Research Article

Patient-shared TCRβ-CDR3 clonotypes correlate with favorable prognosis in chronic hepatitis B

Qiong Jiang*1, Tingting Zhao*1, Wenhong Zheng2, Jijun Zhou3, Haoliang Wang4, Hui Dong1, Yongwen Chen1, Xiaoqin Tang1, Cong Liu1, Lilin Ye1, Qing Mao3, Chunlin Wang4, Jian Han3, Xiaoyun Shang1, Cong Liu1 and Yuzhang Wu1

1 Institute of Immunology, PLA, Third Military Medical University, Chongqing, P. R. China
2 Department of Health, Third Military Medical University, Chongqing, P. R. China
3 Institute of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing, P. R. China
4 Stanford Genome Technology Center, Stanford University, Palo Alto, CA, USA
5 HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA

The presence of shared T-cell clonotypes was found in several different diseases, but its relationship with the progression of disease remains unclear. By sequencing the complementary determining region 3 of T-cell receptor (TCR) β chains from the purified antigen-experienced CD8+ T cells, we characterized the T-cell repertoire in a prospective cohort study among 75 patients with chronic hepatitis B in China, as well as a healthy control and a validation cohort. We found that most T-cell clones from patients harbored the “patient-specific” TCR sequences. However, “patient-shared” TCR clonotypes were also widely found, which correlated with the favorable turnover of disease. Interestingly, the frequency of the “patient-shared” clonotypes can serve as a biomarker for favorable prognosis. Based on the clonotypes in those patients with favorable outcomes, we created a database including several clusters of protective anti-HBV CD8+ T-cell clonotypes that might be a reasonable target for therapeutic vaccine development or adoptive cell transfer therapy. These findings were validated in an additional independent cohort of patients. These results suggest that the “patient-shared” TCR clonotypes may serve as a valuable prognostic tool in the treatment of chronic hepatitis B and possibly other chronic viral diseases.

Keywords: Antigen specific T cells • Chronic viral infection • Hepatitis B virus infection • High throughput sequencing • T-cell repertoire

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

Correspondence: Dr. Xiaoyun Shang and Yuzhang Wu
email: shangxiaoyun@gmail.com; wuyuzhang@tmmu.edu.cn

*These authors contributed equally to this work.
infections can exert both immune protection and collateral tissue damage, possibly depending on their TCR and antigen recognition specificity [3, 4]. Thus, the selection and maintenance of protective CTLs are pivotal in anti-viral immunity [5–7]. Theoretically, a wide TCR diversity is essential to ensure that a certain number of CTLs carry the TCR specificities required for optimal pathogen recognition and clearance [8].

It is commonly known that hepatitis B virus (HBV) is not directly cytopathic but rather, the cytotoxicity of hepatocytes is triggered by the host cellular immune responses [9, 10]. The role of CD8+ T cells in viral clearance and collateral tissue damage during human viral infections, as revealed by conventional immunological methods, has been controversial. Investigations of viral infections, including infection with HIV [6], EBV [11, 12], HBV [9, 13], and HCV [7], have indicated that the strength of antigen-specific CTL response determines viral load, variation in outcome, and whether the immune response results in protection, damage or both [14]. In many cases, the existence of a diverse and robust antigen-specific CTL response anticipates the outcome of viral infection [8]. Nevertheless, it is unclear what exact feature of the CTLs repertoires determines the outcome of anti-viral immune responses in humans.

The configuration of the peripheral lymphocyte repertoire is critical for determining the robustness of an immune response against particular antigens [4, 15]. Each repertoire has millions of TCR sequences with enormous diversity, however, shared clonotypes were found in patients with the same disease in several tumor or chronic viral infections [16–19]. Specifically, TCR usage or featured TCR clonotypes exemplify a signature of tumor or chronic viral infectious diseases [16–19]. Each repertoire has millions of amino acids sequences obtained from patients’ samples was 20,743 (range 3741–95379) without any significant differences identified between this two groups. We found all 46 Vβ gene families among the total TCR repertoire, confirming that our approach covers the entire TCRβ repertoire (Supporting Information Fig. 3). Reproducibility between duplicate samples obtained at the same time point in the same person was high among this set of abundant T-cell clones, indicating that the method is reliable (Supporting Information Fig. 4). Further, there was no significant difference in CDR3 length between the TCRβ repertoire of CHB patients and healthy donors. Taken together, our TCRβ repertoire analysis is highly sensitive and reproducible.

Then, we determined the frequency of which each distinct TCRβ clonotype was present. We found that the top 10% most abundant unique clones accounted for 35–50% of the total reads. In the repertoire of patient HB075, the most frequent clonotype comprises 20.98% of the reads (Supporting Information Fig. 5A). In contrast, the most abundant clonotype in healthy donors comprises just 6.50% of the reads (Supporting Information Fig. 5B). According to the data, it suggested that the antigen reactive T cells existed during chronic HBV infection.

