

Targeted immune activation via mannose-decorated erythrocyte membrane PLGA nanoparticles: Sustained delivery of chuanminshen polysaccharides boosts adaptive immunity

Yao Wang^{1,2,§}, Feng Tang^{1,2,§}, Yanwen Yang^{1,2}, and Haibo Feng^{1,2}  

¹ College of Animal Husbandry and Veterinary Medicine, Southwest Minzu University, Chengdu 610041, China

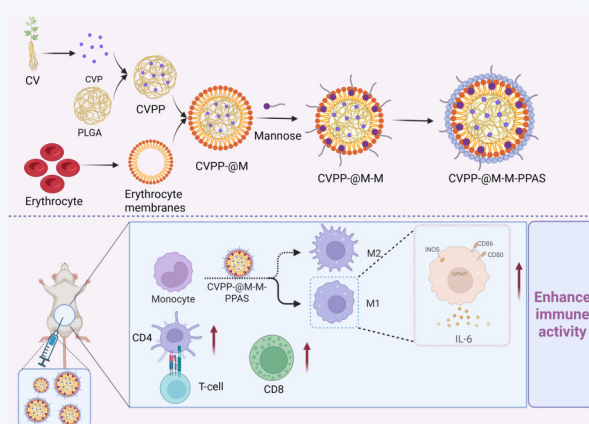
² Institute of Qinghai-Tibetan Plateau, Southwest Minzu University, Chengdu 610041, China

[§] Yao Wang and Feng Tang contributed equally to this work.



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ABSTRACT: The clinical application of *Chuanminshen violaceum* polysaccharides (CVP), a natural immunomodulator with intrinsic antioxidant activity, is constrained by rapid systemic clearance, limited tissue specificity, and short-lived bioactivity. To address these limitations, a multifunctional biomimetic nanoplatform incorporating erythrocyte membrane camouflage, mannose-mediated active targeting, and squalene-stabilized Pickering emulsion technology was developed. CVP-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles (CVPP) were fabricated via solvent evaporation, coated with erythrocyte membranes, and subsequently functionalized with mannose to obtain CVPP@M-M. This dual modification enabled selective recognition by macrophage and dendritic cell (DC) mannose receptors, while the erythrocyte membrane imparted prolonged systemic circulation. Subsequent emulsification for the first time with squalene yielded CVPP@M-M-PPAS, a Pickering emulsion designed for enhanced lymph node delivery. *In vitro*, CVPP@M-M-PPAS significantly promoted macrophage activation, as evidenced by elevated CD80⁺/CD86⁺ expression, compared with free CVP. *In vivo*, intramuscular co-administration with ovalbumin (OVA) antigen induced pronounced DC maturation and T cell polarization in the spleen. This formulation also elicited robust and sustained production of antigen-specific IgG, accompanied by increased upregulation of pro-inflammatory cytokines interleukin-6 (IL-6) and interferon- γ (IFN- γ). *In vivo* imaging demonstrated prolonged lymph node retention (> 336 h) with a near-linear fluorescence decay profile, confirming controlled release kinetics. By integrating stealth properties, receptor-specific targeting, and emulsion-enabled lymphatic trafficking, this nanoplatform effectively circumvents the pharmacokinetic and biodistributional barriers of plant-derived polysaccharides, enabling durable humoral and cellular immune responses. This strategy offers a generalizable framework for translating natural immunomodulators into clinically viable nanotherapeutics.



KEYWORDS: chuanminshen violaceum (CVP), poly(lactic-co-glycolic acid) (PLGA), immune enhancement, mannose receptor

1 Introduction

Chuanminshen violaceum polysaccharides (CVPs) are bioactive macromolecules isolated from the medicinal plant *Chuanminshen violaceum*. Structurally, CVPs are composed primarily of

galacturonic acid, glucose, galactose, and arabinose. Previous studies have demonstrated that root-derived CVPs exhibit strong free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and superoxide anion radicals [1], highlighting their potential as natural antioxidants with anti-aging properties [2]. CVPs extracted from the stems and leaves of *C. violaceum* exhibit comparable pharmacological activities to root-derived CVPs, including significant anti-inflammatory and antioxidant properties [3, 4]. Thus, CVPs from both roots and aerial parts demonstrate consistent bioactivity.

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 Address correspondence to fenghaiborc@126.com

CVPs have demonstrated improved immunoadjuvant properties, including the enhancement of peritoneal macrophage phagocytic capacity, stimulation of splenocyte proliferation, augmentation of natural killer (NK) cell cytotoxic activity, promotion of cytotoxic T lymphocyte (CTL) responses, and elevation of foot-and-mouth disease virus (FMDV)-specific IgG and IgG subclass antibody titers [5]. CVPs engage Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) signaling pathways to induce dendritic cell (DC) maturation, while concurrently downregulating transforming growth factor- β (TGF- β) expression and reducing the frequency of regulatory T cells (Tregs), increasing both cellular and humoral immune responses [5]. Chemical selenylation of CVPs (sCVPs) via the $\text{HNO}_3\text{-Na}_2\text{SeO}_3$ method has been shown to potentiate their immunostimulatory activity, resulting in increased splenocyte proliferation, increased production of interleukin-4 (IL-4) and interferon- γ (IFN- γ), elevated recombinant hepatitis B surface antigen (rHBsAg)-specific IgG titers, augmented T cell proliferation with upregulated IL-2, IL-4, and IFN- γ secretion, and strengthened CD8⁺ T cell-mediated cytotoxicity [6].

Recent progress in nanomaterials science has highlighted nano-adjuvants as promising platforms for next-generation vaccine development, owing to their favorable biocompatibility, nanoscale-associated immunomodulatory effects, high antigen-loading capacity, controlled release profiles, and potential for targeted delivery [7–10]. Poly(lactic-co-glycolic acid) (PLGA) is a biodegradable copolymer composed of lactic acid (LA) and glycolic acid (GA) monomers. It is extensively employed in controlled drug delivery systems due to its low cytotoxicity, excellent tissue biocompatibility, and capacity for site-specific delivery [11–13]. However, PLGA nanoparticles suffer from short systemic circulation and rapid clearance by the reticuloendothelial system (RES) [14].

To address these challenges, cell membrane-camouflaged nanoparticles have been developed as a biomimetic strategy to extend systemic circulation and improve targeting efficiency. Among these, erythrocyte membranes are particularly advantageous due to their enucleated structure and high density of self-recognition markers (e.g., CD47), which facilitated immune evasion and prolonged *in vivo* persistence [15, 16]. CD47 interacts with signal regulatory protein α (SIRP α), transmitting a “don’t eat me” signal that suppresses macrophage-mediated phagocytosis [17, 18]. Despite these advantages, poor targeting remains a major challenge for cell membrane-coated nanoparticles [19].

Mannose surface functionalization has been widely investigated as a strategy to improve targeting specificity and augment immune responses [20]. For example, mannose-conjugated chitosan has been shown to improve gene delivery efficiency through mannose receptor-mediated endocytosis [21]. Mannose receptors expressed on macrophages and DCs can recognize polysaccharide ligands, initiating complement activation, promoting phagocytosis, and stimulating innate immune pathways [8]. Chemically modified mannose ligands have demonstrated efficacy in directing siRNA delivery to macrophages and DCs [22]. The erythrocyte membrane protein CD47 binds to the SIRP α receptor on macrophages, delivering an inhibitory signal that blocks phagosome maturation [23]. This mechanism effectively suppresses phagocytic clearance by the RES, significantly extending the circulation time of nanocarriers [24]. In parallel, mannose ligands incorporated onto the carrier surface selectively engage mannose receptors abundantly expressed on antigen-presenting cells (APCs), including DCs and macrophages, facilitating efficient internalization and targeted

antigen delivery. These design elements create a synergistic “escape-and-target” mechanism: CD47-mediated immune evasion prolongs systemic circulation and promotes accumulation in lymphoid tissues, while mannose-directed recognition ensures precise APC uptake. This integrated approach mitigates immunosuppression, increases adaptive immune activation, and improves the performance of biomimetic nanocarriers in vaccine applications.

Pickering emulsions, stabilized by solid nanoparticles adsorbed at oil–water interfaces, exhibit greater physical stability and immunogenic potential than conventional emulsions. By promoting the recruitment and activation of APCs, they function as potent and well-tolerated adjuvants [25]. Compared with mannose-modified erythrocyte membrane systems, the incorporation of squalene facilitates a more uniform nanoparticle distribution and increases drug-loading capacity, improving overall therapeutic performance. A clinical-stage SARS-CoV-2 nanovaccine designed for lymph node targeting utilizes polymeric nanoparticles decorated with spatially aligned polysialosides. These structures mimic host cell receptors recognized by viruses, acting as “virion-trapping” decoys that bind the SARS-CoV-2 spike protein and prevent its interaction with the ACE2 receptor, independent of conventional immune activation pathways [26]. Clinically, the production of this vaccine is complicated, with a drug encapsulation efficiency of only 8%–20% [26], and the production costs are high. However, our investigational vaccine combines targeted delivery with the ability to elicit durable immune memory at a lower cost, offering a more comprehensive and sustained protective strategy.

Although plant-derived polysaccharides possess significant immunomodulatory and prebiotic properties, their clinical translation is hindered by rapid metabolic clearance, limited tissue specificity, and short *in vivo* half-lives. To overcome these challenges, CVPs were encapsulated within PLGA nanoparticles (CVPP) to achieve sustained release. The particles were further coated with erythrocyte membranes (CVPP@M) to extend systemic circulation, and their surfaces were subsequently functionalized with mannose (CVPP@M-M) to enable active, receptor-mediated targeting. Finally, the resulting nanoparticles were incorporated into a Pickering emulsion (CVPP@M-M-PPAS) to improve physicochemical stability and clinical applicability, amplifying the intrinsic antioxidant and immunoenhancing activities of CVPs.

2 Experimental

2.1 Preparation of CVPP@M-M-PPAS

2.1.1 Preparation of CVPP nanoparticles

The nanoparticles were prepared using the solvent evaporation method. Briefly, 5 mg of CVP (extracted following the protocol by Fan et al., 2017 [1]) was dissolved in 10 mL of 0.5% Poloxamer aqueous solution to form the aqueous phase. Separately, 50 mg of PLGA (50:50; molecular weight 1–2 kDa; Shanghai Yuanye Bio-Technology, Catalog No. S24436) was dissolved in 4 mL of acetone under ultrasonication for 10 min to obtain the oil phase. The oil phase was then added dropwise into the aqueous phase under continuous stirring. The resulting mixture was homogenized by sonication for 180 s to form an emulsion. After 12 h of rotary evaporation to remove organic solvents, the substance gradually solidified and formed nano-sized particles. The nanoparticles were subsequently collected by centrifugation (12,000 rpm, 15 min),

washed three times with deionized water, lyophilized in the presence of a cryoprotectant, and stored as CVPP nanospheres.

2.1.2 Preparation of CVPP@M-M (mannose-modified erythrocyte membrane-coated CVPP)

Erythrocyte membrane extraction: Blood collection & red blood cells (RBC) isolation: 5 mL of mouse orbital blood was collected into an anticoagulant tube and centrifuged (1500 rpm, 3 min, 4 °C). The supernatant and buffy coat (containing leukocytes) were carefully removed.

RBC washing: The packed RBCs were resuspended in a tenfold volume of cold isotonic buffer (or phosphate buffer saline (PBS)), followed by centrifugation at 1500 rpm at 4 °C. The washing process was repeated two to three times until the supernatant became colorless.

