

Next Generation Sequencing-Based Identification of T-Cell Receptors for Immunotherapy Against Hepatocellular Carcinoma

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Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) remains a global health concern, and HBV proteins may be ideal targets for T cell-based immunotherapy for HCC. There is a need for fast and efficient identification of HBV-specific T cell receptors (TCRs) for the development of TCR-transduced T (TCR-T) cell-based immunotherapy. Two widely employed TCR identification approaches, T cell clonal expansion and single-cell sequencing, involve a TCR singularization process for the direct identification of V α and V β pairs of TCR chains. Clonal expansion of T cells is well known to have tedious time and effort requirements due to the use of T cell cultures, whereas single-cell sequencing is limited by the requirements of cell sorting and the preparation of a single-cell immune-transcriptome library as well as the massive cost of the whole procedure. Here, we present a next-generation sequencing (NGS)-based HBV-specific TCR identification that does not require the TCR singularization process. **Conclusion:** Two pairing strategies, ranking-based strategy and α - β chain mixture-based strategy, have proved to be useful for NGS-based TCR identification, particularly for polyclonal T cells purified by a peptide-major histocompatibility complex (pMHC) multimer-based approach. Functional evaluation confirmed the specificity and avidity of two identified HBV-specific TCRs, which may potentially be used to produce TCR-T cells to treat patients with HBV-related HCC. (*Hepatology Communications* 2021;5:1106-1119).

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and causes more than 700,000 deaths annually.⁽¹⁾ At least 50% of HCC is hepatitis B virus (HBV)-related disease.⁽²⁾ HBV infection, cirrhosis, and hepatoma are typical characteristics of HBV-related HCC, especially in Asia.⁽³⁾ Currently, the treatments for HCC mainly include surgery, antiviral drugs, and targeted therapies. The response rate is low, and the

overall efficacy is still limited.^(4,5) Immunotherapy has increasingly become a trend in tumor treatment because of its proven efficacy and clear targeting. T cell receptor (TCR)-transduced T (TCR-T) cell therapy has shown promising efficacy in the clinical treatment of malignant cancers, such as the treatment of metastatic synovial cell sarcoma, melanoma, and non-small cell lung cancer by the TCR-T cell targeting the cancer germline antigen New York esophageal

Abbreviations: ALT, alanine aminotransferase; CD, cluster of differentiation; CDR3, complementarity determining region 3; DMSO, dimethyl sulfoxide; EC50, 50% effective concentration; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immune absorbent spot; FCM, flow cytometry; Hbc, hepatitis B virus core protein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; IFN- γ , interferon-gamma; IL, interleukin; MHC, major histocompatibility complex; mTRBC, mouse T cell receptor beta chain constant region; NGS, next-generation sequencing; PBMC, peripheral blood mononuclear cell; REP, rapid expansion protocol; TCR, T cell receptor; TCR-T, T cell receptor-transduced T; TRAV, T cell receptor alpha chain variable region; TRBV, T cell receptor beta chain variable region.

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squamous cell carcinoma 1 (NY-ESO-1) as well as the treatment of human papillomavirus (HPV)-positive epithelial cancers by a TCR-T cell targeting HPV-derived antigen.^(6,7) HBV antigens, which are expressed in cancer cells of HBV-related HCC and HBV-infected hepatocytes but not in uninfected liver cells and other organ tissues, may be ideal targets for T cell-based immunotherapy.^(8,9)

Currently, the populations covered by reported HBV-specific TCRs are still limited, and there is a need for fast identification of HBV-specific TCRs. Once polyclonal-reactive T cells have been isolated by a peptide-major histocompatibility complex (pMHC) multimer-based approach, limiting dilution is commonly employed to obtain T cell clones for subsequent TCR sequencing.⁽¹⁰⁾ This procedure can reduce the complexity of obtained antigen-specific TCRs and is essentially a TCR singularization process that simplifies the pairing of V α and V β chains. However, this strategy is also known to have tedious requirements of time and effort due to the use of T cell cultures and cloning.⁽¹¹⁾ Recently, great advances have been made in the field of single-cell sequencing, which has proven to be efficient in TCR identification by sorting and directly cloning single V α -V β pairs from a T cell.^(11,12) However, single-cell sequencing is currently limited by the requirements of cell sorting and the preparation of a single-cell immune-transcriptome library as well as the massive cost of the whole procedure.

Bulk sequencing approaches, such as next-generation sequencing (NGS), have been widely employed for

TCR repertoire studies, but they are not considered to be applicable to the identification of TCR pairs.⁽¹³⁾ Although TCR β chains are normally unique for a T cell, the major concern with NGS is that approximately 30% of T cells in peripheral blood mononuclear cells (PBMCs) have the potential to produce two distinct TCR α chain messenger RNAs (mRNAs) because of a different allelic exclusion mechanism that influences the frequency and ranking of TCR α chains in NGS.⁽¹⁴⁾ Despite such limitations, NGS-based pairing has been successfully employed for the identification of neoantigen-targeted TCRs.⁽¹⁵⁾ Furthermore, the peptide-MHC multimer-based purification process can greatly reduce the complexity of the obtained TCR chains in NGS, which makes an α - β chain mixture-based pairing strategy possible. Here, we present a study of NGS-based fast identification of HBV-specific TCRs that may potentially be used to produce TCR-T cells to treat patients with HBV-related HCC.

Materials and Methods

T CELL STIMULATION, ISOLATION, AND RAPID EXPANSION

PBMCs from human leukocyte antigen A (HLA-A)*02-positive (+) donors with chronic HBV infection were isolated through a standard Ficoll gradient.

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Informed consent in writing was obtained from each patient. PBMCs were stimulated by 10 μ M of HBV core₁₈₋₂₇₁ peptide (FLPSDFFPSI; Sangon Biotech, Shanghai, China) for 10 days in X-VIVO 15 (#04-418QCN; Lonza) plus 10% human AB serum, 1% GlutMAX supplement (#35050061; Gibco), and penicillin-streptomycin (#15140122; Gibco). We added 50IU/mL interleukin (IL)-2 (#200-02; PEPROTECH), 10 ng/mL IL-7 (#200-07, PEPROTECH), and IL-15 (#C016; Novoprotein) on day 1 and changed this solution every 2 days. T cells stained with R-phycoerythrin (R-PE)-labeled Pro5 HLA-A*02:01/FLPSDFFPSI pentamer (#F283-2A-G; Proimmune) were isolated by anti-PE MicroBeads (#130-105-639; Miltenyi Biotec) according to the manufacturer's protocol, followed by a rapid expansion protocol.

T CELL REPERTOIRE PREPARATION, HIGH-THROUGHPUT SEQUENCING, AND DATA ANALYSIS

T cells after rapid expansion were collected, and RNA isolation, T cell repertoire preparation, high-throughput sequencing, and data analysis were performed by GENEWIZ (Guangzhou, China). Briefly, total RNA was extracted from the T cell sample using Trizol (#15596018; Invitrogen) according to the user manual. We performed 5' RACE with the SMARTer RACE complementary DNA (cDNA) Amplification Kit (634859; Clontech); total RNA input was 1 μ g. TCR α chain variable region (TRAV) and β chain variable region (TRBV) NGS libraries were made by using NEBNext Ultra DNA Library Prep Kit for Illumina. After quality control on a Bioanalyzer High Sensitivity DNA chip (Agilent), libraries were sequenced on the Illumina Miseq 2 \times 300 platform.