### Results

**Patients, sequencing output, and quality analysis**

The initial clinical features of the 75 patients in test cohort are shown in Table 1. Of the 75 patients, 40 developed HBeAg/HBeAb seroconversion. We applied next-generation sequencing techniques to sequence the TCRβ chains of sorted antigen-experienced CD8+ T cells (CD8+CD45RO–CD27+; CD8+CD45RO+) (Supporting Information Fig. 1). To compare the diversity among patients, a half million sorted cells of each sample were used in each sequencing library. Overall, from 75 enrolled patients in the test cohort and 80 healthy donors, we obtained a total of 199 and 218 million productive TCRβ sequence reads, respectively (Supporting Information Fig. 2A and B). The mean number of the unique CDR3 amino acids sequences obtained from patients’ samples was 20,743 (range 3741–95379) without any significant differences identified between this two groups. We found all 46 Vβ gene families among the total TCR repertoire, confirming that our approach covers the entire TCRβ repertoire (Supporting Information Fig. 3). Reproducibility between duplicate samples obtained at the same time point in the same person was high among this set of abundant T-cell clones, indicating that the method is reliable (Supporting Information Fig. 4). Further, there was no significant difference in CDR3 length between the TCRβ repertoire of CHB patients and healthy donors. Taken together, our TCRβ repertoire analysis is highly sensitive and reproducible.

| Table 1. Baseline characteristics and clinical outcomes of patients in CHB test cohort |
|------------------------------------------|--------------------------|
| Patients without HBeAg seroconversion | Patients with HBeAg seroconversion |
| (n = 35)                                | (n = 40)                 |
| Age at entry (years)                   | 33.3 ± 8.1               | 35.6 ± 8.4               | 0.2366 |
| Sex (male/female)                      | 29:6                     | 32:8                     |
| ALT at entry (U/L)                     | 131.5 ± 50.2             | 214.8 ± 239.3            | 0.0376 |
| ALT at week 76                         | 118.6 ± 186.2            | 43.2 ± 46.4              | 0.0251 |
| HBeAg at entry                         | 322.8 ± 598.3            | 692.7 ± 789.2            | 0.8179 |

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Characterization of patient-shared clonotypes by comparison among patients

To define CHB-enriched clonotypes in the whole CD8+ T-cell repertoire, we proposed three criteria based on our knowledge: (1) the CHB-enriched T cells should be among the most abundant clonotypes as a response to the virus in infected patients; (2) these same CHB-enriched T cells should not exist in individuals not infected by this virus; (3) the CHB-enriched T cells might be shared among patients with same virus infection. Based on these criteria, it would allow the identification of HBV-relevant clonotypes by overlapping the repertoire of CHB patients with that of non-HBV infected patients. When we compared the clonotypes in the repertoire of CHB patient-specific clonotypes in a CHB patient with any other non-HBV infected individuals, three groups of TCRβ clonotypes were defined, CHB+HC−, CHB+HC+, and CHB−HC+ (Fig. 1A). Theoretically, the HBV infection related clonotypes might be included in group CHB+HC−, which included

Figure 1. Classification of CHB patient clonotypes into four groups. (A) Diagrammatic sketch of the overlap between the CD8+ T-cell RNA repertoire of any one CHB patient (black) and one healthy control (red) analyzed using the iRepertoire multiplex primer set. (B) Representative image of the “leave-one-out” method used to analyze the similarity or dissimilarity of each repertoire with others. Every time one sample was taken out from the cohort and its repertoire was compared with all the repertoires of the kept samples pooled together. The process was repeated N times (N = sample numbers in the cohort), leaving out a different sample to use as the single test case each time. By following this method, each sample taken out could be divided into four parts and represented in A, B, C, and D. (C and D) The percentage of abundance (PA) of each group in patients (n = 75) and control (n = 80) was shown. The percentage of abundance refers to the ratio of the combination of the specific clonotypes abundance to the total reads of the sample. Data are from a single experiment representative of one experiment with 75 patients (C) and 80 healthy donor (D) samples per experiment. (E) The percentage of each group in patient and control cohorts. The data are shown as mean ± SD and each plot from a single clinical sample obtained from a particular time representative of 75 patients or 80 healthy controls’ clinical samples. Statistical significance between the two groups was measured by Mann–Whitney U test, with p < 0.05 considered significant. *p < 0.05; **p < 0.01; ****p < 0.0001.
nearly 97% of the clonotypes of his/her CTLs repertoire when compared any other repertoire of non-HBV individuals.