Hemolysis & membrane harvesting: The purified RBCs were lysed in 0.25% hypotonic PBS at a 1:10 ratio for 20 min at 4 °C until the suspension appeared translucent dark red. The lysate was centrifuged at 10,000 rpm for 10 min at 4 °C, and the process was repeated three to four times to pellet the erythrocyte membranes (pink precipitate), which were subsequently stored in liquid nitrogen.

CVPP@M-M fabrication: The erythrocyte membranes were resuspended in PBS and combined with CVPP nanoparticles. The suspension was extruded through a polycarbonate membrane 25 times to facilitate membrane coating, after which it was centrifuged to remove excess membranes. To confer targeting capability, DSPE-PEG-Mannose (DSPE-PEG-MAN) was added at a concentration of 0.1 mg/mL and stirred for 1 h, allowing mannose conjugation to the erythrocyte membrane surface, yielding CVPP@M-M.

2.1.3 Preparation of CVPP@M-M-PPAS (Pickering emulsion)

Squalene was mixed with the CVPP@M suspension at a 9:1 (v/v) ratio and sonicated on ice for 30 s. DSPE-PEG-MAN was then added at a concentration of 0.1 mg/mL and stirred for 1 h to achieve mannose surface modification. The final formulation, CVPP@M-M-PPAS, was obtained as a stable Pickering emulsion. Non-mannosylated control nanoparticles (CVPP-PPAS) were prepared using the same protocol, omitting the mannose modification step.

2.2 Characterization of CVPP@M-M and CVPP@M-M-PPAS

2.2.1 Morphological examination

Transmission electron microscopy (TEM) of CVPP@M-M: TEM was used to confirm erythrocyte membrane coating on CVPP nanoparticles. Briefly, 10 µL of the nanoparticle suspension was deposited onto a copper grid and allowed to air-dry for 5 min. Excess liquid was removed using filter paper, and the sample was negatively stained with 1% phosphotungstic acid (three drops, 10 s). The grid was then dried under an alcohol lamp before imaging.

DSPE-PEG-Mannose conjugation efficiency (%) =

$$\frac{\text{NP@M-M thermal weight loss percentage} - \text{NP@M thermal weight loss percentage}}{\text{Mannose thermal weight loss percentage}} \times 100\% \quad (3)$$

(3) Analysis of mannose coupling ratio by the Phenol-sulfuric acid method

A 1 mg/mL stock solution of polyethylene glycol-mannose

Fluorescence microscopy of CVPP@M-M-PPAS: To verify the formation of a Pickering emulsion, fluorescein isothiocyanate (FITC)-labeled ovalbumin (OVA-FITC) was prepared by incubating OVA with FITC overnight in the dark. The resulting OVA-FITC was encapsulated into CVPP@M and CVPP@M-M-PPAS, and the formulations were subsequently examined by fluorescence microscopy.

2.2.2 Encapsulation efficiency (EE%) and drug loading (DL%) of CVPP@M-M

Standard curve preparation: Phenol solution was prepared by dissolving 5 g of phenol in preheated distilled water to a final volume of 100 mL and stored in an amber glass bottle. A glucose stock solution (0.1 mg/mL) was serially diluted to obtain standard concentrations ranging from 0.5 to 0.03125 mg/mL. Each standard was mixed with 5% phenol solution, followed by the addition of concentrated sulfuric acid, and incubated for 20 min at room temperature. Absorbance was measured at 490 nm using distilled water as the blank.

Nanoparticle analysis: CVPP nanoparticles were centrifuged at 15,000 rpm for 30 min at 4 °C to separate the free CVP from the encapsulated fraction. The concentration of free CVP in the supernatant was determined by the phenol-sulfuric acid method.

Calculations: EE% and DL% were calculated using the following equations (Eqs. (1) and (2)).

$$\text{Encapsulation efficiency (EE\%)} = C_e / C_t \times 100\% \quad (1)$$

where C_e is encapsulated CVP concentration; C_t is total CVP (encapsulated + free) concentration.

$$\text{Drug loading (DL\%)} = W_e / W_p \times 100\% \quad (2)$$

where W_e is mass of encapsulated CVP; W_p is mass of PLGA.

2.2.3 Qualitative and quantitative analysis of mannose in CVPP@M-M

(1) Fourier-transform infrared (FTIR) spectroscopy

Lyophilized samples of blank nanoparticles (CVPP), erythrocyte membrane-coated nanoparticles (CVPP@M), and mannose-modified nanoparticles (CVPP@M-M) were individually mixed with potassium bromide (KBr) and compressed into pellets. FTIR spectra were recorded over the range of 4000–400 cm^{-1} to verify successful mannose conjugation.

(2) Thermogravimetric analysis (TGA)

TGA was performed using an SDT650 instrument (Saugus, USA). Approximately 10 mg each of NP@M-M, mannose, and NP@M samples were heated under a nitrogen atmosphere at a rate of 20 °C/min over the temperature range of 20–420 °C. The thermal weight loss curves were automatically recorded using the instrument software, and data were processed to calculate the mass loss for each sample. The conjugation efficiency of DSPE-PEG-Mannose was estimated according to the following equation (Eq. (3)).

(PEG-mannose) was prepared in deionized water and serially diluted to concentrations of 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL. For each concentration, 250 µL of solution was

transferred to centrifuge tubes, followed by the addition of 150 μL of 5% phenol solution and 625 μL of concentrated sulfuric acid. The mixtures were incubated in an 80 $^{\circ}\text{C}$ water bath for 20 min, then cooled in an ice-water bath for 10 min. Absorbance was measured at 490 nm, and a standard calibration curve was constructed.

For mannose quantification on nanoparticles, CVPP@M-M was resuspended in 10 mL deionized water and placed into a dialysis membrane (MWCO 2000 Da). Dialysis was performed against deionized water, after which the dialysate was collected and concentrated. The mannose concentration in the dialysate was determined using the phenol-sulfuric acid method.

The mannose content on CVPP@M-M was calculated, and the mannose coupling ratio was determined using the following equation (Eq. (4)), with concentration expressed in mg/mL.

$$\text{Mannose coupling rate (\%)} = \frac{\text{Mass of mannose coupled to nanoparticles}}{\text{Total mass of CVPP@M-M added}} \times 100 \quad (4)$$

2.2.4 Particle size and zeta potential measurements

CVPP, CVPP@M-M, CVPP-PPAS, and CVPP@M-M-PPAS were diluted in PBS and analyzed by dynamic light scattering (DLS) for hydrodynamic diameter and zeta potential.

2.2.5 In vitro release profile

Nanoparticles (CVPP, CVPP@M-M, CVPP-PPAS, and CVPP@M-M-PPAS) were enclosed in dialysis membranes and incubated in serum at 37 $^{\circ}\text{C}$ with continuous shaking at 80 rpm. At predetermined time points (2, 4, 6, 8, 10, 24, 48, and 72 h), the released CVP was quantified using the phenol-sulfuric acid method.

2.3 Culture of M ϕ

The cell culture medium for RAW264.7 cells consisted of DMEM, fetal bovine serum, and Penicillin-Streptomycin in a ratio of 88:11:1. For cell resuscitation and culture, cryotubes removed from liquid nitrogen were immediately thawed in a 37 $^{\circ}\text{C}$ water bath for approximately 1 min. When thawing reached the point where a small amount of ice remained, 2 mL of medium was added in a sterile laminar flow hood and mixed thoroughly. The cells were then centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and 2 mL of fresh medium was added and gently resuspended to achieve a homogeneous suspension. The cells were transferred into a culture flask, and the total medium volume was adjusted to 4 mL. The flask was incubated at 37 $^{\circ}\text{C}$ in a 5% CO_2 incubator until the cell density reached 80%–90% for passaging. For passaging, the culture medium was removed, and the flask was rinsed 1–2 times with PBS. Adherent cells were detached using a cell scraper, and the resulting clumps were gently broken up by pipetting several times. The cells were then reseeded and returned to the incubator at 37 $^{\circ}\text{C}$ with 5% CO_2 for continued culture.

2.3.1 Effect of drugs on M ϕ activity

To evaluate the safe concentration range of the drug, its effect on cell viability was assessed using the cell counting kit-8 (CCK-8) assay. M ϕ in the logarithmic growth phase was seeded into 96-well plates at a density of approximately 1×10^4 cells/mL (100 μL per well) and incubated at 37 $^{\circ}\text{C}$ in a 5% CO_2 incubator for 12 h to allow cell attachment. After adherence, the culture medium was removed, and experimental wells, blank wells, and control wells

were prepared. Lyophilized drugs were reconstituted in DMEM to concentrations of 500, 250, 125, 62.5, 31.25, and 15.625 $\mu\text{g/mL}$. Six replicate wells were assigned for each concentration. The cells were incubated with the drugs at 37 $^{\circ}\text{C}$ and 5% CO_2 for 24 h. Subsequently, 10 μL of CCK-8 reagent was added to each well under light-protected conditions and incubated at 37 $^{\circ}\text{C}$ for 30 min. Absorbance at 450 nm was measured using a microplate reader to determine cell viability.

2.3.2 Effect of drugs on NO secretion by M ϕ

Since M ϕ activation induces NO secretion, this experiment was designed to evaluate the effect of the drugs on NO production as an indicator of macrophage immunoreactivity. Cells were cultured as described in Section 2.3 and adjusted to a density of 2×10^5 cells/mL. The cell suspension was seeded into 96-well plates and incubated for 12 h to allow adherence. Subsequently, drugs were added at concentrations of 125, 62.5, 31.25, and 15.625 $\mu\text{g/mL}$, and the cells were incubated at 37 $^{\circ}\text{C}$ in 5% CO_2 for 24 h. The culture supernatants were collected, and the NO content was measured using a commercial NO detection kit according to the manufacturer's instructions.

2.3.3 Effects of drugs on M ϕ cell phenotype

Macrophage M1 polarization mediates pro-inflammatory responses against bacteria and pathogens, primarily characterized by the expression of MHC II, CD80, and CD86, which are critical for host defense against bacterial infections. This experiment was designed to evaluate the effects of drugs on macrophage pro-inflammatory activity by monitoring CD80 and CD86 expression.

Cell culture: Macrophages were cultured as described in Section 2.3 and adjusted to a density of 2×10^5 cells/mL. Cells were seeded into 6-well plates at 2 mL per well and incubated for 12 h. Subsequently, drugs were added at a concentration of 31.25 $\mu\text{g/mL}$, and the cells were cultured for an additional 24 h. Three replicate wells were prepared for each treatment group.

Cell collection: The culture medium was removed, and cells were washed 1–2 times with PBS. Cells were then detached by adding 1 mL PBS and gently pipetting until the suspension appeared homogeneous and transparent.

CD80 and CD86 labeling: A total of 300 μL of the cell suspension was transferred to a 2 mL EP tube, and 300 μL of 5% skimmed milk solution was added for blocking on ice for 30 min. The reaction was terminated by adding 1 mL PBS, followed by centrifugation at 4000 rpm for 2 min. The supernatant was discarded, and cells were washed twice with PBS. Cells were then stained using a single-staining protocol: after discarding the wash supernatant, the appropriate antibody dye was added, and the suspension was gently mixed. The staining reaction was performed on ice for 30 min and terminated by adding 1 mL PBS. Following centrifugation at 4000 rpm for 2 min, the supernatant was discarded, and the cells were washed twice. The final cell pellet was resuspended in 1 mL PBS for flow cytometry analysis.

