

Surface-Engineered Cubosomes Serve as a Novel Vaccine Adjuvant to Modulate Innate Immunity and Improve Adaptive Immunity in vivo

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Objective: Recent studies have revealed the adjuvant activity of cubosomes and their potential utility as an antigen delivery system. In this study, to further enhance the adjuvant activity of cubosomes, two cationic polymers are modified on the surface of cubosomes.

Methods: Here, we exploit the effects of surface chemistry on the adjuvant activity of *Ganoderma lucidum* polysaccharide cubosomes (GLPC) by placing two kinds of molecules, that is, cetyltrimethylammonium bromide (CTAB) and poly(diallyldimethyl ammonium chloride) (PDDAC), on their surface.

Results: CTAB- or PDDAC-modified GLPC were found to significantly promote humoral and cellular immune responses, as well as the proliferation of CD3+ CD4+ or CD3+ CD8+ T cells through the powerful activation of dendritic cells (DCs). The enhanced immune responses of PDDAC-modified GLPC might be attributed to the maturation of DCs into draining lymph nodes and the activation of spleen and cytokines in serum.

Conclusion: PDDAC modification is beneficial for enhancing humoral and cellular immune response, suggesting that PDDAC-GLPC-OVA has the ability to be a potential adjuvant for vaccine.

Keywords: cubosomes, *Ganoderma lucidum* polysaccharide, surface-engineering, immune response, RNA-seq

Introduction

Vaccination is an essential way for protecting the body from bacterial or virus infection and cancer. An effective vaccine should motivate strong humoral immune responses and cellular immune responses. The success of vaccine mainly includes antigen and matching adjuvants, which were necessary for playing the role of protecting the body.

Food and Drug Administration has approved four adjuvants (aluminum salts, MF59, AS03, and AS04), which were applied on a variety of bacteria, virus, and influenza vaccine.¹⁻³ With the development of novel antigen (such as subunit antigen) technology, safer and more effective adjuvant are required. In recent years, nanotechnology and nanomaterial has acquired remarkable progress and been applied on biology and medicine.^{4,5} So far, a variety of nanomaterials have been developed into adjuvants, for example, liposome, PLGA, and cubosomes.⁶⁻⁹ Among these, the adjuvant function of cubosomes was discovered in 2013. The

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researchers found cubosomes have the ability to promote more humoral and cellular immune responses than antigen alone.^{10,11}

The fungal species *Ganoderma lucidum* has been widely used as a traditional Chinese medicine for more than 2000 years. *G. lucidum* polysaccharide (GLP), one of the vital components of *G. lucidum*, plays a crucial role in the biological effects of the fungus. Their structures and pharmacological effects have been extensively studied.¹² Modern research shows that GLP has excellent immunomodulating activities and has the ability to be a potential adjuvant for vaccine.¹³ However, GLP has no ability to target antigen presenting cells, which limits its extensive application as a vaccine adjuvant.

Cubosomes show colloidal dispersions of the bicontinuous cubic liquid crystalline phase and have a larger surface area than spherical nanoparticles, which confers them with a powerful ability of encapsulating drug or antigen.^{14–18} However, their immunogenicity is influenced by their physicochemical properties. Surface engineering of nanoparticles plays a critical role in modification of their physicochemical properties, enhancing vaccine-triggered immune responses and resulting in robust and lasting immune response when applied on subunit vaccine.^{19,20} Recent research has indicated that coating cubosomes with a single layer of poly- ϵ -lysine on enhances their functionality and anticancer properties.²¹ Accordingly, the linking of chemical molecules directly or indirectly on the surface of various nanoparticles has been shown to effectively induce the activation of DCs and enhance both humoral and cellular immune responses.^{22–24}

Our previous studies have shown that cubosomes enhance the targeting and adjuvant activity of GLP.²⁵ However, GLP-cubosomes nanoparticles had shown poorly effects on the carry of subunit antigen and activation of APCs. In this study, we linked cetyltrimethylammonium bromide (CTAB) and poly (diallyldimethyl ammoniumchloride) (PDDAC) directly or indirectly on the surface of *Ganoderma lucidum* polysaccharide cubosomes (GLPC). We hypothesized that CTAB-GLPC and PDDAC-GLPC nanoparticles would function as an effective adjuvant and vaccine system to target and activate the APCs, and induce strong humoral and cell-mediated immune responses. This hypothesis was tested by investigating the effects of CTAB-GLPC and PDDAC-GLPC nanoparticles on the activation of DCs. Furthermore, ovalbumin (OVA) antigens were adsorbed on CTAB-GLPC

and PDDAC-GLPC nanoparticles and their ability to induce humoral and cell-mediated immune responses was investigated. Flow cytometry and RNA-seq were used to measure the activation of DCs in draining lymph nodes and gene expression of the spleen to clarify its adjuvant mechanism.

Materials and Methods

Materials

Phytantriol (98.0%) was obtained from TCI (Tokyo, Japan). Pluronic127 (F127), cetyltrimethylammonium bromide (CTAB), poly (styrene sulfate) (PSS, molecular weight: 70,000) and poly (diallyldimethyl ammoniumchloride (PDDAC, Mw=100,000–200,000)) were purchased from Sigma-Aldrich Co (St Louis, MO, USA). The purified *G. lucidum* polysaccharide ($\geq 98\%$ purity) was purchased from CiYuan Biotechnology Co, Ltd, Shanxi, People's Republic of China. Propylene glycol was purchased from Yuwang Industrial Co, Ltd (Shandong, People's Republic of China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco LLC (Solon, OH, USA). RPMI 1640 was purchased from ThermoFisher Scientific (Waltham, MA, USA).

Characterization of GLP

The carbohydrate content of GLP was determined using the phenol sulfuric acid method. The homogeneity and molecular weight were determined by gel permeation chromatography (GPC). Man, Rha, GlcA, GalA, Glc, Gal, Ara, and Fuc were used as the monosaccharide standards, and the monosaccharide composition was analyzed by High Performance Liquid Chromatography (HPLC).

Synthesis of GLPC and CTAB-GLPC

The preparation of GLPC was assessed as described previously.²⁵ CTAB-GLPC was prepared by adding CTAB (3 mg) to Phytantriol (200 mg) – Ethanol mixture, the subsequent method was used as per the previous description.

Synthesis of PDDAC-GLPC

PDDAC-GLPC was synthesized by layer-by-layer assembly. First, to prepare negatively charged cubosomes, PSS (2‰) solution were added into the aqueous solution of CTAB-GLPC and stirred for 15 minutes at 25°C. The mixture sample was stood for 2 hours at 25°C. Redundant PSS

was removed by a filter membrane and CTAB-GLPC-PSS was obtained. For further synthesis of PDDAC-GLPC, similar steps were applied to coat with PDDAC (2.5%) on the surface of CTAB-GLPC-PSS. Redundant PDDAC was removed by a filter membrane.

