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Immunogenicity evaluation of MS2 phage-mediated chimeric nanoparticle displaying an immunodominant B cell epitope of foot-and-mouth disease virus

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Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals that has caused tremendous economic losses worldwide. In this study, we designed a chimeric nanoparticles vaccine with the predominant epitope of FMDV (VP1 131–160) displayed on the top of the coat protein (CP) of MS2 phage. The recombinant protein was expressed in E. coli and can self-assembled into chimeric nanoparticles (CNPs) with diameter 20-25nm. A tandem repeat peptide epitopes (VP1 131–160) (TRE) was prepared as control. Mice immunized with CNPs and TRE respectively and immunogenicity evaluated show that CNPs stimulated equivalent specific antibody levels to commercialized synthetic peptide vaccines (PepVac), but was significantly higher than TRE groups. Moreover, results from specific IFN-γ responses and lymphocyte proliferation test indicated that CNPs immunized mice exhibited significantly enhanced cellular immune response. These studies suggested that the CNPs constructed in current study could be a potential alternative vaccine in future FMDV control.

Immunogenicity evaluation of MS2 phage-mediated chimeric nanoparticle displaying an immunodominant B cell epitope of foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals that has caused tremendous economic losses worldwide. In this study, we designed a chimeric nanoparticles vaccine with the predominant epitope of FMDV (VP1 131–160) displayed on the top of the coat protein (CP) of MS2 phage. The recombinant protein was expressed in E. coli and can self-assembled into chimeric nanoparticles (CNPs) with diameter 20-25nm. A tandem repeat peptide epitopes (VP1 131–160) (TRE) was prepared as control. Mice immunized with CNPs and TRE respectively and immunogenicity evaluated show that CNPs stimulated equivalent specific antibody levels to commercialized synthetic peptide vaccines (PepVac), but was significantly higher than TRE groups. Moreover, results from specific IFN-γ responses and lymphocyte proliferation test indicated that CNPs immunized mice exhibited significantly enhanced cellular immune response. These studies suggested that the CNPs constructed in current study could be a potential alternative vaccine in future FMDV control.

Subjects Immunology, Biotechnology

Keywords: Foot-and-mouth disease (FMD), MS2 Bacteriophage, chimeric nanoparticles (CNPs), G-H Loop.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) infects more than 70 species of cloven-hoofed animals and has caused enormous economic losses to stockbreeding industry worldwide (Diaz-San Segundo et al. 2016; Pereira 1976). Conventional inactivated FMDV vaccine has been widely used and extremely successful in epidemic control and eradication of FMDV globally. However, there are a number of concerns and limitations with its use in emergency control programs, such as the possibility of virus escape during vaccine production and difficulty in differentiating infected from vaccinated animals (Dong et al. 2015; Parida 2009; Wang et al. 2002). Epitope-based polypeptide vaccines are well known for their abilities to provide more accurate, larger numbers of effective antigens and effectively distinguish between infected and vaccinated animals. However, these epitope vaccines induce limited cellular immune response and immune protection in large host animals (Rodriguez et al. 2003; Taboga et al. 1997).

Neutralizing epitopes of FMDV, which distributed in structural proteins VP1, VP2 and VP3, is critical to neutralizing antibodies induction and effective immune protection. G–H loop has been identified as a primary antigenic epitope on FMDV VP1, which can effectively inducing FMDV specific neutralizing antibodies (Bittle et al. 1982; DiMarchi et al. 1986). Moreover, G–H loop of FMDV has a precise spatial conformation on the surface of natural virus particle and correct conformation is essential for inducing effective immune protection (Acharya et al. 1990; Cao et al. 2016).

Nanoparticle-based antigen display technology provides an approach to improve the cellular immune response and immune protection effect of subunit vaccines (Chackerian 2007; Crisci et al. 2009; Crisci et al. 2012; Xu et al. 2017). MS2 phage is a novel display and delivery platform for foreign peptide epitopes. MS2 phage belongs to the Leviviridae family of small positive-sense single-stranded RNA bacteriophages, which is encapsulated by a icosahedral capsid comprised of 180 copies of coat protein and a single copy of mature protein (AP) (Koning et al. 2016; Wei et

al. 2008). The coat protein of MS2 bacteriophage self-assemble into virus-like particles (VLPs) in E. coli in the absence of viral RNA (Caldeira & Peabody 2011; Lino et al. 2017). The AB loop of coat protein, exposed on the surface of the phage particles, which tolerates exogenous peptides insertion without affecting the self-assembly of coat protein (Fu & Li 2016; Mastico et al. 1993). In the present research, we inserted the G–H loop domain amino acid sequences into the AB loop of MS2 phage coat protein. Chimeric protein was expressed in E. coli and self-assembled into CNPs. Immunoassay test showed CNPs induced higher antibody levels and cellular immune response than TRE immunized group. Importantly, IFN-γ level of CNPs immunized mice were significantly higher than PepVac groups. These results suggest that MS2- mediated CNPs is a useful platform for displaying foreign epitopes and might provide a new insight and approach to develop alternative vaccines for FMDV.

