Supporting Information for

Original article

Enhanced tumor homing of pathogen−mimicking liposomes driven by R848 stimulation: A new platform for synergistic oncology therapy

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1. Mycoplasma membrane (MM) derivation

MM was prepared according to the literature[1]. *Mycoplasma hyopneumoniae* (strain J, ATCC 27715) was cultivated in Friis medium until the beginning of the stationary phase[2]. Then, the mycoplasma suspension was centrifuged at 12,000 g (ST16R, ThermoFisher Scientific, Waltham, MA, USA) for 20 min to remove the culture, and the collected microbe pellet was resuspended in ice-cold water with 1% PMSF and incubated on ice for 15 min. Then, the lysate was alternately immersed in liquid nitrogen and a 37 ℃ water bath for repeated freeze-thawing three times. Thereafter, the complete lysate was centrifuged at 700 g (ThermoFisher Scientific) for 10 min at 4 ℃, and the supernatant was collected and centrifuged at 14,000 g (ThermoFisher Scientific) for 30 min at 4 ℃. The resulting milk-white pellet was purified with 1 × phosphate-buffered saline (PBS), and the concentration was determined using a BCA

assay kit (Beyotime). The products of the membrane were lyophilized and stored at -80 ℃. The lyophilized membrane materials were rehydrated with ultrapure water prior to use.

2. Integrity evaluation of MM-LPs-POD released from neutrophils

To evaluate the integrity of MM-LPs-POD, PMA-treated MM-LPs-POD/neutrophils were centrifuged at 10,000 g (ThermoFisher Scientific) for 10 min, and the supernatant was transferred into a ultrafiltration device (MWCO 30 KD, Millipore), followed by centrifugation at 3,000 g (ThermoFisher Scientific) for 15 min. The amount of free POD in the filtrate was determined using HPLC (Agilent) and the structure of MM-LPs-POD in the upper liquid was observed by TEM (HITACHI).

3. Cytotoxicity of MM-LPs-POD towards neutrophils

The in vitro cytotoxicity of MM-LPs-POD against neutrophils was studied via CCK-8 assay. Isolated neutrophils (1×10^4 cells/well) were seeded in a 96-well plate and cultured in 200 μL FBS-free medium for 1 h. Afterward, MM-LPs-POD or free POD was added to neutrophils at different concentrations for 12 h, followed by the addition of 20 μL CCK-8 solution and incubation for 4 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA, USA). The cell viability was calculated as $(OD_{sample} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$, where OD_{sample}, $OD_{control}$ and OD_{blank} represent the absorbance of the sample wells, control wells and blank wells without cells, respectively.

4. Cytotoxicity of MM-LPs-POD released from neutrophils and free POD to tumor cells

The cytotoxicity of MM-LPs-POD released from neutrophils in response to PMA stimulation towards 4T1 cells was primarily evaluated by CCK-8 assay. 4T1 cells (5×10^3 cells) were seeded in a 96-well plate and incubated for 12 h. Neutrophils with

MM-LPs-POD were pretreated with 100 nmol/L PMA for 4 h, followed by centrifugation at 2,000 rpm (ThermoFisher Scientific) for 5 min. The obtained supernatant containing released MM-LPs-POD was added to 4T1 cells. Untreated MM-LPs-POD as well as free POD was also added to 4T1 cells in the same concentration gradient. After 48 h, a CCK-8 kit was used to estimate the cell proliferation rate. Cell viability was calculated by the formula mentioned above.

5. Pharmacokinetics

To explore the pharmacokinetics profiles of free POD and MM-LPs-POD, five male SD rats weighing 180-220 g in a group received single tail vein injection (1.75 mg kg^{-1} of POD, based on the administration dose of mice). 500 μL anticoagulant blood was collected at preset time intervals and then centrifuged at 5,000 rpm (ThermoFisher Scientific) for 5 min to get plasma. POD was extracted from the plasma by protein precipitation and quantified by UPLC (Nexera UHPLC LC-30A, Shimadzu, Kyoto, Japan). Pharmacokinetic parameters were calculated by DAS 2.0 edited by the Chinese Pharmacological Society mathematical pharmacological professional committee for drug clinical evaluation.

6. Safety evaluation

In consideration of whether the chemotherapy drug POD and immune activator R848 would be toxic to normal tissues, blood samples from mice in in vivo antitumor experiments were collected for the measurement of serological indicators including alanine aminotransferase (ALT), aspartate transaminase (AST), lactic dehydrogenase (LDH-L), and blood urea nitrogen (BUN). Organs including the liver, spleen and kidney were sliced for HE staining.

