LDHs enhanced the immune response of animals to foot-and-mouth disease virus

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Abstract

Background: Foot-and-mouth disease (FMD) is a highly transmissible disease that leads to vast economic losses in many countries. Prevention using inactivated vaccines is one effective measure used to control FMD. Unfortunately, inactivated FMD vaccines provide only short-term protection and require a cold-chain system. In recent years, many studies have shown that layered double metal hydroxides (LDHs) carrying antigens can be used to strongly induce immune responses. In this study, LDH nanoparticles (NPs) were prepared by hydrothermal synthesis. LDH particle size, electric potential, and morphology were measured and observed. The adsorption capacity of LDH NPs to FMDV was tested. The effects of LDH as an adjuvant on inactivated FMDV vaccines were further evaluated and compared with commercial FMDV ISA-206 in BALB/C female mice and Yorkshire pigs.

Results: LDH NPs were successfully prepared with a uniform particle size of ~87.21 nm, regular edges, a loose hexagonal shape and positive zeta charge of 32 mV. The maximum absorption concentration was 0.16-0.31 μg FMDV/μg LDH. In the mouse experiment, antibody of immunized with LDH + FMDV were induced significantly higher from days 42-98 compared to saline + FMDV ($P<0.01$) and significantly higher compared to ISA-206+FMDV on day 56 post-immunization ($P<0.05$). After day14 post-immunization, IFN-γ content was significantly increased ($P<0.05$). In the pig experiment, antibody levels in both the ISA-206 + FMDV and LDH + FMDV were positive and were significantly higher compared with the PBS group on day 7 ($P<0.005$). Antibody levels in 90% pigs were positive on day 56 in the LDH group. The neutralizing antibody levels in the LDH and ISA-206 groups were significantly higher from days 7-28 compared to the PBS control group ($P<0.05$). Thus, LDH NPs were effective at inducing an immune response against FMDV.

Conclusions: LDHs with a loose hexagonal shape and a positive charge were prepared. LDHs can effectively induce humoral- and cell-mediated immune responses in mice and pigs. In addition, the LDHs had a slow-release effect and produced antibodies continuously. LDHs may act as an excellent FMDV adjuvant.

Background

Foot-and-mouth disease (FMD) is a highly contagious disease in cloven-hoofed animals, which spreads rapidly [1]. The disease affects many areas of the world, often causing extensive epizootics in livestock, particularly farmed cattle and swine, although sheep, goats and many wild species are also susceptible [1–2]. High morbidity, a complex host-range and broad genetic diversity make FMD prevention and control exceptionally challenging [2]. In most countries, susceptible livestock are immunized with inactivated foot-and-mouth disease virus (FMDV) vaccine in order to control the disease. Finding truly safe and effective vaccines, especially those that induce cell-mediated immunity, is the key to prevent and control the disease.

Adjuvant development plays a major role in vaccine technology [3]. The reasonable use of adjuvants in vaccines not only lessens the use of antigens, but also stimulates the immune system quickly and enhances the immune response. The choice of adjuvant is particularly important. Several kinds of vaccine adjuvants have been studied for their potency to promote immune responses to FMDV vaccines. These adjuvants include mineral oil (ISA-206 and ISA-201) [4–6], saponins (Quil-A) [7], Toll-like receptor (TLR) ligands (targeting pattern recognition receptors) [8–11], cytokines (IFN-a, IFN-g, IL-1, IL-2, IL-15, IL-18 and GM-CSF) [12–14], liposomes [15]. Current the commercial FMDV adjuvants used include ISA-206 and aluminum. Oil emulsions mainly rely on the strong reactogenicity to induce immunoreactions, which normally trigger severe side effects including hemolysis [16], swelling or necrosis at the injection site [17]. Aluminum hydroxide phosphate (Alum) is approved by the FDA for use in humans because of its safety and efficacy [18]. Nevertheless, Alum typically induces a classical antibody-mediated (Th2) response rather than cell-mediated (Th1) immunity, and therefore is not suitable for vaccination against diseases such as intracellular infections [19].The FDA-approved adjuvant also has undesirable features, it is non-biodegradable and consequently remains *in situ* longer than one year [20]. Alum also frequently produces a strong inflammatory reaction at the injection site [21]. Although some new adjuvants have been developed in recent years, but excellent adjuvant with good safety, efficacy, targeting, stability, controllable release, highly efficient immunity and low cost may be a hot research direction in the future.

Nanoparticles (NPs) and nanomaterials show great potential as next-generation adjuvants with desirable physicochemical features and reduced undesirable drawbacks and side effects [22]. To date, NPs such as mesoporous silica NPs [23], chitosan NPs [24], gold NPs [25], poly (D,L-lactic-co-glycolic acid) (PLGA) NPs [26], clay nanomaterials (i.e. layered double hydroxide and hectorite) [27–28] have proven their capacity to boost immune responses as effective adjuvants.