**Dissection of individual repertoire depending on whether the clones were shared with other patients**

To further characterize the CHB-enriched TCRβ-CDR3 clonotypes in each individual, we compared the pooled repertoires from all the 75 patients in the CHB test cohort (the reference repertoire pool) and that from 80 donors in the healthy control cohort (the control repertoire pool, Fig. 1B). By using the “leave-one-out” method we found that the individual antigen-experienced CD8+ T-cell repertoire of each CHB patient could be classified into four groups (Fig. 1B). CHB-enriched clonotypes were shared between two or more patients of the reference CHB test cohort and absent from the healthy control cohort. Clonotypes of group B were observed in at least two patients of the CHB test cohort and at least one individual of the healthy control cohort. Clonotypes of group C were shared between the control reference pool and at least one patient of the CHB test cohort but absent from the individual repertoire of interest. Clonotypes of group D were unique to a single patient from CHB test cohort or healthy control.

The percentage of abundance of clonotypes from group A (PA(A)), B (PA(B)), C (PA(C)), and D (PA(D)) refers to the total relative representation of clonotypes from each group in the repertoire (Fig. 1C and D). The PA(A) represented the quality and quantity of CHB-enriched clonotypes and enabled the classification of 100% of the samples of CHB patients from non-HBV individuals (Fig. 1E). These data indicated that whole-repertoire sequencing followed by overlap screening of TCRβ-CDR3 repertoires between HBV-infected patients and non-HBV-infected controls could identify CHB-enriched CD8+ T-cell clonotypes effectively, and these CHB-enriched T cells might serve as a valuable independent diagnostic indicator of HBV infection.

**The patient-shared clonotypes positively correlates with seroconversion rates**

Next, we investigated whether CHB-enriched CD8+ T-cell clonotypes contributed to the protective immunity of the host, by determining the relationship between clonotypes and HBeAg/HBeAb seroconversion in patients of the reference CHB cohort. The PA(A) in the full CD8+ T-cell repertoire showed a strong correlation with seroconversion status in patients of the CHB test cohort (Fig. 2A). In particular, by using the cut-off that yields the minimum misclassification error rate, the PA(A) can be used to stratify patients into groups with or without HBeAg/HBeAb seroconversion efficiently and accurately (Supporting Information Fig. 6). A negative

**Figure 2.** The PA(A) positively correlates with seroconversion rates. (A) CD8+ T-cell RNA repertoire of patients was analyzed using the iRepertoire multiplex primer set. The PA (A) in each repertoire of patients with HBeAg/HBeAb seroconversion (n = 40) or not (n = 35) in CHB test cohort. The red line represents the cut-off value that yielded the minimum SE to classify patients into two groups. (B) Correlation between PA(A) value of patients and HBeAg concentration of this patient at Week 76. The HBeAg level, assessed by chemiluminescent microparticle immunoassay (C) or DNA copies (assessed by COBAS TaqMan HBV assay (D) at week 76 in patients larger or smaller than the cut-off value. The data are from a single clinical sample obtained from a given time representative of one experiment with 75 clinical samples per experiment. HBeAg levels (E) and DNA copies (F) over time in patients with PA(A) larger (black) or smaller (gray) than the cut-off value. The data are shown as mean ± SD and from a single clinical sample obtained from a given time representative of one experiment with 75 patients’ clinical samples. Statistical significance was determined by Mann–Whitney U test (A, C, D) or linear regression analysis (B), with p < 0.05 considered significant. **** p < 0.0001.

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correlation was found between the PA(A) and HBeAg (Fig. 2B) or serum alanine aminotransferase (ALT) (Supporting Information Fig. 7A) levels at the end of the follow-up period. There was a significant difference of HBeAg levels between the patients with high or low PA(A) values identified at the end of follow-up (Fig. 2C). However, no significant difference was shown in the DNA copies at the same time (Fig. 2D). These findings suggested that the predominance of CHB-enriched clonotypes in a CHB patient can be used to predict HBeAg/HBeAb seroconversion.

In support of our seroconversion findings, we observed in a time-course analysis that the levels of HBeAg and viral DNA copies decreased in CHB patients with high PA(A) (Fig. 2E and F). Within the 76-week follow-up period, the serum viral DNA load decreased by more than four orders of magnitude in 70% of the patients whose PA(A) was more than 67%. In contrast, no changes were observed in the viral DNA loads in CHB patients with PA(A) under this cutoff value. At Week 76, the serum viral DNA load decreased to below 4 log10 in 60% of the patients with high PA(A) (Fig. 2F). Additionally, in patients with a high PA(A), the serum HBeAg decreased notably in the first few weeks, even before HBeAg/HBeAb seroconversion (Fig. 2E). Taken together, these data indicated that the PA(A) can be used to predict the specific protective immunity of the host and could be a valuable independent prognostic indicator of HBeAg/HBeAb seroconversion and viral clearance.

