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Adjuvanticity of Processed *Aloe vera* gel for Influenza Vaccination in Mice

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ABSTRACT

The effectiveness of current influenza vaccines is considered suboptimal, and 1 way to improve the vaccines is using adjuvants. However, the current pool of adjuvants used in influenza vaccination is limited due to safety concerns. *Aloe vera*, or aloe, has been shown to have immunomodulatory functions and to be safe for oral intake. In this study, we explored the potential of orally administered processed *Aloe vera* gel (PAG) as an adjuvant for influenza vaccines in C57BL/6 mice. We first evaluated its adjuvanticity with a split-type pandemic H1N1 (pH1N1) Ag by subjecting the mice to lethal homologous influenza challenge. Oral PAG administration with the pH1N1 Ag increased survival rates in mice to levels similar to those of alum and MF59, which are currently used as adjuvants in influenza vaccine formulations. Similarly, oral PAG administration improved the survival of mice immunized with a commercial trivalent influenza vaccine against lethal homologous and heterologous virus challenge. PAG also increased hemagglutination inhibition and virus neutralization Ab titers against homologous and heterologous influenza strains following immunization with the split-type pH1N1 Ag or the commercial trivalent vaccine. Therefore, this study demonstrates that PAG may potentially be used as an adjuvant for influenza vaccines.

Keywords: *Aloe vera*; Influenza A virus; Influenza B virus; Vaccines; Immunological adjuvant

INTRODUCTION

Influenza, commonly called “the flu,” is a respiratory illness that heavily impacts public health, leading to 3–5 million severe cases and 290,000–650,000 deaths globally each year (1). These numbers are associated with seasonal influenza caused by influenza A virus (IAV) and influenza B virus (IBV) of the *Orthomyxoviridae* family. IAVs are of zoonotic origin and have high antigenic diversity, with 18 hemagglutinin (HA) and 11 neuraminidase subtypes (2,3). However, only 2 subtypes, A/H1N1 and A/H3N2, currently recur as seasonal influenza. IAVs have also caused 4 pandemics in the past and present centuries: the Spanish Flu (H1N1) in

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

ACM, acemannan; GMT, geometric mean titers; HA, hemagglutinin; HI, hemagglutination inhibition; IAV, influenza A virus; IBV, influenza B virus; i.m., intramuscularly; i.n., intranasally; MBL, mannose-binding lectin; MDCK, Madin-Darby Canine Kidney; PAG, processed *Aloe vera* gel; pH1N1, pandemic H1N1; RDE, Receptor Destroying Enzyme; $TCID_{50}$, 50% tissue culture infectious dose; TIV, trivalent influenza vaccine; VN, virus neutralization

Author Contributions

Conceptualization: Kim JK, Lee CK, Park YI; Data curation: Kim JK, Song EJ, Español E; Formal analysis: Kim JK, Lee CK, Park YI, Shim KS, Shin E; Funding acquisition: Kim JK; Investigation: Song EJ, Nam JH, Kim J, Español E; Methodology: Kim JK, Lee CK; Project administration: Lee CK, Park YI, Shim KS, Shin E; Resources: Shim KS, Shin E; Supervision: Kim JK; Visualization: Song EJ, Español E; Writing - original draft: Kim JK, Song EJ, Español E; Writing - review & editing: Kim JK, Español E, Lee CK.

1918, the Asian Flu (H2N2) in 1957, the Hong Kong Flu (H3N2) in 1968, and, most recently, the pandemic (H1N1) in 2009, which further highlight their public health significance. IBVs, on the other hand, generally infect only humans with occasional spillovers to seals. The 2 known lineages of IBVs (B/Victoria-like and B/Yamagata-like) currently co-circulate as seasonal influenza viruses.

Vaccines are the primary tools for preventing influenza virus infection. Seasonal influenza vaccines are typically inactivated trivalent influenza vaccine (TIV) formulations that carry 2 IAV strains and 1 IBV strain and are generally administered intramuscularly (i.m.). Live-attenuated influenza vaccines and quadrivalent influenza vaccines (carrying 2 IAV and 2 IBV strains) have also been licensed recently. However, the effectiveness of the seasonal influenza vaccine varies from season to season and averages at 50%–60% (4). This is mostly attributed to the high variability of the virus, which necessitates semi-annual reformulation of the vaccine to match the predominant circulating strains. Yet, a study has reported that even with a good match between the recommended vaccine and the currently circulating strains, the vaccine was not significantly preventive of infection (5). Furthermore, vaccine effectiveness is even lower among the elderly who are at high risk of death due to complications that arise from infection (6). Immunity elicited by the vaccine is also short-term, possibly due to the inability of the vaccines to elicit cross-protective T cell responses (7). Additionally, history of previous vaccinations has been linked to reduced effectiveness of subsequent vaccinations (8-10). Taken together, these issues suggest that the currently available influenza vaccines and the current vaccination strategies need to be improved further.

