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Expression of FMD virus-like particles in yeast *Hansenula polymorpha* and immunogenicity of combine with CpG and aluminum adjuvant

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ABSTRACT

Background: Inactivated vaccines are limited in preventing foot-and-mouth disease (FMD) due to safety problems. Recombinant virus-like particles (VLPs) are an excellent candidate for a novel vaccine for preventing FMD, given that VLPs have similar immunogenicity as natural viruses and are replication- and infection-incompetent.
Objectives: The 3C protease and P1 polyprotein of type O FMD virus (FDMV) was expressed in yeast *Hansenula polymorpha* to generate self-resembling VLPs, and the potential of recombinant VLPs as an FMD vaccine was evaluated.
Methods: BALB/c mice were immunized with recombinant purified VLPs using CpG oligodeoxynucleotide and aluminum hydroxide gel as an adjuvant. Cytokines and lymphocytes from serum and spleen were analyzed by enzyme-linked immunosorbent assay, enzyme-linked immunospot assay, and flow cytometry.
Results: The VLPs of FMD were purified successfully from yeast protein with a diameter of approximately 25 nm. The immunization of mice showed that animals produced high levels of FMDV antibodies and a higher level of antibodies for a longer time. In addition, higher levels of interferon-γ and $CD4^+$ T cells were observed in mice immunized with VLPs.
Conclusions: The expression of VLPs of FMD in *H. polymorpha* provides a novel strategy for the generation of the FMDV vaccine.

Keywords: Yeast *Hansenula polymorpha*; food-and-mouth disease; virus-like particles; immunogenicity

INTRODUCTION

Foot-and-mouth disease (FMD) is an acute infectious disease affecting cloven-hoofed animals in Asia, Africa, the Middle East, and South America, seriously influencing the quality of animal products and world trade. FMD virus (FMDV) exists in seven distinct serotypes: A, O, C, Asia 1, and South African Territories-1–3 [1,2]. Type O is the most prevalent serotype

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Conflict of Interest
The authors declare no conflicts of interest.

in the world [3]. The open reading frame (ORF) of the genome of FMDV is translated to generate a polyprotein, which is processed rapidly, both during and after translation, to generate the structural protein precursor (P1-2A) and the precursors of the non-structural proteins, P2 and P3. P1-2A is processed mainly by 3C protease ($3C^{pro}$) to generate the structural proteins VP0, VP1, and VP3, among which VP0 splits further into VP2 and VP4 during the viral particle assembly process. The intact viral capsid has a regular icosahedral structure composed of four structural proteins VP1, VP2, VP3, and VP4, with the VP4 protein located inside [2,4].

Inactivated vaccines for FDM have been forbidden in some countries due to biosafety concerns [5,6]. Virus-like particles (VLPs) maintain similar immunogenicity with native viruses and lack genetic materials, making them promising vaccine candidates for preventing FDM [7]. Several expression systems have been used to generate recombinant VLPs for FMDV [8-11]. Yeast is used widely in cell factories with multiple advantages, such as rapid growth, simple operation, easy fermentation, and high biomass concentration, especially for expressing recombinant proteins that require post-translational modifications for proper folding. The *Hansenula polymorpha* is commonly employed as an expression platform because of its excellent capability like thermotolerance, proper glycosylation of protein, and using methanol as a carbon source [12-14].

This research showed that the type O VLPs of FMD were expressed in *H. polymorpha* by transfecting a construct containing an ORF-encoding P1 structural protein and 3C protease. BALB/c mice were immunized with VLPs together with CpG oligodeoxynucleotide (CpG ODN) and aluminum hydroxide gel to evaluate their potential as an FMD vaccine. The results showed that the purified VLPs exhibited stronger immunogenicity.

MATERIALS AND METHODS

Construction of recombinant yeast

The *H. polymorpha* strain U358 was from Grand Theravac Life Sciences Co., Ltd. (China). ORFs encoding the P1 and 3C protease of FMDV O strain O/BY/CHA/2010 (Gen Bank No. JN998085.1) were synthesized by Anhui General Biology Co., Ltd. (China), which were subcloned into the pMAUR vector (Grand Theravac Life Sciences) to generate recombinant plasmids pMAUR-P1 and pMAUR-3CD. The pMAUR-P1 gene fragment containing the promoter and terminator was cut into the pMAUR-3CD plasmid after enzyme digestion to obtain the pMAUR -P1-3CD plasmid. After linearization with Bgl II, the plasmid pMAUR-P1-3CD was transformed electrically into the *H. polymorpha* strain U358 cells. The positive clones were selected and verified using the following polymerase chain reaction with primers: forward 5′-ACATCAATCTAAAGTACAACACACC-3′, reverse 5′-GTAAACACCCTTGTGGTCGGTTGG-3′ and 5′-GTTACCGCCAGCAGAATGAGTAC-3′.

VLPs purification

After fermentation in YPG medium with 0.8% methanol, the recombinant yeast was harvested by centrifugation and broken down using a low-temperature ultrahigh pressure continuous flow cell disrupter. After re-dissolving into a PEG6000/NaCl solution, the lysates were filtered through an ultrafiltration membrane (> 100 kD, PXB100C50, Pellicon XL; Sigma-Aldrich, USA), followed by sequential purification by anion exchange chromatography (Q Sepharose High Performance [QHP]; GE Healthcare, USA), hydrophobic

interaction chromatography (Butyl Sepharose 4FastFlow; GE Healthcare), and molecular chromatography (Superdex 200 pg; GE Healthcare). The samples were ultracentrifuged with a 15% to 45% sucrose density gradient at 28,000 rpm and 4°C for 22 h. Subsequently, a 500 μL sample was pipetted continuously from the top to bottom of the centrifuge tube, and the absorption at 280 nm (A280) was determined using an ultraviolet spectrophotometer (NANODROP ONE; Thermo Fisher Scientific, USA). The sample with an absorption peak was centrifuged, and the upper layer was taken for testing.

VLPs characterization

For dynamic light scattering (DLS) analysis, the centrifuged sample was placed in a dynamic light scatterer (Nano-ZS; Malvern, UK) and equilibrated at room temperature to determine the particle diameter three times for each sample.

For high-performance liquid chromatography (HPLC) analysis, the sample (150 μL) was injected into the HPLC with the flow rate set to 0.5 mL/min. The purity and homogeneity of the sample were analyzed using a TSKgel G5000PWXL (Tosoh, Japan).