2.3.4 Effect of M ϕ on phagocytosis of drugs

Phagocytosis is a fundamental function of M ϕ . To evaluate the phagocytic activity of M ϕ toward different nano-adjuvant formulations, FITC-labeled ovalbumin (OVA-FITC, green fluorescence) was encapsulated in nanoparticles, and M ϕ were incubated with various OVA-FITC-loaded vaccine formulations for 12 h. Uptake of the nanoparticles by M ϕ *in vitro* was assessed using flow cytometry and fluorescence microscopy.

Cell culture: Mφ were cultured as described in Section 2.3 and adjusted to a density of 2×10^5 cells/mL. Cells were seeded into 6-well plates at 2 mL per well. Experimental treatments included PBS (negative control), free FITC-OVA solution (31.25 μg/mL), and nanoparticles containing an equivalent amount of FITC-OVA. Cells were incubated with these formulations for 12 h.

Sample collection and flow cytometry: Following incubation, cells were detached by gentle pipetting, collected, and centrifuged at 4000 rpm for 2 min. The cell pellets were washed twice with PBS, and the phagocytosis of nanoparticles by macrophages was quantified by flow cytometry.

Fluorescence microscopy: In parallel experiments, after 12 h of incubation, cell nuclei and membranes were stained with 4',6-diamidino-2-phenylindole (DAPI, blue) and DiD (red), respectively. The internalization of the nanoparticles by macrophages was then visualized using fluorescence microscopy.

2.4 Animal grouping and immunization procedures

Mice were randomly assigned to seven groups ($n = 18$ per group): PBS (blank control), Freund's complete adjuvant (FCA, positive control), CVP/OVA, CVPP-OVA, CVPP@M-M-OVA, CVPP-PPAS-OVA, and CVPP@M-M-PPAS-OVA. The concentrations of CVP and OVA were 31.25 and 100 μg/mL, respectively. Immunizations were administered twice, with a second dose given 7 days after the initial immunization at 0.2 mL per animal.

All animal experiments were conducted in strict accordance with the guidelines of the Medical Ethics Review Form of the Animal Ethics Committee (Approval No. 2022MDLS46).

2.4.1 Determination of dendritic cell activation in spleen

To evaluate the activation of DCs in the spleens of mice following drug administration, spleens were harvested on the second day after the initial immunization. The tissues were processed to obtain single-cell suspensions, which were subsequently subjected to double staining using PE- and FITC-conjugated antibodies, specifically anti-FITC-CD11c, anti-PE-CD86, and anti-PE-CD80. Appropriate controls, including unstained (blank) tubes and single-stained samples, were also prepared. The stained cells were then analyzed by flow cytometry to assess DC activation.

2.4.2 Detection of specific IgG antibodies in serum

To assess the antibody titers in mice, a booster immunization was administered seven days after the initial immunization. Blood samples were collected via the orbital sinus on day 14, 28, and 42 post-immunization, followed by centrifugation at $2000 \times g$ for 5 min to obtain serum from the supernatant. The levels of specific IgG antibodies in the serum were subsequently determined using the enzyme-linked immunosorbent assay (ELISA) method, following the manufacturer's instructions.

2.4.3 Detection of splenic T-lymphocyte differentiation

Twenty-one days after the initial immunization, spleens were collected from three mice per experimental group. The tissues were processed into single-cell suspensions, which were subsequently labeled using a double-staining approach with PE- and FITC-conjugated antibodies. Cell surface markers CD3-FITC, CD4-PE, and CD8-PE were applied. The stained cells were transferred to flow cytometry tubes and analyzed using a flow cytometer. Experimental data were processed and analyzed with FlowJo software to complete the study.

2.4.4 Determination of splenic cytokine mRNA expression

Twenty-eight days after the second immunization, spleens were harvested from the mice, and total RNA was extracted using Trizol reagent. The concentration and purity of the RNA were assessed, ensuring an A260/A280 ratio between 1.8 and 2.2 before reverse transcription. Complementary DNA (cDNA) synthesis was performed using Takara reverse transcription reagents, followed by quantitative polymerase chain reaction (PCR) to assess mRNA expression levels of IL-6 and IFN-γ in splenic lymphocytes. Primers were designed and synthesized as listed in Table 1. Relative gene expression was calculated using β-actin as the internal reference (Eq. (5)).

$$\Delta Ct(\text{target gene}) = Ct(\text{target gene}) - Ct(\text{internal reference gene of the same sample}) \quad (5)$$

$$\begin{aligned} \Delta\Delta Ct(\text{target gene}) &= \Delta Ct(\text{target gene}) \\ &\text{in the experimental group} - \\ &\Delta Ct(\text{target gene}) \text{ in the reference group} \end{aligned} \quad (6)$$

The relative multiplicity of the expression of the target gene in the experimental group relative to the control group (experimental group/reference group) equals $2^{-\Delta\Delta Ct}$ (target gene).

2.4.5 Determination of immune organ indexes

Twenty-eight days after the second immunization, three mice from each group were euthanized, and their spleens were collected, weighed, and recorded. The kidneys, spleens, and livers were then fixed in 4% paraformaldehyde for 24 h, dehydrated through a graded alcohol series, embedded in paraffin, and sectioned for histological analysis to evaluate the tissue morphology of the spleens.

2.4.6 Antigen release in vivo

Cy5.5 dye was diluted 100-fold and mixed with the OVA-encapsulated nano-adjuvants at a 1:1 ratio, followed by overnight stirring to enable coupling of Cy5.5 to OVA. The Cy5.5-labeled nano-adjuvant formulations, including the CVPP-OVA, CVPP@M-M-OVA, CVPP-PPAS-OVA, and CVPP@M-M-PPAS-OVA groups, were administered to mice via intramuscular injection. *In vivo* imaging was conducted at 6, 24, 48, 120, and 336 h post-injection. Subsequently, the heart, liver, spleen, lungs, and kidneys were harvested for *ex vivo* imaging. Fluorescence images were analyzed and processed using Living Image software, and quantitative fluorescence data were collected.

2.4.7 Safety experiment

To evaluate the safety of the nano-adjuvant, three mice from each group were randomly selected 28 days after the second immunization for hematological analysis. Following the assessment of immune organ indices, the kidneys, spleens, and livers were

Table 1 Mannose coupling rate

Samples	Mannose input amount (mg)	Mannose coupling rate (%)	Mannose coupling rate (%)
1	100	48.403%	
2	100	43.652%	44.858% ± 2.548%
3	100	42.521%	

harvested and fixed in 4% paraformaldehyde for 24 h. The tissues were then dehydrated, embedded in paraffin, sectioned, deparaffinized, and rehydrated with distilled water in preparation for hematoxylin and eosin (H&E) staining. Tissue sections were immersed in hematoxylin solution for 5 min, rinsed with tap water, differentiated with dilute hydrochloric acid, and rinsed under running water to restore the blue coloration. Sections were subsequently stained with eosin for 2–3 min, rinsed thoroughly, dehydrated, cleared, and mounted. Histological morphology was then examined under a microscope.

3 Results and discussion

3.1 Successful preparation and characterization of CVPP@M-M-PPAS

Nanoparticle-based delivery systems hold significant promise but are often limited by short circulation times and rapid systemic clearance [27]. To overcome these limitations, a dual-functionalized nanoparticle platform was developed, with an erythrocyte membrane coating incorporated to improve biocompatibility [28] and mannose modification to improve targeting specificity and drug utilization [29]. Structural characterization indicated that a well-defined core-shell architecture was exhibited by the CVPP@M-M nanoparticles, as evidenced by TEM imaging (Fig. 1(a)). The spherical PLGA core, encapsulating OVA, was distinctly enveloped by an erythrocyte membrane layer, and the increase in particle size relative to bare PLGA nanoparticles further confirmed successful fabrication.

Fluorescence microscopy analysis (Fig. 1(b)) provided detailed insights into nanoparticle behavior, revealing that CVPP@M-M existed predominantly as discrete particles, whereas the PPAS-containing formulation (CVPP@M-M-PPAS) exhibited organized aggregation. This observation suggests that the incorporation of PPAS increases interparticle cohesion, potentially improving drug-loading capacity. Quantitative assessment of polysaccharide content, performed using a validated phenol-sulfuric acid assay, indicated an encapsulation efficiency of 61% and a drug-loading capacity of 6% for CVP in CVPP@M-M ($R^2 = 0.9913$ for the glucose standard curve, Fig. 1(c)).

FTIR spectroscopy (Fig. 1(d)) confirmed the successful conjugation of mannose, with characteristic absorption peaks observed at 3302.18 cm^{-1} (–OH stretching), $1657.66/1099.79\text{ cm}^{-1}$ (C=O stretching), and a mannose-specific fingerprint at 807.87 cm^{-1} . These findings were further corroborated by TGA analysis (Fig. 1(e)). The thermal mass loss process of the three species shown in the Fig. 1(e) is mainly divided into two stages. In the first stage, the temperature is 100–300 °C, and the mass loss is gentle, which may be caused by the evaporation of water in the substance; the temperature in the second stage is 300–420 °C, with a relatively high rate of quality loss. The residual mass percentages of 1, 2, and 3 in the figure are 6%, –4.43%, and –7.23%, respectively. From this calculation, it can be concluded that the grafting rate of mannose in the nanoparticle complex coated with red blood cell membrane modified with mannose is 42.5%.

As detailed in Table 1, the standard calibration curve was defined by the equation $y = 5.1489x + 0.1134$ ($R^2 = 0.9993$). We determined the average mannose coupling ratio on CVPP@M-M nanoparticles to be $44.858\% \pm 2.548\%$.

The immunological significance of nanoparticle physicochemical

properties is well-documented [30]. The dimensions of our system ($236.2 \pm 3.40\text{ nm}$ for CVPP and $271.87 \pm 7.36\text{ nm}$ for CVPP@M-M, as determined by DLS; Fig. 1(f)) are particularly favorable, as they fall within the 200–300 nm range optimal for APC-mediated lymph node trafficking and sustained immune activation [31]. The increase in particle size following erythrocyte membrane coating was consistently confirmed by both TEM and DLS analyses. When incorporated into squalene-based Pickering emulsions, larger assemblies were formed ($959.67 \pm 2.50\text{ nm}$ for CVPP-PPAS and $1009.33 \pm 8.22\text{ nm}$ for CVPP@M-M-PPAS; Fig. 1(g)), while excellent monodispersity was maintained (poly dispersity index (PDI) < 0.3; Fig. 1(h)).

Surface charge analysis indicated that erythrocyte membrane coating decreased the zeta potential from $-10.27 \pm 0.52\text{ mV}$ (CVPP) to $-7.17 \pm 0.62\text{ mV}$ (CVPP@M-M; Fig. 1(i)), consistent with successful membrane encapsulation. Furthermore, the incorporation of squalene improved colloidal stability, as reflected by significantly more negative zeta potentials ($-20.33 \pm 0.12\text{ mV}$ for CVPP-PPAS and $-15.37 \pm 0.17\text{ mV}$ for CVPP@M-M-PPAS; $P < 0.05$).

The drug release profiles (Fig. 1(j)) exhibited biphasic kinetics for all formulations, characterized by an initial burst release of 20.91%–34.51% within the first 10 h, followed by a sustained release phase reaching 42.53%–53.39% cumulative release over 72 h. Squalene-containing formulations showed moderated release kinetics, indicating their potential for prolonged and controlled drug release.

