Ginseng stem–leaf saponins (GSLS) and mineral oil act synergistically to enhance the immune responses to vaccination against foot-and-mouth disease in mice

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A B S T R A C T

Saponins extracted from ginseng stems and leaves (GSLS) as well as the synergistic effect between GSLS and oil emulsion were investigated for their adjuvant effects on the immune responses of mice to vaccination against foot-and-mouth disease virus (FMDV) serotype Asia 1. In experiment A, ICR mice were subcutaneously immunized twice with FMDV antigen with or without GSLS (0, 1, 5, 10 and 20 μg) at 3 week intervals. Highest FMDV-specific IgG level was observed 2 weeks after the boosting in mice immunized with FMDV antigen plus 10 μg of GSLS. In experiment B, mice were subcutaneously injected with FMDV antigen with or without GSLS (10 μg), or in oil emulsion with or without GSLS (10 μg) on days 1 and 21. Results indicated that when co-administered with a mixture of oil and GSLS, FMDV antigen induced significantly higher IgG titer and IgG1, IgG2a, IgG2b and IgG3 responses, production of IFN-γ (Th1 cytokine) and IL-5 (Th2 cytokine) by splenocytes, as well as T and B lymphocyte proliferation in response to Con A and LPS than when FMDV antigen was used alone or mixed with either GSLS or oil. This suggests that GSLS and oil adjuvant synergistically promote both Th1 and Th2 immune responses. As protection against FMDV requires both cellular and humoral immune responses, the combined effects of GSLS and oil deserve further study in other animals such as cattle and pigs in order to induce effective immunity against FMDV infection.

1. Introduction

Foot-and-mouth disease (FMD) affects the cloven-hoofed animals such as cattle, swine and sheep. The disease is caused by FMDV, which is the prototype member of the *Aphthovirus* genus, *Picornaviridae* [1] and occurs as seven major serotypes: A, O, C, Asia 1, SAT 1, SAT 2, and SAT 3, but a large number of subtypes involved within each serotype [2,3]. Financial losses due to FMD can be huge, and it is believed to be the most economically cost animal disease in the world. Direct losses result from loss of milk and meat. However, in young animals, infection of the heart muscles may result in severe myocardial necrosis and death [4]. Most FMD vaccines contain oil emulsions as an adjuvant to improve their efficacy [5,6] and have been shown to induce neutralizing antibodies, protect animals from FMDV and play a key role in control campaigns and eradication of FMD [7]. However, some currently available FMD vaccines for pigs in some districts in China have been reported to induce poor immune responses in swine [8,9]. For example, Xie et al. [8]

observed that only 20.9% of piglets produced immune responses with antibody titers high surficient for protection following vaccination against FMD in the Ningxia province of China. Hao et al. [9] analyzed 91 serum samples of pigs having received vaccination against FMD and found that only 31.9% of the samples had antibody titers required for immune protection immunity. Therefore, there is a need to find a new approach to the improvement of FMD vaccines.

Our previous investigation has found that supplement of saponin adjuvants such as Quil A and extract of *Cochinchina momordica* seeds (ECMS) in a commercial FMD vaccine can significantly enhance immune responses [10,11]. Such enhancement may result from the synergistic effect of saponin and oil. Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a traditional medicine in oriental countries for thousands of years [12,13]. Ginseng saponins, ginsenosides, are believed to be the main pharmacologically active constituents in *P. ginseng* [14]. Ginsenosides have a broad range of biological activities, including anti-inflammatory activity [15], antioxidant, anti-tumor effects [16,17], as well as adjuvant property with low haemolytic activity [18–24]. In addition to the roots, the stems and leaves of *P. ginseng* have also been found containing saponins (GSLS) with the pharmacological activity similar to the root [14]. In the past, these parts of ginseng plants were discarded as waste after harvesting

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the roots. Compared to the saponins isolated from the root, the cost of saponins made from the stem and leaf is much low. The present study was designed to investigate GSLS for its adjuvant and the synergistic effects with oil emulsion on the immune responses induced with FMD vaccine in mice by measuring FMDV-specific IgG and the subclasses as well as proliferation of and IL-5 and IFN-γ production by mouse splenocytes.