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the samples collected through each purification process were mixed with 5× loading buffer and boiled for 10 min, followed by 12% SDS-PAGE (TGX FastCast Acrylamide Kit; Bio-Rad, USA). After electrophoresis, the gel was stained with Coomassie brilliant blue, and the decolorized samples were photographed and analyzed in the gel imager.

For western-blot analysis, the protein was transferred to a polyvinylidene difluoride membrane (Millipore, USA), which then was blocked with 5% nonfat milk, and incubated with the rabbit-type O FMDV antibody (donated by Lanzhou Veterinary Research Institute, China). After washing with TBST, the membrane was added with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit antibody (Abcam, UK) for incubation. After washing with TBST, the protein was detected using an ECL solution.

For electron microscopy, the samples identified by sucrose-density-gradient ultracentrifugation were adsorbed onto carbon-coated copper webs, negatively stained with 2% phosphotungstic acid, dried, and observed by transmission electron microscopy (80.0kV, HT7700; Hitachi, Japan).

Immunogenicity of FMDV empty-capsid-like particles in mice

BALB/c mice (six–eight-week-old, female) were purchased from Shanghai Lingchang Biotechnology Co., LTD. (License SCXK(Shanghai)2018-003, Certificate 20180003019425; China). All animal tests were approved by the Laboratory Animal Ethics Committee of Grand Theravac Life Sciences Co., Ltd. All mice were divided randomly into four groups, with five mice in each group. Two groups were injected with 100 μL sterile PBS, and 200 μL bivalent inactivated swine FMD type O and type A vaccine as the control group. The other two groups were treated with the preparation for VLPs, CpG ODN, and aluminum hydroxide gel adjuvant (ALHYDROGEL 2%, Croda, Denmark) (VLPs:ALHYDROGEL:CpG = 2:10:1) containing 5 μg and 10 μg VLPs respectively, through injection into the thigh muscles of mice. The time of immunization was 2 wk apart. No blood was collected before the first immunization, but blood was collected from the orbit the day before. The first and second blood samples were collected 2 wk apart, and the third and fourth samples were collected 4 wk apart. At the end of the experiment, the mice were sacrificed, and the splenocytes were ground and suspended.

Enzyme-linked immunosorbent assay (ELISA)

The antibody titer against type O FMD was determined using the FMD type O antibody liquid phase blocking ELISA detection kit (improved) (Lanzhou Shouyan Biotechnology Co., LTD., China) according to the manufacturer's instruction.

The serum immunoglobulin G (IgG) level was measured by diluting the VLPs into carbonate–bicarbonate buffer (2 μg/mL) and then pre-coated them in a reader plate. After blocking with 5% nonfat milk and washing with PBST, the serum with gradient was added to the plate. The sample was then incubated with HRP-conjugated antibody against mouse IgG (sheep anti-mouse IgG/IgG1/IgG2a-HRP) and viewed using DAB regent. The absorbance at 450 nm/630 nm was recorded using a microplate reader (TECAN SPARK; Tecan, Switzerland), and the antibody titer was calculated.

Enzyme-linked immunospot assay (ELISpot assay)

Diluted total anti-mouse interferon (IFN)-γ was added to an ELISpot plate and incubated overnight. The VLPs served as a peptide library and were added to the splenocyte suspension (4×10^5 cells/mL) to a final concentration of 10 μg/mL. Concanavalin A (20 μg/mL) was used as a positive control. After incubation with the detection antibody and HRP-labeled antibody, the samples were viewed using a DAB regent. The number of spots was recorded using an enzyme-linked spot reader.

Flow cytometry

For cytokines analysis, spleen cell (2×10^7cells/well) was seeded on the microplates. The B cells were detected by incubating the samples with anti-mouse CD16/32 (eBioscience, USA) to block the Fc fragment, the antibodies for cell surface proteins, including CD45R-eFluor 450, CD38-FITC, CD138- PC, and GL7-PE (eBioscience) were added for 30 min in the dark. The T cell & T cell cytokines were detected by culturing the spleen cells with the peptide library for 5 h and adding the antibodies for cell surface proteins, including CD3-AF700, CD4-BV510, CD8a-FITC (eBioscience), for 30 min in the dark. After cell fixation with an IC buffer overnight at 4°C, the antibodies for cytokines, including TNF-α-eFluor450, IL-2-APC, IL-4-BV711, and IL-17A-PE/Cy7 (eBioscience), were added and incubated for 1 h in the dark. The cells underwent flow cytometry analysis (Attvne NXT; Thermo Fisher Scientific). FlowJo software (FlowJo, LLC, USA) was used for data analysis.

Statistical analysis

The data were plotted using GraphPad Prism 5 software (GraphPad Software, Inc., USA), and the significance was analyzed by one-way analysis of the variance followed by Tukey's multiple comparison test.

RESULTS

VLPs were successfully expressed and purified by *H. polymorpha*

As shown in **Fig. 1**, the sample from *H. polymorpha* was purified through QHP, Butyl Sepharose 4FastFlow, and Superdex 200 pg, respectively. The purified samples were then determined using western blot analysis. The protein bands were observed at approximately 25–35 KD, as expected. The results suggested that the target protein was effectively separated. As shown in **Fig. 2**, homogeneous spherical particles were observed by transmission electron microscopy. They showed a similar diameter of approximately 25 nm to that determined by DLS analysis.