Given that drug loading efficiency, release behavior, and nanoparticle stability are critical determinants of nano-adjuvant performance, the PLGA nanoparticles (CVPP@M-M) achieved an encapsulation efficiency of 61% for CVPP with a drug loading capacity of 6%. Recognizing that drug release directly influences immune response efficacy, squalene was incorporated to optimize release kinetics. A soft-core Pickering emulsion (CVPP@M-M-PPAS) was formulated using squalene as the oil phase and CVPP@M-M or CVPP nanoparticles as the outer aqueous phase.

Following incorporation with squalene, changes in particle size, zeta potential, and PDI were assessed. The zeta potential of the squalene-containing formulations was higher than that of the nanoparticles without squalene, indicating that squalene incorporation substantially increases nano-adjuvant stability. Moreover, *in vitro* release studies demonstrated that the presence of squalene significantly prolonged the drug release profile.

A novel vaccine adjuvant platform, the CVPP@M-M-PPAS Pickering emulsion system, was developed, with uniform CVPP@M-M nanoparticles incorporated as its core. Mannose-targeting conjugation was achieved with an efficiency of 42.5% through a precise fabrication process, and excellent physicochemical properties were exhibited, including a uniform particle size distribution (PDI < 0.3) and size-optimized design, providing an ideal carrier framework for vaccine delivery. Physical stability of the nanoemulsion was substantially improved through the incorporation of squalene, and sustained-release functionality was imparted, resulting in an extended antigen presentation window and prolonged immunostimulation. Targeting precision, structural integrity, and controlled release were integrated through this multilevel, synergistic design, enhancing antigen presentation via mannose-mediated active targeting and enabling spatiotemporally programmed release of vaccine components. This intelligent delivery platform is considered a significant advancement

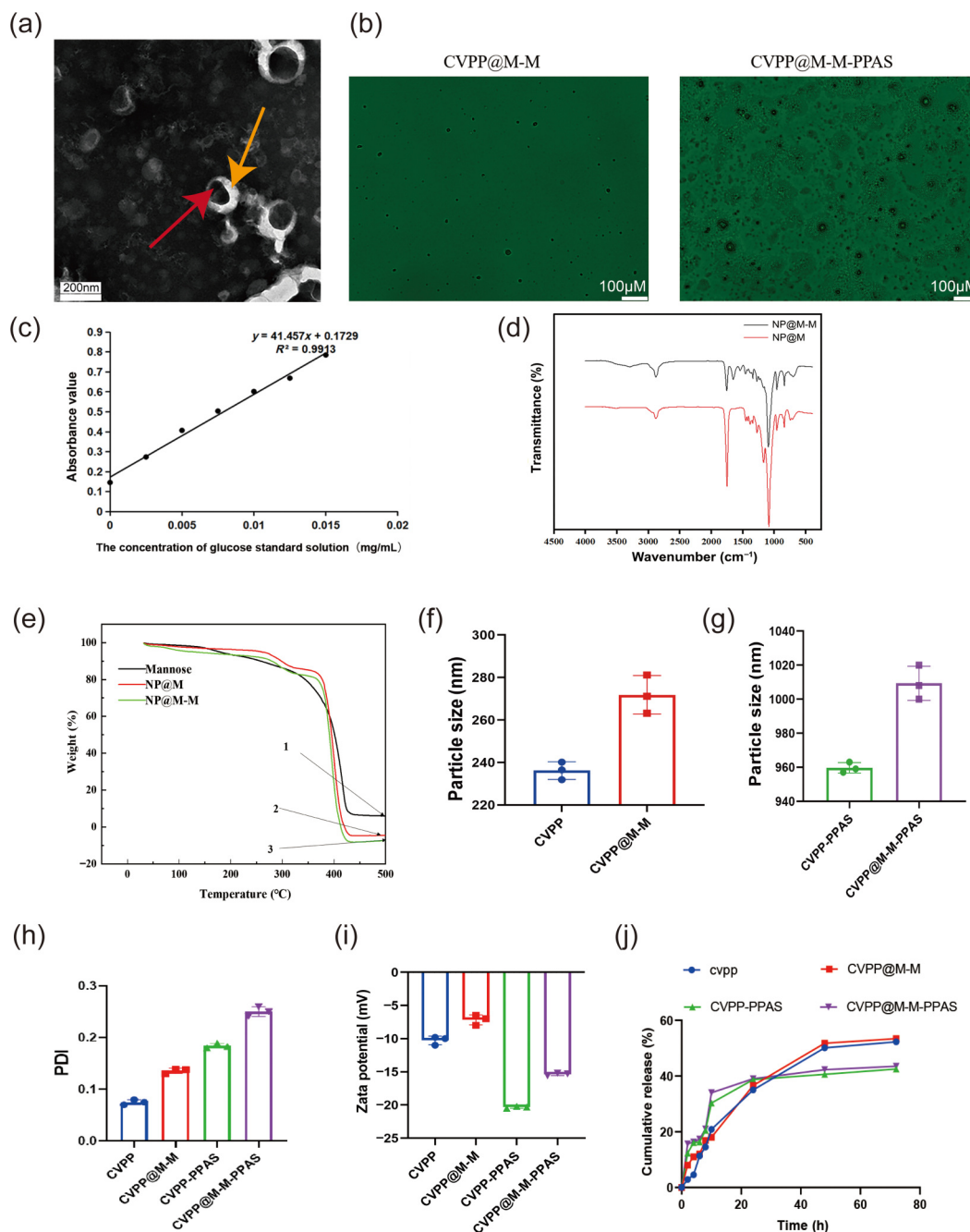


Figure 1 CVPP@M-M-PPAS characterization and *in vitro* release. (a) CVPP@M-M electron microscopy morphology of nanoparticles. Scale bars: 200 nm. The red arrow indicates that the inner layer is PLGA nanoparticles wrapped in the OVA, and the yellow arrow indicates that the outer layer is a translucent red blood cell membrane structure. (b) CVPP@M-M-PPAS and CVPP@M-M fluorescence observation. Scale bars: 100 μm . (c) Glucose standard curve. (d) CVPP@M-M The infrared spectrum. (e) Thermogravimetric analysis. (f) The particle size of nanoparticles CVPP and CVPP@M-M. (g) The particle size of nanoparticles CVPP-PPAS and CVPP@M-M-PPAS. (h) PDI of four types of nanoparticles. The average PDI of CVPP is 0.075. The average PDI of CVPP@M-M is 0.135. The average PDI of CVPP-PPAS is 0.184. The average PDI of CVPP@M-M-PPAS is 0.250. (i) Zeta potential of nanoparticles CVPP, CVPP@M-M, CVPP-PPAS and CVPP@M-M-PPAS. (j) *In vitro* release curves of four types of nanoparticles encapsulating CVP. All statistical analyses were presented as mean $\pm$ standard deviation (SD), $n = 3$.

in next-generation vaccine development, with strong potential for clinical translation.

3.2 CVPP@M-M-PPAS enhances macrophage phagocytosis *in vitro*

Mannose-functionalized nanoparticles were strategically engineered to facilitate targeted delivery to macrophages, key APCs that mediate crosstalk between innate and adaptive immunity [32]. The

CVPP@M-M-OVA nanoparticles exhibited significantly increased macrophage uptake via mannose receptor-mediated phagocytosis, an effect further enhanced by the inclusion of squalene, which contributes both to local drug enrichment and intrinsic immunostimulatory activity [33]. This targeted engagement promoted pronounced polarization of macrophages toward the pro-inflammatory M1 phenotype, as indicated by elevated CD80⁺/CD86⁺ surface marker expression [34] and increased nitric

oxide (NO) production, a multifunctional mediator known to exert antimicrobial and antitumor effects through inhibition of DNA synthesis and disruption of mitochondrial electron transport [35].

Biocompatibility evaluation revealed concentration-dependent cell viability profiles (Fig. 2(a)). Squalene-containing formulations, CVPP-PPAS (91.5%) and CVPP@M-M-PPAS (94.3%), maintained excellent cytocompatibility at 31.25 $\mu\text{g/mL}$. However, higher concentrations in these formulations led to viability reductions below 90%, whereas non-squalene formulations (CVPP and CVPP@M-M) exhibited increased cytotoxicity even at 15.625 $\mu\text{g/mL}$, establishing 31.25 $\mu\text{g/mL}$ as the optimal safe concentration. Immunological assessment via NO secretion (Fig. 2(b)) highlighted the adjuvant role of squalene, with CVPP-PPAS and CVPP@M-M-PPAS eliciting significantly higher NO production compared to non-squalene formulations at

concentrations above 31.25 $\mu\text{g/mL}$ ($P < 0.05$), confirming the concentration-dependent activation of pro-inflammatory pathways.

Flow cytometric analysis (Figs. 2(c)–2(f)) confirmed the polarization effects, identifying CVPP@M-M-PPAS as the most potent inducer of the M1 phenotype ($P < 0.05$ compared with other experimental groups), a shift critical for effective pathogen clearance. Complementary assessment of phagocytic activity using fluorescently labeled OVA (Figs. 2(g)–2(i)) demonstrated increased uptake across all CVP-containing formulations relative to OVA controls ($P < 0.05$). Squalene-containing systems (CVPP-PPAS-OVA and CVPP@M-M-PPAS-OVA) outperformed non-squalene formulations, and mannose-modified CVPP@M-M-OVA exhibited higher internalization compared with unmodified nanoparticles ($P < 0.05$).

