In vivo therapeutic effects of affinity-improved-TCR engineered T-cells on HBV-related hepatocellular carcinoma

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ABSTRACT

Background In patients with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC), virus-specific cytotoxic T lymphocytes (CTLs) fail to eliminate HCC cells expressing HBV antigens. As the expression of viral antigen in HBV-associated HCC may decrease to allow tumor to escape immune attacks, we hypothesized that an HBV surface antigen (HBsAg)-specific affinity-improved-T-cell receptor (TCR) will enable T cells to target HCC more effectively than corresponding wild-type-TCR. We also postulated that TCR promiscuity can be exploited to efficiently capture HBV variants that can hinder CTL-based therapeutics.

Methods We applied flexi-panning to isolate affinity-improved TCRs binding to a variant antigen, the human leukocyte antigen (HLA)-A*02:01-restricted nonapeptide HBs370-379-SIVSPFIPLL, from libraries constructed with a TCR cloned using the decapetide HBs370-379-GVSPFIPLL. The potency and safety of the affinity-improved-TCR engineered T-cells (AI-TCR-T) were verified with potentially cross-reactive human and HBV-variant peptides, tumor and normal cells, and xenograft mouse models.

Results AI-TCR-T cells retained cognate HBV antigen specificity and recognized a wide range of HBV genotypic variants with improved sensitivity and cytotoxicity. Cell infusions produced complete elimination of HCC without recurrence in the xenograft mouse models. Elevated infusions produced complete elimination of HCC without recurrence in the xenograft mouse models.

Conclusion The in vitro and in vivo studies demonstrated that HBsAg-specific AI-TCR-T cells had safety profiles similar to those of their wild-type counterparts and significantly enhanced potency. This study presents an approach to develop new therapeutic strategies for HBV-related HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks as the sixth most common cancer and fourth leading cause of cancer-related death worldwide, accounting for nearly 800,000 deaths per year. In HCC cases, almost 80% are associated with chronic hepatitis B or C virus (HBV or HCV, respectively) infection. Current treatments for HCC have a 10-year actual survival rate of only 7.2%, and the relapse rate for HBV-positive HCC is high following liver transplantation. There is a great demand for potent and tolerable therapies in clinics worldwide.

Although immunotherapies have been considered for HCC, virus-specific T cells are deleted or dysfunctional, and immune attack to eliminate cancer cells is often retarded in patients with HBV-related HCC. This problem may be overcome with T-cell receptor (TCR)-engineered T cell (TCR-T cell) therapy, because the viral DNA frequently integrates into the liver cell genome. Whether HCC cells can present any HBV peptide antigens remains controversial, as there is often no HBV protein detected in HCC cells with immunohistochemistry (IHC). However, Tan et al recently demonstrated that HBV-related HCC cells might not express whole HBV antigens but could transcribe short HBV messenger RNAs encoding epitopes recognized by HBV-specific T cells. Additionally, HBV surface antigen (HBsAg) has been used as a target for TCR-T cells to treat HBV-related HCC. An HBs385-191-specific wild-type (WT) TCR, which was isolated from a human leukocyte antigen (HLA)-A2 patient who had resolved an HBV infection, was shown to recognize HCC cells integrated with natural HBV-DNA and mediate HBV-specific T cell immunity in vitro and in vivo. As a result, clinical studies of HBsAg-specific T cell therapy have demonstrated good tolerability and safety with wild-type T-cell receptor (TCR)-engineered T cells (WT-TCR-T). The efficacy of the treatment revealed a volume reduction of several pulmonary HCC metastatic lesions in one of the two patients.

Although HBV antigen-negative HCC cells can present HBV peptide antigens, the density may be relatively low. TCR-T cells engineered with a wild-type TCR, whose natural affinities are limited to ~1 to 100 μM, may not effectively target cells with down-regulated tumor antigen presentation.
However, affinity-improved TCRs can recognize tumor cells presenting low-density antigens. On the other hand, liver transplantation-treated HCC patients still have a large number of liver tumor cells outside the liver, which may present sparse antigens and contribute to HCC relapse during a later stage. Therefore, we hypothesized that in the treatment of HBV-related HCC, especially in liver-transplanted patients, affinity-improved TCR-engineered T-cells (Ai-TCR-T) will produce better outcomes than WT-TCR-T.

Furthermore, nine HBV genotypes and many variants that have different peptide antigens have been identified to date, and it would be valuable to create one TCR that can specifically recognize several HBV-variant peptide antigens. It is well documented that a TCR can show promiscuity for recognizing a set of variant antigens, but the T cell maintains physiological specificity in the peripheral immune system. We then questioned whether this feature can be harnessed for the generation of affinity-improved TCRs that are capable of recognizing several HBV variants.

