cell repertoires in HBV-ACLF subjects. A significant oligoclonal expansion of CD8 T cells and its association with outcome in HBV-ACLF patients was observed, indicating the important role of CD8 T cell in HBV-ACLF pathogenesis.

Materials and methods

Subjects

Five HBV-ACLF patients were enrolled from Guangzhou Eight People's Hospital between February and December 2016. All patients were hepatitis B surface antigen positive longer than 6 months and met the definition of ACLF as defined by the Consensus Recommendation on ACLF issued by the Asian Pacific Association for the Study of the Liver (APASL) criteria: jaundice [serum bilirubin ≥ 5 mg/dL (85 μmol/L) and coagulopathy (international normalized ratio, INR) ≥ 1.5 or prothrombin activity (PTA) < 40%] complicated within 4 weeks by clinical ascites and/or encephalopathy in a patient with or without previously diagnosed chronic liver disease [14]. The major precipitating factor for ACLF of the five patients was the reactivation of HBV infection. Two longitudinal samples were collected from each patient. The first was collected at the time of clinical diagnosis, and the timing of the second was dependent on patient outcome. If total bilirubin (TBIL) decreased, we collected the samples when the TBIL level decreased by ~ twofold. If TBIL increased, we collected the second sample at the last observation during hospitalization. The average duration between time points was 22 days (range 7–48 days). All patients received conservative management including nutritional support, hepatoprotective drugs, nucleotide analog antiviral therapy (entecavir 0.5 mg/d), and prevention and control of complications. No glucocorticoid treatment was administered between sample collections.

Exclusion criteria included co-infection with hepatitis A, C, D, E or human immunodeficiency virus, alcoholic liver, autoimmune liver diseases, and tumors. Patients who were treated with immunomodulatory therapy within the previous 6 months were also excluded.

Clinical and laboratory parameters

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), TBIL, albumin (ALB), PTA (%), prothrombin time (PT), and INR were measured with commercial kits. Serum HBV DNA was tested with the Taqman polymerase chain reaction (PCR) assay (DaAn Gene, Guangzhou, China) with a detection limit of 100 IU/mL. HBV serological markers were measured by chemiluminescence immunoassays (Roche Diagnostics, Risch-Rotkruez, Switzerland). The Model for End-Stage Liver Disease (MELD) score, which is used to assess the severity and prognosis of end-stage liver disease, was calculated as

$$10 \times [0.957 \times \ln(\text{serum creatinine}) + 0.378 \times \ln(\text{TBIL}) + 1.12 \times \ln(\text{INR}) + 0.643]$$

[15]. Hepatic encephalopathy was graded from I to IV as previously described [16].

TCR sequencing

Peripheral blood mononuclear cells (PBMCs) were isolated from 5 mL fresh EDTAK2 anti-coagulant treated blood by Lymphoprep (Axis-Shield Diagnostics, Dundee, Scotland) gradient centrifugation and frozen in liquid nitrogen until the samples were used. $CD4^+$ and $CD8^+$ T cells were positively sorted by CD4 and CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of sorted cells was > 93% as demonstrated by fluorescence-activated cell sorting (FACS Canto II; BD Biosciences, Franklin Lakes, NJ, USA) (data not shown). The total RNA was extracted from the sorted cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse transcribed by 5′ rapid amplification of cDNA ends [17]. TCR β chains were amplified by nest PCR as described previously [18]. The TCR libraries were generated with NEBnext Ultra DNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol and deep sequenced using the Illumina HiSeq platform (Illumina, San Diego, CA, USA) with a read length of 2 × 150 bp.

Data processing and TCR repertoire analysis

The FASTQ files obtained by sequencing were imported to MiXCR software (v2.1.8) [19] for sequence alignment; identification of V, D, and J gene segments and CDR3 sequences; and correction of PCR and sequencing errors after filtering out low-quality and unproductive reads as previously described [20]. The relative degree of clonal expansion was assessed by calculating the cumulative percentage of the repertoire that was constituted by the top 100 clonotypes (the most 100 abundant TCRβ amino acid sequences in the total repertoire of each individual). To address TCR repertoire diversity in each sample, we calculated the normalized Shannon diversity entropy (NSDE), an index which is the normalized Shannon entropy by $\log_2$ (number of unique TCR clones) and represents the clonal nature of a sample irrespective of samples of different sequencing depth [21]. NSDE values range from 0 (least diverse) to 1 (most diverse). The NSDE was calculated using the diversity function in R package vegan. The Morisita-Horn similarity index (MHSI) was calculated for the similarity of any two TCR repertoires, with a value from 0 (no overlap) to 1 (identical overlap) [22]. The exact algorithm of MHSI is