These results establish CVPP@M-M-PPAS as a multifunctional

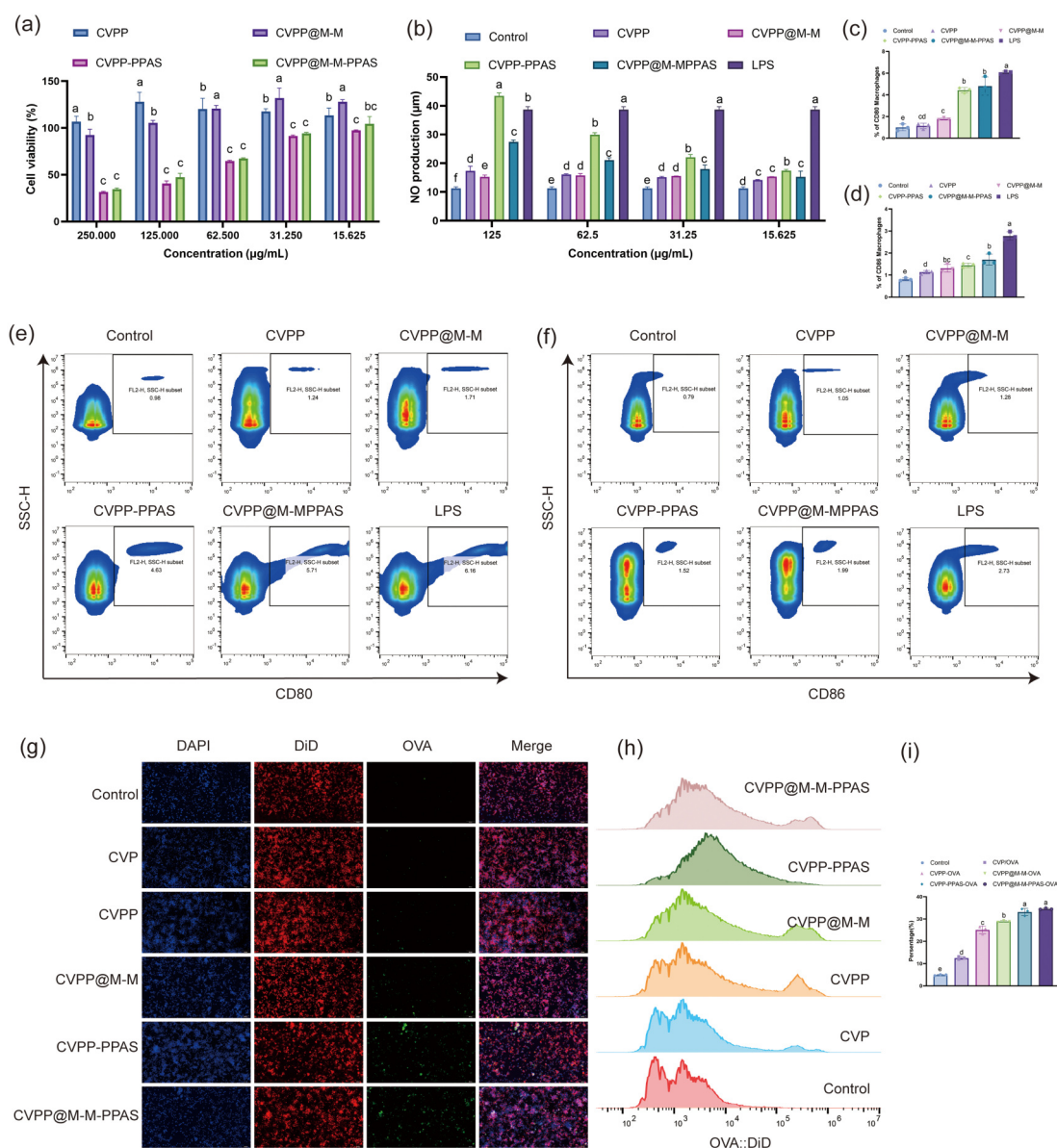


Figure 2 Effects of drug group on M1 polarization and phagocytosis of macrophages. Functional evaluation of macrophages across experimental groups: (a) Macrophage activity and (b) NO secretion were assessed. Alongside flow cytometry analysis of (c) CD80 and (d) CD86 expression, with representative histograms shown in (e) and (f), respectively. Phagocytic function was evaluated through (g) fluorescence imaging and further quantified by (h) and (i) flow cytometry. Note: Bars labeled with identical letters denote no significant differences, whereas those with different letters indicate statistically significant differences ($P < 0.05$).

nanoplatfrom that integrates mannose-mediated targeting specificity, squalene-enhanced immunostimulation, and precisely controlled activation kinetics. The observed concentration-dependent balance between biocompatibility and immunogenicity highlights the importance of formulation optimization. Moreover, the platform's ability to increase antigen uptake, induce M1 macrophage polarization, and sustain nitric oxide-mediated effector functions underscores its potential as a next-generation vaccine adjuvant. This approach is consistent with current advances in precision immunomodulation strategies [29, 36], offering a versatile platform for improving both prophylactic and therapeutic vaccine approaches through spatiotemporal regulation of immune activation and targeted delivery.

3.3 Enhanced immune cell proliferation by drug formulations in mice

The maturation status of DCs, a pivotal factor in initiating adaptive immune responses, was quantitatively evaluated via surface

expression of CD80⁺ and CD86⁺, established markers of DC functional maturation [37]. Comparative analysis demonstrated a significant increase in CD80⁺ expression across all drug-treated groups relative to controls ($P < 0.05$, Fig. 3(a)), with squalene-containing formulations showing improved effects. The CVPP-PPAS-OVA and CVPP@M-M-PPAS-OVA groups induced higher CD80⁺ levels than non-squalene formulations (CVP/OVA, CVPP-OVA, CVPP@M-M-OVA; $P < 0.01$), while mannose-modified CVPP@M-M-PPAS-OVA exhibited a significant advantage over its non-targeted counterpart, CVPP-PPAS-OVA ($P < 0.05$), confirming the synergistic benefits of squalene adjuvantation and carbohydrate-mediated targeting. A similar pattern was observed for CD86⁺ expression (Fig. 3(b)), with all drug-treated groups showing significant elevations versus controls ($P < 0.05$), and squalene-containing formulations maintaining their superiority over non-squalene groups ($P < 0.05$). The degree of DC maturation induced by squalene-incorporated nanoparticles was comparable to that achieved with FCA, the gold-standard immunostimulant,

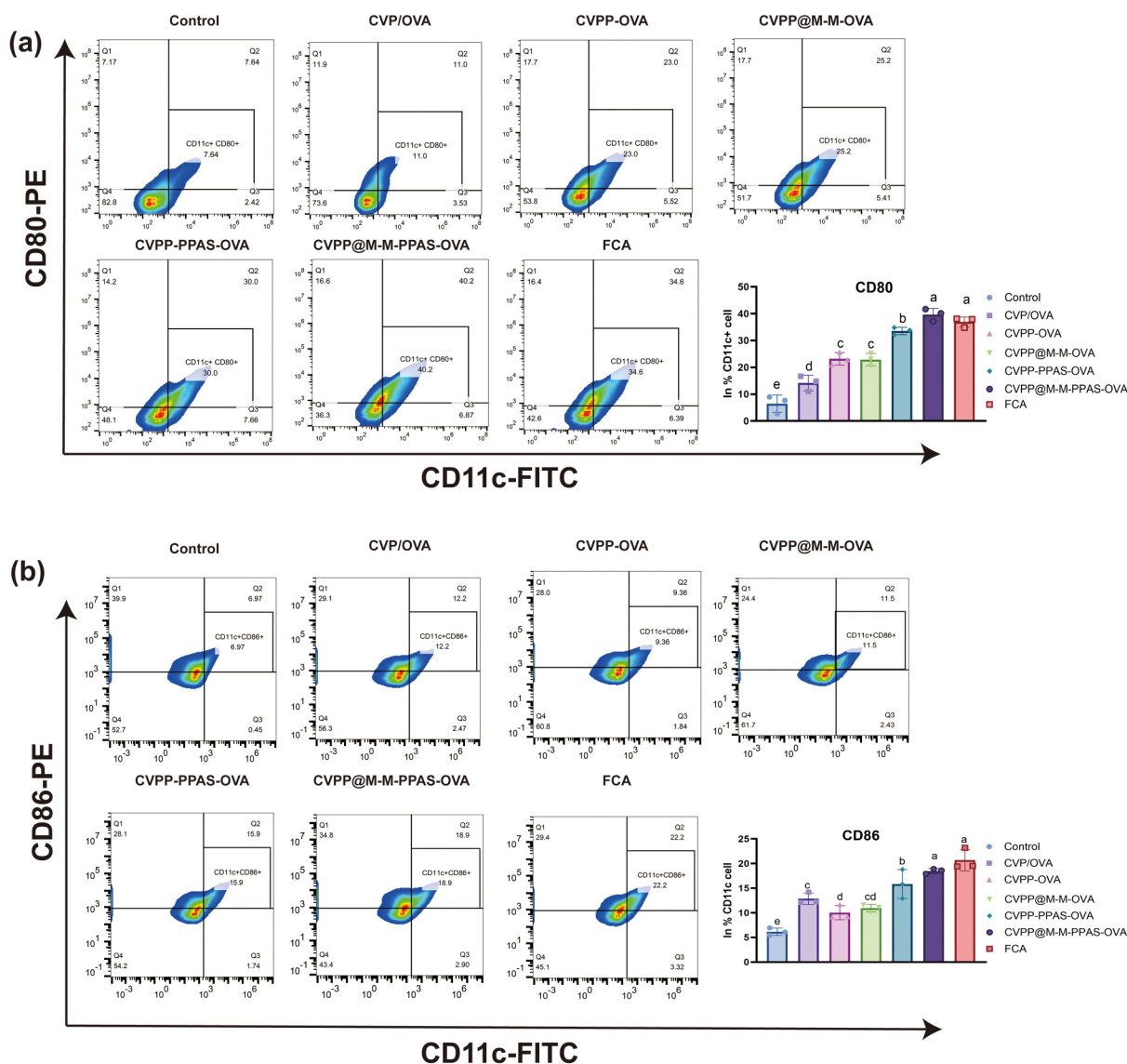


Figure 3 The effect of the drug group on immune cells in mice. (a) Representative flow cytometry data and expression statistics of CD80⁺ on dendritic surface molecules in the mouse spleen. (b) Representative flow cytometry data and expression statistics of CD86⁺ on dendritic surface molecules in the mouse spleen. Note: The letters atop the bars indicate statistical significance. Identical letters denote no significant difference, while different letters denote significant differences ($P < 0.05$).

underscoring squalene's dual role as both a stabilizing agent and a potent immunopotentiator. The coordinated upregulation of both co-stimulatory markers ($CD80^+/CD86^+$) in squalene-containing formulations, particularly in the mannose-functionalized CVPP@M-M-PPAS-OVA, indicates synergistic activation of DC maturation pathways through combined pathogen-associated molecular pattern (PAMP) recognition and enhanced antigen presentation. These results establish the CVPP@M-M-PPAS system as a multifunctional immunostimulatory platform capable of recapitulating natural immune activation cascades while maintaining formulation stability—a critical advancement in the design of next-generation vaccine adjuvants.

3.4 Drug formulations enhance immune responses in mice

Evaluation of splenic immunocompetence revealed a significant increase in spleen indices across all treatment groups compared with blank controls ($P < 0.05$, Fig. 4(a)). The mannose-modified CVPP@M-M-OVA displayed optimal performance relative to its non-targeted counterpart, CVPP-OVA, demonstrating the immunostimulatory advantage conferred by carbohydrate-mediated targeting. Squalene-containing formulations (CVPP-PPAS-OVA and CVPP@M-M-PPAS-OVA) further increased splenic responses compared with non-emulsified groups ($P < 0.05$), confirming the synergistic adjuvant effect of squalene.

