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Improved immune responses and safety of foot-and-mouth disease vaccine containing immunostimulating components in pigs

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ABSTRACT

Background: The quality of a vaccine depends strongly on the effects of the adjuvants applied simultaneously with the antigen in the vaccine. The adjuvants enhance the protective effect of the vaccine against a viral challenge. Conversely, oil-type adjuvants leave oil residue inside the bodies of the injected animals that can produce a local reaction in the muscle. The long-term immunogenicity of mice after vaccination was examined. ISA206 or ISA15 oil adjuvants maintained the best immunity, protective capability, and safety among the oil adjuvants in the experimental group.

Objectives: This study screened the adjuvant composites aimed at enhancing foot-and-mouth disease (FMD) immunity. The C-type lectin or toll-like receptor (TLR) agonist showed the most improved protection rate.

Methods: Experimental vaccines were fabricated by mixing various known oil adjuvants and composites that can act as immunogenic adjuvants (gel, saponin, and other components) and examined the enhancement effect on the vaccine.

Results: The water in oil (W/O) and water in oil in water (W/O/W) adjuvants showed better immune effects than the oil in water (O/W) adjuvants, which have a small volume of oil component. The W/O type left the largest amount of oil residue, followed by W/O/W and O/W types. In the mouse model, intramuscular inoculation showed a better protection rate than subcutaneous inoculation. Moreover, the protective effect was particularly weak in the case of inoculation in fatty tissue. The initial immune reaction and persistence of long-term immunity were also confirmed in an immune reaction on pigs.

Conclusions: The new experimental vaccine with immunostimulants produces improved immune responses and safety in pigs than general oil-adjuvanted vaccines.

Keywords: Foot-and-mouth disease; immunologic adjuvants; safety; immunostimulants

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INTRODUCTION

The FMD virus, which belongs to the genus *Aphthovirus*, family *Picornaviridae*, mainly causes blisters on cloven-hoofed animals [1]. The size of the genome is approximately 8.5 kb, and it is a positive-sense single-stranded RNA [2]. The FMD virus has 7 serotypes, A, O, Asia 1, C SAT1, SAT2, and SAT3, as well as more than 60 subtypes [2]. The FMD virus can spread a long distance in a short time through airborne transmission. FMD results in a decrease in the productivity of pigs, cattle, sheep, and goats [3-5], resulting in the need for the culling of infected animals. Moreover, FMD has become a serious social problem because of the reduced farm income due to the decreased or restricted sales and exports resulting from FMD infections [6,7].

Among the methods used to prevent FMD, vaccines are most effective. On the other hand, this requires an immunity formation time ranging from a minimum of 7 days [8] to as long as a month for the antibody titer to be formed by the vaccine. The development of a scheme that induces instant and long-term immunity would have a significant impact on preventing the spread of the disease. Vaccine adjuvants maximize the formation of immunity in animals, but the safety of a vaccine can be affected by the type of adjuvant.

FMD vaccines have used oil as an adjuvant. Selecting the optimal oil adjuvant is very important because the effects of the FMD vaccine vary according to types of oil adjuvant [9]. Vaccines that contain oil were first developed in the 1910s. Initially, lanolin was used as a component, and paraffin oil was later added to enhance the immunocompetence of the vaccines. On the other hand, abscesses or granuloma formed in the injection area, and other adjuvants were sought to reduce these side effects. In previous study, they reported that abscesses or granuloma on the neck cause economic losses of approximately 7.9 dollars per pig [10]. Because of the shortcoming of the adjuvants, an aluminum gel was introduced as an alternative in vaccines in the 1960s. Aluminum gel has the advantage in that the antigen is released slowly as it is absorbed into the gel made of aluminum phosphate ($AlPO_4$) or aluminum hydroxide [$Al(OH)_3$], but the immune-stimulating effect of these vaccines is relatively weak. Despite providing more safety for animals, the gel adjuvant was inadequate for vaccines against diseases requiring the immediate formation of immunity due to rapid diffusion, such as FMD. A previous study compared the immune effect of an oil vaccine with an aluminum gel vaccine against FMD. They reported that the neutralization antibody titer continued to increase to 1 month after inoculation with the oil vaccine in growing calves with pre-existing maternal antibodies. In contrast, antibody formation continued to decrease after inoculation when an aluminum gel vaccine was used [11]. Oil is currently used as the primary vaccine adjuvant composite because oil vaccines are effective for FMD prevention despite the local reaction caused by the small residual amounts of oil [11]. Among the candidate vaccine-composite materials aimed at a stronger effect, the PPR ligands (TLR3, TLR5, TLR 7/8, and TLR9) were recently reported to improve the effectiveness of the FMD vaccine [12]. The formation of granuloma in the vaccination site is considered an inevitable phenomenon because of the characteristics of the oil component in the FMD vaccine. On the other hand, efforts should be made to minimize granuloma formation and reduce the residue of the vaccine adjuvant. More studies on the inoculation scheme, vaccination site, and the number of inoculations are required to ensure vaccine safety. Improvement of the immune-boosting effects of the FMD vaccine through the selection of materials that can enhance the immunity antibody levels from among the adjuvants applied to pigs will provide an opportunity to control FMD more efficiently.

Moreover, protection against FMD was needed not only on type O occurring in Korea but also on type A and Asia1 that appeared in neighboring countries around Korea. For this reason, the adjuvants were selected using several types of antigen, and the antigenicity of the vaccine produced with the adjuvant and antigen was confirmed in mice and pigs.

This study chose an adjuvant that could improve the effect, safety, and stability of the FMD vaccine. Before applying it to pigs as the target animal, the vaccine was tested thoroughly to screen the effective adjuvants and composites using an experimental mouse model. In this way, the possibility of developing an effective vaccine for pigs was confirmed.

