Exploring the usage of inactivated whole virus particle vaccines for influenza and COVID-19

（インフルエンザおよびCOVID-19不活化ウイルス全粒子ワクチンにより誘導される免疫効果の検討）

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Abbreviations

A/Brisbane (H1N1)	A/Brisbane/02/2018 (IVR-190E) (H1N1) pdm09
A/California (H1N1)	A/California/7/2009 (X-179A) (H1N1) pdm09
A/Hokkaido (H5N1)	A/duck/Hokkaido/Vac-3/2007 (H5N1)
A/Singapore (H3N2)	A/Singapore/INFIMH-16-0019/2016 (IVR-186) (H3N2)
A/Singapore (H1N1)	A/Singapore/GP1908/2015 (IVR-180) (H1N1) pdm09
ANOVA	analysis of variance
APC	antigen-presenting cell
B/Maryland (Victoria)	B/Maryland/15/2016 (NYMC BX-69A) (Victoria lineage)
B/Phuket (Yamagata)	B/Phuket/3073/2013 (Yamagata lineage)
Ccl2	C-C motif chemokine ligand 2
Co WPV	COVID-19 inactivated whole virus particle vaccine
COVID-19	coronavirus disease 2019
Csf2	colony stimulating factor 2
Csf3	colony stimulating factor 3
Cxcl10	C-X-C motif chemokine ligand 10
DMEM	Dulbecco's modified Eagle medium
dpi	days post infection
FBS	fetal bovine serum
Flu WPV	monovalent influenza inactivated whole virus particle vaccine
H&E	hematoxylin and eosin
HA	hemagglutinin
HI	hemagglutination inhibition

Icam	intercellular adhesion molecule 1
Ifng	interferon gamma
IIV	inactivated influenza vaccines
Il10	interleukin 10
Il1b	interleukin 1 beta
Il6	interleukin 6
MDCK	Mardine-Darby canine kidney
mRNA	messenger RNA
NA	neuraminidase
NI	neuraminidase inhibition
NIID	National Institute of Infectious Diseases
OD	optical density
PBS	Phosphate buffered saline
PFU	plaque forming unit
qFlu WPV	quadrivalent influenza inactivated whole virus particle vaccine
RDE	receptor destroying enzyme
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcriptase polymerase chain reaction
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SEM	standard error of the mean
SV	split virus vaccine
TMPRSS2	transmembrane protease serine 2
Tnf	tumor necrosis factor
TPCK	N-Tosyl-L-phenylalanine chloromethylketone
WHO	World Health Organization

Notes

1. The contents of Chapter I have been published in *Vaccines* as follows;

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2. A manuscript is in preparation based on the contents of Chapter II.

Preface

Respiratory viruses are among pathogens causing human diseases with profound public health impacts. In particular, influenza and coronavirus disease 2019 (COVID-19) are important since these diseases provoked severe pandemics that have caused enormous damages globally. Worldwide, approximately, 3–5 million people are infected each year with seasonal influenza viruses while as of June 2023, severe acute respiratory syndrome virus 2 (SARS-CoV-2) has claimed over 6.9 million lives and infected over 767 million people since its emergence in 2019. Influenza viruses and SARS-CoV-2 are enveloped viruses whose surface proteins undergo antigenic changes under selective host immune pressure that could lead to variants with immune-evading capabilities. The co-circulation of seasonal influenza viruses with SARS-CoV-2 is particularly dreadful due to the unpredictability of the emergence of these immune-escaping variants/subtypes. Accordingly, the World Health Organization (WHO) recommends vaccination to control the prevalence of both diseases.