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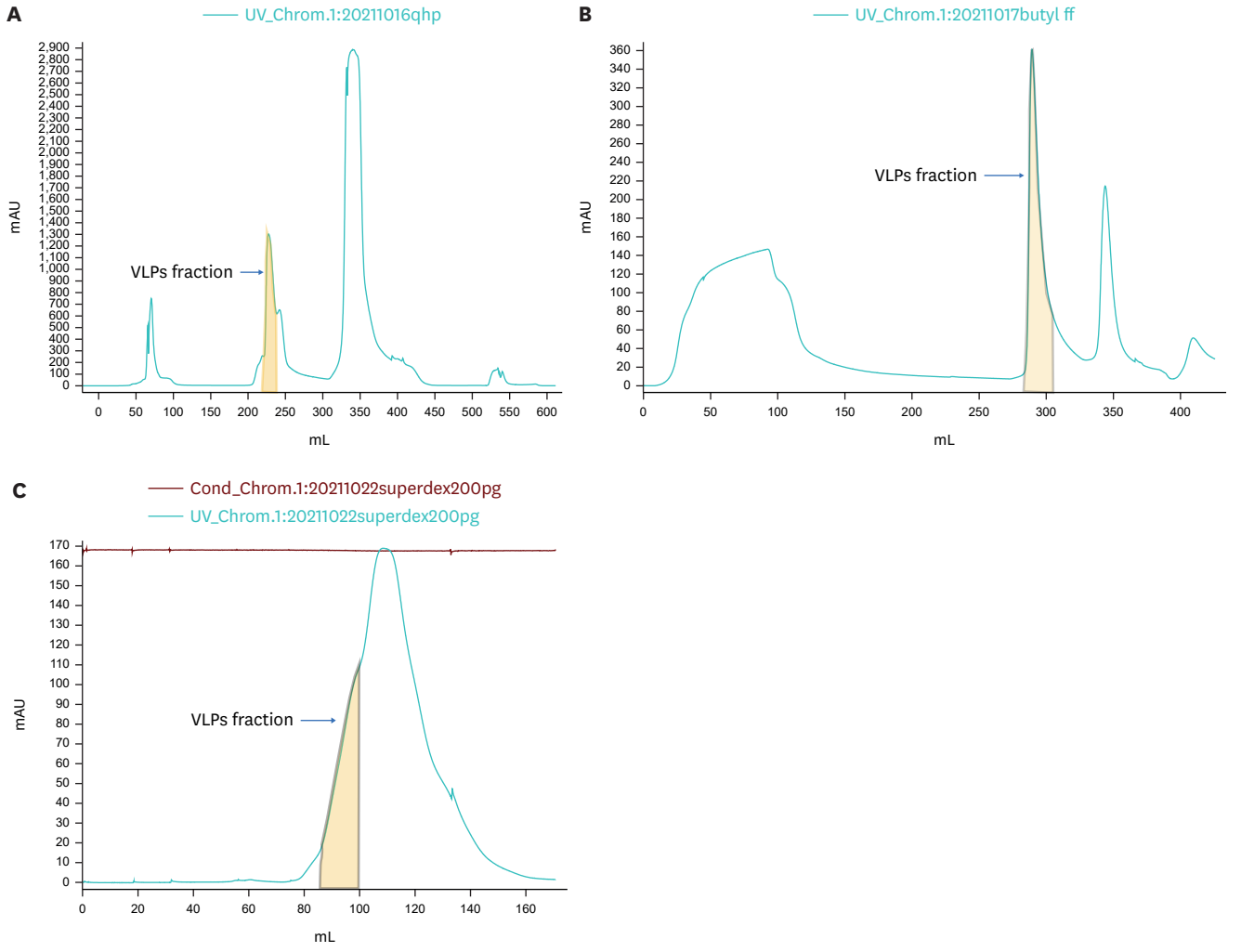

Fig. 1. The target protein was further purified by three-step chromatography. (A) Anion exchange chromatography (Q Sepharose High Performance; GE Healthcare). (B) A hydrophobic interaction chromatograph (Butyl Sepharose 4FastFlow; GE Healthcare). (C) Based on molecular chromatography (Superdex 200 pg; GE Healthcare). The shaded part in the figure is the purified VLPs collected by chromatography.
VLP, virus-like particle.

The VP0, VP1, and VP3 proteins were detected using specific antibodies by western blotting, suggesting that the P1 polyprotein was cleaved successfully by 3C protease in *H. polymorpha.* The absorption peak of the sample at A280 was observed at 16.855 min with a purification above 98%.

Immunization of BALB/c mice

The VLPs and inactivated vaccines induced the mice to produce high levels of antibodies against type O FMDV compared to the control group after immunization (**Fig. 3**). There was no significance between VLPs and inactivated vaccines treatment group after the first immunization. However, the level of antibodies for the VLPs group was higher than inactivated vaccine group after the following boost. Moreover, VLPs immunization maintained a persistently high antibody level, whereas the antibody decreased slightly in the group of inactivated vaccine treatment after the last immunization. Hence, the antibody duration and titer levels were weaker in the inactivated vaccine group than in the VLPs group (**Fig. 3**).