MATERIALS AND METHODS

Animal and commercial vaccine

Six-week-old female Kunming mice were provided by Laboratory Animal Center, Zhengzhou University. This study was performed with the approval of the Animal Experiment Committee of Henan Academy of Agricultural Sciences (Approval number SYXK 2014-0007). All animals used in this study were humanely maintained and euthanized according to the animal ethics procedures and guidelines of China.

PepVac (peptide 2600+2700+2800) was purchased from Shen Lian Biotechnology Corporation (Shang Hai, China). The anti-polypeptide （140-160 of VP1）monoclonal antibodies was prepared by our laboratory.

Plasmid construction

cDNAs of A and CP sequences of MS2 were reversely transcribed from its mRNAs (purchased from Sigma) and cloned into T-Vector pMD^{TM}19 (Simple) using a One-Step RT-PCR Kit (TaKaRa) following manufacturers' instructions. The G-H loop chimeric MS2 sequence was constructed by overlap extension PCR (OE-PCR). Sequences of primers for OE-PCR were listed in Table 1. Briefly, after the upstream and downstream sequences were amplified by MS2-F/IN-R and MS2-R/IN-F primer pairs, respectively, the full-length chimeric gene containing the MS2 phage A gene, CP gene and G-H loop gene of VP1 was amplified by MS2-F/MS2-R primer pairs and cloned into pET28a vectors (pCP-EP$_{131-160}$). A tandem repeats of G–H loop domain sequence (131–160) connected by GSGSGS were cloned into pET28a vector (p-EP$_{131-160}$). Recombinant plasmids of pCP-EP$_{131-160}$ and p-EP$_{131-160}$ were identified by restriction analysis and sequencing.

Recombinant protein expression and purification

Recombinant plasmids pCP-EP$_{131-160}$ and p-EP$_{131-160}$ were separately transformed into E. Coli BL21 (DE3), respectively. A single clone was selected from LB agar plate and cultured in LB medium supplemented with 50 μg/ml kanamycin. Until the optical absorbance OD$_{600}$ reached 0.8, target proteins were induced by 0.3 mM of isopropyl β-D-thiogalactoside (IPTG). After 16 h induction at 20℃, the cells were harvested and lysed by sonication. The recombinant proteins were analyzed by SDS-PAGE and Western blot. Chimeric protein in supernatant was purified as below: DNase I and RNase A with a final concentration of 1 μg/ml were added into the

supernatant at room temperature for 30 min, then 1 M solid NaCl was added and incubated on ice for 1 hour. After centrifugation at 9,000rpm/min for 10 min, PEG8000 was added into supernatant to a final concentration of 10% w/v and stored the mixture for at least 1 hour . Centrifuged again, the pellet was resuspended in PBS buffer. After incubated with an equal volume of chloroform and vortex the mixture gently for 30 seconds, the aqueous phases containing CNPs were collected by centrifuged at 4500rpm/min for 10 minutes. CNPs were further purified by gel filtration chromatography (Capto Core 700, GE). Briefly, preliminary purified CNPs were pumped onto PBS buffer equilibrated chromatography column and the effluent containing target protein was collected directly.

Besides, TRE was purified by Ni-NTA column (Merck, Germany). TRE was expressed as inclusion body, so the inclusion bodies were dissolved in 8M urea and loaded onto Ni-NTA column equilibrated with 0.05 M carbonate buffer（pH=9.0）containing 8M urea. After washing with 10 beds of carbonate buffer (50 mM imidazole, 8 M urea), the inclusion body was eluted with carbonate buffer (100 mM imidazole, 8 M urea). The purified protein was gradient dialyzed with 0.05 M carbonate buffer (pH 9.0) containing continuously decreased urea concentration (from 8 to 0 M) for 72 h. The concentration of purified CNPs and TRE was calculated using Micro BCA™ protein assay kit (Thermo Scientific, USA) following the manufacturer's protocol.

Identification of recombinant proteins

The purified CNPs was further characterized by transmission electronic microscopy (TEM) using the negative staining method, and particle size distribution was analyzed by dynamic light scattering (DLS) as described before (Chandramouli et al. 2013). The reactivity of purified recombinant proteins was analyzed by Dot-ELISA. Purified CNPs and TRE were blotted onto a nitrocellulose membrane. Inactivated FMDV was used as positive controls. The NC membrane was blocked with 5% skimmed milk for 2h at 37 ℃, and then incubated with guinea pigs anti-FMDV/O hyperimmune serum or anti-polypeptide monoclonal antibodies as the primary antibodies, followed by a HRP-conjugated goat anti guinea pigs or mouse as secondary antibody (Abcam). The Dot-ELISA was visualized using the AEC substrate.

Vaccine preparation and immunization

The purified CNPs or TRE was emulsified with adjuvant Montanide ISA 50V2 (Seppic, France) for animal vaccination. The ratio of aqueous antigen to the oil adjuvant was 1:1 (V/V). A total of 20 female Kunming mice of 4-6 weeks old were randomly divided into 4 groups with 5 animals per group and were inoculated subcutaneously with TRE 30ug, CNPs 15ug, 100ul of PepVac and 100ul of PBS, respectively. All mice received boost vaccination at 28 days after first immunization.