To address both questions, we constructed TCR libraries with a wild-type TCR that was isolated initially with an HLA-A*02:01-restricted HBV genotype-A HBs371-379 (SIVSPFIPPLL, named vrt0) decameric peptide antigen complex from a resolved HBV patient. Because HBV genotype C is closely related to an elevated HCC risk, we isolated high-affinity TCRs binding to a genotype B/C/D HBs371-379 (ILSPFLPLL, named vrt1) nonameric peptide antigen from the TCR libraries. An affinity-improved mutant TCR could retain HBsAg specificity similar to the specificity of the wild-type TCR. The mutant TCR constructed Ai-TCR-T cells showed enhanced sensitivity and cytotoxicity in vitro. In addition, Ai-TCR-T cells recognized not only vrt1 and vrt0 but also nine other peptide variants found at high frequencies in HBV species. However, both WT-TCR-T cells and Ai-TCR-T cells were hardly activated by irrelevant peptide-pulsed or potentially cross-reactive human peptide-pulsed cells, human normal primary cells and healthy peripheral blood mononuclear cells (PBMCs), indicating that the Ai-TCR-T cells have a good safety profile. The Ai-TCR-T cells were also demonstrated to eliminate HCC tumors without recurrence in vivo.

**METHODS**

**HBs peptides and HBV genotypes**

HBs371-379 and HBs370-379 epitope sequences were retrieved from 5855 unique full-length HBV surface antigen (HBs) polyproteins (389, 399 and 400AA HBs polyprotein sequences) collected from UniProt around May 2019. First, there were 134 unique nonameric or 190 unique decameric peptides extracted respectively from HBs371-379 and HBs370-379 epitope sequences with Strawberry Perl 5.26.1.1 (http://strawberryperl.com) after removing duplication. Second, the most common 20 peptides of HBs371-379 and HBs370-379 were statistically analyzed from unique 5657 (97% of 5855) and 5528 (94% of 5855) full-length HBs polyprotein sequences separately. Finally, the most common 20 peptides that already categorized the HBV genotypes in UniProt were statistically analyzed with HBV genotypes. The corresponding HBV genotypes of the most common eight peptides of HBs371-379 (vrt1 to vrt8) and HBs370-379 (vrt0, vrt9 to vrt16) were analyzed with Package ‘phemap’ (https://cran.r-project.org/web/packages/phemap/index.html) based on statistical software R V.3.6.1 (https://www.r-project.org/).

**Identification of potential cross-reactive human peptides of vrt1 and vrt0**

Potential cross-reactive human peptides were identified by Expitope webserver 2.0 (http://webclu.bio.wzw.tum.de/expitope2/) designed to predict the similar peptides and its potential presentation by the HLA allele of interest.

For identifying vrt1 and vrt0 related human cross-reactive peptides, we combined the results of the alanine scanning to determine the critical TCR binding position of vrt1 and vrt0. The maximum allowed number of mismatches were set as three for vrt1 and four for vrt0 for the primary screening. The chosen allele was HLA-A*02:01. The default parameters were used for the other options. We also carried out a second screening without fixing the critical positions, and the maximum allowed number of mismatches were set two for vrt1 and three for vrt0. Those potential peptides derived from proteins with expression data were proceeded the third step of T cell activation assays.

**Antigen peptides**

The 16 most frequent HBs371-379 (HBs371) and HBs370-379 (HBs370) antigenic peptides were retrieved from 5855 full-length HBsAg polyproteins. There were eight HBs371 nonameric peptides, that is, vrt1-ILSPFIPPLL, vrt2-ILSPFIPPLL, vrt3-ILSPFIPPLL, vrt4-ILSPFIPPLL, vrt5-IVSPFIPPLL, vrt6-IVSPFIPPLL, vrt7-ILSPFIPPLL and vrt8-IVSPFIPPLL, and eight HBs370 decameric peptides, that is, vrt9-NILSPFIPPLL, vrt10-NILSPFIPPLL, vrt11-NILSPFIPPLL, vrt12-NILSPFIPPLL, vrt13-NILSPFIPPLL, vrt14-SILSPFIPPLL, vrt9-SIVSPFIPPLL and vrt16-NILSPFIPPLL, that were analyzed (online supplemental figure S1, tables S1.1 and S1.2).

The irrelevant peptide-HLA complexes (pHLAs) (online supplemental table S2) were used to verify the specificity of the TCR mutants. Alanine-scanning peptides and potentially cross-reactive human peptides related to vrt1 and vrt0 (online supplemental tables S3.1 and S3.2) were used to test the binding site and TCR specificity, respectively. All peptides were purchased from Genscript and validated with a mass spectrometer, and the purities were confirmed to be greater than 95%.

**Cell lines, PBMC and human normal primary cells**

T2, J.RT3-T3.5 cell, 293T and HepG2 were purchased from ATCC. HCC cell lines, PLC/PRF/5 were provided from ATCC. HCC cell lines, PLC/PRF/5 were provided from ATCC. HCC cell lines, PLC/PRF/5 were provided from ATCC.

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by Dr Xiaoping Chen from Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. PBMC, provided by informed consent healthy donors, are listed in online supplemental table S4 and 12 sets of human primary cells from ScienCell are listed in online supplemental table S5, including human bronchial epithelial cells. Cell culture dish was treated with Poly-L-Lysine before culturing. All cell lines were authenticated using short tandem repeat profiling and regularly tested negative for mycoplasma contamination throughout the whole duration of this study.

HLA-A2 negative HCC cell line PLC/PRF/5 has multiple, random integrations of HBV DNA and can express HBsAg. PLC/PRF/5 cells were transduced with HLA-A*02:01 subtype with pLenti-CMV-puro lentivirus and puromycin (BD Biosciences). Cells were stained with APC-conjugated anti-human-HLA-A2 monoclonal antibody (BioLegend) and positive cells were isolated by sorting on a FACS Aria III flow cytometer (BD Biosciences).