Layered double hydroxide (LDH) is hydrotalcite-like clay, represented by the chemical formula $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{X+}+[A^{n-}]_{x/n}{\cdot}mH_2O$ [29]. LDHs are formed by weathering of basalt in the nature [30]. LDH is a layered structure: the laminates have a structural positive charge, and the interlayers are composed of anions and water molecules. The interlayers are bound together by electrostatic interaction. Recent results have demonstrated that dispersion-stable LDH NPs are efficient vaccine carriers, stimulate higher levels of antibodies for a longer period, maturate dendritic cells (DCs) and promote stronger specific T cell immune responses [31]. For example, antigen BSA-Cy7 loaded LDH complexes generate loosely structured agglomerates either in solution or within nodules formed at the injection site and recruit immune cells into injection nodules and over a prolonged period [32]. LDH-adjuvanted multiple-antigen vaccine formulations can efficiently stimulate strong humoral, cellular and mucosal immune responses that are capable of preventing *E. coli* from adhering to mammalian cells more efficiently than the commercial adjuvant formulation [27, 33]. A dispersion-stable LDH-based vaccine induced stronger cytotoxic T-lymphocyte (CTL) responses and significantly inhibited tumor growth [34].

Therefore, LDH is a promising adjuvant vaccine due to the small particle size, stable dispersion, large specific surface area, positive charge, large cargo load, sustained release, easy absorption, low toxicity, low cost, and significantly improved the cellular immune response.

FMD vaccines not only focus on antigens but also focus on the adjuvant technology. Efficacy, source, cost and safety should be taken into account in adjuvant selection. In this study, LDH NPs were prepared by hydrothermal synthesis, and their properties analyzed. The mouse is a typical model animal and one of the representatives of typical mammals. The immune effects in mice were initially easy to observe. Pigs are susceptible to foot-and-mouth disease. It is practical to evaluate the effects of vaccines and adjuvants. The effects of LDH as adjuvant on inactivated FMDV vaccines were further evaluated compared with the commercial ISA-206 adjuvant in mice and pigs. LDH may be an effective and safe adjuvant improve FMD vaccine efficacy.

Results

Physicochemical properties of LDH NPs

LDH NPs (Mg_2Al-Cl-LDH) were synthesized via rapid precipitation followed by hydrothermal treatment. The particle size of LDH had only one peak at 87.21 nm, which showed homogeneously dispersed suspension (Fig. 1A). The equivalent mean hydrodynamic diameter was 70.96 nm, and LDH size was no greater than 200 nm. The test LDH NPs were positively charged (zeta potential 32 mV). The TEM image showed that the LDH crystallites were well crystallized with a typical hexagonally-shaped morphology (Fig. 1B). The final concentration of LDH was 48.62 mg/ml.

Effective adsorption of LDHs for FMDV

The adsorption capacity of LDH NPs to FMDV 146 s was determined by the amount of free virions in the supernatant after the binding of LDHs and FMDV. After 1536 μg inactivated FMDV was added to 100 μl (4862 μg) of LDHs particles, a large number of viruses began to appear in the supernatant as observed by OD260/OD280 (Fig. 1C). The LDH NPs were able to absorb the maximum FMDV 768–1536 ug, that is, the maximum absorption concentration was 0.16–0.31 μg FMDV/μg LDH.

Cytotoxicity of LDH NPs

LDH toxicity was evaluated using BHK-21, MDBK, and SKC cells. Incubation with different concentrations of LDH with 38 h had no effect on any of the three cell types, and there was no statistically significant difference when compared with the PBS control group (Fig. 2). The results showed that LDH NPs can be used as an adjuvant in organisms.

Evaluation of LDHs + inactivated virus immunized BALB/C mice

The LDH was formulated with inactivated FMDV to determine its potential adjuvant efficacy. IL-4 and IFN-γ secretions were measured 14d post-immunization. The IFN-γ level was significantly higher different in three groups vaccinated with FMDV + LDHs, FMDV + ISA-206 and FMDV + saline compared to pre-immunization ($P < 0.01$). There was no difference in IL-4 level among the groups (Fig. 3A, B). Th1 and Th2 represent the two extremes of the adaptive immune response [35]. In general, IL-4 is secreted by activated Th2 cells, and IFN-γ is secreted by Th1 cells. The results showed that the adjuvant LDH and ISA-206 caused strong cellular immunity, but the level of humoral immunity was still weak in the early stage. The average antibody titer in different stages was measured and results showed a significantly higher difference in antibodies induced by LDHs + FMDV compared to the saline group from day 42 to 98 ($P < 0.01$) and a significantly higher compared to the ISA-206 group on day 56 post-immunization ($P < 0.05$) (Fig. 3C). The humoral immune effect reached the highest on day 56 and remained at a high level on days 70, 84, and 98 (Fig. 3C). FMDV VP1 antibody levels on day 70 post-immunization showed that the VP1 antibody titer of the LDH group was significantly higher compared to the control group immunized virus alone ($P < 0.05$). In addition, the positive rate in LDH group was 1/2, the ISA-206 group was 1/3, and the virus only group was negative compared with the positive serum (Fig. 3D). The antibody titer of the liquid phase blocking ELISA was basically the same as that of FMDV VP1. The results showed that LDH as an adjuvant not only induced cellular and humoral immunity, but also had sustained release.