Characterization of Cubosomes-Based Nanoparticles

Zeta potential, sizes, and polydispersity index (PDI) of various cubosomes-based nanoparticles were measured using the Zetasizer Nano ZS90 instrument (Malvern Instruments Co., Ltd.). Small-angle X-ray scattering (SAXS) of various cubosomes-based nanoparticles were performed by a Bruker NanoSTAR SAXS. After equilibration at 25°C for 30 minutes, measurement was performed and a scattering pattern was collected for 30 minutes. Cryo-field emission scanning electron microscopy (Cryo-FESEM) was used to measure the surface and morphology of cubosomes-based nanoparticles. The various cubosomes-based nanoparticles were viewed using a microscope containing cryochamber at -140°C with an accelerating voltage of 3 kV.

The Effects of Cubosomes-Based Nanoparticles on the Maturation of Dendritic Cells (DCs)

DCs from the bone marrow of BALB/c mice were collected and cultured according to the previous described method.²³ All animal handling procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and followed the guidelines of the Animal Welfare Act. The Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University approved all animal procedures (Approval ID: 2011BAD34B02). Briefly, the bone marrow of the femur and tibia were collected and red blood cells were removed. Then, 3 mL cells were added into 6-well plates at 1×10^6 per well, which contain 20 ng/mL GM-CSF (Peprotech/Tebu, Germany) and 20 ng/mL IL-4 (Peprotech/Tebu). On days 2 and 4, all medium was removed and 3 mL of fresh medium was added to the cell well. On day 6, 1.5 mL medium was removed and 1.5 mL of fresh medium was added to the cell well. On day 8, DCs were obtained and ready for subsequent experiment. DCs were treated with GLP, Cub, GLPC, CTAB-GLPC, or PDDAC-GLPC, respectively, for 12 hours. The concentrations of various cubosomes-based nanoparticles were fixed at 10 µg/mL

(phytantriol content), while that of GLP was 1 µg/mL. Lipopolysaccharide (LPS, Sigma) at 5 µg/mL was used as the positive control while untreated DCs was used as the negative control. DCs were collected and stained with anti-CD11c-PE-Cy7, anti-CD86-FITC, and anti-CD80-PE antibodies (eBioscience) for 30 minutes at 4°C. After removing redundant antibodies by washing, cells were analyzed for DCs mature.

Immunization Protocol

Female BALB/c mice (6–8 weeks old) were injected subcutaneously with 0.2 mL of either OVA alone or various cubosome-based nanoparticles/OVA complex. The various cubosomes-based nanoparticles/OVA complex was prepared by simple mixing and then incubated for 30 minutes. The concentrations of different components in various cubosomes-based nanoparticles/OVA were fixed at 100 µg/mL (phytantriol content) and OVA 50 µg/mL, while that of GLP was 10 µg/mL. Freund adjuvant (FCA) was used as a positive control. Immunization were processed twice at 2-week intervals. Blood were collected 14 days following the second immunization, and serum was collected for OVA-IgG, IgG subtype, and cytokines analysis. Spleens were collected 14 days following the second immunization for cellular immune analysis and RNA-seq.

Analysis of DCs from Lymph Nodes

The activation of DCs in draining lymph nodes was measured using a previously described method.^{25,26} After injection with OVA or cubosome-based nanoparticles/OVA at 24 and 48 hours, draining lymph nodes were collected and transferred into 1.5 mL Eppendorf tubes containing Collagenase D (Roche Applied Science) for preparation of cell suspensions. Then, cell suspensions were filtered with a 40 µm Cell Strainer (BD Biosciences) to obtain a single cell suspension. The collected cells were washed and stained with antibodies against CD11c, MHC-II, CD80, and CD86 for 30 minutes at 4°C. Then, redundant antibodies were removed by washing twice and cells were analyzed using a flow cytometer.

T Cell Proliferation Assay

Splenocytes from mice were harvested²⁵ and cells were washed twice with RPMI 1640 medium containing 10% FBS and then incubated with OVA at 50 µg/mL for 68 hours. The MTT method was performed for proliferation assay.

Differentiation of CD4+ and CD8+ T Cells and Cytokines Analysis

Splenocytes were treated as described above. After incubation with OVA for 68 hours, resulting cells were washed and stained with antibodies against CD3, CD4, and CD8 for 30 minutes at 4°C and supernatant were collected for cytokines analysis. Then, cells were washed and analyzed by Flow cytometry instrument.

OVA-IgG, IgG Subtype, and Cytokine Analysis

The detection of OVA-IgG and IgG subtype were performed as previously described.²⁷ The suspension of splenocytes medium and serum collected from mice were diluted and analyzed with cytokines ELISA kits (R&D, Co., USA).

RNA-Seq Analysis in Spleen

Gene expression levels were measured in spleen by RNA-seq sequencing. Spleen from mice were harvested and the total RNA were extracted. A total amount of 2 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations, and index codes were added to attribute sequences to each sample.

Clustering and Sequencing

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq 3000/4000 PE Cluster Kit Box1 (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 4000 platform and 150bp paired-end reads were generated.

Statistical Analysis

The experimental data were plotted by GraphPad Prism 6. Statistical significance was calculated by the one-way ANOVA. All results are presented as mean±SEM.

Results and Discussion

Characterization of GLP and GLPC Nanoparticles

The carbohydrate content of GLP was 99.0%, as determined by the phenol-sulfuric acid method. According to

GPC analysis, the average molecular weight of GLP was 408 Da, a single symmetrical peak was also detected by GPC analysis (Figure 1A). As shown in Figure 1B, GLP was composed of Glc, Gal, and Ara in a molar ratio of 4.87:1.99:1.07. The results of GPC and HPLC chromatogram analysis indicated that GLP is an homogeneous oligosaccharide.

The changes in the cubosomes after surface engineering and adsorption of OVA were assessed using a Zetasizer Nano ZS instrument. After surface engineering, the zeta potential of nanoparticles often changes as an indicator of successful modification. Following the addition of CTAB, the zeta potential of CTAB-GLPC increased from -16.97 ± 1 to 29.17 ± 0.24 mV (Figure 2A). This indicated that the zeta potential happened inversion, which enabled OVA adsorption. The slow addition of OVA to CTAB-GLPC was performed for the preparation of CTAB-GLPC-OVA, and the zeta potential was measured. As shown in Figure 2A, the zeta potential of CTAB-GLPC-OVA decreased from 29.17 ± 0.24 to 19.77 ± 0.24 mV, which was considered to be caused by the adsorption of negatively charged OVA on the CTAB-GLPC surface. The effective size decreased from 324.4 ± 2.1 to 303.1 ± 0.5 nm after CTAB modification (Figure 2B).

To link PDDAC to the surface of GLPC, PSS was used as transition. Following the linking of PSS on the surface of CTAB-GLPC, the zeta potential of CTAB-GLPC-PSS was negative (-30.97 ± 0.33 mV), which created the conditions for PDDAC modification. Then, PDDAC was modified on the surface of CTAB-GLPC-PSS and zeta potential of PDDAC-GLPC increased from -30.97 ± 0.33 to 22.97 ± 0.23 mV (Figure 2A). After OVA adsorption, zeta potential of PDDAC-GLPC-OVA was 22.13 ± 0.03 mV and showed no obvious change, which attributed to larger surface area of PDDAC-GLPC likely. PDI is necessary to study the stability of the nanoparticles. As shown in Figure 2C, PDI of CTAB-GLPC was lower compared to other cubosomes-based nanoparticles. The results indicated Surface-engineering has no effect on the stability of the nanoparticles (Figure 2C). The adsorption efficiency of OVA on CTAB-GLPC and PDDAC-GLPC was investigated using a BCA kit and found to be 52.3% and 80.9%, respectively.