One of the ways to increase vaccine effectiveness is through the use of adjuvants, a diverse class of compounds that can enhance the immune response elicited by an Ag. Influenza vaccine formulations that include adjuvants use aluminum salts (i.e., alum) or MF59, an oil-in-water emulsion. Alum has been correlated with mild to severe adjuvant-related local side-effects and has a bias for eliciting the Th2 response, which is more critical for extracellular pathogens rather than viral infections. MF59, on the other hand, has been shown to be safe in children (11). AS03 is an adjuvant included in a pandemic influenza vaccine formulation; however, it has been associated with narcolepsy (12,13). A pandemic influenza vaccine containing a heat-labile enterotoxin adjuvant had to be discontinued due to correlations with Bell's palsy (14). As such, the number of adjuvants currently used for influenza vaccines is limited by their safety. Therefore, the discovery and characterization of new and safe adjuvants can help enhance the effectiveness of influenza vaccines.

Aloe vera or aloe has been used medically for thousands of years. *Aloe vera* gel extracts have been reported to have a host of biological and pharmacological activities such as antibacterial, anti-inflammatory, antiproliferative capacity and so on (15-17). Oral administration of *Aloe vera* gel has been found to have immunostimulatory activity in mice (18). Acemannan (ACM), the major polysaccharide in *Aloe vera* gel, has been reported to have immunomodulatory effects (19). The growing evidence for the immune-enhancing properties of *Aloe vera* gel and its components suggests that *Aloe vera* gel can potentially be used as an adjuvant. Indeed, ACM has been demonstrated to have adjuvant activity in poultry vaccines, while *Aloe vera* gel has been reported to have adjuvant activity against oral submucous fibrosis (20,21).

Adjuvants are not approved independently (i.e., an adjuvant is only approved as a part of a vaccine formulation), and the effectivity of an adjuvant with one formulation or with one Ag does not guarantee effectivity with others. Therefore, adjuvants have to be tested for each

formulation. In this study, we aimed to demonstrate the feasibility of orally administered processed *Aloe vera* gel (PAG) as an adjuvant to influenza vaccines. We used PAG in combination with a split pandemic H1N1 (pH1N1) Ag and a commercial TIV in mice, and we characterized the adjuvant effects of oral PAG by monitoring the health and survival of mice after homologous and heterologous influenza challenge. We also measured immune correlates of protection after immunization. Our results suggest that PAG has potential to be used as an adjuvant in influenza vaccination.

MATERIALS AND METHODS

Preparation of PAG

PAG was prepared by processing the gel of *Aloe vera* as previously described (22). Briefly, *Aloe vera* gel was incubated with cellulase, which was then terminated by heating. The cellulose-treated gel was filtered through a charcoal column to remove anthraquinone and other coloring matters. The molecular weight distribution of polysaccharides in PAG was determined as previously described (22). PAG was dissolved in PBS for all experiments in this study.

Viruses and reagents

Influenza virus strains, including A/Korea/01/2009 (H1N1) and A/Philippines/2/1982 (H3N2) viruses, were grown in 10-day-old embryonic chicken eggs at 37°C or in Madin-Darby Canine Kidney (MDCK) cells. After 48 h or after showing cytopathic effects, the allantoic fluid or cell culture supernatant was harvested and stored at −80°C until use. Aluminum hydroxide (alum) and AddaVax® were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and InvivoGen (San Diego, CA, USA), respectively.

Mouse source and maintenance

All animal experiments were performed in biosafety level 2-plus facilities at the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Daejeon, South Korea). Six-week-old C57BL/6 mice were purchased from Korea Animal Technology (Pyeongtaek, South Korea), and ten mice per group were used. General animal care was provided as required by the Institutional Animal Care of Use Committee (IACUC) of the KRIBB (approval No. KRIBB-AEC-10046).

Mouse immunization with pH1N1 Ag and homologous challenge

Two groups of mice (n=10/group) were administered PAG (800 mg/kg of mouse body weight; oral, p.o.) once daily for a total of 4 wk. Of these, 1 group was immunized i.m. with A/California/07/2009 (H1N1) virus Ags (800 ng/mouse) 2 and 4 wk from the start of PAG administration (Ag + PAG group), while the other was maintained without immunization (PAG group). As positive controls, 2 groups of mice were likewise immunized with the pH1N1 Ags with either alum (Ag + alum group) or with AddaVax® (Ag + AddaVax group) as adjuvant. As negative controls, 2 groups of mice were immunized twice with the pH1N1 Ags in the absence of adjuvant (Ag group) or injected twice with PBS (PBS group) at 2-wk intervals. Two wk after the second immunization, all mouse groups were intranasally (i.n.) administered with 10 LD_{50} of A/Korea/01/2009 (H1N1) virus. Body weight and mortality were monitored daily for 2 wk.

Mouse immunization with trivalent commercial vaccine and homologous challenge

A group of mice (n=10) was administered PAG (800 mg/kg of mouse body weight; p.o.) once daily for a total of 6 wk and immunized (i.m.) with a single dose (3 μg/mouse) of

a commercial trivalent seasonal influenza vaccine for use in the 2014–2015 influenza season, containing A/California/07/2009 (H1N1)-like, A/Texas/50/2012 (H3N2)-like, and B/Massachusetts/02/2012-like viruses (Green Cross, Yongin, South Korea) 4 wk from the start of PAG administration (vaccine + PAG group). As positive control, 1 group of mice was likewise immunized (i.m.) with the vaccine using alum (vaccine + alum group) as adjuvant. As negative controls, 2 groups of mice were immunized once with the vaccine in the absence of adjuvant (vaccine group) or injected once with PBS (PBS group). Three wk after immunization, all mouse groups were administered (i.n.) with 10 LD_{50} of A/Korea/01/2009 (H1N1). Body weight and mortality were monitored daily for 2 wk.