MATERIALS AND METHODS

Mice, viruses, and vaccines

Seven-week-old, 17–19 g C57BL/6 mice were purchased from the KOSABIO Co., Ltd. (Korea). After inactivating the recombinant FMD viruses expressing the P1 protein of A22, Asia1/MOG/2015 (Asia1 MOG), O Taiwan 97 (O TWN), and O Thailand 60 (O THI) FMD virus with 3 mM BEI through a 24-h treatment process, the viral antigens were purified through sucrose density gradient centrifugation. A test vaccine was made by mixing the purified antigen, 10% Al(OH)$_3$, and saponin (3 mg/2 mL or 0.5 mg/mL) with an adjuvant (ISA15, ISA28, ISA61, ISA71, ISA161611, ISA1616102, ISA201, ISA 206, and ISA207, respectively). This study examined whether the oil adjuvant candidates react similarly to be applicable for each serotype.

Immunization of mice for oil adjuvant selection

To select a superior oil adjuvant through a comparison of the immunogenicity, A22 antigen (10 μg/2 mL, 1 dose), 10% Al(OH)$_3$, and saponin (3 mg/2 mL, 1 dose) were mixed with 1 of 10 adjuvants (ISA15, ISA28, ISA61, ISA71, ISA161611, ISA1616102, ISA201, ISA 206, and ISA207). C57BL/6 7-week-old mice (5 mice/group) were inoculated intramuscularly (IM) with a 1/20 dose (0.1 mL). The animals were treated according to the ethical guidelines of the Animal Welfare Committee of the Animal and Plant Quarantine Agency (APQA). The animals were kept and cared for at the APQA animal facility and used for the experiment after approval from the Committee (Approval No. 2017-357). The antibody against A-type FMD virus in the serum of the mice 21 days post-vaccination was measured using SP-ELISA (Priocheck, Prionics, Switzerland) for type A, following the directions of the kit manufacturer. Briefly, in the PrioCHECK FMDV SP-ELISA, plates are coated with the FMDV type-specific antigen. The key reagent of the test is an anti-FMDV specific monoclonal antibody (mAb) conjugated to an enzyme that generates a color signal. Binding of the conjugate to the immobilized antigen was blocked by anti-FMDV antibodies present in the test sample. The signal was then measured. A lack of color formation indicated that the antibodies in the sample have competed for the viral protein coated on the plate. Samples with a percent inhibition (PI) value of ≥ 50% showed an immune response to the foot-and-mouth disease virus type-specific.

Asia 1 MOG antigen (15 μg/2 mL, 1 dose), 10% Al(OH)$_3$, and saponin (3 mg/2 mL, 1 dose) were mixed with each of the adjuvants (ISA15, ISA61, ISA1616102, and ISA 206). A 1/20 dose (0.1 mL) was inoculated intramuscularly (IM) in C57BL/6 7-week-old mice (5 mice/group). The antibody against FMD virus Asia 1 in the mice serum was measured 30, 60, and 90 days after the vaccination using SP-ELISA (Priocheck, Prionics) for type Asia1, according to the directions of the kit manufacturer. A 100 LD$_{50}$ of Asia 1 Shamir virus was administered

intraperitoneally (IP) 90 days after the vaccination, and the survival rate and rate of body weight changes were observed for 7 days.

Measurement of vaccine composite residue in the injected site in mice following FMD vaccination

To measure the immune response and oil residue in the vaccination site, the O TWN antigen (15 µg/mL, 1 dose), 10% Al(OH)$_3$, and saponin (0.5 mg/mL/dose) were mixed with each adjuvant (ISA15, ISA 206) [13]. A 1/10 dose of vaccine was administered to C57BL/6 7-week-old mice (5 mice/group) vis the intramuscular (IM), subcutaneous (SC) inoculation, and the fatty tissue (inguinal fat) route. After 7 or 21 days post-vaccination, 100 LD$_{50}$ of O Vietnam 2013 (ME-SA topotype) FMD virus was administered by intraperitoneal (IP) inoculation. The survival rate and rate of body weight change were observed for 7 days. The surviving mice (approximately 14, 28, or 100 days after vaccination) were assigned a score from 0 to 3 according to the level of vaccine composite residue as follows: 0 = no residue-oil, 1 = small residue (less than 0.1 mm), 2 = medium residue (0.1–0.3 mm), and 3 = maximum residue (more than 0.3 mm) in the vaccination area. The residue index score of the subjects in each inoculation group was summed.

Composition of the vaccine with immunostimulants, adjuvants, and antigen

The test vaccine was mixed at a 1/50 dose (O THI) or 1/5,000 dose (A22) of antigen, 10% Al(OH)$_3$, 0.5 mg/mL saponin and immunoadjuvants, which were 25 µg/mL heat-killed *Mycobacterium tuberculosis* (HKMT: Mincle and TLR2 agonist), 25 µg/mL Zymosan (TLR2 and Dectin-1 agonist), or 50 µg/mL Resiquimod (R848: TLR7 and TLR8 agonist) with oil adjuvant ISA15. The mice (5 mice/group) were challenged with 100 LD$_{50}$ O Vietnam, A Malay97, or Asia1/Shamir by IP 10 days after being inoculated with the test vaccine by IM. The survival rate and body weight changes were monitored for 7 days.

Immunogenicity of the experimental vaccine in pigs

ISA 15 was mixed with A22 antigen (15 µg/mL, 1 dose), 10% Al(OH)$_3$, and saponin (0.5 mg/mL, 1 dose) in 2 groups, 1 added with 500 µg/mL R848 and the other added with 250 µg/mL HKMT (InvivoGen Co., USA) or 250 µg/mL Zymosan. The pigs were treated according to the ethical guidelines of the Animal Welfare Committee of the Animal and Plant Quarantine Agency (APQA). The pigs were kept and cared at the APQA animal facility and used for the experiment after approval from the Committee (Approval No. 2017-357). One dose was inoculated IM to five 10-week-old farm pigs, and 28 days after the first inoculation, an additional inoculation was administered IM. After 0, 14, 28, 42, 56, and 70 days, the antibody titer against A-type FMD virus in pig serum was measured using an SP-ELISA (Priocheck, Prionics) kit and VN test. The SP-ELISA was used based on the directions of the kit manufacturer. The VN test was performed in the pig sera immunized with the commercial vaccine (Boehringer Ingelheim, France, 2 mL/dose) containing the A22 antigen or the experimental vaccines.