FLOW CYTOMETRY

T cells were analyzed by flow cytometry (FCM), as described in the Supporting Methods.

RETROVIRAL TRANSDUCTION

TCR chains were codon optimized and synthesized by Sangon Biotech, fused by a furin plus P2A element when needed, substituted with murine constant domains, as described, and cloned into

the retroviral vector MSGV1 (#107227; Addgene). We used 293T cells for transfection of the retrovirus plasmids TCR plasmid, VSV-G, and Gag-Pol by Lipofectamine 2000 (#11668019; Invitrogen) according to the manufacturer's protocol. Retrovirus supernatant was centrifuged at 2000g for 2 hours in nontissue culture plates precoated with 20 μ g/mL RetroNectin (#T100A; Takara). The retrovirus supernatant was then removed, and prestimulated PBMCs were added and cultured overnight in a 5% CO₂ incubator at 37°C. When the α - β chain mixture-based pairing strategy was employed, retrovirus supernatant of TCR α chains and β chains were generated individually and mixed at a volume ratio of 1:1 for cotransfection of T cells.

ENZYME-LINKED IMMUNE ABSORBENT SPOT ASSAY

Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immune absorbent spot (ELISpot) assay were performed as described in the Supporting Methods.

V δ 2+ T CELL CULTURE AND TCR TRANSDUCTION

Healthy donor PBMCs were selectively activated to culture V δ 2+ T cells by adding 1 μ mol/L zoledronic acid (#SML0223; Sigma-Aldrich) and transduced as described in the Supporting Methods. For the cytotoxicity assay, cells were stained with anti-human TCR V δ 2-BV605 (clone B6; #331430; Biolegend) and sorted by fluorescence-activated cell sorting (FACS) using BD FACS Aria II before resting.

CYTOTOXICITY ASSAY

In vitro cytotoxicity of TCR-T cells was evaluated by lactate dehydrogenase (LDH) release of target cells according to the manufacturer's protocol (#C0017; Beyotime), as described in the Supporting Methods.

Results

Nearly 96% of chronic HBV infections are caused by five HBV genotypes (A-E), although

genotype C is the most common.⁽¹⁶⁾ HBV core 18-27V (FLPSDFFPSV) is a proven epitope derived from HBV genotypes A, D, and E, which are the dominant genotypes in Africa, Europe, and Western Asia, respectively. The hepatitis B core (HBc) proteins of HBV genotypes B and C contain a slightly different epitope in the same region (FLPSDFFPSI), 18-27I in this study.⁽¹⁷⁾ As HBV genotypes B and C are endemic in Eastern Asia, we attempted to focus on searching for TCRs targeting core proteins derived from these genotypes. Furthermore, considering that V27 epitope-specific cytotoxic T lymphocytes may not cross-react with the I27 epitope and may be inhibited by the simultaneous presentation of V27 and I27 epitopes,^(17,18) an HBV core_{18-27I} pentamer was used to isolate HBV-specific T cells from PBMCs of patients who were A*02+/HBV+. A proportion of pentamer-positive T cells was identified from 1 patient with a chronic HBV infection (Fig. 1A). Pentamer-stained T cells were then isolated by magnetic beads and expanded by a rapid expansion protocol (REP) (Fig. 1A). The expanded T cells

were purified by pentamer staining again for TCR sequencing. Instead of limiting dilution or single-cell sequencing, NGS was employed for TCR identification. The top five abundant TCR V α chains accounted for 99.6% of all the presented V α chains, which in order were TRAV12-2, TRAV14/DV4, TRAV12-2, TRAV8-3, and TRAV38-2/DV8 (Fig. 1B). Similarly, the top five abundant TCR V β chains accounted for 99.9% of all the presented V β chains, which in order were TRBV27, TRBV4-1, TRBV9, TRBV19, and TRBV29-1. The ranking of the α complementarity determining region 3 (α CDR3) and β CDR3 of these TCR chains was correlated with the ranking of the corresponding V α and V β chains (Fig. 1C).

Theoretically, an equimolar endogenous expression of TCR α and β chains would lead to an equal abundance of V α and V β chains in NGS when all T cells express a unique TCR. Therefore, a ranking-based pairing strategy was employed. Given that the purity of pentamer staining-based isolation is approximately 90%, TRAV12-2 and TRBV27 as well as TRAV14/DV4 and TRBV4-1, which account for