The clonotypes shared between patients and controls conversely correlated with seroconversion rates

Also, we investigated whether there is any correlation between clonotypes of group B and immune-mediated HBV control or liver damage. We examined the relationship between the PA(B) in an individual repertoire and the corresponding clinical information, such as HBeAg/HBeAb seroconversion (Fig. 3A), serum HBeAg concentration (Supporting Information Fig. 8A), serum DNA loads (Supporting Information Fig. 8B), and ALT concentration (Fig. 3B). In contrast to what was observed with CHB-enriched clonotypes, there was an inverse correlation between the PA(B) and the HBeAg/HBeAb seroconversion status in patients of the reference CHB cohort (Fig. 3A). Particularly, the use of the cut-off that yielded the misclassification error rate, the PA(B) allowed for the stratification of patients into groups with and without HBeAg/HBeAb seroconversion (Fig. 3A, Supporting Information Fig. 6).

Interestingly, there was a positive correlation between the PA(B) and ALT concentration at the end of the follow-up period (Fig. 3B). This is in contrast to the observation that the ALT concentration decreased in patients with high PA(A) during the same follow-up period. In a time-course analysis, we observed that patients with a high PA(B) have sustained or further increased ALT compared to patients with a low PA(B) (Fig. 3C). Thus, these findings indicated that clonotypes in group B might be responsible for liver damage, which was inverse with that in group A (Fig. 3D).

Hence, these data indicated that clonotypes in group B played a negative role in the viral control and chronic liver injury.

Character of CDR3 sequences between Group A and B

To further explore the feature of clonotypes in group A and B and their relationship with clinical outcome, clonotypes were compared between patients with high or low PA(A) values. CHB-enriched clonotypes were the preferential in vivo dominant clonotypes, with TCR Vβ chains belonging to the TCRβ V20, TCRβ V27, TCRβ V28, and TCRβ V6-3 families, while most TCR Vβ families used in Group B belonged to the TCRβ V27, TCRβ V6-3, TCRβ V28, and TCRβ V20 families (Fig. 4A). The CDR3 size distributions in both groups A and B demonstrated a Gaussian pattern similar to the results shown in the total repertoire, however, the average length of the CDR3 in CHB-enriched clonotypes was significantly larger than that in group B in CDR3 length of 13, 14, or 15 amino-acids (Fig. 4B). Shared clonotypes were defined by the presence of an identical TCR V-CDR3-J amino-acids sequence found in at least two unrelated individuals. Although none of the other patients presented a high frequency of the immunodominant clones, these clones were present at the fundamental level in patients with CHB among almost all cases. In CHB-enriched clonotypes, the most
Figure 4. Summary of TCRβ sequence features in group A and B. (A) Vβ gene usage of TCRβ CDR3 amino acids sequences observed in groups A and B was calculated in MiXCR software. The data and statistics shown are pooled from all TCRβ clonotypes in CHB-enriched (Group A, black) or CHB-HC shared population (Group B, gray) in 75 CHB patients. (B) CDR3β length variation in clonotypes of group A (red) and B (blue) was analyzed and counted in MiXCR software. The data are shown as mean + SD and from pooled TCRβ clonotypes in CHB-enriched (A, red) or CHB-HC shared population (B, blue) in 75 CHB patients. Statistical significance was determined by Mann–Whitney U test in each size, with p < 0.05 considered significant (**** p < 0.0001). (C and D) Cluster analysis of the top 200 most abundant clonotypes in group A (C) and those in group B (D). Hierarchical cluster analysis was performed based on multiple sequence alignment of these selected amino acid sequences. The six clustered group in CHB-enriched clonotypes were labeled with A1–A6. (E) Percentage of (in vitro expanded) antigen-specific T cells (assessed by flow cytometry), in response to different peptides of the HBV peptide library, or other peptide. The data are shown as mean + SD and from a single clinical sample obtained from a particular time representative of one experiment with five patients' clinical samples. (F) Antiviral responses were measured in the other three patients using a panel of four independent pentamers by flow cytometry. Each plot represented a pentamer in a different patient. The data are shown as mean + SD and from a single clinical sample obtained from a particular time representative of one experiment with three patients’ clinical samples. (G) Rank of the most abundant clones of each tetramer in the CD8 population. Median rank is 16th (range 1st–118th). The data are shown as mean value and from a single clinical sample of patient HB143 obtained from a particular time representative of one experiment.
common shared clonotype was shared by 23 patients of the test cohort, while the most common shared clonotype in group B was shared by all individuals of the healthy control cohort (Supporting Information Fig. 9A and C). Nearly 8% of the CHB-enriched clonotypes were found at more than ten individuals, while more than half clonotypes were found only twice in these patients approximately. We performed hierarchical cluster analyses of 200 top abundance clonotypes and identified five major motifs shared by TCR derived from patient cohort individuals (Fig. 4C and D). A representative cluster of identified CHB-enriched clonotypes was shown in Supporting Information Fig. 10A and conserved CDR3 motif analysis was shown in Supporting Information Fig. 10B. The expression level of shared clonotypes in CHB-enriched clonotypes varied in different individuals, which range from 0.01% to 20% (Supporting Information Fig. 9B), while the expression level of most common shared clonotypes in group B below 0.1% (Supporting Information Fig. 9D). Together, our data showed evidence for a high level of character similarity of patient-shared CD8+ T cells among CHB patients.