Phyтанtriol is known to form a double-diamond cubic phase (Pn3m space group) in excess water or when dispersed as nanoparticles stabilized by pluronic F127. The double-diamond cubic phase (Pn3m) was formed when

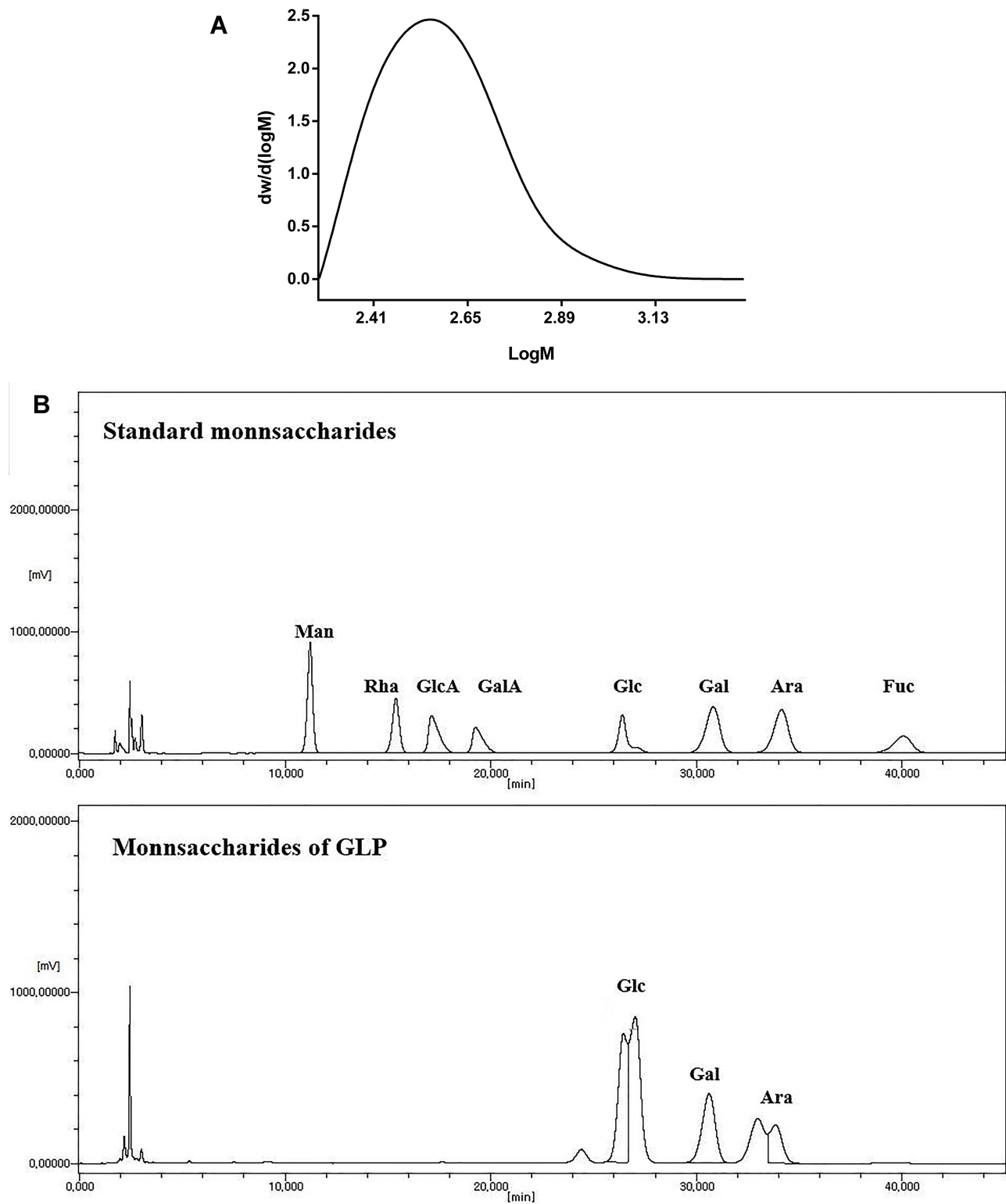


Figure 1 Characterization of GLP. (A) Molecular weight of GLP was determined using GPC. (B) HPLC chromatogram of hydrolysate from GLP.

phytatriol and pluronic F127 were dispersed in excess water. Our previous studies have indicated that GLPC shows a Pn3m internal structure.²⁸ To determine whether

surface engineering affects the internal structure of GLPC, SAXS was performed. The SAXS curves were collected at 25°C for 30 minutes. As shown in Figure 3, although the

