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RESEARCH ARTICLE

Mucosal Stimulation with D-Galacto-D-Mannan Enhances Systemic Immunity Elicited by the Foot-and-Mouth Disease Vaccine

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A B S T R A C T

Foot-and-mouth disease (FMD), caused by the FMD virus, remains a significant threat to livestock worldwide. While current vaccines primarily induce systemic immunity, they have limitations, including delayed antibody (Ab) responses and low titers. To address these shortcomings, we investigated the potential of D-galacto-D-mannan (DGDM) as an oral immunomodulator to enhance both mucosal and systemic immune responses following FMD vaccination. The safety of DGDM was evaluated in mice and pigs through food efficiency ratios and biochemical analyses, confirming its safety for *in vivo* use. In mice, DGDM administration improved survival rates and preserved body weight after viral challenge. In both mice and pigs, DGDM intake led to a marked increase in both serum Ab and virus-neutralizing (VN) Ab titers, alongside a notable enhancement in the production of secretory IgA (SIgA), a key marker of mucosal immunity. Furthermore, in pigs, DGDM administration also upregulated mucosal immune-related gene expression. These findings demonstrate that DGDM effectively stimulates mucosal immunity, enhancing the overall systemic immune response to FMD and providing a long-lasting immune response. This study underscores the critical role of mucosal immunity in optimizing the efficacy of FMD vaccines, offering a promising strategy for improving vaccine-induced systemic immunity and host protection.

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease that affects cloven-hoofed livestock such as cattle, sheep, pigs, and goats. Clinical signs of FMD include blistering, drooling, anorexia, and fever. FMD virus (FMDV), that causes FMD is a positive-sense RNA virus classified under the genus *Aphthovirus* within the family *Picornaviridae* (Azeem *et al.*, 2020). FMDV has four structural proteins (VP1, VP2, VP3, and VP4), and seven serotypes (O, A, C, Asia1, SAT1, SAT2, and SAT3) have been identified based on the VP1 protein sequence. All serotypes are further subdivided into subtypes, with no cross-protection occurring between the different serotypes (Belsham *et al.*, 2020; Aslam and Alkheraije, 2023). In many countries at risk of FMD outbreaks, susceptible livestock are vaccinated as a preventive measure. However, commercially available FMD vaccines used on farms have several shortcomings, including a short duration of immunity and the need for frequent vaccination (Robinson *et al.*, 2016).

We hypothesized that these shortcomings may be because commercial FMD vaccines induce only systemic immunity and not mucosal immunity. The mucosa is the first line of defense against foreign antigens. Mucosa-associated lymphoid tissue (MALT) is rich in dendritic cells (DCs), macrophages (MΦs), T cells, and B cells; hence, stimulating mucosal immunity leads to potent cellular and humoral immunity (Longet *et al.*, 2018; Cho *et al.*, 2021). Immune cells activated at the mucosal sites circulate through the bloodstream and stimulate systemic immunity, thereby establishing crosstalk between mucosal and systemic immunity (Song *et al.*, 2024). D-galacto-D-mannan (DGDM) is a polysaccharide extracted from plant cell walls that acts as a DC-associated C-type lectin-2 (Dectin-2) agonist. DGDM is a natural product with potent antioxidant properties and is considered safe for inclusion in the human diet (Geshi *et al.*, 2010; Pristov *et al.*, 2011). Dectin-2 is found on several types of immune cells, including Langerhans cells, MΦs, neutrophils, and DCs, and recognizes mannose structures as ligands (Scur *et al.*, 2023).

The commercial FMD vaccine currently available on farms is an inactivated vaccine that uses whole inactivated virus as an antigen and aluminum hydroxide (AL), Quil-A, and ISA206 as adjuvants (Lu *et al.*, 2022). AL is widely used in veterinary vaccines because of its low cost and favorable safety profile (Hogenesch, 2012). However, AL has the disadvantage of inducing antibody (Ab)-mediated immunity, which lacks cell-mediated immunity (Jiang *et al.*, 2018). Quil-A is a saponin-type adjuvant produced by purifying crude saponin. Existing crude saponin adjuvants have safety concerns, such as inducing toxicity at the injection site; hence, Quil-A is used as an alternative to crude saponins. Compared with crude saponins, Quil-A elicits a stronger immune response and is considered safer (Sun *et al.*, 2009). ISA206 is a mineral oil-based adjuvant that induces a long-lasting immune response; however, oil adjuvants can cause serious side effects such as necrosis and lesions. Moreover, ISA206 promotes the degradation of inactivated antigens (Harmsen *et al.*, 2015; Rathogwa *et al.*, 2021). We formulated test vaccines with the same composition and ratio as commercial FMD vaccines and adjusted the vaccination dose (1 mL) accordingly. In our previous study, potent long-term immunity and high serum IgA concentrations were induced in pigs vaccinated intramuscularly with a vaccine containing DGDM (Kim *et al.*, 2024). Therefore, we speculated that since intramuscular DGDM induced mucosal immunity, oral administration of DGDM could induce even stronger mucosal immunity. In this study, we demonstrated that the combination of intramuscular FMD vaccine and oral DGDM induces robust systemic and mucosal immunity.

MATERIALS AND METHODS

Animals: C57BL/6N female mice and landrace pigs were purchased from KOSA BIO Inc. (Gyeonggi-do, Republic of Korea) and BARON BIO Inc. (Gyeongsangbuk-do, Republic of Korea), respectively. The animals were housed in a biosafety level 3 (BSL-3) facility (Kim *et al.*, 2023). Saliva samples were collected from the mice following the intraperitoneal injection of pilocarpine hydrochloride (100 µg/dose; Sigma-Aldrich, MO, USA) (Yamamoto *et al.*, 1998).