The clones against HBV identified by epitope-specific pentamers

To facilitate the test of the hypothesis that CHB-enriched T cells are abundant and unique to the disease, we generated HBV peptide specific T-cell lines and measured the response against HBV using four different peptide-pentamer complexes: HLA-A0201-FLLSLGHIL (HBp573, derived from the HBV polymerase protein 573–581), HLA-A0201-FLLTRILT1 (HBe183, derived from the HBV envelope protein 183–191), HLA-A0201-FLLPSDFPPSV (HBc18, derived from the HBV core antigen protein 18–27), and HLA-A1101-YVNVNMGLK (Hbc88, derived from the HBV core antigen protein 88–96). A combination of FACS and TCR8 cloning was used to quantitatively characterize epitope-specific antiviral responses (Fig. 4E and F). The clones were defined on the basis of their distinct TCR8-sequence within the analyzed pentamer-sorted population. By using this method, we could detect the antivirus-specific clones in the entire CD8+ T-cell repertoire. It was observed that lots of the HBV-specific clones could be identified among CHB-enriched clonotypes, considered as the most abundant clones in the CD8+ T-cell repertoire. In patient HB088, the 2nd most abundant clones targeted against the HBc18 peptide, whereas the 11th and 13th highest-frequent clones recognized the HBe183 peptide. These findings were confirmed in other patients, like HB143, where the 4th clone within the CD8+ T-cell repertoire was directed against the HBe183 pentamer, as the same as the other six clones within the 50 most abundant clones in group A (Fig. 4G). Overall, a median of 56% (range 25–100%) of the pentamer responsive clonotypes could be ascertained among the 100 most abundant clones in group A. According to our data, it was able to utilize the NGS-approach to identify the individual antiviral T-cell expansions in the CD8+ T-cell repertoire. Thus, these CHB-enriched CD8+ T cells were validated to be the antigen reactive clones, and their abundance was related to a favorable prognosis.

Validation on another cohort

Another independent cohort included 60 CHB patients from another hospital in China to confirm the data obtained from the first series. Patients of the validation CHB cohort were recruited while at the immune active phase. These patients were also followed for 76 weeks. The CHB enriched TCRβ-CDR3 sequences in each patient in the CHB validation cohort were analyzed using the same method as shown in Fig. 1B. The repertoire of each patient in the CHB validation cohort could also be classified into four groups (Fig. 5A). The ratio of the number of unique reads versus the abundance of this set of clonotypes to clonotypes in a single individual reflects the clonotypic expansion status of each of the clonotype groups, and CHB-enriched clonotypes had the highest expansion rates in CHB patients compared to the healthy control cohort (Fig. 5B). The PA(A) represented the quality and quantity of HBV-specific CTLs response and enabled the classification of 100% of the samples from patients in the CHB validation cohort from non-HBV individuals (Fig. 5C). The PA(A) (Fig. 5D) and PA(B) in the full CD8+ T-cell repertoire showed a strong correlation with seroconversion status in patients in the CHB validation cohort. Based on a time-course analysis, we found a negative correlation between the PA(A) and HBeAg viral DNA levels during the follow-up period (Fig. 5E). Further, we observed that patients with a high PA(B) have sustained or further increased ALT compared with patients with a low PA(B). Analysis of this cohort validated the first data series.