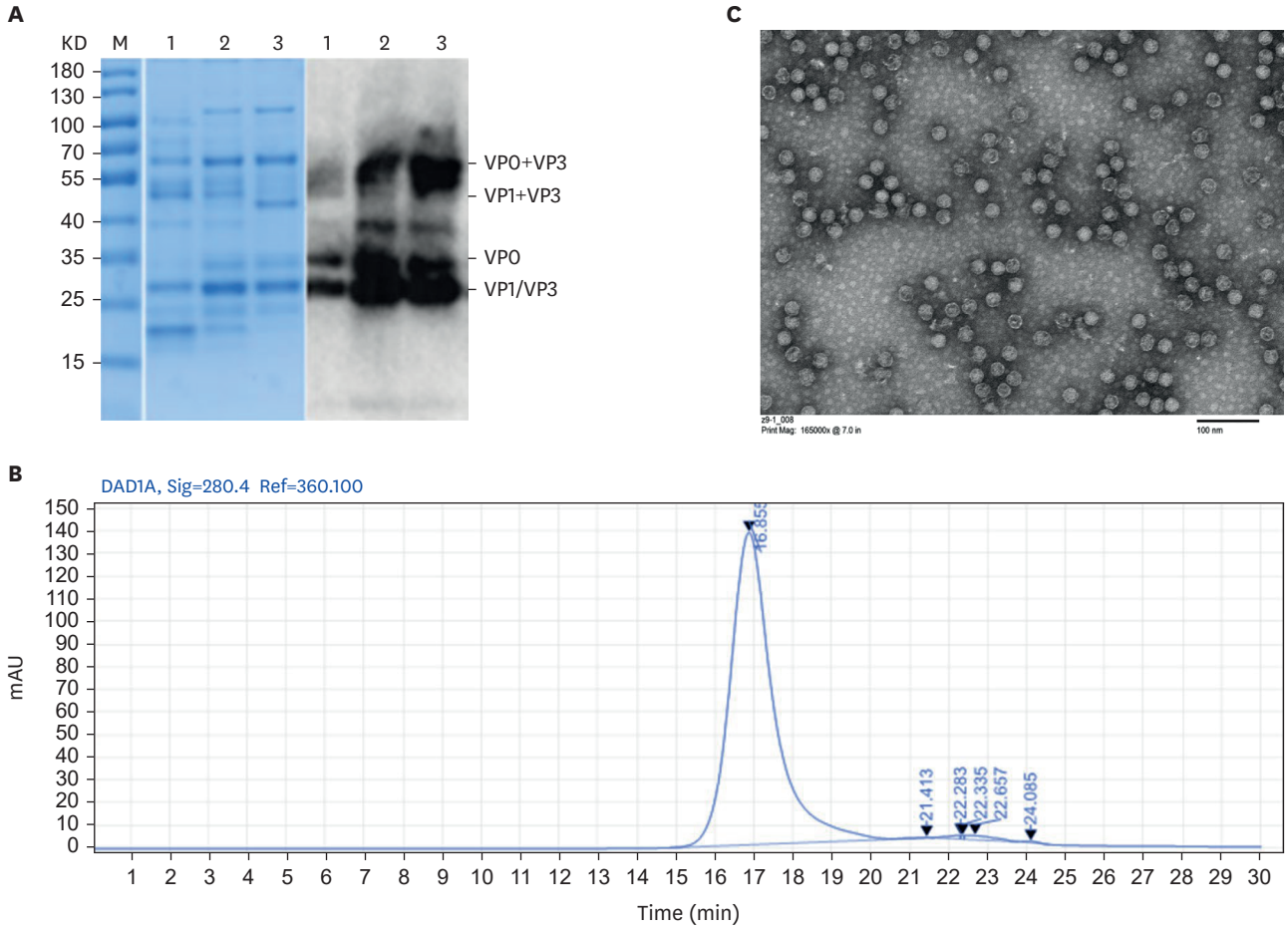

Fig. 2. Identification of virus-like particles after ultracentrifugation. (A) The protein curve of collected samples determined by A280. The sodium dodecyl sulfate polyacrylamide gel electrophoresis and WB detection results of samples near the peak. WB analyses of recombinant proteins using rabbit type O foot-and-mouth disease virus antibody (lane M is the protein Marker; lanes 1, 2, and 3 are the three sample tubes at the peak, respectively). (B) The purity of the peak sample (lane 2 sample) measured by high-performance liquid chromatography. (C) The peak sample (lane 2 sample) with particles around 25 nm observed by electron microscopy.
WB, Western blot.

On the other hand, the inactivated vaccine and VLPs increased the serum IgG, IgG1, and IgG2a production after immunization compared with the control group. In contrast, it is lower in inactivated vaccine group than the VLPs group, as shown in **Fig. 4**. The IgG1/IgG2a ratio represents either humoral or cellular immune response when it is positive or negative accordingly. As shown in **Fig. 4**, the inactivated vaccine can activate humoral immunity after multiple immunizations, but the VLPs group displayed a balance between humoral and cellular immunity.

VLPs increased IFN-γ production in splenocytes significantly compared to the FMD-inactivated vaccine group. The ELISpot results showed that the positive conversion rate of the VLPs group was 100%, and the average spot number was 1,418.5 and 1,644, while the positive conversion rate of the inactivated vaccine group was only 40%, and the average spot number was 22, as shown in **Fig. 5**. In addition, the number of B cells in the inactivated vaccine group was higher than that in the VLPs group, and there was no change in terms of the Germinal center B cell and memory B cell. In contrast, VLPs immunization increased the number of Plasma B cells compared to the inactivated vaccine group (**Fig. 6**). Furthermore, VLPs largely

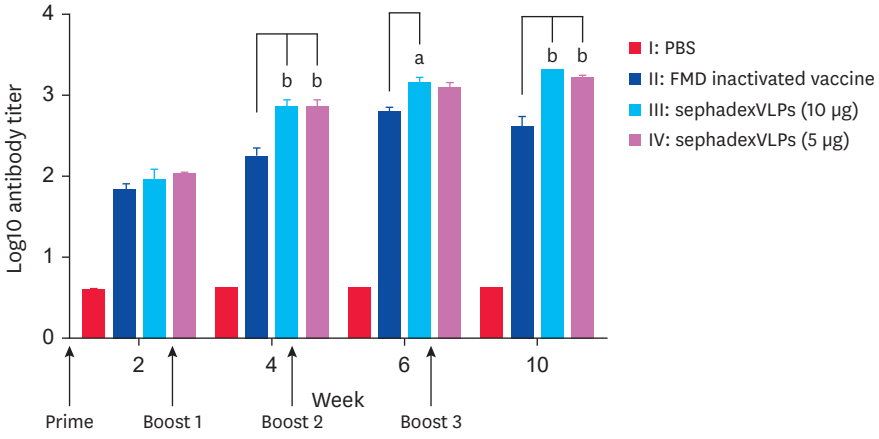

Fig. 3. Liquid phase blocking ELISA of FMD type O antibody. BALB/c mice were immunized every 2 wk for a total of four times. Blood samples were collected 2 wk after immunization for antibody detection. The last blood samples were collected 4 wk after the last immunization for antibody detection by FMD type O antibody liquid phase blocking ELISA. The antibody titers of each group were transformed logarithmically, and the bars represent mean ± standard error of the mean. I: PBS represents the immune PBS solution, II: FMD inactivated vaccine represents the immune inactivated vaccine group, III: sephadexVLPs (10 μg) represents the immune group containing 10 μg VLPs, IV: sephadexVLPs (5 μg) represents the immunized group containing 5 μg VLPs. Titer = 0.6 means the minimum dilution assay in which no positive signal was detected.
ELISA, enzyme-linked immunosorbent assay; FMD, foot-and-mouth disease; PBS, phosphate buffered saline; VLP, virus-like particle.
[a]$p < 0.05$; [b]$p < 0.001$.