Seasonal influenza vaccines are in two main forms: live virus vaccines composed of an attenuated form of influenza virus and inactivated influenza virus vaccines (IIVs), where the virus is killed such as split virus vaccine (SV), whole virus particle vaccines (WPV), and subunit vaccines. SV consists of viral components that are the products of purified virions that are disrupted with ether or detergent, while subunit vaccine undergoes an additional purification step for the further enrichment of the surface proteins, hemagglutinin (HA) and neuraminidase (NA). By contrast, the WPV comprises intact viruses which are inactivated by chemical methods such as formaldehyde and β-propiolactone. Following the start of the COVID-19 pandemic, several platforms were used to develop COVID-19 vaccines including the revolutionary messenger RNA (mRNA) vaccines, DNA vaccines, recombinant protein vaccines and WPVs. mRNA vaccines played a crucial role in impeding the COVID-19 pandemic.

Although seasonal influenza and COVID-19 vaccines are available, they have several issues that need to be addressed. The SV, for example, is broadly used to prevent seasonal influenza but induces a poor cross-reactive immune response and its immunogenicity can be modest in vulnerable groups such as children and the elderly leading to suboptimal protection. The suboptimal immunogenicity of SV has resulted in development of other forms of vaccine.

In particular, re-evaluation of the classical WPV as an alternative for controlling seasonal influenza has been recently investigated. Meanwhile, development of COVID-19 vaccine accelerated at an unprecedented rate as billions of doses were required globally to impede the pandemic. Among them, mRNA vaccines are the first nucleic acid vaccines to receive emergency approval in history and are now widely used in high income countries. While they are quick and easy to manufacture once the sequence of the epitope is determined, they have a challenge of maintaining the cold chain for storage and transportation. Moreover, mRNA vaccines are associated with marked adverse effects such as high fever and muscle aches. In view of this, mRNA vaccines may be appropriate for pandemic scenarios when an effective vaccine is urgently needed to halt disease transmission, but they may not be desirable for annual vaccination. Therefore, there is a need for safe, cheap, and effective vaccine options other than mRNA vaccines. Our laboratory has proposed that WPV could help to resolve the issues of current influenza and COVID-19 vaccines. Here, I further explored the application of WPV in inducing immunity against influenza and COVID-19.

In Chapter I, the cross-immunogenicity and protectivity of WPV and SV against a heterologous influenza H1N1 virus were investigated *in vivo*. Furthermore, in Chapter II, the influenza WPV was co-formulated with a COVID-19 WPV to make a two-in-one influenza/ COVID-19 WPV and analyzed in terms of antibody induction and protection from virus challenge.

CHAPTER I: Inactivated whole virus particle influenza vaccine induces anti-neuraminidase antibodies that may contribute to cross-protection against heterologous virus infection

Introduction

Seasonal influenza continues to be a threat to public health accounting for 290,000–650,000 deaths and up to 3–5 million illnesses annually (1). It is mainly caused by influenza A and B viruses belonging to the *Orthomyxoviridae* family comprising negative sense segmented RNA genome. On the basis of the two surface glycoproteins HA and NA, influenza A viruses are classified according to subtypes. Currently, there are 18 HA and 11 NA subtypes and of these, only the H1N1, H2N2, and H3N2 are known to have caused pandemics in humans (2–4), but occasionally, H5N1, H7N9, and H9N2 have infected humans (5–7). Seasonal influenza leads to absenteeism from workplaces resulting in low productivity and also negatively impacts the economy through the cost of medical care which could potentially be higher when a pandemic occurs (8,9). Seasonal influenza viruses infect individuals of all age groups, but the burden is significantly higher in children below 5 years and elderly individuals over 65 years owing to naïvety and senescence of the immune systems in these groups, respectively (10,11).

Vaccination is the cornerstone for combating seasonal influenza. Due to the continuous antigenic changes of their viral surface proteins (12–14), WHO selects vaccine strains that closely match predominant strains in the forthcoming influenza season based on global surveillance data. While vaccination against seasonal influenza has been ongoing for decades, efficacy varies depending on the antigenic relatedness between vaccine strains and predominant strains and may sometimes be low. Nonetheless, vaccination still accounts for a significant reduction in influenza-associated morbidities and mortalities (15,16).