Cells, viruses, and antigens: Fetal porcine kidney, fetal goat tongue epithelium (ZZR 127), baby hamster kidney (BHK-21), and FMDV type O (O PA2; GenBank accession No. AY593829.1) and A (A YC; GenBank Accession No. KY766148.1) were used. Cells and viruses were cultured in Dulbecco's Modified Eagle's medium (HyClone, UT, USA). The BHK-21 cells were infected with FMDV (O, PA2; A, YC). The antigens were purified and quantified as previously described (Kim *et al.*, 2023).

Food efficiency ratio (FER) in mice, and liver and kidney function tests in pigs: As previously described (López-Varela *et al.*, 1995), the FER was calculated using the following equation:

$$\text{FER} = \frac{\text{Weight gain (grams)}}{\text{Food intake (grams)}} \times 100$$

DGDM safety profile was assessed using liver and kidney function tests performed at KLS BIO, Inc. (Gyeonggi-do, Republic of Korea). Serum albumin (ALB), blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALB/globulin (A/G), total protein (TP), lactate dehydrogenase (LDH), and creatinine (CREA) levels were evaluated using a HITACHI Automatic Analyzer 3100 (Hitachi High-Tech Corporation, Tokyo, Japan).

Serological assays: Serum Ab titers were assessed using PrioCheck™ FMDV kits (Prionics AG, Schlieren, Switzerland) following the manufacturer's instructions (Kim *et al.*, 2023). The virus neutralization (VN) assay was performed as outlined by the World Organization for Animal Health.

Secretory IgA (SIgA) concentration assays: Serum concentrations of murine and porcine SIgA were assessed using an SIgA ELISA kit (CUSABIO®, Wuhan, China) according to the manufacturer's instructions. The concentration of SIgA in mouse saliva was assessed using an SIgA ELISA kit (Cloud-Clone Corp., TX, USA) according to the manufacturer's instructions (Zhang *et al.*, 2022; Yang *et al.*, 2023).

Animal experiments: Animals in the vaccine-only (positive control) and DGDM-fed (experimental) groups were administered the test vaccine intramuscularly. An equal volume of PBS was administered to the negative controls. In the DGDM-fed groups, mice (100 µg/dose) and pigs (20 mg/dose) were orally administered DGDM (Sigma-Aldrich) daily until 28 days post-vaccination (dpv) and then weekly until 56 dpv. The pigs received a booster vaccination at 28 dpv. Blood samples were collected for serological analysis. Long-term host defense was assessed by challenging the mice with FMDV [100 lethal dose 50% (LD_{50}) O/VET/2013] intraperitoneally at 84 dpv (Kim *et al.*, 2023). Body weights (BWs) and survival rates were recorded for approximately 7 days post-challenge (dpc).

All animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Animal and Plant Quarantine Agency (certification no. IACUC-2025-1608). Throughout the experimental period, all animals were monitored daily for clinical signs, body condition, behavior, and feed and water intake. Any abnormal signs were promptly addressed according to the approved protocol. Humane endpoints and euthanasia procedures were clearly defined and strictly followed to minimize pain and distress. All procedures complied with the "3Rs" principle (Replacement, Reduction, and Refinement), and every effort was made to ensure animal welfare during vaccination, sample collection, and oral administration of DGDM.

Peripheral blood mononuclear cells (PBMCs) isolation and quantitative reverse transcription-PCR (qRT-PCR): PBMCs were isolated and stored in TRIzol reagent (Invitrogen, CA, USA), as previously described (Kim *et al.*, 2023). RNA was extracted from TRIzol reagent (Invitrogen) with the RNeasy Mini Kit® (QIAGEN, CA, USA) following the user manual. cDNA was synthesized using the GoScript Reverse Transcription System (Promega, WI, USA) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Green Supermix (Bio-Rad, CA, USA). All primers used in the experiments are listed in Table 1.

Table 1: List of primer sequences for qRT-PCR

Target	Forward/ Reverse	Sequence (5'-3')	Length (mer)
IL-2	IL-2 F	AAGCTCTGGAGGGAGTGCTA	20
	IL-2 R	CAACAGCAGTTACTGTCTCATCA	23
IL-4	IL-4 F	CTCACCTCCCAACTGATCCC	20
	IL-4 R	TGTGTCCGTGGACGAAGTTG	20
IL-12p40	IL-12p40 F	GGAGTATAAGAAGTACAGAGTGG	23
	IL-12p40 R	GATGTCCCTGATGAAGAAGC	20
IL-17A	IL-17A F	CTCGTGAAGGCGGGAATCAT	20
	IL-17A R	GGTGTGCTCCGGTTCAAGAT	20
IL-18	IL-18 F	AGCTGAAAACGATGAAGACCTG	22
	IL-18 R	AAACACGGCTTGATGTCCCT	20
IL-23p19	IL-23p19 F	CCATATCCAGTGCGGGGATG	20
	IL-23p19 R	AGGCCTTGGTGGATCCTTTG	20
IL-23R	IL-23R F	TCCCTCATTGCAAAGCACAA	20
	IL-23R R	GCATCTCCTCTTGCAAGCAAAT	22
IFNγ	IFN-γ F	GCCATTCAAAGGAGCATGGAT	21
	IFN-γ R	CTGATGGCTTTGCGCTGGAT	20
HPRT	HPRT F	CCCAGCGTCGTGATTAGTGA	20
	HPRT R	GCCGTTCAGTCCTGTCCATA	20

Statistical analysis: Unless otherwise noted, all results are presented as the mean ± standard error of the mean (SEM). Survival curves were generated using the Kaplan–Meier method, and differences were analyzed with the log-rank test. Differences between groups, including NC, PC, and Exp groups, were analyzed using one-way or two-way analysis of variance (ANOVA), followed by Tukey's or Dunnett's post-hoc tests. These *post-hoc* tests were specifically applied to correct multiple comparisons following ANOVA. Statistically significant differences were indicated using *, **, *** and **** corresponding to $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$, respectively. All statistical analyses were performed using GraphPad Prism 10.2.3 (GraphPad, CA, USA).