$$p_i^l = \frac{n_i^l}{\sum_i n_i^l} p_i^c = \frac{n_i^c}{\sum_i n_i^c}$$

$$\mathrm{MHSI} = \frac{2\sum_i p_i^l p_i^c}{\sum_i \left(p_i^l\right)^2 + \sum_i \left(p_i^c\right)^2}.$$

where n^l or n^c defined the abundance (or the number) of one unique CDR3 aa clonotype in sample l or sample c, respectively (The sample l and sample c were taken together to have i kinds of clonotypes); thus, p^l or p^c was the frequency of one unique CDR3 aa clonotype in its repertoire of sample l or sample c, respectively.

The fold change (FC) of T cell clone frequency was defined as a TCR sequence frequency at the second time point divided by the sequence frequency at the first time point. A TCR sequence was categorized as increased for FC ≥ 2, unchanged for FC < 2 and > 0.5, and decreased for FC ≤ 0.5. All detectable TCR sequences at any time point were included in the analysis. For TCR sequences that were not detectable at the first time point but available at the second time point, the lowest frequency value in the same sample was used [23].

Statistical analysis

Statistical analysis was performed using SPSS version 20 (IBM Corp., Armonk, NY, USA). The data are expressed as medians. Wilcoxon signed-rank tests were used to compare paired samples. Spearman's rank order correlation coefficient was used to calculate correlations. All data were analyzed using two-tailed tests, and differences with p value less than 0.05 were considered statistically significant.

Results

Patient summary

Baseline characteristics at hospital admission and the clinical characteristics at the second time point are shown in Table 1. Two of the five patients with hepatic encephalopathy and hepatorenal syndrome died, and the other three subjects survived. Blood draws were conducted twice after admission. ALT and ALB levels of all subjects were significantly decreased and increased during hospitalization, respectively. No differences were found in the values of TBIL, PT, PTA, INR, Cr, or MELD scores between time points (Fig. S1).

TCRβ repertoire profiling

A total of 163,259,321 clean reads were obtained from PBMCs of 5 HBV-ACLF subjects (20 samples). Each CD4 and CD8 TCR repertoire has an average of 7782,177 and 5907,150 productive reads per sample, respectively. Overall,

Table 1 Clinical characteristics of HBV-ACLF patients during the hospitalization

Patient no.	P1		P2		P3		P4		P5	
Outcome	Non-survivor		Survivor		Survivor		Non-survivor		Survivor	
Age	63		42		29		55		36	
Duration of sample collection (day)	16		48		18		7		24	
Time point	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
HBsAg (±)	+	N.D.	+	N.D.	+	N.D.	+	N.D.	+	N.D.
HBeAg (±)	–	N.D.	+	N.D.	–	N.D.	–	N.D.	+	N.D.
HBeAb (±)	+	N.D.	–	N.D.	+	N.D.	+	N.D.	–	N.D.
HBV DNA ($\log_{10}$ IU/ml)	8.70	N.D.	7.70	< 2.0	5.27	< 2.0	6.43	N.D.	5.66	3.35
ALT (U/L)	1971	71	1498	22	291	60	319	85	408	56
TBIL (mg/dL)	8.51	29.51	11.85	5.27	27.30	12.0	25.00	30.58	15.13	6.46
PTA (%)	24.58	7.92	24.81	43.0	35.0	58.80	20.25	25.53	38.88	67.01
PT(s)	29.40	74.60	29.20	20.20	19.60	16.90	34.00	28.60	21.50	15.80
INR	2.70	9.18	2.87	1.74	1.67	1.39	3.59	2.84	1.87	1.26
Cr (mg/dL)	0.88	1.47	0.76	0.69	0.89	0.66	1.65	1.74	0.74	0.92
PLT ($\times 10^9$/L)	107	127	93	51	194	151	189	166	105	106
WBC ($\times 10^9$/L)	11.44	15.4	3.39	1.84	7.13	4.0	6.44	10.92	5.15	3.01
MELD score	24.46	47.79	24.94	15.36	23.63	15.49	37.76	36.42	20.78	15.24

ALT alanine aminotransferase, *Cr* serum creatinine, *N.D.* not determined, *INR* international normalized ratio, *MELD* model for end-stage liver disease, *PLT* platelet count, *PT* prothrombin time, *PTA* prothrombin activity, *TBIL* total bilirubin, *T1* the first time point, *T2* the second time point, *WBC* white blood cell

296,548 and 51,320 unique CDR3 nucleotide clonotypes from CD4 and CD8 T cells were collected, respectively, encoding 27,769 and 4834 unique CDR3 amino acid (aa) clonotypes per sample, respectively. The sequence profiles of the TCR repertoires are shown in Table S1.