7. Supplementary figures and tables

Table 1. Mouse sequenses for qRT-PCR:

Gene (Mouse)		Pramer sequence (5´-3´)
Gapdh	F	TCCCTTTTGGGTAGAGGGGT
	R	CCTTGTCCCAAGTCACTGTCA
Cxcl1	F	GACTCCACAGGAACCGACAG
	R	TTGGGGACACCTCTGCAATC
Cxcl2	F	CAGGCTACAGGTGGGATTCG
	R	ACATGACTTCTGTCTGGGCG
Cxcl5	F	TCCTCAGTCATAGCCGCAAC
	R	GGGTGACTGAAGAATCCGGG

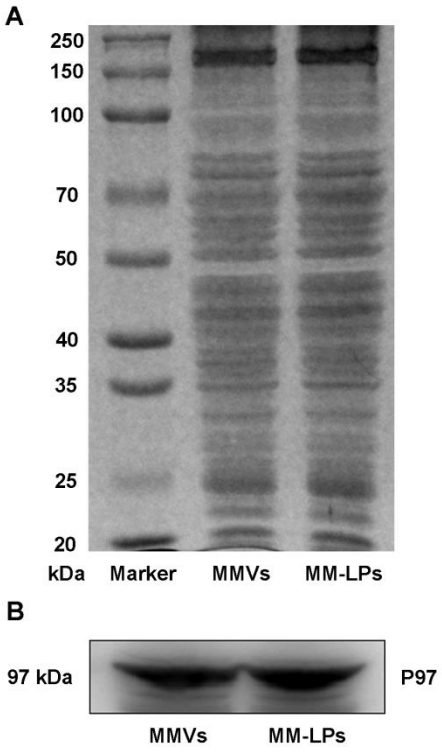


Figure S1 Characterization of membrane proteins of MM-LPs. (A) SDS-PAGE analysis of MM-LPs and MMVs. (B) Western blotting of P97 in MM-LPs and MMVs.

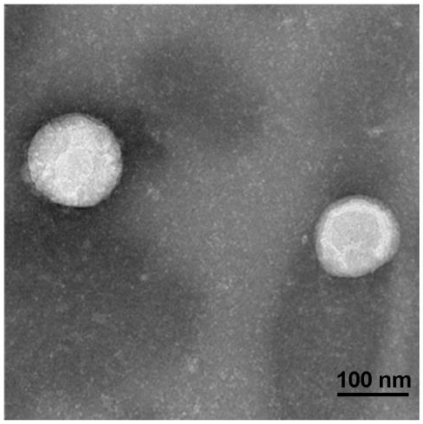


Figure S2 The TEM imaging of MMVs.

