Personalized neoantigen vaccine combined with PD-1 blockade increases CD8$^+$ tissue-resident memory T cell infiltration in preclinical hepatocellular carcinoma models

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Supplementary Methods

Cell line

Murine HCC cell lines Hepa1-6 cells were obtained from ATCC. Luciferase-expressing Hepa1-6 (Hepa1-6-luc) cells were established through transfection of lentivirus expressing luciferase reporter gene (Shanghai Genechem Co., Ltd) for 48h. Following lentiviral infection, 2μg/mL of Puromycin was used to screen

the stable expressed cells. Hepa1-6 cells and Hepa1-6-luc cells were cultured in Dulbecco's modified eagle medium (DMEM) medium with 10% FBS at 37°C with 5% CO_2.

Neoantigen Identification and immunogenicity validation

DNA and RNA were extracted from Hepa1-6 cells and C57BL/6 mouse tail tissue were subjected to whole exome sequencing and transcriptomic sequencing using Genomic DNA kit (Tiangen biotech, China) and EasyPure RNA kit (Transgen Biotech, China) according to the manufacturer's instructions. Those DNA samples were subjected to DNA library preparation and whole-exome capture using SureSelect XT Mouse All Exon Kit and RNA samples were subjected to RNA library preparation using VAHTS Stranded mRNA-seq Library Prep Kit. Then DNA and RNA library sequencing were performed by Berry Genomics (Bejing) on illumina Novoseq 6000 (paired end, 150bp).

Mutation variants of Hepa1-6 cells were called using VarScan software with mouse genome mm10 as reference and only mutations with variant allele frequency (VAF) >10% and depth more than 20× were retained. Remaining variants were annotated with wANNOVAR to filter nonsynonymous mutations. To acquire high quality mutations for identifying neoantigen, mutations with less than 20 variant allele reads were excluded and then mutations with variant allele frequency higher than 60% were also discarded to filter out possible germline mutations.

All mutations were further validated at RNA level by bam2R algorithm from R package DeepSNV (v1.24.0), and mutations with ≥ 20× depth as well as VAF ≥ 10%

in RNA-seq data and located in genes with adequate expression (TPM>1) were retained.

Immunogenicity of all mutations were evaluated using NetMHCpan binding affinity predictor, and mutations that produce 9-mer mutant peptides with IC50 < 500nM to H2-K^b allele were considered as candidate neoantigens. Then these candidates were assessed in C57BL/6 mouse tail tissues via DeepSNV, and mutations with a depth more than 20× and no variant allele detected in all three mouse tail tissues were further included in the downstream study.

Neoantigen immunogenicity validation

To identify potential neoantigen peptides, 26 neoantigen mutations from Hepa1-6 cells were selected and 20 long peptides (17 amino acids in length) of them were successfully synthesized by the standard solid-phase synthetic peptide chemistry (> 95% purity, Genscipt Biotechnology co., Ltd, China). For neoantigen immunogenicity validation, 20 neoantigen peptides were randomly divided into 2 pools (2μg/peptide) and then mixed with 50μg Poly(I:C) (Guangdong South China Pharmaceutical. co., Ltd) for subcutaneously immunizing of male C57BL/6 mice at the lateral flank on day 0, day 4 and day 8, respectively. Then, the mice were sacrificed on day 14 and the splenic T cells were freshly harvested by generating a single-cell suspension through passing the spleen through a 40-μm filter in RPMI-1640 medium (1% penicillin/streptomycin) and then isolated by Ficoll density gradient centrifugation. Erythrocytes were removed by 1 mL Red Blood Cell Lysis Solution (Gibco), and the resulting splenic T cells were washed twice by PBS and counted for ELISPOT assay.