**Generation and analysis of affinity-improved TCR mutants**

We constructed Vα and Vβ chain single-chain TCR variable domain (scTv) evolution libraries using TCR0, which was isolated previously with vrt0-SIVSPFIPLL. The nonapeptide variant vrt1-ILSPFLPLL HBs371-HLA-A*02:01 complex was used as the antigen for bio-panning to isolate affinity-improved TCR variants from the libraries using methods described previously. Briefly, the scTv libraries were displayed on the surface of the filamentous bacteriophage M13, and affinity-improved scTv variants were selected with biotinylated vrt1-phLA captured on streptavidin-coated Dynabeads (Life Technologies). Binding improved scTvs were analyzed in soluble heterodimeric TCR molecules that were purified after refolding Escherichia coli-produced inclusion bodies. The TCR affinities were measured by surface plasma resonance with ProteOn and Biacore.

**TCR-T cell construction**

TCR α/β chain transgene lentiviral vectors contained a picornavirus 2A sequence. In addition, the mouse TCR constant domains replaced the human constant domains to reduce mispairing.

PBMCs, provided by healthy human volunteer donors under Institutional Review Board approved informed consent, were prepared with human Lymphoprep reagent (Axis-Shield). Peripheral blood lymphocytes (PBLs) prepared with the PBMCs were activated with Human T-Activator CD3/CD28 Dynabeads (Life Technologies) at a bead-to-cell ratio of 1:1 in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 IU recombinant human IL-2. Lentiviral vectors for WT HBsAg-TCR, affinity-improved HBsAg-TCRs and control-TCR-A6 (a TCR specific for the HTLV-1 Tax11-19 (LLFGYPVV)-HLA-A*02:01 complex) were transduced into PBLs, which were supplemented with protamine (10 µg/µl). After proliferation, T cells were stained with FITC-conjugated or APC-conjugated antibodies (BioLegend) for human CD3, CD8, TCR Vβ13.1 or mouse TCR β-chain, and a PE-conjugated HTLV-1 Tax11-19 phLA or vrt1-phLA tetramers to verify TCR expression.

**T cell activation**

Interferon gamma (IFN-γ) secretion was measured with ELISPOT kits (BD Biosciences). Briefly, 2×10⁶ positive TCR-T cells were incubated with T2 cells (HLA-A*02:01) pulsed with vrt0-vrt16 or irrelevant peptides from human antigens (ranging from 10⁻⁶ to 10⁻¹³ M), HCC cells (HLA-A*02:01 PLC/PRF/5 or HLA-A*02:01 PLC/PRF/5 cells), PBMCs or human normal primary cell lines at an effectorto-target (E:T) ratio of 1:10. To compare specificity between WT and affinity-improved TCRs, TCR-T cells were incubated with T2 cells pulsed with the irrelevant antigen SLLMWITQCHLA-A*02:01 in a range of 10⁻⁶ to 10⁻¹³ M. The other two negative controls were unpulsed T2 cells and HLA-A*02:01 HBsAg+ HepG2 cells.

**T-cell-mediated cytotoxicity**

Cell lysis was determined by releasing lactate dehydrogenase (LDH) with a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). T2 cells (5×10⁴) pulsed with vrt0-vrt16 (ranging from 10⁻⁶ to 10⁻¹³ M) were incubated with an equal amount of TCR-T cells overnight. Unpulsed T2 and 10⁻⁶ M irrelevant SLLMWITQCHLA-A*02:01 were set as negative controls. Cell-line assays were carried out with the HCC cell line PLC/PRF/5 transduced with HLA-A*02:01 at an E:T ratio of 1:1 and 3:1, and the controls were HLA-A*02:01 PLC/PRF/5 cells and HBsAg+ HLA-A*02:01 HepG2 cells. Experiments were performed in triplicate.

**Mouse xenograft models**

Immunodeficient male NOD/SCID IL2γ−/− (NSI) mice aged 6 to 9 weeks were provided by Dr Peng Li at the Guangzhou Institutes of Biomedicine and Health, CAS. Mice were maintained with daily monitoring under controlled sterile environment conditions on a 12-hours light/dark cycle, and received a rodent diet sterilized with 60Co-γ-ray irradiation and sterilized water. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Each mouse was injected subcutaneously with 3.0×10⁷ HLA-A*02:01 PLC/PRF/5 HCC cells on day 0. After the tumors reached nearly 60 mm³ (day 13), the mice were randomly assigned to groups and treated with phosphate-buffered solution (PBS), 3.0×10⁷ TCR-A6-T cells, 3.0×10⁷ TCR0-T cells or 3.3×10⁶, 1.0×10⁷ or 3.0×10⁷ TCR15-T cells via tail vein injection. Tumor volumes were measured every 3 days. From the criteria of IACUC, for subcutaneous tumors the maximum allowable size is 20 mm in diameter for a mouse. So, on day 34, the tumor-bearing mice were...
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euthanized by cervical dislocation, and tumor tissues were resected and weighed.

Detection of human T cells in tumors
Histological HCC tumor sections were stained with H&E or antibodies for IHC. Primary anti-human CD3 (Dako) and anti-human CD8 (ZSGB-BIO) monoclonal antibodies and Dako REAL EnVision Detection System were used for IHC, and DAPI (blue) stained nuclei. The sections were imaged with a LAIKA2500, and cell numbers were calculated with Image-Pro Plus 6.0.