Presently, the IIVs, particularly SV is widely used for annual vaccination. SV contains ether- or detergent-disrupted virions that are highly purified with minimum reactogenicity. Another type of IIV, WPV differs from SV because the integrity of the virion structure, surface and internal proteins, and viral RNA is preserved during its preparation,

making it the most immunogenic among contemporary unadjuvanted IIVs. Previously the immunogenicity of a single inoculation of SV or WPV in mice and macaques was evaluated. The results indicated that WPV was superior to SV in priming naïve animals and protection from homologous virus infection (17–19), which reasonably answers the concern with low immunogenicity of currently used SV. Furthermore, it was demonstrated that the priming potency of WPV may be linked to its ability to activate the maturation of antigen-presenting cells (APCs) through the viral RNA that is contained inside of virions of WPV but not in SV (17). Because of the excellent potency in priming, our laboratory has recommended WPV as an alternative vaccine, particularly for naïve populations.

Given the antigenic variation of influenza viruses between seasons, in addition to the priming potency, the ability to produce cross-reactive antibodies should be considered when assessing influenza vaccines. The widely accepted primary assessment for seasonal influenza vaccine efficacy is the induction of antibodies against HA by HI assay, and a titer ≥1:40 is believed to correlate with a 50% reduction in contracting severe disease (20–23). However, because vaccine-induced virus neutralizing antibodies are predominantly against HA, HA may undergo higher selective pressure than other viral proteins which may result in a faster rate of antigenic drift (13,24). Given the importance of antigenic matching between vaccine strains and prevalent strains, induction of antibodies against influenza virus proteins other than HA, which are more antigenically stable, such as NA, may be necessary to solve the problem of antigenic mismatch (25). Unlike SV, WPV which contains all viral components within each virion is expected to function as a better vaccine that presents a wide range of antigens and thus may induce better cross-immunity.

In this study, monovalent WPV or SV of influenza virus A/California/7/2009 (X-179A) (H1N1) pdm09 [A/California (H1N1)] was evaluated in generating cross-reactive immune responses against heterologous influenza viruses; A/Singapore/GP1908/2015 (IVR-180) (H1N1) pdm09 [A/Singapore (H1N1)], A/Brisbane/02/2018 (IVR-190E) (H1N1) pdm09 [A/Brisbane (H1N1)], and A/duck/Hokkaido/Vac-3/2007 (H5N1) [A/Hokkaido (H5N1)]. A/Singapore (H1N1) and A/Brisbane (H1N1) are drifted from A/California (H1N1), the original virus that caused the 2009 pandemic and have been used as vaccine strains during the 2017–2019 and 2019/2020 influenza seasons respectively. Here, the present study has shown that WPV induces anti-HA antibodies against A/Singapore (H1N1)

and anti-NA antibodies against A/Singapore (H1N1), A/Brisbane (H1N1), and A/Hokkaido (H5N1), while SV induced only anti-HA antibodies against A/Singapore (H1N1). The use of WPV could potentially reduce the impact of immune evasion not only by antigenically drifted seasonal influenza strains but also by pandemic viruses of related subtypes to contemporary influenza strains.

Materials and methods

Viruses

Influenza viruses, A/California (H1N1), A/Singapore (H1N1), and A/Brisbane (H1N1) were kindly provided by the National Institute of Infectious Diseases in Japan (NIID). A/Hokkaido (H5N1) was kindly provided by the Laboratory of Microbiology at the Faculty of Veterinary Medicine, Hokkaido University, Japan. The viruses were propagated in 10-day-old embryonated chicken eggs, incubated at 35°C for 48 hours, and chilled at 4°C overnight, and the allantoic fluid was harvested and stored at −80°C until use.

