



Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Silencing PTPN2 with nanoparticle-delivered small interfering RNA remodels tumor microenvironment to sensitize immunotherapy in hepatocellular carcinoma



Fu Wang^{a,b,†}, Haoyu You^{a,†}, Huahua Liu^b, Zhuoran Qi^b, Xuan Shi^b,
Zhiping Jin^c, Qingyang Zhong^b, Taotao Liu^b, Xizhong Shen^b,
Sergii Rudiuk^d, Jimin Zhu^{b,*}, Tao Sun^{a,*}, Chen Jiang^a

^aKey Laboratory of Smart Drug Delivery Ministry of Education, Minhang Hospital, State Key Laboratory of Medical Neurobiology and MOE Frontiers Center for Brain Science, Institutes of Brain Science, Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai 201203, China

^bDepartment of Gastroenterology and Hepatology and Shanghai Institute of Liver Diseases, Zhongshan Hospital, Fudan University, Shanghai 200032, China

^cPharmacy Department, Zhongshan Hospital, Fudan University, Shanghai 200032, China

^dPASTEUR, UMR8640, Department of Chemistry, PSL University, Sorbonne Université, CNRS, Ecole Normale Supérieure, Paris 75005, France

Received 28 September 2024; received in revised form 3 December 2024; accepted 10 January 2025

KEY WORDS

Immune checkpoint
blockades;
Liver cancer;
Protein tyrosine
phosphatase
nonreceptor type 2;
Type II interferon
signaling;

Abstract Protein tyrosine phosphatase nonreceptor type 2 (PTPN2) is a promising target for sensitizing solid tumors to immune checkpoint blockades. However, the highly polar active sites of PTPN2 hinder drug discovery efforts. Leveraging small interfering RNA (siRNA) technology, we developed a novel glutathione-responsive nano-platform HPssPT (HA/PEI_{ss}@siPtpn2) to silence PTPN2 and enhance immunotherapy efficacy in hepatocellular carcinoma (HCC). HPssPT showed potent transfection and favorable safety profiles. PTPN2 deficiency induced by HPssPT amplified the interferon γ signaling in HCC cells by increasing the phosphorylation of Janus-activated kinase 1 and signal transducer and activator of transcription 1, resulting in enhanced antigen presentation and T cell activation. The nano-platform was also able to promote the M1-like polarization of macrophages *in vitro*. The unique tropism

*Corresponding authors.

E-mail addresses: zhu.jimin@zs-hospital.sh.cn (Jimin Zhu), sunt@fudan.edu.cn (Tao Sun).

[†]These authors made equal contributions to this work.

Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

<https://doi.org/10.1016/j.apsb.2025.03.015>

2211-3835 © 2025 The Authors. Published by Elsevier B.V. on behalf of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Tumor-associated
macrophages;
Tumor microenvironment

of HPssPT towards tumor-associated macrophages, facilitated by hyaluronic acid coating and CD44 receptor targeting, allowed for simultaneous reprogramming of both tumor cells and tumor-associated macrophages, thereby synergistically reshaping tumor microenvironment to an immunostimulatory state. In HCC, colorectal cancer, and melanoma animal models, HPssPT monotherapy provoked robust antitumor immunity, thereby sensitizing tumors to PD-1 blockade, which provided new inspiration for siRNA-based drug discovery and tumor immunotherapy.

© 2025 The Authors. Published by Elsevier B.V. on behalf of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Hepatocellular carcinoma (HCC) comprises 75%–85% of primary liver cancer, ranking among the most prevalent and deadly malignancies worldwide¹. While curative surgery is an option for patients diagnosed at an early stage, 40%–70% experience recurrence within 5 years, necessitating further treatment². Emerging immunotherapies, such as immune checkpoint blockades (ICBs), have revolutionized the treatment paradigm for solid tumors. ICBs disrupt receptor–ligand interactions that suppress T cell activation or function, such as the PD-1 and CTLA-4 pathways³. Although substantial clinical benefits are observed in some patients, the overall objective response rate to ICBs in HCC remains below 20%, largely due to the immunosuppressive tumor microenvironment (TME)^{4,5}. This limited efficacy underscores the urgent need for novel therapeutic strategies.

Protein tyrosine phosphatase nonreceptor type 2 (PTPN2) has emerged as a promising target for tumor immunotherapy⁶. It is a ubiquitously expressed protein tyrosine phosphatase that can dephosphorylate Janus-activated kinase 1 (JAK1) and signal transducer and activator of transcription 1 (STAT1) upon interferon (IFN) γ stimulation^{7,8}. Phosphorylated STAT1 binds to the IFN γ activation site sequences, inducing the transcription of IFN-stimulated genes (ISGs) that encode numerous proteins to elicit antitumor effects, such as suppression of tumor cell proliferation and promotion of tumor antigen presentation^{9,10}. Antigen presentation is essential for the activation of T cell-mediated adaptive immunity. Thus, this deficiency has been widely considered a critical reason for antitumor immunity injury and tumor immunotherapy resistance^{11,12}. Preclinical studies in melanoma, colorectal, and breast cancers have shown that tumor cell-specific PTPN2 deletion markedly enhances immunotherapy efficacy by activating IFN γ -mediated growth suppression and antigen presentation, suggesting PTPN2 is a critical checkpoint target, but its role in HCC remains elusive^{13–15}. Meanwhile, the highly polar active sites of PTPN2 have hindered drug discovery at the protein level¹⁶.

Small interfering RNA (siRNA) technology, a nucleic acid-based approach for gene silencing, offers significant advantages in efficacy, safety, and ease of preparation. However, the short half-life and instability under physiological conditions limit its applications¹⁷. A common strategy for protecting and delivering siRNA involves combining negatively charged siRNA with positively charged materials through electrostatic interactions. Classic cationic polymers used for this purpose include polyethyleneimine (PEI), polylysine, polyarginine, and dendritic polymers. Among these, PEI 25k is considered the gold standard for gene delivery, showing extremely high transfection efficiency *in vitro*¹⁸. However, the high molecular weight of PEI 25k impedes its metabolism and excretion, leading to *in vivo* accumulation and

considerable physiological toxicity¹⁹. On the other hand, lower molecular weight PEI is more biocompatible but suffers from insufficient positive charge density, which restrains its gene compaction efficiency²⁰. Consequently, there is an urgent need for a novel strategy to achieve both efficient gene editing and high biological safety.

Herein, we describe the discovery and preclinical characterization of HA/PEI_{ss}@si *Ptpn2* (HPssPT), a novel nano-platform loaded with si*Ptpn2* that showed superior transfection efficiency compared with commercial reagents. PTPN2 deficiency induced by HPssPT potently sensitized HCC cells to IFN γ and enhanced the JAK-STAT pathway, increasing antigen presentation and T cell activation. Beyond its effects on tumor cells, HPssPT exhibited an affinity for multiple tumor microenvironment (TME) components, notably tumor-associated macrophages (TAMs), promoting their polarization towards an immune-activated M1-like phenotype. In well-defined mouse models, HPssPT exhibited excellent tolerance and significantly reduced tumor burden when combined with PD-1 blockade by reshaping the immunosuppressive TME. These findings provide new insights for siRNA-based drug discovery and tumor immunotherapy development.

2. Materials and methods

2.1. Materials

Bis (sulfosuccinimidyl) 3,3'-dithiobis (propionate) (DTSSP) and agarose were purchased from Merck (Shanghai, China). Hyaluronic acid (HA, MW = 10,000) was obtained from Meilun Co., Ltd. (Dalian, China). Ethylenediamine branched PEI (MW = 800), O'RangeRuler 10 bp DNA ladder, and 6 × Orange DNA loading dye were acquired from Sigma–Aldrich (St. Louis, MO, USA). Dialysis bags (8000 MWCO) were sourced from Yuanze Co., Ltd. (Shanghai, China).

2.2. Preparation of PEI_{ss}, PssPT and HPssPT

PEI_{ss} Synthesis: Equimolar amounts of PEI and DTSSP were reacted in an air shaker at 37 °C under argon protection for 48 h; the resulting solution was dialyzed against deionized water for 24 h using a dialysis bag with a molecular weight cutoff of 8000. The product, designated as PEI_{ss}, was obtained as a white flocculent substance after lyophilization. The structure of PEI_{ss} was characterized by one-dimensional proton nuclear magnetic resonance (Mercury Plus, Varian, CA, USA).

PssPT Preparation: Solutions of si*Ptpn2* (0.264 mg/mL) and PEI_{ss} (at various concentrations) were prepared in DEPC-treated water. Equal volumes of these solutions were mixed, vortexed

for 30 s at room temperature (RT), and incubated in an ice bath for 30 min to obtain PssPT complexes. The optimal mass ratio of PEIs to siPtpn2 for efficient complexation was determined by agarose gel electrophoresis and dynamic light scattering (DLS) analysis (3600 Nano ZS, Malvern Panalytical, UK).

HPssPT Formulation: HA solutions of varying concentrations were prepared in DEPC-treated water. Equal volumes of HA and PssPT were mixed, vortexed for 30 s at RT, and left to stand in an ice bath for 30 min to obtain HPssPT nanoparticles. The optimal mass ratio of HA to PssPT was determined based on DLS (Malvern Panalytical) results.

2.3. Transmission electron microscopy (TEM) sample preparation and imaging

A 10 μ L aliquot of the prepared HPssPT solution was deposited onto a carbon-coated copper grid and absorbed for 1 min at RT. The excess solution was carefully removed from the edge of the grid using filter paper. Subsequently, the grid was inverted onto a 10 μ L droplet of 3% uranyl acetate staining solution placed on the lid of a Petri dish and incubated for 1 min. The grid was then washed twice with deionized water and dried at RT. Transmission electron microscope imaging was performed to visualize the nanoparticle morphology.

2.4. In vitro stability of HPssPT

The stability of HPssPT nanoparticles was assessed under physiologically relevant conditions. Equal volumes of freshly prepared HPssPT solution and $2 \times$ phosphate-buffered saline (PBS) were mixed. The resulting solution was stored at 4 °C, and the hydrodynamic diameter and zeta potential of the nanoparticles were monitored daily using DLS (Malvern Panalytical) and electrophoretic light scattering, respectively.