2. Materials and methods

2.1. Animals

Female ICR mice were purchased from Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China), and housed in polypropylene cages with sawdust bedding in hygienically controlled environment. Feed and water were supplied *ad libitum*. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

2.2. Antigen, adjuvants and vaccine

Inactivated FMDV type Asia 1 antigen was kindly provided by Lanzhou Veterinary Research Institute (LVRI), China. Standardized ginseng saponins (GSLS) from the stems and leaves of *Panax ginseng* C.A. Meyer was supplied by Hongjiu Ginseng Industry Co. Ltd. (Jilin, China) and contained Rb1 (2.63%), Rb2 (4.73%), Rc (3.67%), Rd (12.05%), Re (19.02%) and Rg1 (7.25%) as determined by high performance liquid chromatography (HPLC) analysis. Mineral oil #10 was purchased from Hangzhou Refinery China Petrochemical Co. (light white oil).

GSLS was dissolved in physiological saline solution (1 mg/ml) and sterilized by passing through a 0.22 μm filter before use. Mineral oil was emulsified in FMDV antigen with or without GSLS at a ratio of 1:1 (v/v) by pushing back and forth between two syringes connected with a plastic tube for 15 min as described elsewhere [10]. The endotoxin level in above solutions was less than 0.5 endotoxin unit (EU)/ml by a gel-clot Limulus amebocyte lysate assay (Bath no. 0708271, Zhanjiang A&C Biological Ltd., Zhanjiang, China).

2.3. Immunization and sample collection

In experiment A, 42 ICR mice were randomly divided into 7 groups with 6 mice in each. The animals were subcutaneously (s.c.) immunized twice with 100 μl of FMDV antigen plus 100 μl of physiological saline solution containing GSLS (0, 1, 5, 10 and 20 μg) at 3 week intervals. Blood samples were collected 2 weeks after the boost for detection of FMDV-specific IgG. In experiment B, 50 mice were randomly distributed in 5 groups of 10 individuals each. The animals were injected s.c. with 100 μl of FMDV antigen plus 100 μl of physiological saline solution with or without GSLS (10 μg), or plus 100 μl of oil emulsion with or without GSLS (10 μg) on days 1 and 21. The control mice were injected with physiological saline solution (200 μl) in the same manner. Two weeks after the boost, blood samples were collected for measurement of serum FMDV-specific IgG titers and the isotypes. Splenocytes were collected for determination of lymphocyte proliferation and production of IFN-γ and IL-5.

2.4. FMDV type Asia 1 specific IgG and the IgG subclasses

Serum samples were analyzed for measurement of serum IgG and the isotypes by an indirect double antibody sandwich enzyme-linked immunosorbent assay. The wells of polyvinyl 96-well microtiter plates were coated with 50 μl rabbit anti-FMDV serotype Asia 1 antibody (LVRI, China) diluted in 0.05 M carbonate/bicarbonate buffer (1:800), pH 9.6 and incubated overnight at 4 °C. After five washes with phosphate buffer saline containing 0.05% Tween-20 (PBST), the wells were blocked with 5% skimmed milk and incubated at 37 °C for 2 h. Thereafter, 50 μl FMDV type Asia 1 antigen (LVRI) (1:3 dilution) was added and incubated at 4 °C for 2 h. After five washes, 50 μl of serum (diluted serially for IgG analysis or diluted 1:50 in PBS 5% skimmed milk for isotype analysis) was added to each well and incubated at 37 °C for 1 h. Plates were then washed five times in PBST. For IgG titer detection, 50 μl of goat anti-mouse IgG (1:500) (Kirkegaard, Perry Lab., Maryland, USA) was added to the wells and incubated at 37 °C for 1 h. Plates were washed again with PBST. Fifty microliters of 3,3′,5,5′-tetramethyl benzidine solution (100 μg/ml of 0.1 M citrate-phosphate, pH 5.0) was added to each well and incubated for 15 min at 37 °C. The reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. The optical density of the plate was read by an automatic ELISA plate reader at 450 nm. Values above the cut-off background level (mean value of sera from unimmunized mice multiplied by a factor of 2.1) were considered positive. Titers were depicted as reciprocal end-dilutions. For subclasses, 50 μl of biotin conjugated goat anti-mouse IgG1 or IgG2a or IgG2b or IgG3 (1:600) (Santa Cruz Biotechnology Inc., CA, USA) was added to corresponding plate and then incubated for 1 h at 37 °C. After washing, 50 μl of horseradish peroxidase conjugated anti-biotin (BD Biosciences, Pharmingen, USA) diluted 1:4000 in PBST was added to each well and incubated for 1 h at 37 °C. Incubations, washing and development were as described above for detection of FMDV-specific IgG. The optical density of the plate was read at 450 nm.