Detection of Anti-FMDV-Specific Antibodies

Serum samples were collected weekly from the tail vein after the first immunization. FMDV specific antibodies were detected by ELISA. Briefly, a rabbit polyclonal antibody against FMDV was coated on 96 well ELISA plate with 100ul per well and incubated at 4 ℃ overnight. The plate was blocked with 5% skimmed milk, and incubated with working concentration of inactivated viral at 37 ℃ for 1 h. After well washed with PBST, the serum samples were added

and incubated at room temperature for 1 h. Then the plate was washed thoroughly, goat anti-mice IgG-HRP was added to each well and incubated for 1 h at 37 ℃. After being washed five times, the reaction substrate was respectively added to each well and incubated at 37 ℃ for 10 minutes. Then, the reaction was stopped by 2M H_2SO_4, and the OD_{450} values were measured by a spectrophotometer.

Spleen lymphocyte proliferation assay

The spleen lymphocytes were isolated from immunized mice at 28 days after booster immunization using a lymphocyte separation kit (Solarbio, Beijing, China). Briefly, The spleen lymphocytes were resuspended in RPMI-1640 medium containing 10% FBS, and incubated in triplicate in 96-well plate with a density of 5×10^5 cells/well at 37 ℃ for 24 h. Then, the cells were stimulated with 50 μL of inactivated FMDV (20 μg/mL). Concanavalin A (ConA, 5μg/ml) and unstimulated wells were used as the positive control and negative control. After incubation at 37 ℃ for 48 h, WST-8 (10 ul/well) was added to each well and incubated at 37 ℃ for 1 h. The absorbance of each well was measured at 450 nm. T lymphocyte proliferation were expressed as the stimulation index (SI), which was the ratio of the mean reading of triplicate stimulated wells to unstimulated wells.

Cytokines detection

Splenic lymphocytes culture supernatants used in the proliferation assay were collected for evaluating IL-2, IL-4 and IFN-γ concentration. The assay and data calculation were performed by the commercially available ELISA kit (Bogoo, Shanghai, China) following manufacturers' instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 statistical software. Unless otherwise indicated, two-way ANOVA method was employed for significance test. Dates are shown as the mean ± SEM, and a $p < 0.05$ was considered statistically significant.

RESULTS

Construction of recombinant vectors

Recombinant vectors s, pCP-$EP_{131-160}$ and p-$EP_{131-160,}$ were confirmed by PCR, restriction digestion and sequence analysis. The upstream, downstream and the full length chimeric genes were amplified by PCR with the expected molecular size of 1260 bp, 487 bp and 1734 bp (Fig.1A), which showed that the $LOOP_{131-160}$ was successfully inserted into CP of MS2. This result was also confirmed by restriction digestion (Fig.1B) and sequencing (data not shown).

Expression and purification of CNPs and TRE

The expression of CNPs and TRE was verified by SDS-PAGE and Western blot. The result of SDS-PAGE indicated that approximately 50% of the chimeric proteins (18 kDa) are expressed in soluble form and TRE (10 kDa) are expressed as inclusion body (Fig.2A). The recombinant proteins were recognized specifically by the anti-polypeptide （140-160 of VP1）monoclonal

antibodies(Fig.2B). The purity of purified CNPs and TRE was estimated to be over 90% and 85% (Fig.2C), separately.

Reactivity of the CNPs and TRE

The reactivity of CNPs and TRE was analyzed by Dot-ELISA. The results showed that CNPs and TRE could react strongly with anti-FMDV hyper-immune serum (Fig.3A) and anti-polypeptide (140-160 of VP1) monoclonal antibodies (Fig.3B). These results suggested that the G-H loop$_{131-160}$ of VP1 correctly displayed on the surface of A-B loop of MS2, and CNPs and TRE had a good immune reactivity with hyper-immune serum against FMDV.

Physical characterization of CNPs

To verify the self-assembly of chimeric protein into nanoparticles in vitro, the purified CNPs was analyzed by TEM. Results showed that the purified chimeric proteins assembled into nanoparticles with a diameter of 25-30nm at pH 8.0 in 150 mM of NaCl (Fig.4A), which were conform to the size of MS2 phage. In addition, similar results were observed by DLS (Fig.4B).

Antibody induction in immunized mice

To evaluate the immunogenicity of CNPs and TRE in vivo, FMDV antibody titer in sera samples was evaluated by ELISA. As shown in Fig.5, the antibody titers increased with time and specific antibodies of recognizing inactivated virus could be detected at 14 days post-vaccination(dpv) in experimental group and PepVac group. The CNPs group induced the highest antibody levels throughout the experiment, but no significantly different was found between CNPs group and PepVac group ($p > 0.05$). On day 28 dpv and 56 dpv, mice immunized with CNPs induced significantly higher antibody titer than TRE group and PBS group($p < 0.05$). After booster immunization, antibody titers further increased significantly except for PBS group. In summary, These results indicated that CNPs, TRE and PepVac could induce antibodies to react with inactivated FMDV, and CNPs could induce the highest antibodies after prime and booster vaccination in mice.

T lymphocyte proliferation

The spleen lymphocytes were isolated from mice at 28 days after booster immunization and stimulated in vitro with inactivated FMDV. As shown in Fig.6, The specific lymphocyte response levels of CNPs, PepVac and TRE groups were significantly higher than PBS groups. The group of CNPs elicited higher lymphocyte proliferation responses than the TRE group ($P<0.05$), while no significant differences were observed between CNPs and PepVac groups ($p > 0.05$).