RESULTS

Oral DGDM with FMD vaccine is safe for the host: To assess the safety of orally administered DGDM, food intake and BW were monitored, and FER was calculated based on the oral administration schedule. The BWs of the mice were measured weekly until 28 dpv and monthly until 56 dpv (Table 2). The DGDM-fed group showed no significant differences in FER compared with the control group (Table 3). These results demonstrate that the DGDM did not cause any side effects in mice. To evaluate the side effects of DGDM in pigs, the liver and kidney function tests were performed. No significant differences in the liver and kidney functional indices (ALB, BUN, ALT, AST, A/G, TP, LDH, and CREA) at each time point (28, 56, and 84 dpv) were observed between the experimental group and the other control groups (Table 4), indicating the safety of DGDM in pigs.

Table 2: Body weight gain of mice treated with or without D-galacto-D-mannan by oral administration for 56 days post vaccination (dpv). Experiments were performed according to the mice experimental strategies described in Fig. 1A. Data are represented as the mean ± SEM of triplicate measurements (*n*=5/group). Statistical analyses were performed using two-way ANOVA, followed by Tukey's *post-hoc* test. dpv, days post-vaccination

Group	0 dpv	7 dpv	14 dpv	21 dpv	28 dpv	56 dpv
Negative control group	18.68± 0.28	19.93± 0.38	20.85± 0.42	21.39± 0.43	21.54± 0.31	23.13± 0.60
Positive control group	19.10± 0.34	20.84± 0.31	21.00± 0.49	21.57± 0.45	21.70± 0.46	24.04± 0.36
Experimental group	19.31± 0.23	21.19± 0.21	21.38± 0.36	22.17± 0.40	21.65± 0.29	24.84± 0.20

Table 3: Weight gain, food intake, and food efficiency ratio (FER) of mice treated with D-galacto-D-mannan by oral administration for 56 days post vaccination. Experiments were performed according to the mice experimental strategies described in Fig. 1A. FER, food efficiency ratio {FER=Body weight gain (g/dpv) / food intake (g/dpv) *100}. Data are represented as the mean ± SEM of triplicate measurements (*n*=5/group). Statistical analyses were performed using two-way ANOVA, followed by Tukey's *post-hoc* test. dpv, days-post vaccination; FER, food efficiency ratio

Group	Weight gain (g/56pv)	Food intake (g/56 dpv)	FER
Negative control group	4.45±0.61	121.57±3.45	3.66±0.50
Positive control group	4.85±0.58	152.86±2.68	3.17±0.38
Experimental group	4.42±0.38	125.93±2.46	3.51±0.32

DGDM induces a potent adaptive immunity in mice: To investigate humoral immunity induced by DGDM, experiments were performed as shown in Fig. 1A. The DGDM-fed group showed a steeper increase in Ab titers in the early stages and an Ab positivity at 14 dpv following FMD vaccination than the vaccine-only group. The DGDM-fed group also showed potent adaptive immunity and maintained Ab positivity until 84 dpv (Fig. 1B and C). Similar to the Ab titers, VN titers specific for FMDV increased more rapidly in the DGDM-fed group and were maintained for a longer period than those in the vaccine-only group (Fig. 1D and E). These results suggest that DGDM enhances the FMDV-induced immune response, leading to robust humoral immunity.

Table 4: Safety of oral DGDM in pigs verified using serum biochemical tests. The pigs were divided into three groups: negative control (PBS), positive control (FMD vaccine only), and experimental (FMD vaccine + oral DGDM). Data are presented as the mean ± SEM of triplicate measurements (*n*=5–6/group). Statistical analyses were performed using one-way ANOVA, followed by Tukey's post-hoc test. Different superscripts represent significant differences at $P<0.05$. PBS, phosphate-buffered saline; FMD, foot and mouth disease; SEM, standard error of the mean; ANOVA, analysis of variance; DGDM, D-galacto-D-mannan

Group	Days post-Vaccination (dpv)	ALT (U/L)	AST (U/L)	BUN (mg/dL)	CREA (mg/dL)	LDH (U/L)	TP (mg/dL)	ALB (mg/dL)	A/G ratio
Negative control group	0	41.20±3.68	40.00±2.06	5.90±0.37	0.75±0.02	421.28±45.74	2.58±0.10	2.90±0.06	1.22±0.04
	28	56.80±4.03	46.33±12.25	9.56±1.08	1.01±0.05	496.23±40.97	6.08±0.15	3.36±0.05	1.26±0.08
	56	49.60±1.85	43.20±13.28	10.50±1.21	1.15±0.02	333.10±13.02	6.72±0.15	3.14±0.08	0.89±0.05
	84	47.00±1.74	46.00±14.04	17.26±1.47	1.46±0.05	266.48±2.76	6.06±0.07	3.62±0.04	1.49±0.05
Positive control group	0	37.20±1.73	32.60±0.61	7.50±1.43	0.68±0.02	340.30±13.22	5.34±0.07	2.98±0.07	1.26±0.08
	28	48.40±1.85	45.50±3.32	9.30±0.67	0.93±0.02	456.34±17.62	6.30±0.14	3.32±0.11	1.12±0.07
	56	53.80±3.33	48.60±9.20	13.98±0.95	1.16±0.05	361.90±26.52	6.80±0.10	3.36±0.08	0.98±0.02
	84	43.60±2.79	47.75±5.86	16.78±1.60	1.30±0.07	254.12±8.38	6.34±0.12	3.72±0.09	1.44±0.08
Experimental group	0	44.80±7.36	44.40±6.55	7.12±0.19	0.86±0.03	426.50±50.54	5.52±0.11	2.80±0.11	1.07±0.10
	28	55.20±2.50	40.20±4.13	6.10±0.57	1.01±0.04	523.48±56.14	6.40±0.27	3.16±0.08	0.99±0.06
	56	56.60±1.66	37.20±1.71	10.52±0.71	1.18±0.04	449.00±13.60	6.90±0.11	3.50±0.09	1.04±0.06
	84	35.20±3.75	27.20±1.18	13.70±1.41	1.40±0.03	232.24±10.72	5.92±0.22	3.54±0.25	1.52±0.17