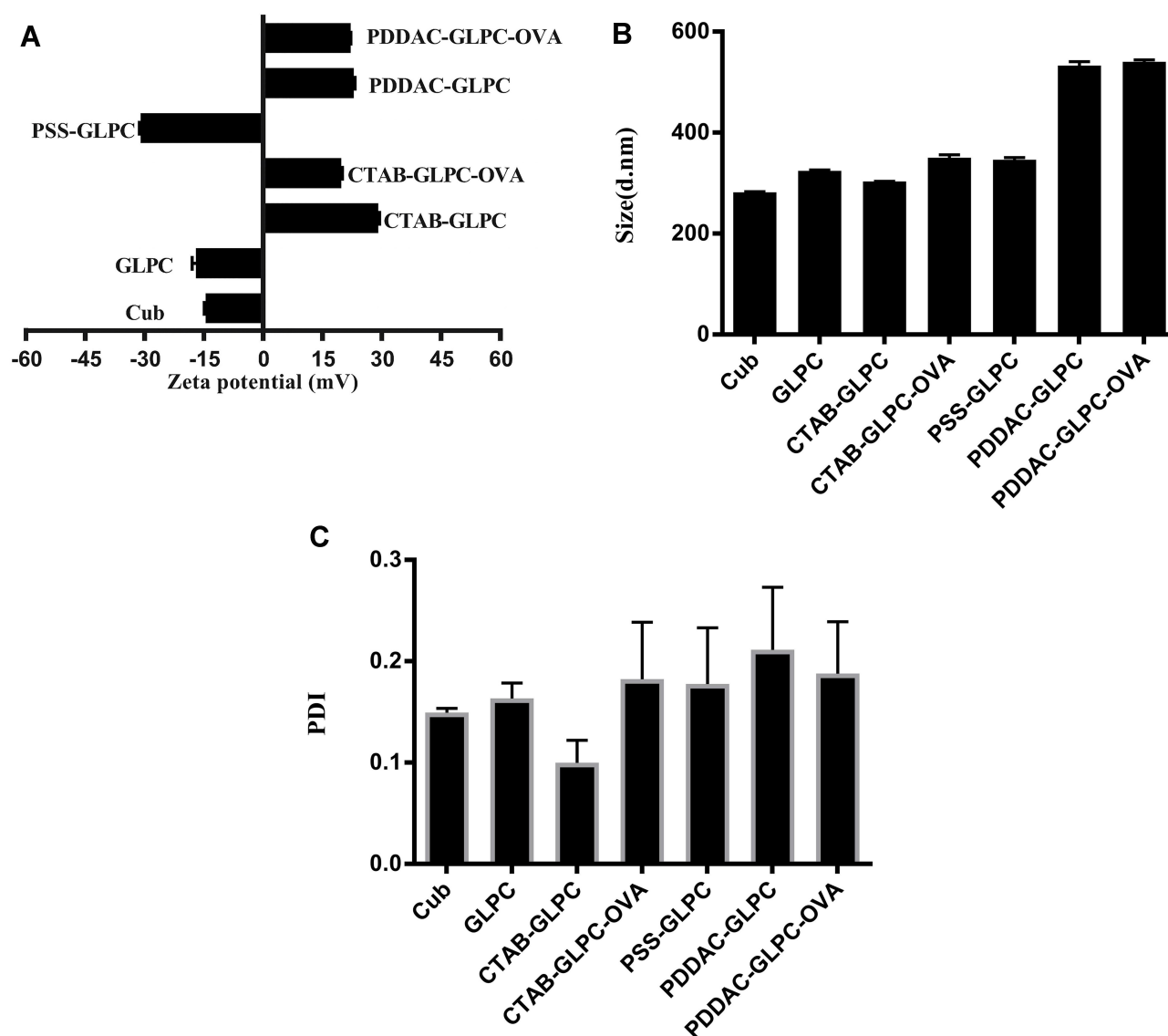


Figure 2 Characterization of Cubosomes-based Nanoparticles. (A) Zeta potential, (B) Size, (C) PDI.

peaks of PDDAC-GLPC and PDDAC-GLPC-OVA moved slightly to the right, three diffraction peaks (relative positions, $\sqrt{2}:\sqrt{3}:\sqrt{4}$) were observed, which indicated a Pn3m nanostructure.²⁸ The Pn3m nanostructure was retained upon Surface-Engineering and OVA adsorbing. Cryo-FESEM was performed to investigate the surface structures of cubosome-based nanoparticles. As shown in Figure 4, the nanoparticles showed a cubic morphology and the size of PDDAC-GLPC and PDDAC-GLPC-OVA increased. Cryo-FESEM provides valuable insights into the morphological features of bicontinuous cubic liquid crystalline systems. Some spheres may be formed by freezing water.

Analysis of DCs Maturation

DCs, which are APCs, play a pivotal role in triggering, enhancing, and linking humoral and cellular immunity.^{29–34} GLPC with different surface coatings (CTAB and PDDAC) were examined to determine whether they could promote the maturation of DCs. Co-stimulatory molecules, such as B7 molecules (CD40 and CD86), play a critical role in regulating T-cell activation and differentiation.^{34–37} Since vaccine was injected and APC (macrophages, DCs, etc.) will be enriched, cubosomes-based nanoparticles would interact with DCs.^{37–40} To test this interaction, DCs were incubated with CTAB-GLPC-OVA and PDDAC-GLPC-OVA, followed by antibody (CD11c) staining for DCs screening or antibody (CD40 and

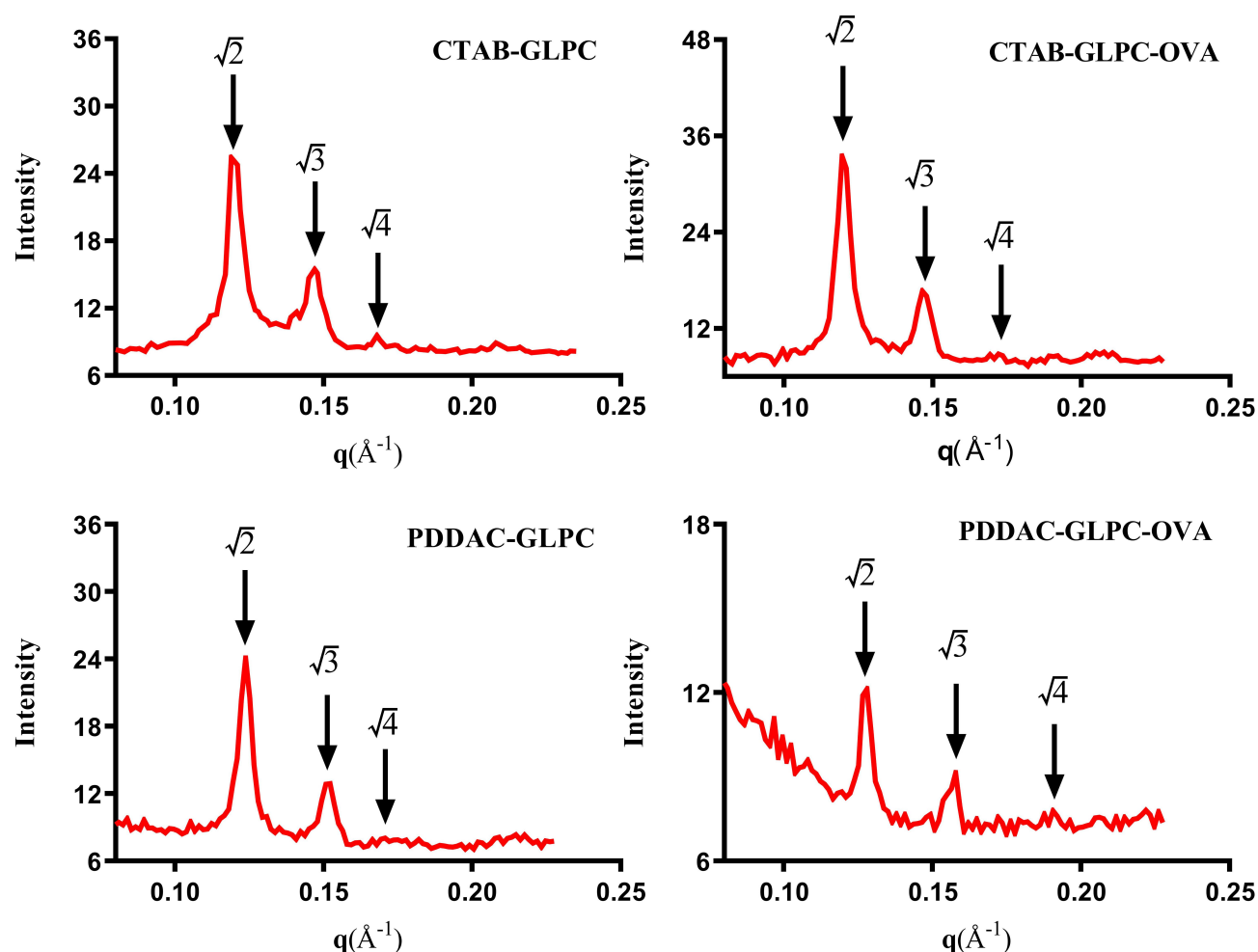


Figure 3 SAXS patterns of CTAB-GLPC, CTAB-GLPC-OVA, PDDAC-GLPC, and PDDAC-GLPC-OVA.