Cytokine assay

To assess the cytokine secretion of spleen lymphocytes after stimulation, IFN-γ, IL-2 and IL-4 concentrations in culture supernatants were evaluated by ELISA. As shown in Fig.7, CNPs group, PepVac group and TRE group induced marginally greater IFN-γ and IL-2 levels than PBS group($p<0.05$) (Fig.7 A, B). However, there are no significant differences in the production of IL-4 among three groups (Fig.7C). Notably, the CNPs immunized group produced significantly higher IFN-γ levels than TRE group and PepVac group ($p<0.05$).

DISCUSSION

FMDV remains a significant threat to cloven-hoofed animals in developing countries(Sobrino et al. 2001), and a safe and effective vaccine is urgent need. Infectious bursal disease subviral particles, Hepatitis B virus core particles and porcine parvovirus subviral particles have been used as a delivery and display platform for FMDV epitopes (Pan et al. 2016; Pumpens et al. 1995; Remond et al. 2009), but these chimeric particles were obtained by eukaryotic cells. Conveniently, the CP of MS2 phage could be expressed and assembled in E.coli, and this makes it possible to achieve a rapid and low-cost production. Previous studies have shown that MS2 phage is a good platform for displaying and delivering epitope peptides (Fu & Li 2016; Heal et al. 1999; Lino et al. 2017). However, the tolerance of MS2 phage to this insertion is limited, and insertion of too long amino acids often results in misfolded, aggregated or degraded proteins(Caldeira & Peabody 2011; Peabody 1997). In this study, we demonstrated that a chimeric vaccine based on MS2 phage, which display the G-H loop domain sequences of VP1 on the CP of MS2, can be readily produced in the E. coli expression system.

The highly conserved RGD motif of G-H loop recognizes and adsorbs the integrin αvβ6 on the cell surface, which facilitates FMDV adsorption and invasion into cells(Knowles et al. 2001). The G-H loop (140-160) of VP1 is the main immunogenic epitopes for inducing neutralizing antibodies (Morgan & Moore 1990; Ochoa et al. 2000). Based on G-H loop and c-terminal sequence (200-213) of VP1, researchers have developed a variety of epitope vaccines which can also elicit high neutralizing antibodies titers in small animals such as mice and guinea pigs(Su et al. 2007). But these epitope vaccines induce limited antibody levels and immune protection in host animals, which may be due to the lack of appropriate T-helper cell epitopes and low molecular weight of peptides (Cao et al. 2016; Rodriguez et al. 2003). MS2 mediated VLPs vaccine displaying G-H loop (141-160) of FMDV conferred 65% in guinea pigs and 60% in pigs protection against FMDV challenge (Dong et al. 2015). The flanking sequences of G-H loop can further strengthen the immune response (Fang et al. 2015). In the present research, the predominant epitope 131-160 of VP1 was intensively presented on the CNPs surface. Meanwhile, the AB loop of coat proteins of MS2 makes the insertion sequence to form a circular structure which is similar to the natural virus. So the natural conformation of the G-H loop may be maintained and stabilized in CNPs. CNPs could react strongly with hyper-immune serum in Dot-ELISA showed that the 131-160 sequence of VP1 appeared on the surface of the MS2 coat protein in the correct conformation. CNPs stimulated higher stronger humoral immune response than TRE in mice also confirmed that the insertion sequence was displayed on MS2 surface with high density and correct structure.

CNPs as a special form of VLPs had the capability of inducing extensive cell-mediated immune responses and potentially enhancing the activation of innate immune systems (Fu & Li 2016; Ong et al. 2017). We observed a stronger lymphocyte proliferation response in CNPs immunized mice than TRE group. Moreover, the secretion of IFN-γ in CNPs group was significant higher than the TRE group and PepVac group and the IL -2 levels in CNPs group was higher than the TRE group. Cytokine IFN-γ and IL -2 were associated with cell-mediated immunity, so the results indicate that CNPs could elicit higher cell immune response than TRE and PepVac.

At present, the purification processes of macromolecules such as virus or virus-like particles are mainly density gradient centrifugation and gel filtration chromatography, but these methods are

too expensive and waste time and energy(Dong et al. 2015; Liu et al. 2017; Pan et al. 2016). The new media Capto Core 700, with the capable of both size separation and capture of small molecules, is designed for purification of viruses and other large biomolecules. In this study, we used gel filtration chromatography (Capto Core 700) for CNPs purification. Without flow restriction and multiple elution, the effluent containing CNPs was collected directly, and the purity of CNPs was over 90%.

CONCLUSIONS

In conclusion, We have developed a MS2 phage mediated CNPs, with displaying predominant epitope (131-160 of VP1) of FMDV on the CP of MS2 phage, which could be expressed and self-assembled into nanoparticles in E.coli. In addition, the CNPs had a better immunogenicity compared with PepVac and TRE, which could elicit higher specific antibody titers and stronger cellular immune response than TRE in mice. Therefore, these results indicated that this novel CNPs had the potential to be a safe, efficient and cost-effective subunit vaccine in future FMDV eradication.