Enzyme-linked immunospot (ELISPOT) assay

IFN-γ secretion of mouse splenic T cells were detected by ELISPOT kit (Mabtech，3321-4APT-10). Bone marrow derived-DCs (BMDCs) were obtained as follows: Femurs and tibias from 6-8 weeks old naive C57BL/6 mice were isolated. After removal of residual soft tissue and epiphyses, the marrow was collected by flushing the canals with PBS and centrifuged at 800g for 5min at room temperature. The precipitated cells were lysed with 1mL Red Blood Cell Lysis Solution (Gibco) for 4 minutes, centrifuged at 800g for 5 minutes, and washed twice with PBS. At day 0, 2 million cells/well were added into a 6-well plate and cultured with 2 mL RPMI-1640 medium/well (10 ng/mL IL-4, 404-ML-010/CF and 20 ng/mL mGM-CSF, R&D systems, 415-ML-020/CF) to obtain BMDCs at 37°C with 5% CO_2. Half volume of medium was changed at day 3. At day 6, BMDCs were pulsed with neoantigen peptide pool (4μg in total，0.55μg per peptide) or each peptide (4μg) for 48h. For ELISPOT assay, 3×10^4 BMDCs (pulsed with neoantigen peptide as described previously) were co-incubated with 3×10^5 splenic T cells or 3×10^4 sorted splenic $CD8^+$/$CD4^+$ T cells in a multiscreen 96-well filtration plate (Mabtech，3321-4APT-2) at 37℃ with 5% CO_2 for another 48h. The plates were washed and subsequently incubated with detection antibody (R4-6A2-biotin,1 μg/mL, 100μl/well) for 2 hours at room temperature. The plates were washed again and then incubated with Streptavidin ALP (1:1000 dilution, 100μl per well) for 1 hour at room temperature. Subsequently, 3, 3', 5, 5'-T etramethylbenzidine (TMB) substrate solution was added to each well and incubated for 4-8 min at room temperature before adding deionized water to stop

the reaction. Finally, IFN-γ spot-forming cells were imaged and analyzed by ELISPOT Analysis System (AT-Spot-2200, Beijing Antai Yongxin Medical Technology Co., Ltd).

In vivo antitumor efficacy evaluation

To establish subcutaneous HCC model, 3×10^6 Hepa1-6 cells were transplanted subcutaneously into the armpits of mice for 10 days. The orthotopic HCC mouse model was established as follows: C57BL/6 mice were intraperitoneally anesthetized with 50mg/kg of pentobarbital. Then, the skin was prepared aseptically and midline laparotomy was performed after the abdomen was shaved. Afterwards, liver subcapsular inoculation with 3×10^5 Hepa1-6-luc tumor cells mixed with the matrigel plugs. Finally, the abdominal muscle and skin were fully-layer sutured.

For neoantigen vaccine optimization and combinational treatment, subcutaneous HCC model and/or the orthotopic HCC model were randomly divided into 4~10 groups (n=5) as indicated and then subcutaneously injected with identified neoantigen peptides (2μg/peptide) mixed with each adjuvant (Pam3CSK4: 10μg per mouse; Poly(I:C): 50μg per mouse; Mpla: 25μg per mouse; Flagellin: 4μg per mouse; R848: 10μg per mouse; CpG ODN: 10μg per mouse) or Poly(I:C) alone in 200μl volume on day 0, day 4 and day 8, respectively. α-PD1 (BioLegend, 124328) was injected intravenously (50μg per mouse). Mice treated with PBS were used as control. Tumor burden in subcutaneous HCC model was monitored every 3 days by vernier caliper. Tumor burden in orthotopic HCC model was monitored every 10 days by IVIS Spectrum animal imaging system (PerkinElmer, USA) after intraperitoneal injection

of fluorescent substrates (D-luciferin potassium salt, Xenolight^{TM}) about 5 min, then the mice were exposed for 2s to obtain bioluminescence images.

Immunofluorescence.

Tumor isolated from mouse as mentioned above was formalin-fixed and paraffin-embedded. Sections of 4-μm thickness were prepared on slides, and air dried at room temperature. Following blocking, slides were incubated with primary antibodies (rabbit anti-mouse CD4 mAb, Servicebio, GB13064-1), rabbit anti-mouse CD8 mAb (Servicebio, GB13068) overnight at 4℃ and secondary antibodies (Wuhan servicebio technology CO., LTD) for 30min at room temperature. The immuno-stained slides (5 slides per mouse; 3 mice) were digitized at 10/40 magnification (objective lens) and crop images were collected by fluorescent microscope (NIKON ECLIPSE C1).

Immunohistochemistry.

Tumor of each mouse was formalin-fixed and paraffin-embedded. Sections of 4-μm thickness were prepared on slides, and air dried at room temperature. Following blocking, slides were incubated with primary antibody (rabbit anti-mouse PD-L1 mAb, Cell Signaling Technology, 64988) overnight at 4℃ and secondary antibody (Fuzhou Maixin Biothech CO., Ltd) for 30min at room temperature. The immuno-stained slides (5 slides/mouse; 3 mice) were digitized at ×10/40 magnification (objective lens) and crop images were collected.