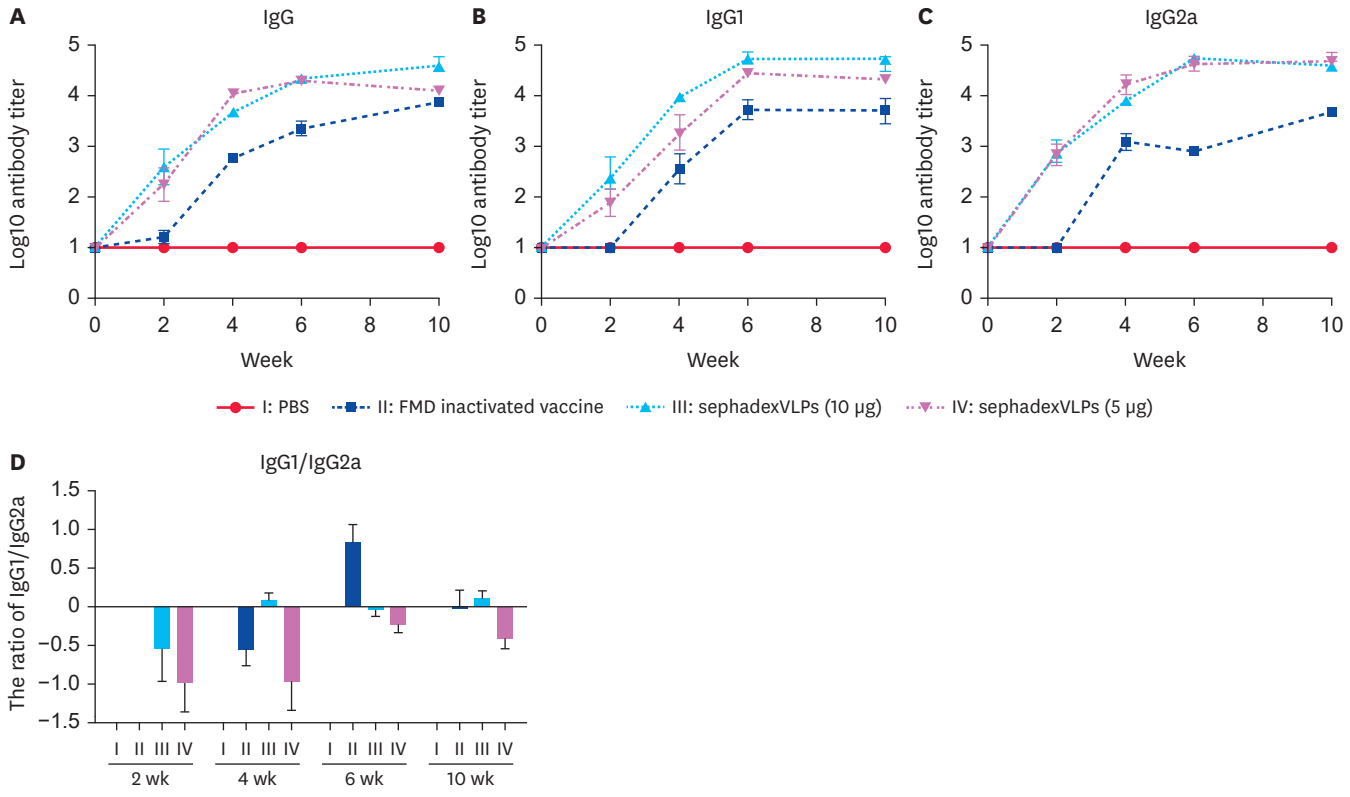

Fig. 4. ELISA results of mouse serum IgG, IgG, IgG2a, and IgG1/IgG2a. BALB/c mice were immunized every 2 wk for a total of four times. Blood samples were collected 2 wk after immunization for antibody detection, and the last blood samples were collected 4 wk after the last immunization for antibody detection by ELISA. The antibody titers of each group were transformed logarithmically, and the bars represent mean ± standard error of the mean. (A-C) The production of serum IgG, IgG1, and IgG2a after immunization. (D) The IgG1/IgG2a ratio after multiple immunizations. I: PBS represents the immune PBS solution; II: FMD inactivated vaccine represents the immune inactivated vaccine group; III: sephadexVLPs (10 μg) represents the immune group containing 10 μg VLPs; IV: sephadexVLPs (5 μg) represents the immunized group containing 5 μg VLPs. Titer = 1 means the minimum dilution assay in which no positive signal was detected.
ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; FMD, foot-and-mouth disease; PBS, phosphate buffered saline; VLP, virus-like particle.

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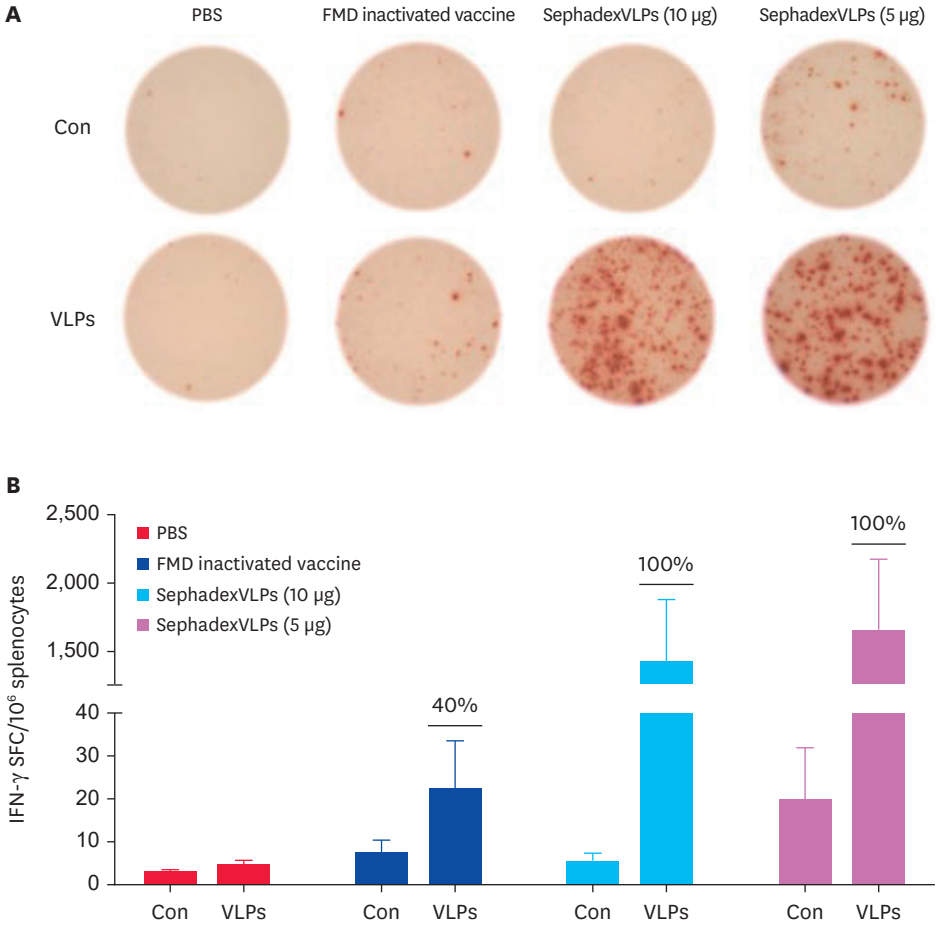