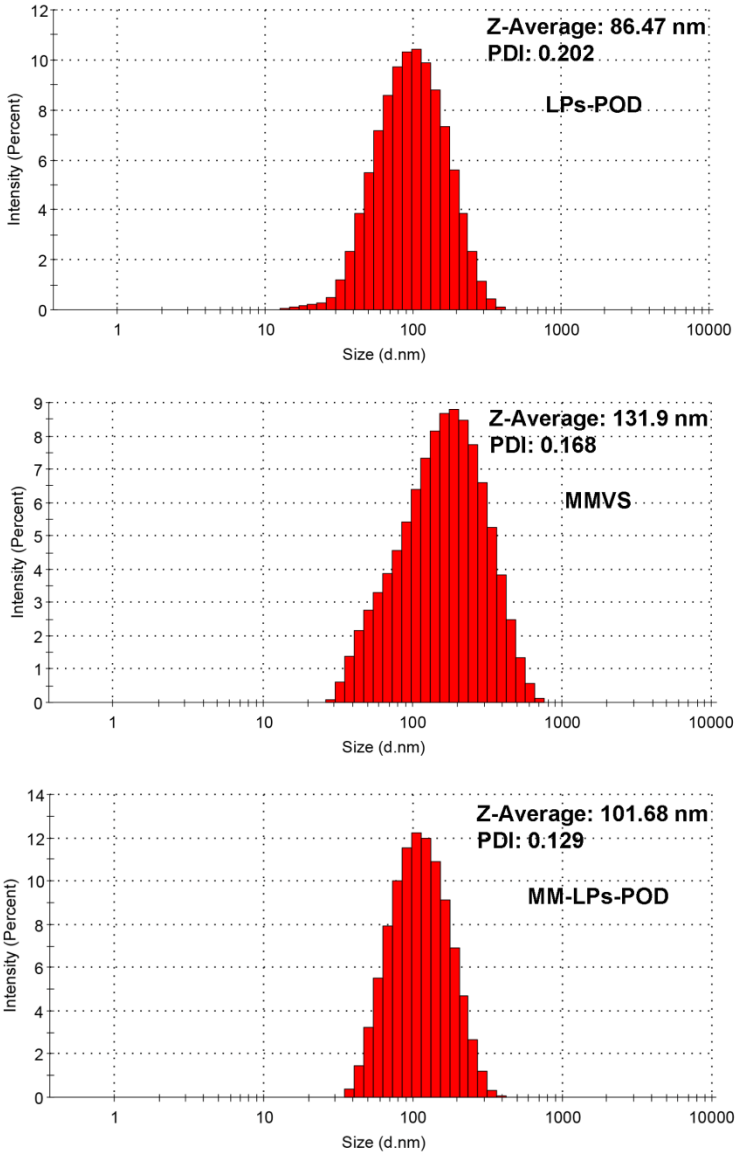


Figure S3 Size distribution of LPs-POD, MMVs and MM-LP-POD.

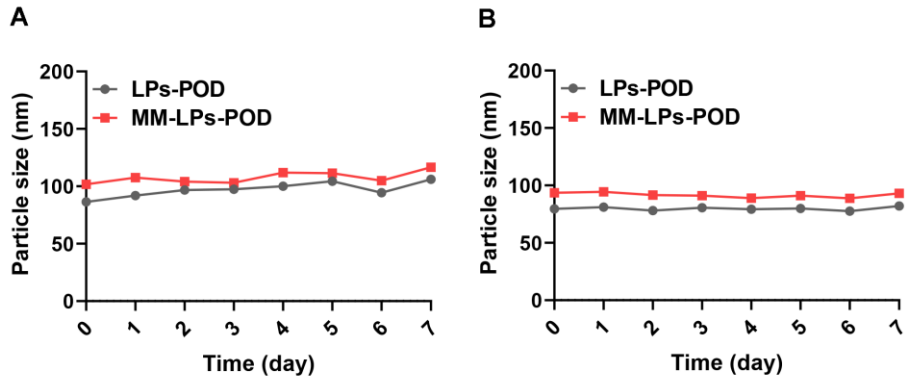


Figure S4 Particle sizes of LPs-POD and MM-LPs-POD at 4 ℃ suspended (A) in saline and (B) 10% FBS by DLS instruments.

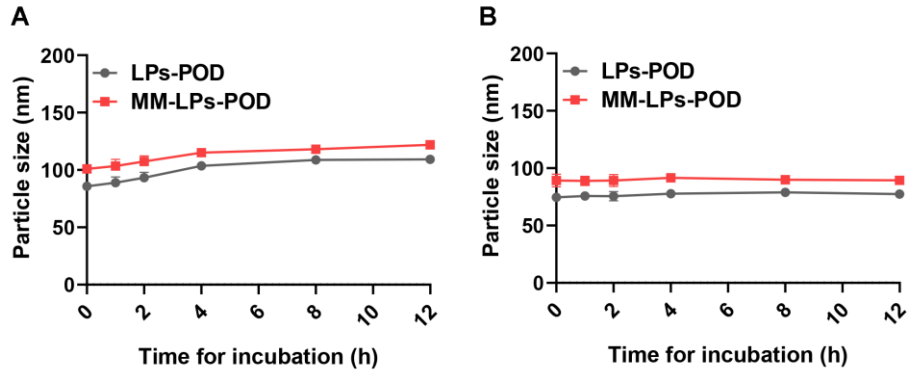


Figure S5 Particle sizes of LPs-POD and MM-LPs-POD at 37 ℃ incubated (A) in saline and (B) 10% FBS by DLS instruments.

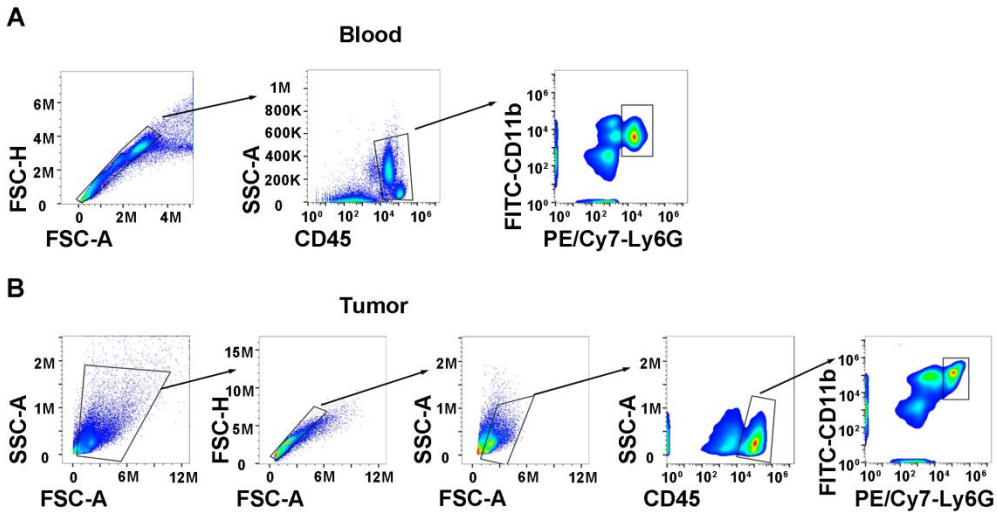


Figure S6 Graphical account for flow cytometry gating strategies. Gating strategy to sort $CD11b^+$ and $Ly6G^+$ neutrophils in $CD45^+$ leukocytes in (A) blood samples and (B) tumor tissues.