DGDM elicits long-term robust host defense in mice: To evaluate the long-term host defense elicited by DGDM in mice, experiments were conducted as shown in Fig. 1A. The DGDM-fed, vaccine-only, and negative groups showed 100%, 40%, and 0% survival rates against FMDV type O infection, respectively (Fig. 2A). In the challenge experiment, there was no significant difference in BW between the groups (Fig. 2B). These results suggest that DGDM contributes to long-term host defense against viral infections.

DGDM induces potent adaptive immunity in pigs: To assess humoral immunity elicited by DGDM, experiments were conducted as shown in Fig. 3A. The DGDM-fed and vaccine-only groups showed similar Ab titers, with a slight increase up to 14 dpv. From 21 dpv (type O) and 28 dpv (type A), the DGDM-fed group gradually became Ab-positive, whereas the vaccine-only group maintained or even showed decreasing Ab titers. After the second vaccination, the DGDM-fed group showed higher Ab titers than the other groups up to 84 dpv. The DGDM-fed group also maintained elevated Ab titers over a long period without a rapid decline. The vaccine-only group also showed an increase in Ab titers after the second vaccination; however, the levels were significantly lower than those in the DGDM-fed group (Fig. 3B and C). For VN titers specific to FMDV type O (O PA2) or type A (A YC), the overall VN titers were similar to the Ab titers (Fig. 3D). The DGDM-fed group showed a steady increase in VN titers from 7 dpv and high VN titers until 84 dpv, whereas the vaccine-only group showed an increase in VN titers from 21 dpv and a gradual decrease from 42 dpv. These results suggest that DGDM induces adaptive immunity, thereby stimulating memory immune responses.