HE staining

HE staining was conducted according to routine protocols. Briefly, after

deparaffinization and rehydration, organ sections including heart, liver, spleen, lung and kidney, were stained with hematoxylin solution (Servicebio, G1004) for 5 min followed by 5 dips in 1% acid ethanol (1% HCl in 75% ethanol) and then rinsed in distilled water. Then the sections were stained with eosin solution (Servicebio, G1001) for 3 min and followed by dehydration with graded alcohol and clearing in xylene. The HE-stained slides (5 slides/mouse; 3 mice) were digitized and crop images were collected.

Tissue processing for flow cytometry.

Tumor specimens from mice were cut into pieces and then minced with surgical scissors at day 12 after initial treatment. Minced specimens were digested in 5 mL of RPMI-1640 medium, containing 1mg/mL collagenase type II (Sigma, C6885) and 2 mg/mL DNase I (Sigma, DN25), at 37 °C for 1h with gentle stirring. After digest, an equal volume of cold flow buffer (PBS with 5% BSA) was added. Samples were then filtered through a 40μm cell strainer. The single-cell suspension was centrifuged at 800g for 20 min and resuspended in flow buffer at a concentration of 1×10^7 cells per 1mL. Tissue and mononuclear cells were isolated by using Ficoll density gradient centrifugation.

To isolate spleen cells, the spleens were excised and smashed with a syringe plunger. Mononuclear cells were isolated by using Ficoll density gradient centrifugation; afterwards, the single-cell suspension was centrifuged at 800g for 5 min and resuspended in flow buffer at a concentration of 1×10^7 cells per 1mL.

For the lymph node lymphocyte isolation, the lymph nodes were mashed on a

40μm cell strainer with a syringe plunger. The single-cell suspension was centrifuged at 800g for 5 min and resuspended in flow buffer at a concentration of 1×10^7 cells per 1mL.

Flow cytometry and tetramer staining.

For flow cytometry analysis, single-cell suspensions from tumor (as described above), spleen and lymph nodes were stained in PBS (0.5% BSA) for 30 min with following antibodies for 45 min, rotating in the dark, including anti-mouse CD69-APC mAb (eBioscience™, 17-0691-82), anti-mouse CD3-APC/FITC mAb (eBioscience™, 17-0032-82/11-0037-42), anti-mouse CD8-PE/FITC mAb (eBioscience™, 12-0081-82/11-0081-82), anti-mouse CD62L-PerCP/Cy5.5 mAb (eBioscience™, 45-0621-82), anti-mouse CD44-PE-Cyanine7 mAb (eBioscience™, 25-0441-82), anti-mouse CD80-PE mAb (eBioscience™, 12-0801-82), anti-mouse CD86-PE-Cyanine7 mAb (eBioscience™, 25-0862-82), anti-mouse CD11c-APC mAb (eBioscience™, 17-0114-82), anti-mouse CD137-APC mAb (eBioscience™, 17-1371-82) and anti-mouse CD279-PE-Cyanine7 mAb (eBioscience™, 25-9985-82). Flow cytometry and the fluorescence minus one (FMO) controls were performed on a flow cytometer (BD FACSVerse™, USA) and data were analyzed using FlowJo v.10.

The anti-mouse peptide-specific tetramer-PE was generated with QuickSwith™ Quant Tetramer Kit (MBL international, TB-7400-K1) according to the manufacturer's instruction. Briefly, 50μl Tetramer (50μg/mL) was mixed with 1μl peptide solution (10nM) and 1μl proprietary Peptide Exchange Factor and incubated for 4 hours at room temperature in dark. The exchanging efficiency was evaluated by

a flow cytometer (BD FACSVerse™, USA) as described in the manual and qualified peptide-MHC I-tetramers were stored at 4℃ until use. For tetramer staining, single-cell suspensions from tumor (as described above) were blocked with mice Cohn fraction IgG (20μl per sample) for 5 min and then stained with 20μl of anti-mouse Tetramer-PE for epitopes ($Ptpn2_{376-384}$ (RWLYWQPTL):H-$2K^b$, MBL) with 30 min at room temperature. After tetramer preincubation, cells were stained with anti-mouse CD8-FITC mAb (eBioscience™, 12-0081-82) for 20min, rotating in the dark. Flow cytometry was performed on a flow cytometer (BD FACSVerse™, USA) and data were analyzed using FlowJo v.10.