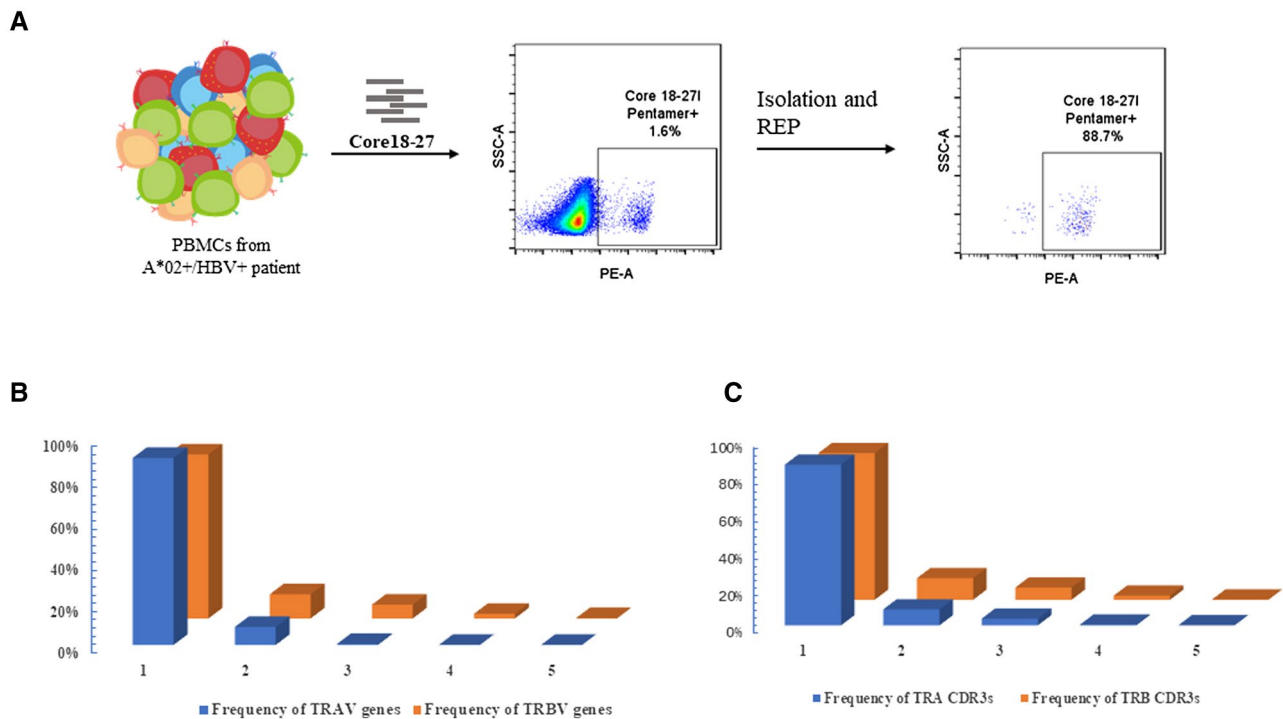


FIG. 1. Isolation of polyclonal HBV-specific T cells and NGS. (A) HBV-specific T cells were isolated using a peptide-MHC tetramer and expanded by an REP, after which the expanded T cells were purified by pentamer staining again for NGS. (B) Frequency of the top five abundant TCR V α and V β genes. (C) Frequency of the top five abundant α CDR3s and β CDR3s. Abbreviations: PE, phycoerythrin; SSC, side scatter.

approximately 90% of V α and V β chains, were paired directly based on their abundances and ranking in NGS (Fig. 2A) and were constructed into one retroviral vector with respective modified mouse constant regions (Fig. 2B).⁽¹⁹⁾ We termed the TRAV12-2 and TRBV27 pair TCR1 and the TRAV14/DV4 and TRBV4-1 pair TCR2. Most cluster of differentiation (CD)8+ T cells transduced with both TCRs (TCR1-T and TCR2-T cells) can be stained simultaneously by the HBV core₁₈₋₂₇₁ pentamer and anti-mouse β chain antibody (Fig. 2C), further confirming the reliability of the pairings of V α and V β chains based on NGS abundances and rankings.

At the same time, an α - β chain mixture-based pairing strategy was also employed for the identification of possible TCR pairs. First, the top four V β chains, which account for 99.7% of all the sequenced TCR β chains, were chosen. Considering that the frequency

of pentamer-positive T cells in NGS-sequenced polyclonal T cells is approximately 90%, these top four V β chains should be able to cover all possible functional TCRs. When dual α chain T cells are present in pentamer-purified polyclonal T cells, their influence on the overall abundance of V α chains can be difficult to predict. However, given that the two α chains from the same T cell will most likely result in equal abundance in NGS, its influence can be easily accommodated by including extra V α chains in ranking-based selection of V α chains. When the frequency of dual receptor T cells is approximately 30%,⁽¹⁴⁾ the possible number (according to the Poisson probability distribution) of dual α chain T cells (0-4) that correspond to the four selected V β chains and their possibilities (p) are p(0) = 0.3, p(1) = 0.36, p(2) = 0.22, p(3) = 0.09, and p(4) = 0.03. Therefore, selection of the top six V α chains can cover approximately 90% of all possibilities. These chains were

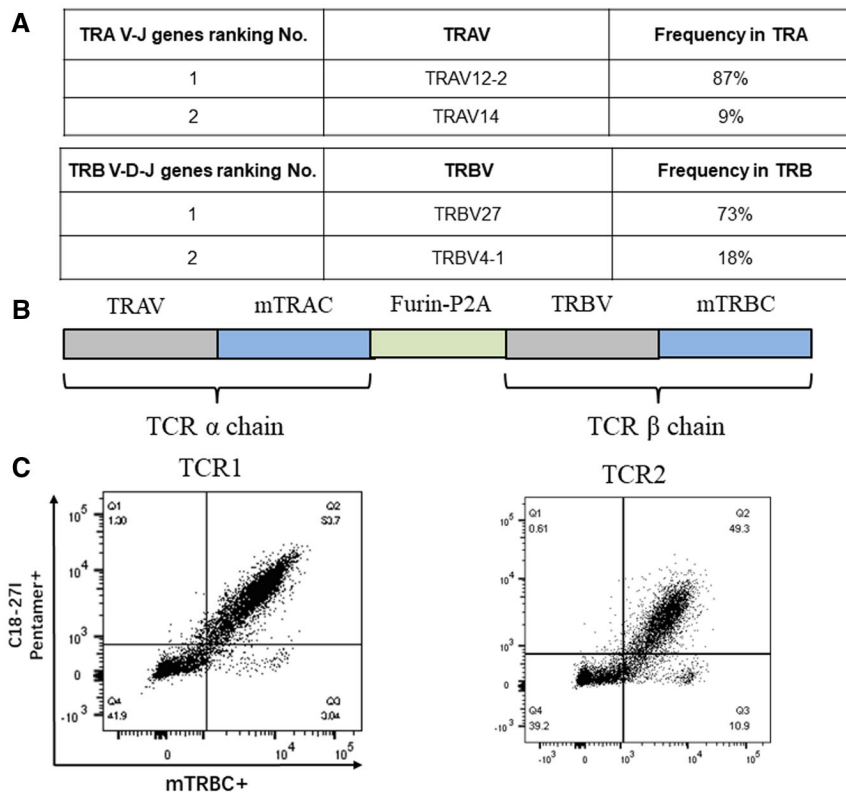


FIG. 2. Ranking-based pairing strategy for TCR identification. (A) TRAV12-2 and TRBV27 as well as TRAV14/DV4 and TRBV4-1, which account for approximately 90% of V α and V β chains, were paired directly based on their abundances and ranking in NGS. (B) Schematic representation of TCR α and β chains cloned into one transgene cassette. Constant regions were replaced by corresponding modified mouse TCR constant chains (mTRAC and mTRBC) and were fused by a furin-P2A element. (C) FCM analysis of CD8+ T cells transduced with both TCRs (TCR1-T and TCR2-T cells) and stained simultaneously by the HBV core18-271 pentamer and anti-mouse β chain antibody. Abbreviation: LTR, long-terminal repeat.

inserted into an MSGV vector. All 24 possible α - β pairs were tested by cotransfecting T cells with 24 different mixtures of $V\alpha$ virus and $V\beta$ virus. Only two engineered T cells showed specific recognition of the core 18-27I epitope (Fig. 3A,B). Further, FCM analysis showed that activation markers of T cells, such as 4-1BB, tumor necrosis factor receptor superfamily member 4 (OX40), and programmed death 1 receptor (PD-1), were also up-regulated (Fig. 3C,D). The two transfected $V\alpha$ and $V\beta$ pairs were TRAV12-2 and TRBV27 and TRAV14/DV4 and TRBV4-1, the same TCR pairs identified by the ranking-based pairing strategy.

Both TCRs recognized the core 18-27I and core 18-27V epitopes presented on HLA-A*02:01 molecules (Fig. 4A). Neither showed recognition of the polymerase 575-583 epitope. Similar results were obtained in the ELISpot assay (Fig. 4B); many more TCR1-T cells showed recognition of both the core 18-27I and core 18-27V epitopes than TCR2-T cells,

whereas neither responded to T2 cells (Fig. 4B). In addition, both TCR-T cells also recognized intrinsically processed epitopes (core 18-27V) by HepG2.2.15 (Fig. 4C). Furthermore, this recognition was blocked by the HLA-I blocking antibody (Fig. 4C).