Vβ and Jβ gene segment usage

Overall, 63 Vβ and 13 Jβ gene segments were identified. Heatmaps showed that the usage pattern of Vβ or Jβ genes was heterogeneous in different samples (Figs. S2, S3). Though some Vβ gene segments such as T cell receptor beta variable (TRBV) 4-2, TRBV6-9 and TRBV7-5, TRBV10-1 showed an increased usage frequency in the second time point in CD4 and CD8 T cells, respectively, the changes are not consistently occurring in all individuals (Figs. S4, S5).

Dynamic changes of TCRβ repertoire diversity during HBV-ACLF

Because the TCR and peptide-MHC complex binding sites must be considered in the context of protein, we only investigated unique CDR3 aa clonotypes in this analysis. Differences in clonotype numbers were longitudinally assessed during ACLF. For CD8 T cells, the median number of clonotypes significantly decreased over time (5559 vs. 3687, $p = 0.043$; Fig. 1b). We used the NSDE, which is independent of the total number of T cells sequenced, to assess TCR repertoire diversity. In parallel, we observed reduced CD8 TCR repertoire diversity in all 5 patients (0.65 vs. 0.50, $p = 0.043$; Fig. 1d). For CD4 T cells, both the median number of clonotypes (24,891 vs. 26,029, $p = 0.686$; Fig. 1a) and NSDE values (0.77 vs 0.79, $p = 0.345$) (Fig. 1c) were comparable between both time points. These results imply that CD8 T cells had a significant clonal expansion but it is not seen for CD4. Interestingly, when we analyzed the results separately based on patient outcome, the three survivors showed decreases while the two non-survivors showed increases in clonotypes and NSDE value for CD4 T cells (Fig. 1a, c).

We then compared TCR clonal diversity between CD4 and CD8 T cells at the same time point. NSDE values were higher in CD4 than in CD8 T cells at clinical diagnosis (0.77 vs. 0.65, $p = 0.043$), but no difference was observed at the second time point (Fig. 1e, f). The number of unique CD4 clonotypes was at least 4.4 times higher than that for CD8 T cells at both time points (T1, 24,891 vs. 5559; T2, 26,029 vs. 3687). For comparison, our previous study [20] demonstrated that the number of unique clonotypes in CD4 was < 1.4 times that of CD8 T cells in treatment-naive CHB patients (22,232 vs. 16,422). These data suggest that a portion of CD8 T cells undergoes more dramatic oligoclonal expansion than CD4 T cells in HBV-ACLF patients.