CD86) staining for the upregulation of surface markers to measure DCs maturation. DCs accounts for 90% of the proportion of the total cell population based on CD11c fluorescence intensity. We found that the percentage of CD40 and CD86 was significantly enhanced ($P < 0.01$) when DCs were treated with PDDAC-GLPC-OVA compared with that when they were treated with GLPC (Figure 5 and [Supplementary Figure S1](#)). CD40 and CD86 were up-regulated ($P < 0.05$) when DCs were treated with CTAB-GLPC-OVA relative to GLPC. These results indicate that PDDAC-GLPC-OVA enhanced DC maturation more effectively.

PDDAC-GLPC-OVA Induced Antigen-Specific Immunity

GLPC with different surface coatings (CTAB and PDDAC) were examined to determine whether they could induce antigen-specific immunity after OVA

adsorption. When the PDDAC-GLPC-OVA groups were immunized by subcutaneous injection, the results for specific antibodies that are typically representative of humoral immunity showed that the OVA-IgG level was enhanced significantly ($P < 0.05$) compared with that in the GLPC group (Figure 6). However, the CTAB-GLPC-OVA groups did not show stronger immune responses than the GLPC group.

The rapid induction of humoral and cellular immune responses mounts a defensive response against infection. The proliferative ability of splenic lymphocytes is an indirect indicator of an organism's immune status.^{41–43} As shown in Figure 7A, the proliferation index revealed that the PDDAC-GLPC-OVA and CTAB-GLPC-OVA groups had superior lymphocyte proliferation indices than the GLPC group (Figure 7A). Furthermore, T cell differentiation was examined, and the results showed that CTAB-GLPC-OVA and

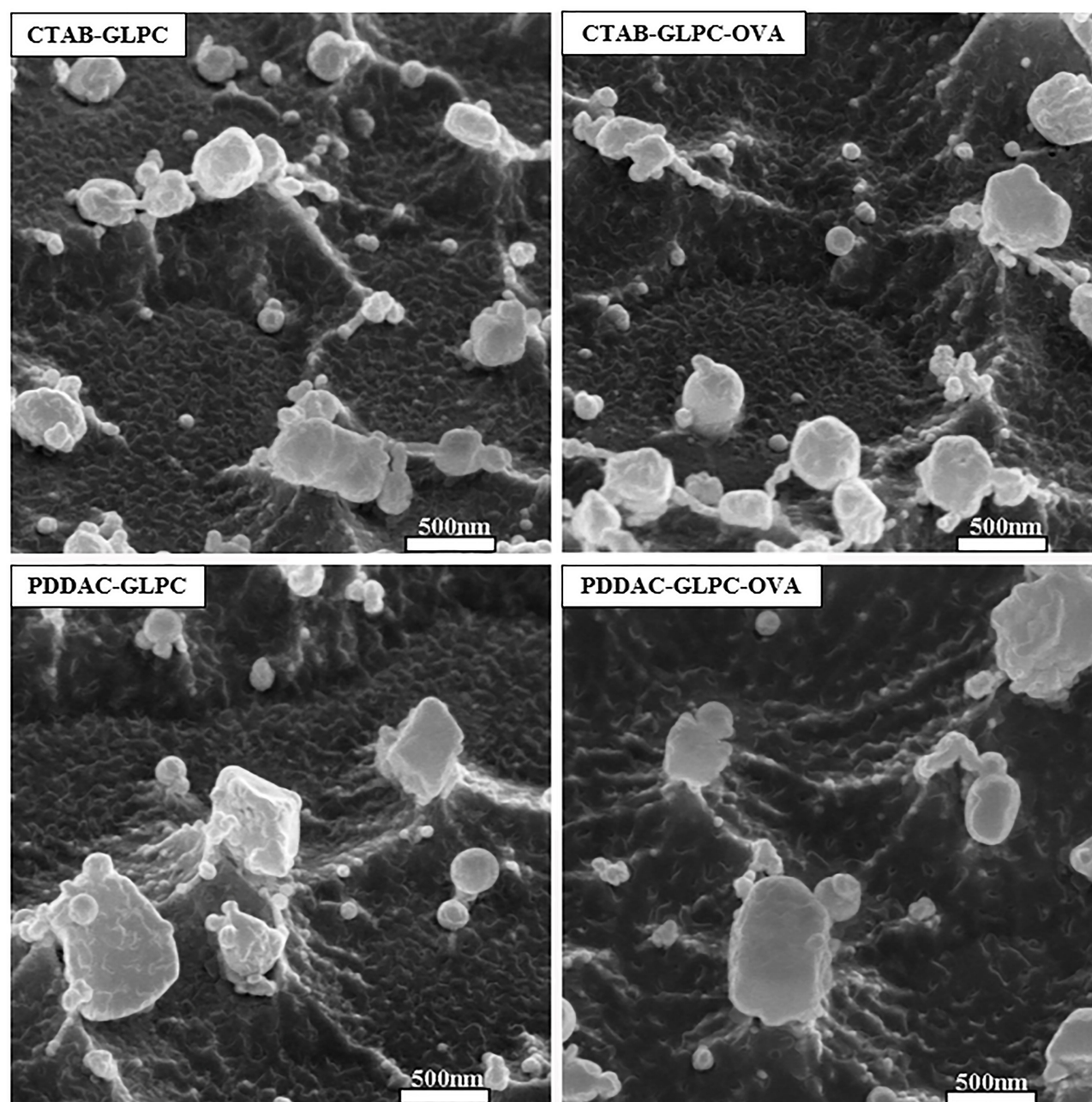


Figure 4 Cryo-FESEM micrographs of CTAB-GLPC, CTAB-GLPC-OVA, PDDAC-GLPC, and PDDAC-GLPC-OVA.

PDDAC-GLPC-OVA have no effect ($P>0.05$) on the ratio of CD4⁺/CD8⁺ compared with that in the GLPC group (Figure 7B and Supplementary Figure S2). However, after immunization with PDDAC-GLPC-OVA or CTAB-GLPC-OVA, the T helper cells (CD3⁺ CD4⁺) could increase timely and further caused B-cell activation. In other words, after immunization with PDDAC-GLPC-OVA, CD3⁺ CD8⁺ T-cells showed rapid proliferation and secretion of various cytokines involved in immune function (Figure 7C and D). The supernatants of splenic lymphocytes were collected after OVA

treatment at 68 hours. IFN- γ secretion level was determined by ELISA. When the CTAB-GLPC-OVA and PDDAC-GLPC-OVA groups were immunized, the IFN- γ level was clearly enhanced ($P<0.05$) compared with that in the single GLPC group (Figure 7E). The result indicates that PDDAC-GLPC-OVA can generate a rapid and powerful humoral and cellular immune response compared with naked cubosome-based nanoparticles.