2.5. Splenocyte proliferation assay

Spleen collected from the FMDV-immunized ICR mice under aseptic conditions, in Hank's balanced salt solution (HBSS, Sigma), was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. After centrifugation (380 × g at 4 °C for 10 min), the pelleted cells were washed three times in HBSS and resuspended in complete medium (RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated FCS). Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as described previously [24]. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen cultures divided by the absorbance value for non-stimulated cultures.

2.6. IFN-γ and IL-5 production by splenocytes in vitro

Single cell suspensions were adjusted to a concentration of 2.5×10^6 cells/ml in complete medium. To a 96-well flat-bottom microtiter plate (Nunc), 100 μl of the cell suspension and equal volume of Con A solution (final concentration 5 μg/ml) were added. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. After that, the culture supernatants were collected for cytokine assay. The concentrations of IFN-γ and IL-5 were determined by a commercial capture ELISA kit (R & D Systems Inc., Minneapolis, USA). Concentrations of cytokines were calculated from interpolation of the cytokine standard curve.

2.7. Statistical analysis

Data analysis was performed with SPSS software (SPSS, Version 11.5, SPSS Inc., Chicago, IL, USA). ANOVA with Fisher's LSD post hoc
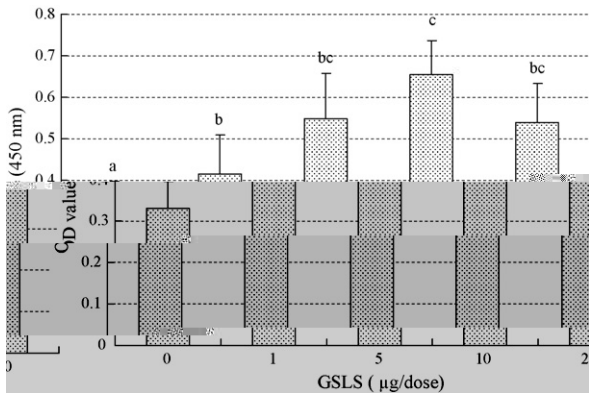
Fig. 1. FMDV-specific IgG responses. Mice were immunized s.c. with 100 µl of FMDV antigen plus physiological saline solution containing GSLS (0,1, 5, 10 and 20 µg) on days 1 and 21. Serum was collected 2 weeks after the boosting. Serum (1:50 dilution) FMDV-specific IgG was measured by an indirect ELISA. The values are presented as mean ± S.D. (n = 6). Bars with different letters are P < 0.05.

test was used for multiple comparisons between groups. Values were expressed as the mean ± standard deviation (S.D.). P-values of less than 0.05 were considered statistically significant.

3. Results

3.1. FMDV-specific IgG and isotypes

IgG levels were significantly higher in mice immunized with FMDV antigen plus GSLS than in mice immunized with FMDV antigen alone (P < 0.05) (Fig. 1). Highest IgG response was found in mice immunized with FMDV antigen plus 10 µg of GSLS. Thus, 10 µg of GSLS per dose was chosen for further investigation.

Fig. 2. indicated that IgG response was significantly higher in mice immunized with FMDV antigens in oil emulsion combined with GSLS (10 µg) than in mice immunized with FMDV antigen alone or adjuvanted with oil or GSLS (P < 0.05). The IgG titer in group of GSLS + oil (1:1714) almost doubled the IgG titer in group of oil (1:918). The ratio of the two titers was 1.88. Fig. 3 showed that IgG1, IgG2a, IgG2b and IgG3 responses were significantly higher in group of GSLS + oil than in other groups (P < 0.05).