Potential antitumor function was evaluated by the LDH detection assay. We first generated a HepG2 cell line that stably integrated the HBc coding sequence (18-27I) derived from HBV genotypes B and C. Both TCR1-T cells and TCR2-T cells specifically lysed HepG2-HBc cells in a dose-dependent manner (Fig. 5A) but did not lyse wild-type HepG2 cells (Fig. 5B). As shown by intracellular staining (Fig. 5C,D), only T cells transduced with TCRs (mouse TRB constant region positive [mTRBC+]) specifically recognized HepG2-HBc cells and did not respond to HepG2 cells.

The functional avidity of the two TCRs was further evaluated by a peptide dilution assay. The 50%

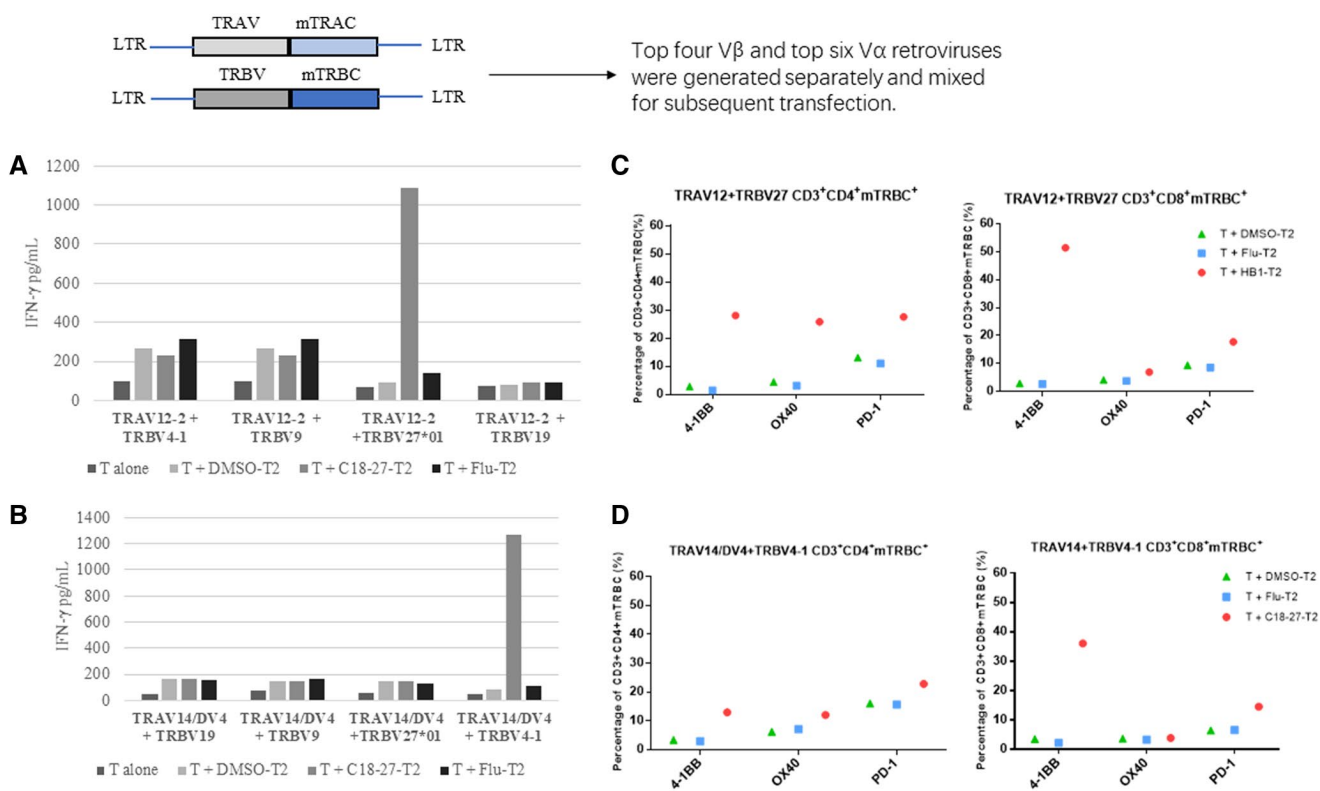


FIG. 3. An α - β chain mixture-based pairing strategy for TCR identification. (A,B) Top four $V\beta$ and top six $V\alpha$ retroviruses were generated separately and mixed for subsequent transfection of T cells. T cells transfected with 24 different α - β chain mixtures were tested by ELISA for the secretion of IFN- γ . TRAV12-2 and TRAV14/DV4 showed specific recognition of core 18-27I epitope when paired with TRBV27*01 and TRBV4-1, respectively. (C,D) FCM analysis of the activation markers (4-1BB, OX40 and PD-1) of CD4 + and CD8 + T cells. Abbreviations: OX40, tumor necrosis factor receptor superfamily, member 4; PD-1, programmed death 1 receptor.