Mouse immunization with trivalent commercial vaccine and heterologous challenge

A group of mice (n=10) was administered PAG (800 mg/kg of mouse body weight; p.o.) once daily for a total of 6 wk and immunized (i.m.) 3 times with 3 μg/mouse of a commercial trivalent seasonal influenza vaccine for use in the 2014–2015 influenza season, containing A/California/07/2009 (H1N1)-like, A/Texas/50/2012 (H3N2)-like, and B/Massachusetts/02/2012-like viruses (Green Cross), at 2, 4, and 6 wk from the start of PAG administration (vaccine + PAG group). As positive controls, 2 groups of mice were likewise immunized (i.m.) with the vaccine with either alum (vaccine + alum group) or AddaVax® (vaccine + AddaVax group) as adjuvant. As negative controls, 2 groups of mice were immunized 3 times with the vaccine in the absence of adjuvant (vaccine group) or injected 3 times with PBS (PBS group) at 2-wk intervals. Two wk after the third immunization, all mouse groups were administered (i.n.) with 5 LD_{50} of A/Philippines/2/1982 (H3N2) virus. Body weight and mortality were monitored daily for 2 wk.

Mouse blood sampling

Blood samples were collected through retro-orbital bleeding from mice in all groups prior to immunization and 2 wk after the last immunization. Blood samples were stored at −20°C until use.

Hemagglutination inhibition (HI) assay

The HI assay was performed on mouse blood samples as previously described (23). To remove any non-specific inhibitors of hemagglutination, serum samples were treated with Receptor Destroying Enzyme (RDE; Denka Seiken, Tokyo, Japan) by incubation overnight at 37°C and then for 30 min at 56°C. Serially diluted serum samples were mixed with 4 hemagglutination units in PBS of virus (A/Korea/01/2009 [H1N1] or A/Philippines/2/1982 [H3N2], depending on the samples being analyzed), and then incubated with serial 2-fold dilutions of the RDE-treated serum samples at room temperature for 30 min. After incubation, 0.5% turkey or chicken RBCs was added to test for HA activity, and HI titers were recorded after another 30 min. HI Ab titers were determined according to the World Health Organization manual using turkey erythrocyte (24). HI Ab titers are described as geometric mean titers (GMT).

Virus neutralization (VN) assay

The VN assay was performed as described previously (25). To determine the VN Ab titer, MDCK cells were plated into 96-well culture plates. Heat-inactivated serum samples were serially diluted 2-folds. Virus was diluted to contain 2,000 50% tissue culture infectious dose ($TCID_{50}$)/ml in serum-free MEM medium (0.3% BSA and 1.0 μg/ml of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin; Worthington Biochemical Corporation,

Lakewood, NJ, USA). The diluted sera were mixed with 2,000 $TCID_{50}$/ml virus at 37ºC for 1 h. The Ag-Ab mixture was transferred into each well of MDCK cells in 96 well plates, and the plates were incubated at 37ºC for 72 h. The supernatant (50 µl) was harvested and tested for HA assay by the addition of 0.5% chicken RBCs. GMT were calculated for each group of serum samples.

Statistical analysis

Data analysis was performed using GraphPad Prism, and differences were evaluated using the 1-way ANOVA. The p-values less than 0.05 ($p<0.05$) were considered to be statistically significant.

RESULTS

PAG enhances the protective efficacy of the pH1N1 Ag against lethal challenge in mice

Split-virus Ags, prepared by chemical dissociation of virions to expose viral proteins, are typically used in inactivated influenza virus vaccine formulations. In this study, we first evaluated the ability of PAG to enhance the protectivity of a split-type pH1N1 Ag in mice. Six-week-old C57BL/6J mice were orally administered with PAG (800 mg/kg of body weight) once daily for 4 wk (**Fig. 1A**). The dosage of PAG used in mice was based on the dose of PAG used as functional food (in supplement form) for human consumption. Two wk from the start of PAG administration, the mice were vaccinated i.m. with the pH1N1 Ag (800 ng/mouse). As positive controls, 2 groups of mice were immunized i.m. with a mixture of the pH1N1 Ag and either AddaVax® (an oil-in-water emulsion similar to MF59) or alum. As negative control groups, 3 groups of mice were given only the pH1N1 Ag, PAG, or PBS. A booster injection of each formulation was performed 2 wk after the first immunization. At 2 wk after the booster immunization, the mice were challenged with 10 LD_{50} of a homologous virus (A/Korea/01/2009, H1N1). Body weight (**Fig. 1B**) and survival (**Fig. 1C**) were monitored daily for 14 days.

As shown in **Fig. 1B and C**, no significant body weight changes and no death were observed in the PAG-, alum- and AddaVax®-adjuvanted groups of mice. However, the group of mice vaccinated with only the pH1N1 Ag had 60% survival, and the groups of mice given either PAG or PBS showed 10% survival. These results show that the oral administration of PAG significantly increased the protective capacity of the pH1N1 Ag in mice to levels that are comparable to AddaVax® and alum, indicating the potential of PAG for enhancing the protectivity of the pH1N1 Ag in mice.