Fig. 5. Detection of the IFN-γ secretion level and positive conversion rate in splenocytes by ELISpot. After the mice were sacrificed, splenocytes were taken and stimulated with peptide pools. The number of IFN-γ-producing T cells was assessed by ELISpot. The Bars are presented as the mean ± standard error of the mean. (A) The representative spot plot. (B) The corresponding statistical results. PBS represents the immune PBS solution, FMD inactivated vaccine represents the immune inactivated vaccine group, sephadexVLPs (10 μg) represents the immune group containing 10 μg VLPs, sephadexVLPs (5 μg) represents the immunized group containing 5 μg VLPs.
IFN, interferon; con, control; ELISpot, enzyme-linked immunospot assay; IgG, immunoglobulin G; FMD, foot-and-mouth disease; PBS, phosphate buffered saline; VLP, virus-like particle.

elevated the number of $CD4^+$ T cells but decreased the number of $CD8^+$ T cells. Therefore, the $CD4^+/CD8^+$ ratio in the VLPs group was higher than that in the inactivated vaccine group (**Fig. 7**). Consistently, $CD4^+$ T cell-specific cytokines, such as IL-2, TNF-α, and IL-17A were upregulated significantly in the VLPs group (10 μg) than in the other groups. In contrast, IL-4 expression in the $CD4^+$ T cells was downregulated in the inactivated vaccine and VLPs groups compared to the control group (**Fig. 8**).

DISCUSSION

The recombinant FMD VLPs requires viral structural proteins VP0, VP3, and VP1, which can be expressed alone or generated from P1-2A polyprotein cleavage by 3C protease [8]. Thus far, there are no reports on the expression of FMD type O VLPs in yeast *H. polymorpha*. In this study, VLPs were formed autonomously in cells by co-expressing FMD P1 and 3CD proteins. The immunized mice with the VLPs showed stronger immunogenicity. These results showed that the FMD type O VLPs were expressed in yeast *H. polymorpha*.

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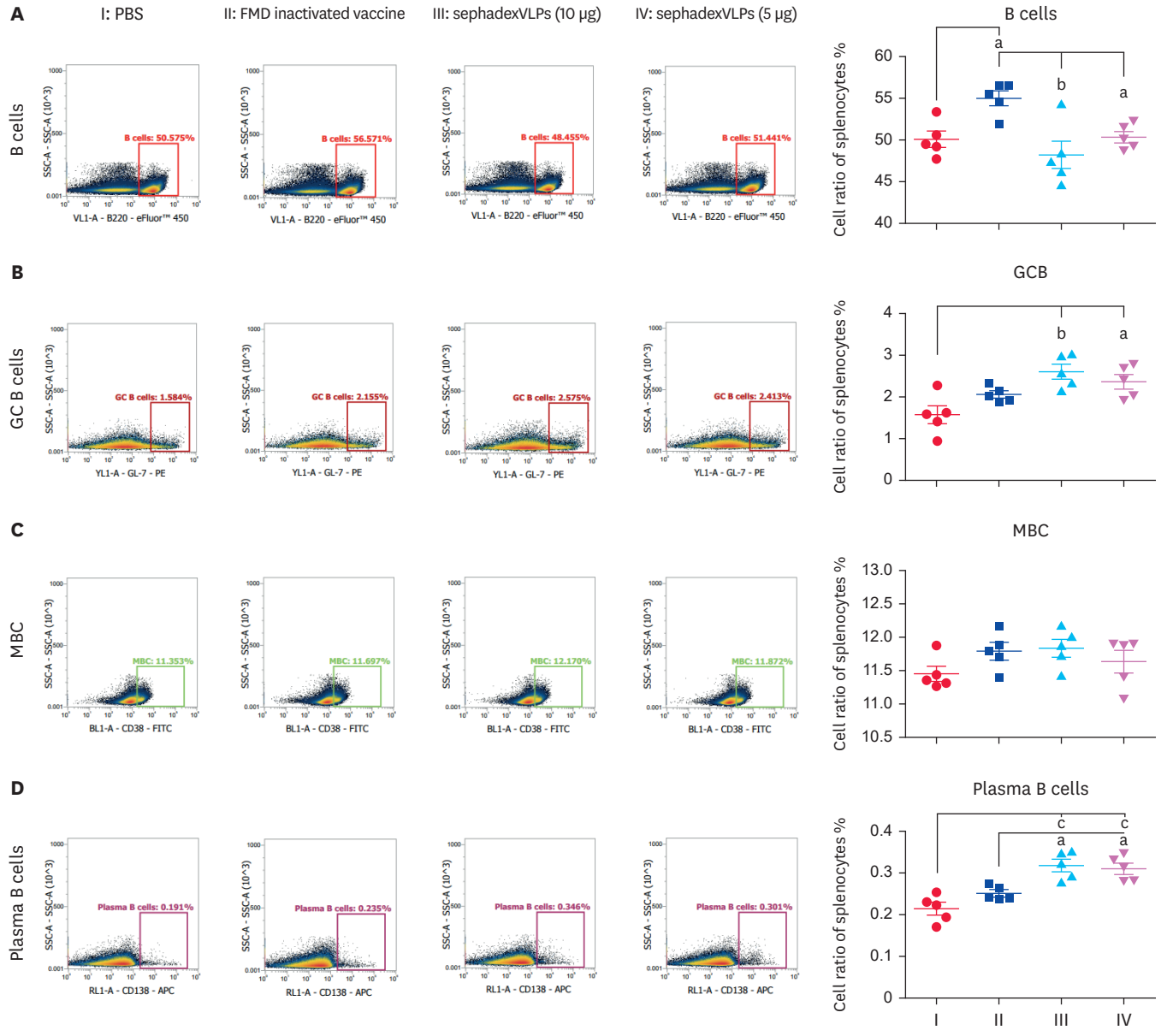

Fig. 6. Flow cytometry results of mouse splenocytes B cells. Fluorescence-activated cell sorting were conducted 28 d after the last injection to analyze the B cells, GC B, MBC, and plasma B cells, respectively. (A) The representative B cell flow cytometry and the statistical results of the B cell data. (B) The representative GC B flow cytometry and the statistical results of the GC B data. (C) The representative MBC flow cytometry and the statistical results of the MBC data. (D) The representative plasma B cells flow cytometry and the statistical results of plasma B cells data. The data are presented as the mean ± standard error of the mean. I: PBS represents the immune PBS solution, II: FMD inactivated vaccine represents the immune inactivated vaccine group, III: sephadexVLPs (10 μg) represents the immune group containing 10 μg VLPs, IV: sephadexVLPs (5 μg) represents the immunized group containing 5 μg VLPs.
GC B, Germinal center B cell; MBC, memory B cell; FMD, foot-and-mouth disease; PBS, phosphate buffered saline; VLP, virus-like particle.
[a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.