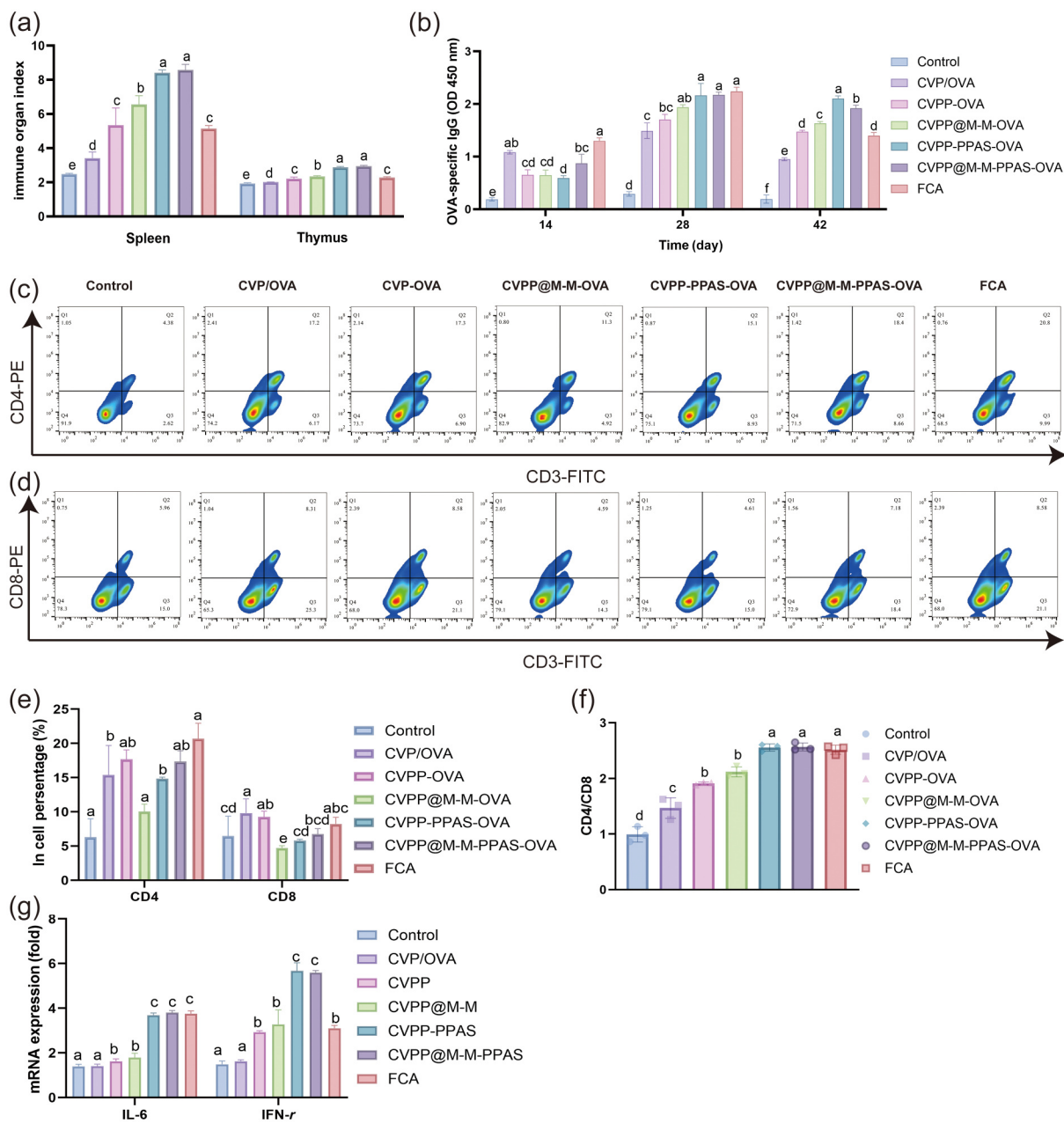


Figure 4 Effect of drug group on immune activity in mice. (a) Organ index of spleen and lymph node immune organs. (b) Changes in serum IgG levels in mice. (c) Representative flow cytometry data of $CD4^+$ in mouse splenic lymphocytes treated with different drugs. (d) Representative flow cytometry data of $CD8^+$ in mouse splenic lymphocytes treated with different drugs. (e) Statistical analysis of $CD4^+$ expression in mouse spleen lymphocytes treated with different drugs. (f) Statistical analysis of $CD4^+$ expression in mouse spleen lymphocytes treated with different drugs. (g) The mRNA expression levels of IL-6 and IFN- γ in the spleen. Note: The letters atop the bars indicate statistical significance. Identical letters denote no significant difference, while different letters denote significant differences ($P < 0.05$).

Humoral immune responses, assessed by longitudinal IgG quantification (Fig. 4(b)), showed that all treatment groups achieved significantly higher antibody titers than controls by day 14 ($P < 0.05$). By day 28, squalene-containing formulations reached IgG levels comparable to those induced by FCA ($P > 0.05$ vs. positive control) and significantly exceeded the titers of other nanoparticle groups ($P < 0.05$). IgG levels in the squalene-formulated groups remained stable through day 42, whereas titers declined in the CVP/OVA and FCA groups ($P > 0.05$ vs. day 28), indicating that the PPAS-mediated system provides sustained antigen release via high payload capacity combined with controlled liberation kinetics.

Cellular immunity assessment focused on splenic T-lymphocyte dynamics-critical determinants of adaptive immune responses [38, 39]. All nanoformulations produced a significant elevation in CD4⁺/CD8⁺ ratios compared to controls ($P < 0.05$, Figs. 4(c) and 4(d)), with squalene-containing groups exhibiting significantly higher ratios than their non-emulsified counterparts ($P < 0.05$). This Th-biased polarization was further substantiated by cytokine analysis (Figs. 4(e) and 4(f)), which demonstrated that squalene-based formulations elicited pronounced IL-6 and IFN- γ secretion 21 days post-boost (Fig. 4(g)), serving as critical mediators of Th1-oriented immune responses [40]. The coordinated enhancement of both helper (CD4⁺) and cytotoxic (CD8⁺) T cell populations, coupled with Th1 cytokine induction, suggests comprehensive immune activation through: (1) Mannose-mediated APC targeting, (2) Squalene-driven costimulatory signaling, and (3) nanoparticle-enabled sustained antigen presentation.

These immunological correlations align with established DC maturation patterns [37] and T cell differentiation mechanisms [41–43], validating the capacity of CVPP@M-M-PPAS-OVA to orchestrate coordinated engagement of both humoral and cellular branches of adaptive immunity. The concurrent maintenance of CD4⁺/CD8⁺ ratios with sustained IgG production positions this platform as a balanced adjuvant system capable of supporting durable immune memory while mitigating risks of excessive polarization, a critical consideration in rational vaccine design [44]. The observed coordination between splenic cellular responses and sustained systemic antibody production highlights the potential of this formulation to confer durable protective immunity through multimodal immune activation. However, certain limitations remain: antigen-specific T-cell responses were assessed exclusively at the gene expression level, without subsequent validation at the protein level.

3.5 Sustained-release drug formulations prolong immune activation *in vivo*

Real-time biodistribution and drug release kinetics were systematically monitored using *in vivo* imaging system (IVIS) live-animal imaging, revealing distinct formulation-dependent patterns (Figs. 5(a)–5(d)). All groups displayed biphasic fluorescence dynamics, characterized by an initial increase in signal intensity followed by gradual attenuation. Non-emulsified formulations (CVPP and CVPP@M-M) exhibited rapid pharmacokinetics, with peak fluorescence observed at 24 h and significant signal decline by 336 h ($P < 0.05$), consistent with substantial OVA release. However, squalene-stabilized systems (CVPP-PPAS and CVPP@M-M-PPAS) maintained stable fluorescence signals with minimal fluctuations, indicative of prolonged antigen retention and controlled release behavior.

Organ-level analyses further demonstrated formulation-dependent biodistribution. Conventional nanoparticles (CVPP/CVPP@M-M) initially localized to hepatic, pulmonary, and lymphoid tissues (0–48 h) before shifting toward predominant renal accumulation after 48 h. Persistent renal fluorescence was observed in CVPP, whereas CVPP@M-M underwent near-complete systemic clearance by 336 h, leaving only residual nodal signals. By contrast, squalene-formulated adjuvants exhibited exclusive lymph node (LN) tropism across all time points, with LN signal intensities consistently surpassing those of non-emulsified counterparts. The absence of hepatic and renal accumulation in these groups confirms the role of squalene in directing nanoparticles toward lymphoid reservoirs while circumventing systemic clearance pathways.

The 24 h fluorescence maxima across formulations likely reflect redistribution of antigen from intramuscular depots to more superficial, detection-accessible tissues. Squalene-based systems demonstrated sustained LN residency extending beyond 14 days post-injection, a phenomenon attributable to optimized size-dependent lymphatic drainage and surface-mediated retention effects. This prolonged antigen depot within secondary lymphoid organs corresponds with the improved humoral and cellular immune responses observed in earlier experiments, providing mechanistic support for sustained immune activation via controlled antigen presentation. The differential biodistribution profiles highlight the critical influence of nanocarrier design on *in vivo* fate, with targeted LN accumulation emerging as a defining factor in adjuvant performance.

3.6 Drug formulations exhibit no toxicity to organs or tissues *in vivo*

Biosafety was confirmed through comprehensive histopathological and hematological assessments. H&E-stained tissue sections (Fig. 6(a)) showed no pathological alterations, inflammatory infiltrates, or structural abnormalities in vital organs (liver, spleen, kidneys) across all treatment groups, indicating systemic tolerance. Quantification of splenic germinal centers (Fig. 6(b)) further demonstrated that the CVPP@M-M-PPAS group exhibited significantly greater germinal center proliferation compared to the blank control, reflecting improved immune capacity in the spleen. Squalene-formulated groups (CVPP-PPAS and CVPP@M-M-PPAS) displayed increased follicle proliferation and germinal center activity, histomorphological correlates of adaptive immune activation, without evidence of lymphoid toxicity.

Hematological profiling (Figs. 6(c)–6(g)) corroborated these findings, with all complete blood count (CBC) parameters (leukocyte counts, hemoglobin levels, platelet indices) remaining within normal physiological ranges. Variations in neutrophil counts among groups are likely attributable to formulation-specific immunomodulatory effects rather than toxicity, consistent with earlier immune response indicators such as cytokine induction and antibody kinetics.

The rational integration of erythrocyte membrane cloaking, mannose-mediated targeting, and squalene-stabilized Pickering emulsions effectively addressed classical limitations of polysaccharide adjuvants, namely, rapid clearance and limited bioavailability. This engineered design endowed CVPP@M-M-PPAS with dual functionality: (i) Receptor-targeted delivery to mannose-expressing APCs (macrophages/DCs), and (ii) sustained lymphoid tissue retention to prolong immune activation. The

