Supplementary information

Oral exosome-like nanovesicles from *Phellinus linteus* suppress metastatic hepatocellular carcinoma by reactive oxygen species generation and microbiota rebalance

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Materials and Regents. *Phellinus linteus* (mulberry) was provided by a local farmers' market. DMSO, triton X-100, MTT, *N*-nitrosodiethylamine, and diethylnitrosamine (DEN) were purchased from Sigma-Aldrich (St Louis, MO, USA). Biological reagents, such as 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), 1,1′-dioctadecyl-3,3′,3′-tetramethylindotricarbocyanine iodide (DiR), foetal bovine serum (FBS) and 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were obtained from Beyotime Institute of Biotechnology (Nanjing, Jiangsu, China). H&E staining kit, TUNEL staining kit, TBAs, AST, BUN, and ALT kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Preparation of Fluorescence-Labelling P-ELNs. To visually track the P-ELNs, we fluorescently labeled them using DiO or DiR. Briefly, P-ELNs (10 mg in 1 mL PBS) were mixed with 2 μL DiO or DiR (Sigma, 10 mM in DMSO) and incubated at 37 ^{o}C for 30 min. The unlabelled dye was subsequently removed through centrifugation at 100,000 g for 60 min, followed by resuspension of the labelled P-ELNs pellets in PBS.

Physicochemical Characterization of P-ELNs. The physical and chemical properties of P-ELNs were comprehensively studied to realize the preponderance and therapeutic mechanism of P-ELNs significantly. First, the hydrodynamic size and zeta potential of P-ELNs were confirmed by dynamic light scattering (Brookhaven Instruments Corp, Holtsville, NY, USA). Further, the morphology and surface structure of P-ELNs were displayed by atomic force microscopy (AFM; SPA 400 AFM instrument, Chiba, Japan) and transmission electron microscopy (TEM; HT7800，Hitachi, Co. Ltd, Tokyo

Japan). Subsequently, the lipid composition of the P-ELNs was determined by a triple quadrupole mass spectrometer (Applied Biosystems Q-TRAP, Applied Biosystems, Foster City, CA). Last, to analyze the contents of flavonoid glycoside, P-ELNs were dissolved in methanol and centrifuged at 12,000 g for 10 min to extract the supernatants. The produced supernatants were examined by high-performance liquid chromatography (HPLC; Shimadzu Corporation, Kyoto, Japan).

Proteins from P-ELNs were analyzed by Majorbio BioPharm Technology Co, Ltd (Shanghai, China). Proteins were identified and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using Orbitrap mass spectrometry (Thermo Fisher Scientific, Bremen, Germany). Finally, the data were analyzed based on the National Center for Biotechnology Information (NCBI) database.

***In Vitro* Live/Dead Staining of P-ELNs.** Hepa 1-6 cells were seeded into 12-well plates at a density of 1×10^5 per well and cultured for 12 h. The DMEM medium containing P-ELNs (protein concentration = 8 μg/mL) was added and cultured for 12 h. Subsequently, cells were stained with calcein-AM and PI for 30 min, washed with cold PBS for 3 times, and imaged by fluorescence microscope (Olympus FV3000, Tokyo, Japan).

Assays for Invasion and Migration. The assessment of cell migration and invasion was conducted using a 24-well plate Transwell system equipped with a matrigel invasion chamber and an 8 μm pore size polycarbonate filter. In brief, the CT-26 cells were inoculated into the upper chamber of the Transwell instrument at a density of 2×10^4 cells per well using serum-free medium and incubated overnight. The apparatus's

lower chamber was then filled with conventional growth media containing 10% FBS. Upper chambers were replaced with serum-free media containing P-ELN suspensions (protein concentration: 8 μg/mL) for 12 or 24 hours. The unit was then incubated at 37 ^{o}C in a 5% CO_2 environment.

A scratch test was used to assess the anti-migration properties of P-ELNs against Hepa-2 cells. The cells were seeded onto 6-well plates at a density of 1×10^6 per well and cultured for 24 hours. A sterile p10 pipette tip was used to wound the confluent cell monolayers. These cells were gently washed, and wounds were examined before and after adding P-ELN solutions (protein concentration: 8 μg/mL, 2 mL) using an inverted fluorescent microscope (Olympus FV3000, Tokyo, Japan). The relative wound area covered was determined using Image J software (Wayne Rasband, Bethesda, MD, USA).