Neutralizing antibodies and neutralizing antibody-mediated opsonization are major immune responses protecting animals from FMD, whereas cellular immune responses also play an essential role against FMDV infections [15]. VLPs served as empty viral particles because the absence of genetic materials can induce B and T cells to multiply and enhance cell-mediated immunity (CMI) [7]. The vaccine adjuvants used in this study are CpG ODN and aluminum hydroxide gel. CpG ODN can promote the humoral and cellular immune responses by activating the TLR9 signal pathway. In addition, CpG ODN could directly activate B cells and

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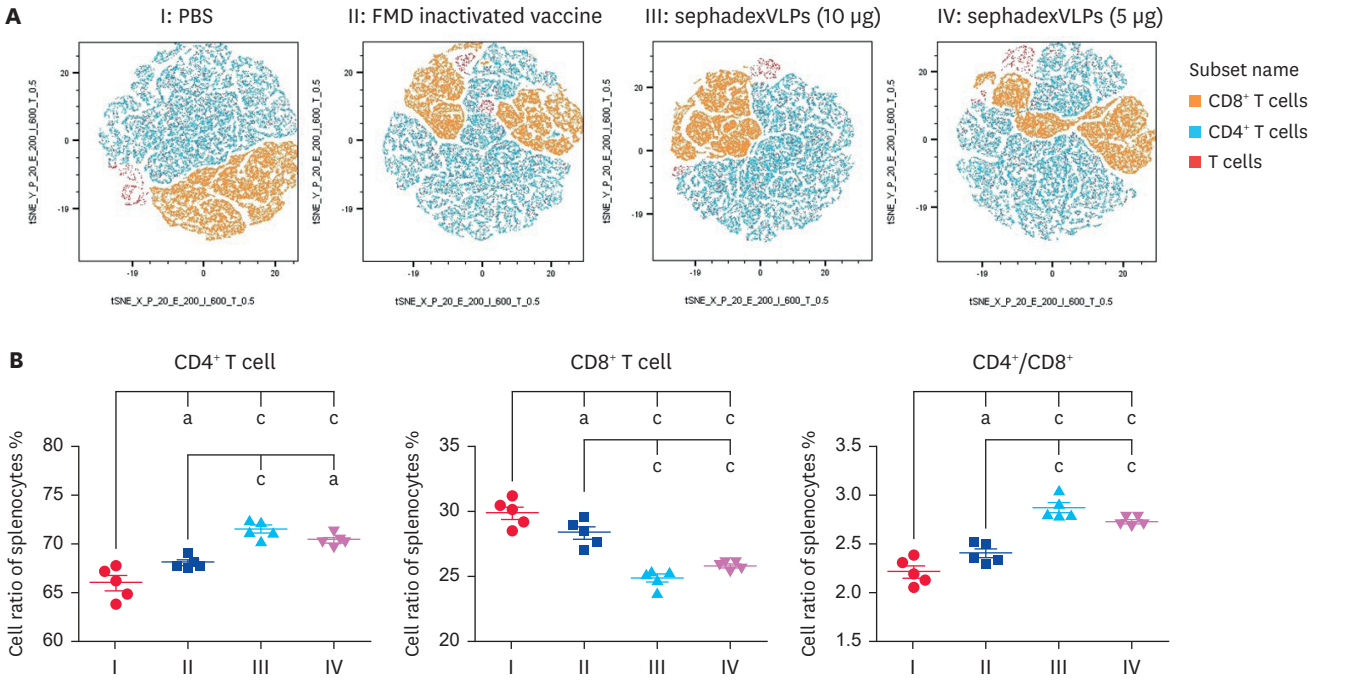

Fig. 7. Flow cytometry results of T cells in mouse splenocytes. T cells, $CD4^+$ T cells, and $CD8^+$ T cells were labeled with CD3-AF700, CD4-BV510, and CD8a-FITC (eBioscience), respectively. (A) The flow data of T cells analyzed by FlowJo with TSNE dimension reduction. (B) The number of $CD4^+$ T cells, $CD8^+$ T cells, and the ratio of $CD4^+/CD8^+$. The data are presented as the mean ± standard error of the mean. I: PBS represents the immune PBS solution, II: FMD inactivated vaccine represents the immune inactivated vaccine group, III: sephadexVLPs (10 μg) represents the immune group containing 10 μg VLPs, IV: sephadexVLPs (5 μg) represents the immunized group containing 5 μg VLPs.
FMD, foot-and-mouth disease; PBS, phosphate buffered saline; VLP, virus-like particle.
$^a p < 0.05$; $^b p < 0.01$; $^c p < 0.001$.

plasmacytoid dendritic cells, inducing the production of Th1 and proinflammatory cytokines, particularly cellular immunity [16,17]. Alum salts are a relatively weak adjuvant and cause little cellular immune response. On the other hand, this adjuvant slows the rate of antigen release and increases the time the antigen interacts with the immune system, enhancing the immune response against the antigen [18]. Specific immune stimulation to antigens was increased markedly using alum as a coadjuvant with CpG ODN over using alum and antigen alone. Moreover, the immune response was predominantly a Th1-type response [19]. In mice, Th1 cells produce IFN-γ resulting in IgG2a induction. In contrast, Th2 cells produce IL-4 and IL-5, inducing IgG1 responses [20].