RESULTS
Selection and analysis of affinity-improved TCRs
Commonly, phage display bio-panning for affinity improvement applies the antigens used for the isolation of WT molecules to maintain specificity. However, TCR binding shows a certain degree of promiscuity. Therefore, the nonameric antigen vrt1-ILSPFLPLL pHLA, the variant of decameric antigen vrt0-SIVSPFIPLL used to isolate the WT TCR, was employed for affinity evolution, and we named this kind of selection flexi-panning. Nineteen TCR variants with CDR3 mutations were identified and showed affinity-improved binding to the vrt1-pHLA (table 1).

Compared with TCR0-T cells (WT), TCR6-T, TCR11-T, TCR14-T, TCR15-T, TCR17-T and TCR19-T cells were Ai-TCR-T cells that could be specifically activated by T2 cells pulsed with relatively low-level antigens (figure 1A,B). Four TCR variants demonstrated stronger capabilities than WT TCR0 for redirecting T cells, with TCR14-T, TCR15-T, TCR17-T and TCR19-T cells showing significantly better half maximal effective concentration (EC50) values. TCR14-T cells and TCR15-T cells showed the best activation with the HLA-A*02:01+ PLC/PRF/5 cell line (figure 1C) despite showing relatively little improvement in the EC50 by peptide titration (online supplemental table S6).

Characteristics of TCR15 and enhanced cytotoxicity of TCR15-T cells
TCR14-T and TCR15-T cells exhibited strong cytotoxicity, as measured by assessing the lysis of vrt1-pulsed T2 cells (figure 2A,B). However, only TCR15-T cells showed specific cytotoxicity with minimal background cytotoxicity. The EC50 of vrt1-pulsed T2 cell lysis by TCR15-T cells was nearly 38-fold better than that by TCR0-T cells (online supplemental figure S2). When HBsAg-specific cytotoxicity was investigated by targeting HLA-A*02:01+ PLC/PRF/5 HCC cell lines, both WT-TCR-T cells and Ai-TCR-T cells exhibited dose-dependent target cell lysis.

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Table 1  The affinities of mutated TCRs isolated from TCR0 (WT) mutation libraries*

| TCR no. | TCR and the vrt1-pHLA | Kd (1/Ms) | Kd (1/s) | Kd (M) | Kd-TCR0/Kd-TCR15 | Mutated sequences of TCRα CDR3 |
|---------|----------------------|-----------|----------|--------|-----------------|-------------------------------|
| TCR0 (WT) | 2.58E+05 8.78E-01 3.40E-06 1.0 NLYAG |
| TCR1    | 3.04E+05 6.21E-02 2.04E-07 16.7 QDPSR |
| TCR2    | 2.54E+05 3.85E-02 1.51E-07 22.5 ADQSR |
| TCR3    | 2.55E+05 1.11E-01 4.36E-07 7.8 QDPSK |
| TCR4    | 3.35E+05 7.88E-02 2.35E-07 14.5 QDPSM |
| TCR5    | 2.13E+05 1.10E-01 5.17E-07 6.6 QDPSQ |
| TCR6    | 1.37E+05 3.44E-01 2.51E-06 1.4 QDPTN |
| TCR7    | 1.66E+05 2.42E-01 1.46E-06 2.3 QDSSR |
| TCR8    | 2.76E+05 3.47E-01 1.26E-06 2.7 QDPAK |
| TCR9    | 2.37E+05 2.57E-01 1.09E-06 3.1 QEPSR |
| TCR10   | 3.27E+05 4.11E-01 1.26E-06 2.7 QDPTK |
| TCR11   | 2.62E+05 1.41E-01 5.38E-07 6.3 AGGWR |
| TCR12   | 3.56E+05 5.53E-01 1.55E-06 2.2 QSPDR |
| TCR13   | 1.27E+05 3.27E-01 2.58E-06 1.3 QHPAT |
| TCR14   | 2.59E+05 1.57E-01 6.09E-07 5.6 ADPSK |
| TCR15   | 3.84E+05 1.77E-01 4.61E-07 7.4 AHPSK |
| TCR16   | 1.73E+05 2.97E-01 1.72E-06 2.0 QSPDQ |
| TCR17   | 1.17E+05 2.82E-01 2.42E-06 1.4 QDPS |
| TCR18   | 1.30E+05 2.27E-01 1.74E-06 2.0 QDPSH |
| TCR19   | 9.50E+04 2.34E-01 2.46E-06 1.4 QDPS |

*The antigen binding affinities of TCR mutants were determined with ProteOn analysis, and the change over the WT-TCR affinity is indicated.

pHLA, peptide-HLA complexes; TCR, T-cell receptor; WT, wild-type.
and TCR15-T cells caused significantly enhanced LDH release compared with TCR0-T cells (figure 2C).

These data demonstrated TCR15 transduced T cells with acceptable activation, killing potency and specificity. Peptide vr0-isolated TCR0 was compared with vr1-pHLA-isolated TCR15 for binding to the two distinct antigens by Biacore. The mutant TCR15 had over sevenfold and fivefold higher binding affinities for the variant vr1-pHLA and the original vr0-pHLA antigens, respectively (table 2). TCR15 also demonstrated modest affinity enhancement for binding to vr0-pHLA, despite vr1-pHLA being used as the ligand for molecular evolution. This result revealed that flexi-panning could maintain the original affinity pattern of the TCR.