Safety of the experimental vaccine in pigs

One hundred and ten days after vaccinating the pigs with the test vaccine, the lesions, adjuvant residue, and size were determined by slicing the neck muscle of pigs at 1 cm intervals at the injection site of the vaccine using the methods reported elsewhere [10,17]. The diameter of the vaccine residue in the neck muscle was measured.

RESULTS

Immune response and lesions in vaccinated mice

In most cases, a high antibody was induced 3 weeks after vaccination when an adjuvant was used in the vaccine formation (**Fig. 1A**). The O/W type ISA 28 showed an antibody positive rate of 60%, whereas ISA25 showed an antibody positive rate of 80%. Antibody formation was induced in all vaccine formulations of the W/O/W and W/O type. Each mouse was assigned an oil residue score from 0 to 3 according to the amount of oil residue remaining in the vaccination area 21 days after vaccination, and the scores were summed in each group (n = 5, **Fig. 1B**). No oil residue was observed when ISA25 was used. ISA 1616101 showed the largest oil residue scores. The W/O/W type of ISA201, 206, and 207 showed a small cumulative sum of oil residue scores under 5. ISA15, ISA206, and ISA207 were the adjuvants, whose ELISA antibody PI values were approximately 60% with summed residue scores under 5.

For the candidate adjuvants, the long-term changes in the antibody titers were observed for up to 90 days. In most cases, the inclusion of aluminum gel (AL gel) resulted in lower antibody levels than the inclusion of an oil adjuvant (**Fig. 2A**). The antibody level of the vaccine with the added oil adjuvant reached a peak approximately 60 days after inoculation, and the level decreased gradually afterward (**Fig. 2A and B**). The antibody positive rates of the oil adjuvants ISA61, ISA1616102, and ISA206 reached their peak 60 days after vaccination. Finally, ISA 206 showed the highest antibody positive rate of approximately 40% in the 90-day experiment after vaccination (**Fig. 2B**). The survival rate of the mice increased to more than 80% when any adjuvant, either oil or gel, was used (**Fig. 2C**), and the body weight of the mice showed no change (**Fig. 2D**). The vaccine containing Asia 1 antigen maintained the survival rate of the mice, even though it contains gel and saponin only (**Fig. 2C**). On the other hand, ISA 1616102 and 206, which showed a high antibody positive rate, resulted in the formation of
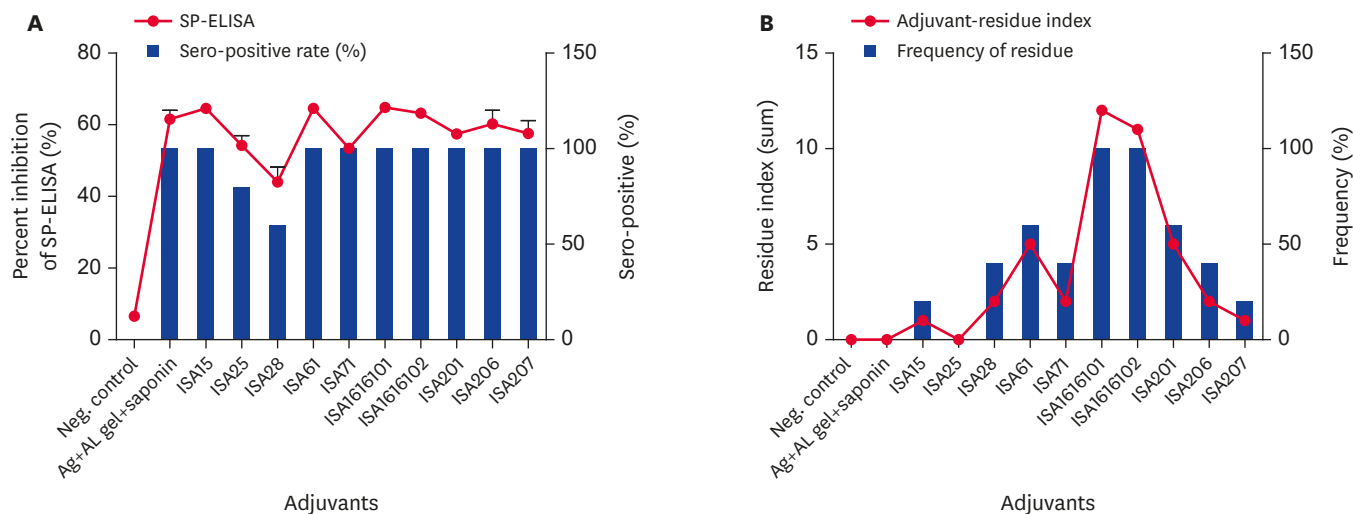