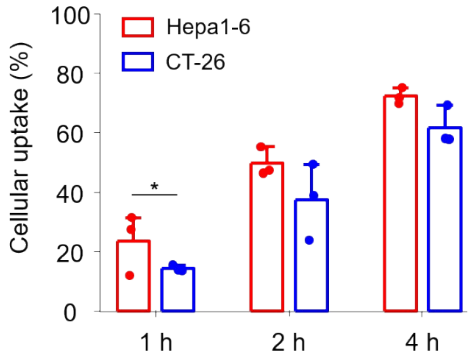

Figure S1 . Cellular uptake percentages of DiO-P-ELNs by Hepa 1-6 cells and CT-26 cells.

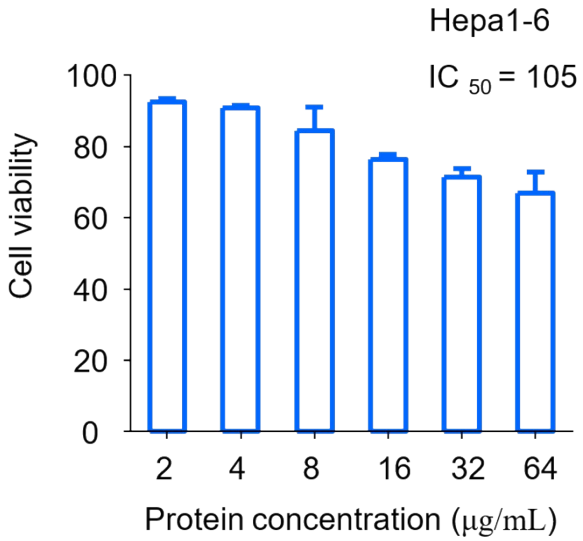

Figure S2. Viabilities of Hepa1-6 cells receiving the treatment of PL with various protenin concentrations for 24 h.

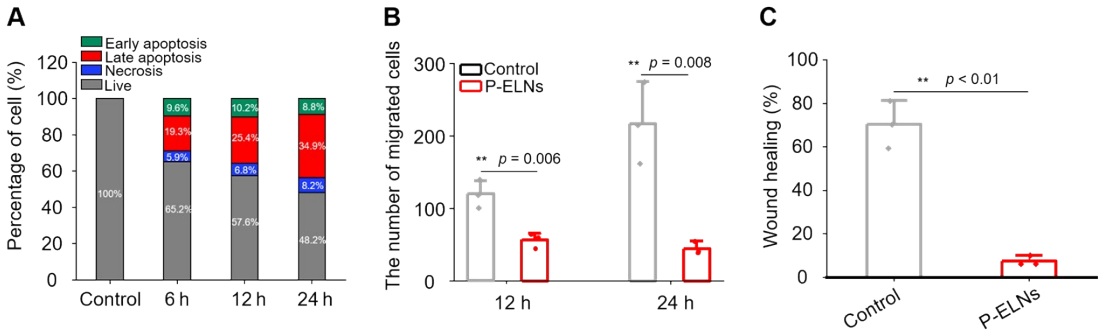

Figure S3. (A) Proportions of necrosis, early apoptotic, late apoptotic, and viable Hepa 1-6 cells receiving the treatment of P-ELNs for 6, 12, and 24 h. Data are expressed as means ± S.E.M. (n = 3). (B) Migration and (C) invasion of Hepa 1-6 cells with or without the treatment of P-ELNs for 12 or 24 h, and their corresponding semi-quantitative results. Data are expressed as means ± S.E.M. (n = 3). Statistical significances were calculated *via* Student's *t*-test, *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