Evaluation of LDHs + inactivated virus immunized pigs

To better evaluate the potential efficacy of LDH as an adjuvant, 20 pigs were immunized with LDH NPs + inactivated FMDV O/MYA/BY/2010 or ISA-206 + inactivated FMDV O/MYA/BY/2010. On day 7 post-immunization, antibody levels in both the ISA-206 adjuvant group and the LDH adjuvant group were positive and were significantly higher ($P < 0.005$) compared to the PBS group, in which antibody level was negative (Fig. 4A). There was no significant difference between the LDH group and the ISA-206 group. In the LDH group, antibody levels of 9 heads (10 in total) were positive on day 7. The number of positive heads decreased on day 28, but returned to 9 on day 56 (Fig. 4B), which may indicate that LDH was continuously releasing antigen. In conclusion, the FMDV antibody level detected by ELISA was not very high, which may have been due to the low immune dose.

Results of the virus micro-neutralization test showed that antibody levels of pigs immunized with LDH + FMDV and ISA-206 + FMDV ranged from 1:4 to 1:32, and were significantly higher from day 7 to day 28 compared to the PBS control group ($P < 0.01$) (Fig. 4C). The pig numbers of the LDH group with positive titers was higher than the ISA-206 adjuvant group on day 28 post-immunization. These results demonstrate that the efficacy of LDH NPs for inducing specific antibody responses against FMDV approximated that of commercial ISA-206 adjuvant in pigs.

Discussion

LDHs have a very strong load capacity [36] and can carry proteins, DNA and other substances. The positive charge property can combine with the negative charge group of the cell membrane to enter the cell smoothly [37]. Compared with aluminum salt adjuvant, nano-adjuvant has a better effect on spleen cell activation and cytokine secretion [38]. When the nano-adjuvant is combined with virus, the carrier effect of the conventional adjuvant can be avoided and antigens can be protected. LDHs have the dual effects of immunostimulant and antigen carrier. This study showed that LDH had a loose hexagonally-shaped structure and a positive charge, and could effectively carry FMDV virus.

The current study also demonstrated that sustained antigen release can be accomplished using an LDH nano-adjuvant, which promotes antigen presentation and produces a long-lasting and efficient memory immune response, thus reducing the number of vaccinations and the amount of antigen [31–34]. In this study, antibody levels in mice immunized with LDH + FMDV were significantly higher from day 42 to day 98 compared to the saline group ($P < 0.01$) and were significantly higher compared with the ISA-206 group on day 56 ($P < 0.05$). The humoral immune effect remained at a high level on day 98, indicating that LDH had a slow-release effect and continuously produced antibodies. FMDV combined with LDH enhanced the immunogenicity. IFN-γ and IL-4 are important immunomodulators that have multiple biological functions. IFN-γ is a Th1 cytokine produced by activated T cells and NK cells, which has a variety of biological activities. IFN-γ is anti-viral, anti-parasitic and inhibits cell proliferation, which can induce at Th1 type immune response [39]. IL-4 is a cytokine produced by activated Th2 cells, which can enhance the interaction between B cells and T cells, promote the humoral immune response, and induce mononuclear-macrophages to express MHC-II molecules [40]. Th1 cells induce cell-mediated immunity whereas Th2 cells induce strong antibody responses [36].After day14 post-immunizing mouse, IFN-γ content increased significantly, indicating that LDH induced cellular immune response at an early stage. In addition, in pigs, average antibody levels in both the ISA-206 and LDH group were positive and were significantly higher ($P < 0.005$) compared with the PBS group on day 7. Furthermore, the number of pigs with positive antibody titers returned to 9 on day 56, indicating that LDH has sustained release behavior. The neutralizing antibody levels of LDH and ISA-206 groups were induced significant higher from day 7 to day 28 compared to the PBS control group ($P < 0.01$). Both the ELISA and cell suspension measurements showed that LDH NPs had effective induced specific antibody responses against FMDV in mice and pigs, which may be also applied to cattle and sheep.

The preparation process of LDH is relatively simple, and provides a basis for large-scale industrial production. Due to the low production cost of LDH, the use of it as an adjuvant will greatly reduce the breeding cost to farmers. As an inorganic substance, with stable dispersion properties, LDHs can be stored for a long time. Because of the production and preparation through high temperature and high pressure, the product is sterile, which is a necessary condition for the production of vaccines.

Conclusion

In this study, LDH NPs were synthesized using hydrothermal treatment. The LDHs had an average particle size of 70.96 nm, a loose hexagonal shape and a positive charge. The maximum absorption concentration was 0.16–0.31 μg FMDV/μg LDH. In order to verify the effectiveness of LDH as an adjuvant to FMDV vaccine, mice and pigs were immunize with LDH + FMDV and compared to mice and pigs immunized with commercial FMDV adjuvant ISA-206. LDH had a slow-release effect, produced antibodies continuously and effectively induced humoral- and cell-mediated immune responses in both mice and pigs. This research demonstrates the potential of LDH NPs as an effective nano-adjuvant for FMDV.