Fig. 1. Immunogenicity and vaccine residue-frequency of various adjuvants with foot-and-mouth disease virus, type A antigen at 21 dpv.
The 10 adjuvants (ISA15, ISA25, ISA28, ISA61, ISA71, ISA161611, ISA1616102, ISA201, ISA206, ISA207) containing 10% Al(OH)$_3$ gel and saponin (3 mg/2 mL) and the FMDV A22 antigen (1/20 dose vaccine of 10 μg/2 mL) were tested for SP-ELISA antibody induction and vaccine residue-frequency 21 dpv. The immunogenicity and safety test were performed with C57BL/6 mice (5 mice/group). (A) The percent inhibition ratio of SP-ELISA (left y-axis) and positive rate of SP-antibody (right y-axis) 21 dpv in vaccinated mice (n = 5). The antibody levels and positive rate of the mice were tested using type A SP-ELISA (Priocheck) 21 dpv. (B) The mice were tested for adjuvant-residue and frequency (%), and the residue in the muscle was counted in the vaccinated groups. The percent inhibition ratio of SP-ELISA (left y-axis) and positive rate of SP-antibody (right y-axis) in vaccinated mice (n = 5) 21 dpv.
dpv, days post-vaccination; ELISA, enzyme-linked immunosorbent assay.
*O/W, ISA15 (15% of oil), ISA25 (25%), ISA28 (25%); †W/O, ISA61 (60%), ISA71(70%), ISA1616101 (60%), 1616102 (60%); ‡W/O/W (50%), ISA201, ISA206, ISA207.

large oil residue in the mice. Among the adjuvants, the O/W type ISA15 showed the lowest oil residue score of 2 (**Fig. 2E**). Despite this, it showed a relatively weak antibody response and a low survival rate (**Fig. 2A and B**).
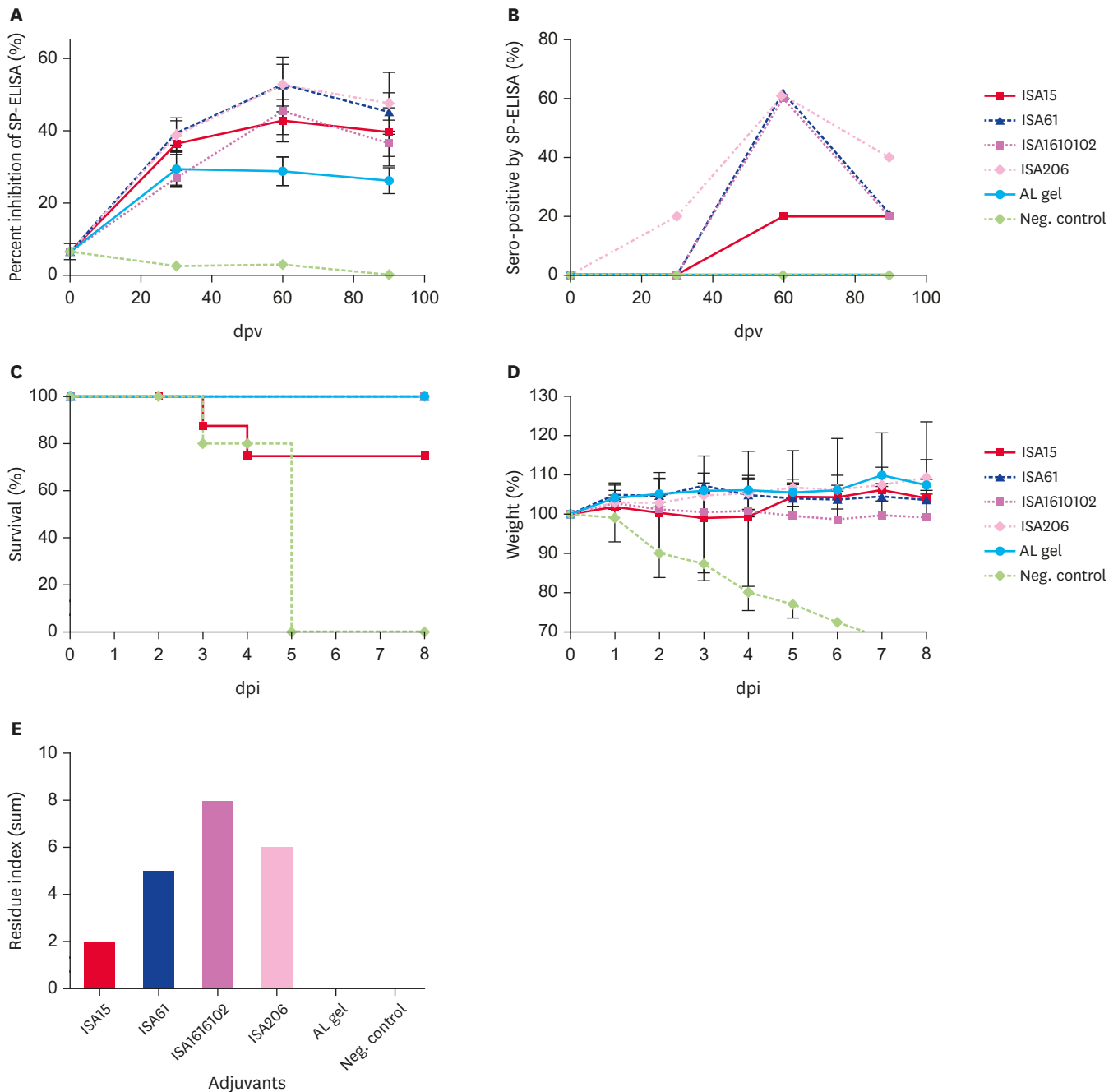
Fig. 2. Immunogenicity through vaccination of various adjuvants with foot-and-mouth disease virus, type Asia1 antigen in mice 90 dpv. The adjuvants were mixed with 10 µg/2 mL Asia 1 MOG antigen, 10% Al(OH)$_3$ gel, and saponin (3 mg/2 mL). The mice were vaccinated with 1/20 dose and then challenged with Asia1 Shamir 90 days after vaccination. (A) SP-antibody by type Asia1 SP-ELISA for 90 days after vaccination 1/20 dose vaccine (1.5 µg), (B) Positive rate (%) of SP-antibody in vaccinated groups (n = 5), (C) Survival rates until 7 days post-challenge with 1/20 dose vaccine (1.5 µg). (D) Weight loss of challenged mice. The 1/20 dosage (for cattle use) groups were protected without weight loss. (E) The mice were tested for adjuvant-residue and index (0–3 points/mouse by the degree of residue size), and the residue in the muscle was counted in the vaccinated groups.
dpi, days post-infection; dpv, days post-vaccination; ELISA, enzyme-linked immunosorbent assay.