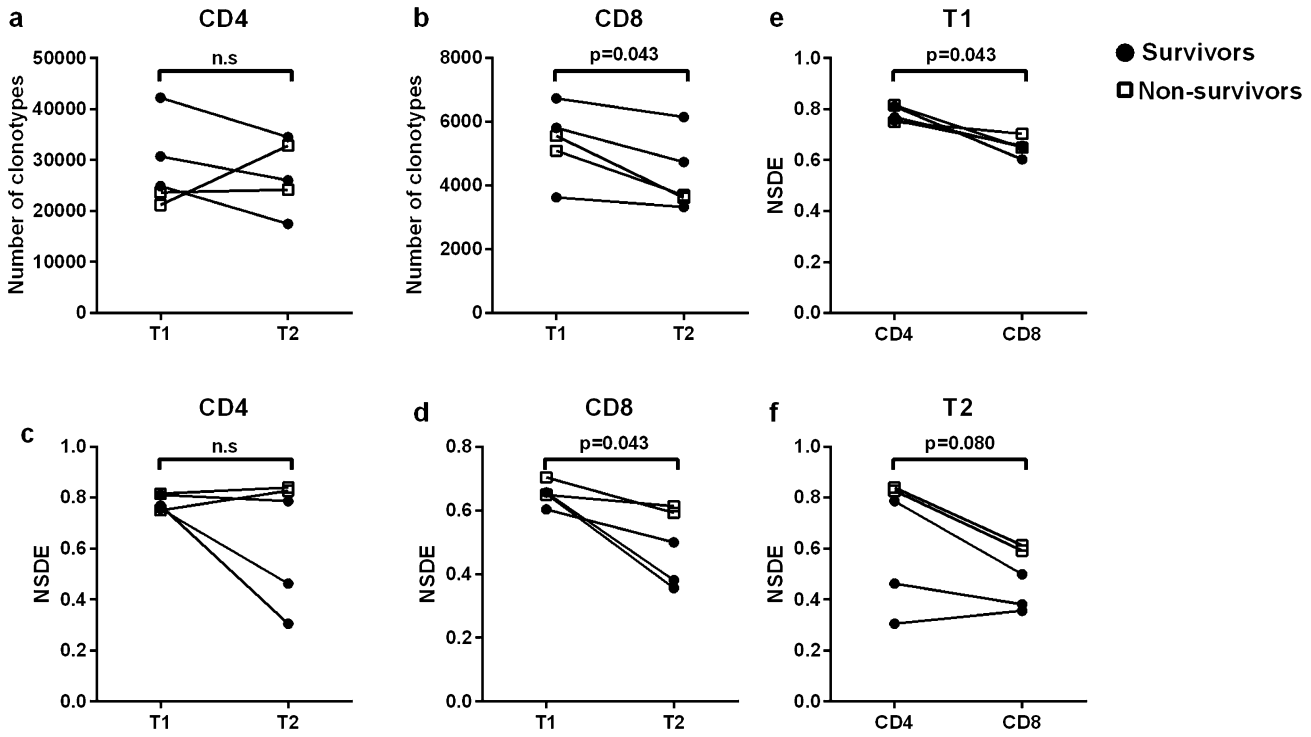

Fig. 1 Diversity of CD4 and CD8 T cell repertoires during HBV-ACLF. Longitudinal analysis of CDR3 aa clonotypes and normalized Shannon diversity entropy (NSDE) values for CD4 (**a**, **c**) and CD8 (**b**, **d**) T cells in patients with HBV-ACLF, respectively; (**e**, **f**) NSDE values of CD4 and CD8 TCR repertoires at each time point. *p* values less than 0.05 are shown. The *x* axis represents treatment time points. T1, first time point; T2, second time point

Tracking T cell clones in HBV-ACLF patients

To better understand the potential mechanism behind changes in TCR repertoire diversity, we examined how the circulating TCR repertoire was affected during ACLF. To measure the alterations in TCR sequence frequency over time, each TCR clonotype in T cell repertoire was tracked during the hospitalization and sorted into one of the three categories: increased ($FC \geq 2$), unchanged ($0.5 < FC < 2$), or decreased ($FC \leq 0.5$) according to the FC of its T cell clone frequency for time point 2 relative to time point 1 (details in Materials and Methods). For both CD4 and CD8 T cells, the percentage of unique increased clones was the lowest, followed by unchanged and decreased clones (Fig. 2a, c). The cumulative frequency of these three categories clones at the second time point showed a reverse trend (Fig. 2b, d).

We wondered whether the change profile was associated with patient outcome. The most obvious difference in the two patient groups was the cumulative frequency per percentage unit, compared to the percentage of unique clonotypes (Fig. 2a, d) and the cumulative frequency of T cell repertoires in both cell subsets (Fig. 2b, e). Survivors exhibited higher cumulative frequency per percentage unit of increased CD4 (Fig. 2c) and CD8 (Fig. 2f) clones than non-survivors, although there were no statistically significant differences due to the small sample sizes. However, less heterogeneity was observed in both unchanged and decreased clones between patients with different outcomes. These results indicate that the greater decrease of TCR repertoire diversity in survivors may be due to more intensive and robust clonal expansion during ACLF.

Highly expanded T cell clonotypes during HBV-ACLF

Next, we investigated the relative degree of clonal expansion. Here, we defined the highly expanded clones as the most 100 abundant clonotypes (top 100 clonotypes) (Tables S2, S3). The cumulative frequencies of the top 100 clonotypes increased in CD8 repertoires during treatment (56.56% vs. 76.22%, $p = 0.043$; Fig. 3b), suggesting that CD8 T cells underwent oligoclonality during hospitalization. Different tendencies of cumulative frequencies of the top 100 CD4 clonotypes were observed between survivors and non-survivors, increasing and decreasing, respectively. Overall, no significant differences were observed during disease progression (15.0% vs. 16.18%, $p = 0.50$; Fig. 3a). These results were in agreement with the diversity profiles of CD4 repertoires mentioned above. When comparing the cumulative frequencies of the top 100 clonotypes between CD4 and CD8 repertoires at the same time point, we also