PAG enhances humoral immune responses to the pH1N1 Ag in mice

To determine the effects of oral PAG administration on mouse Ab responses to the pH1N1 Ag, we obtained sera from all mice prior to vaccination and after the second vaccination. We then evaluated the HI and VN Ab titers of the sera. Prior to vaccination, all the mice had undetectable (<10) HI and VN titers (**Fig. 1D and E**). The HI and VN Ab titers significantly increased after the second immunization when the mice were given PAG compared to when the mice were administered the pH1N1 Ag alone. Both the AddaVax® and alum groups also had higher HI and VN Ab titers than the Ag-only group. These results suggest that oral PAG administration contributed to elicit higher humoral immune responses, which would be responsible for enhancing the protectivity of the pH1N1 Ag.

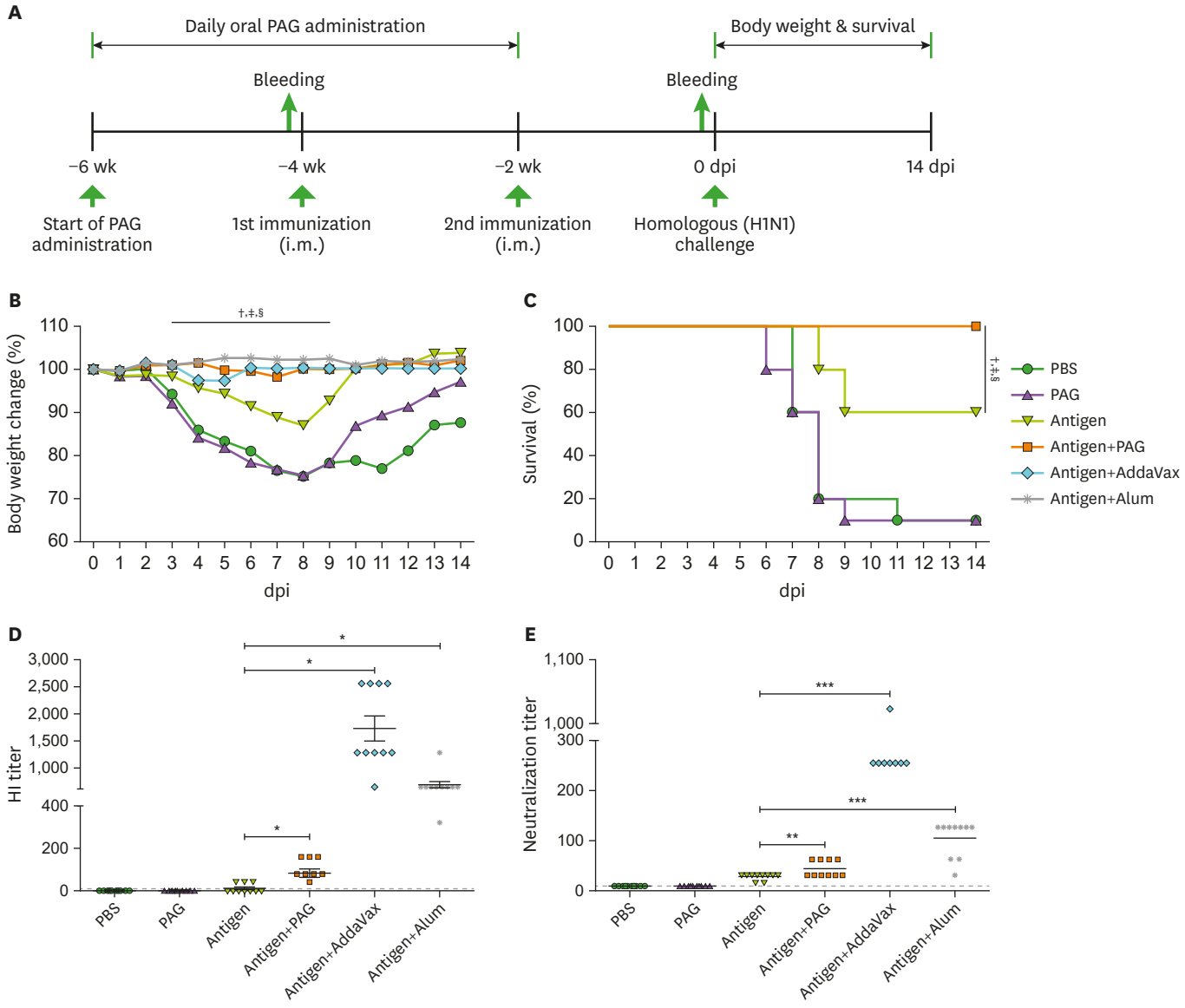

Figure 1. Adjuvant effects of orally administered PAG in mice immunized with the pH1N1 Ag. (A) C57BL/6J mice (n=10 per group) were orally administered with PAG (800 mg/kg of body weight) once daily for 4 wk and immunized i.m. with the pH1N1 Ag (800 ng/mouse) twice at 2-wk intervals. Mice were infected by intranasal administration of 10 LD_{50} of A/Korea/01/2009 (H1N1) virus 2 wk after vaccination, and changes in (B) body weight and (C) mouse survival were monitored for 14 dpi. $^{\dagger,\ddagger,\S}p<0.05$, statistically significant difference between the non-adjuvanted and adjuvanted groups. (D) HI and (E) VN Ab titers against the challenge virus (pH1N1) were determined from blood samples collected prior to vaccination and 2 wk after final immunization. Bars indicate GMT of the HI-positive serum samples (≥10), and comparisons of the statistical data were carried out using 1-way ANOVA.
dpi, days post-infection.
†Ag only vs. PAG-adjuvanted; ‡Ag only vs. Ag + AddaVax; §Ag only vs. Ag + alum.
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with the non-adjuvanted Ag group.