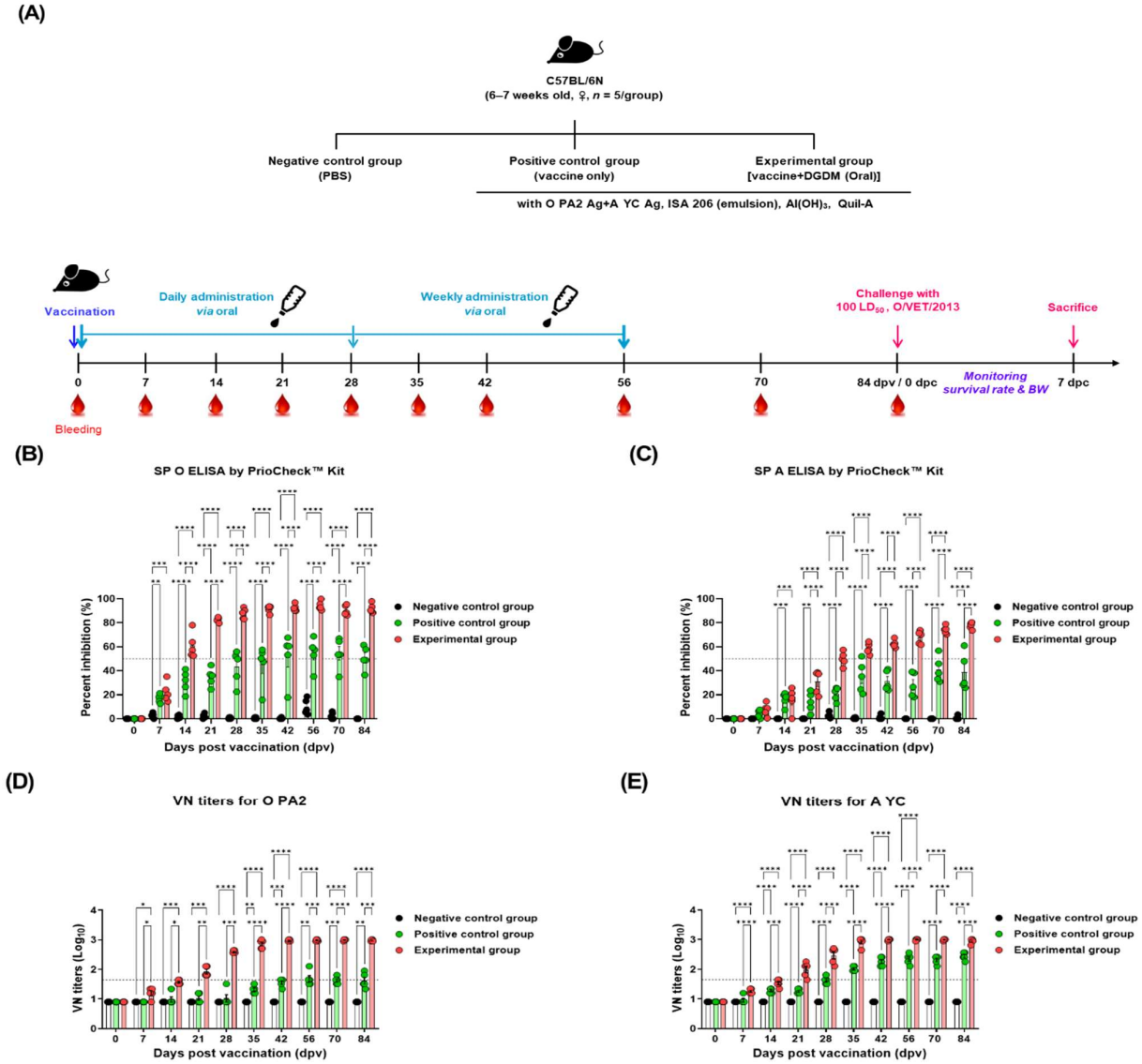

Fig. 1: Oral DGDM combined with parenteral vaccination induces potent and long-lasting immunity in mice. Blood samples were collected at 0, 7, 21, 28, 35, 42, 56, 70, and 84 dpv for serological analyses using SP ELISA and VN tests. (A–E) Experimental strategy (A); antibody titers using SP O ELISA kits (B); SP A ELISA kits (C); VN titers for O PA2 (D); VN titers for A YC (E). Data are presented as the mean ± SEM of triplicate measurements (n=5/group). Statistical analyses were performed using two-way ANOVA, followed by Tukey's *post-hoc* test. *P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001. PBS, phosphate-buffered saline; FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; DGDM, D-galacto-D-mannan; VN, virus neutralization; SEM, standard error of the mean.

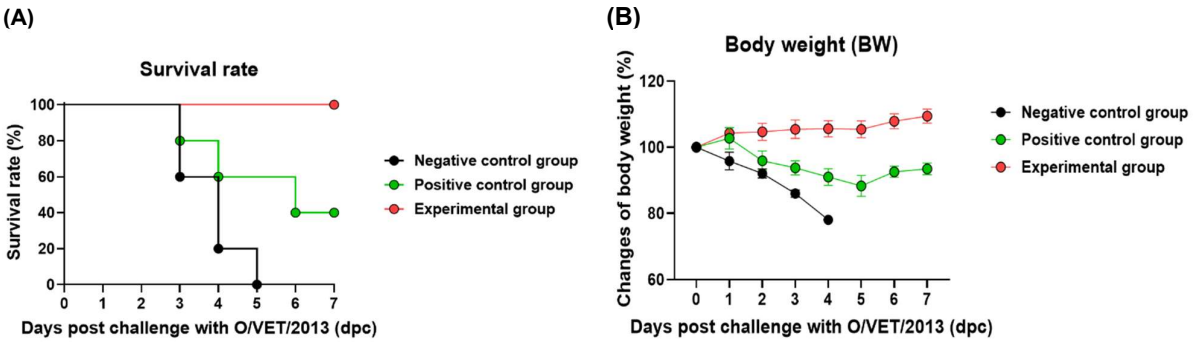

Fig. 2: Oral DGDM combined with parenteral vaccination induces robust host defense in mice. Mice were intraperitoneally challenged with FMDV type O (100 lethal dose 50% [LD_{50}] O/VET/2013) at 84 dpv as shown in Figure 1A. The survival rates and body weights were monitored at 7 dpc. (A, B) Survival rates post-challenge with O/VET/2013 (A); and changes in body weight post-challenge with O/VET/2013 (B). Data are presented as mean ± SEM (*n*=5/group).