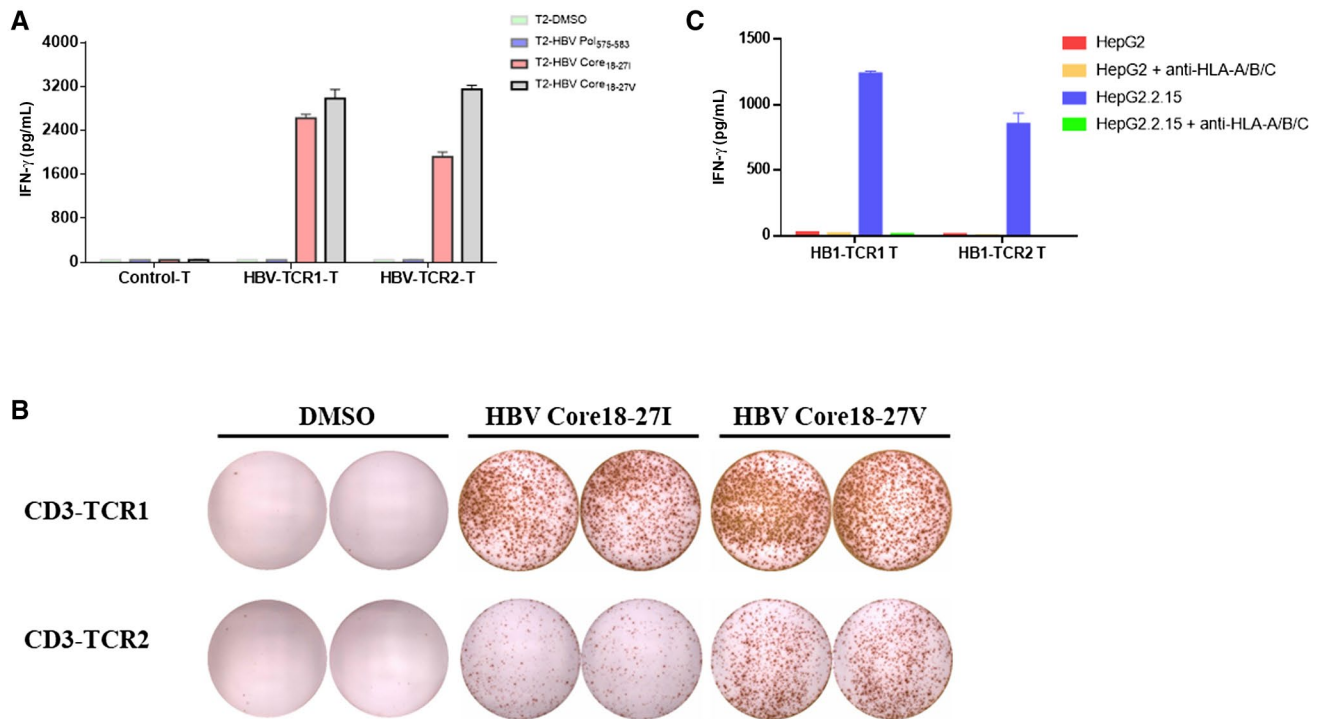


FIG. 4. Functional evaluation of T cells transduced with TCR1 and TCR2. (A) T2 cells loaded with different epitopes or DMSO control were incubated with TCR1-T and TCR2-T cells overnight. The supernatant was tested by ELISA for the amount of IFN- γ . (B) T2 cells loaded with different epitopes or DMSO control were incubated with TCR1-T and TCR2-T cells overnight in an anti-IFN- γ antibody-coated ELISpot plate for the detection of IFN- γ secreting T cells. (C) HepG2 cells and HepG2.2.15 cells were incubated with TCR1-T and TCR2-T cells overnight with or without the addition of anti-HLA-A/B/C antibody (clone W6/32). Supernatants were taken for the quantification of IFN- γ by ELISA. Data in (A,B) represent mean \pm SD.

effective concentration (EC_{50}) values of TCR1-T and TCR2-T cells for the core 18-27I epitope were 1.263×10^{-6} mol/L and 2.218×10^{-6} mol/L, respectively, (Fig. 6A,B). For the core 18-27V epitope, the EC_{50} values were 7.711×10^{-11} mol/L and 3.145×10^{-10} mol/L, respectively. Neither showed recognition of the polymerase 575-583 epitope. Furthermore, the recognition of core 18-27 epitopes on the most frequent HLA-A*02 subtypes in China was also evaluated. Based on the common and well-documented alleles in Chinese, the top 10 most frequent HLA-A*02 subtypes (HLA-A*02:01, A*02:07, A*02:03, A*02:05, A*02:06, A*02:09, A*02:10, A*02:11, A*02:48, and A*02:53N) were constructed and transduced into Cos-7 cells. Both TCRs recognized their cognate peptides derived from the HBV genotype or A/D/E (core 18-27V) or B/C (core 18-27I) not only when presented by HLA-A*02:01 but also when presented by 02:05, 02:06, 02:07, 02:09, 02:10, and 02:11 (Fig. 6C,D).

The use of mouse constant regions can reduce the mispairing of TCR chains⁽¹⁹⁾ but may lead to immunologic rejection when used *in vivo*. Therefore, the functions of the two paired HBV-specific TCRs were further verified by transduction to $\gamma\delta$ -T cells, which can also greatly reduce potential mispairings of TCR chains because of the employment of different constant chains.⁽²⁰⁾ Furthermore, it is known that a subgroup of V δ 2+ T cells expresses its coreceptor CD8 through an α - α homodimer instead of an α - β heterodimer at a relatively low level of constant expression; this makes them an ideal tool for the functional evaluation of the dependence TCRs on coreceptors. V δ 2+ T cells transduced with both TCRs (Supporting Fig. S1) specifically recognized the core 18-27V epitope loaded on T2 cells (Fig. 7A,B). In contrast, non-transduced V δ 2+ T cells did not show any recognition of target cells. This specific recognition was further confirmed by intracellular interferon- γ (IFN- γ)