PAG enhances the protective efficacy of a TIV against homologous lethal challenge in mice

Next, we evaluated the adjuvant effects of orally administered PAG in mice immunized with a commercial trivalent seasonal influenza vaccine (GC Flu®) containing A/California/07/2009 (H1N1)-like, A/Texas/50/2012 (H3N2)-like and B/Massachusetts/02/2012-like viruses. We first compared the protective efficacy of the vaccine against lethal challenge with a homologous pH1N1 virus (A/Korea/01/2009) in the presence or absence of oral PAG administration. Six-week-old C57BL/6J mice were orally administered with PAG (800 mg/kg of body weight) once

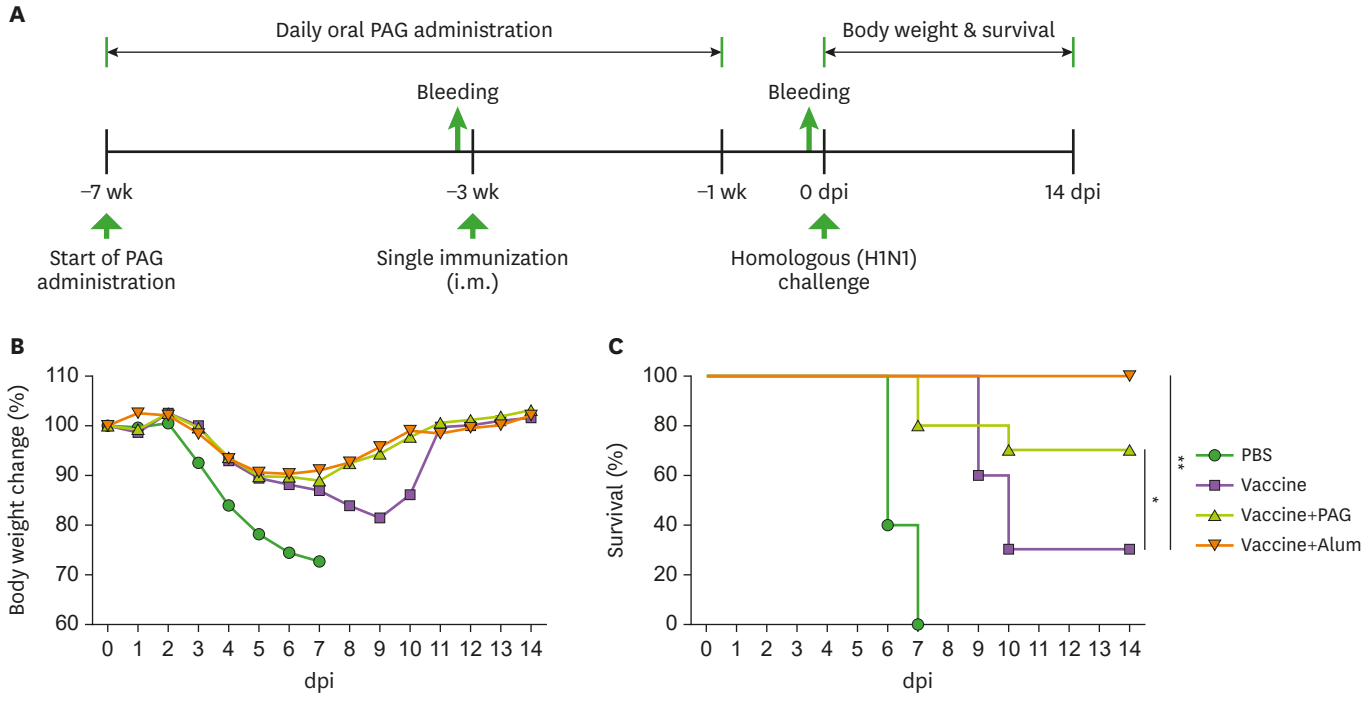

Figure 2. Adjuvant effects of orally administered PAG in mice immunized with a commercial trivalent vaccine. (A) C57BL/6J mice (n=10 per group) were orally administered with PAG (800 mg/kg of body weight) once daily for 6 wk and immunized (i.m.) with a single dose (3 μg/mouse) of a commercial TIV 4 wk from the start of PAG administration. Mice were infected by intranasal administration of 10 LD_{50} of A/Korea/01/2009 (pH1N1) virus 3 wk after vaccination, and changes in (B) body weight and (C) mouse survival were monitored for 14 dpi.
dpi, days post-infection.
*p<0.05, **p<0.01, compared with the non-adjuvanted vaccine group, Student's *t*-test.

daily for 6 wk and immunized as shown in **Fig. 2A**. Four wk into PAG administration, one mouse group (vaccine + PAG) was immunized with the trivalent vaccine. No significant body weight changes were observed when the mice were immunized in combination with either alum or PAG (**Fig. 2B**). Oral administration of PAG significantly enhanced the survival rate of mice immunized with the commercial trivalent seasonal influenza vaccine to 70% (vaccine + PAG) from 30% in the vaccine-only group (**Fig. 2C**), which was quite similar to the results of pH1N1 split-type Ag + PAG. The alum group (vaccine + alum) had the highest mouse survival rate at 100%. These results suggest that PAG enhances the protectivity of the trivalent vaccine to homologous strains of influenza.