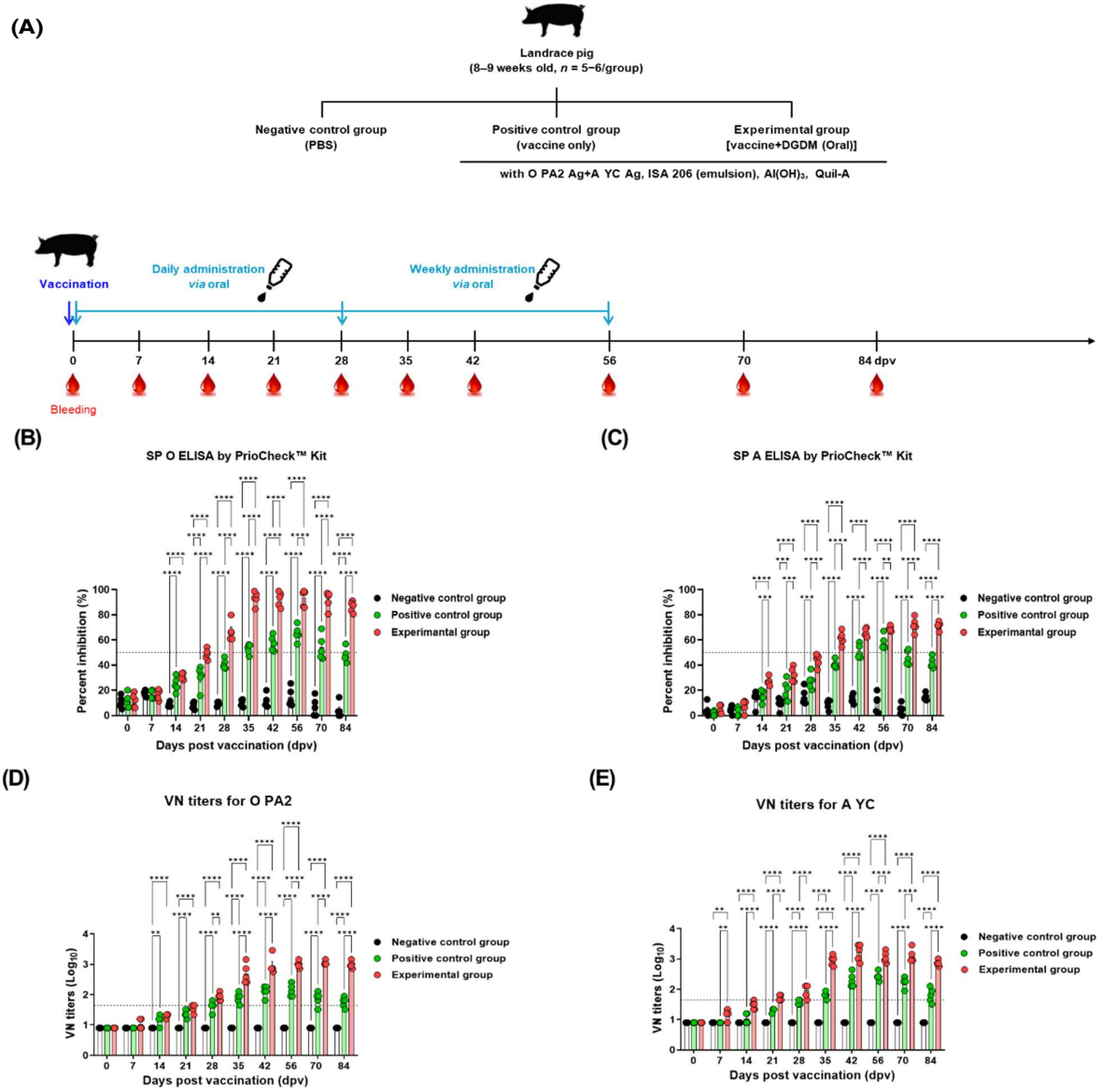

Fig. 3: Oral DGDM combined with parenteral vaccination induces potent and long-lasting immunity in pigs. Blood samples were collected from the pigs at 0, 7, 14, 21, 28, 35, 42, 56, 70, and 84 dpv for serological assays. (A–E) Experimental strategy (A); antibody titers using SP O ELISA kits (B); SP A ELISA kits (C); VN titers for O PA2 (D); and VN titers for A YC (E). Data are presented as the mean ± SEM of triplicate measurements (*n*=5–6/group). Statistical analyses were performed using two-way ANOVA, followed by Tukey's *post-hoc* test. *P< 0.05; **P< 0.01; ***P< 0.001; and ****P< 0.0001. PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; DGDM, D-galacto-D-mannan; VN, virus neutralization; SEM, standard error of the mean.

DGDM upregulates SIgA secretion: To evaluate the mucosal immune response induced by DGDM, SIgA secretion was detected in mice and pigs. SIgA concentrations measured in mouse sera and saliva were significantly higher in the DGDM-fed group than in the control group at 56 dpv (Fig. 4A, 4B). SIgA concentrations measured in pigs were also significantly higher in the DGDM-fed group than in the control group at 56 dpv (Fig. 4C). These results indicate that DGDM activated mucosal immunity, thereby upregulating SIgA secretion.

DGDM elicits the expression of mucosal and systemic immune-related genes: To measure cytokines related to mucosal immunity induced by DGDM, the gene expression of cytokines was evaluated using qRT-PCR (Fig. 5A−H). Gene expression data were normalized to HPRT (reference gene) levels and expressed as fold change relative to the control group. The samples were collected at 14 dpv (Fig. 3A). The expression of mucosal and systemic immune-related cytokines, including IL-2 (Fig. 5A), IL-4 (Fig. 5B), IL-12p40 (Fig. 5C), IL-17A (Fig. 5D), IL-18 (Fig. 5E), IL-23p19 (Fig. 5F), IL-23R (Fig. 5G), and interferon (IFN)γ (Fig. 5H) showed significant differences between the DGDM-fed group and other control groups at 14 dpv. These results suggest that DGDM intake induces robust cellular and humoral immunity through mucosal immune activation.

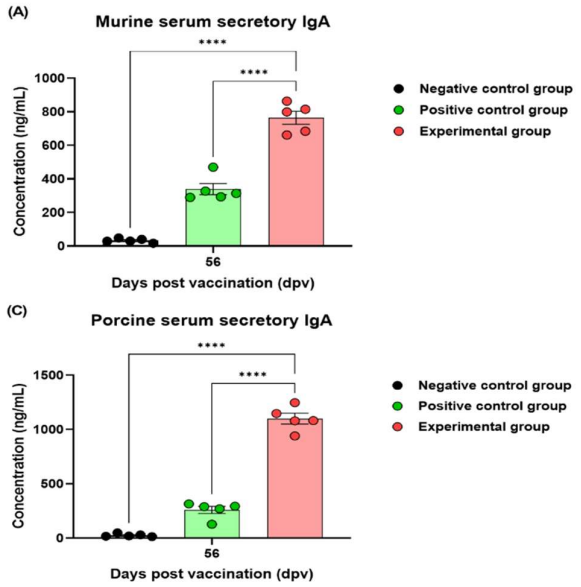

Fig. 4: Oral DGDM combined with parenteral vaccination stimulates the secretion of SIgA in mice and pigs. The experimental strategy and methods are described in the legends of Fig. 1A and 3A, respectively. (A–C) Murine serum (A); murine saliva (B); and porcine serum (C) SIgA concentrations. Data are presented as the mean ± SEM of triplicate measurements (n=5–6/group). Statistical analyses were performed using two-way ANOVA, followed by Tukey's *post-hoc* test. *P<0.05 and ****P<0.0001. ANOVA, analysis of variance; DGDM, D-galacto-D-mannan; SEM, standard error of the mean; SIgA, secretory IgA.