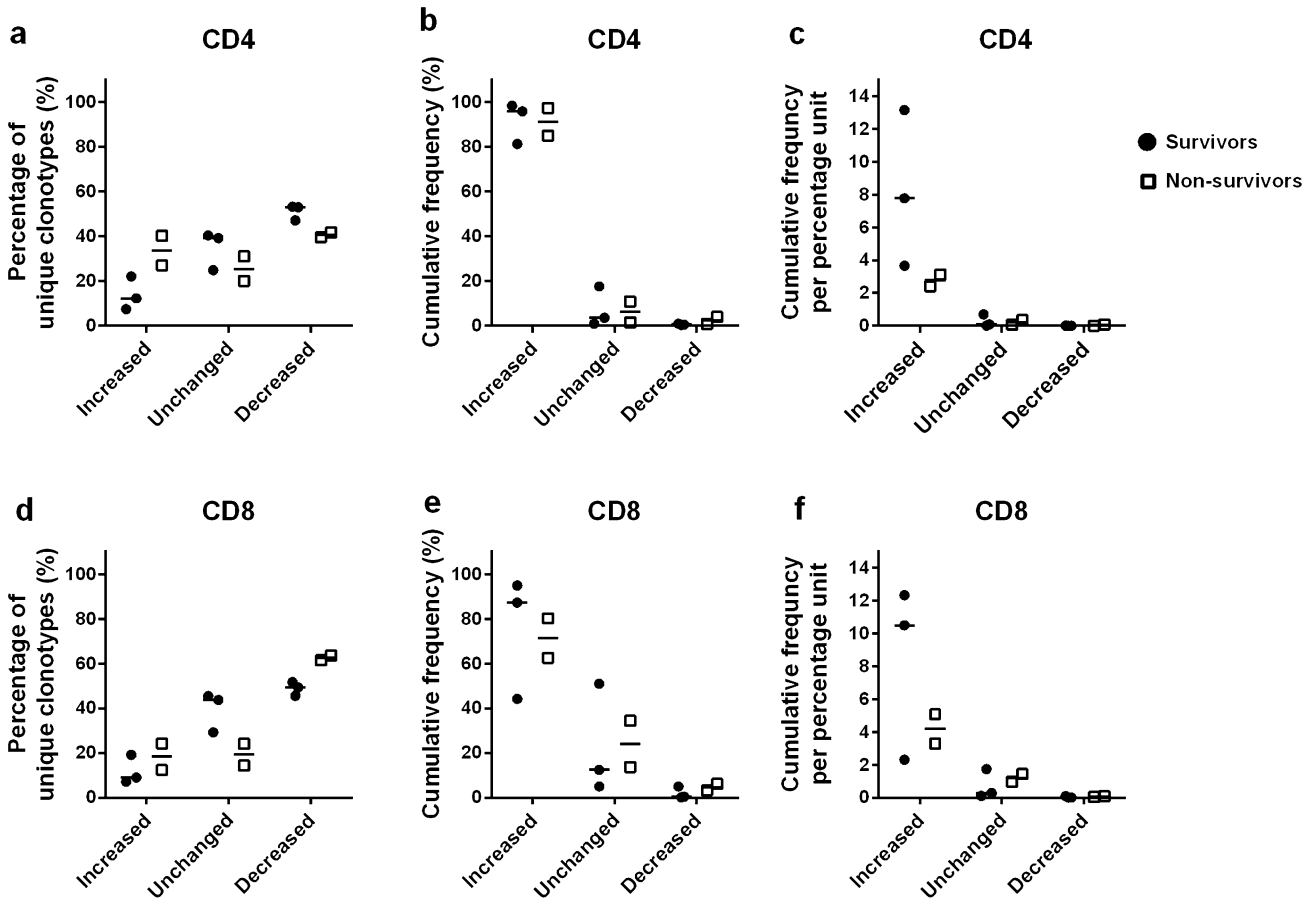

Fig. 2 Tracking T cell clones in ACLF patients with different outcomes. Dynamic change patterns of all detectable T cell clones were analyzed in each patient during ACLF. The FC in TCR sequence frequency at the second time point versus baseline was categorized as increased (FC ≥ 2), unchanged (0.5 < FC < 2), or decreased (FC ≤ 0.5). The percentage of unique clonotypes (**a** CD4 T cells; **d** CD8 T cells), the cumulative frequency (**b** CD4 T cells; **e** CD8 T cells), and the cumulative frequency per percentage unit (**c** CD4 T cells; **f** CD8 T cells) of each dynamic change category were plotted, respectively. The cumulative frequency per percentage unit was calculated as the cumulative frequency of the all clonotypes in each category divided by the percentage of unique clonotypes in same category