Methods

Materials

The FMDV AKT-III (Serotype A) and O/MYA/BY/2010(Serotype O) inactivated virus and ISA-206 adjuvant were provided by Tiankang Biotechnology (Urumqi, China). The FMD antibody ELISA test kits were purchased from the Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (Lanzhou, China). Mouse IFN-γ ELISA kit (SEKM-0031) and IL-4 detection kit (SEKM0005) were purchased from Solarbio Science & Technology (Beijing China). RecombiVirus FMDV VP1 (Serotypes O + A + A1) IgG ELISA Kit (Cat. # RV-400750-1, 96 tests) was purchased from Alpha Diagnostic International Inc (San Antonio, USA). The MTS Cell Proliferation Colorimetric Assay Kit (Catalog # K300-500) was purchased from BioVision (Milpitas, USA). Fetal bovine serum and DMEM medium were purchased from Gibco. $MgCl_2.6H_2O$, $AlCl_3.6H_2O$, and NaOH were purchased from Sigma.

Cells and animals

Hamster kidney cells (BHK-21 cells) and bovine kidney cells (MDBK cells) were purchased from Cell Resource Center, IBMS, CAMS / PUMC (Beijing, China). Sheep kidney cells (SKCs) and mouse macrophages were provided by Xinjiang Key Laboratory of Local and Ethnic Diseases, Shihezi University(Shihezi, China). The 2-months-old BALB/C female mice weighed ~ 20 g. The 3-month-old Yorkshire pigs weighed ~ 20 kg. The mice and pigs were healthy and had not been used for the other experiments. The mice and pigs were provided by Huaxing Laboratory Animal Farm (Zhengzhou, China).

Preparation and characterization of LDH nanoparticle suspension

Mg_2Al-Cl-LDH NPs were prepared by rapid precipitation and subsequent hydrothermal treatment [35, 41]. After mixing 0.7 M $MgCl_2$ solution with 0.3 M $AlCl_3$ solution uniformly, 0.45 M NaOH solution was quickly added, stirred for 10 min, and put in the reaction kettle at 100 °C for 16 h. The chemical formula of LDH is $Mg_{1.9}Al\ (OH)_{5.8}\ (1/2CO^{2-}_3,Cl)_{1.0}\cdot 5H_2O$. The particle size and potential of the LDHs samples were analyzed using the Malvern particle size analyzer. Scanning electron microscope (SEM) was used to observe the morphology of the nanometer adjuvant, the acceleration voltage was 80 kV, and the magnification rate was 30,000×.

FMDV adsorption by LDH NPs

To quantify the adsorption of FMDV on LDH NPs, 100 μl LDH adjuvant (48.62 mg/ml) was added to each EP tube, and FMDV 146 s (6 mg/ml) was added by volume 0 μl, 8 μl, 16 μl, 32 μl, 64 μl, 128 μl, 256 μl, 512 μl, 1024 μl, and 1400 μl. Deionized water was then added to a volume of 1500 μl per tub. After shaking the mixture for 10 min, the nanoparticle/virus complexes were recovered by centrifugation at 5000 rpm for 20 min and the amount of unbound FMDV in the supernatant was estimated via nanodrop at 280 nm (A280) and 260 nm (A260).

Cytotoxicity test

BHK-21, MDBK, and SKC were resuscitated with warm water at 37 °C, and cell culture fluid (90% DMEM + 10% FBS) was added. A total of 6×10^3 cells were transferred into 96-well plates and incubated for 3 h. Triplicate wells were treated with LDH at final concentrations of 5 μg/ml, 10 μg/ml, 20 μg/ml, and 40 μg/ml. The plates were incubated at 37 °C in 5% CO_2 for 38 h, and 20 μl/well MTS reagent was then added and wells were incubated for 3 h at 37 °C. The absorbance was measured at 492 nm after oscillation.

LDH and inactivated virus immunized BALB/C mice

All mouse experiments were performed according to the guidelines of the Animal Ethics Committee of the Shihezi University. The BALB/C mice were SPF grade. The mice were kept in a cage and sterilized wood dust as the bedding material. The mice were allowed free access to clean water and food. The ambient temperature was ~ 27 °C. After the experiment, the mice were euthanized by intraperitoneal injection of excessive sodium pentobarbital (200 mg/kg weight). The mice were randomly divided into three groups, as follows: 125 μl inactivated FMDV AKT-III + 125 μl saline(control group, n = 6), 125 μl inactivated FMDV AKT-III + 125 μl ISA-206 adjuvant (n = 6), 125 μl inactivated FMDV AKT-III + 125 μl LDH adjuvant (n = 6). The final virus concentration was 7.08 μg/ml all three groups. Before injection, inactivated FMDV was mixed 1:1 with commercial ISA-206 adjuvant and stirred at 600 rpm for 10 min. The other two groups were mixed uniformly and injected. Six female mice in each group were injected subcutaneously. Blood was collected from submandibular vein under abdominal anesthesia with sodium pentobarbital (40 mg/kg). Blood samples were collected on days 0, 14, 28, 42, 56, 70, 84, and 98 and the serum collected. Antibodies were detected using the Lanzhou Veterinary Research Institute liquid phase blocking kit according to the instructions. FMDV VP1 antibody levels were measured on day 70 post-immunization using the RecombiVirus FMDV VP1 (Serotypes O + A + A1) IgG ELISA kit. Secretion levels of IL-4 and IFN-γ were detected using the Solarbio mouse IL-4/IFN-γ assay kit according to the manufacturer's protocol.