Recurrence and metastasis rechallenge experiments

The long-term immune memory efficacy of combinational therapy was studied by using orthotopic Hepa1-6-luc tumor bearing mice model as described above. Ten days after cured by NeoVAC plus α-PD1, all mice were injected with 1×10^5 of Hepa1-6-luc cells orthotopically again (naive mice were included as the control). Fifty days after the first treatment, all mice were injected with 2×10^6 of Hepa1-6-luc cells via tail vein. The Hepa1-6-luc tumor burden in C57BL/6 mice was monitored by the IVIS Spectrum animal imaging system as mentioned above.

Flow cytometric sorting

Single-cell suspensions (as described above) underwent Fc block with RPMI-1640 containing 5% BSA for 10 min on ice. Samples were then stained with 1:100 dilutions of anti-mouse CD3-FITC mAb, (eBioscience™, 17-0032-82), anti-mouse CD8-PE mAb (eBioscience™, 12-0081-82) for ELISPOT assay to test

which T cell subset was activated by each neoantigen peptide, anti-mouse CD45-APC mAb, (eBioscience™, 17-0451-82) for single cell RNA-sequencing (RNA-seq) and anti-human/anti-mouse CD3-FITC mAb, (eBioscience™, 11-0037-42/17-0032-82), anti-human/anti-mouse CD8-PE mAb (eBioscience™, 12-0088-42/12-0081-82) and anti-human/anti-mouse CD69-APC mAb, (BioLegend/eBioscience™, 310910/17-0691-82) for in vivo T cell cytotoxicity assay and adoptive cell therapy. Samples were stained in the dark for 30 min on ice, washed twice with flow buffer and resuspended in PBS with 0.5% BSA. Sorting was performed on a Fusion cell sorter (BD FACSAria^{TM}, USA). Tumor specimens were sorted in their entirety. All samples from a single mouse were sorted on the day of specimen collection.

Single-cell RNA sequencing (scRNA-seq) library construction and data analysis.

$CD45^+$ T cells were sorted from HCC tumor in orthotopic HCC model treated with Neoantigen and/or α-PD1 by FACS and subjected to scRNA-seq libraries preparation by using the 10×Genomics Chromium Single Cell 5' Library & Gel Bead reagent kit and Chromium Single Cell V(D)J Enrichment Kit. scRNA-seq libraries were prepared by using the 10×Genomics Chromium single cell 5' v2 reagent kit. scRNA-seq reads acquired from 10×genomics platform were aligned using Cell Ranger (v5.0.1, 10×Genomics) to reference genome (mm10) with default parameters. After mapping, qualified UMI reads and cells were further filtered with Seurat (v4.0.3) in R (v4.1.0) using following criteria: genes detected in less than 3 cells; cells with less than 500 genes detected; cells with more than 5000 genes detected; cells with more than 5% UMI reads mapped to mitochondrial genes; cells with corresponding

highest expressed gene count for more than 20% UMI reads.

After removing low-quality cells and likely doublets, a total of 5982, 8424, 7351 and 11007 cells were retained for combinational therapy, neoantigen vaccine treatment alone, α-PD1 treatment alone and control group respectively. To gain an intuitive comparison and visualization, 5900 cells of each group was randomly selected and included for following analysis. Then the gene expression levels were normalized using the NormalizeData function with the LogNormalize method. The t-SNE dimensionality reduction was conducted and Seurat functions FindNeighbors and FindClusters were used for cell clustering. Then cell clusters were annotated by SingleR package (v1.6.1) using the annotation from combining Immgen data set and MouseRNAseq data set. To further analyze the heterogeneity of T cells, T cell clusters (c1 and c2) were extracted and re-clustered using t-SNE algorithm in Seurat.

Single-cell V(D)J analysis

T-cell library was analyzed using Cell Ranger (v5.0.1, 10× Genomics). The "vdj" command was used to generate sequence annotations with default parameters using reference refdata-cellranger-vdj-GRCm38-alts-ensembl-5.0.0 downloaded from 10×-genomics website. Then "filtered_contig_annotations.csv" of each sample produced by Cell Ranger was future loaded and reanalyzed by R package scRepertoire (v1.3.2). **$CD8^+$ T cell developmental trajectory**

The cell lineage trajectory of $CD8^+$ T cell clusters and corresponding trajectory plot were derived by Monocle2. After extraction of $CD8^+$ T cell clusters, genes with expression detected in less than 10 cells were removed. Then genes with q-value <

0.01 in ‘‘differentialGeneTest’’ function were used to order the cells in pseudotime analysis.