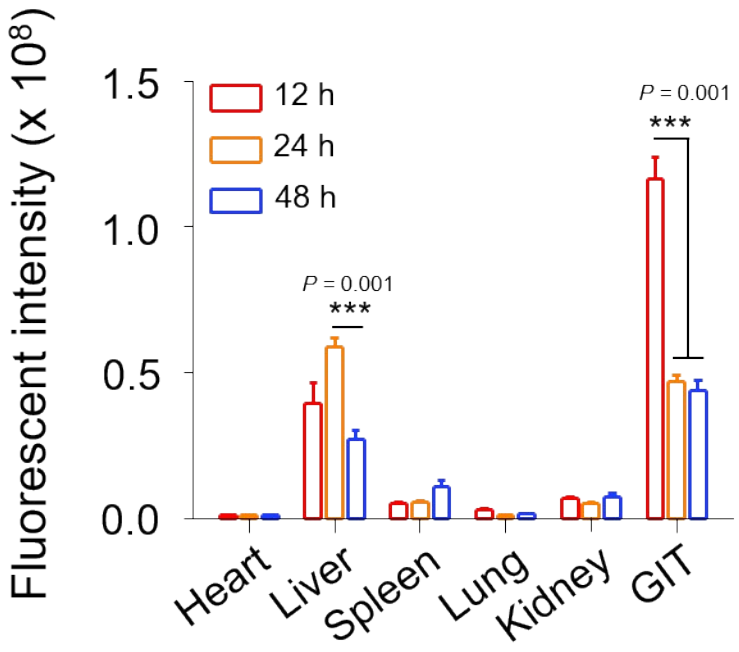

Figure S4. MFIs of the major organs (heart, liver, spleen, lung, kidney, and GIT) of the mice following oral administration of P-ELNs at 12, 24, and 48 h. Data are expressed as means ± S.E.M. (n = 3). Statistical significances were calculated *via* one-way ANOVA, *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

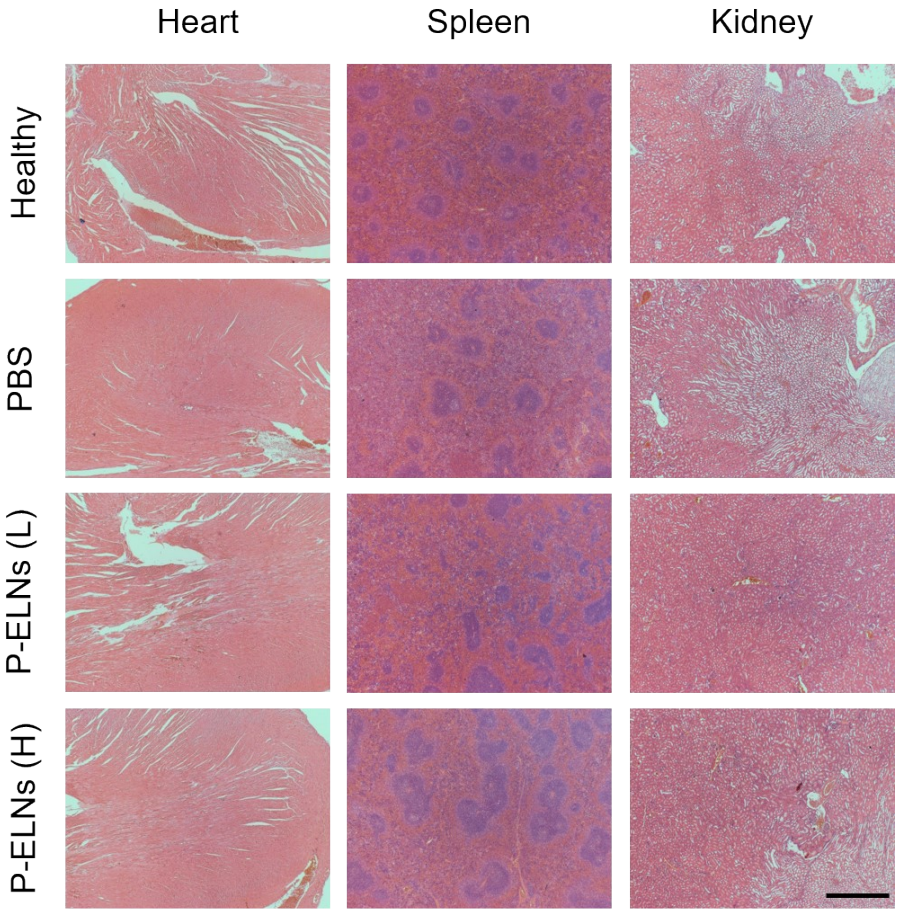

Figure S5. H&E staining images of the main organs (heart, spleen, and kidney) from various mouse groups at the end of treatments (scale bar = 200 μm).