The balance of Th1 and Th2 cells was evaluated by comparing the IgG1 and IgG2a levels, which are surrogates of the Th1 and Th2 responses. The IgG1/IgG2a ratio showed that after being inoculated many times, the immune response of mice immunized with mixed adjuvants tended to be cellular immunity or the balance between cellular immunity and humoral immunity. In contrast, the inactivated vaccine group in the control group tended to be humoral immunity. Moreover, VLPs immunization worked better in the immune response of mice regardless of humoral and cellular immune than in inactivated vaccine group, implying its more protective effect on FMDV infection. On the other hand, the antigen content of the commercially available inactivated vaccine used in the control was not determined. The antigen content of the VLPs group in this trial may be much higher than that of the inactivated vaccine group. In a study, guinea pigs immunized with VLP + CpG ODN vaccine showed markedly higher CMI compared to the conventional vaccine group, as evidenced by higher levels of IgG2 than IgG1 [21,22]. On the contrary, the vaccine using CpG

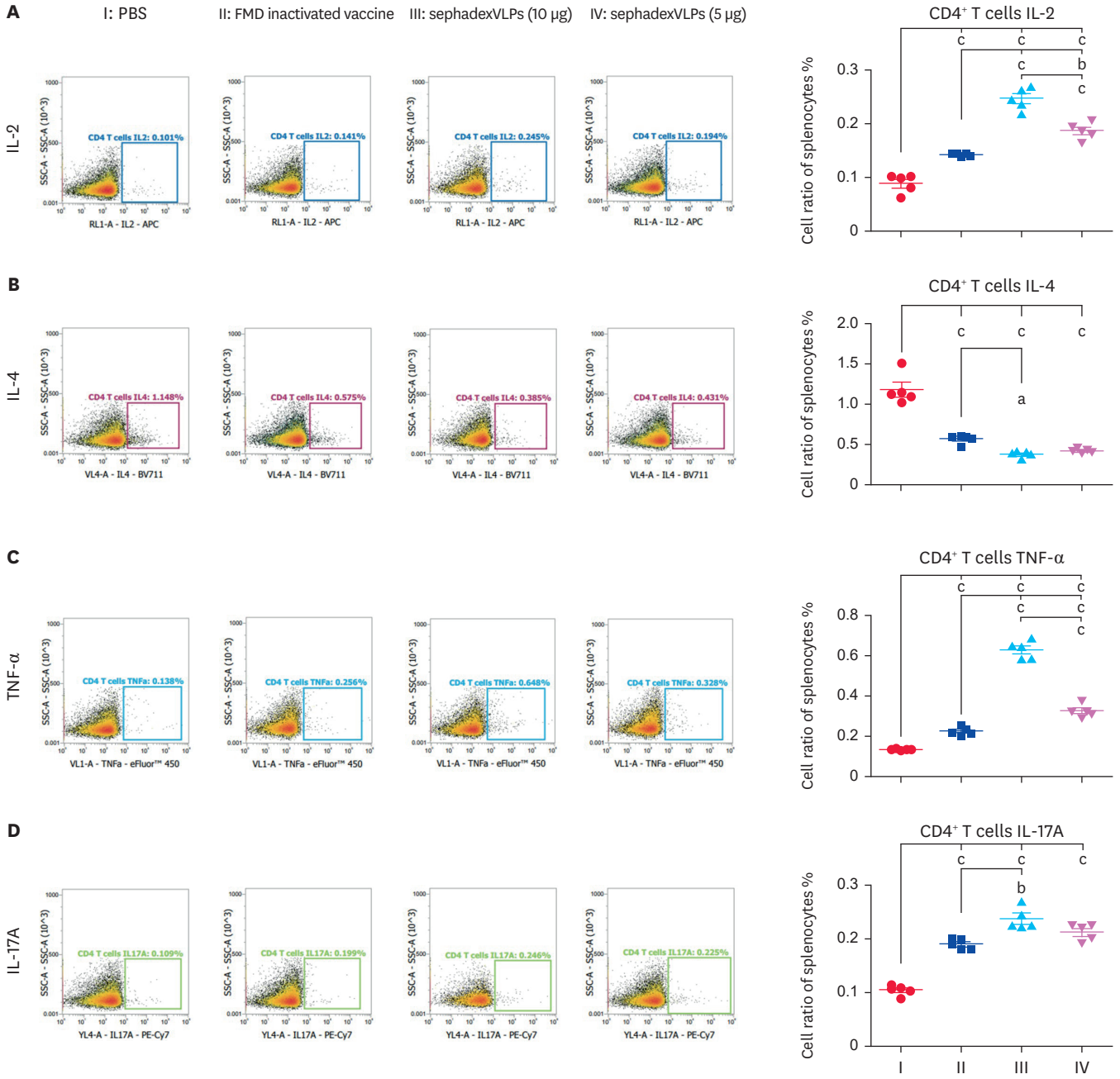

Fig. 8. Results of mouse splenocyte-specific cytokine assay by flow cytometric. Fluorescence-activated cell sorting were conducted 28 d after the last injection to analyze the FMD-specific CD4+ cells producing IL-2 (A), IL-4 (B), TNF-α (C), and IL-17A(D), including the representative cells flow cytometry and the statistical results, respectively. The data are presented as the mean ± standard error of the mean. I: PBS represents the immune PBS solution; II: FMD inactivated vaccine represents the immune inactivated vaccine group; III: sephadexVLPs (10 μg) represents the immune group containing 10 μg VLPs; IV: sephadexVLPs (5 μg) represents the immunized group containing 5 μg VLPs.
FMD, foot-and-mouth disease; PBS, phosphate buffered saline; VLP, virus-like particle.
[a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.

ODN as an adjuvant failed to protect pigs from FMDV infection earlier. The FMD-inactivated vaccine and CpG ODN did not improve the efficiency of the vaccine itself [23,24]. Therefore, how the preparation of VLPs, CpG ODN, and aluminum hydroxide gel as adjuvants have a protective effect on susceptible animals remains unclear.

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These results suggest no significant difference between the high and low doses of VLPs in activating the immune responses, indicating that using CpG ODN and aluminum hydroxide gel as adjuvants together with 5 μg VLPs could induce strong immunity after multiple immunizations. Increasing the VLPs did not improve the immune response significantly.

VLPs with more than 98% purity were obtained through a multi-step purification process. Given that FMDV could be purified only through Butyl Sepharose 4FF and Superdex 200 pg to collect 98% pure particles [25], the purification process for VLPs expressed in yeast *H. polymorpha* needs to be modified further to make it more economical. Nevertheless, it is still instructive for the purification of FMD VLPs. In conclusion, the expression of VLPs in *H. polymorpha* and the purification process provide novel strategies for optimizing VLPs synthesis and FMD vaccine development.

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