Specificity and safety of TCR15-T cells

The specificity of TCR0 and TCR15 was verified, and these TCRs showed no binding with a panel of irrelevant pHLAs (online supplemental table S2). A three-step approach for an in-depth study was carried out for potential cross-reactivity on relevant pHLAs; the first step involved alanine scanning of vr0 and vr1, the second step involved searching for cross-reactive antigens in the available human proteome expression data (online supplemental tables S3.1 and S3.2), and the third step involved verifying these antigens with pulsed T2 cells. Safety was measured with a panel of normal cells. Based on the cell activation showed in the alanine scanning, the residue positions F5 and L8 of vr1 and F6 and L9 of vr0 are crucial for maintaining the interactions between the TCR and HBsAg peptide-pulsed HLA*A-02:01+ T2 cells (online supplemental figure S3A,B). We identified a total of 33 vr1-related potentially cross-reactive human peptides, 1 of which contained 2 mismatch residues and 32 of which contained 3 mismatches plus invariable F5 and L8 residues. For vr0, we identified 2 sequences containing 2 or 3 mismatches and 10 sequences
noted that most of these peptides, except high concentration (10^{-6} and 10^{-7} M) vrt1- L1, vrt1- L5, vrt1- L12, and vrt1- L19, which mainly derived from poorly expressed proteins, showed no agonist capability (figure 3A,B, online supplemental figure S4). TCR0-T cells but not TCR15-T cells appeared to be activated by T2 cells pulsed with vrt1- L13 derived from a poorly expressed protein.

To verify TCR-T cell specificity with primary cells, TCR15-T and TCR0-T cells were tested and showed no activation by any PBMCs from 30 different healthy donors (figure 3C, online supplemental table S4). Further verification of the TCR15-T cell interactions with antigens presented by normal cells was carried out with 12 sets of human primary cells (online supplemental table S5). These normal cells could not activate TCR0-T or TCR15-T cells (figure 3D).

In summary, these results demonstrated that TCR15-T cells showed HBsAg specificity similar to that of WT-TCR-T.

Table 2: The affinities of TCR0 and TCR15 for the vrt1-pHLA and vrt0-pHLA

| TCR no.      | K_a (1/Ms) | K_d (1/s) | K_D (M) | K_D^-TCR0/K_D^-TCR15 |
|--------------|------------|-----------|---------|----------------------|
| TCR0 (WT)    | 4.91E+05   | 2.68E+00  | 5.46E−06| 1.0                  |
| TCR15        | 4.19E+05   | 2.99E−01  | 7.14E−07| 7.6                  |
| TCR0 (WT)    | 3.58E+05   | 7.76E−01  | 2.17E−06| 1.0                  |
| TCR15        | 4.28E+05   | 1.67E−01  | 3.90E−07| 5.6                  |

*The affinities of the TCRs were determined with Biacore 4000, and the change over the affinity of the wild-type molecule TCR0 is indicated. pHLA, peptide-HLA complexes; TCR, T-cell receptor; WT, wild-type.
cells and at least an equivalent safety profile under a range of measurements.

**TCR15-T cells had improved recognition of HBs370 and HBs371 variants**

The specificity of flexi-panning-generated TCRs was measured with HBs371 and HBs370 variants (online supplemental figure S1, tables S1.1 and S1.2). Approximately 5855 unique full-length HBsAg protein sequences were collected from UniProt, and 16 of the most frequent HBs371- and HBs370-variant peptides represented approximately 90% of the sequences, that is, vrt1 to vrt8 were nonamer HBs371 variants, and vrt0, vrt9 to vrt16 were decamer HBs370 variants. Results for IFN-γ (figure 4A,B) and LDH release (figure 4C,D) showed that both WT and affinity-improved TCRs were able to endow T cells with the ability to recognize 11 of the 16 variants, excluding vrt4, vrt7, vrt8, vrt13 and vrt16. Epitope sequence analysis of these five non-stimulating peptides indicated that the
Ser at position three in the nonapeptide and position four in the decapeptide would be the key in the epitope. Ser is allowed at this position, while Asn or Arg will abolish the binding to the TCR. Additionally, comparing EC_{50} values made it very clear that the variant-mediated activation and cytotoxicity of TCR15- T cells were better than those of TCR0- T cells. This was consistent with the TCR affinity improvement (online supplemental table S7). Especially for vrt6, TCR15- T cells showed over 1000-fold enhancement than TCR0- T cells, which indicated no control of infection with HBV containing vrt6 variant. In conclusion, TCR15- T cells exhibited enhanced killing of T2 cells presenting the 11 most frequent variant peptides.

**Figure 4** Determination of TCR-T cell activation by and cytotoxicity to T2 cells pulsed with viral peptide variants. T2 cells pulsed with an HBsAg epitope variant peptide (10^{-6} to 10^{-13} M) were incubated with TCR-T cells overnight at an E:T ratio of 1:10. IFN-γ release was detected for vrt1-8 (A) and vrt0, vrt9-16 (B) with an ELISPOT kit. Measuring LDH release showed the percentages of specific lysis of vrt1-8 (C) and vrt0, vrt9-16 (D) (10^{-6} to 10^{-13} M) pulsed T2 cell, which were incubated with TCR0-T or TCR15-T cells at an E:T ratio of 1:1 overnight. E:T, effector-to-target; HBsAg, HBV surface antigen; HBV, hepatitis B virus; IFN-γ, interferon gamma; LDH, lactate dehydrogenase; TCR,T-cell receptor; TCR-T, TCR-engineered T cell.