Immunogenicity according to the vaccine injected sites

After vaccinating the mice via the IM, SC, or fatty tissue (inguinal fat) route, the mice were challenged with the FMD virus 7 days after inoculation. In the case of vaccination via the fatty tissue route, ISA 15 showed a survival rate of approximately 33%, whereas ISA 206 showed a survival rate of approximately 66% (**Fig. 3A**). The ISA 15 and 206 in SC or fatty tissue route resulted in a severe decrease in body weight in the mice (**Fig. 3B**). When challenged with the FMD virus 21 days after vaccination, the survival rate of the mice was 100%, regardless of the vaccinated site (IM, SC, or fatty tissue) (**Fig. 3C**). When ISA 15 was administered to the fatty tissue, the weight change rate of mice was approximately 5%. On the other hand, the inoculation of ISA206 caused almost no change in the body weight of mice when administered SC or in the fatty tissue (**Fig. 3D**). The level of oil residue in the vaccination area 2 or 4 weeks after vaccination was measured by scores ranging from 0 to 3 (n = 3) and summed (**Fig. 3E**). The W/O/W type of ISA 206 showed the same level of oil residue in the case of IM and SC up to 4 weeks after inoculation. The sum oil residue score decreased by 2 from the second week after inoculation to the fourth week when the vaccine was administered in the fatty tissue. In the case of the O/W type of ISA 15, the sum of the oil residue score decreased by 2–4 weeks after vaccination in both cases of IM and SC. The W/O/W type left oil residue more persistently inside the body.

Protection in the immunized mice with adjuvant and immunostimulants

Considering the mouse survival rate and body weight change rate, the immunostimulants, R848 and HKMT, were more effective on mouse survival than Zymosan in O THI and A22 of FMDV antigen (**Fig. 4**). As a result, a combination of HKMT, R848, or Zymosan with the vaccine antigen was found to be most effective for protection against the FMD virus.

In type O FMDV, the survival rate of mice was increased to 80% when using HKMT or R848 mixed with ISA15. The body weight rate of the mice showed almost no change after inoculation with the virus when R848 was used, and there was a significant difference from the fourth day of vaccination compared to the negative control (**Fig. 4A and B**).

In type A FMDV, the survival rate of the mice was improved in HKMT or R848 with ISA15. There was almost no change in the body weight of mice when the immunomodulator HKMT or R848 was used. The group of HKMT and R848 were significantly different from the negative control on day 2 after viral inoculation, and the Zymosan group was significant on day 4 (**Fig. 4C and D**).

Long-lasting immunity in the immunized pigs

The vaccine containing A22 antigen and ISA15, which was proven to provide immunity in the experiment above, was administered twice to the experimental pigs (0 days, 28 days). Type A was selected as an antigen model for pigs because the type A antigen induces lower antibody levels than other serotype antigens in pigs in a previous study.