2.5. Glutathione (GSH) responsiveness of HPssPT

The GSH responsiveness of HPssPT nanoparticles was evaluated under simulated extracellular and intracellular redox conditions. Freshly prepared HPssPT solution was mixed in equal volumes with either 4 μ mol/L or 20 mmol/L GSH solutions, representing extracellular and intracellular GSH concentrations, respectively. The mixtures were incubated at 37 °C with gentle agitation for 2 h. Changes in the hydrodynamic diameter of the nanoparticles were monitored using DLS (Malvern Panalytical) to assess the GSH-induced disassembly of the nanoparticles.

2.6. Cell lines and culture conditions

Murine Hepa1-6 HCC cells, B16–F10 melanoma cells (referred to as B16), CT26 colorectal cancer cells, Raw264.7 macrophages, bEnd.3 endothelial cells and NIH3T3 fibroblasts were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). Hepa1-6, B16, Raw264.7, bEnd.3, and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah, USA). CT26 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone). All media were supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA). Cells were

cultured at 37 °C in a humidified atmosphere containing 5% CO₂ (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Biodistribution

An orthotopic HCC mouse model was established using Hepa1-6 cells stably expressing Luciferase (Hepa1-6-Luc). HPssPT nanoparticles loaded with Cy5-labeled siRNA (0.825 mg/kg) were administered to the mice *via* tail vein injection. The bio-distribution of the nanoparticles was monitored using the IVIS Spectrum CT small animal *in vivo* imaging system (PerkinElmer, MA, USA) at 6 h post-injection. Immediately following 24 h post-injection, mice were anesthetized and perfused. Major organs were then harvested and imaged *ex vivo* to quantify fluorescence intensity using the same imaging system.

2.8. In vitro cellular uptake

Time-dependent uptake: Hepa1-6 cells were seeded at a density of 1.5×10^4 cells per well in a 96-well plate. Cells were treated with HPssPT nanoparticles loaded with Cy5-labeled siRNA at a constant dose for varying incubation periods time (0.5, 1, 2, 3, 4, 5, and 6 h). Following incubation, cells were harvested and analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). Data were processed using FlowJo software (Becton, Dickinson and Company, version 10.8.1).

Cell type-specific uptake: Hepa1-6, RAW264.7, bEnd.3, and NIH3T3 cells were seeded at a density of 1.5×10^4 cells per well in 96-well plates. Cells were treated with HPssPT nanoparticles loaded with FAM-labeled siRNA for 2 h. After incubation, cells were harvested and analyzed by flow cytometry as described above.

2.9. Uptake pathway of HPssPT on HCC cells

Hepa1-6 cells were seeded in 12-well plates at approximately 60% confluence. Cells were pre-treated with various endocytic inhibitors diluted in Hank's Balanced Salt Solution (HBSS): filipin (1 μ g/mL), chlorpromazine (25 μ g/mL), or colchicine (5 μ g/mL). Following inhibitor treatment, cells were incubated with HPssPT nanoparticles loaded with FAM-labeled siRNA for 30 min. Cells were then harvested and analyzed by flow cytometry as described above.

2.10. Intracellular trafficking of HPssPT

Hepa1-6 cells were seeded at a density of 5×10^3 cells per well in confocal dishes. Cells were treated with HPssPT nanoparticles loaded with Cy3-labeled siRNA at a constant dose for various incubation periods (1, 2, 4, and 6 h). Following incubation, the cells were stained with Hoechst (5 μ g/mL) and LysoTracker (50 nmol/L) for an additional 15 min, then washed with HBSS and imaged using a spinning disk confocal microscope to visualize the intracellular trafficking of HPssPT nanoparticles.

2.11. In vitro cytotoxicity and cell viability assays

Cell Viability Assay: Hepa1-6 cells were seeded at a density of 5×10^3 cells per well in a 96-well plate. Cells were treated with HPssNC at various concentrations (0, 10, 25, 50, 100 nmol/L) for

24 h. Cell viability was determined using the Cell Counting Kit-8 (CCK8) Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell proliferation assay: Hepa1-6 cells were seeded at a density of 2×10^3 cells per well in a 96-well plate. Cells were treated with either HPssPT or HPssNC (both at 25 nmol/L) for various incubation times (0, 24, 48, and 72 h). Cell proliferation was evaluated using the CCK8 Assay Kit, and the absorbance was measured at 450 nm using a microplate reader.

Live/Dead Cell Staining: Hepa1-6 cells were seeded in a 6-well plate and incubated with either HPssPT or HPssNC (both at 25 nmol/L) for 24 h. Cells were then co-stained with Calcein AM and propidium iodide (PI) using a Live/Dead staining kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's protocol. Fluorescence images were photographed using fluorescence microscopy (Olympus, Tokyo, Japan).

Apoptosis assay: Hepa1-6 cells were seeded in 6-well plates and treated with either HPssPT or HPssNC (both at 25 nmol/L) for 24 h. Cells were then co-stained with Annexin V-FITC and PI using an Annexin V-FITC detection kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's protocol. Stained cells were harvested and analyzed using flow cytometry, as described above.

2.12. siRNA transfection and PTPN2 knockdown

To achieve PTPN2 knockdown, Hepa1-6 cells were transfected with siRNA targeting PTPN2 (siPtpn2) using Lipofectamine 3000 (Thermo Fisher Scientific). The siRNA: Lipofectamine 3000 ratio was maintained at 0.05% w/v. After 48 h of transfection, cells were harvested for quantitative reverse transcription PCR (qRT-PCR) and Western blot analysis to assess the knockdown efficiency of PTPN2. The sequence of siPtpn2 was as follows: 5'-CCAGCUUAGUUGACAUGA-3' (forward) and 5'-UCUAU GUCAACUAAGCUGG-3' (reverse).

2.13. RNA extraction and qRT-PCR

Total RNA was extracted from cells using RNAiso PLUS (Takara, Tokyo, Japan). Reverse transcription was conducted using an RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green (Yeason, Shanghai, China) on an ABI Prism 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). β -Actin was used as the housekeeping gene, and relative gene expression was calculated using the comparative $\Delta\Delta C_T$ method. The sequences of primers designed were as follows. *Ptpn2*: GCAGTGAGAGCATTCTACGGA (forward) and TGACACAAACCCCATCTTAGTGA (reverse). *H2-k1*: ACCAGCAGTACGCCTACGA (forward) and AACCAGAACAG CAACGGTCG (reverse). *H2-d1*: TGGTGCTGCAGAGCATT ACA (forward) and TGTGCCTTTGGGGAATCTGT (reverse). *H2-i*: GATGCAGAGCATTACAGGGC (forward) and GCCAGGT CAGGGCAATGTC (reverse). *Tap1*: GGACTTGCCCTTGTT CCGAGAG (forward) and CAGCATCCGACACAGCATGT (reverse). β -Actin: GGCTGTATCCCTCCATCG (forward) and CCAGTTGGTAACAATGCCATGT (reverse).

2.14. Western blot analysis

Western blot analysis was performed as previously described²¹. Briefly, cells were lysed in RIPA buffer (Beyotime) supplemented

with 1% phenylmethylsulfonyl fluoride, protease, and phosphatase inhibitor cocktail (Beyotime). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membrane was then incubated with primary antibodies against the following proteins: PTPN2 (1:2000), JAK1 (1:2000), *p*-JAK1 (1:2000), STAT1 (1:2000), *p*-STAT1 (1:2000; all from Cell Signaling Technology, Danvers, MA, USA), or Vinculin (1:2000; Abclonal, Wuhan, China). Following incubation with a secondary antibody, protein bands were visualized using a Tanon-5200 Chemiluminescent Image System (Tanon, Shanghai, China) for imaging.

2.15. Co-culture of OT-I T cells and OVA-expressing Hepa1-6 cells

CD8⁺ T cells were isolated from OT-I mouse splenocytes using magnetic separation (BioLegend, San Diego, CA, USA). The isolated T cells were then stimulated with plate-bound anti-CD3 (2.5 μ g/mL) and anti-CD28 (2.5 μ g/mL) antibodies (BioXcell, Lebanon, NH, USA). Hepa1-6 cells were transfected with SIINFEKL-expressing plasmid using Lipofectamine 3000 according to the manufacturer's protocol. The transfected Hepa1-6 cells were then treated with either HPssNC or HPssPT (both at 25 nmol/L) in the presence of IFN γ (100 ng/mL) for 24 h. Subsequently, these treated Hepa1-6 cells were co-cultured with the stimulated OT-I CD8⁺ T cells at a ratio of 1:1 for 48 h.

2.16. Acquisition and analysis of patient data

RNA-Seq Data Analysis: RNA-seq data were obtained from The Cancer Genome Atlas (TCGA) database (<https://www.cancer.gov/tcga>) to generate CD44 expression profiles across various solid tumors, including colorectal adenocarcinoma (COAD), rectal adenocarcinoma (READ), liver hepatocellular carcinoma (LIHC), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), kidney renal clear cell carcinoma (KIRC), kidney chromophobe (KICH), and ovarian serous cystadenocarcinoma (OV).

Single-cell RNA (scRNA)-seq data analysis: scRNA-seq data were obtained from the Gene Expression Omnibus (GEO) database (accession number: GSE202642), comprising 7 HCC and 4 adjacent non-tumor samples. Data analysis was performed using the Seurat package (version 4.3.1) in R software. Genes with a normalized expression between 0.125 and 3 and a quantile-normalized variance exceeding 0.5 were considered highly variable. Dimensionality reduction was performed using Uniform manifold approximation and projection (UMAP) and t-distributed stochastic neighbor embedding (t-SNE). Known biological cell types were identified using canonical marker genes and putative CNV signals, as previously described²².

2.17. Extraction and differentiation of mouse bone marrow-derived macrophages (BMDMs)

Bone marrow cells were extracted from tibias and femurs of 6-week-old male C57BL/6 wild-type mice. Following isolation, red blood cells were lysed using a standard lysis buffer. The remaining cells were cultured in DMEM supplemented with 10% FBS and 50 ng/mL recombinant mouse macrophage colony-stimulating factor (M-CSF; Novoprotein, Suzhou, China). The culture medium was replenished on Day 2. On Day 6, interleukin-4 (IL-4; Novoprotein) was added to

the medium to induce M2 polarization. The macrophage differentiation and polarization process were completed on Day 8.