To definite the tendency of the immune response, IgG2a/IgG21 ratio was used as the indicator of Th1/

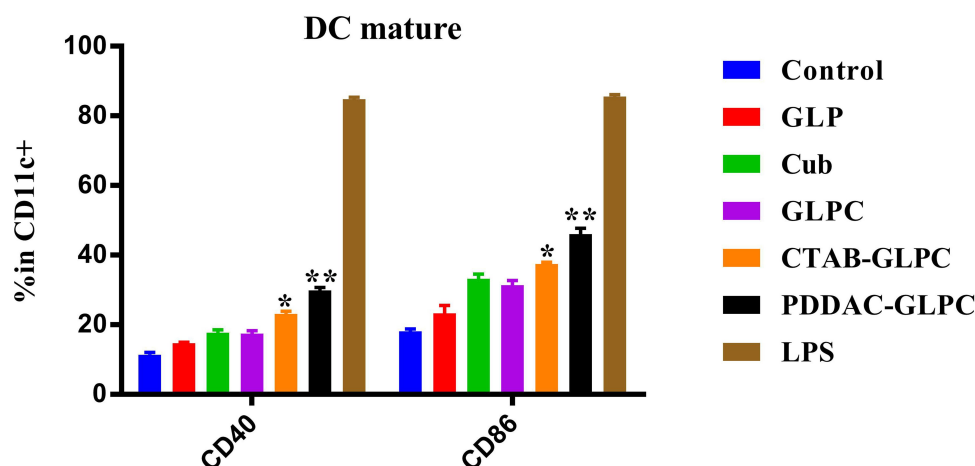


Figure 5 The effects of cubosomes-based nanoparticles on dendritic cell maturation. Data are expressed as the mean \pm SEM. * P <0.05 and ** P <0.01 vs the GLPC group.

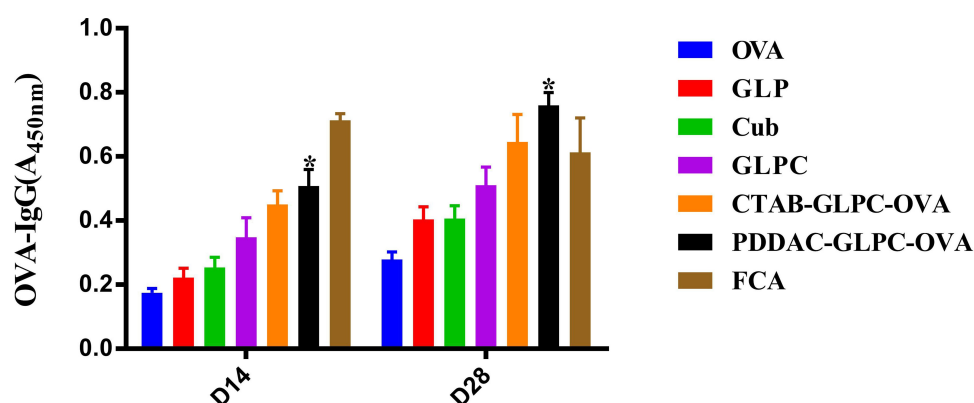


Figure 6 The OVA specific antibody level was measured after final immunization. Data are expressed as the mean \pm SEM. * P <0.05 vs the GLPC group.

Th2.⁴⁴ Th1 cells could induce cellular immunity to target cells and clear intracellular pathogens (virus or bacterium), while Th2 cells induce humoral immune response to clear extracellular pathogens. IgG2a/IgG21 in the cubosomes-based nanoparticles increased significantly compared to the naked OVA, which indicates a Th2 immune response. However, the value of IgG2a/IgG21 has no significant (P >0.05) difference among GLPC, CTAB-GLPC-OVA, and PDDAC-GLPC-OVA group (Figure 7F). The result indicated the tendency of the immune response had not been changed by Surface-Engineering.

DCs Maturation in Draining Lymph Nodes

The results showed that cubosomes-based nanoparticles with different modification showed different adjuvant activity effects in vivo. The mechanism underlying the action of PDDAC-GLPC-OVA as an effective adjuvant and the effect

of modifications on triggering the immune response were explored. As the initial site at which the immune response to a vaccine occurs, draining lymph nodes play an important role in triggering the immune response. After the uptake of vaccine antigens at the injection site, DCs migrate to nearby draining lymph nodes and mature during the process of migration. Then, mature DCs interact with T and B lymphocytes via their surface molecules. We next investigated how surface engineering affects adjuvant activity in terms of activating DCs in the draining lymph nodes. The activation markers (CD80, CD86, and MHC II) of DCs were examined in the lymph node after primary immunization at 24 hours and 48 hours. The expression of CD80, CD86, and MHC II in CTAB-GLPC-OVA and PDDAC-GLPC-OVA groups was higher than in the naked OVA group (P <0.05). At 24 hours, only the expression of CD80 in the CTAB-GLPC-OVA and PDDAC-GLPC-OVA groups was higher than in the GLPC group (Figure 8A and Supplementary Figure S3). However, CD80 and