PAG enhances the protective efficacy of a trivalent influenza vaccine against heterologous lethal challenge in mice

We next assessed the ability of PAG to enhance the protective efficacy of the commercial trivalent vaccine against lethal heterologous challenge. A group of mice was orally administered with PAG for 6 wk and immunized 3 times at 2-wk intervals, beginning from 2 wk after the start of PAG administration (**Fig. 3A**). At 2 wk from the third immunization, the mice were challenged with 5 LD_{50} of A/Philippines/2/1982 (H3N2), which comes from a different phylogenetic A/H3N2 clade compared to that of A/Texas/50/2012 (H3N2)-like virus included in the vaccine (**Supplementary Fig. 1**). Groups of mice for the positive adjuvant controls (AddaVax® and alum), the vaccine-only control, and the PBS control were similarly immunized and challenged. We monitored body weight and survival of the mice daily for 2 wk. As shown in **Fig. 3B**, the vaccine + PAG group had 20% decrease in mean body weight after challenge. Using PAG, AddaVax® or alum as adjuvant significantly improved survival

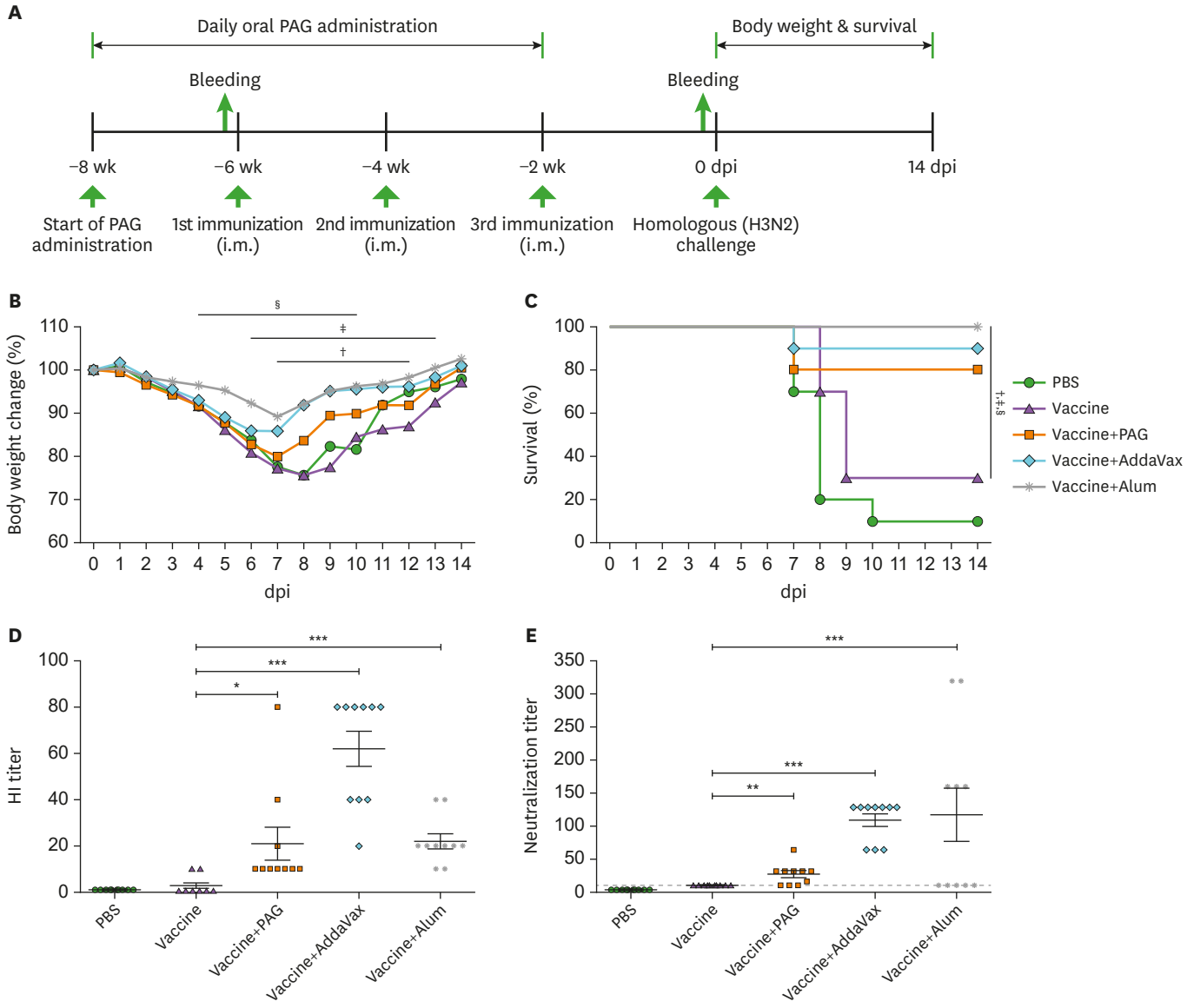

Figure 3. Adjuvant effects of orally administered PAG in mice immunized with a commercial TIV. (A) C57BL/6J mice (n = 10 per group) were orally administered with PAG (800 mg/kg of body weight) once daily for 6 wk and immunized (i.m.) with a commercial TIV (3 μg/mouse) 3 times at 2-wk intervals. Mice were infected by intranasal administration of 5 LD_{50} of A/Philippines/2/1982 (H3N2) virus 2 wk after vaccination, and changes in (B) body weight and (C) mouse survival were monitored for 14 dpi. (B) $^{\dagger,\ddagger,\S}p<0.05$, (C) $^{\dagger}p<0.05$, $^{\ddagger,\S}p<0.01$, statistically significant difference between the non-adjuvanted and adjuvanted groups. (D) HI and (E) VN Ab titers against the challenge virus (pH1N1) were determined from blood samples collected prior to vaccination and 2 wk after final immunization. Bars indicate GMT of the HI-positive serum samples (≥10), and comparisons of the statistical data were carried out using the 1-way ANOVA.
dpi, days post-infection.
†Vaccine only vs. PAG-adjuvanted; ‡Vaccine only vs. AddaVax-adjuvanted; §Vaccine only vs. alum-adjuvanted.
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the non-adjuvanted vaccine group.

rates of mice to 80%, 90%, and 100%, respectively, compared to the 30% survival rate of mice in the vaccine-only control group (**Fig. 3C**). These results imply that PAG could improve the cross-protection of the commercial influenza vaccine in mice.

PAG enhances mouse Ab response to heterologous influenza strain

To determine the ability of PAG to enhance the immunogenicity of a trivalent commercial vaccine against a heterologous A/H3N2 strain, we collected sera from the different mouse groups pre-vaccination and after the third vaccination. Prior to vaccination, all the mice

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had undetectable (<10) HI (**Fig. 3D**) and VN (**Fig. 3E**) Ab titers against the A/Philippines/2/82 (H3N2) virus. However, after the third immunization, the HI and VN Ab titers against the A/H3N2 virus significantly increased in the PAG group compared to those in the group immunized with the vaccine alone. Consistent with our results using the pH1N1 split-type Ag, these results suggest that PAG could enhance the humoral immune response elicited by the vaccine.

DISCUSSION