2.18. Flow cytometry

For *in vitro* experiments, cells were harvested *via* trypsinization, re-suspended in PBS, and stained with the following antibodies: APC anti-mouse H2K(b) bound to SIINFEKL (1:200; BioLegend), APC anti-mouse H2K(b)/H2D(b) (1:200; BioLegend), PE anti-mouse IFN γ (1:200; BD Biosciences), and FITC anti-mouse MHC-II (1:200; BioLegend).

For *in vivo* experiments, fresh tumor tissues were digested with collagenase IV (1 mg/mL; Sigma–Aldrich) and DNase I (1×10^{-3} U/L; Invitrogen, Carlsbad, CA, USA). Tumor-infiltrating lymphocytes were isolated by gradient centrifugation using Percoll (Yeaston). Isolated cells were stained with the following antibodies: Live&Dead-Fluor506 (1:1000; Invitrogen), BUV395 anti-mouse CD45 (1:250; BD Biosciences), PE Cy7 anti-mouse CD3 (1:250; BioLegend), Fluor450 CD4 (1:250; BD Biosciences), PerCP Cy5.5 anti-mouse CD8 (1:250; BD Biosciences), PE anti-mouse IFN γ (1:250; BD Biosciences), PerCP Cy5.5 anti-mouse CD11b (1:250; BioLegend), APC anti-mouse Gr-1 (1:250; BioLegend), PE anti-mouse F4/80 (1:250; BioLegend), and FITC anti-mouse MHC-II (1:250; BioLegend). Stained cells were analyzed using a CytoFLEX flow cytometer. Data were processed using the FlowJo software.

2.19. Immunofluorescence staining and analysis

Tumor tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were then deparaffinized and rehydrated following standard protocols. Heat-induced epitope retrieval was performed by immersing the specimens in 10 mmol/L sodium citrate buffer (pH 6.0) and boiling for 10 min. Specimens were blocked with 10% serum at RT for 30 min and then incubated overnight at 4 °C with primary antibodies: anti-CD8 α (1:100; Abcam, Boston, MA, USA) and anti-granzyme B (1:100; Abcam). The following day, specimens were incubated with HRP-conjugated secondary antibodies at RT for 60 min. Nuclei were counterstained with DAPI. Stained sections were examined using a fluorescence microscope (Olympus, Tokyo, Japan) at the appropriate excitation wavelengths for each fluorophore.

2.20. Animal studies

All animal experiments were conducted in accordance with guidelines evaluated and approved by the Institutional Animal Care and Use Committee of Zhongshan Hospital, Fudan University. Male C57BL/6 mice and BALB/c wild-type mice (6 weeks old) were purchased from Biocytogen (Shanghai, China). OT-I mice were kindly gifted by Dr. Yikun Yao at the Chinese Academy of Science (Shanghai, China).

To establish the HCC orthotopic mouse model, about 1×10^6 Hepa1-6 cells were suspended in 150 μ L PBS and injected subcutaneously into the right flank of C57BL/6 mice. After two weeks, the mice were euthanized, and the subcutaneous tumors were excised, cut into 1 mm³ cubes, and implanted into the liver lobe of new C57BL/6 mice under anesthesia. At the study endpoint, orthotopic tumors and major organs, including the heart,

lung, liver, kidney, spleen, and intestine, were harvested for further analysis. Organ samples were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Pulmonary metastases were evaluated microscopically by two experienced pathologists.

For the xenograft mouse model, about 1×10^6 cells of B16 or CT26 were suspended in 150 μ L PBS and injected subcutaneously into the right flank of C57BL/6 or BALB/c mice, respectively. Tumor volume was measured every 3 days using Eq. (1):

$$\text{Volume} = \text{Length} \times \text{Width}^2 \times \pi/6 \quad (1)$$

At the endpoint, subcutaneous tumors were harvested for further analysis.

For drug intervention studies, HPssNC (0.8 mg/kg, i.v.), HPssPT (0.8 mg/kg, i.v.), and anti-PD-1 antibody (5 mg/kg, i.p.; BioLegend) were administered twice weekly, either alone or in combination. Sterile PBS and isotype IgG₂ served as vehicle controls.

2.21. Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 9.0). Data are presented as means $\pm$ standard deviation (SD). Two-group comparisons were analyzed using unpaired Student's *t*-tests. For comparisons among multiple groups, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference (HSD) *post-hoc* test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Preparation and characterizations of the GSH-responsive nano-platform loaded with siRNA

The cationic network polymer PEIss was synthesized by cross-linking PEI with GSH-responsive linkers, as confirmed by nuclear magnetic resonance spectroscopy (Fig. 1A and Supporting Information Fig. S1A). Agarose gel electrophoresis experiments were conducted to assess the gene loading capacity of PEIss. Results showed complete compression of siRNA at a 5:1 mass ratio of PEIss to siRNA (Fig. 1B). A ratio of 6:1 was chosen for further formulation optimization to prevent potential gene leakage.

To improve the *in vivo* fate of the positively charged nanocore PssPT and prevent potential aggregation caused by negatively charged substance adsorption, PssPT was coated with a layer of negatively charged, biodegradable hyaluronic acid (HA). This formulation was designated as HA/PEIss@siPtpn2 (abbreviated as HPssPT; Fig. 1A). As HA content increased, the surface charge flipped from positive to negative, stabilizing at a ratio of HA:PEIss:siRNA of 15:6:1 (*w/w/w*), indicating whole encapsulation of PssPT by HA (Fig. 1C and Fig. S1B). Based on particle size and zeta potential analyses, the ratio of 15:6:1 was selected as the final formulation (Fig. 1D and Fig. S1C).

TEM revealed that HPssPT was uniformly sized, well-formed spheres with a diameter of approximately 180 nm and a zeta potential of approximately -22.3 mV (Fig. 1E–G). The particle size and polydispersity index of HPssPT stabilized in PBS solution

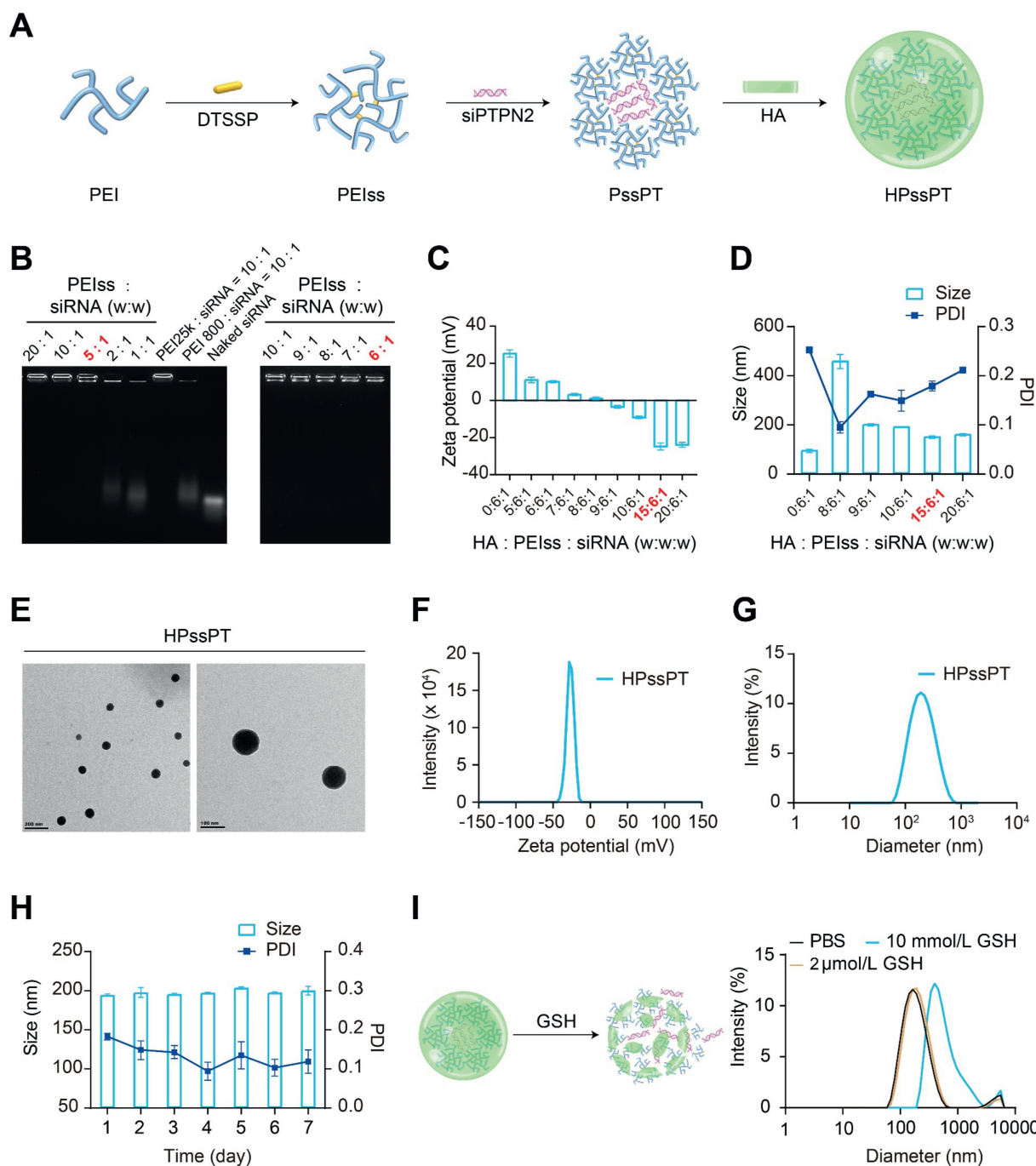


Figure 1 Synthesis and characterizations of the GSH-responsive siRNA delivery nanoplatfom HPssPT. (A) Schematic of the HPssPT preparation process. (B) Agarose gel electrophoresis to determine the optimal mass ratio of PEI_{ss} for condensing siRNA. (C) Changes in zeta potential and (D) particle size of HPssPT with increasing HA content at a fixed PEI_{ss}:siRNA ratio. (E) Representative TEM images of HPssPT. Scale bars, 200 nm (left) and 100 nm (right). (F) Zeta potential and (G) particle size of HPssPT with the final formulation. (H) Colloidal stability of HPssPT in PBS at 4 °C over time, as assessed by changes in particle size. (I) DLS analysis of HPssPT particle size in solutions containing different GSH solutions. Data are presented as means $\pm$ SD, $n = 3$.

at 4 °C for 7 days, demonstrating excellent *in vitro* stability (Fig. 1H).