Discussion

By comprehensively analyzing the TCRβ repertoire profile of 40 controllers, compared to those of 35 progressors, we obtained compelling evidence that effective immune control in CHB is mediated by some characterized protective T-cell clonotypes, providing a rational target for CD8+ T-cell based vaccine design or adoptive transfer therapy. In this study, a significantly higher proportion of CHB-enriched CTLs was archived in patients with a condition of the developing spontaneous HBeAg seroconversion during the long-term follow-up. According to the data, it suggested that CHB-enriched CTLs (clonotypes in group A shown in Fig. 1B) might represent the protective immunity against HBV and contribute to beneficial long-term outcomes probably. The dominant protective immunity had also been shown to correlate with the decreased serum ALT levels. In this regard, the presence of a dominant protective immunity indicates the effective host immune control over HBV replication. The set of identified protective clonotypes covered multiple TCRβ families and were characterized by longer CDR3 regions and more frequently in individuals. Since a number of shared TCR common to patients of nearly half cohort have been characterized, it would be important to identify the
The PA(A) can predict clinical outcome in CHB patients in validation cohort. (A) CD8⁺ T-cell RNA repertoire of patients in the CHB validation cohort was analyzed using the iRepertoire multiplex primer set, and the percentage of each group was calculated using "leave-one-out" method as described in Fig. 1. As mentioned above, the percentage of abundance refers to the ratio of the combination of the specific clonotypes abundance to the total reads of the sample. The data are from a single clinical sample obtained from a particular time representative of one experiment validated with samples of 60 cohort patients per experiment. (B) The expansion rates of each group in all patients in the test cohort. (C) The PA(A) in each repertoire of patients in the CHB validation cohort (n = 60) and that of the healthy control cohort (n = 80). The data are shown as mean ± SD and each plot from a single clinical sample obtained from a particular time representative of one experiment validated with samples of 60 cohort patients or 80 healthy donors. (D) The PA(A) in each repertoire of patients with HBeAg/HBeAb seroconversion (n = 29) or not (n = 31) in the CHB validation cohort. Each point represents the PA(A) value of a single patient. The red line represents the cut-off value that yielded the minimum SE to classify patients into the two groups. The data are shown as mean ± SD and each plot from a single clinical sample obtained from a particular time representative of one experiment validated with clinical samples of 60 cohort patients. (E) Correlation between PA(A) and HBeAg concentrations. Statistical significance was determined by Mann–Whitney U test (C, D) or linear regression analysis (E), with \( p < 0.05 \) considered significant. The data are from a single clinical sample obtained from a particular time representative of one experiment validated with samples of 60 cohort patients per experiment.

corresponding antigen that might be broadly used as immunotherapeutic targets [26]. These observations argued for the inclusion of specific protective clonotypes in immunotherapy, rather than the whole T-cell repertoire transfer therapy that may result in further immune damage, and suggested that a hallmark of effective immune control is the presence of a dominant protective immune response. These findings provide the method to identify protective immune response in the whole T-cell repertoire.

The improvement of high-throughput sequencing promotes the development of a novel and potent technology for the investigation of the adaptive immune system [13, 16]. In this study, we devised a disease cohort and case-control study by using new tools and methods, based on high-throughput TCRβ repertoire sequencing. Our analysis yielded hundreds of thousands of sequence that reads with tens of thousands of unique sequences. Our data showed that the CHB-enriched CTLs could be identified by comparing the repertoire of the CHB patient cohort to that of the healthy control cohort. The identified clonotypes in group A represented CHB-enriched CTLs responses and enabled the group classification of 100% of the samples of CHB patients in both the test and validation cohorts. These data also suggested that TCRβ repertoire profiling has value for infectious disease diagnosis.

CTLs have been heavily involved with both the mediators of protection and the principal effectors of the liver damage [1, 2]. In this study, CHB-enriched clonotypes were identified in each patient with CHB and might relate to HBeAg seroconversion. Further, these CHB-enriched clones were validated by antigen-specific tools. These data indicated that whole-repertoire sequencing followed by overlapping the repertoire of HBV-infected patients with that of non-HBV-infected controls is an effective method to define the antigen-specific T-cell response at a whole-repertoire level. Given HBV-specific T cells measured by HLA-A2 restricted epitopes and pentamers, the difference in the number of HBV-specific T cells in the liver was not significant between individuals with normal or raised ALT. Compared with the patients with normal ALT, the number of non-HBV-specific CD8⁺ T cells was increased in individuals with elevated ALT [27]. In the previous study, it was implicated that bystander immune response has been embroiled in the clinical manifestations of several chronic liver diseases [28]. Consistently, we found that dominant non-HBV specific immunity correlated with unfavorable clinical outcomes of CHB. In clinical practice, the use of prolonged antiviral agents is correlated with significant cost and risk of antiviral resistance [25]. Our findings might be beneficial to physicians’ decision making on whether
to keep patients under clinical observations for the avoidance of the unnecessary costs and risks of intervention, or alternatively to implement anti-virus therapy to prevent the repetitive immune activity and liver damage.

In conclusion, we focused on furthering the understanding and exploitation of the specificity of T cell-mediated immune response in this study, to predict or manipulate the results of immunological diseases or vaccine responses. The technique presented here is getting ready to enable the comprehensive analyses of T-cell responses finally. Our data indicated that classification of patients into positive and negative outcome subgroups based on the TCRβ repertoire prognosis profile might serve as a useful approach of guiding treatment strategies selection in patients with CHB. This approach should also reduce the rate of mistreatment (both over- and undertreatment) by enhancing the selection of patients who would benefit from the immune-related treatment.