LDHs and inactivated virus immunized pigs

All pig experiments were performed according to the guidelines of the Animal Ethics Committee of the Shihezi University. The pigs were CL grade and were farmed on the ground with sufficient light. The pigs were allowed free access to clean water and limit complete diet pellets. All treatments were aseptic and clean feeding. The breeding temperature was ~ 27 °C. After the experiment, the pigs were euthanized by intraperitoneal injection of excessive sodium pentobarbital (200 mg/kg weight). All pigs were randomly divided into groups. Five pigs were raised together without bedding. The PBS control group (n = 5) was injected with 1 ml PBS/pigs. The ISA-206 group (n = 10) was injected with 0.5 ml inactivated virus O/MYA/BY/2010 + 0.5 ml ISA-206 adjuvant /pigs. The LDH group (n = 10) was injected with 0.5 ml inactivated virus O/MYA/BY/2010 + 0.5 ml LDH NPs /pigs. The final O/MYA/BY/2010 virus concentration in immunized groups was 6 μg/ml. All injections were administered intramuscularly. Blood was collected from anterior vena cava in pigs under abdominal anesthesia with sodium pentobarbital (40 mg/kg). Day 0 prior to immunization and subsequently at day 14, 21 and 28 for serum collection. The FMD antibody level was detected using the Lanzhou Veterinary Institute FMDV type O liquid phase blocking diagnostic kit. The virus micro-neutralization test (VNT) was performed by Tiankang Biotechnology Co., Ltd. in P3 laboratory.

Virus micro-neutralization test (VNT) procedure

The valence of serum neutralizing antibody was determined via the cell suspension method.

1. The virus valence of O/MYA/BY/2010 was calibrated. Maintenance fluid (50 μl/well) was added to the 96-well plate. The virus was diluted 10 times in series, and diluted virus was added 50 μl /well from high to low with 8 wells at each dilution. Cell suspension (50 μl) was added and incubated at 37°C in 5% CO_2 for 48 h. Cytopathic changes were observed. The results were calculated using the Reed-Muench method.
2. Convalescence pig serum was used as the standard positive serum, and antibody valence was calibrated. Pig serum without FMD antibodies was used as the negative serum.
3. The serum from each stage was inactivated by placing in a 56°C water bath for 30min.
4. Valence of neutralizing antibody was tested in immunized pig serum. Maintenance fluid of 50 μl/well was added to the 96-well plate. The inactivated serum was diluted two times in series and 50 μl/well was added to each dilution. According to the virus valence, 100 $TCID_{50}$ viruses

were added at 50 ul/well. After incubation at 37°C in 5% CO_2 for 1 h, The well-growing BHK21 cell suspension was added and further cultured in the incubator. The cytopathic conditions were observed under a microscope after 48 h. The valence of serum antibody was calculated according to the lesions.

Data Statistics and Analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 8 software, and significant differences were analyzed by Mann-Whitney U test (* P <0.05, ** P <0.01, ***P <0.005).

List Of Abbreviations

LDHs: Layered double metal hydroxides；FMDV: Foot-and-mouth disease virus; NPs: Nanoparticles; BHK-21: Hamster kidney cells; MDBK: bovine kidney cells; SKC: sheep kidney cells; VNT: virus micro-neutralization test; OD: optical density; TLR: Toll-like receptor; GM-CSF: granulocyte-macrophage colony-stimulating factor; CTL: cytotoxic T-lymphocyte.

Declarations

Ethical approval and consent to participate

All animal experiments were approved by the Animal Welfare Institute of Shihezi University, and all operations were performed in accordance with animal welfare requirements (the written document, reference number A2018-163-01).

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CC designed the study, led collection of samples. PW designed the study and drafting of the manuscript. YZ performed data analysis and wrote the manuscript. XY contributed to cell culture and animal test. YH and QZ performed data regarding the mice and pigs serum samples. All authors approved the final manuscript.