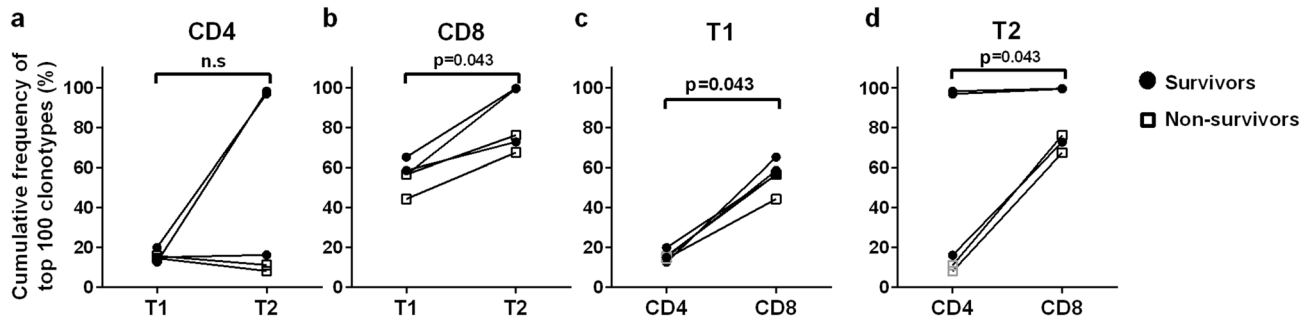

Fig. 3 Comparisons of highly expanded clonotypes. The change of the cumulative frequency of top 100 CD4 (**a**) and CD8 (**b**) T cell clonotypes during ACLF. **c**, **d** Comparison between the cumulative frequency of top 100 CD4 and CD8 T cell clonotypes at each time point

found that CD8 consistently showed a higher percentage of the clonally expanded population than CD4 T cells for both time points (T1, 56.56% vs. 15.0%, $p = 0.043$; T2, 76.22% vs. 16.18%, $p = 0.043$; Fig. 3c, d). Overall, the

degree of clonal expansion was higher for CD8 as compared to CD4.

The degree of clonal expansions in CD8 T cell repertoires was related to HBV-ACLF progression

Finally, we analyzed the correlation between MELD scores and overall repertoire diversity, as well as the proportion of highly expanded clonotypes in HBV-ACLF patients. The MELD scores were positively correlated with CD8 repertoire diversity ($r=0.90$, $p=0.037$; Fig. 4a) and negatively correlated with the cumulative frequencies of top 100 clonotypes ($r=-0.90$, $p=0.037$; Fig. 4b) at clinical diagnosis. However, there was no relationship between MELD score and CD4 T cell repertoire diversity at admission (Fig. 4d, e). Moreover, the change of diversity of CD8 repertoires during ACLF was positively correlated with the change of MELD values ($r=0.9$, $p=0.037$; Fig. 4c), but this tendency was not observed for CD4 T cells (Fig. 4f). These results indicate that HBV-ACLF patients who underwent greater clonal expansion of CD8 T cells in an early stage had better prognoses. This suggests that clonal expansions in CD8 T cell repertoires play an essential role in HBV-ACLF pathogenesis and may improve patient outcomes.

Discussion

Two of the key unmet needs in HBV-ACLF clinical management are predictive prognostic biomarkers and definite immunomodulatory strategies. T cell responses play critical roles in virus control and immune-mediated liver damage. Limited longitudinal studies have shown dynamic changes of T cell immunity in HBV-ACLF patients. Most of the studies investigated peripheral blood immune cell counts, phenotypes, or cytokines during disease progression rather than quantitatively assess the composition and perturbation of T cell repertoires. In this study, we longitudinally investigated the characteristics of peripheral CD4 and CD8 TCR repertoires in HBV-ACLF patients. To the best of our knowledge, this is the first NGS data on dynamic changes in TCR repertoires in HBV-ACLF patients based on massive CDR3 sequences.

Previous studies demonstrated cellular immunity overactivation in the early stage of ACLF, accompanied by