Materials and methods

Patients and study design

A test cohort of 75 patients with HBeAg-positive CHB in total was followed in the Department of Infectious Diseases, Southwest Hospital, China, from 2010 through 2013 after obtaining their informed consent. All patients were diagnosed with CHB according to the criteria defined in “2010 Hepatitis B: diagnosis and treatment” [29]. Additional criteria used for the recruitment of patients included an initial serum HBV-DNA concentration higher than 10⁵ copies/ml, testing positive for HBeAg, testing negative for hepatitis B s antibody (HBsAb), serum alanine aminotransferase (ALT) levels at 2–10 times the normal upper limit, and testing positive for HLA-A2. The exclusion criteria were coinfection with HCV or HIV-1, serological markers suggestive of autoimmune disease, daily intake of alcohol >50 g, or recent exposure to hepatotoxic drugs. Patients that had ever received anti-HBV therapy (PEGylated alfa-2a or lamivudine) were also excluded. After enrolled in this study, all patients were given an anti-HBV therapy or immunotherapy approved by the Ethics Board of the hospital, which is a peptide-based therapeutic vaccine against HBV (ClinicalTrials.gov Identifier: NCT00869778). They were followed every 3 months, or more frequently if clinically indicated, and their serum samples were monitored for liver biochemistry and serologic markers of HBV infection, including HBsAg, HBeAg, anti-HBe, HBV DNA, and HBeAg. Serum samples were stored at –20°C until use. Whole heparin anti-coagulated blood samples were obtained by venipuncture from patients. Another independent validation cohort of 60 CHB patients with the same inclusion and exclusion criteria were followed in an additional independent hospital (XiangYa Hospital, Central South University) from 2010 through 2013, and all these patients were given an immunotherapy approved by the Ethics Board of the hospital or anti-HBV therapy such as nucleotide analogs or PEGylated IFN-α. The control cohort of 80 healthy people tested negative for HBsAg, HBeAg, HBeAb, HBsAb, and HBeAg (No hepatitis B vaccinations in the past 10 years). Patient information and study design are summarized in the supplemental data. The protocols involved in this study were approved and monitored by the Southwest Hospital Research Ethics Board, and all patients signed informed consent forms by the Declaration of Helsinki.

Cell preparation and flow cytometry

PBMCs were obtained by using the Ficoll Paque density gradient centrifugation and frozen until use. For phenotypic analysis or cell subset sorting, the following anti-human monoclonal antibodies were used: CD3 APC-eFluor® 780 (SK7, ebioscience), CD4 PerCP-Cyanine5.5 (OKT4, ebioscience), CD8 PE/Cy7 (HIT8a, Bilegend), CD27 PE (O323, Bilegend), CD45RO FITC (UCHL1, ebioscience), and HBV relevant pentamer (ProImmune, Oxford, UK). Frozen PBMCs were thawed with RPMI-1640 containing 10% FBS, 1% Pen/Strep, 2 mM Glutamine and 25 U/ml Benzonase, then washed with FACS Buffer (HBSS containing 0.5% BSA, 5 mM MgCl2, 30 μg/mL DNase) and stained with the monoclonal antibody mixture for 30 minutes at 4°C. Cells were then washed and suspended in FACS Buffer for immediate acquisition. Samples were analyzed and sorted using a FACS Aria I (BD, Bioscience). Flow cytometry data were analyzed with FlowJo (Tree Star). As shown in Supporting Information Fig. 1, the sort purity was above 96% for all the samples. Sorted cells were immediately lysed and homogenized with Buffer RL1 (Qiagen), then kept at ~80°C until future use. Full gating strategy of antigen-experienced CD8 lymphocytes was shown in Supporting Information Fig. 1.

TCR repertoire library construction and sequences analysis

Total RNA was extracted by using AllPrep DNA/RNA Micro Kit (Qiagen), and cDNA was constructed using a Qiagen Onestep RT PCR kit. The CDR3 region of TCRβ chain was amplified by using the iRepertoire multiplex primer set (iRepertoire, Inc.) as described the previous study [30]. A set of nested primers specific for various V and C elements of the TCRβ was involved in the first amplification cycles, followed by an amplification using commumal primers based on the manufacturer’s protocol of TCRβ sequencing (iRepertoire Inc.). Agilent Bioanalyzer traces were used to determine the library molarity and quality prior to loading of the flow cell. Purified PCR products were sequenced using an Illumina 2500 (Illumina Inc.). Raw DNA sequences were first analyzed to identify V and J genes by using an edited version of MiXCR [31]. MiXCR efficiently considers sequence quality, corrects PCR errors and identifies germline hypermutations. Sequences were screened for alignment using the “align” command. The “assemble” command was then used to identify clones from the pool of aligned sequences and to assign each clone to a particular V or J segment in the second round of alignment. Multiple alignments and
hierarchical clustering of conserved amino acid sequences were analyzed as the previous study [30].