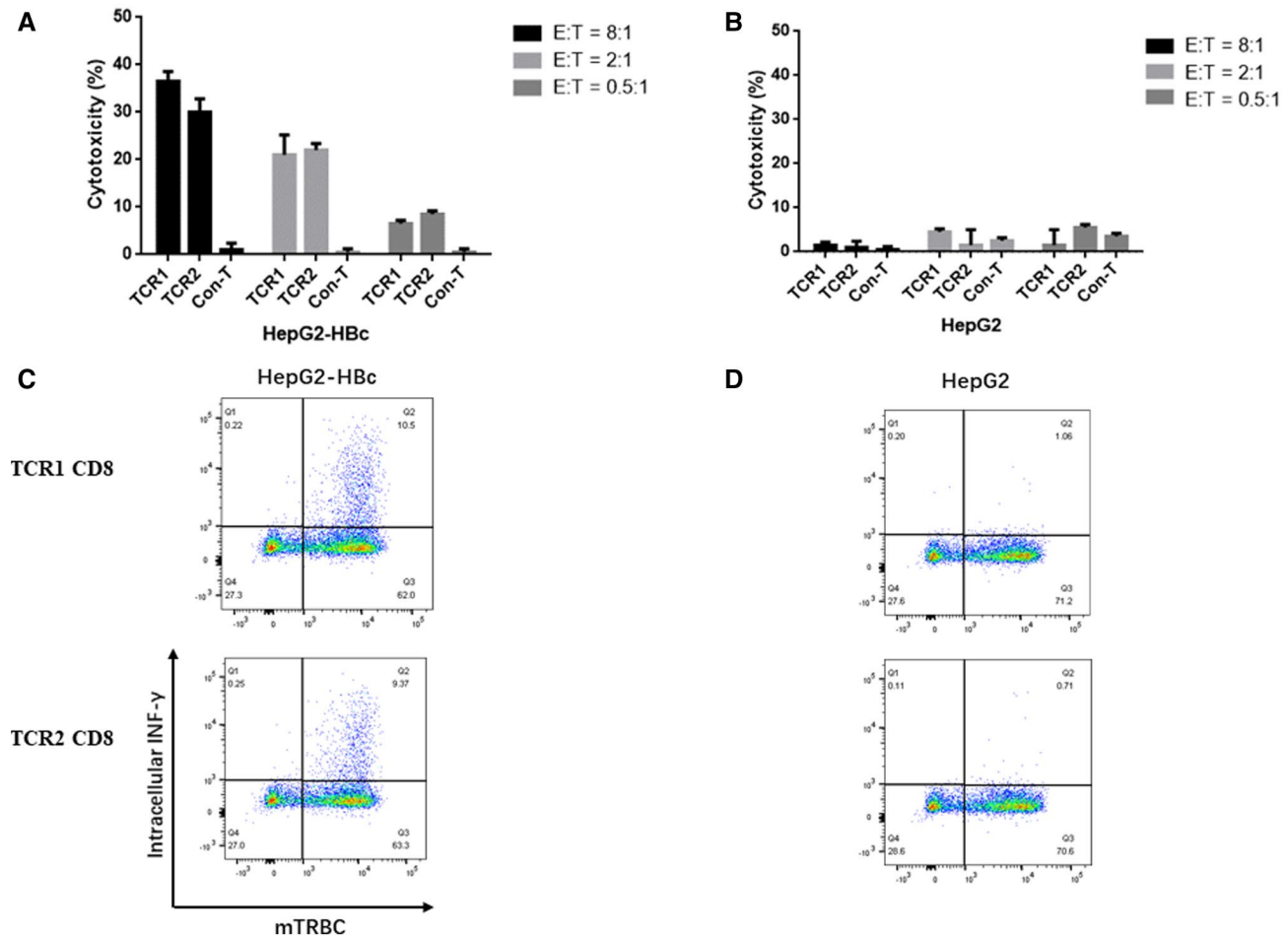


FIG. 5. Recognition of intrinsically processed core 18-27I epitope. (A) HepG2 stably transfected with HBV core antigens derived from genotypes B and C (HepG2-HBc) or (B) HepG2 wild type were incubated with TCR1-T and TCR2-T cells. The specific lysis of target cells was measured by LDH assay. Data represent mean \pm SD. (C,D) FCM analysis of intracellular IFN- γ of both TCR1-T and TCR2-T cells after incubating overnight with (C) HepG2-HBc or (D) HepG2. Abbreviations: Con-T, control T cells; E:T, effector to target cell ratio.

staining (Fig. 7B,C). Moreover, the proportion of IFN- γ -positive V δ 2⁺ T cells was much higher than the proportion of V δ 2⁺ CD8⁺ T cells, which suggests that the TCR functioned without coreceptors. The recognition of intrinsically processed epitope was further verified. V δ 2⁺ T cells transduced with TCR1 lysed HepG2.2.15 cells dose dependently, while the nontransduced V δ 2⁺ control T cells only showed moderate cytotoxicity against HepG2.2.15 (Fig. 7D). This is not surprising as V δ 2⁺ T cells is known for non-TCR-mediated activity, such as natural killer group 2 member D (NKG2D)/ligand interaction-triggered cytotoxicity.^(21,22)

Discussion

In this study, polyclonal T cells specific to the HLA-A*02-restricted 18-27 epitope of the HBV core antigen were purified by a peptide-MHC multimer-based approach and TCRs were sequenced by NGS, a bulk sequencing approach. Without the typically involved TCR singularization processes, such as limiting dilution for single-cell expansion or single-cell sequencing, the V α and V β chains were paired based solely on NGS. We suggest that NGS-based pairing strategies (ranking-based and α - β chain mixture-based strategies) are feasible for

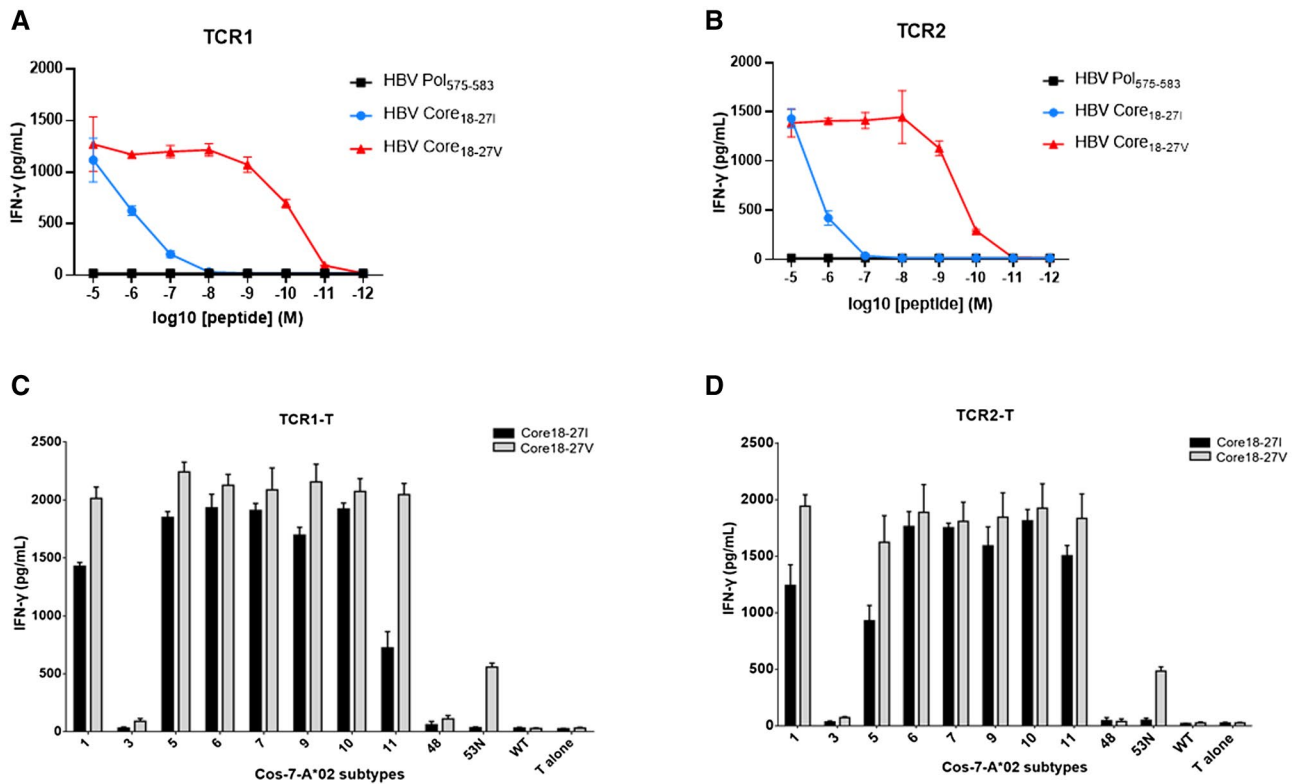


FIG. 6. Functional affinity and recognition of different HLA-A*02 subtypes. (A,B) T2 cells loaded with a serial dilution of different epitopes were incubated with (A) TCR1-T and (B) TCR2-T cells overnight. Supernatants were taken for quantification of IFN- γ by ELISA. (C,D) Cos-7 cells transduced with different A*02 subtypes were loaded with 18-27I and 18-27V epitopes and then incubated with (C) TCR1-T and (D) TCR2-T cells overnight for the detection of IFN- γ in the supernatant by ELISA. Data represent mean \pm SD. Abbreviations: HBV Pol, HBV polymerase; WT, wild type.

the fast and efficient identification of HBV-specific TCRs.