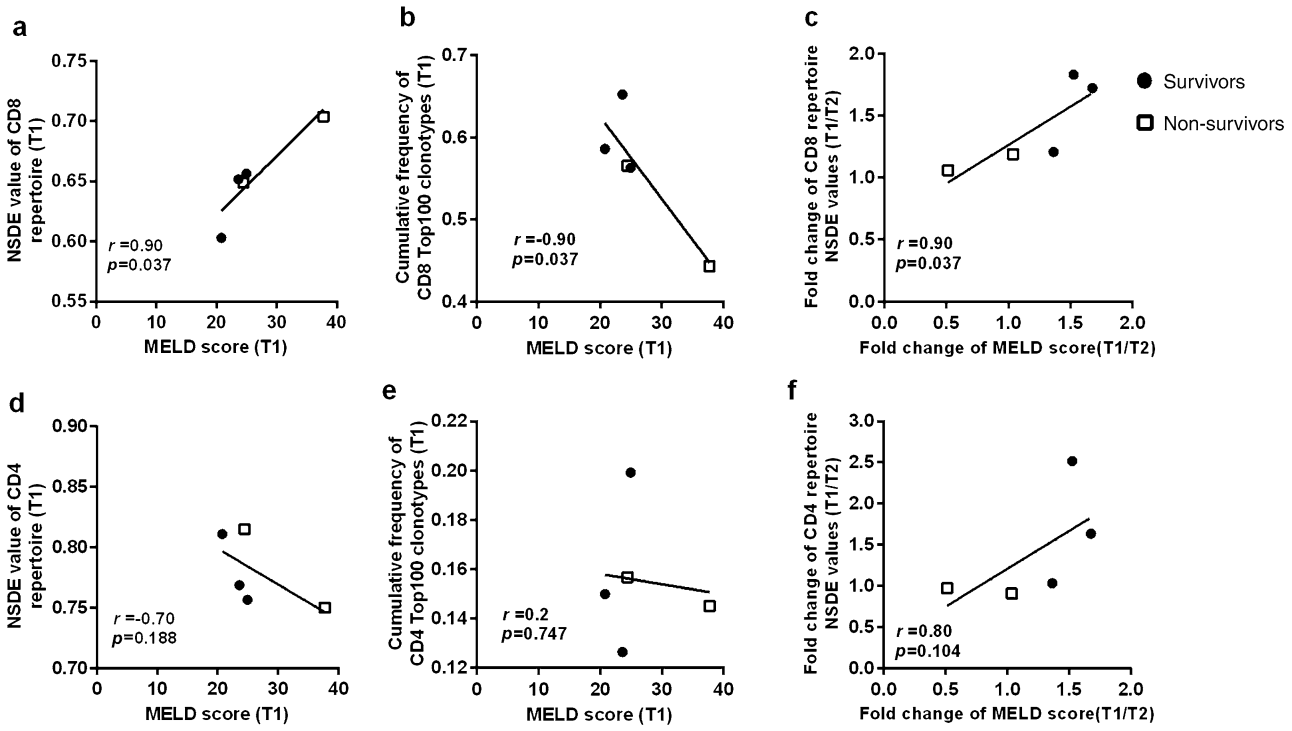

Fig. 4 Correlation between the clonality of T cell repertoires and the disease progression. The correlations were calculated between the NSDE values of T cell repertoire (**a** CD8; **d** CD4) or cumulative frequencies of top 100 clonotypes (**b** CD8; **e** CD4) and the MELD scores at the first time point. The correlations were also investigated between the fold change of NSDE values of T cell repertoire (**c** CD8; **f** CD4) and the fold change of MELD scores during the observation, calculating as the value of a given item at the first time point divided by that at second time point. The correlations were calculated by Spearman's rank order correlation coefficient for samples during ACLF in the five patients; *r*: correlation coefficient. *NSDE* Normalized Shannon diversity entropy

immune suppression in late stages. The progression of the disease is very fast within 1 month. T cell subsets, cell counts, and activation status have been investigated during HBV-ACLF, but the findings remain controversial. For example, some studies reported that the T cells response was depressed, while others observed over-activated cellular immunity in HBV-ACLF [8, 10]. The discrepancies may be attributable to the rapid changes in immune status, phenotypes, or cytokine expression levels in T cells, as well as the use of different T cells staining markers in HBV-ACLF patients. The TCR is unique to each T cell clone even when they undergo clonal expansion upon activation. Therefore, by analyzing TCR repertoires in HBV-ACLF patients, T cell composition and perturbation during disease progression can be quantitatively assessed without in vitro manipulation. Compared with CD4, TCR NGS sequencing showed significant oligoclonal expansion in CD8 T cells in patients with HBV-ACLF at admission and during hospitalization. In treatment-naive CHB patients in our previous study [20], we obtained similar cell counts and clean reads, and the number of unique clonotypes in CD4 was comparable to that in CD8 T cells. In this HBV-ACLF study, the number of unique CD4 clonotypes was at least 4.4 times higher than that in CD8 T cells. On the other hand, CD8 T cells showed greater clonal expansions compared to CD4 T cells at the same time point, as there were lower diversity and a higher proportion of the top100 clonotypes in CD8 repertoires relative to CD4 T cells. Furthermore, in the condition of the comparable cell counts of CD8 T cells between two time points, CD8 T cell repertoires diversity decreased while the proportions of top 100 CD8 clonotypes increased during hospitalization. Consistent with these results, two cross-sectional studies reported that the numbers of clonal expansion TCR Vβ families in $CD8^+$ T cells were significantly higher than those in $CD4^+$ T cells in patients with chronic severe hepatitis B, indicating a major role of $CD8^+$ T cells in disease pathogenesis [13, 24]. Another more recent study demonstrated the higher levels of Tc17 cells in HBV-ACLF than in CHB patients, and a positive correlation between Tc17 cell frequency and MELD score [10]. Our present study indicated the significant CD8 T cells clonal expansion but the negative correlation with MELD, suggesting some other CD8 T cells subsets or clonotypes expansion except for Tc17. Further studies are needed to determine the function and antigen specificity of these highly expanded CD8 T cells in HBV-ACLF.