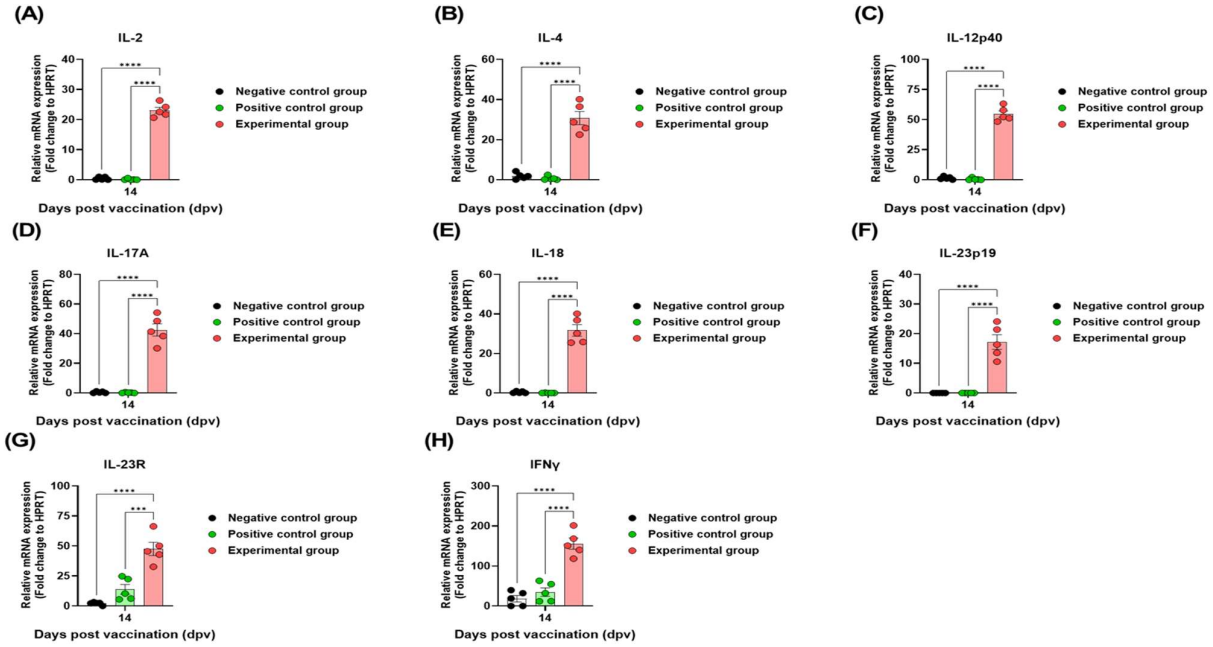

Fig. 5: Oral DGDM combined with parenteral vaccination upregulates the expression of mucosal and systemic immune-related genes. Porcine PBMCs isolated from the whole blood of vaccinated pigs (n=5–6/group), as described in Fig. 3A, were analyzed using qRT-PCR. Gene expression levels were normalized to those of HPRT and presented as ratios relative to the control levels. (A–H) Gene expression levels of IL-2 (A); IL-4 (B); IL-12p40 (C); IL-17A (D); IL-18 (E); IL-23p19 (F); IL-23R (G); and IFNγ (H). Statistical analyses were performed using two-way ANOVA, followed by Tukey's *post-hoc* test. *P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001. PBMCs, peripheral blood mononuclear cells; ANOVA, analysis of variance; DGDM, D-galacto-D-mannan; IL, interleukin; qRT-PCR, quantitative real-time polymerase chain reaction; IFN, interferon

DISCUSSION

One advantage of inducing mucosal immunity is that it prevents transmission between hosts through viral shedding at mucosal sites (oral and nasal) (Leung, 2021). FMDV infects and multiplies in the respiratory mucosa and is shed through mucosal droplets. However, commercial intramuscularly administered FMD vaccines are designed to induce a systemic immune response and thus fail to suppress viral infection and replication at mucosal sites. The movement of systemic Abs induced by intramuscularly administered vaccines to the mucosal surface area is limited; therefore, it is necessary to develop strategies to directly stimulate the mucosa (Jang *et al.*, 2023). Antigens administered via the mucosa are recognized and processed by the MALT located in the mucosa. MALT cells contain many mucosal immune-related cells, including DCs, MΦs, and tissue-resident immune cells that mediate innate and adaptive immunity (Dwivedy and Aich, 2011). MALT cells also produce and secrete mucosal Abs (IgA and IgG) to combat external pathogens. IgA, which is more abundant than IgG, is secreted in the mucosa in monomeric or dimeric forms and can resist proteolytic enzymes in mucosal secretions. Dimeric IgA has a higher neutralizing ability than monomeric IgA (Steffen *et al.*, 2020). Mucosal Abs and tissue-resident immune cells can only be induced by mucosal immunity and not by systemic immunity (Pilapitiya *et al.*, 2023).

In the present study, a combination of orally administered DGDM and parenterally administered FMDV stimulated mucosal immunity and elicited a robust systemic immune response. The DGDM-fed group exhibited higher Ab and VN titers than the other control groups. DGDM enhanced mucosal and systemic immunity, inducing the production of Abs specific to the FMDV antigen (Fig. 1). Inducing mucosal immunity via mucosal stimulation stimulates tissue-resident and systemic-circulating immune MALT cells, thereby enhancing humoral immune responses. Furthermore, mice in the DGDM-fed group showed robust long-term survival against viral infections following parenteral vaccination. Mucosal immunity not only enhances humoral immunity to induce a potent Ab-mediated immune response but also stimulates immune cells, contributing to host defense against viral infection (Fig. 2). In the case of other respiratory viral diseases, such as coronavirus disease 2019, parenteral vaccination does not induce mucosal immunity and thus does not suppress viral infection through the respiratory tract (Azzi *et al.*, 2022; Tang *et al.*, 2022). To overcome these limitations, a strategic combination of oral adjuvant administration and parenteral vaccination has been developed. A combined vaccination program of intranasal administration of recombinant IFNα and parenteral vaccine simultaneously induced mucosal and systemic immunity, thereby blocking respiratory tract invasion of the virus (Bessière *et al.*, 2021; Fraser *et al.*, 2023). Thus, the combined oral administration of adjuvants and parenteral vaccines enhances mucosal and systemic immunity, thereby enhancing the innate and adaptive immunity of the host. During the mouse experiment, there was no significant difference in BW change and FER between the DGDM-fed group and the other control groups; therefore, the side effects were considered very mild or non-existent (Tables 2 and 3).