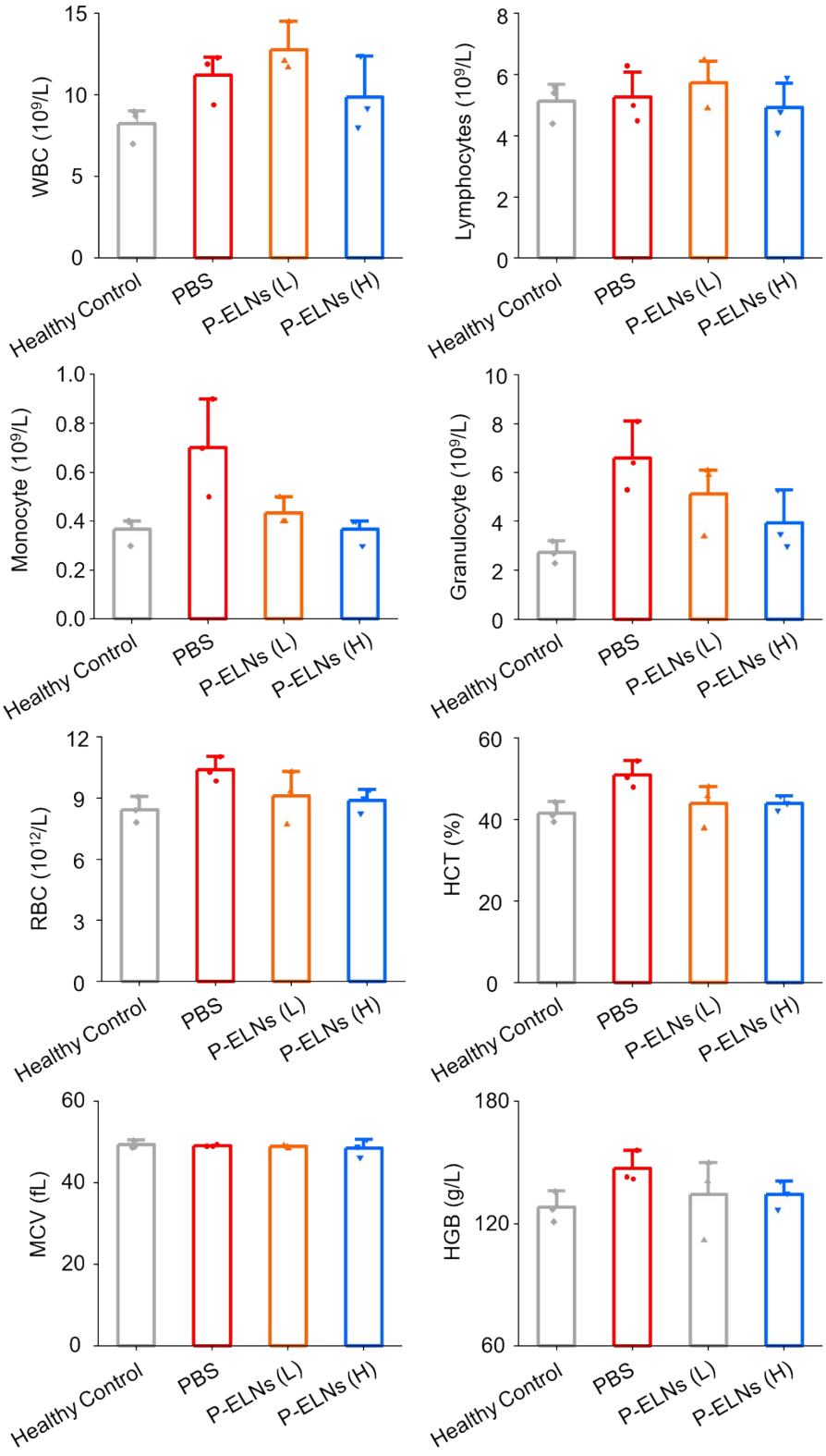

Figure S6. Hematological parameters of mice receiving various treatments at the end of treatments. Data are expressed as means ± S.E.M. (n = 3).