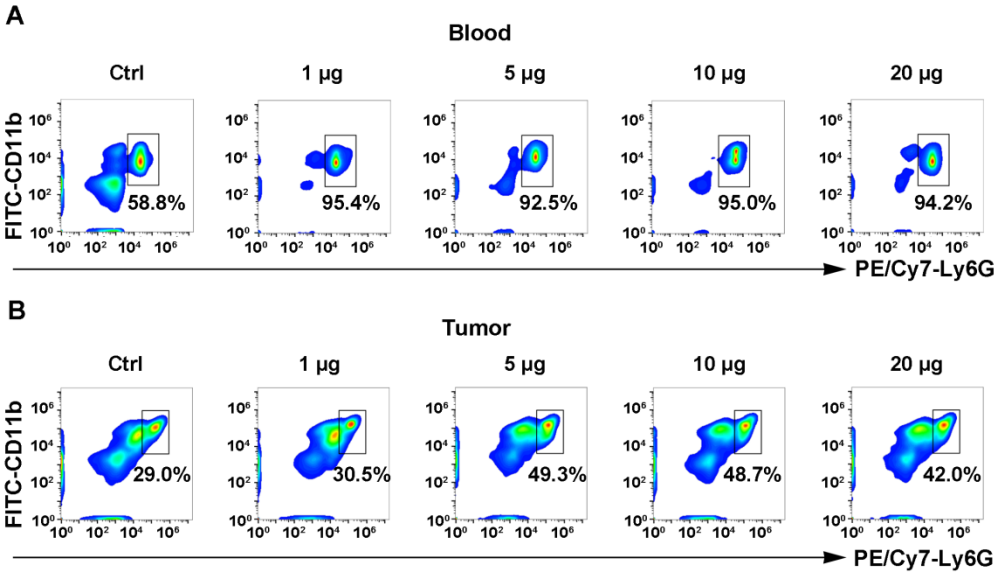


Figure S7 Flow cytometric analyses of the percentage of neutrophils in $CD45^+$ leukocytes in (A) blood samples and (B) tumor tissues after treatment with different doses of LPs-R848 (*n*=5).

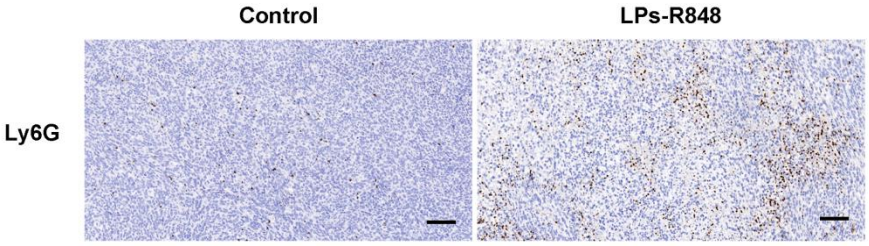


Figure S8 Representative images of Ly6G staining of tumor tissues obtained from control group or LPs-R848 treated group at 4 h after injection (*n*=3). Scale bar: 100 μm.

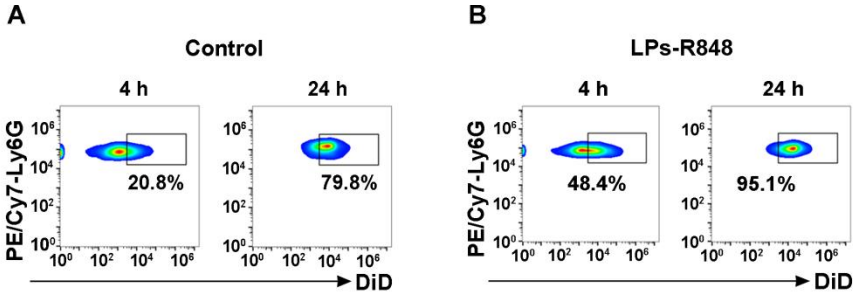


Figure S9 Flow cytometric analyses of the change in DiD^+ neutrophil percentage in

(A) the control group or (B) LPs-R848 pretreated group of tumors after injection of LPs-DiD (*n*=5).