3.2. Proliferation of splenocytes isolated from FMDV-immunized mice

The effects of oil and GSLS on splencyte proliferative responses to Con A and LPS stimulation are shown in Fig. 4. Supplement of
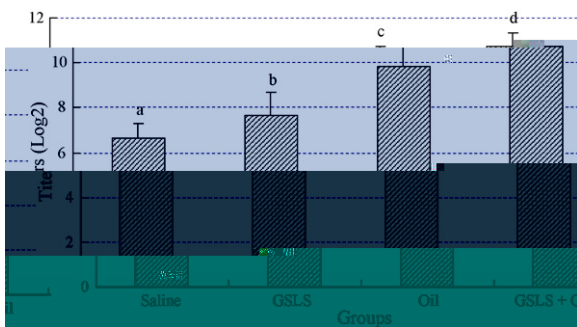

Fig. 2. FMDV-specific IgG titers. Mice (n = 10) were immunized s.c. with 100 µl of FMDV antigen plus physiological saline solution with or without GSLS (10 µg) or plus 100 µl of oil emulsion with or without GSLS (10 µg) on days 1 and 21. Serum was collected 2 weeks after the boosting. Serum FMDV-specific IgG was measured by an indirect ELISA. Values above the cut-off background level (the mean from unimmunized mice as negative control multiplied by a factor of 2.1) were considered positive. Titers were depicted as reciprocal end-dilutions. Bars with different letters are P < 0.05.
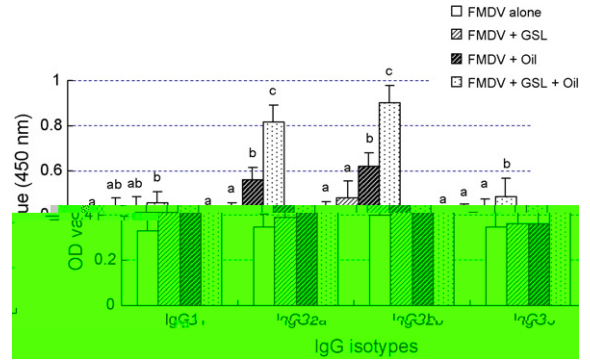

Fig. 3. FMDV-specific IgG isotypes. Mice were immunized s.c. with 100 µl of FMDV antigen plus 100 µl of physiological saline solution with or without GSLS (10 µg) or plus 100 µl of oil emulsion with or without GSLS (10 µg) on days 1 and 21. Serum was collected 2 weeks after the boosting. Serum (1:50 dilution) FMDV-specific isotypes were measured by an indirect ELISA. The values are presented as mean ± S.D. (n = 10). Bars with different letters are P < 0.05.
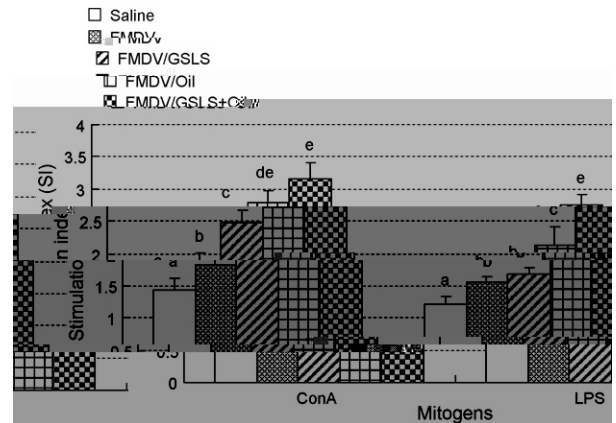

Fig. 4. Splenocyte proliferative responses to Con A and LPS. Mice were immunized s.c. with 100 µl of FMDV antigen plus 100 µl of physiological saline solution with or without GSLS (10 µg) or plus 100 µl of oil emulsion with or without GSLS (10 µg) on days 1 and 21. The control mice were injected with physiological saline solution (200 µl) in the same manner. Splenocytes were prepared 2 weeks after the last immunization and cultured with Con A (5 µg/ml) or LPS (5 µg/ml) or RPMI 1640. Splenocyte proliferation was measured by the MTT method as described in the text, and shown as a stimulation index. The values are represented mean ± S.D. (n = 10). Bars with different letters are P < 0.05.