To investigate the GSH responsiveness of HPssPT, GSH solutions of 2 μmol/L and 10 mmol/L were used to simulate the extracellular and intracellular GSH environments, respectively. HPssPT particle size remained unchanged in the 2 μmol/L GSH

solution but rapidly disintegrated in the 10 mmol/L GSH solution, suggesting superior intracellular GSH responsiveness (Fig. 1I). Collectively, a novel siRNA delivery nano-platform, HPssPT, was developed based on HA, PEI, and disulfide-containing linkers. The internal disulfide bonds of HPssPT were designed to break under high intracellular GSH levels, leading to nanoparticle

disintegration and substantial siRNA release, potentially eliciting a potent gene-silencing effect in target cells.

3.2. HPssPT delivers siRNA into HCC cells with potent transfection efficiency and favorable safety performance

To investigate the potential mechanism of cell uptake, tumor cells were treated with HPssPT and various uptake pathway inhibitors. Flow cytometry analysis revealed significant inhibition of cell uptake at 4 °C and with chlorpromazine treatment, suggesting an energy-dependent and clathrin-mediated endocytosis mechanism for HPssPT (Fig. 2A). This implies that HPssPT likely enters the lysosome before escaping into the cytoplasm. A time-dependent pattern of cell uptake was observed, with uptake reaching saturation after a 5-h incubation (Fig. 2B).

Confocal microscopy studies using Cy3-labeled HPssPT and LysoTracker staining revealed the intracellular trafficking of nanoparticles. After 1 h, minimal uptake was observed with no colocalization in lysosomes. Significant colocalization and cytoplasmic presence were noted after 2 h. At 4 h, substantial colocalization persisted, with some particle escape evident. By 6 h, colocalization decreased markedly, indicating predominant lysosomal escape (Fig. 2C). These results demonstrate that HPssPT can enter the cytoplasm *via* lysosomal escape and subsequently disintegrate, releasing siRNA under high intracellular GSH concentrations.

To assess PTPN2 inhibition efficiency and determine the optimal concentration, HCC cells were treated with 10, 25, or 50 nmol/L siRNA using HPssPT or a commercial reagent. HPssPT significantly attenuated the mRNA and protein expression of PTPN2 in HCC cells, displaying superior transfection capacity than Lipofectamine 3000, particularly at low siRNA concentrations (Fig. 2D and E). No enhanced transfection capacity was observed between 25 and 50 nmol/L; therefore, 25 nmol/L was identified as the working concentration for subsequent studies.

Next, the cytotoxicity of HPssPT was evaluated. To avoid the impact of PTPN2 inhibition, siRNA with the chaotic sequence was utilized as negative control (siNC). HA/PEI_{ss}@siNC (HPssNC) at various concentrations revealed no significant changes in cell viability (Fig. 2F). PTPN2, the target of HPssPT, is known as a potential therapeutic target for regulating tumor-immune cell interactions. Still, its intrinsic role in tumor cell growth remains uncertain. Cell proliferation assay was conducted to explore the “immunity-independent” effect of HPssPT, and no impact of HPssPT on cell proliferation was observed within 3 days (Fig. 2G). Live/Dead cell staining corroborated the absence of cell death induction by HPssPT (Fig. 2H). Flow cytometry analysis of apoptosis markers demonstrated no significant early apoptosis (Annexin V⁺/PI⁻), later apoptosis (Annexin V⁺/PI⁺), or other nonapoptotic cell death (Annexin V⁻/PI⁺), with no notable difference in live cell ratios (Annexin V⁻/PI⁻) between HPssPT and HPssNC groups (Fig. 2I). These findings suggest that HPssPT exhibits exceptional transfection efficiency and excellent safety performance *in vitro*, with no direct impact on cell viability by targeting PTPN2.

3.3. HPssPT significantly increases IFN γ sensing by HCC cells

Genetic ablation of PTPN2 in tumor cells has been shown to enhance the phosphorylation of JAK1 and STAT1, augmenting downstream

IFN γ responses, including cell growth suppression, antigen presentation, and T cell stimulation⁶ (Fig. 3A). Therefore, the effect of HPssPT on the IFN γ signal transduction was investigated.

PTPN2 inhibition by HPssPT promoted the phosphorylation of JAK1 and STAT1 in HCC cells under IFN γ treatment (Fig. 3B). Notably, cells did not exhibit increased JAK1 and STAT1 phosphorylation without IFN γ treatment, suggesting that HPssPT does not spontaneously activate the IFN γ signaling (Fig. 3B). Consistently, HPssPT treatment significantly enhanced downstream IFN γ responses, such as IFN γ -driven growth suppression *in vitro* (Fig. 3C). It also led to increased expression of IFN γ -induced genes for antigen presentation *via* major histocompatibility complex class I (MHC-I; Fig. 3D). Consistently, cells treated with HPssPT and IFN γ had increased membrane MHC-I expression compared with control-treated cells (Fig. 3E).

To confirm the improved antigen presentation by PTPN2 inhibition, the exogenous protein ovalbumin (OVA) in HCC cells was expressed. Flow cytometry analysis showed increased mean fluorescence intensity (MFI) of the OVA-derived SIINFEKL epitope presented by MHC-I (H2-Kb) on cells treated with HPssPT and IFN γ , suggesting that PTPN2 inhibition in HCC cells after IFN γ stimulation promoted functional antigen-loaded MHC-I (Fig. 3F). To explore whether PTPN2 inhibition by HPssPT enhances T cell recognition, OVA-expressing HCC cells were co-cultured with OT-I CD8⁺ T cells, which specifically recognize the SIINFEKL epitope (Fig. 3G). Pretreatment of OVA-expressing HCC cells with HPssPT and IFN γ enhanced CD8⁺ T cell activation status, as evidenced by increased intracellular IFN γ production (Fig. 3H). Taken together, HPssPT treatment *in vitro* sensitizes tumor cells to IFN γ , increasing antigen presentation and potentiating T cell activation.

3.4. HPssPT remarkably enhances the immune-activated function of macrophages

CD44, the major surface receptor for HA²³, may determine the tumor-targeting ability of HPssPT. Analysis of the TCGA database revealed generally elevated CD44 expression in tumor tissues compared to peritumor tissues across multiple cancers (Supporting Information Fig. S2). Furthermore, scRNA-seq analysis of the GEO database indicated CD44 expression not only on tumor cells but also on immune cells, endothelial cells, and fibroblasts, indicating potential affinity of HPssPT for multiple components in the TME (Fig. 4A and B). Quantitative analysis revealed relatively higher CD44 expression in CD8⁺ T cells and macrophages (Fig. 4C and D). HPssPT remarkably inhibited PTPN2 expression in mouse bone marrow-derived macrophages (BMDMs), possibly due to their potent phagocytic capacity (Fig. 4E). However, no significant siRNA transfection effects were observed in CD8⁺ T cells (Supporting Information Fig. S3). Cell uptake assays in CD44-expressed cells showed that tumor cells (Hep1-6) had higher uptake efficiency than endothelial cells (bEnd.3) and fibroblasts (NIH3T3), indicating preferential uptake of HPssPT by tumor cells (Fig. 4F). Additionally, macrophages (Raw264.7) also exhibited high uptake efficiency, consistent with the notably decreased PTPN2 expression in HPssPT-treated macrophages (Fig. 4F).

TAMs, which tend to exert an immunosuppressive M2-like phenotype, are predominant immunocytes in HCC and potential therapeutic targets due to their phenotype plasticity²⁴. To explore the effect of HPssPT on macrophage function, the expression of