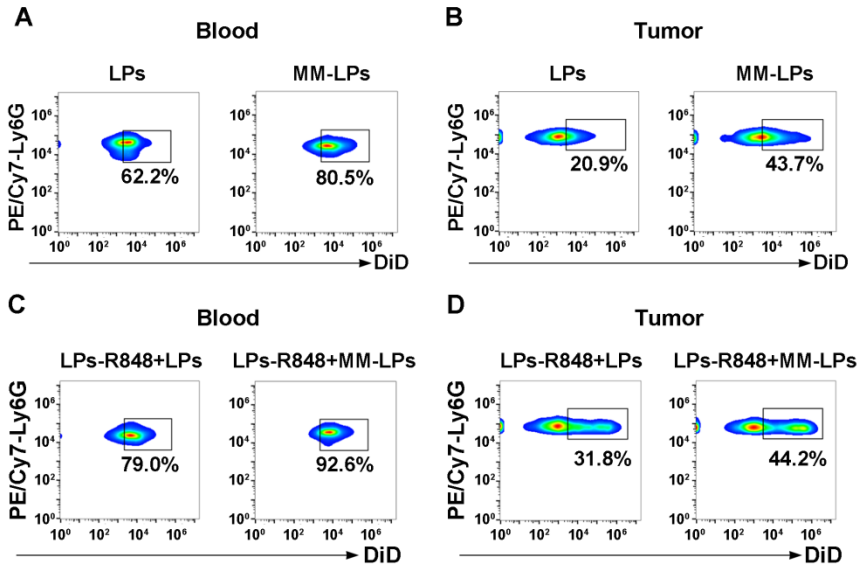


Figure S10 Flow cytometric analyses of the percentage of neutrophils that internalized LPs or MM-LPs. (A) Blood and (B) tumors collected at 4 h after nanoparticle injection without LPs-R848 pretreatment (*n*=5). (C) Blood and (D) tumors collected at 1 h after nanoparticles injection with LPs-R848 pretreated (*n*=5).

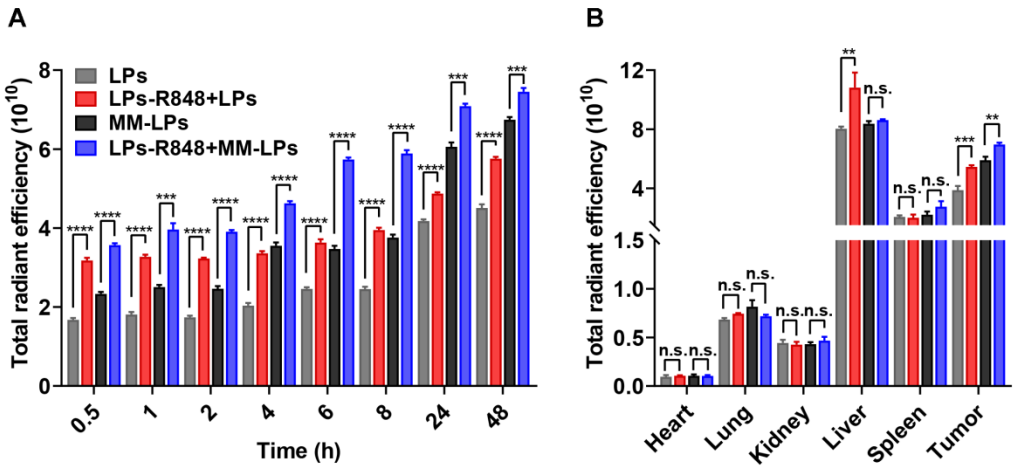


Figure S11 Corresponding quantitative analysis of (A) tumoral fluorescence *in vivo* and (B) fluorescence intensity of tumors and major organs *ex vivo* excised at 48 h post injection (*n*=3). $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. n.s., not significant.

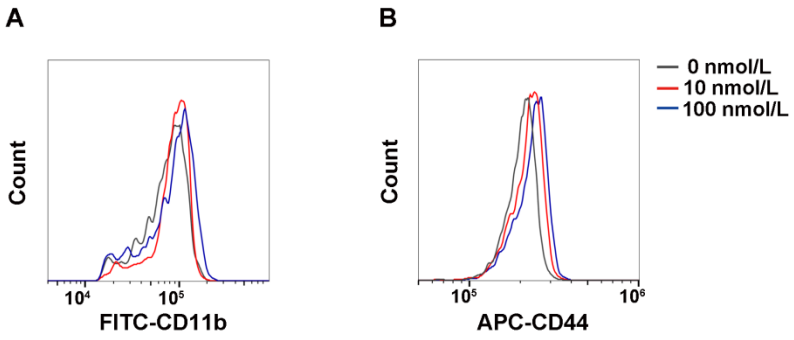


Figure S12 (A) CD11b and (B) CD44 expression on neutrophils was determined by flow cytometry (BD) after PMA stimulation.