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Table 1(on next page)

Sequences of primers for OE-PCR

Primer name	Primer sequence (5′-3′)
MS2-F	CG*GGATCC*GTGCGAGCTTTTAGTACCCTTGA
MS2-R	CCC*AAGCTT*TGTTGTCTTCGACATGGGTAATCCTC
IN-F	**CTGACCAACGTGCGTGGCGATCTGCAAGTCCTGGCACAGAAAGCTGCACGTC CTCTGCCT**ACTGGCGACGTGACTGTCGCCCCAAGCAA
IN-R	**TGCCAGGACTTGCAGATCGCCACGCACGTTGGTCAGGGAACCCTCAGCGTAC TTGCAGTTCCCGTT**TCCGCCATTGTCGACGAGAACGAAC

Notes

The restriction sites BamH I and Hind III are shown in italics in primers MS2-F and MS2-R; Synthetic oligonucleotides in bold type used to generate the GH loop (131-160) of VP1 for insertion into the MS2 coat protein gene, and 36 reverse complementary base pairs between oligonucleotides IN-F and IN-R underlined.

Figure 1

Products of amplifying and cleaving were analyzed by gel electrophoresis.

(A) The upstream, downstream and the whole fragments were amplified. M, DNA Marker; Lane 1, downstream fragment; Lane 2, upstream fragment; Lane 3, SOE-PCR product of whole fragment.(B) Recombinant plasmids were digested. M, DNA Marker; Lane 1, Digested of vector p-EP 131-160 ; Lane 2, Digested of vector pCP-EP 131-160 .

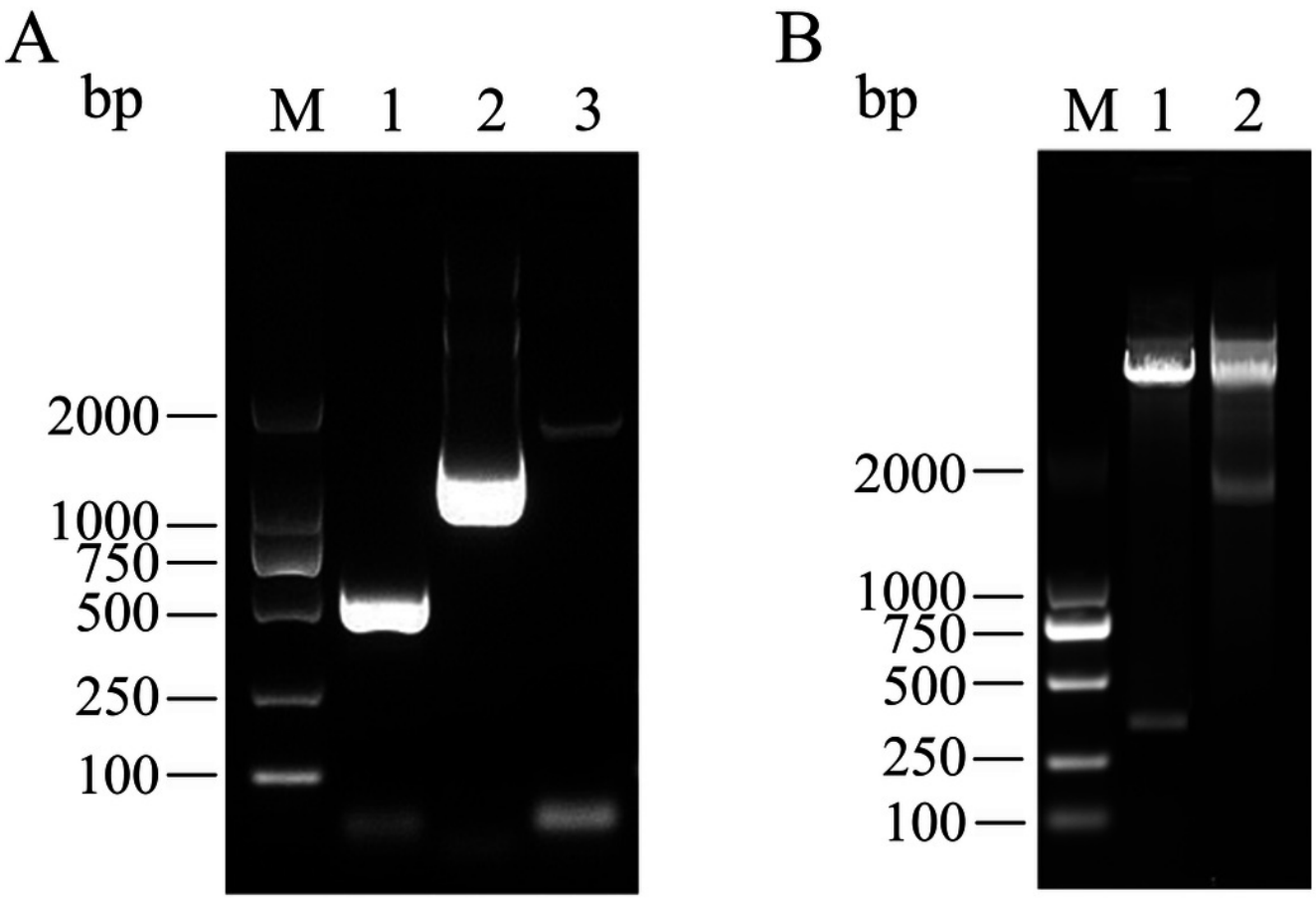

Figure 2

Expression and purification of CNPs and TRE.