Cell-cell interaction analysis

To identify potential cell-cell interactions between $CD8^+$ T_{RMs} and other T cell clusters, we used Cellchat to identify significant ligand-receptor pairs. The ligand-receptor pairs with $p < 0.05$ were considered as significantly interacted in two cell types.

Prognostic assessment using TCGA data

RNA-seq and clinical data of HCC patients in The Cancer Genome Atlas (TCGA) was downloaded from GDC data portal. 371 patients with available transcriptome data for tumor samples were included. Patients were stratified into two groups with median expression level of CD69_CD8A two-gene signature, which was assessed by ssGSEA. Survival curves between two groups were estimated using the Kaplan-Meier method, and the log-rank test was performed to determine the significance.

Bulk RNA-seq data analysis

For RNA-seq data of orthotopic tumor treated as described above, the raw sequencing reads were first preprocessed with fastp (v0.21.0) to remove unqualified reads. Remaining reads were aligned to mm10 mouse genome using STAR (v2.7.8a). To quantify the expression levels for each gene, TPM was calculated by RSEM (v1.3.0).

In vitro T cell cytotoxicity assay on Hepa1-6 cells.

To assess the tumor-killing ability of $CD8^+CD69^+$ T cells in vitro, orthotopic

HCC tumor-bearing mice were treated with NeoVAC plus and α-PD1 as described above and were sacrificed 12 days after initial treatment. $CD8^+CD69^-$/$CD8^+CD69^+$ T cells in single-cell were sorted from fresh tumor excisions as described above. And then, $CD8^+CD69^-$/$CD8^+CD69^+$ T cells (3×10^4 cells per well) and Hep1-6 cells (1×10^4 cells per well) were incubated for 48 hours in a 96-well plate with IL-2 (10ng/ml, R&D systems, MX2918061) at 37°C with 5% CO_2. The cells were collected and co-stained by anti-mouse CD3-APC mAb (eBioscience™, 17-0031-82), annexin V-FITC and Propidium Iodide at 1.5 μg/mL, and detected the apoptosis of CD3-negative cells by using flow cytometer (BD FACSVerse™, USA). Culture medium were filtered and collected for ELISA analysis (TNF-α, Boster, EK0527; IFN-γ, Boster, EK0375) according to the manufacturer's instruction.

In vivo T cell therapy experiment

For in vivo T cell therapy experiment, orthotopic HCC model was constructed as described above and then tumor-bearing mice were treated with FACS sorted $CD8^+CD69^-$ T cells and $CD8^+CD69^+$ T cells (2×10^5 per mouse, mice in control group were treated with PBS). The T cells were sorted from tumor-bearing mice treated with combinational therapy (described above). Control mice receiving PBS treatment were included as the control. All treated animals received daily injections of 1×10^3 IU IL-2 infusion for 5 days. All tumor measurements were performed double-blinded by an independent investigator. The Hepa1-6-luc tumor burden in C57BL/6 mice was monitored by the IVIS Spectrum animal imaging system (PerkinElmer, USA) as mention above.

In vitro T cell cytotoxicity assay on PDCs.

For PDC culture, fresh tumor excisions of HCC patients were obtained from surgical resection. The human primary hepatoma cells were isolated by collagenase perfusion and centrifugation as previously described. Briefly, the HCC tissues were washed several times in pre-cooled sterile PBS buffer (1% penicillin/streptomycin) to remove blood and connective tissue; GBSS mixed enzyme solution was used for digestion. The cells were centrifuged, and the supernatant was discarded. Cells viability and counting were performed using trypanosoma blue staining with cell filtrate, and cultured in a 96-well plate containing complete medium heavy suspension at 37°C with 5% CO_2. After the cell adheres to the wall, the cell morphology was identified.