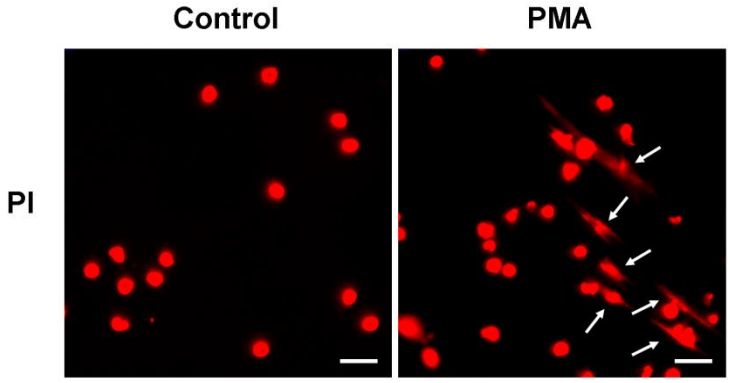


Figure S13 Confocal fluorescent images of propidium iodide (PI)-stained neutrophils cultured under normal or inflammatory conditions (100 nmol/L PMA) for 8 h. Scale bar: 20 μm. Data are representative of three independent experiments.

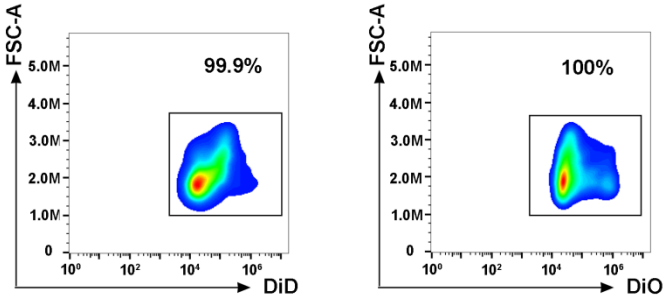


Figure S14 Analysis of neutrophils-DiD/MM-LPs-DiO by flow cytometry (BD). Isolated neutrophils were stained with DiD and then incubated with MM-LPs-DiO.

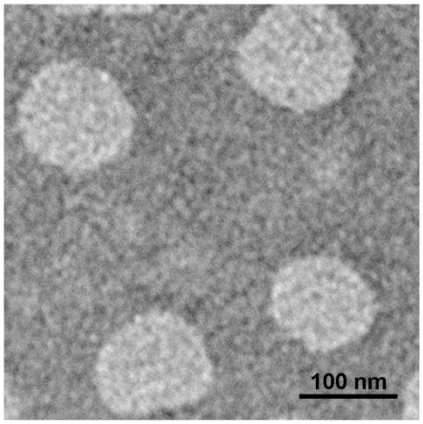


Figure S15 TEM images of MM-LPs-POD released from neutrophils after treatment with PMA for 4 h. The intact MM-LPs-POD, which were secreted from neutrophils after the PMA treatment, were collected in the medium.

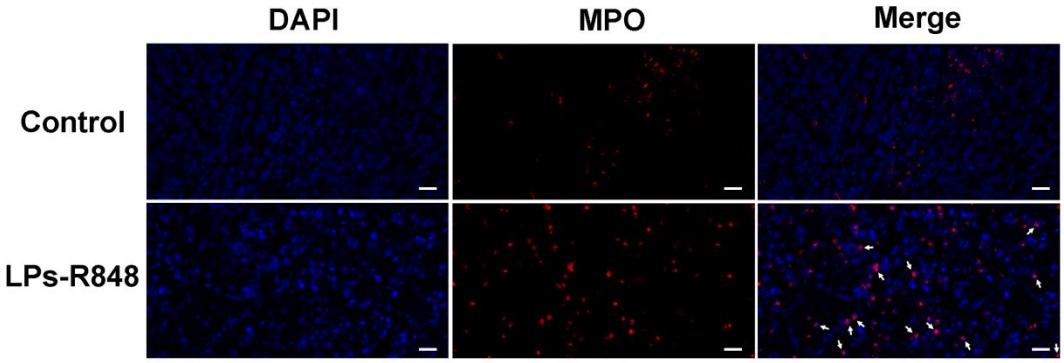


Figure S16 *In vivo* NETs formation in LPs-R848-treated tumor. 4T1 tumor-bearing mice were left untreated or treated with LPs-R848. After 24 h, the tumors were excised and were stained with MPO antibody and DAPI to analyze *in vivo* NETs formation. Scale bar: 20 μm. Data are representative of three biological replicates.