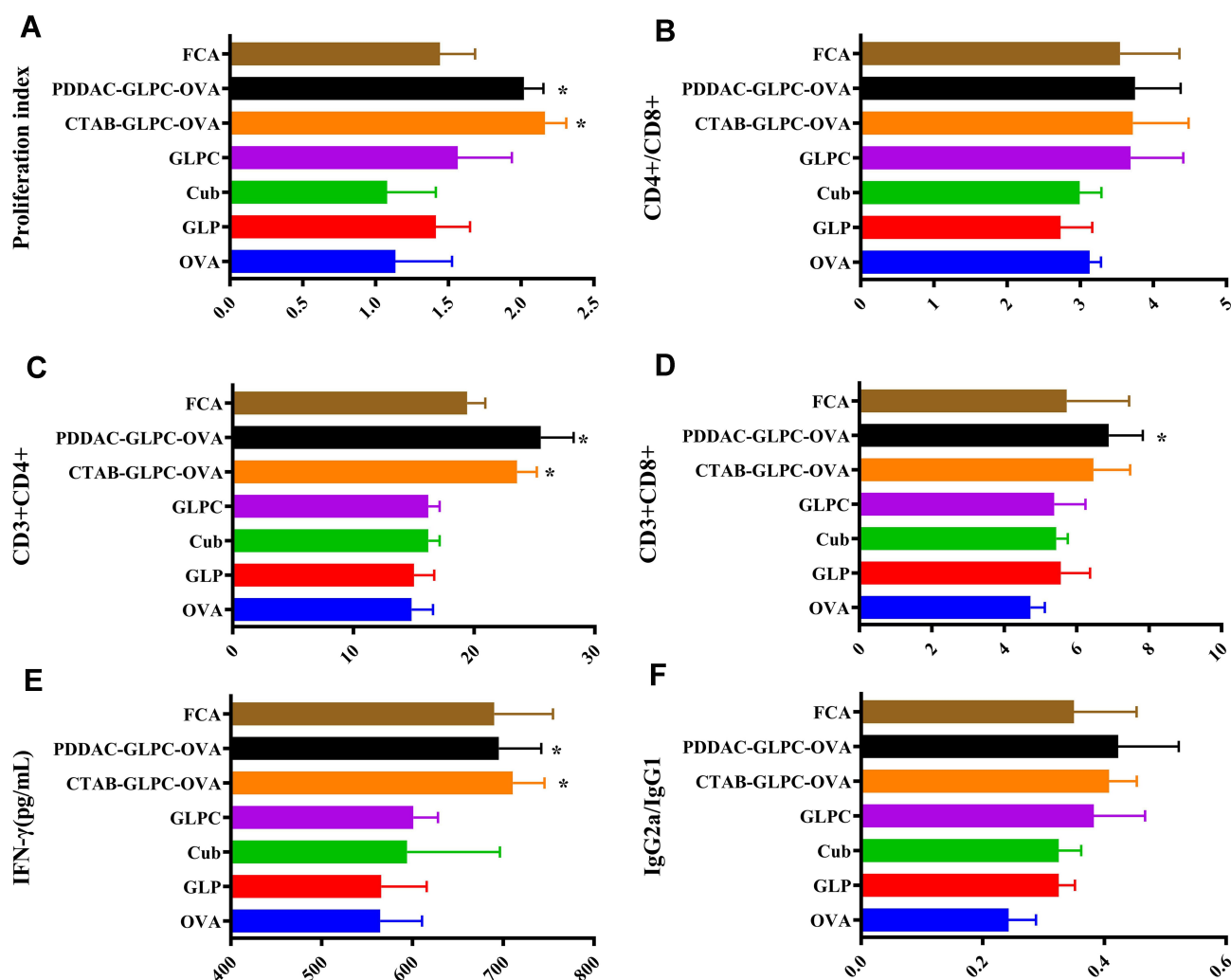


Figure 7 The effects of cubosome-based nanoparticles on splenic T-cells after OVA re-stimulation, and the level of IgG2a/IgG1 in serum. **(A)** Spleen lymphocyte proliferation index after OVA re-stimulation. **(B)** CD4+ to CD8+ T-cell ratio. **(C)** CD3+CD4+ T-cell proliferation. **(D)** CD3+CD8+ T-cell proliferation. **(E)** IFN- γ secretion by splenocytes after OVA re-stimulation. **(F)** IgG2a to IgG1 ratio in serum; data are expressed as the mean \pm SEM; * P <0.05 vs. the GLPC group.

CD86 were significantly more highly expressed in mice injected with CTAB-GLPC-OVA and PDDAC-GLPC-OVA than in those injected with naked GLPC at 48 hours (Figure 8B and Supplementary Figure S4) (P <0.05), which indicates that CTAB-GLPC-OVA and PDDAC-GLPC-OVA can induce the activation and maturation of DCs more effectively than naked cubosome-based nanoparticles in vivo.

Cytokine Levels in Serum

The change of serum cytokines has an important value to assess the activity of adjuvant in systemic circulation.⁴⁵ To measure the immune response in systemic circulation, the levels of IFN- γ , TNF- α , IL-4, and IL-6 in serum were measured by ELISA at D14 after final vaccination. As shown in Figure 8C and D, mice injected with PDDAC-

GLPC-OVA generated higher production of IFN- γ , TNF- α , and IL-4 compared to GLPC. However, mice injected with CTAB-GLPC-OVA only generated higher (P <0.05) production of IL-4. These results suggested that PDDAC-GLPC-OVA could significantly enhance both the Th1 and Th2 immune response. Overall, serum cytokines were enhanced after Surface-Engineering in systemic circulation, which suggested PDDAC modification can effectively cause a systemic immune response.

General Genetic Expression of the Spleen

Immune function is the most important function of the spleen, which contains a variety of immune cells that interact with immune factors.⁴⁶ The spleen plays specific roles in the immune response through cell-mediated cellular and humoral immunity.⁴⁷ The function of the spleen

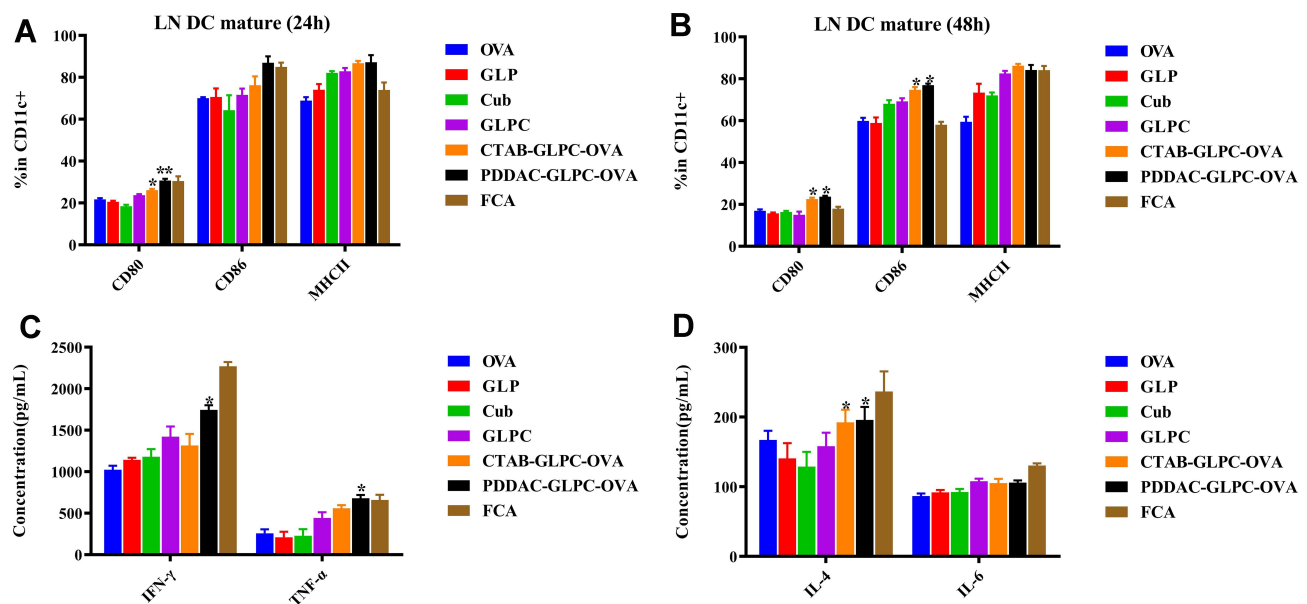


Figure 8 Activating DCs in the draining lymph nodes at 24 hours (A) and 48 hours (B) after the first immunization. Production of IFN-γ and TNF-α in the serum of mice (C), IL-4, and IL-6 (D) in the serum of mice. Data are expressed as the mean±SEM. * $P<0.05$ and ** $P<0.01$ vs the GLPC group.

is indirectly influenced by draining lymph nodes. Then, the function of the spleen interacted with systemic circulation. We have found PDDAC-GLPC-OVA can induced promoted the more effective promotion of the activation of DCs compared to more effectively than did naked cubosomes-based nanoparticles in vivo; further, they promoted the production of OVA-IgG in the serum. How they affected draining lymph nodes and further affected systemic circulation. Spleens were collected at day 14 after final immunization, and the total RNA extracted from the OVA, GLPC, and PDDAC-GLPC-OVA group was analyzed using RNA-seq.