The abundance of V α chains showed a correlation with the abundance of the corresponding α CDR3s in NGS, suggesting that there are no shared α CDR3s by several different V α chains or one shared V α chain by different α CDR3s, which is also true for V β chains and β CDR3s. Nevertheless, as mentioned earlier, a proportion of T cells may encode two mRNAs of two different TCR α chains. Dual-receptor T cells may present TCRs at a lower quantity on the cell surface than single-receptor T cells, which may lead to less clonal expansion when encountering repeated antigen stimulation in a chronic virus infection, such as HBV.⁽²³⁾ This means that the outgrowth of single-receptor virus-reactive T cells may account for a much larger proportion than 70%. Equimolar expression of endogenous TCR α and β chains would theoretically lead to

equal abundances of V α and V β chains in NGS, but there could always be some deviations considering that the V α and V β chains are normally cloned separately by different primer sets and that two different NGS libraries are generated. However, the ranking of the TCR chains presented in their own library should not be affected by such different manipulations. Therefore, it is also important to correlate the ranking of different V α and V β chains when processing the pairing. Among the two identified TCR pairs, the rankings of V α chains (TRAV12-2 and TRAV14/DV4) are the same as the corresponding V β chains (TRBV27 and TRBV4-1), whereas their abundances showed differences of 17% and 9%, respectively. Pentamer staining confirmed that both V α and V β chains paired correctly when introduced on the surface of T cells.

Interestingly, the α - β chain mixture-based pairing strategy identified the same two TCR pairs as

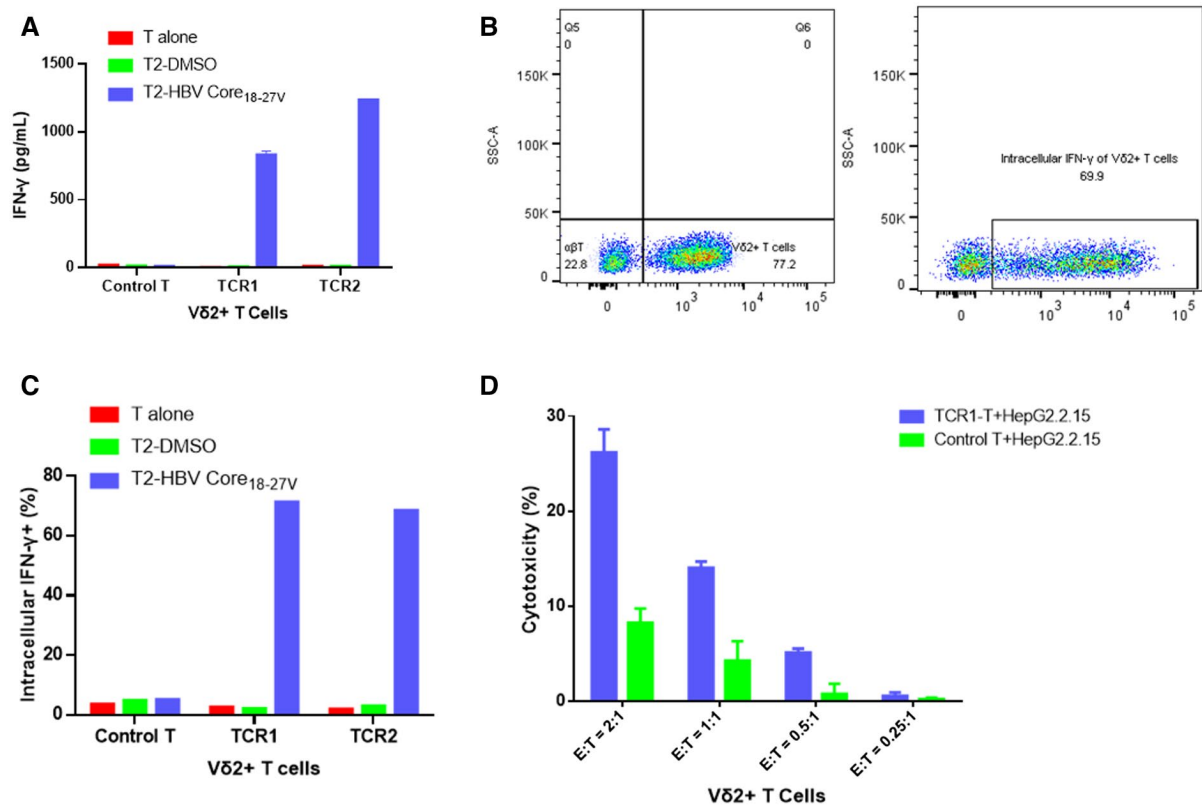


FIG. 7. Functional evaluation of Vδ2 T cells transduced with TCR1 and TCR2. (A) Vδ2+ T cells transduced with both TCRs were incubated overnight with T2 cells loaded with core 18-27V epitopes or DMSO. Supernatants were taken for the quantification of IFN-γ by ELISA. (B) An illustration of the FCM analysis of intracellular IFN-γ of Vδ2+ T cells after overnight incubation with T2 cells loaded with core 18-27V or DMSO. (C) Statistics summary of the proportion of intracellular IFN-γ positive Vδ2+ T cells after overnight incubation with T2 cells loaded with core 18-27V or DMSO. (D) HepG2.2.15 cells were incubated overnight with Vδ2+ T cells transduced with TCR1 or the nontransduced control T cells. Supernatants were taken for cytotoxicity assay. Data in (A,C,D) represent mean ± SD. Abbreviations: E:T, effector to target cell ratio; SSC, side scatter.

the ranking-based pairing, which suggests that the NGS frequency-based selection of Vβ chains and dual-receptor T cell frequency-based selection of Vα chains can cover most functional T cells. Furthermore, the results of the α-β chain mixture-based pairing strategy suggest that the two identified TCRs are paired endogenously, although they are not obtained from single cells (single-cell sequencing or limiting dilution for single-cell expansion). As demonstrated previously,⁽²⁴⁾ consistent and repeated antigenic stimulation in chronic infection continues to narrow the TCR repertoire, which results in the vast majority of antigen-specific responses being clonal. Additionally, both our *in vitro* stimulation and the REP processes may have contributed to the narrowing of the HBV-specific TCR repertoire, given that not all T cells

are expandable under the same conditions.^(25,26) Our results highlight the potential value of the α-β chain mixture-based pairing strategy for fast TCR identification when there is a limited number (e.g., <10) of Vα and Vβ chains presented, which is normally true for peptide-MHC multimer-purified polyclonal virus-specific T cells from donors with a chronic virus infection.^(10,27) Certainly, this strategy is not suitable for TCR identification from T cell mixtures that do not undergo peptide-MHC multimer purification because neither the potential functional Vβ chains nor the corresponding Vα chains can be selected.⁽¹¹⁾ A reasonable concern about this strategy is that the virus mixtures we used here led to a limited cotransfection ratio, which was approximately 2% in our case, as detected by mTRBC in FCM analysis. The reasons

for this result are multiple. One is that the titer of viruses available for transfection is limited when they are generated in 24-well plate wells for feasible manipulation. Another is that the nonpaired transferred chains are internalized quickly. Either way, the high sensitivity of functional assays, such as ELISA and ELISpot, can accommodate such a low frequency of functional T cells.^(28,29) Moreover, the plasmid-based TCR-introducing approach can employ much simpler manipulation processes (e.g., mixture of plasmids and electroporation for transposon-based gene transfer) than virus-based approaches, which further increases the applicability of this strategy.⁽³⁰⁾