(A) SDS-PAGE analysis the expression of recombinant protein. M, protein marker; Lane 1, pET28a-CP-$EP_{131-160}$ cell lysate supernatant; Lane 2, pET28a-CP-$EP_{131-160}$ cell lysate precipitation. Lane 3, pET28a-$EP_{131-160}$ cell lysate supernatant; Lane 4, pET28a-$EP_{131-160}$ cell lysate precipitation. (B) Western-blot analysis of purified CNPs and TRE with anti-G-H loop monoclonal antibody, M, protein marker; Lane 1-4,the same with SDS-PAGE.(C) SDS-PAGE analysis of purification protein. M, protein marker; Lane 1, purified CNPs after PEG8000 centrifugation; Lane 2, further purified CNPs with gel filtration chromatography (Capto Core 700); Lane 3, purified TRE.

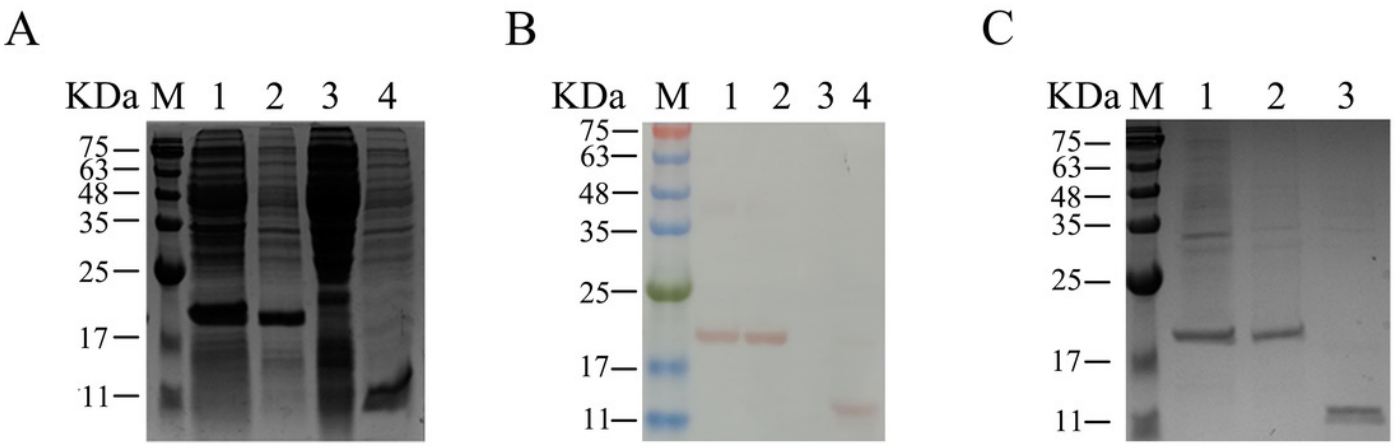

Figure 3

Identify the immunogenicity of CNPs and TRE.

(A) Dot-ELISA immune assay with pigs anti-FMDV hyper-immune serum. (B) Dot-ELISA immune assay with anti- G-H lOOP monoclonal antibody. 1, inactivated FMDV; 2, purified CNPs; 3, purified refolding TRE.

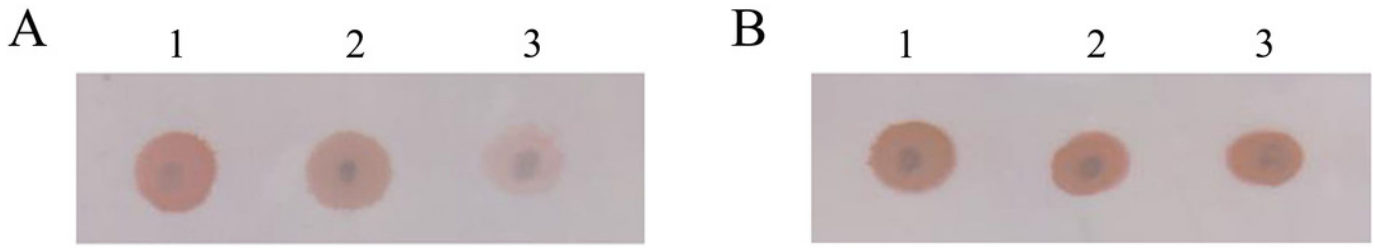

Figure 4

Physical characterization of CNPs.

(A) Transmission electron microscope (TEM) image of negative staining CNPs. These nanoparticles are approximately 25 ± 5 nm in diameter. (B) Dynamic light scattering results of CNPs in buffer containing 200 mM of NaCl at pH 8.0.