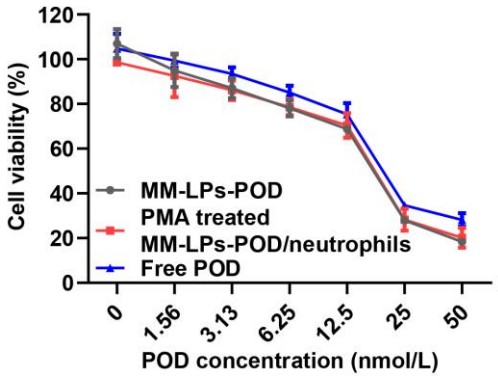
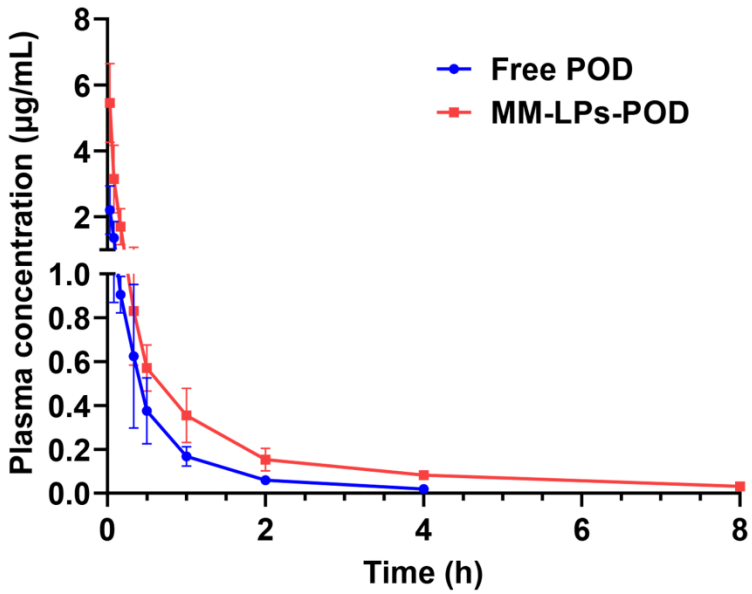


Figure S17 Cytotoxicity of MM-LPs-POD released from PMA-stimulated neutrophils against 4T1 cells for 48 h, compared with MM-LPs-POD and free POD. Neutrophils containing MM-LPs-POD were pretreated with PMA (100 nmol/L) for 4 h followed by centrifugation. The supernatant was incubated with 4T1 cells for 48 h. Data are shown as the mean ±SD (*n*=3).

A



B

Parameters	Free POD	MM-LPs-POD
$t_{1//2}$ (h)	0.857±0.298	2.345±0.549
$AUC_{(0-\infty)}$ (mg/L.h)	0.891±0.129	2.158±0.525
CL (L/h/kg)	1.995±0.268	0.845±0.179

Figure S18 (A) *In vivo* pharmacokinetics studies of free POD and MM-LPs-POD with a single *i.v.* injection of 1.75 mg kg^{-1} POD. (B) Pharmacokinetic parameters of free POD and MM-LPs-POD. Data are shown as the mean ±SD (*n*=5).

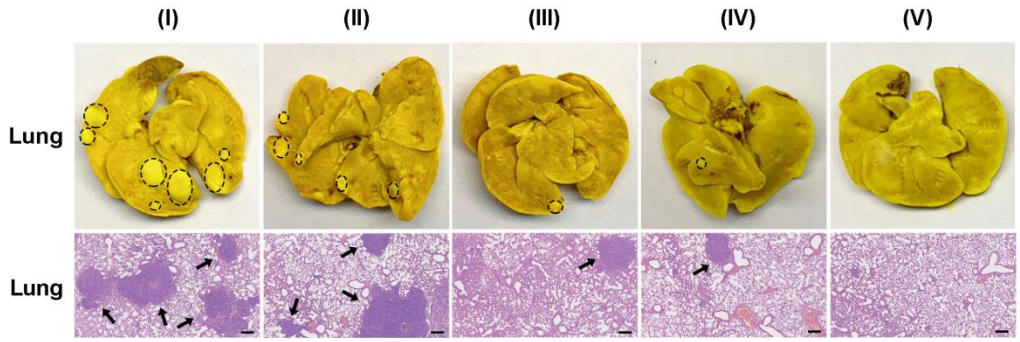


Figure S19 Photos and HE staining of lungs collected from 4T1 tumor-bearing mice. Black circles and arrows represent the sites of metastasis. Scale bar: 200 μm.