The specificity of both TCRs was verified by the recognition of HBV core 18-27I and HBV core 18-27V epitopes but not nonrelevant HBV polymerase 585-593 epitopes in ELISA. Although no significant differences in functional avidity were detected between the two TCRs by ELISA, the ELISpot assay clearly showed a much higher functional avidity of TCR1 for both core 18-27V and 18-27I epitopes. This result may be explained by the higher sensitivity of the ELISpot assay and highlights its potential value in the comparison of the functional avidity of different TCRs. Additionally, the function of the two paired TCRs was further confirmed by showing recognition of the endogenously presented epitope (core 18-27V) by HepG2.2.15. The blocking of recognition by anti-HLA-I blocking antibody suggests that this recognition is HLA-I molecule restricted. The peptide dilution assay also revealed a much higher functional avidity of both TCRs for the core 18-27V epitope in contrast to the core 18-27I epitope. This result may be attributed to the stronger binding affinity between the core 18-27V epitope and HLA-A*02 subtypes and also to the fact that while the 27I reduces the binding of the epitope to HLA-A*02, it does not alter TCR interaction.^(17,31) It is suggested that binding stability between the epitope and HLA molecule is a better indication of immunogenicity than affinity, considering that the epitope should be able to bind to the MHC molecule and remain bound for long enough to be presented to and recognized by T cells to elicit an immune response.^(32,33) As described,⁽³⁴⁾ the comparatively lower binding stability of the 18-27I epitope with A*02:03 may partially explain the inactivity of both TCRs. Similar low-binding stability of the 18-27I epitope with A*02:48 was predicted by using NetMHCstabpan version1.0 (data not shown).⁽³⁵⁾

This suggests that other epitopes from HBV genotypes B/C are required when performing TCR identification for these two types of HLAs (A*02:03 and A*02:48). Furthermore, the activity of both TCRs for multiple HLA-A2 subtypes makes them potentially applicable to a wide range of populations.

Both TCRs showed a similar pattern for dose-response curves toward two epitopes, but TCR1 (TRAV12-2 and TRBV27) was more potent with a lower EC₅₀ value. The ELISpot assay further confirmed the higher functional avidity, as previously described. Interestingly, the prevalent expression of TRAV12-2 was identified in different TCRs targeting HLA-A*02-restricted epitopes derived from viruses as well as cancer antigens.^(10,36-38) Structural analysis revealed an unusual “ α -centric” TCR binding mode in which the invariable CDR1 α loop, not the CDR3 loops, dominated the interaction with the HLA-A*02 molecule. This finding suggests that TCRs expressing the TRAV12-2 could have an intrinsic advantage when binding to cognate antigens restricted by HLA-A*02 due to the innate recognition of residues on the MHC surface.⁽³⁶⁾ This advantage may explain why the two V chains of TCR1 are mostly abundant in NGS. A higher functional avidity may lead to the outgrowth of TCR1-T cells *in vivo* following repeated antigenic stimulation and when stimulated *in vitro* using the core 18-27I peptide. Given that as the abundance of TCRs increases so does their chances of being covered using NGS-based pairing strategies, this positive association between the abundances of TCR chains and their functional avidity lends further credence to the potential value of an MHC multimer-aided NGS-based strategy for the fast identification of TCRs with superior functional avidity. Additionally, the NGS abundance of TCR chains can be valuable information in the choice of TCRs with variable affinities, such as an intrinsic higher abundance; hence, the higher avidity of TCR1 supports its potentially better efficacy for TCR-T cell therapy.⁽³⁹⁾ Although the strategy based on the abundance of TCR reads is shown to work in a single patient and for a single epitope in the present study, it has been successfully and repeatedly applied in our laboratory to identify functional TCRs specific to epitopes derived from other types of viruses, including Epstein-Barr virus and cytomegalovirus (unpublished data). In addition, it would be interesting to determine whether single-cell analysis is able to verify the

pair of TCR α and TCR β detected by calculating the abundance of TCR reads in bulk sequencing. We will address this issue in future studies.

The possible mispairing of endogenous and introduced TCR chains that do not experience negative selection in the thymus may potentially lead to auto-immune reactions; therefore, V α and V β chains were expressed from one construct with the modified mouse TCR constant domains by adding an extra disulfide bond. As reported,⁽¹⁹⁾ this strategy can reduce the chance of mispairings. However, this strategy may lead to allergic reactions when used *in vivo* and therefore is not widely employed in clinical TCR-T cell therapies. An alternative approach to avoid mispairing would be transducing TCRs to $\gamma\delta$ -T cells, such as V δ 2+ T cells, which employ different TCR constant regions from $\alpha\beta$ T cells.⁽²⁰⁾ The superior functional avidity of both TCRs was further verified by the activities of V δ 2+ T cells without the expression of coreceptors. In addition to directly acting on target cells, V δ 2+ T cells can also function as antigen-presenting cells following activation for the recruitment of reactive CD4 and CD8 T cells to bridge innate and adaptive immunity.^(22,40) Furthermore, V δ 2+ T cells, as the first-line defenders of infections, can defend against infection by secreting a unique panel of antiviral cytokines following activation.^(41,42) For example, V δ 2+ T cells have been found to be capable of uniquely secreting the dendritic cell-inducing cytokine granulocyte-macrophage colony-stimulating factor following activation,⁽⁴¹⁾ which has been proven to eliminate hepatitis B surface antigen-positive hepatocytes.⁽⁴³⁾ This is the first study to confirm the activities of HBV-specific TCRs when introduced into $\gamma\delta$ -T cells, which have the potential to contribute uniquely to the treatment of HBV-related diseases, including HCC.

A reasonable concern about HBV-specific TCR-T cell therapy is the potential liver toxicity induced by the lysis of HBV-infected yet functional liver cells. However, as demonstrated in bone marrow transplantation, the transfer of HBV-specific immune cells into patients with chronic HBV can lead to HBV clearance while causing limited and acceptable liver toxicity.⁽⁴⁴⁻⁴⁶⁾ In a humanized mouse model, the data suggest that when a minor proportion of liver cells is infected by HBV, the adoptive transfer of TCR-T cells only leads to moderate toxicity.⁽⁴⁷⁾ In addition, antiviral therapies, including nucleotide analogs as well as upcoming RNA interference therapies, can

greatly reduce the antigen presence in normal liver cells, which can potentially be combined with TCR-T cell therapies for patients with HCC.⁽⁴⁸⁾ Furthermore, TCR-T cell therapy has already been shown to be a safe approach for the treatment of metastatic HCC in patients with a liver transplant.⁽⁹⁾ Transient introduction of HBV-specific TCRs has also been proven to be a safe approach to avoid robust and consistent T cell reactions.^(49,50)

In summary, we present an example of fast and efficient NGS-based identification of HBV-specific TCRs. Functional evaluation confirmed their specificity and avidity, which means that they may be able to be used to generate TCR-T cells to treat patients with HBV-related HCC.

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