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Figures

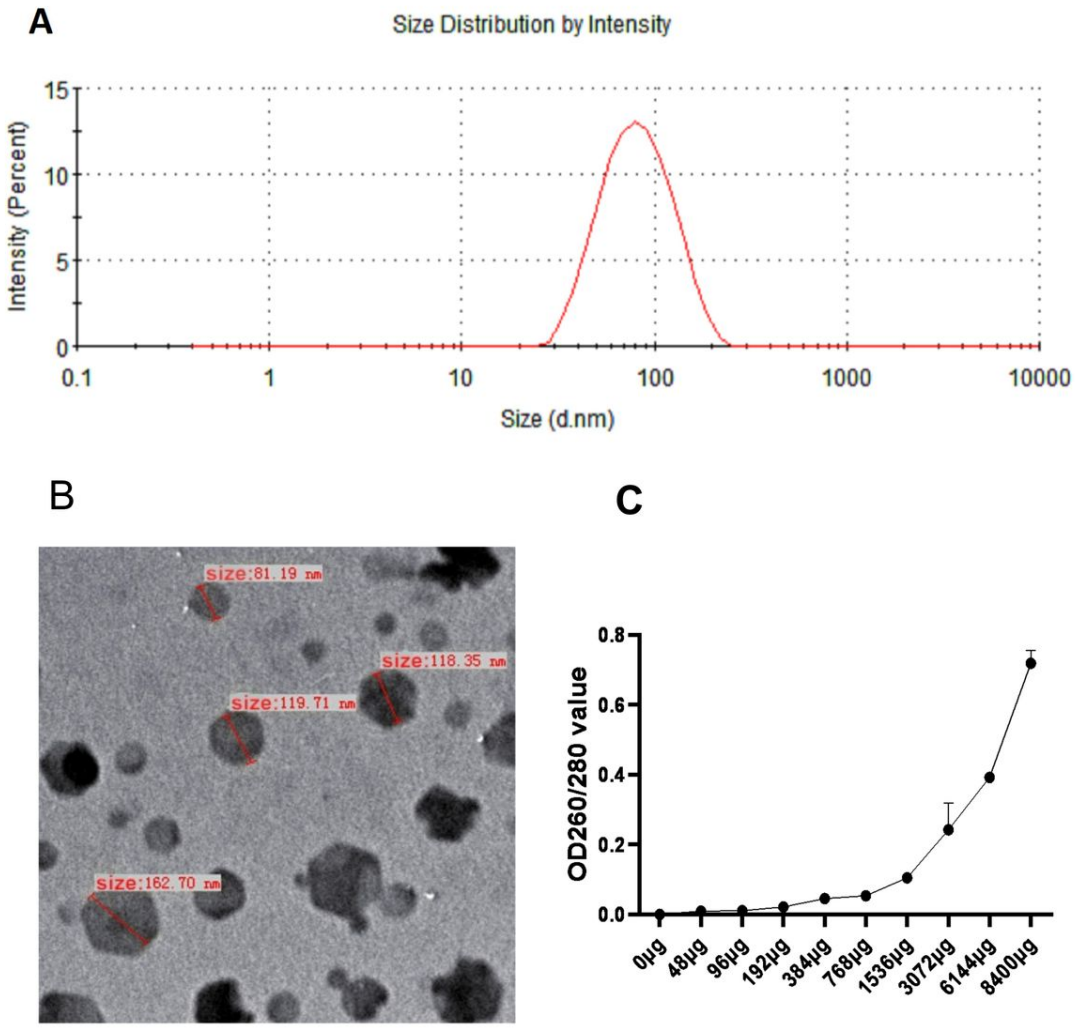

Figure 1

Electron micrograph of LDHs and adsorption capacity of LDHs. (A) Distribution of particle size (B) Electron micrograph of LDHs (C) FMDV absorption of LDHs.