As shown in Figure 9A, 368 differentially expressed genes were identified, including 292 up-regulated and 76 down-regulated genes ($P<0.05$) in the PDDAC-GLPC-OVA group relative to the GLPC group (Figure 9A). Hierarchical clustering showed that the mechanism of action underlying the effect of PDDAC-GLPC-OVA on the spleen was significantly different from that of GLPC or OVA (Figure 9B). The results indicate the mechanism of action of cubosome-based nanoparticles changes after PDDAC modification.

The immune-related signaling pathways were screened by KEGG pathway analysis. As shown in Figure 9C, the adjuvant activity of the PDDAC-GLPC-OVA in the spleen was induced by the activation of leukocyte transendothelial migration, cytokine–cytokine receptor interaction, and complement and coagulation cascades. We were not sure if

these cytokines identified in this study were OVA specific, but we believe they did regulate the immune cell homing in response to antigen. The leukocyte transendothelial migration pathway was also found in the KEGG enrichment in this study, which confirmed that intensive immune cell migration had occurred in immune response. Further study will be needed to identify the exact role of the cytokine–cytokine receptor interaction and leukocyte transendothelial migration identified in this study, and to identify what immune cells are involved and how they are attracted to the immune response after vaccine.

Conclusion

In this study, we investigated the adjuvant activity of surface-engineered GLP cubosomes, and demonstrated that the adjuvant activity of GLPC was influenced by surface engineering. For cubosome-based nanoparticles, CTAB-GLPC-OVA and PDDAC-GLPC-OVA showed a stable state and retained their Pn3m nanostructure. Because of the PDDAC modification, the compound had excellent features and promoted DC maturation and antigen uptake. The results showed that CTAB-GLPC-OVA failed to promote the humoral immune response relative to GLPC and PDDAC-GLPC-OVA, which were both found to activate and promote humoral and cellular immune responses. PDDAC-GLPC-OVA strongly promoted the proliferation of CD3+ CD4+ or CD3+ CD8+ T-cells; furthermore, it effectively modulated the expression of some cytokines,

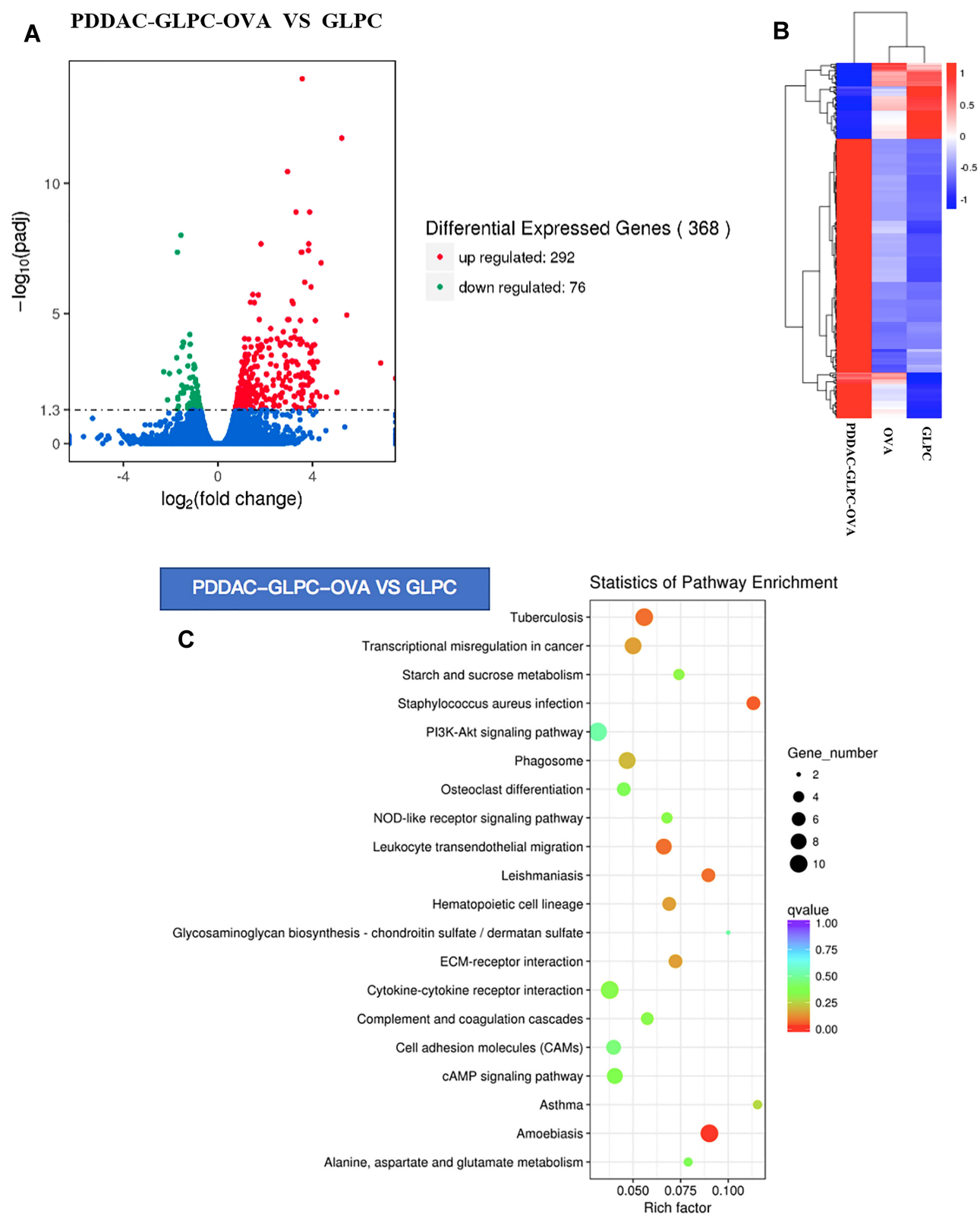


Figure 9 (A) Expression profiles of mRNAs in the spleen. (B) Hierarchical clustering of genes differentially expressed in PDDAC-GLPC-OVA vs OVA vs GLPC. (C) KEGG pathway analysis in the PDDAC-GLPC-OVA group vs the GLPC group.

including IFN- γ , TNF- α , IL-4, and IL-6. The enhanced immune responses of PDDAC-GLPC-OVA might be attributed to the maturation of DCs into draining lymph nodes, activation of the spleen, and secretion of cytokines into systemic circulation. The current work indicates that PDDAC modification is beneficial for enhancing the humoral and cellular immune response, suggesting that PDDAC-GLPC-OVA is a potential adjuvant for protein-antigen vaccines.

Abbreviations

GLP, *Ganoderma lucidum* polysaccharide; GLPC, *Ganoderma lucidum* polysaccharide cubosomes; CTAB, cetyltrimethylammonium bromide; PDDAC, poly (diallyldimethyl ammoniumchloride); PSS, poly(styrenesulfate); GPC, gel permeation chromatography; HPLC, High Performance Liquid Chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDI, polydispersity index; SAXS, X-ray scattering; Cryo-FESEM, cryo-field emission scanning electron microscopy; DCs, dendritic cells.

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Disclosure

The authors report no conflicts of interest for this work.

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