To assess the tumor-killing ability of $CD8^+CD69^+$ T cells on PDCs, $CD8^+CD69^-$/$CD8^+CD69^+$ T cells were sorted from fresh tumor excisions of HCC patients as described above. And then, $CD8^+CD69^-$/$CD8^+CD69^+$ T cells (3×10^4 cells per well) and PDCs (1×10^4 cells per well) were incubated for 48 hours in a 96-well plate with IL-2 (10ng/mL, R&D systems, MX2918061) at 37°C with 5% CO_2. The cells were collected and co-stained by anti-human CD3-APC mAb (eBioscience™, 17-0031-82), annexin V- FITC and Propidium Iodide at 1.5 μg/mL, and the apoptosis of CD3-negative cells was detected by using flow cytometer (BD FACSVerse™, USA)).

Institutional review board–approved written, informed consent was obtained from HCC patients to perform for analysis. All human sample studies were performed

in accordance with ethical regulations, and preapproved by the Ethics Review Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University (KESHEN 2021_110_01).

Supplementary Figure legends：

Figure S1. Flow cytometry gating strategies. A. Representative images of gating strategy and fluorescence minus one (FMO) controls to assess the percentage of infiltrating $CD8^+$ T cells expressing PD-1 and 4-1BB in subcutaneous HCC model. B. Representative images of gating strategy and FMO controls to assess the percentage of matured DCs (CD11c+) in LNs. C. Representative images of gating strategy and FMO controls to assess the percentage of central memory $CD8^+$ T cells (T_{CM}, $CD44^+$ and $CD62L^+$) in spleen. D. Representative images of gating strategy and FMO controls to assess the percentage of $CD8^+$ T cells expressing PD-1 and 4-1BB in EILSPOT assay. E. Representative images of gating strategy and FMO controls to assess the percentage of infiltrating $CD8^+$ T cells expressing PD-1 and 4-1BB in orthotopic HCC model. F. Representative images of gating strategy and FMO controls to assess the percentage of $Ptpn2_{376\text{-}384}$ (RWLYWQPTL):H-$2K^b$ specific $CD8^+$ T cells in infiltrating $CD8^+$ T cells. G. Representative images of gating strategy and FMO controls to assess the percentage of $Ptpn2_{376\text{-}384}$:H-$2K^b$ specific $CD8^+$ T cells in blood. H. Representative images of gating strategy and FMO controls to assess the percentage of $CD8^+$ T_{RMs} in infiltrating $CD8^+$ T cells and the percentage of $Ptpn2_{376\text{-}384}$:H-$2K^b$ specific $CD8^+$ T cells in infiltrating $CD8^+$ T_{RMs}. I. Representative images of gating strategy and FMO controls to assess the percentage of apoptotic cells induced by $CD8^+CD69^+$ or $CD8^+CD69^-$ TILs. J. Representative images of gating strategy and FMO controls to assess the percentage of apoptotic cells induced by $CD8^+CD69^+$ or $CD8^+CD69^-$ TILs in PDCs. The corresponding FMO controls are represented in the

curve boxes.

Figure S2. T cell subsets primarily activated by each neoantigen peptide. A. ELISPOT assay showing neoantigen specific-reactivity of splenic $CD8^+$ or CD4+ T cell subset against each neoantigen peptide.

Figure S3. Immune response evaluation for adjuvant optimization. A. ELISPOT assay showing neoantigen specific-reactivity of splenic T cells from each group treated with neoantigen peptides pulsed with different adjuvants against the pool of 7 neoantigen peptides pool. B. ELISPOT assay showing neoantigen specific-reactivity of splenic T cells against 7 neoantigen peptides in mice treated with Poly(I:C) alone. C. Total radiance changes of tumor burden in mice treated as indicated (n=5 for all the groups) by bioluminescence imaging. Data collection of a group stopped when deaths were observed in the group. Neo, neoantigen peptides. The statistical analysis was performed with ANOVA analysis, Results are shown as mean ± SEM.

Figure S4. Antitumor efficacy of each neoantigen peptide in orthotopic HCC model. A. Treatment timeline for each neoantigen peptide pulsed with Poly(I:C) treatment in orthotopic HCC model. B. Tumor burden monitoring of each group (n=5) treated with each single neoantigen peptides (Mapk3_S284F, Lmf1_F523V, Samd91_K752M, Traf7_C403W, Dtnb_K40T, Lbr_A341P, Ptpn2_I383T) or all 7 peptides pulsed with Poly(I:C) by bioluminescence imaging. Data collection of a group stopped when deaths were observed in the group. Neo, neoantigen peptides. C. Total radiance changes as indicated, n=5, The statistical analysis was performed with ANOVA analysis. Results are shown as mean ± SEM.