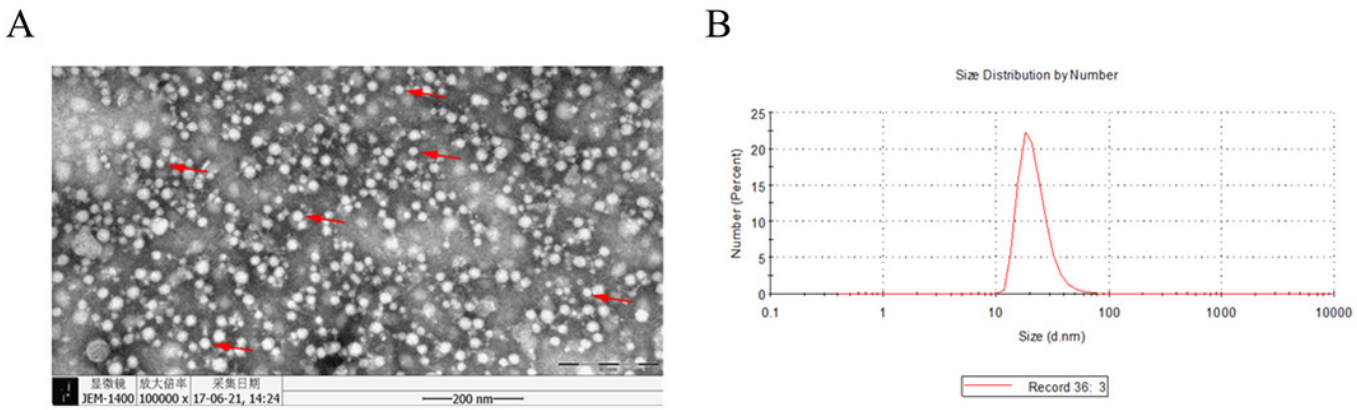

Figure 5

The vaccines elicit specific antibodies in mice.

Sera were collected at 0, 7, 14, 21, 28, 35, 42, 49 and 56 dpv and tested at a 1:20 dilution for antibodies against the inactivated virus. Date are shown as mean ± SEM. Number of asterisks indicate significant difference between groups (*p < 0.05, ****p < 0.0001, n=5).

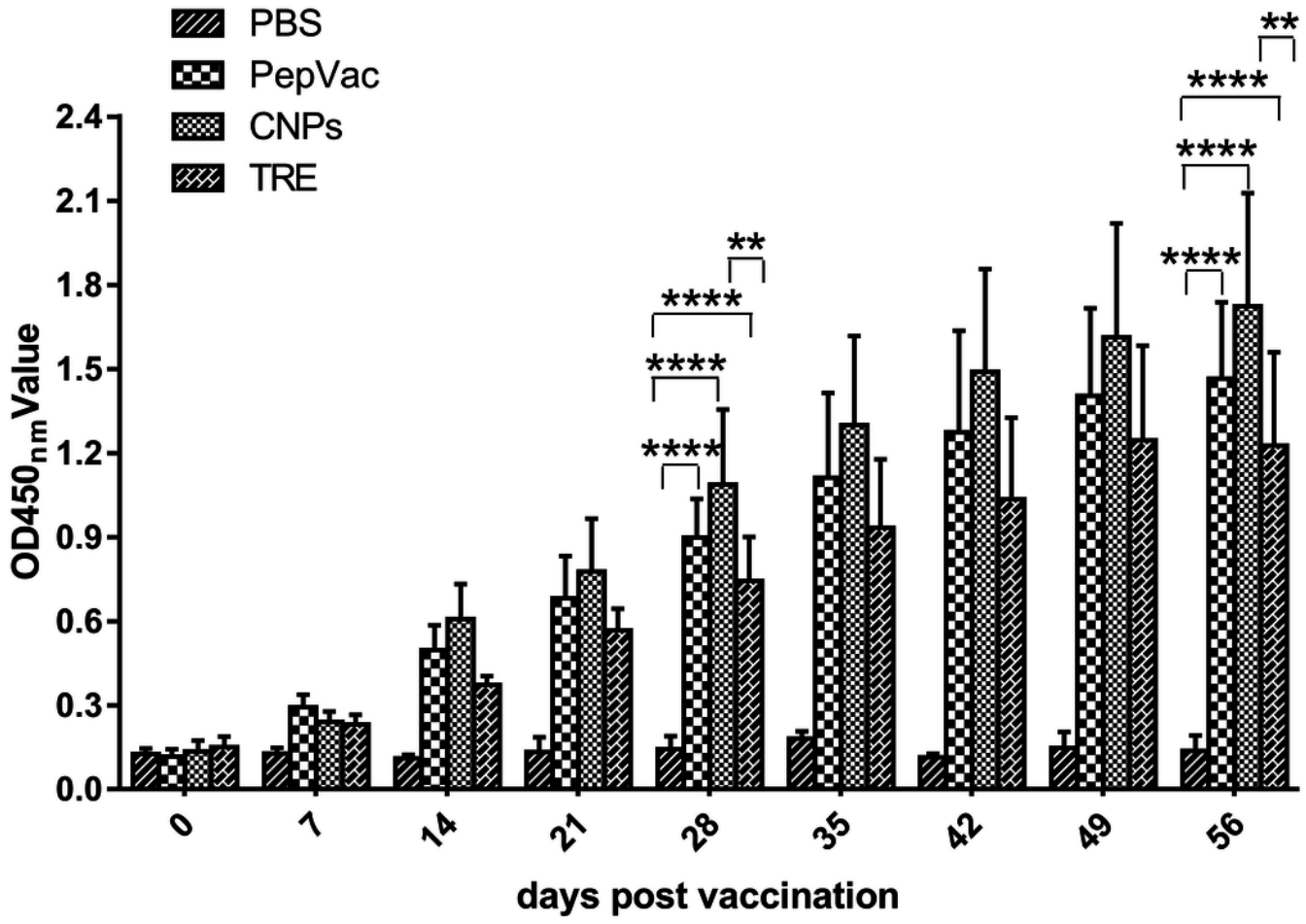

Figure 6

The T-lymphocyte proliferation in mice.