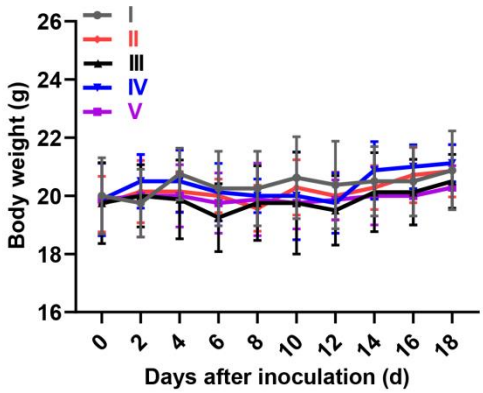


Figure S20 Change in body weight of 4T1 tumor-bearing mice (*n*=6).

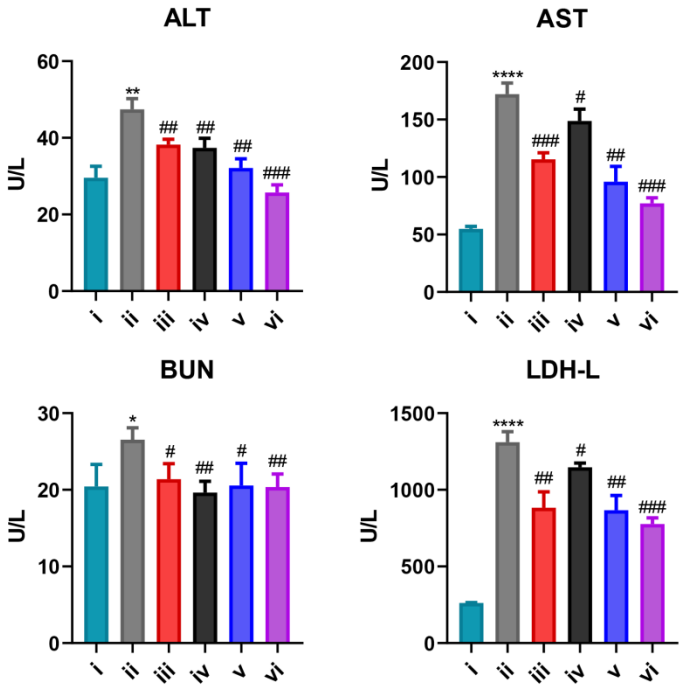


Figure S21 Alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and lactic dehydrogenase (LDH-L) of mice in the antitumor assay (n=3). i) blank, ii) control, iii) free POD, iv) LPs-R848, v) MM-LPs-POD, vi) LPs-R848+MM-LPs-POD. For all data, $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$ vs. blank (group i); $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. control (group ii).

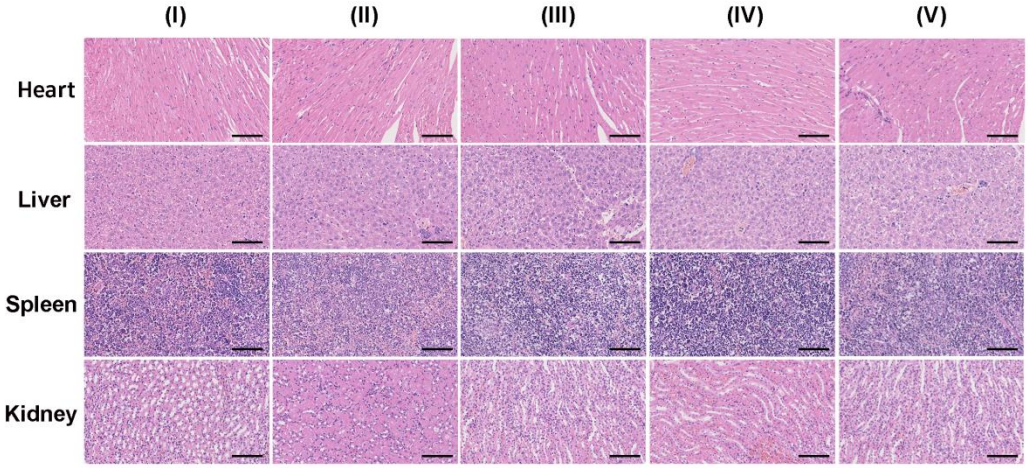


Figure S22 HE staining of major organs. Scale bar: 100 μm.

Reference

1. Liu WL, Zou MZ, Liu T, Zeng JY, Li X, Yu WY, et al. Cytomembrane nanovaccines show therapeutic effects by mimicking tumor cells and antigen presenting cells. *Nat Commun* 2019; **10**: 3199.
2. Zhang ZZ, Wei YN, Liu BB, Wu YZ, Wang HY, Xie X, et al. Hsp90/Sec22b promotes unconventional secretion of mature-IL-1β through an autophagosomal carrier in porcine alveolar macrophages during Mycoplasma hyopneumoniae infection. *Mol Immunol* 2018; **101**: 130-9.