Although vaccination is considered the best form of prophylaxis against influenza virus infection, the effectiveness of the seasonal influenza virus vaccine varies from season to season, between age groups, and among people with different influenza vaccination histories. Influenza vaccine effectiveness is also considered suboptimal. Therefore, there is a general consensus that the current influenza vaccines and vaccination strategies need to be improved. Including an adjuvant in a vaccine formulation is one of the ways to increase vaccine effectiveness by increasing the magnitude, breadth, and duration of immune responses elicited by the vaccine. In the context of an influenza vaccine, an adjuvant can: 1) improve the overall protectivity of the vaccine, especially in children, the elderly, and the immune-compromised, who are most susceptible to infection; 2) lower the overall Ag and dosage requirement for vaccination; and 3) allow the vaccines to be immunogenic in other routes of administration.

Currently, only 2 adjuvants are approved for the seasonal influenza vaccine: alum and MF59. Alum is the oldest adjuvant in use and is easily approved in formulations due to its history. However, alum has been associated with mild to severe local side effects. Additionally, alum's effects are biased toward the Th2 Ab response, which is less capable of combatting intracellular pathogens than the Th1 response; other adjuvants that lean towards the Th1 response may prove to be more beneficial in an influenza vaccine than alum. On the other hand, MF59 has been shown to elicit a diverse Ab response against influenza; to enhance the influenza virus effectiveness in children and adults; and to be generally safe (11,26,27). Given the suboptimal effectiveness of the influenza vaccine with the current repertoire of adjuvants, the discovery of other adjuvants will be beneficial to the improvement of influenza vaccines.

However, safety regulations for licensing vaccines that include adjuvants are very strict, thereby limiting the pool of currently approved adjuvants used in vaccine formulations. As in the case with AS03 and an enterotoxin-based adjuvant in pandemic influenza vaccines, using adjuvants may cause side effects that can outweigh the benefits of the formulation or that may require reformulation of the vaccines. New and safe adjuvants will have to be discovered not only for the improvement of influenza vaccines but also for the development of other vaccines. Deriving adjuvants from sources that are considered safe would reduce the likelihood of adverse effects. Among these sources would be natural products that have long history of human use. *Aloe vera* or aloe, a succulent of the *Liliaceae* family, has been used in traditional medicine for thousands of years for a variety of applications such as wound healing, treating fungal infections, skin moisturization, and as a laxative. More in depth studies have also revealed several immune-related activities of aloe gel. The gel of aloe leaf has been commercialized in edible form, and studies have shown that oral intake of aloe juice or gel do not have toxic effects in mice (28,29). Given the immune-enhancing properties of aloe, we decided to evaluate its adjuvant effects with influenza vaccines.

Our results show that PAG enhances the protectivity of an influenza split-type Ag or a trivalent commercial vaccine in mice against homologous or heterologous challenge as indicated by increased mouse survival. Evaluation of the HI and VN Ab titers showed that PAG is able to increase mouse Ab response to influenza. This indicates that PAG improved the protectivity of the Ag and the vaccine by enhancing the protective humoral response in mice. Ab titers, particularly those of HI, are correlated with protectivity in humans. Whether this apparent adjuvant activity of PAG in mice will translate to enhanced humoral responses and enhanced vaccine effectiveness in humans will have to be determined in future studies.