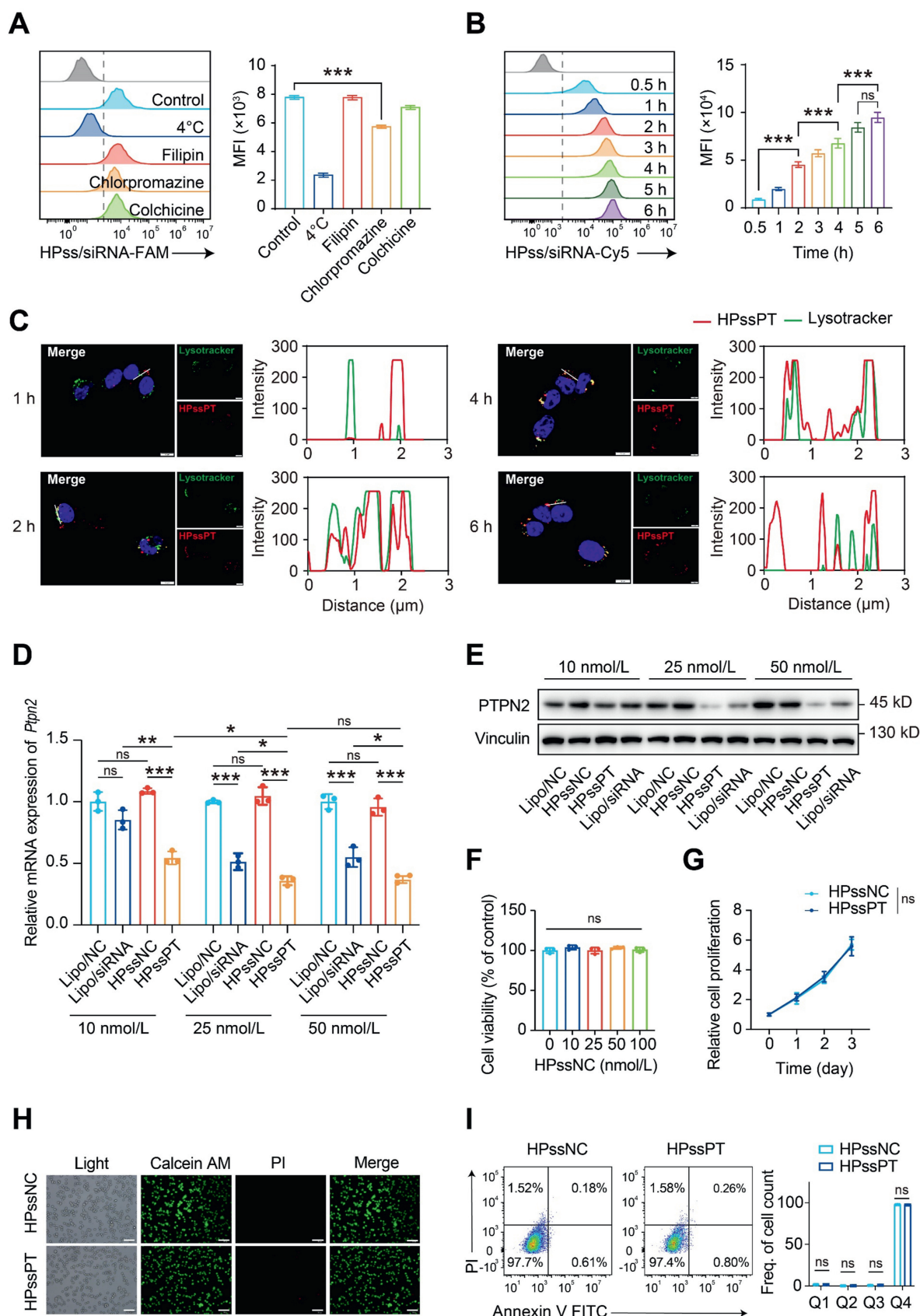


Figure 2 Cellular uptake, transfection efficiency, and cytotoxicity of HPssPT in HCC cells. (A) Flow cytometry analysis of HPssPT uptake mechanisms in HCC cells using different endocytic pathway inhibitors, with corresponding semi-quantitative results. (B) Time-dependent uptake of HPssPT by HCC cells analyzed using flow cytometry, with semi-quantitative results. (C) Spinning disk confocal microscopy to investigate the

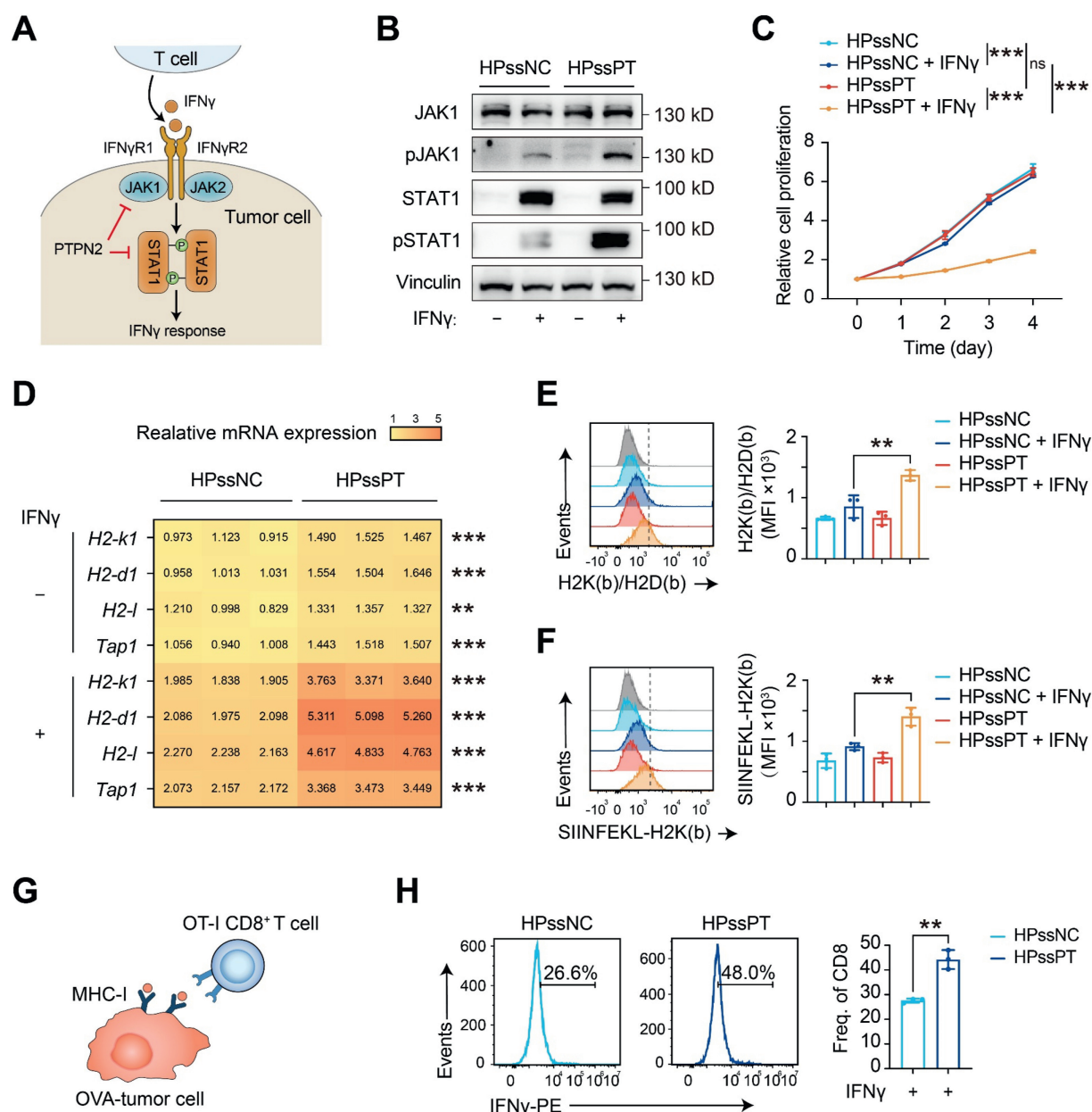


Figure 3 HPssPT sensitizes tumor cells to IFN γ and improves IFN γ -mediated growth suppression and T cell stimulation. (A) Schematic of the IFN γ signaling pathway. (B) Western blot analysis of JAK1, pJAK1, STAT1, pSTAT1 levels in Hepa1-6 cells treated with HPssPT or HPssNC. (C) *In vitro* growth curves of Hepa1-6 cells under indicated treatment conditions. (D) qRT-PCR analysis of MHC-I-related gene expression in Hepa1-6 cells. (E) Flow cytometry analysis of MHC-I (H2K(b)/H2D(b)) expression on Hepa1-6 cells with quantification of mean fluorescence intensity (MFI). (F) Flow cytometry analysis of OVA-derived SIINFEKL epitope presentation on OVA-expressing Hepa1-6 cells with MFI quantification. (G) Schematic illustration of the co-culture model using OVA-expressing Hepa1-6 cells and OT-I CD8 $^{+}$ T cells. (H) Flow cytometry analysis of IFN γ levels in CD8 $^{+}$ T cells after co-culture, with MFI quantification. Treatment conditions: Hepa1-6 cells were treated with HPssPT (25 nmol/L) or HPssNC (25 nmol/L) $\pm$ IFN γ (100 ng/mL) for 24 h, and OVA-expressing Hepa1-6 cells were treated with HPssPT (25 nmol/L) or HPssNC (25 nmol/L) plus IFN γ (100 ng/mL) for 24 h before co-culture. Data are presented as means $\pm$ SD, $n = 3$. ** $P < 0.01$ and *** $P < 0.001$. ns, not significant.

intracellular fate of HPssPT over time using Cy3-labeled siRNA. Lysosomes were stained green with LysoTracker. Scale bars, 10 μ m. (D) qRT-PCR and (E) Western blot analysis of PTPN2 expression in Hepa1-6 cells treated with HPssPT or commercial transfection reagent Lipofectamine 3000 loaded with different siRNA concentrations for 24 h. (F) Cell viability assessment after 24 h treatment with varying concentrations of HPssNC. (G) Impact of HPssPT or HPssNC (both at 25 nmol/L) treatment on cell proliferation over 72 h. (H) Live/Dead cell staining to assess cell death following HPssPT or HPssNC treatment. Scale bars, 50 μ m. (I) Flow cytometry analysis of apoptosis in HCC cells treated with HPssPT or HPssNC. Data are presented as means $\pm$ SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ns, not significant.