The FMDV-structural protein (SP) antibody level reached a peak on the 56th day and decreased gradually thereafter (**Fig. 5A**). From the 28th day of vaccination, the ISA15+ R848+HKMT group and the ISA15+R848+Zymosan group were significantly different from the ISA15 group. The mean antibody titers of the ISA 15+R848+Zymosan groups were highest at 42 and 56 days after vaccination. The neutralizing antibody level was low after 56 days following the initial inoculation (**Fig. 5B**). After 42 days, the neutralizing antibody titer was approximately 1:100 greater than the prevention antibody level, and a relatively high level was maintained afterward. The VNT was implemented using pig serum (**Fig. 5B**). The neutralizing antibody titer began to increase after the second inoculation and reached its
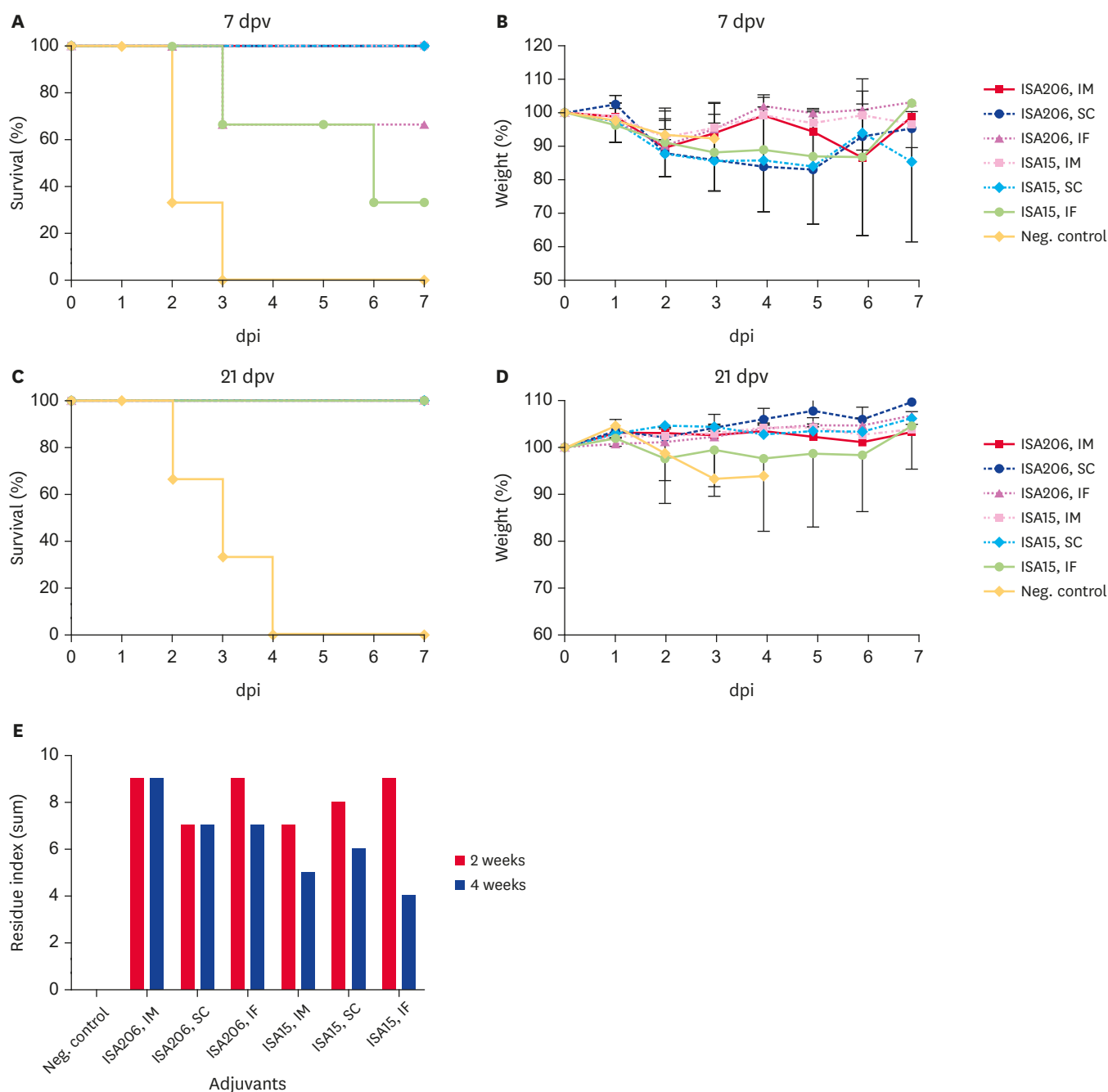
Fig. 3. Protection and immunization of the selected-oil adjuvants with FMDV type O antigen (O Taiwan 97) strain. Survival rate and weight loss after immunization (1/1,000 dose of O TWN for cattle use, 1 dose = 15 µg/mL antigen, 10% Al(OH)$_3$, and 0.5 mg/mL saponin) and challenge along with different injection routes and oil adjuvants. The mice (n = 3) were challenged with FMD O Vet 2013 strain, (A) and (B); 7 dpv, (C) and (D); 21 dpv.
The survival rate in the mice injected by IM, SC, and inguinal fatty tissue (IF) route with ISA206 or ISA15 as an adjuvant. (B) The weight loss rate in the mice injected by IM, SC, and IF route with ISA206 or ISA15 as an adjuvant. (C) The survival rate in the mice injected by IM, SC, and IF route with ISA206 or ISA15 as an adjuvant. (D) Weight loss rate in the mice injected by IM, SC, and IF route with ISA206 or ISA15 as an adjuvant. (E) Frequency of adjuvant-residue through vaccination (14 or 28 dpv) of different adjuvants by various routes (3 mice/group). The mice were tested for their levels of adjuvant-residue and frequency (sum), and the residue in the muscle was counted in the vaccinated groups (0–3 points/mouse).
dpi, days post-infection; dpv, days post-vaccination.

peak on the 42nd day, followed by a gradual decrease. More significant differences in the ISA 15+R848+Zymosan groups compared to the ISA15 control group were observed on the 28th and 70th days of vaccination.
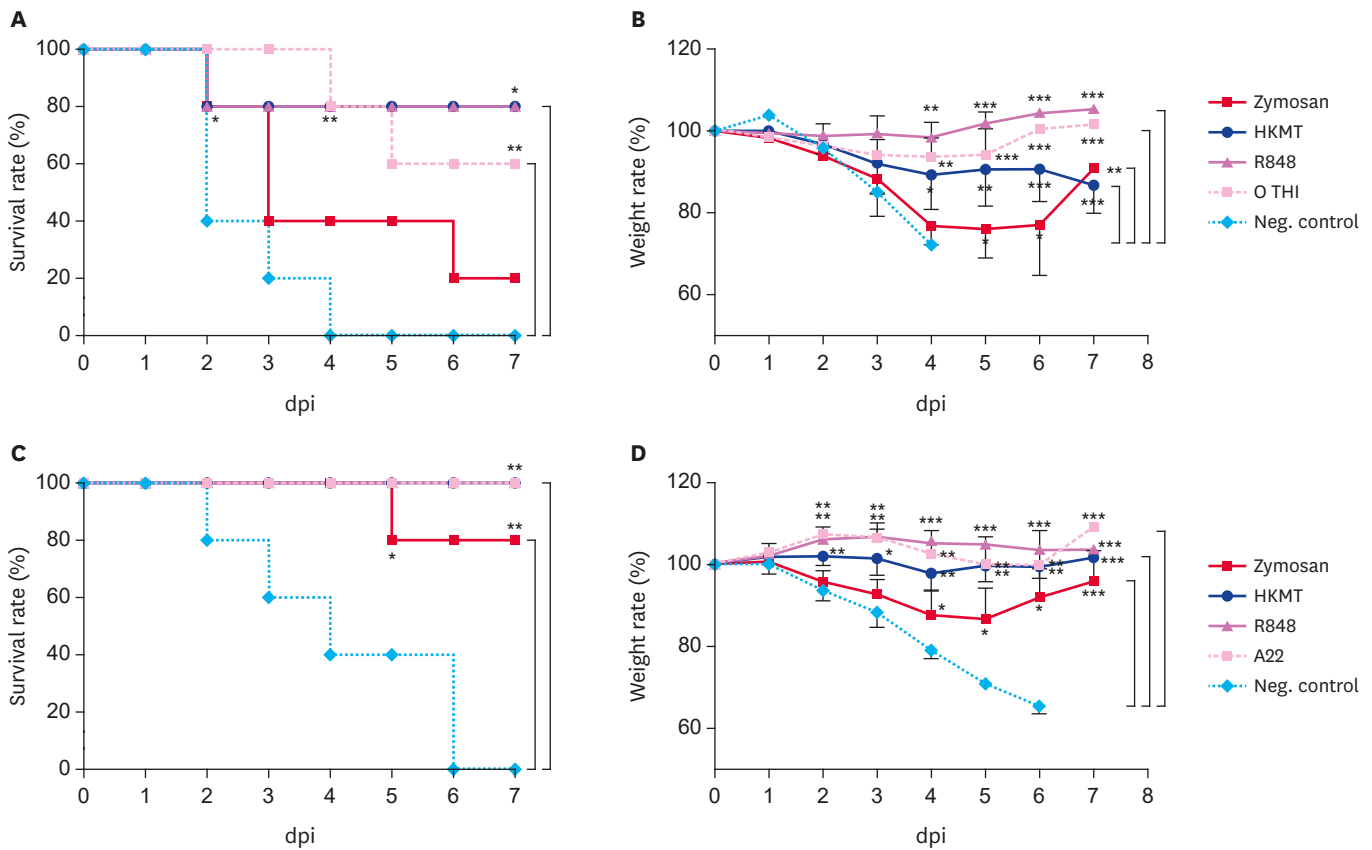
Fig. 4. Survival and weight loss rate through vaccination of various adjuvants with foot-and-mouth disease virus antigens in mice. Survival rate and weight loss after immunization (1/50 dose of O THI-R antigen [A, B], or 1/5,000 of A22 antigen [C, D] with ISA 15) with various stimulants (Zymosan, HKMT, or R848) as vaccine adjuvant. One dose for cattle use contains 15 µg/mL antigen, 10% Al(OH)$_3$, and 0.5 mg/mL saponin. The mice were challenged, along with different adjuvants. The mice (n = 5) were challenged with O Vet 2013 strain [A, B] or A Malay 97 strain [C, D], (A), and (C); survival rate, (B) and (D); weight rate.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to negative control.
dpi, days post-infection.