Figure S5. Exploration of the resistant mechanism to NeoVAC. A. Flow cytometry showing the percentage of infiltrating $CD8^+$ T cells expressing PD-1. Blue represents control and red represents TILs from NeoVAC treated mice. B. Immunohistochemistry showing the expression of PD-L1 in tumor tissues. Scare bars, 100μm (10×), 40μm (40×).

Figure S6. Tumor therapeutic effect of NeoVAC plus α-PD1 treatment in orthotopic HCC model. A. Tumor burden monitoring of PBS, NeoVAC alone, α-PD1 alone and NeoVAC plus α-PD1 treated mice by bioluminescence imaging (n=5). B. Tumor weight of each group treated with PBS, NeoVAC alone, α-PD1 alone and NeoVAC plus α-PD1 (n=10) at the end of the experiment or the time mice died spontaneously. Results are shown as mean ± SD. C. The HE staining of tumors from one of the mice received different treatment as indicated. Scare bars, 20μm (2×), 100μm (20×).

Figure S7. In vivo safety evaluation of NeoVAC plus α-PD1 treatment. A-M. Blood plasma analysis for ALB (albumin), TBIL (total bilirubin), ALT (alanine aminotransferase), AST (aspartate aminotransferase), γ-GGT (Gamma-glutamyltransferase), AKP (alkaline phosphatase), CK (creatine kinase), Urea, SCr (serum creatinine), Glu (glucose), TG (triglyceride), TCHO (total cholesterol) during treatment for all groups (n=5) as indicated, N. HE staining for organs including heart, liver, spleen, lung and kidney in mice treated with NeoVAC plus α-PD1, Scare bars, 100μm (10×), 40μm (40×). O. Body weight change during treatment for all groups (n=10) as indicated. The statistical analysis was performed

with ANOVA analysis, Results are shown as mean ± SD.

Figure S8. Immune activation by NeoVAC plus α-PD1 treatment. A. Flow cytometry analysis showing the percentage of matured DCs (CD11c+) in LNs after treated with PBS, NeoVAC alone, α-PD1 alone and NeoVAC plus α-PD1 at day 12 after treatment. B. Flow cytometry analysis showing the percentage of central memory $CD8^+$ T cells (T_{CM}, $CD44^+$ and $CD62L^+$) in spleen at day 12 after treatment. C. The histogram of absolute number of $Ptpn2_{376-384}$ (RWLYWQPTL):H-$2K^b$ specific $CD8^+$ T cells in infiltrating $CD8^+$ T cells. The statistical analysis was performed with ANOVA analysis, n=3 for all the groups. Results are shown as mean ± SD.

Figure S9. Long-term immune memory induced by NeoVAC plus α-PD1 treatment. A. ELISPOT analysis showing the neoantigen specific-reactivity of splenic T cells against neoantigen pools from combinational therapy treated mice at the 90th day after treatment. B. The histogram of absolute number of $Ptpn2_{376-384}$:H-$2K^b$ specific $CD8^+$ T cells in blood at 2 days after orthotopic recurrence and metastasis rechallenge. The statistical analysis was performed with ANOVA analysis, n=3 for all the groups. Results are shown as mean ± SD.

Figure S10. Depiction of tumor immune microenvironment via scRNA-seq data. A. The group distribution of all $CD8^+$ T cell (left) and $CD4^+$ T cell (right) clusters. B. Single cell gene set enrichment analysis of T13 cluster showing significant enrichment in genes response to alpha and gamma interferon. C. Scatterplot showing the correlation between the two gene signatures (Cd69 and Cd8a) and top upregulated genes from T13 cluster.

Figure S11. The statistical analysis of the number of neoantigen-specific $CD8^+$ T_{RMs} and the antitumor efficacy of $CD8^+$ T_{RMs}. A. The histograms of absolute number of $Ptpn2_{376-384}$:H-$2K^b$ specific $CD8^+$ T cells in infiltrating $CD8^+$ T_{RMs} (n=5). The statistical analysis was performed with ANOVA analysis. Results are shown as mean ± SD. B. Total radiance changes of tumor burden in mice after adoptive $CD8^+CD69^+$ or $CD8^+CD69^-$ T cells therapy by bioluminescence imaging, n=4. The statistical analysis was performed with ANOVA analysis. Results are shown as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.