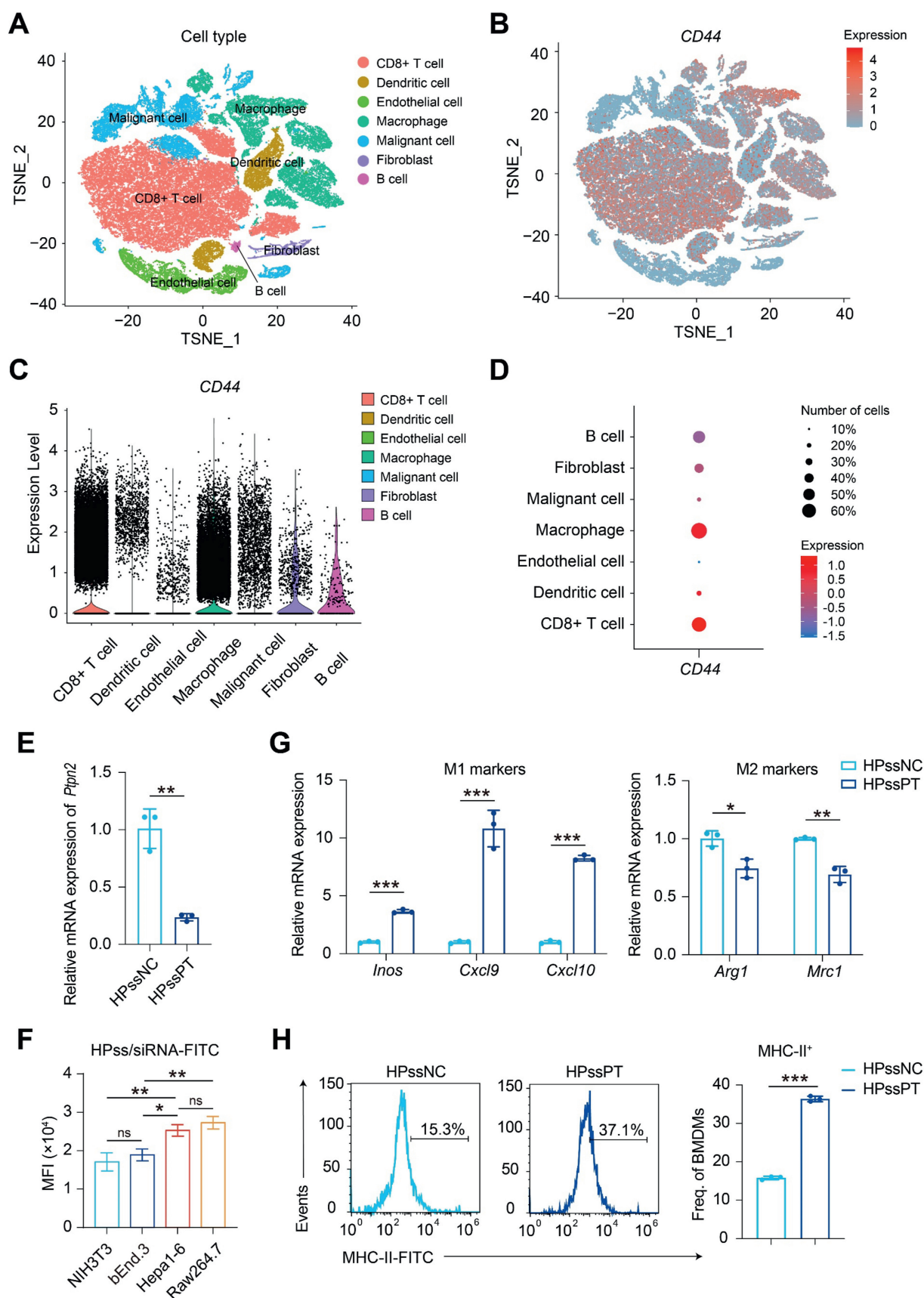


Figure 4 HPssPT targets macrophages and induces an immune-activated phenotype. (A) TSNE plot illustrating the cell composition of human HCC. (B) TSNE plot showing the distribution of CD44⁺ cells in human HCC. (C) Violin plots and (D) bubble plots depicting CD44

M1 and M2 gene markers was investigated in BMDMs pre-treated with IL-4. HPssPT treatment considerably increased the mRNA expression of M1 markers (Inos, Cxcl9, and Cxcl10) and decreased the mRNA expression of M2 markers (*Arg1* and *Mrc1*) in the M2-like BMDMs (Fig. 4G). It also notably promoted the previously inhibited antigen-presenting function of M2-like BMDMs, as measured by MHC-II expression levels (Fig. 4H). In conclusion, macrophages are susceptible to HPssPT, which can induce immune activation upon HPssPT treatment. These findings suggest that HPssPT may have potential applications in reprogramming the immunosuppressive TME, particularly through its effects on macrophages.

3.5. HPssPT reshapes the TME and sensitizes HCC to immunotherapy

Prior to exploring *in vivo* efficacy, the distribution of HPssPT was investigated in orthotopic HCC-bearing nude mice using Cy5-labeled HPssPT. After 6 h, a pronounced accumulation of HPssPT was observed in D-luciferase pre-labeled tumors (Supporting Information Fig. S4A). *Ex vivo* organ analysis at 24 h displayed a maximum accumulation of HPssPT at the tumor site, except the metabolic organs like the kidneys and liver, suggesting HPssPT's excellent capability to reach and continuously accumulate at the tumor site for an extended period (Fig. S4B and S4C). Meanwhile, besides the tumor, a general distribution of HPssPT across various organs was observed, which was likely due to the wide expression of CD44 in various tissues. Among them, there was a maximum accumulation of HPssPT in the metabolic organs, especially the kidneys, suggesting that they might be the major metabolic and excreted organs of HPssPT.

To investigate the antitumor capacity of HPssPT and whether it could inflame the TME *in vivo*, orthotopic HCC mouse models were established and treated with: 1) IgG, 2) IgG plus HPssNC, 3) IgG plus HPssPT, 4) anti-PD-1, 5) anti-PD-1 plus HPssNC, or 6) anti-PD-1 plus HPssPT (Fig. 5A). Moderate tumor growth suppression and lung metastasis restriction were observed for HPssPT or anti-PD-1 monotherapy, while the combination therapy of HPssPT and anti-PD-1 significantly inhibited tumor growth and lung metastasis (Fig. 5B–E). Besides, the combination therapy or HPssPT monotherapy was well-tolerated by mice, as shown by stable weight growth and unchanged indices of hepatic and renal function compared with untreated mice (Supporting Information Fig. S5A and S5B). H&E staining of major organs confirmed no systemic damage after treatment with HPssPT alone or combined with anti-PD-1 (Fig. S5C).

Flow cytometry analysis revealed that both HPssPT monotherapy and the combination with anti-PD-1 significantly stimulated the infiltration of MHC-II⁺ TAMs compared with control groups (Fig. 5F, Supporting Information Fig. S6A and S6B). Analysis of tumor-infiltrating lymphocytes spotted a pronounced enhancement in intratumor infiltration of CD4⁺ and CD8⁺ T cells in mice treated with HPssPT (Fig. S6C and S6D). However, no

obvious differences in tumor-infiltrating CD4⁺ and CD8⁺ T cells were observed among mice receiving anti-PD-1 antibodies with or without HPssPT, suggesting that the maximum of T cell infiltrating might have been reached due to anti-PD-1 therapy (Fig. S6D). As mentioned before, HPssPT treatment potentiated T cell activation by increasing intracellular IFN γ production *in vitro*. Consistently, HPssPT treatment with or without anti-PD-1 also notably increased intracellular IFN γ production in tumor-infiltrating CD4⁺ and CD8⁺ T cells (Fig. 5G and H, Fig. S6E and S6F). Additionally, the number of cytotoxic T cells increased the most under the combination treatment compared to other groups, and HPssPT monotherapy also enhanced the infiltrating of cytotoxic T cells, suggesting an immune-activated TME induced by HPssPT (Fig. 5I). Furthermore, HPssPT treatment was applied in other immunotherapy-resisted tumors, including colorectal cancer and melanoma. As expected, HPssPT also showed successful synergy with anti-PD-1 in suppressing the growth of colorectal tumors (Supporting Information Fig. S7A–S7C) and melanoma (Fig. S7D–S7F) and remarkably inflamed the melanoma TME, characterized by enhanced cytokine production and increased cytotoxic T cell infiltration (Fig. S7G and S7H). Overall, these data further support PTPN2's role in remodeling the TME from an immunosuppressive state to an immunostimulatory state, thus enhancing immunotherapy response in immunotherapy-resisted tumors.

4. Discussion

Immunotherapy for HCC is promising yet particularly challenging due to the unsatisfactory response rate, which is associated with the quantity and quality of immune cells within TME^{25–27}. Herein, this study presents the design and application of a novel siRNA delivery nano-platform, HPssPT, for delivering siRNA targeting the protein tyrosine phosphatase PTPN2 to sensitize solid cancers to immunotherapy. The key findings demonstrate the potent transfection efficiency of HPssPT in delivering siRNA to tumor cells and TAMs, leading to pronounced antitumor immunity and remodeling of the immunosuppressive TME.

Based on the previous research on gene delivery systems^{28–30}, we developed HPssPT, a novel nano-platform loaded with si*Ptpn2* that was created by crosslinking low molecular weight PEI into a network and frangible polymer *via* disulfide linkers with functional groups at both ends. The nano-platform was characterized by a high positive charge density, which facilitated the protection and delivery of siRNA. Upon cellular entry, HPssPT rapidly disintegrates due to the diminished electrostatic interactions triggered by the cleavage of disulfide linkers by specific reactive groups. Thereby, HPssPT showed outstanding transfection efficiency compared with market reagents.

PTPN2 has previously been reported to regulate multiple immune responses as well as immune cell differentiation and to increase the production of cytokines, including IFN γ , TNF, and IL-17 in mice^{31,32}. In our study, PTPN2 inhibition by HPssPT

expression across different cell types in HCC. (E) qRT-PCR analysis of transfection efficiency of HPssPT (25 nmol/L) in M2-like BMDMs after 24 h treatment. (F) Semi-quantitative flow cytometry analysis of cellular uptake of siRNA-FITC-loaded HPssPT in various cell types: tumor cells (Hepa1-6), macrophages (Raw264.7), endothelial cells (bEnd.3) and fibroblasts (NIH3T3). (G) qRT-PCR analysis of M1 and M2 marker gene expression in M2-like BMDMs treated with HPssPT (25 nmol/L) for 24 h. (H) Flow cytometry analysis of MHC-II expression on M2-like BMDMs treated with HPssPT (25 nmol/L), with quantification of MHC-II⁺ cell frequency. Data are presented as means $\pm$ SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ns, not significant.