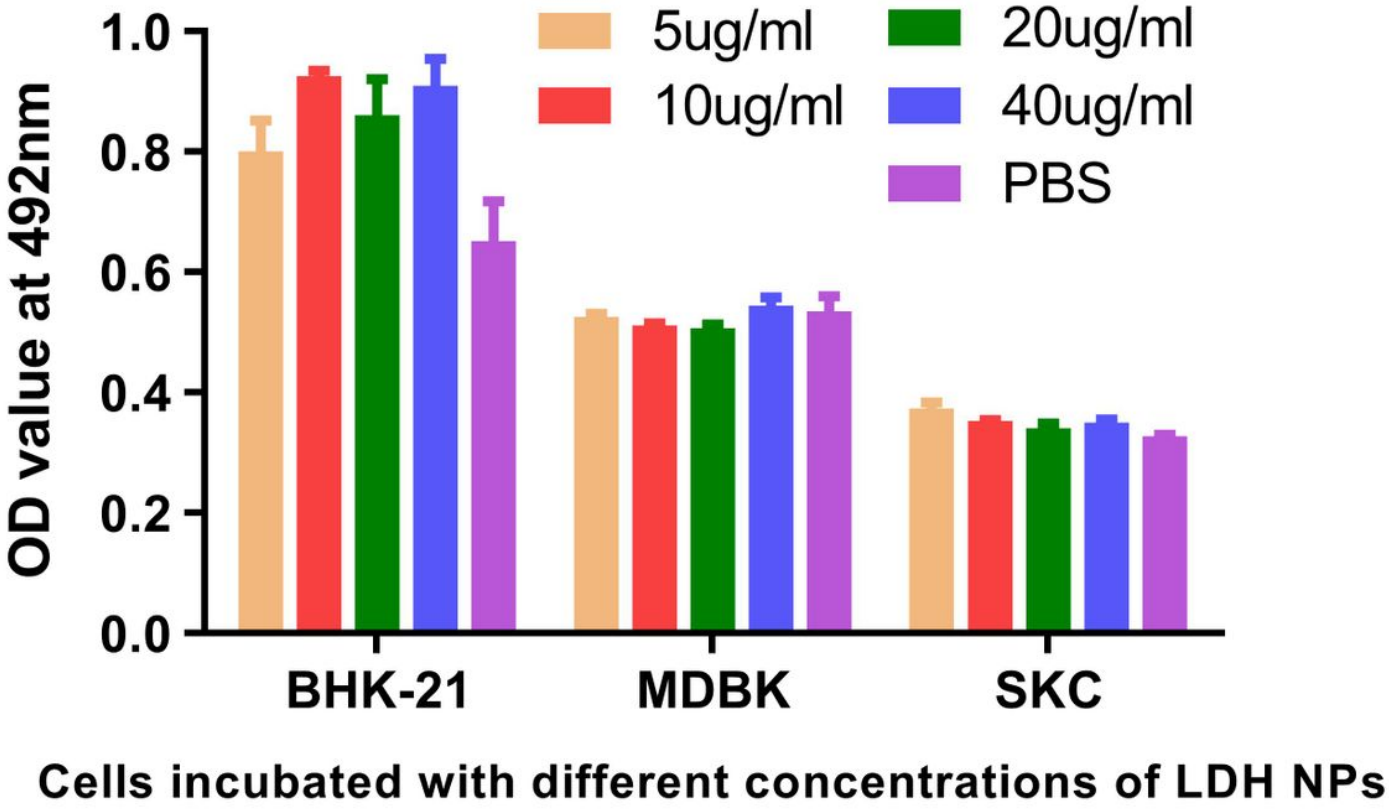

Figure 2

LDH NPs cytotoxicity in BHK-21, MDBK and SKC cells. Data are represented as the mean ± SD.