TCR15-T cells could eradicate HCC xenograft tumors

To verify whether improving TCR affinity affects in vivo tumor suppression, TCR0-T and TCR15-T cells were evaluated in xenograft mouse models established with...
HLA-A*02:01+ PLC/PRF/5 HCC cells, which expressed the HBs371 nonapeptide antigen vrt2 and HBs370 decapeptide antigen vrt10 (confirmed with reverse transcription-PCR, data not show) as the agonist antigens for both TCRs (figure 4). The mice bearing tumors were injected with TCR-T cells on day 13 (figure 5A). All tumors volume enlarged on the third day after the injection; however, on the ninth day, they showed different changes. The full-dose of TCR0-T cells (3.0×10^7) produced only slight tumor-suppressive effects that were similar to those found in the mice receiving the low-dose of TCR15-T cells (3.3×10^6). However, the medium-dose of TCR15-T cells (1.0×10^7) produced significant tumor suppression. The mice in this group had only residual tumors detected on day 25, despite the tumors increased to approximately 107 mm^3 on day 34. On the other hand, more than 50% (7/14) and 85% (12/14) of the mice that received the full-dose of TCR15-T cells (3.0×10^7) showed no detectable tumors on day 23 and day 25, respectively. All tumors were eliminated by day 28 in the full-dose group, and no relapse occurred during an additional 17 days of monitoring. Thus, TCR15-T cells demonstrated superior efficacy and dose-dependent tumor suppression (figure 5B–E). These results confirmed that Ai-TCR-T cells (TCR15-T cells) had a significant advantage over WT-TCR-T cells (TCR0-T cells) in the suppression and elimination of HCC in vivo.
TCR15-T cells efficiently infiltrated tumors

Human CD3+ and CD8+ T cell infiltration was detected in tumor sites by IHC. Examining H&E-stained (figure 6A) and IHC (figure 6B,C) samples revealed that significant CD3+ and CD8+ T cell staining was observed in the tumor sections from mice treated with 3.3×10^6 (CD3+:~25.9% and CD8+:~16.8%) or 1.0×10^7 (CD3+:~57.1% and CD8+:~38.6%) TCR15-T cells. In contrast, the positive rates for CD3+ and CD8+ T cell populations detected in tumors were only ~12.1% and ~4.1%, respectively, for mice receiving 3-fold or ~10-fold more TCR0-T cells (figure 6D). It was obvious that elevated TCR15-T cell accumulation occurred in a dose-dependent manner. Furthermore, we found it intriguing that the percent of CD8+ cells in the CD3+ cell population was significantly different between the groups. Only 35% of the human T cells detected in the tumor sections of the mice infused with 3.0×10^7 TCR0-T cells were CD8+ cells. On the other hand, 66% or 72% of the human T cells detected in the tumors of the mice infused with 3.3×10^6 or 1.0×10^7 TCR15-T cells, respectively, were CD8+ cells (figure 6E). However, the initial CD8+ percentages of all tested TCR-T cells, including TCR-A6-T cells, were very similar, with an approximate average of 65.4% (online supplemental figure S5). The increased CD8+ populations were consistent with the tumor reduction data for the models. We concluded that a large quantity of CD8+ TCR15-T cells accumulated in tumor sites.

DISCUSSION

Treating HBV-related HCC has been a major challenge in the clinic. There are many ways for HCC cells to evade available treatments. One of the fundamental problems is immune escape, which ultimately hinders HCC eradication. In particular, poorly differentiated cells can...
effectively escape by downregulating antigen presentation to avoid CTL attacks, so strategies are needed to address this issue. Immunotherapies have been considered for HCC, HBV antigen-specific TCR-T and AFP-specific TCR-T are ongoing trials to address these clinic needs. We consider HBV-specific TCR-T can not only treat HCC, may also be used to treat HBV hepatitis with careful manipulated clinic practices.

Although the downregulation of pHLA presentation in tumor cells is well documented in many cancers, whether HCC cells present pHLAs at sufficient levels to activate a CTL response is still under debate. Recent studies by Dr Bertolotti’s team demonstrated that HBV antigens could be presented by HCC cells despite these antigens not being detectable by IHC techniques.

In an immune synapse, the threshold for triggering the T cell is determined by the available number of agonist ligands interacting with the TCR cluster formed at the T-cell-target-cell junction. Every TCR cluster unit requires two or four agonist pMHCs for reaching the activation threshold, which is a requirement that evolved to balance central tolerance towards self-antigens with wild-type TCRs possessing relatively low affinities for pMHCs (K_\text{d} in the range of 1 to 100 \mu M). The \text{EC}_{50} of TCR0-T cell activation by vrt1-pHLA was approximately 6.3 nM (online supplemental figure S2), and this result indicated that effective activation of this TCR might need 50 to 100 pHLAs presented per cell, which could be reduced further during the cancer development. It offers a chance for HCC to escape attacks by TCR0-T cells.