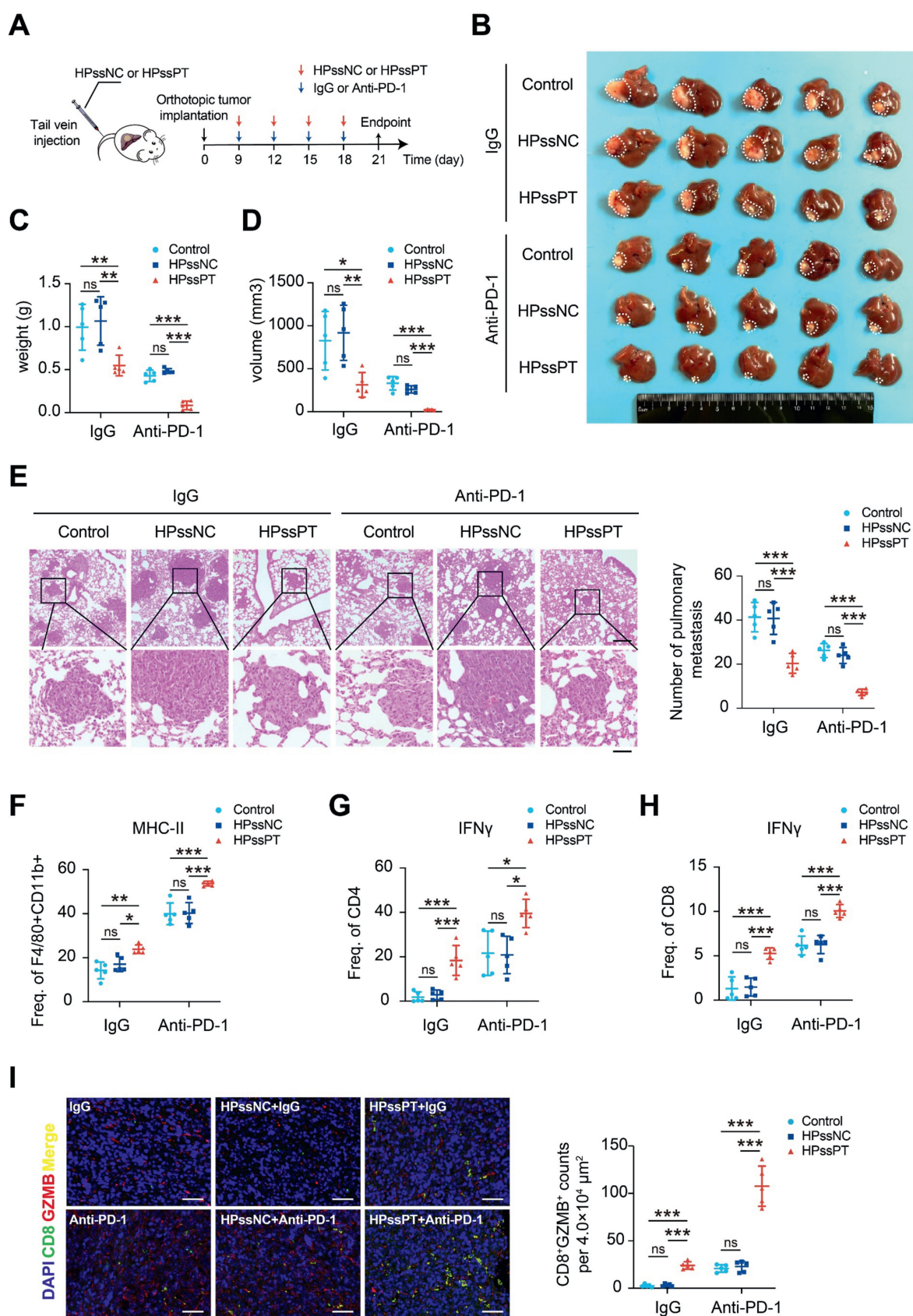


Figure 5 HPssPT reshapes the tumor microenvironment and sensitizes HCC to immunotherapy in the orthotopic HCC mouse model. (A) Schematic illustration of the treatment schedule for HPssNC, HPssPT, anti-PD-1, or the combination therapy in mice bearing orthotopic HCC tumors. (B) Representative gross liver images from mice bearing orthotopic HCC tumors after various treatments. (C) Tumor weight and

amplified IFN γ signaling in tumor cells, as evidenced by increased phosphorylation of JAK1 and STAT1 upon IFN γ stimulation. Phosphorylated STAT1 binds to the IFN γ activation site sequences, inducing the transcription of IFN-stimulated genes that encode numerous proteins to elicit antitumor effects, such as suppression of tumor cell proliferation and activation of T cell function *via* increased MHC expression^{9,10}. Consistently, HPssPT treatment enhanced IFN γ -driven HCC growth suppression and promoted MHC-I-dependent antigen presentation in HCC cells. Furthermore, the increased antigen-presenting capacity prompted a more activated T cell effector function, such as IFN γ production, which in turn amplified the HPssPT effect *via* a positive feedback loop involving the IFN γ signaling pathway. These findings corroborate previous reports highlighting the tumor cell-intrinsic role of PTPN2 as a negative regulator of IFN γ responsiveness and antitumor immunity³³. Notably, HPssPT did not spontaneously activate the IFN γ pathway in the absence of exogenous IFN γ , suggesting that PTPN2 inhibition alone is insufficient to trigger IFN γ signaling and requires an additional inflammatory stimulus.

Beyond its direct effects on tumor cells, HPssPT also targeted TAMs within the TME. TAMs constitute a significant component of the immunosuppressive TME and tend to exhibit an M2-like phenotype that supports tumor progression and immune evasion^{34,35}. Remarkably, HPssPT treatment reprogrammed TAMs towards an immunostimulatory M1-like phenotype, characterized by increased expression of M1 markers, decreased M2 marker expression, and enhanced antigen-presenting capacity *via* upregulation of MHC-II. This repolarization of TAMs likely contributed to the remodeling of the immunosuppressive TME observed upon HPssPT treatment, promoting an inflammatory and immune-activated state conducive to T cell infiltration and antitumor immunity.

In the HCC, colorectal cancer, and melanoma animal models, HPssPT monotherapy exhibited moderate antitumor activity, while its combination with anti-PD-1 blockade resulted in significant tumor growth inhibition. These findings are consistent with previous studies demonstrating that PTPN2 deletion enhances the efficacy of immunotherapy in melanoma, colorectal, and breast cancers^{6,13,36}. The combination therapy was well-tolerated, highlighting the safety profile of HPssPT. Mechanistically, HPssPT treatment promoted infiltration of MHC-II⁺ TAMs and CD4⁺/CD8⁺ T cells into the tumor, with a pronounced increase in intracellular IFN γ production by T cells, indicating an enhanced effector function. Moreover, the combination therapy led to a remarkable increase in the number of cytotoxic T cells within the TME, suggesting a potent antitumor immune response. The broad applicability of HPssPT was further demonstrated in melanoma and colorectal cancer models, where it synergized with anti-PD-1 therapy to suppress tumor growth and inflame the TME, characterized by increased cytokine production and cytotoxic T cell infiltration. These findings suggest that PTPN2 inhibition by HPssPT may represent a promising combination strategy to overcome resistance to immunotherapy across multiple tumors. Notably, previous studies have reported that PTPN2 deletion in

cancer cells can enhance antitumor immunity by activating IFN γ -mediated effects on growth suppression and antigen presentation^{37,38}. However, the highly polar active sites of PTPN2 have hindered drug discovery efforts at the protein level¹⁶. The siRNA-based approach employed by HPssPT circumvents this limitation, enabling precise and effective inhibition of PTPN2 in both tumor cells and TAMs.

A unique aspect of the HPssPT nano-platform is its ability to target multiple components of the TME, facilitated by the HA coating and the widespread expression of the HA receptor CD44³⁹. Notably, HPssPT exhibited a remarkable tropism towards TAMs, which are known to express high levels of CD44^{40,41}. By simultaneously reprogramming both tumor cells and TAMs, HPssPT was able to synergistically remodel the TME to an immunostimulatory state, thereby sensitizing tumors to immunotherapy. This dual-targeting approach may offer advantages over strategies that solely focus on modulating tumor cells or immunocytes, as it addresses the complex interplay between these components within the TME.

Although we observed the accumulation of HPssPT in the tumor site and verified the outstanding RNA interfering capability of HPssPT in HCC cells and TAMs, the reason why HPssPT had no impact on CD8⁺ T cells and the susceptibility of HPssPT to other immunocytes remains to be clarified. Especially previous studies have reported that PTPN2 deletion in T cells and dendritic cells may result in increased immune activation⁴²⁻⁴⁴. Hence, a PTPN2-targeted therapy may induce multiple antitumor mechanisms by acting on varied components in TME, revealing the immense potential of the therapeutic target of PTPN2 and directing future studies on HPssPT.

It is worth noting that while HPssPT monotherapy exhibited moderate antitumor activity, the most profound therapeutic benefit was achieved in combination with anti-PD-1 blockade. This observation aligns with the proposed mechanism of action, wherein PTPN2 inhibition by HPssPT potentiates the IFN γ signaling, antigen presentation, and T cell activation, thereby enhancing the sensitivity of tumors to immune checkpoint inhibitors. Notably, the combination therapy was well-tolerated, suggesting that the potential toxicities associated with systemic PTPN2 inhibition may be mitigated by the targeted delivery approach employed by HPssPT.

5. Conclusions

In summary, our study provides compelling evidence for the pivotal role of PTPN2 in orchestrating an immunosuppressive TME and highlights the therapeutic potential of targeting PTPN2 to sensitize tumors to immunotherapy. We developed a novel system for siRNA delivery based on electrostatic complexes of siRNA with disulfide-crosslinked short PEI covered with hyaluronic acid and successfully overcame the challenges associated with targeting undruggable proteins like PTPN2, leveraging the specificity and potency of siRNA-mediated gene silencing.

(D) tumor volume at the experiment endpoint for each treatment group. (E) Representative images of H&E staining for the lungs and quantification of the number of metastases per lung. Scale bars, 200 μ m (top) and 50 μ m (bottom). (F) Flow cytometry quantification of MHC-II expression on TAM across treatment groups. (G) Flow cytometry analysis of IFN γ levels on tumor-infiltrating CD4⁺ T cells. (H) Flow cytometry analysis of IFN γ levels on tumor-infiltrating CD8⁺ T cells. (I) Representative immunofluorescence images of orthotopic HCC tumors stained for GZMB⁺CD8⁺ T cells, with quantification of cell numbers per field of view. Scale bars, 50 μ m. Data are presented as means $\pm$ SD, $n = 5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ns, not significant.

Moreover, the unique tropism of HPssPT towards TAMs, facilitated by the HA coating and CD44 receptor targeting, allowed for simultaneous reprogramming of both tumor cells and TAMs, thereby synergistically remodeling the TME to an immunostimulatory state. This study provides a strong rationale for pursuing PTPN2 inhibition as a combination strategy to enhance the efficacy of cancer immunotherapy and paves the way for future translational efforts in this domain.

Acknowledgments

This work was supported by the Natural Science Foundation of Shanghai Science and Technology Innovation Plan (No. 22ZR1414100), Oriental Youth Talent Program (to T. Sun, China), Key Special Project of the Ministry of Science and Technology (2023YFC2510000, China), National Natural Science Foundation of China (No. 81872808, 81972234, 82103252, 82121002, 82173122, and 82273027), Open Grant from the Pingyuan Lab (2023PY-OP-0106, China), and Shanghai Municipal Science and Technology Major Project (No. 2018SHZDZX01) and ZJLab, Shanghai Science and Technology Committee (Nos. 20Y11908100, and 22ZR1411800), and Shanghai Sailing Program (No. 21YF1407400).