The results of our study corroborate with other reports on the immune-enhancing capacities of aloe gel. In particular, a study has demonstrated that the production of anti-sheep RBC Abs in mice was enhanced by treatment with orally administered aloe gel (18). This study has also shown that aloe gel enhances splenocyte response to phytohemagglutinin, which is a strong T cell mitogen. A study in rabbits has also shown that oral administration of aloe extracts in combination with a vaccine against myxomatosis increased the production of $CD8^+$ and $CD4^+$ T cells and raised serum IgG and IgM levels (30). As shown by our results, the combination of PAG and the vaccine or Ag increased serum Ab titers against influenza compared to immunization with the unadjuvanted vaccine or Ag. This increase in Ab titers may be partially responsible for the improved protectivity conferred by the vaccine or Ag upon administration with PAG. However, we did not measure any indicator of cell-mediated immune response and therefore cannot state whether PAG also enhances cellular immunity against influenza. Interestingly, in the PAG + vaccine group challenged with homologous pH1N1, we observed seroconversion (based on HI Ab titers) in only 2 out of 10 mice (**Supplementary Table 1**); however, this group still had a higher survival rate than the vaccine-only group. This may indicate that an enhanced humoral immune response due to PAG is not solely responsible for the increased protectivity of the vaccine in mice, suggesting that, as other studies have shown, PAG may also enhance influenza vaccine protectivity by improving the cellular immune response. Notably, recent studies have shown that cell-mediated immunity is important in influenza clearance, in preventing influenza symptoms, in maintaining memory for longer-lasting protection, and in cross-protection against influenza (31-34). As such, stimulating the cellular, as well as the humoral, immune response has become one of the goals of new influenza vaccine formulations. Whether PAG does improve the cell-mediated immune response will be an interesting topic for future studies as both alum and MF59 have been generally correlated with enhanced Ab responses and both have limited evidence for enhancing cell-mediated immunity.

Most of the immune-enhancing properties of aloe gel is attributed to ACM, the major polysaccharide in aloe gel. ACM has been shown to have immunomodulatory activities (19). It has been reported to induce maturation of dendritic cells, which are important in the activation of T cell responses (35); to enhance the generation of cytotoxic T-cells in response to an alloantigen (36); and to upregulate cytokine production in different model systems (19,37). Taken together, these previous reports suggest mechanisms through which ACM enhances the immunogenicity of the influenza vaccine. However, ACM is not the only component of aloe gel that has been reported to have immune-enhancing capabilities. Lectins, also called Aloctins, have been extracted from aloe and found to stimulate IL-2, IL-3, and IFN-γ production in T cells (38). Another type of lectin from aloe (ATF1011) was also found to activate helper T cells (39). Other polysaccharide and glycoprotein fractions of the aloe gel have also been reported to have immunoreactive capabilities (40). Evidently, aloe gel is a complex mixture of compounds that have immune-enhancing capabilities. Some

have proposed that these components work synergistically and will have to be used together instead of in isolation to maximize their pharmacological capacities (41); the PAG form may therefore be better than using ACM alone as an adjuvant. However, this will have to be confirmed in studies comparing the adjuvant effects of PAG and ACM.

Another potential mechanism through which PAG may enhance the immune response to influenza Ags is by activating complement through the lectin pathway. Influenza has been found to activate the 3 complement pathways, and complement has been found to neutralize influenza. Additionally, the absence of mannose-binding lectin (MBL), an initiator of complement through the lectin pathway, increased mouse susceptibility to influenza infection (42). ACM, which is a type of mannan, may be able to bind MBL and be recognized as a pathogen associated molecular pattern to start the lectin pathway cascade. While this is still largely unexplored, this may be worth investigating as a possible mechanism for the adjuvanticity of PAG.

Taken together, in this study, we have shown that orally administered processed aloe gel can act as an adjuvant to influenza Ags or vaccine in mice as indicated by mouse survival and Ab titers. Human studies will have to be performed to verify the adjuvanticity of PAG with influenza vaccines. Furthermore, we only investigated the effects of PAG on Ab correlates of protectivity against influenza, namely the VN and HI Ab titers. Studies on other indicators of immune response and on the specific pathways of immunity that PAG activates will have to be performed to fully understand the mechanisms for its adjuvanticity, and to maximize its benefits on influenza vaccines. Additionally, we only used orally administered PAG in powder form; adjuvants are typically included in vaccine formulations and administered together with the vaccine components. Future studies may then be performed using PAG or aloe gel components in influenza vaccine formulations and through other routes of administration. Interestingly, a different study has shown that ACM administered through the nasopharyngeal route acts as an adjuvant to the hepatitis B virus surface Ag in mice, suggesting that ACM, and potentially PAG, may be used in this manner in the context of an influenza vaccine as well (43). Overall, we have been able to show that PAG is a new, naturally derived potential adjuvant that can help improve the effectiveness of influenza vaccines.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Serum HI Ab GMT following single-dose vaccination with the trivalent influenza vaccine

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IMMUNE NETWORK

Supplementary Figure 1

Phylogenetic relationship between the vaccine strain (A/Texas/50/2012 [H3N2]) and the virus used for heterologous challenge in mice (A/Philippines/2/1932 [H3N2]).

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