When the VN titer was compared in pigs, the ISA15 and ISA15+R848+Zymosan group was significantly different at 28 days. At 42 days after vaccination, the VN antibody in all groups, except for one in ISA15, reached high levels. After 70 days, the ISA15+R848+Zymosan group maintained a high VN antibody titer.

Lesions in the vaccine injection site in pigs

Granulomatous lesions (n = 10) were observed at the vaccination sites. All groups were examined with the lesions. The granuloma in the injected sites occurred in approximately 20% of the tested samples, and the detected sizes were 0.3 to 2.0 in diameter (**Table 1**).

Table 1. Virus neutralizing antibody titers and lesion size in injected site after the vaccination by type A antigen and various adjuvants in pigs

Adjuvants with type A (A22) antigen	VN titers (Log 10)			Lesions in the injected site	
	28 days	42 days	56 days	No. of detected/no. of tested	Size in detected muscle (cm)
ISA15	1.488 ± 0.31*	2.53 ± 0.67*	2.23 ± 0.27*	2/10	1, 1.5
ISA15+R848+HKMT	1.27 ± 0.18	2.44 ± 0.26†	1.99 ± 0.30*,‡	2/10	0.5, 2
ISA15+R848+Zymosan	1.34 ± 0.23†	2.53 ± 0.39§	2.35 ± 0.39*	2/10	0.3, 2
Commercial vaccine	1.07 ± 0.17	1.69 ± 0.33	1.99 ± 0.20	n.d	n.d

n.d., not done.
*$p < 0.05$, †$p < 0.01$, §$p < 0.001$ when compared to commercial vaccine containing A22 antigen; ‡p value = 0.033.
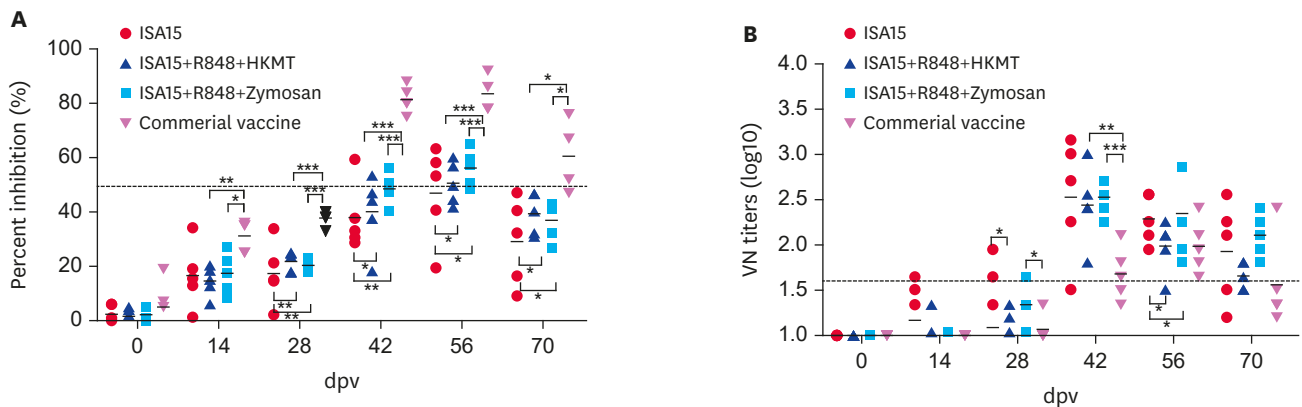
Fig. 5. Long-lasting immunogenicity in the pigs immunized with the vaccine containing various immunostimulants.
The components of experimental vaccine commonly consist of FMDV type A, A22 antigen (15 μg/dose), 0.5 mg/mL of saponin, 10% Al(OH)$_3$, and selectively consist of 500 μg/mL R848, 250 ug/ml HKMT, 250 μg/mL Zymosan, and The SP-ELISA and VN test were performed in the pigs immunized with the commercial vaccine (Boehringer Ingelheim, 2 mL/dose) containing A22 antigen or the experimental vaccines.
The vaccines were injected 0 and 28 dpv in field pigs (n = 5); (A) Percent inhibition value (%) according to SP-ELISA for the vaccinated groups. The dotted lines in SP-ELISA show 50% inhibition (PI), which would be positive in a test. (B) VN antibody titer after vaccination. The dotted lines in the VN test represent 1.65 log VN titer (1:45).
dpv, days post-vaccination; ELISA, enzyme-linked immunosorbent assay; VN, virus-neutralizing.