In summary, the in vitro and in vivo studies demonstrate HBsAg-specific Ai-TCR-T with significantly enhanced potency of HCC treatment, and also showed good safety profiles. We found that Ai-TCR-T were safe with no activity against human normal primary cells, and had good potency showing effective tumor infiltration and tumor clearance in mouse xenograft models. In addition, flexi-panning can generate TCR-T cells that cover several HBV antigen variants, which should facilitate the development of TCR-T cell therapy for HCC in a larger population. This is the first example using affinity-improved-TCR to redirect HBV specific T cells for addressing the problem
of HBV-related HCC. Our studies will provide supports for clinical investigations of the Ai-TCR-T for treating HBV-associated HCC.

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REFERENCES

1. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Allen C, et al. Global regional, and National cancer incidence, mortality, years of life lost, years lived with disability, and Disability-Adjusted life-years for 32 cancer groups, 1990 to 2015: a systematic analysis for the global burden of disease study. *JAMA Oncol* 2017;3:524–48.

2. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 38 cancers in 185 countries. *CA Cancer J Clin* 2018;68:394–424.

3. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012;142:1264–73.

4. de Martel C, Maucourt-Bouch D, Plummer M, et al. Worldwide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma. *Hepatology* 2015;62:1190–200.

5. Gluer AM, Cocco N, Laurence JM, et al. Systematic review of actual 10-year survival following resection for hepatocellular carcinoma. *HPB* 2012;14:285–90.

6. Faria LC, Giguou M, Roque-Afonso AM, et al. Hepatocellular carcinoma is associated with an increased risk of hepatitis B virus recurrence after liver transplantation. *Gastroenterology* 2008;134:1890–9.

7. Gehring AJ, Ho ZZ, Tan AT, et al. Profile of tumor antigen-specific CD8 T cells in patients with hepatitis B virus-related hepatocellular carcinoma. *Gastroenterology* 2009;137:882–90.

8. June CH. Adaptive T cell therapy for cancer in the clinic. *J Clin Invest* 2007;117:1466–76.

9. Brechot C, Pourcel C, Louise A, et al. Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. *Nature* 1980;286:533–5.

10. Dhanasekaran R, Nault J-C, Roberts LR, et al. Genomic medicine and implications for hepatocellular carcinoma prevention and therapy. *Gastroenterology* 2019;156:492–509.

11. Buschow SI, Sprenger D, Wolman AM. To target or not to target viral antigens in HBV-related HCC? *J Hepatol* 2015;62:1440–50.

12. Tan AT, Yang N, Lee Krishnamoorthy T, et al. Use of expression profiles of HBV-DNA integrated into genomes of hepatocellular carcinoma cells to select T cells for immunotherapy. *Gastroenterology* 2019;156:1862–76.

13. Qasim W, Brunetto M, Gehring AJ, et al. Reply to: “To target or not to target viral antigens in HBV related HCC?”. *J Hepatol* 2015;62:1450–2.

14. Gehring AJ, Xue S-A, Ho ZZ, et al. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. *J Hepatol* 2018;69:103–10.

15. Koh S, Shimasaki N, Suvarnasuk R, et al. A practical approach to immunotherapy of hepatocellular carcinoma using T cells redirected against hepatitis B virus. *Mol Ther Nucleic Acids* 2021;3:e114.

16. Barros N, Chia A, Ho ZZ, et al. Building and optimizing a virus-specific T cell receptor library for targeted immunotherapy in viral infections. *Sci Rep* 2014;4:4166.

17. Qasim W, Brunetto M, Gehring AJ, et al. Immunotherapy of HCC metastases with autologous T cell receptor redirected T cells, targeting HBsAg in a liver transplant patient. *J Hepatol* 2015;62:486–91.

18. Koh S, Tan AT, Li L, et al. Targeted therapy of hepatitis B virus-related hepatocellular carcinoma: present and future. *Diseases* 2016;4:10.

19. Wang Y, Wu M-C, Sham JST, et al. Different expression of hepatitis B surface antigen between hepatocellular carcinoma and its surrounding liver tissue, studied using a tissue microarray. *J Pathol* 2002;197:610–6.

20. Tantiwetrueangdet A, Panvichian R, Sornmayura P, et al. Insights from deep sequencing of the HBV Genome- Unique, tiny, and misunderstood. *Sci Rep* 2015;5:1019–27.

21. Varela-Rohena A, Molloy PE, Dunn SM, et al. Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nat Med* 2008;14:1390–5.

22. Liddy N, Bossi G, Ansari MA, et al. Monoclonal TCR redirected-tumor killing. *J Exp Med* 2015;203:1643–9.

23. Rao AN, Chacko C, Tron TP, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin Cancer Res* 2015;21:1019–27.

24. McNaughton AL, D’Arienzo V, Ansari MA, et al. Targeted therapy of hepatitis B virus-related hepatocellular carcinoma: present and future. *Diseases* 2016;4:10.

25. Robbins PF, Kassim SH, Tran TLN, et al. Directed evolution of human T-cell antigen recognition. *Annu Rev Immunol* 2018;35:127.

26. van der Merve PA, Davis SJ. Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol* 2003;21:659–84.

27. Li Y, Mossey R, Molloy PE, et al. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nat Biotechnol* 2006;24:349–54.

28. Selin LK, Welsh RM. Plasticity of T cell memory responses to viruses. *Immunity* 2004;20:5–16.

29. Hao Y, Legrand N, Freitas AA. The clone size of peripheral CD8 T cells is regulated by TCR promiscuity. *J Exp Med* 2006;203:1643–9.