Author contributions

Fu Wang: Writing — original draft, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Haoyu You: Writing — original draft, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Huahua Liu: Writing — original draft, Validation, Investigation. Zhuoran Qi: Project administration, Investigation. Xuan Shi: Writing — review & editing, Software, Formal analysis, Data curation. Zhiping Jin: Writing — review & editing, Resources, Formal analysis, Data curation. Qingyang Zhong: Software, Resources, Project administration, Data curation. Taotao Liu: Software, Resources, Formal analysis, Data curation. Xizhong Shen: Software, Formal analysis, Data curation. Sergii Rudiuk: Writing — review & editing, Validation. Jimin Zhu: Writing — review & editing, Validation, Funding acquisition, Conceptualization. Tao Sun: Writing — review & editing, Supervision, Funding acquisition, Conceptualization. Chen Jiang: Writing — review & editing, Supervision, Funding acquisition.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.03.015>.

References

- Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;**71**:209–49.
- Zhou J, Sun HC, Wang Z, Cong WM, Wang JH, Zeng MS, et al. Guidelines for the diagnosis and treatment of hepatocellular carcinoma (2019 edition). *Liver Cancer* 2020;**9**:682–720.
- Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* 2015;**27**:450–61.
- El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017;**389**:2492–502.
- Finn RS, Ryoo BY, Merle P, Kudo M, Bouattour M, Lim HY, et al. Pembrolizumab as second-line therapy in patients with advanced hepatocellular carcinoma in KEYNOTE-240: a randomized, double-blind, phase III trial. *J Clin Oncol* 2020;**38**:193–202.
- Manguso RT, Pope HW, Zimmer MD, Brown FD, Yates KB, Miller BC, et al. *In vivo* CRISPR screening identifies PTPN2 as a cancer immunotherapy target. *Nature* 2017;**547**:413–8.
- Kleppe M, Soulier J, Asnafi V, Mentens N, Hornakova T, Knoops L, et al. PTPN2 negatively regulates oncogenic JAK1 in T-cell acute lymphoblastic leukemia. *Blood* 2011;**117**:7090–8.
- Pike KA, Tremblay ML. TC-PTP and PTP1B: regulating JAK-STAT signaling, controlling lymphoid malignancies. *Cytokine* 2016;**82**:52–7.
- Decker T, Kovarik P, Meinke A. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. *J Interferon Cytokine Res* 1997;**17**:121–34.
- Ivashkiv LB. IFN γ : signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nat Rev Immunol* 2018;**18**:545–58.
- Pishesha N, Harmand TJ, Ploegh HL. A guide to antigen processing and presentation. *Nat Rev Immunol* 2022;**22**:751–64.
- Reeves E, James E. Antigen processing and immune regulation in the response to tumours. *Immunology* 2017;**150**:16–24.
- Goh PK, Wiede F, Zeissig MN, Britt KL, Liang S, Molloy T, et al. PTPN2 elicits cell autonomous and non-cell autonomous effects on antitumor immunity in triple-negative breast cancer. *Sci Adv* 2022;**8**:eabk3338.
- Katkeviciute E, Hering L, Montalban-Arques A, Busenhardt P, Schwarzfischer M, Manzini R, et al. Protein tyrosine phosphatase nonreceptor type 2 controls colorectal cancer development. *J Clin Invest* 2021;**131**:e140281.
- LaFleur MW, Nguyen TH, Cox MA, Miller BC, Yates KB, Gillis JE, et al. PTPN2 regulates the generation of exhausted CD8(+) T cell subpopulations and restrains tumor immunity. *Nat Immunol* 2019;**20**:1335–47.
- Mullard A. Phosphatases start shedding their stigma of undruggability. *Nat Rev Drug Discov* 2018;**17**:847–9.
- de Brito ECD, Frederico ABT, Azamor T, Melgaco JG, da Costa Neves PC, Bom A, et al. Biotechnological evolution of siRNA molecules: from bench tool to the refined drug. *Pharmaceuticals* 2022;**15**.
- Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. *Nat Rev Genet* 2014;**15**:541–55.
- Liu HM, Liu CY, Ye L, Ma D, He XZ, Tang QY, et al. Nanoassemblies with effective serum tolerance capability achieving robust gene silencing efficacy for breast cancer gene therapy. *Adv Mater* 2021;**33**:2003523.
- Liu WJ, Cao JP, Ma CC, Zhou H, Guo TY. Zinc(II)-quinoline module and ROS cleavable crosslinker functionalized self-fluorescent siRNA vectors for selective gene silencing of cancer cells. *J Control Release* 2024;**366**:366–74.
- Wang F, Xu WQ, Zhang WQ, Xu RC, Sun JL, Zhang GC, et al. Transferrin receptor 1 promotes hepatocellular carcinoma progression and metastasis by activating the mTOR signaling pathway. *Hepatology* 2023;**78**:636–50.
- Zhu GQ, Tang Z, Huang R, Qu WF, Fang Y, Yang R, et al. CD36(+) cancer-associated fibroblasts provide immunosuppressive microenvironment for hepatocellular carcinoma via secretion of macrophage migration inhibitory factor. *Cell Discov* 2023;**9**:25.

23. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990;**61**: 1303–13.
24. Sprinzl MF, Reisinger F, Puschnik A, Ringelhan M, Ackermann K, Hartmann D, et al. Sorafenib perpetuates cellular anticancer effector functions by modulating the crosstalk between macrophages and natural killer cells. *Hepatology* 2013;**57**:2358–68.
25. Li Q, Han JJ, Yang YL, Chen Y. PD-1/PD-L1 checkpoint inhibitors in advanced hepatocellular carcinoma immunotherapy. *Front Immunol* 2022;**13**:1070961.
26. Paijens ST, Vledder A, de Bruyn M, Nijman HW. Tumor-infiltrating lymphocytes in the immunotherapy era. *Cell Mol Immunol* 2021;**18**: 842–59.
27. Rimassa L, Finn RS, Sangro B. Combination immunotherapy for hepatocellular carcinoma. *J Hepatol* 2023;**79**:506–15.
28. An S, He D, Wagner E, Jiang C. Peptide-like polymers exerting effective glioma-targeted siRNA delivery and release for therapeutic application. *Small* 2015;**11**:5142–50.
29. Guo Q, Li C, Zhou WX, Chen XL, Zhang Y, Lu YF, et al. GLUT1-mediated effective anti-miRNA21 pompon for cancer therapy. *Acta Pharm Sin B* 2019;**9**:832–42.
30. Zhao ZH, Zhang YJ, Li C, Li XW, Chu YC, Guo Q, et al. Microenvironment-tailored micelles restrain carcinoma-astrocyte crosstalk for brain metastasis. *J Control Release* 2022;**349**:520–32.
31. Heinonen KM, Nestel FP, Newell EW, Charette G, Seemayer TA, Tremblay ML, et al. T-cell protein tyrosine phosphatase deletion results in progressive systemic inflammatory disease. *Blood* 2004;**103**: 3457–64.
32. Spalinger MR, Manzini R, Hering L, Riggs JB, Gottier C, Lang S, et al. PTPN2 regulates inflammasome activation and controls onset of intestinal inflammation and colon cancer. *Cell Rep* 2018;**22**:1835–48.
33. Liang SW, Tran E, Du X, Dong JJ, Sudholz H, Chen H, et al. A small molecule inhibitor of PTP1B and PTPN2 enhances T cell antitumor immunity. *Nat Commun* 2023;**14**:4524.
34. Liu JY, Geng XF, Hou JX, Wu GS. New insights into M1/M2 macrophages: key modulators in cancer progression. *Cancer Cel Int* 2021; **21**:389.
35. Sun JL, Zhang NP, Xu RC, Zhang GC, Liu ZY, Abuduwaili W, et al. Tumor cell-imposed iron restriction drives immunosuppressive polarization of tumor-associated macrophages. *J Transl Med* 2021;**19**: 347.
36. Zhao W, Hao L, Jia LZ, Wang JS, Wang B, Huang YQ, et al. TAFs contributes the function of PTPN2 in colorectal carcinogenesis through activating JAK/STAT signaling pathway. *Am J Cancer Res* 2021;**11**:3085–97.
37. Du W, Frankel TL, Green M, Zou WP. IFN γ signaling integrity in colorectal cancer immunity and immunotherapy. *Cell Mol Immunol* 2022;**19**:23–32.
38. Baumgartner CK, Ebrahimi-Nik H, Iracheta-Vellve A, Hamel KM, Olander KE, Davis TGR, et al. The PTPN2/PTPN1 inhibitor ABBV-CLS-484 unleashes potent anti-tumour immunity. *Nature* 2023;**622**: 850–62.
39. Choi KY, Han HS, Lee ES, Shin JM, Almquist BD, Lee DS, et al. Hyaluronic acid-based activatable nanomaterials for stimuli-responsive imaging and therapeutics: beyond CD44-mediated drug delivery. *Adv Mater* 2019;**31**:e1803549.
40. He HS, Chen SZ, Fan ZC, Dong YP, Wang Y, Li SY, et al. Multi-dimensional single-cell characterization revealed suppressive immune microenvironment in AFP-positive hepatocellular carcinoma. *Cell Discov* 2023;**9**:60.
41. Narunsky L, Oren R, Bochner F, Neeman M. Imaging aspects of the tumor stroma with therapeutic implications. *Pharmacol Ther* 2014; **141**:192–208.
42. Hering L, Katkeviciute E, Schwarzfischer M, Busenhardt P, Gottier C, Mrdjen D, et al. Protein tyrosine phosphatase non-receptor type 2 function in dendritic cells is crucial to maintain tissue tolerance. *Front Immunol* 2020;**11**:1856.
43. Spalinger MR, Kasper S, Chassard C, Raselli T, Frey-Wagner I, Gottier C, et al. PTPN2 controls differentiation of CD4(+) T cells and limits intestinal inflammation and intestinal dysbiosis. *Mucosal Immunol* 2015;**8**:918–29.
44. Wiede F, Shields BJ, Chew SH, Kyparissoudis K, van Vliet C, Galic S, et al. T cell protein tyrosine phosphatase attenuates T cell signaling to maintain tolerance in mice. *J Clin Invest* 2011;**121**:4758–74.