DISCUSSION

Improvement of the short- and long-term immune effects and safety is the main issue related to a FMD vaccine, particularly in pigs. In general, the FMD vaccine requires re-vaccination 1 month after the initial vaccination, and an additional vaccination is required after 4 to 6 months. Not only is the frequent inoculation inconvenient, but the frequent administration of vaccine-containing oil also results in inevitable granuloma formation. Designing a vaccine that leaves little residue but has a strong immune effect by reducing the number of inoculations or the volume of vaccine administration is a very important issue in the national FMD control policy.

Even in cases of the same amount of FMD vaccine injected, immunity is weakly formed in pigs, while cattle show relatively strong immunity. Efforts have been made to solve the problem of low formation of the FMD antibody titer or low protection level in pigs. Oil adjuvants are used as the main component of FMD vaccines, and ISA206 is the most frequently used oil adjuvant. On the other hand, a recent study reported that a new adjuvant of ISA201 increased the FMD protection rate in pigs by stimulating cell-mediated immunity [14].

In these experiments, the existing oil adjuvants were screened under the same conditions to find the optimal adjuvant in terms of immunogenicity and safety in pigs. The antibody titer increased only when oil adjuvant was used, but oil residue, in this case, was observed within the short term of vaccination. Among the adjuvants, ISA206 had the best effect of immunogenicity with the addition of gel and saponin. In addition, the saponin concentration improved vaccine safety while the gel increased the immune response quite effectively. Vaccines containing ISA206 or ISA15 showed persistent stability regardless of the concentration of additional gel.

The levels of oil residue in the ISA15 and 206 vaccinated groups were relatively low in the case of their inoculation in the inguinal fatty tissue of mice, assuming they were inoculated in the fatty tissue around the muscle of the target animals, compared to intramuscular inoculation.

On the other hand, inoculation of the vaccine in fatty tissue resulted in the formation of weak immunity against the FMD virus within 7 days. The body weight of the mice that were challenged with the FMD virus 7 days after the intramuscular inoculation of vaccine recovered within 3 days. In the case of FMD virus challenge after mid-term of 21 days post-vaccination, the body weight showed almost no change. Intramuscular inoculation of the various routes tested showed the best immune effect in this study.

Currently, research on improving immunity or long-term immunity by adding components that can stimulate TLR (immune adjuvant) to oil is underway. For example, one study produced a vaccine by combining the immune adjuvant of CpG ODN RW03, which stimulated B cell and T cell epitopes, with the ISA206 oil adjuvant and injected the vaccine into cattle. The vaccine was proven effective in inducing long-term immunity [15]. A previous study reported that lipopolysaccharides (LPS) activate Th1 cells by secreting interleukin as a TLR4 agonist, and Flagellin (TLR5 agonist) induces a strong antibody titer against the FMD virus [16]. After nationwide vaccination against FMD in Korea in 2011, many pig producers complained that the economic damage to the farmers was significant because of granuloma lesions, including inflammatory reactions, in pigs inoculated with the foot-and-mouth disease vaccine [17].

In this experiment, Mincle, TLR, and Dectin-1 agonists were proven to enhance the protective immunity of the vaccine. In a comparison of the survival rates and rate of body weight changes after vaccinating the mice with additional immune adjuvants and challenging them with the FMD virus, a combination of HKMT, R848, and Zymosan was proven to be most effective for protection against the FMD virus. This reaction can be attributed to the increased immune reaction resulting from the relevant cytokine, which was induced by the improved response in dendritic cells or macrophages [18]. In the experiment on pigs, a test vaccine with the A22 antigen showed weak long-term immunity persistency, but the addition of HKMT, R848, and Zymosan caused a constant high antibody titer and persistent immunogenicity. In **Fig. 5A**, the foot-and-mouth disease antibodies in pig serum were measured using ELISA, and the results reacted with the virus antigen regardless of the live active and inactive viruses. **Fig. 5B** shows the antibody response to only the live virus as a result of VNT. For this reason, inconsistencies in **Fig. 5A and B** can occur. In many cases, the VNT results indicate protection from FMD because the protection-related immunogenicity is better correlated with neutralization against the live virus. In this pig-immunization experiment, there was a limitation as evidence for protection. In the previous study [19], however, it would be protected in VN antibody titers log 1.65 (1:45) or higher against the FMDV. **Fig. 5** indicates the antibody levels to be protected against FMDV since 28 dpv. In the experiment on mice and pigs, the ISA15 group containing gel and saponin showed good short-term protection and antibody formation to the FMDV while leaving a small oil residue. The 73.7% pigs vaccinated twice in Korea had granuloma in the FMD vaccine injection site, and the economic loss from discarding abnormal neck muscle was approximately US 241 million dollars per year [17]. On the other hand, this would be expected to reduce the economic loss of approximately 45% by applying the vaccine using the adjuvants and immunity-stimulants used in this study. Additional experiments on large numbers of pigs will be needed to determine the exact economic profit.

In conclusion, O/W type ISA15 adjuvant containing gel and saponin showed short- and long-term antibody persistency, despite the low level of granuloma by the oil adjuvant. A combination of C-type lectin and Mincle agonist to the oil adjuvant was effective in

improving the immunity of the FMD vaccine, demonstrating early antibody induction and long-term immunity.

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