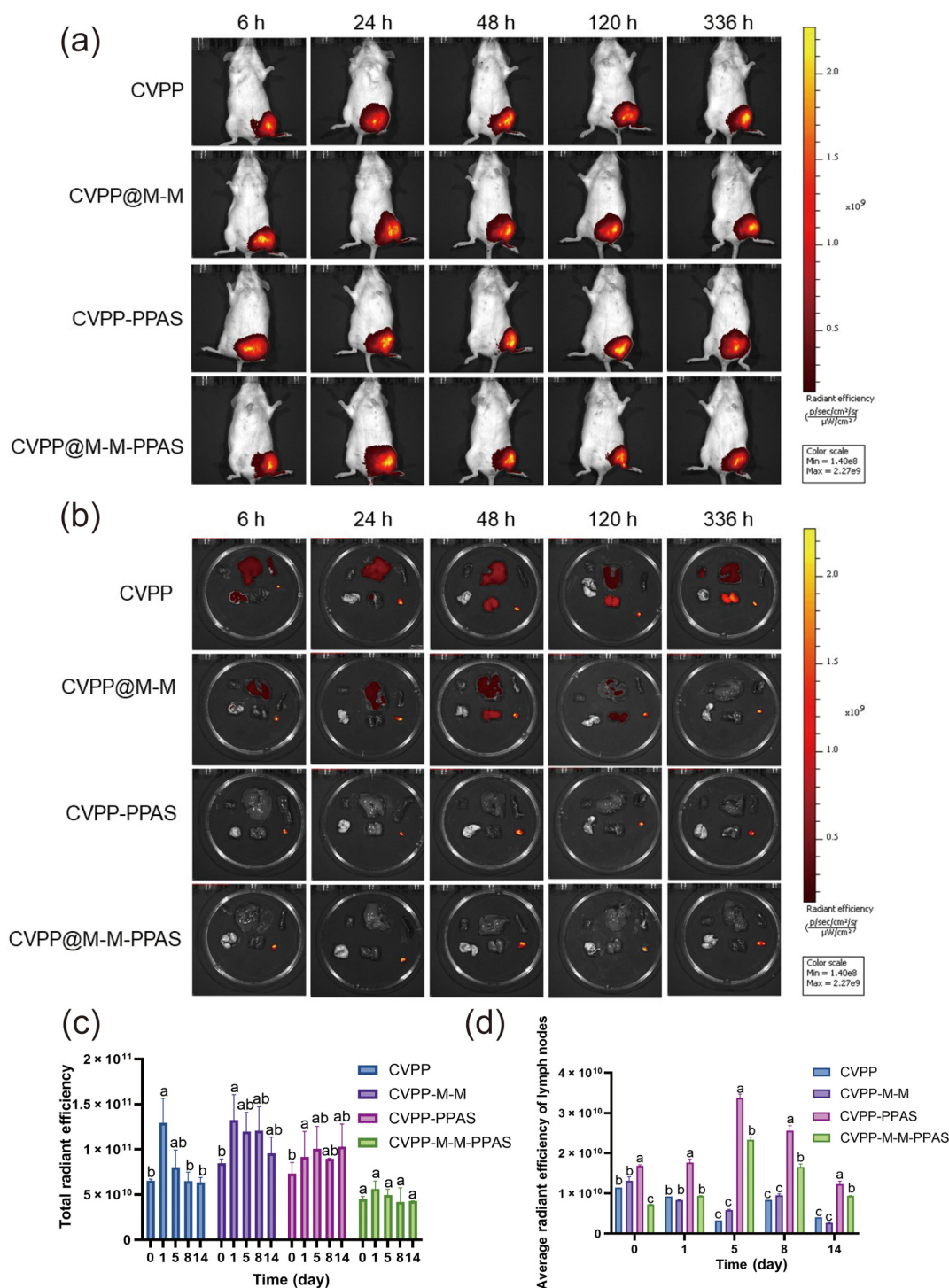


Figure 5 *In vivo* imaging of mice in different drug groups. (a) Live images of mice injected with nano adjuvant at different time points. (b) Fluorescence images of *ex vivo* organs taken at different time points after intramuscular injection of nano adjuvant. (c) Fluorescence intensity of the mouse body surface. (d) Lymph node fluorescence intensity. Note: The letters atop the bars indicate statistical significance. Identical letters denote no significant difference, while different letters denote significant differences.

absence of organotoxicity together with preserved hematological homeostasis positions this system as a translationally viable adjuvant platform, balancing robust immunostimulation with stringent biosafety, an essential advancement in vaccine nanotechnology.

Long-term evaluation remains outstanding, as a 42-day efficacy study was not conducted. Consequently, persistence of immune

protection at later stages could not be assessed. However, early-phase quantification of splenic germinal centers at Day 7 demonstrated that CVPP@M-M-PPAS elicited increased immunogenicity, evidenced by significantly higher germinal center proliferation. These findings warrant further investigation in subsequent developmental stages.

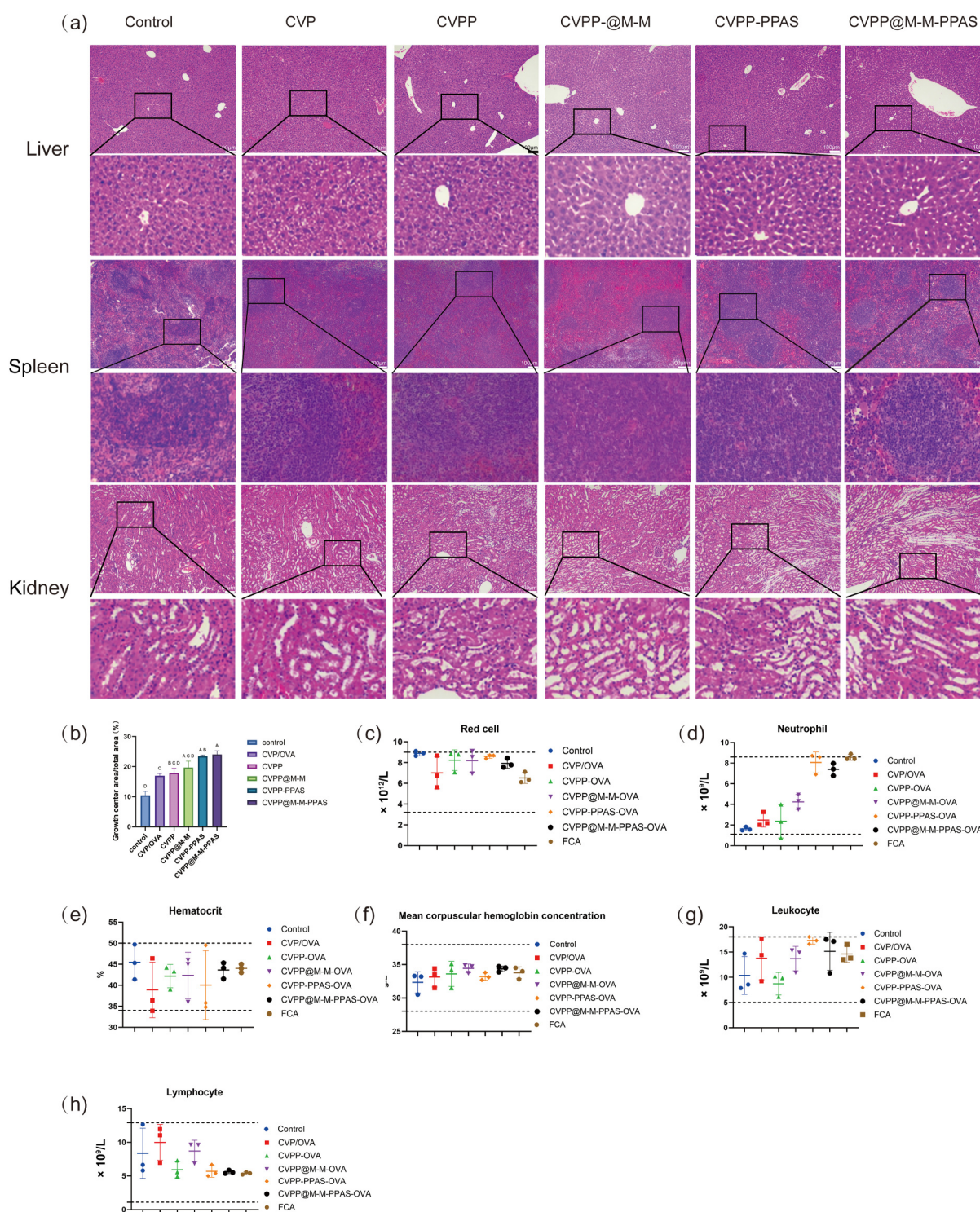


Figure 6 Histopathological and hematological evaluation of major organs in mice. (a) H&E sectional images of liver, kidney, and spleen. (b) Percentage of the area of hair growth centers in different groups compared to the total area. Note: The capital letters atop the bars indicate statistical significance. Identical capital letters denote no significant difference, while different letters denote significant differences. (c) The number of red blood cells in the blood of each group of mice. (d) The number of neutrophils in the blood of each group of mice. (e) Red blood cell volume in the blood of each group of mice. (f) Red blood cell accumulation in the blood of each group of mice. (g) The number of leukocyte in the blood of each group of mice. (h) The number of lymphocyte in the blood of each group of mice.

4 Conclusions

To enhance immunogenicity, an immunomodulatory CVP complex was engineered and encapsulated within PLGA nanoparticles to enable sustained release. This core was

subsequently conjugated with mannose moieties for targeted delivery and cloaked with erythrocyte membranes to achieve improved immune evasion. Finally, squalene was incorporated to form a homogenized emulsion, optimizing immune potentiation. All components were derived from non-pyrogenic and non-

cytotoxic biomaterials, and their biocompatibility was validated through comprehensive safety assessments, including hemocompatibility testing and cytokine storm risk profiling. Relative to mannose-modified erythrocyte membrane systems, the incorporation of squalene was shown to promote more homogeneous distribution and greater drug-loading capacity, improving therapeutic efficacy. In current polymer nanoparticle vaccines, where polysialosides are precisely aligned on the surface, immune responses are induced independently of conventional pathways; however, such systems exhibit limited immunological memory and necessitate repeated administrations to maintain protection. By contrast, the CVPP@M-M-PPAS formulation integrated both targeted delivery and durable immune memory, achieving sustained activation through lymph node-focused delivery, improved APC targeting, and balanced Th1/Th2 polarization. These findings position CVPP@M-M-PPAS as a multifunctional vaccine platform that provides a blueprint for the design of next-generation adjuvants with improved safety and efficacy.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

Y. W.: Data curation, project administration, validation, writing manuscript, experimental design; F. T.: Data curation, project administration, validation, experimental design; Y. W. Y.: Project administration, writing manuscript; H. B. F.: Project administration, funding acquisition, writing manuscript, experimental design. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

This experiment was performed in strict accordance with the Medical Ethics Review Form of the Animal Ethics Committee (Ethics No.: 2022MDLS46).

Use of AI statement

None.

References

- [1] Fan, J.; Feng, H. B.; Yu, Y.; Sun, M. X.; Liu, Y. R.; Li, T. Z.; Sun, X.; Liu, S. J.; Sun, M. D. Antioxidant activities of the polysaccharides of *Chuanminshen violaceum*. *Carbohydr. Polym.* **2017**, *157*, 629–636.
- [2] Zou, Y. F.; Jize, X. P.; Li, C. Y.; Zhang, C. W.; Fu, Y. P.; Yin, Z. Q.; Li, Y. P.; Song, X.; Li, L. X.; Zhao, X. H. et al. Polysaccharide from aerial part of *Chuanminshen violaceum* alleviates oxidative stress and inflammatory response in aging mice through modulating intestinal microbiota. *Front. Immunol.* **2023**, *14*, 1159291.
- [3] Lin, S.; Li, H. Y.; Wang, Z. Y.; Liu, X.; Yang, Y.; Cao, Z. W.; Du, G.; Zhao, L.; Zhang, Q.; Wu, D. T. et al. Analysis of methanolic extracts and crude polysaccharides from the leaves of *Chuanminshen violaceum* and their antioxidant activities. *Antioxidants* **2019**, *8*, 266.
- [4] Jize, X. P.; Fu, Y. P.; Li, C. Y.; Zhang, C. W.; Zhao, Y. Z.; Kuang, Y. C.; Liu, S. Q.; Huang, C.; Li, L. X.; Tang, H. Q. et al. Extraction, characterization and intestinal anti-inflammatory and anti-oxidative activities of polysaccharide from stems and leaves of *Chuanminshen violaceum* M. L. Sheh & R. H. Shan. *J. Ethnopharmacol.* **2024**, *332*, 118357.
- [5] Feng, H. B.; Fan, J.; Qiu, H.; Wang, Z. H.; Yan, Z. Q.; Yuan, L. H.; Guan, L.; Du, X. G.; Song, Z. H.; Han, X. F. et al. *Chuanminshen violaceum* polysaccharides improve the immune responses of foot-and-mouth disease vaccine in mice. *Int. J. Biol. Macromol.* **2015**, *78*, 405–416.
- [6] Feng, H. B.; Fan, J.; Bo, H. Q.; Tian, X.; Bao, H.; Wang, X. H. Selenylation modification can enhance immune-enhancing activity of *Chuanminshen violaceum* polysaccharide. *Carbohydr. Polym.* **2016**, *153*, 302–311.
- [7] Gu, P. F.; Wusiman, A.; Zhang, Y.; Liu, Z. G.; Bo, R. N.; Hu, Y. L.; Liu, J. G.; Wang, D. Y. Rational design of PLGA nanoparticle vaccine delivery systems to improve immune responses. *Mol. Pharm.* **2019**, *16*, 5000–5012.
- [8] Nam, J.; Kim, A.; Kim, K.; Moon, J. H.; Baig, J.; Phoo, M.; Moon, J. J.; Son, S. Engineered polysaccharides for controlling innate and adaptive immune responses. *Nat. Rev. Bioeng.* **2024**, *2*, 733–751.
- [9] Souto, E. B.; Blanco-Llamero, C.; Krambeck, K.; Kiran, N. S.; Yashaswini, C.; Postwala, H.; Severino, P.; Priefer, R.; Prajapati, B. G.; Maheshwari, R. Regulatory insights into nanomedicine and gene vaccine innovation: Safety assessment, challenges, and regulatory perspectives. *Acta Biomater.* **2024**, *180*, 1–17.
- [10] Wu, J. S.; Wang, X. P.; Wang, Y. D.; Xun, Z.; Li, S. Application of PLGA in tumor immunotherapy. *Polymers* **2024**, *16*, 1253.
- [11] Park, J. S.; Jeon, H.; Lee, Y.; Cheon, S. Y.; Lee, D.; Lim, S. G.; Koo, H. Rapid DNA repair in mesenchymal stem cells and bone regeneration by nanoparticle-based codelivery of Nrf2-mRNA and dexamethasone. *ACS Nano* **2024**, *18*, 31877–31890.
- [12] Seegobin, N.; Abdalla, Y.; Li, G.; Murdan, S.; Shorthouse, D.; Basit, A. W. Optimising the production of PLGA nanoparticles by combining design of experiment and machine learning. *Int. J. Pharm.* **2024**, *667*, 124905.
- [13] Makadia, H. K.; Siegel, S. J. Poly Lactic-co-Glycolic Acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers* **2011**, *3*, 1377–1397.
- [14] Dupuis, V.; Cerbu, C.; Witkowski, L.; Potarniche, A. V.; Timar, M. C.; Žychska, M.; Sabliov, C. M. Nanodelivery of essential oils as efficient tools against antimicrobial resistance: A review of the type and physical-chemical properties of the delivery systems and applications. *Drug Deliv.* **2022**, *29*, 1007–1024.
- [15] Liu, L. Z.; Bai, X.; Martikainen, M. V.; Kårlund, A.; Roponen, M.; Xu, W. J.; Hu, G. Q.; Tasciotti, E.; Lehto, V. P. Cell membrane coating integrity affects the internalization mechanism of biomimetic nanoparticles. *Nat. Commun.* **2021**, *12*, 5726.
- [16] Fang, R. H.; Gao, W. W.; Zhang, L. F. Targeting drugs to tumours