Spleen cells were isolated at 56 dpv and stimulated with inactivated FMDV and CoA, respectively. Proliferation was analyzed using the CCK-8 colorimetric assay. SI means the ratio of stimulated sample : unstimulated sample at OD_{450} nm. Significant values (*p < 0.05) are indicated by an asterisk.

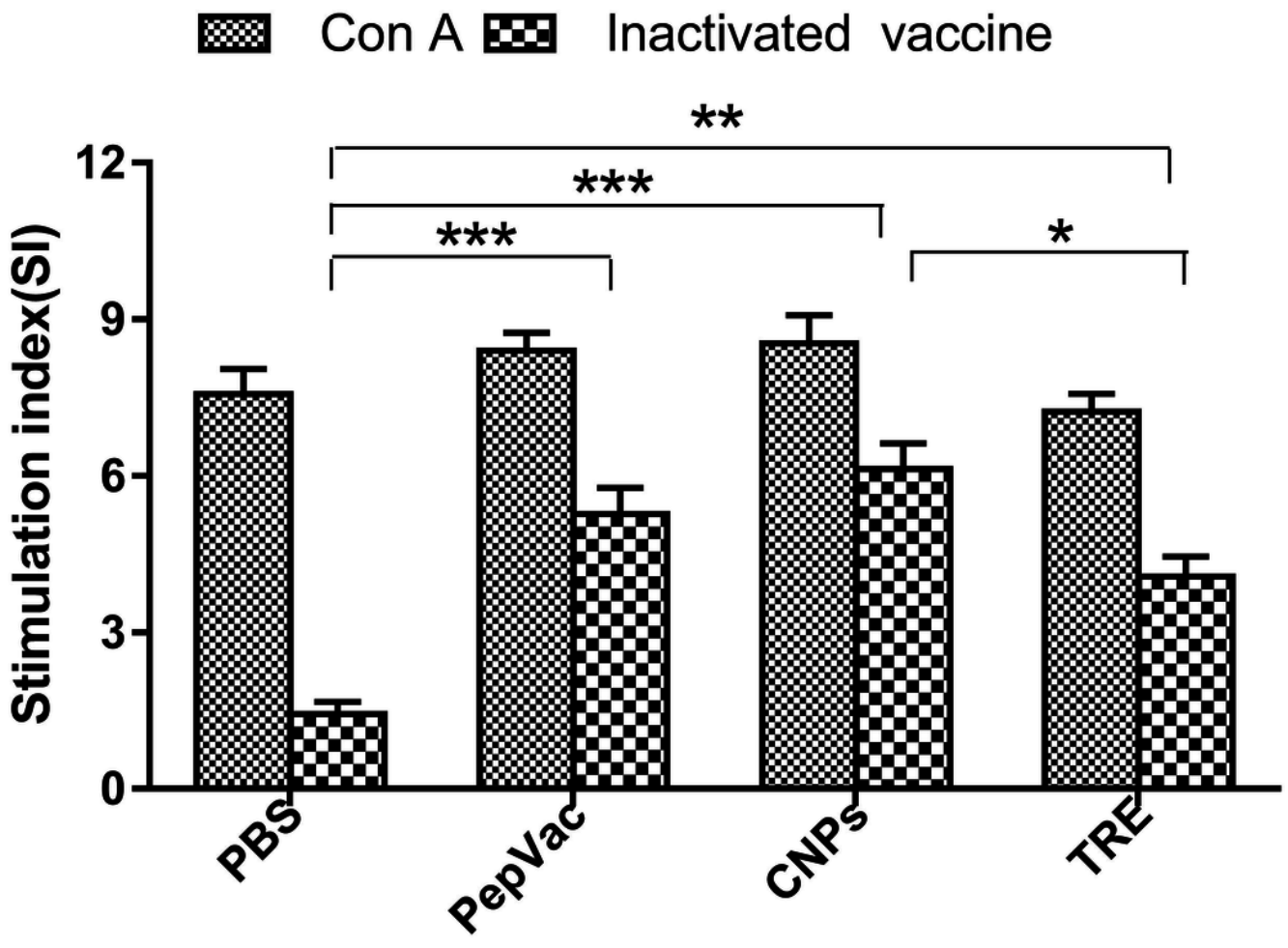

Figure 7

The vaccines elicit cytokines levels in mice.

Spleen cells were isolated at 56 dpv and stimulated with inactivated FMDV. The culture supernatants were collected and calculated by ELISA. (A), (B) and (C) are the concentrations (pg/ml) of IFN-γ, IL-2 and IL-4 in the supernatants, respectively. Data are shown as mean±SEM . Significant values (*P < 0.05) are indicated by one asterisk.