30. Hoof I, Pérez CL, Buggert M, et al. Interdisciplinary analysis of HIV-specific CD8+ T cell responses against variant epitopes reveals restricted TCR promiscuity. *J Immunol* 2010;184:5383–91.

31. Zanker D, Quinn K, Waltham J, et al. T cells recognizing a 11mer influenza peptide complexed to H-2D(b) show promiscuity for restricted TCR binding. *J Hepatol* 2015;62:1440–50.

32. Liu Q, et al. J Immunother Cancer 2020;8:e001748. doi:10.1136/jitc-2020-001748
34 Yang H-J, Yeh S-H, Chen P-J, et al. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. *J Natl Cancer Inst* 2008;100:1134–43.
35 Liu S, Zhang H, Gu C, et al. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *J Natl Cancer Inst* 2009;101:1066–82.
36 Haase K, Raffegerst S, Schendel DJ, et al. Expitope: a web server for epitope expression. *Bioinformatics* 2015;31:1854–6.
37 Jaravine V, Mösch A, Raffegerst S, et al. Expitope 2.0: a tool to assess immunotherapeutic antigens for their potential cross-reactivity against naturally expressed proteins in human tissues. *BMC Cancer* 2017;17:892.
38 Zhang H, Zhang J, Chen L, et al. Targeting naturally occurring epitope variants of hepatitis C virus with high-affinity T-cell receptors. *J Gen Virol* 2017;98:374–84.
39 Ye W, Jiang Z, Li G-X, et al. Quantitative evaluation of the immunodeficiency of a mouse strain by tumor engraftments. *J Hematol Oncol* 2015;8:59.
40 Zhu W, Peng Y, Wang L, et al. Identification of α-fetoprotein-specific T-cell receptors for hepatocellular carcinoma immunotherapy. *Hepatology* 2018;68:574–89.
41 Docta RY, Ferronha T, Sanderson JP, et al. Tuning T-cell receptor affinity to optimize clinical risk-benefit when targeting Alpha-Fetoprotein-Positive liver cancer. *Hepatology* 2019;69:2061–75.
42 Ogino T, Shigyo H, Ishii H, et al. Hla class I antigen down-regulation in primary laryngeal squamous cell carcinoma lesions as a poor prognostic marker. *Cancer Res* 2006;66:9281–9.
43 Kageshita T, Ishihara T, Campoli M, et al. Selective monomorphic and polymorphic HLA class I antigenic determinant loss in surgically removed melanoma lesions. *Tissue Antigens* 2005;65:419–28.
44 Hicklin DJ, Marincola FM, Ferrone S, Hla class I antigen down-regulation in human cancers: T-cell immunotherapy revives an old story. *Mol Med Today* 1999;5:178–86.
45 Lei W-Y, Hsing S-C, Wen S-H, et al. Total HLA class I antigen loss with the downregulation of antigen-processing machinery components in two newly established Sarcomatoid hepatocellular carcinoma cell lines. *J Immunol Res* 2018;2018:1–12.
46 Kurokohchi K, Carrington M, Mann DL, et al. Expression of HLA class I molecules and the transporter associated with antigen processing in hepatocellular carcinoma. *Hepatology* 1996;23:1181–8.
47 Fujiwara K, Higashi T, Nousu K, et al. Decreased expression of B7 costimulatory molecules and major histocompatibility complex class-I in human hepatocellular carcinoma. *J Gastroenterol Hepatol* 2004;19:1121–7.
48 Shen Y, Xia M, Zhang J, et al. Irf-1 and p65 mediate upregulation of constitutive HLA-A antigen expression by hepatocellular carcinoma cells. *Mol Immunol* 2009;46:2045–53.
49 Zhou J, Ma P, Li J, et al. Improvement of the cytotoxic T lymphocyte response against hepatocellular carcinoma by transduction of cancer cells with an adeno-associated virus carrying the interferon-γ gene. *Mol Med Rep* 2016;13:3197–205.
50 Dustin ML, Depoil D. New insights into the T cell synapse from single molecule techniques. *Nat Rev Immunol* 2011;11:672–84.
51 Manz BN, Jackson BL, Petit RS, et al. T-Cell triggering thresholds are modulated by the number of antigen within individual T-cell receptor clusters. *Proc Natl Acad Sci U S A* 2011;108:9089–94.
52 Bossi G, Gerry AB, Paston SJ, et al. Examining the presentation of tumor-associated antigens on peptide-pulsed T2 cells. *Oncoimmunology* 2013;2:e26840.
53 McMahon BJ, The natural history of chronic hepatitis B virus infection. *Hepatology* 2009;49:S45–55.
54 Bijen HM, van der Steen DM, Hagedoorn RS, et al. Preclinical strategies to identify off-target toxicity of high-affinity TCRs. *Mol Ther* 2018;26:1206–14.
55 Border EC, Sanderson JP, Weissensteiner T, et al. Affinity-enhanced T-cell receptors for adoptive T-cell therapy targeting MAGE-A10: strategy for selection of an optimal candidate. *Oncoimmunology* 2019;8:e1532759.
56 Coles CH, Mulvaney RM, Malla S, et al. TCRs with distinct specificity profiles use different binding modes to engage an identical Peptide-HLA complex. *J Immunol* 2020;204:1943–53.