- using cell membrane-coated nanoparticles. *Nat. Rev. Clin. Oncol.* **2023**, *20*, 33–48.
- [17] Bian, Z.; Shi, L.; Guo, Y. L.; Lv, Z. Y.; Tang, C.; Niu, S.; Tremblay, A.; Venkataramani, M.; Culpepper, C.; Li, L. M. et al. Cd47-Sirpa interaction and IL-10 constrain inflammation-induced macrophage phagocytosis of healthy self-cells. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E5434–E5443.
- [18] Malhotra, S.; Dumoga, S.; Sirohi, P.; Singh, N. Red blood cells-derived vesicles for delivery of lipophilic drug camptothecin. *ACS Appl. Mater. Interfaces* **2019**, *11*, 22141–22151.
- [19] Krishnan, N.; Jiang, Y.; Zhou, J. R.; Mohapatra, A.; Peng, F. X.; Duan, Y. O.; Holay, M.; Chekuri, S.; Guo, Z. Y.; Gao, W. W. et al. A modular approach to enhancing cell membrane-coated nanoparticle functionality using genetic engineering. *Nat. Nanotechnol.* **2024**, *19*, 345–353.
- [20] Yousefpour, P.; Ni, K. Y.; Irvine, D. J. Targeted modulation of immune cells and tissues using engineered biomaterials. *Nat. Rev. Bioeng.* **2023**, *1*, 107–124.
- [21] Yang, H.; Wang, Z. Y.; Li, L. X.; Wang, X.; Wei, X.; Gou, S.; Ding, Z. M.; Cai, Z. H.; Ling, Q. J.; Hoffmann, P. R. et al. Mannose coated selenium nanoparticles normalize intestinal homeostasis in mice and mitigate colitis by inhibiting NF- κ B activation and enhancing glutathione peroxidase expression. *J. Nanobiotechnol.* **2024**, *22*, 613.
- [22] Uehara, K.; Harumoto, T.; Makino, A.; Koda, Y.; Iwano, J.; Suzuki, Y.; Tanigawa, M.; Iwai, H.; Asano, K.; Kurihara, K. et al. Targeted delivery to macrophages and dendritic cells by chemically modified mannose ligand-conjugated siRNA. *Nucleic Acids Res.* **2022**, *50*, 4840–4859.
- [23] Logtenberg, M. E. W.; Scheeren, F. A.; Schumacher, T. N. The CD47-SIRP α immune checkpoint. *Immunity* **2020**, *52*, 742–752.
- [24] Jia, X.; Yan, B. J.; Tian, X. Q.; Liu, Q.; Jin, J. H.; Shi, J. J.; Hou, Y. Z. CD47/SIRP α pathway mediates cancer immune escape and immunotherapy. *Int. J. Biol. Sci.* **2021**, *17*, 3281–3287.
- [25] Xia, Y. F.; Wu, J.; Wei, W.; Du, Y. Q.; Wan, T.; Ma, X. W.; An, W. Q.; Guo, A. Y.; Miao, C. Y.; Yue, H. et al. Exploiting the pliability and lateral mobility of Pickering emulsion for enhanced vaccination. *Nat. Mater.* **2018**, *17*, 187–194.
- [26] Ross, K. A.; Kelly, S.; Phadke, K. S.; Peroutka-Bigus, N.; Fasina, O.; Siddoway, A.; Mallapragada, S. K.; Wannemuehler, M. J.; Bellaire, B. H.; Narasimhan, B. Next-generation nanovaccine induces durable immunity and protects against SARS-CoV-2. *Acta Biomater.* **2024**, *183*, 318–329.
- [27] Wang, J.; Zhu, M. T.; Nie, G. J. Biomembrane-based nanostructures for cancer targeting and therapy: From synthetic liposomes to natural biomembranes and membrane-vesicles. *Adv. Drug Deliv. Rev.* **2021**, *178*, 113974.
- [28] Liu, H. Y.; Lu, Y. M.; Zong, J. B.; Zhang, B.; Li, X. L.; Qi, H. Z.; Yu, T.; Li, Y. Engineering dendritic cell biomimetic membrane as a delivery system for tumor targeted therapy. *J. Nanobiotechnol.* **2024**, *22*, 663.
- [29] Yang, Y. X.; Wang, K.; Pan, Y. W.; Rao, L.; Luo, G. X. Engineered cell membrane-derived nanoparticles in immune modulation. *Adv. Sci.* **2021**, *8*, 2102330.
- [30] Wu, Y.; Liang, X. Y.; Mao, C. Q.; Jiang, Y. H. The distinct properties of polysaccharide nanoparticles tune immune responses against mRNA antigen via stimulator of interferon genes-mediated autophagy and inflammasome. *ACS Nano* **2023**, *17*, 21782–21798.
- [31] Roth, G. A.; Picece, V. C. T. M.; Ou, B. S.; Luo, W.; Pulendran, B.; Appel, E. A. Designing spatial and temporal control of vaccine responses. *Nat. Rev. Mater.* **2022**, *7*, 174–195.
- [32] Rodríguez-Morales, P.; Franklin, R. A. Macrophage phenotypes and functions: Resolving inflammation and restoring homeostasis. *Trends Immunol.* **2023**, *44*, 986–998.
- [33] Guo, Y. C.; Li, Y. J.; Zhang, M. Z.; Ma, R.; Wang, Y. Y.; Weng, X.; Zhang, J. J.; Zhang, Z. Z.; Chen, X. Y.; Yang, W. J. Polymeric nanocarrier via metabolism regulation mediates immunogenic cell death with spatiotemporal orchestration for cancer immunotherapy. *Nat. Commun.* **2024**, *15*, 8586.
- [34] Chen, S. Z.; Saeed, A. F. U. H.; Liu, Q.; Jiang, Q.; Xu, H. Z.; Xiao, G. G.; Rao, L.; Duo, Y. H. Macrophages in immunoregulation and therapeutics. *Signal Transduct. Target. Ther.* **2023**, *8*, 207.
- [35] Arnold, P. K.; Jackson, B. T.; Paras, K. I.; Brunner, J. S.; Hart, M. L.; Newsom, O. J.; Alibeckoff, S. P.; Endress, J.; Drill, E.; Sullivan, L. B. et al. A non-canonical tricarboxylic acid cycle underlies cellular identity. *Nature* **2022**, *603*, 477–481.
- [36] Zhu, Y.; Huan, S. Q.; Bai, L.; Ketola, A.; Shi, X. T.; Zhang, X.; Ketoja, J. A.; Rojas, O. J. High internal phase oil-in-water pickering emulsions stabilized by chitin nanofibrils: 3D structuring and solid foam. *ACS Appl. Mater. Interfaces* **2020**, *12*, 11240–11251.
- [37] Wolkow, P. P.; Gębska, A.; Korbut, R. *In vitro* maturation of monocyte-derived dendritic cells results in two populations of cells with different surface marker expression, independently of applied concentration of interleukin-4. *Int. Immunopharmacol.* **2018**, *57*, 165–171.
- [38] Skisiel, G. D.; Mirsoian, A.; Minnar, C. M.; Crittenden, M.; Curti, B.; Chen, J. Q.; Blazar, B. R.; Borowsky, A. D.; Monjaze, A. M.; Murphy, W. J. Differential phenotypes of memory CD4 and CD8 T cells in the spleen and peripheral tissues following immunostimulatory therapy. *J. Immuno Ther. Cancer* **2017**, *5*, 33.
- [39] Hensel, J. A.; Khattar, V.; Ashton, R.; Ponnazhagan, S. Characterization of immune cell subtypes in three commonly used mouse strains reveals gender and strain-specific variations. *Lab. Invest.* **2019**, *99*, 93–106.
- [40] Xie, L. Y.; Wang, N.; Yin, Q. Q.; Zhang, X. M.; Chen, L.; Hao, C. N.; Liu, T. T.; Wang, S. W.; Duan, J. L.; Du, K. et al. Rapid monitoring of T-lymphocyte CD4⁺/CD8⁺ ratio in patient immune management by infrared spectroscopy of whole leukocytes. *Microchem. J.* **2024**, *204*, 111070.
- [41] Philip, M.; Schietinger, A. CD8⁺ T cell differentiation and dysfunction in cancer. *Nat. Rev. Immunol.* **2022**, *22*, 209–223.
- [42] Giles, J. R.; Globig, A. M.; Kaech, S. M.; Wherry, E. J. CD8⁺ T cells in the cancer-immunity cycle. *Immunity* **2023**, *56*, 2231–2253.
- [43] Shah, K.; Al-Haidari, A.; Sun, J. M.; Kazi, J. U. T cell receptor (TCR) signaling in health and disease. *Signal Transduct. Target. Ther.* **2021**, *6*, 412.
- [44] Zhang, N. R.; Ji, Q. T.; Liu, Z. Z.; Tang, K. M.; Xie, Y. B.; Li, K. C.; Zhou, J.; Li, S. S.; Shang, H. T.; Shi, Z. C. et al. Effect of different adjuvants on immune responses elicited by protein-based subunit vaccines against SARS-CoV-2 and its delta variant. *Viruses* **2022**, *14*, 501.



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