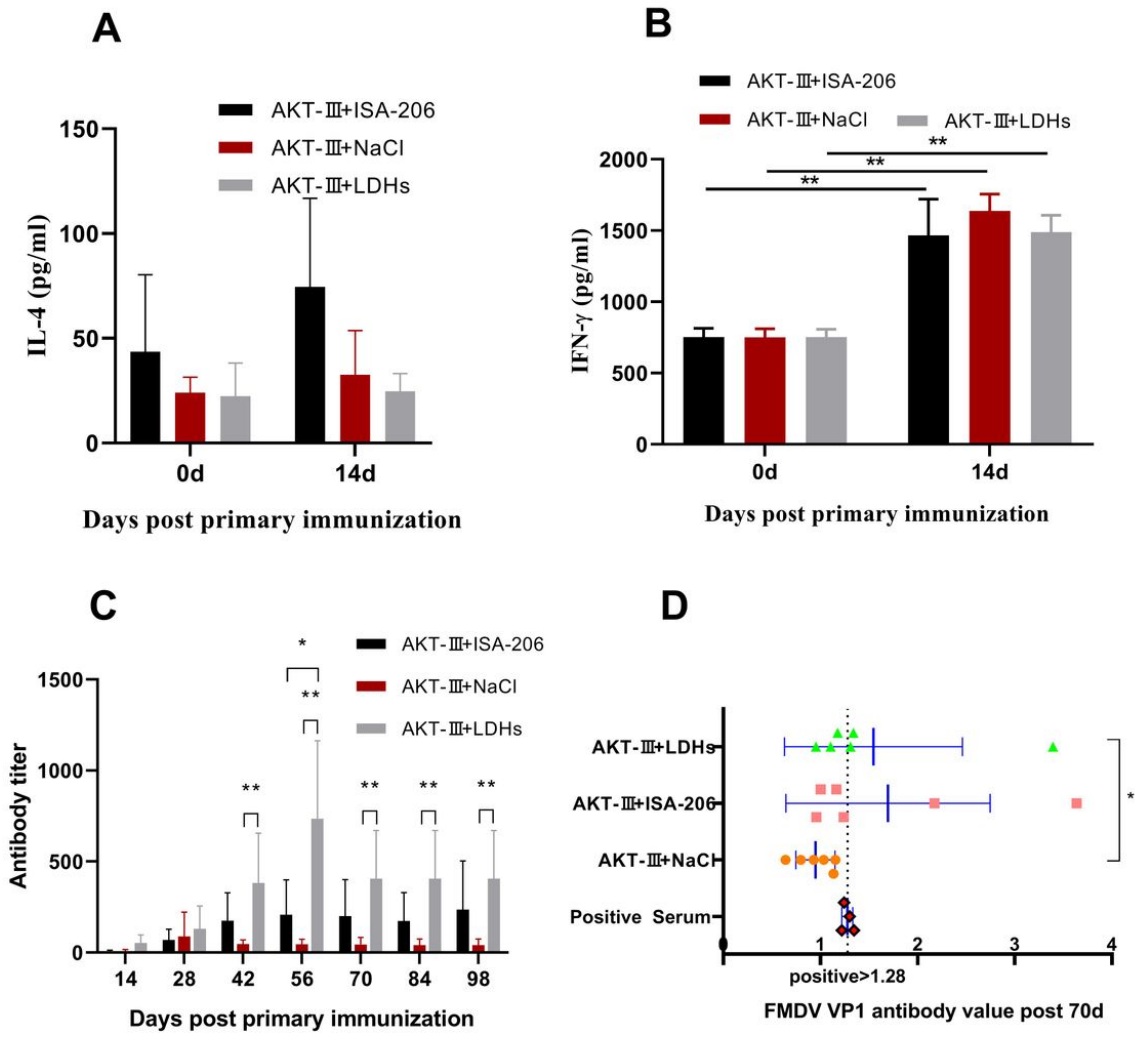

Figure 3

Results from immunized mice. (A)IL-4 levels of immunized mice. (B) IFN-γ levels of immunized mice. (C) FMDV antibody levels of mice immunized at different times. (D)FMDV-VP1 antibody levels of immunized mice on the day 70. Data are expressed as mean ± SD (n = 6). * p <0.05, ** p <0.01.

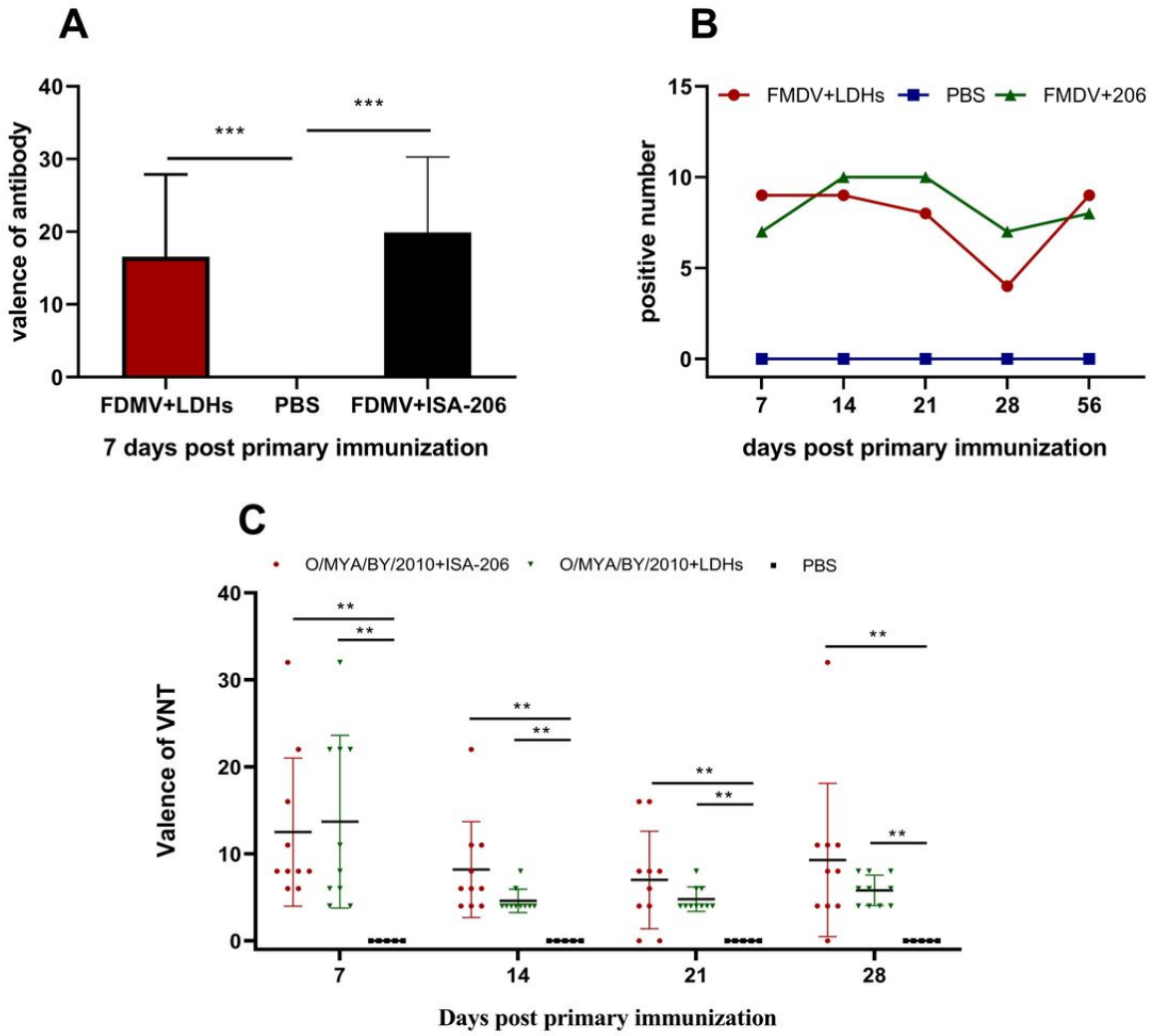

Figure 4

Results of immunized pigs. (A)Antibody level of immunized pig on the day 7. (B) Positive number of pigs at different times as showed by ELASE results. (C) Valence of VNT at different times. Data are expressed as mean ± SD (n = 10). * p <0.05, ** p <0.01, *** p <0.005.

Supplementary Files

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