Similar to that in mice, potent humoral immunity was induced in pigs in the DGDM-fed group, resulting in high Ab and VN titers. The combined oral administration of adjuvants and a parenteral vaccination induced higher Ab and VN titers, suggesting that simultaneous stimulation of mucosal and systemic immunity is more advantageous in maintaining long-lasting immunity (Fig. 3). The FMD vaccine evaluation criteria were mainly Ab and VN titers. Previous studies have reported that VN titers above 1.5 $\log_{10}$ are associated with protective immunity against FMDV infection (Gubbins *et al.*, 2022). In this study, the combination of DGDM treatment and intramuscular FMD vaccination resulted in VN titers that surpassed the threshold associated with protective immunity. Based on these results, it is reasonable to hypothesize that this combination could offer protective immunity against FMDV infection in pigs. However, further confirmation through FMDV challenge studies is needed to validate this hypothesis.

Liver and kidney function tests were conducted during the experiment, and all test indices in all groups were within the normal range, indicating no adverse effects of DGDM (Table 1) (Zhang *et al.*, 2022; Meissner *et al.*, 2024). Humoral immune-related MALT cells activated by external stimuli produce Abs (IgA and IgG) that are secreted outside the mucosa or circulate throughout the body via blood vessels (Alu *et al.*, 2022). The DGDM-fed group had higher concentrations of SIgA in saliva droplets and sera than the other control groups. Mucosal SIgA plays a pivotal role in the immune system (Fig. 4). Mucosal SIgA, which is mainly present in the dimeric form, has a high affinity for pathogens and thus has excellent neutralizing ability (Li *et al.*, 2020). High concentrations of SIgA in saliva droplets and serum prevent the invasion of respiratory viruses, thereby blocking viral infection and shedding at the mucosal site and neutralizing and eliminating viruses from the blood.

Oral administration of DGDM combined with parenteral vaccination simultaneously stimulated mucosal and systemic immunity, thereby inducing a robust adaptive immune response. We aimed to elucidate immune mechanisms by evaluating the gene expression of cytokines involved in mucosal and systemic immunity (Fig. 5). IL-2 contributes to the maturation and differentiation of various immune cells and regulates innate and adaptive immunity (Bendickova and Fric, 2020). Within the mucosa, IL-4 is primarily produced and secreted by T helper (Th) 2 cells, and is involved in tissue repair and anti-inflammatory immunity (Allen, 2023). IL-12 comprises two subunits: p35 and p40. IL-12p40 combines with IL-23p19 to form IL-23 (Lupardus and Garcia, 2008). IL-12 interacts with several cytokines to upregulate the host immune response. IL-12 and IFNγ form a positive feedback loop, stimulating their production and secretion (Elsner and Shlomchik, 2025). The synergistic effect of IL-12 and IL-18 elicits IFN-γ secretion from a wider range of immune cell types (Cole *et al.*, 2020). IL-23 induces the proliferation of Th17 cells and tissue-resident memory T cells that secrete IL-17A. In response to external stimuli,

MALT immune cells promote the IL-23/IL-17A axis and IL-23 receptor expression, thereby achieving host defense against pathogens and contributing to mucosal tissue repair and maintenance of function (Lee *et al.*, 2015; Krueger *et al.*, 2024). In summary, DGDM harmoniously induced Th1-mediated inflammatory immunity and Th2-mediated anti-inflammatory immunity, thereby improving the overall immune response and maintaining homeostasis.

This study is limited in that pigs were not subjected to FMDV challenge. Although our study demonstrated promising results, including VN titers exceeding the 1.5 $\log_{10}$ threshold commonly associated with protection, the lack of direct challenge data in pigs means that the true efficacy of the combination of oral DGDM and FMD vaccination remains unconfirmed. A pig challenge experiment is essential to demonstrate that the combination of DGDM intake and parenteral vaccination contributes to the induction of host defense against viral infection. There are several reasons for the absence of pig challenge experiments, including limitations on the number of experiments to prevent virus transmission, restrictions on the number of experimental animals, and permission for researchers to enter. Our institution's BSL-3 animal facility is the only one in Korea capable of conducting large-animal challenge experiments (e.g., cattle, pigs) for high-risk viral infections, making facility reservations difficult. In subsequent studies, we will evaluate whether oral DGDM contributes to achieving host protection against FMDV infection using various indicators (serum viremia, viral titers in oral swabs, and clinical symptoms).

Conclusions: Oral DGDM combined with parenteral vaccination induced potent cellular and humoral immunity compared with parenteral vaccination alone. This combined approach also elicits robust mucosal and systemic immunity by inducing harmonious inflammatory and anti-inflammatory immune responses. In this study, we demonstrated that DGDM has potential as an oral vaccine adjuvant and that the simultaneous stimulation of mucosal and systemic immunity induces a robust adaptive immune response. This study serves as a foundation for future studies on mucosal vaccine adjuvants. In this study, oral DGDM combined with parenteral vaccination simultaneously stimulated systemic and mucosal immunity, thereby enhancing both cellular and humoral immune responses in mice and pigs. These findings provide new insights into the potential of DGDM as an oral vaccine adjuvant. Future studies under field conditions and in other animal species may further expand its applicability and explore its use against other diseases, supporting the continued development of mucosal vaccine adjuvants.

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