Serological assays of HBeAg, HbeAb, ALT, and HBV-DNA

A commercial automated biochemical analyzer (Olympus AU640, Olympus, Japan) was used for measuring ALT. A chemiluminescent microparticle immunoassay (Abbott i2000, AltaVista, VA, USA) was applied to identify HBeAg and anti-HBc. HBV DNA was detected via the COBAS TaqMan HBV assay (CAP-CTM; Roche Molecular Systems, Inc., Branchburg, NJ, USA), as per the manufacturer’s protocol. An Applied Biosystems Inc. (Carlsbad, CA) real-time PCR 7500 system was used. Screening for the positivity of HLA-A2 was performed by staining PBMC with an HLA-A0201 antibody (BD, San Diego, CA, USA) and analyzed by flow cytometry.

Generation of HBV peptide library-specific T-cell lines

Generation of T-cell lines from the HBV peptide library was described as mentioned before [27]. All T-cell lines were generated in the same way: heparinized, fresh venous blood was obtained from the patients. Ninety-six microcultures were set up with 2500 T cells, isolated by rosetting, autologous PBMC and HBV peptides were added in 200 μl complete medium as follows: HLA-A0201-FLISLGIIHL (HBp18, derived from the HBV core antigen protein 18–27), HLA-A0201- FLTRILTI (HBe183, derived from the HBV envelope protein 183–191), HLA-A0201-FLLSLGIHL (HBp573, derived from the HBV polymerase protein 573–581), HLA-A0201-FLLTRILTI (HBe183, derived from the HBV envelope protein 183–191), HLA-A0201- FLPDFPSV (Hbc18, derived from the HBV core antigen protein 18–27), HLA-A1101-YVNVMGLK (Hbc88, derived from the HBV core antigen protein 88–96). The rIL-2 (human, 5 U/ml; Eurocetus, Leiden, The Netherlands) was added three times per week to each culture after 5 days. The cultures were restimulated with antigen and autologous PBMC and HBV peptides were added in 200 μl complete medium as follows: HLA-A0201-FLISLGIIHL (HBp573, derived from the HBV polymerase protein 573–581), HLA-A0201- FLTRILTI (HBe183, derived from the HBV envelope protein 183–191), HLA-A0201- FLPDFPSV (Hbc18, derived from the HBV core antigen protein 18–27), HLA-A1101-YVNVMGLK (Hbc88, derived from the HBV core antigen protein 88–96). The rIL-2 (human, 5 U/ml; Eurocetus, Leiden, The Netherlands) was added three times per week to each culture after 5 days. The cultures were restimulated with antigen and autologous in every two weeks, irradiated PBMC as APC after 8 weeks, the specificity of the viable T-cell cultures was analyzed by HBV relevant pentamers (ProImmune, Oxford, UK). DNA of pentamer positive T cells was used as templates for detecting TCRβ cloning via PCR product sequencing (Sangon Biotech, Shanghai, China).

Statistical analysis

All data were analyzed via GraphPad Prism 6.0 software (San Diego, CA, USA). Statistical differences between groups were identified by using the nonparametric Mann–Whitney U test. Data from the same individuals were compared via the Wilcoxon matched pairs test. Correlations between variables were assessed by linear regression analysis. For all tests, a p-value of less than 0.05 was considered to be a significant difference. The similarity or dissimilarity of each repertoire with others was analyzed by using the “leave-one-out” method. This method works as follows, every time we take out a sample from the cohort, and pooled all the repertoire of the kept samples together, and compare the repertoire of left-out samples with the pooled repertoire. Now, repeat the process N times (N = sample numbers in the cohort), leaving out a different sample to use as the single test case each time. All calculations were performed using version 2.8.1 of the statistical programming language R.

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Data and materials availability: The sequence data published in this paper can be found in the Sequence Read Archive, accession no. SRA178440.

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Immunity to infection


Abbreviations: ALT: alanine aminotransferase · CHB: chronic hepatitis B · HBV: hepatitis B virus · HBeAg: hepatitis B virus e antigen · HBeAb: hepatitis B virus e antibody · PA: percentage of abundance

Full correspondence: Dr. Xiaoyun Shang, Institute of Immunology, PLA, Third Military Medical University, Chongqing 400038, P. R. China. Fax:+862368752230 e-mail: shangxiaoyun@gmail.com

Additional correspondence: Yuzhang Wu, Institute of Immunology, PLA, Third Military Medical University, Chongqing 400038, P. R. China. Fax:+862368752230 e-mail: wuyuzhang@tmmu.edu.cn

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