GSLS in experimental FMDV vaccine significantly enhanced the proliferative response to Con A when compared to the group of FMDV alone (P < 0.05). The proliferative responses to Con A and LPS stimulation were significantly higher in groups of oil emulsion or oil emulsion + GSLS than in other groups (P < 0.05) with highest stimulation index found in group of oil emulsion + GSLS.

3.3. IFN-γ and IL-5 release by splenocytes isolated from FMDV-immunized mice

Cytokine secretion in the supernatants are shown in Fig. 5. IFN-γ and IL-5 production was significantly higher in the cultures of cells isolated from the mice immunized with FMDV antigen in oil emulsion with or without GSLS than the control group (P < 0.05). Highest IFN-γ and IL-5 production was found in the group of oil + GSLS.

4. Discussion

Adjuvant properties, particularly of the combined oil and GSLS, but also of GSLS have been demonstrated in mice. When
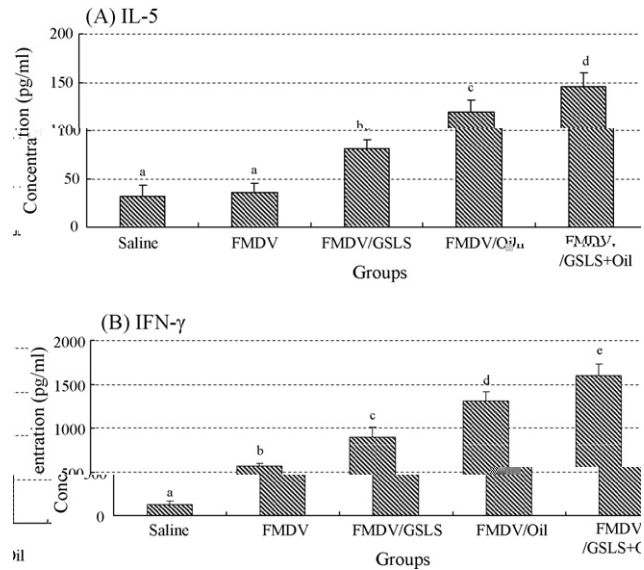
Fig. 5. IL-5 (A) and IFN-γ (B) in the supernatants of splenocytes stimulated with Con A (5 μg/ml). Mice were immunized s.c. with 100 μl of FMDV antigen plus 100 μl of physiological saline solution with or without GSLS (10 μg) or plus 100 μl of oil emulsion with or without GSLS (10 μg) on days 1 and 21. The control mice were injected with physiological saline solution (200 μl) in the same manner. Splenocytes were prepared 2 weeks after the last immunization and cultured with Con A (5 μg/ml). The culture supernatants were harvested after 48 h incubation of the splenocytes with Con A. The values are presented as mean ± S.D. ($n = 10$). Bars with different letters are $P < 0.05$.

co-administered with GSLS, FMDV antigen induced significantly higher IgG response than when FMDV antigen was used alone. Especially, when co-administered with a mixture of oil and GSLS, FMDV antigen induced significantly higher IgG and the subclass responses, production of IFN-γ and IL-5 by splenocytes, as well as splenocyte proliferation in response to Con A and LPS than when FMDV antigen was used alone or mixed with either GSLS or oil.

Adjuvant effects of ginseng saponins (GS) have been reported in other studies. Rivera et al. [19] observed an increased antibody response with a bias towards production of IgG2 isotype to vaccination against porcine parvovirus (PPV) in guinea pigs when PPV antigen was administered with a GS. Hu et al. [21] reported an increased antibody response induced by *Staphylococcus aureus* bacterin in dairy cattle when the bacterin was mixed with a ginseng extract or ginsenoside Rb1. As GS used in previous studies was isolated from the root of *P. ginseng*, which grows very slowly, and usually takes 5–7 years to produce a quality root, GS is quite expensive for use in domestic animals. In comparison with the root, the stem and leaf of the plant are cheap as they are usually discarded as a waste after harvesting the root by the farmers. Adjuvant properties of GSLS similar to GS have been discovered in present study. HPLC analysis showed that GSLS used in this study contained Rb1 (2.63%), Rb2 (4.73%), Rc (3.67%), Rd (12.05%), Re (19.02%) and Rg1 (7.25%). We have recently compared protopanaxadiols (Rg3, Rd, Rc, Rb1 and Rb2) and protopanaxatriols (Rg1, Re and Rg2) from *P. ginseng* root [24], and found that Rg1, Re, Rg2, Rg3 and Rb1 have potent adjuvant properties. The fractions Rb1, Re and Rg1 contained in GSLS may contribute to the adjuvant activities of GSLS as presented in this study.