CD4 T cells exhibited a stronger response than the CD8 population in CHB patients with nucleos(t)ide analog treatment [20]. CD8 T cells showed more significant clonal expansion in HBV-ACLF than in CHB patients, suggesting different immune responses in these diseases. Limited studies have focused on CD4 and CD8 TCR repertoires among different clinical types of chronic HBV infection. One study found no significant difference in skewed TCRBV expression rates in PBMCs between patients with CHB and chronic severe hepatitis B [25]. Another study demonstrated that ACLF patients showed fewer activated CD8 T cells compared to non-ACLF patients, implying exhaustion or suppression of lymphocytes, especially activated CD8 T cells [8]. Previous study has shown the equal reduction of $CD3^+$ T lymphocytes in patients with HBV-ACLF and septic shock, while the HBV-ACLF patients with or without sepsis did not significantly affect the immune status [26]. Whether patients with HBV-ACLF and septic shock show the similar or different TCR repertoire profile and diversity deserves further investigation.

Unlike CD8, CD4 T cells repertoire changes in longitudinal analysis were not evident among the entire group. The survivors showed a decrease, while the non-survivors showed a slight increase in clonotypes and CD4 repertoire diversity during hospitalization, suggesting clonal expansion in the survivors. However, we were unable to perform statistical analyses on TCR repertoires between survivors and non-survivors due to the small sample size. On the other hand, since it is difficult to obtain samples in ACLF patients, we only detected the repertoires of bulk CD4 T cells instead of different functional CD4 T cell subsets (e.g., Tregs). It was reported that the frequency of Tregs, which suppress the immune response, was significantly increased in HBV-ACLF patients as compared with healthy donors and CHB patients [6, 9]. Another investigation reported that the elevation was not due to the absolute count of $CD4^+$ Tregs ($CD4^+$ $CD25^{++}$ $FoxP3^+$) increase but rather a significant reduction in conventional $CD4^+$ T cells ($CD4^+CD25^{-/+}$) [26]. Two studies demonstrated that higher peripheral Tregs were associated with more severe liver diseases in ACLF patients, with non-survivors and survivors showing increasing and decreasing trends of Treg frequency, respectively [6, 9]. Whether the declines in clonotypes and diversity in survivors in the current study reflected recovery from hepatocytes injuries accompanied by down-regulation of the Tregs or migration of peripheral Tregs to the liver requires further investigation.

The very small sample size, as well as the detection of bulk CD4 and CD8 T cell repertoires, is the limitation of this study. Furthermore, this study is also limited by the only analysis of peripheral, rather than the comparison of intrahepatic T cells repertoire change. It would be better to collect blood and liver tissue samples of HBV-ACLF patients in prospective rather than cross-sectional studies. More patients, control groups (e.g., healthy volunteers, patients with CHB or liver cirrhosis), and specific cell subsets (rather than global CD4 or CD8 cells) should be assessed in future investigations.

Conclusion

In summary, we showed that HBV-ACLF patients display significant expansions in CD8 but no significant change in the CD4 T cell repertoires. These findings will improve our understanding of the pathogenesis of cellular immune dysfunction in HBV-ACLF and could be used to develop a predictive marker for disease outcome.

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Data availability The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics and Bioinformatics 2017) in BIG Data Center (Nucleic Acids Res 2019), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession number CRA002112 that is publicly accessible at https://bigd.big.ac.cn/gsa.

Compliance with ethical standards

Conflict of interest Guojun Shen, Shuilin Sun, Huang jie, Haohui Deng, Ying Xu, Zhanhui Wang, Xiong Tang, Xiaodong Gong declare that they have no conflict of interest.

Ethical approval The study was approved by the Ethics Committee of the Guangzhou Eight People's Hospital. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Informed consent Written informed consent was obtained from all patients for being included in the study.

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