Compared to GSLS alone, GSLS in combination with oil produced stronger adjuvant activity. Adjuvant combination has been found earlier. The best-known adjuvant combination is Freund's complete adjuvant [25], which combines the immunomodulatory properties of *Mycobacterium tuberculosis* with the depot effect of water-in-oil emulsion. This adjuvant generates very strong Th1 and Th2 responses. Although the combination of saponin-like adjuvant with oil is not common, the synergism between them for immunopotentiation has been reported previously. Gerber [26] found higher immune responses in guinea pigs vaccinated against canine parvovirus, in pigs vaccinated against pseudorabies virus, and in cats vaccinated against feline infectious virus when Quil A together with oil was used as an adjuvant than when Quil A or oil was used alone. Martínez-Fernández et al. [27] reported an increased immune response induced by Fh12 FABP of *Fasciola hepatica* in oil emulsion containing Quil A in sheep. Xiao et al. [10,11] reported significantly enhanced immune responses in pigs by supplement of saponin adjuvants such as Quil A and ECMS in a commercial FMD vaccine. Oil emulsion provides the depot effect with progressive release of the antigens at the site of injection. GSLS has immunomodulatory capacity. The synergism between GSLS and oil may result from the depot effect of oil emulsion in combination with the immunomodulatory effect of GSLS.

The lymphocyte proliferative response varied depending on the mitogen used. In GSLS-adjuvanted group, significantly enhanced proliferation was induced by Con A but not LPS, indicating that T cells but not B cells were activated by GSLS. In GSLS/oil-adjuvanted group, both Con A- and LPS-induced proliferations were significantly enhanced, suggesting that T as well as B cells were activated by GSLS/oil. In order to induce antibody production, triggered B lymphocytes are required for clonal expansion. The enhanced lymphocyte responses to Con A or LPS stimulation paralleled the increased serum IgG responses detected in the mice injected with FMDV antigen plus GSLS or GSLS/oil.

There are different subclasses of IgG immunoglobulins such as IgG1, IgG2a, IgG2b, and IgG3 that provide the bulk of immunity to most infectious agents. During a T cell dependent immune response, there is a progressive change in the predominant immunoglobulin class of the specific antibodies. This change is influenced by T cells and their cytokines. In mice, IL-4 and IL-5 preferentially switch activated B cells to the IgG1 isotype (Th2 type immune response); IFN-γ enhances IgG2a and IgG3 responses (Th1 type); transforming growth factor-β (TGF-β) induces the switch to IgA or IgG2b [28]. Data in Fig. 3 indicated that oil alone promotes the production of IgG2a and IgG2b while the mixture of GSLS and oil promotes significantly higher production of all IgG subclasses tested. Enhanced production of all IgG subclasses may be explained by increased release of both IL-5 and IFN-γ from splenocytes as shown in Fig. 5. All these suggested that both Th1 and Th2 immune responses were activated.

In conclusion, GSLS as well as the combined GSLS and oil significantly enhanced immune response of mice to FMDV vaccination. Importantly, when co-administered with oil/GSLS, FMDV antigen induced significantly higher IgG and the subclass responses, production of IFN-γ and IL-5 by splenocytes, as well as splenocyte proliferation in response to Con A and LPS than when FMDV antigen was used alone or mixed with either GSLS or oil, suggesting GSLS and oil synergistically act as an adjuvant to augment Th1/Th2 responses. As both cellular and humoral immune responses are required for protection against FMDV, the combined effects of GSLS and oil deserve further study in other animals such as cattle and pigs in order to induce effective immunity against FMDV infection.

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