Vaccine preparation

Monovalent WPV and SV were prepared from the same batch of purified virus as previously described (26,27). Briefly, the A/California (H1N1) was inoculated in 10-day-old embryonated chicken eggs, incubated at 35°C for 48 hours, and chilled at 4°C overnight, and the allantoic fluids were harvested. Virus particles were concentrated by high-speed centrifugation at 53,000 × *g* for one and half hours, at 4°C and then purified by centrifugation at 107,000 × *g* for one and half hours at 4°C through a 10–50% sucrose density gradient. The purified virus was divided into two fractions; one fraction was treated with 0.02% formalin (Kanto Chemical Co., Inc., Tokyo, Japan) and placed at 4°C for a week to prepare WPV. For SV, one-tenth of the total virus volume of 0.05% tween 80 (Sigma Aldrich, St. Louis, MO, USA) and diethyl ether (1:1 ratio with total virus volume) (Sigma Aldrich) were added, and placed at room temperature for 30 minutes, followed by centrifugation at 1750 × *g* for 15 minutes. The aqueous phase was collected, and diethyl ether was evaporated using nitrogen gas. The protein concentrations of both WPV and SV were measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Cells

Madin-Darby canine kidney (MDCK) cells were grown in RP10 [Roswell Park Memorial Institute medium (RPMI 1640; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK), 50 µM of 2-mercaptoethanol (Merck, Darmstadt, Germany), 1 nM of sodium pyruvate

(Thermo Fisher Scientific), 100 µg/mL streptomycin (Thermo Fisher Scientific), 100 U/mL of penicillin (Thermo Fisher Scientific) and 20 µg/mL gentamicin (Thermo Fisher Scientific)]. These cells were used for plaque and neutralization assays.

Animals, vaccinations, and virus challenge

Eight-week-old female C57BL/6 mice were purchased from Hokudo, Co., Ltd. (Sapporo, Japan) and housed in a BSL-2 animal room at the International Institute for Zoonosis Control, Hokkaido University (temperature of 22°C ± 2°C, humidity of 50% ± 10%, and a 12 hours/12 hours light/dark cycle). All mice were given food pellets (CE-2; CLEA Japan, Sapporo, Japan) and water *ad libitum*. Mice were subcutaneously vaccinated twice or three times with 9 µg/100 µL of either WPV or SV at three-week intervals. All vaccinations were performed under inhalation anesthesia with isoflurane. Serum samples were collected on days 19, 41, and 52 to estimate HI antibody titers. On day 41, some mice were intranasally challenged with a lethal dose of 3,000 plaque-forming units (PFU) of the A/Singapore (H1N1) under the same anesthetic conditions. Daily body weight was measured after the infection with a pre-determined humane endpoint of 25% weight loss of the initial measurement. Mice were humanely euthanized by cervical dislocation at 3-, 5-, 7-, and 14-days post-infection (dpi), and lung samples were collected and homogenized in $RPMI_{anti}$ (RPMI 1640 medium supplemented with 20 µg/mL gentamicin, 100 U/mL penicillin, and 100 µg/mL streptomycin). Following lung homogenization and centrifugation at 800 × *g* for 10 minutes, the supernatants were collected and stored at −80°C until use.

Hemagglutination inhibition (HI) assay

HI assay was performed as previously described (17). Briefly, serum samples were first treated with receptor-destroying enzyme II (RDE) (Denka Seiken, Tokyo, Japan) to inactivate non-specific inhibitors. Serum was treated at a ratio of 1:3 (serum:RDE) and incubated at 37°C for 15 hours, followed by inactivation of RDE by heating at 56°C for one hour. Phosphate-buffered saline (PBS) was added at four times the volume of the serum to give a final dilution of 1:10. To carry out HI, RDE-treated sera were serially diluted two-fold in a 96-well plate and mixed with 8 hemagglutination units of the virus antigens A/California (H1N1) or A/Singapore (H1N1). The plates were incubated at room temperature for 30

minutes for antibody-antigen reaction. An equal volume of 0.5% chicken red blood cells was added to the mixtures and further incubated for 30 minutes. The HI antibody titer was determined as the reciprocal of the highest dilution that inhibited hemagglutination.

NA and Neuraminidase inhibition (NI) assays

The NA assay was used to determine the optimal virus concentration for NI assay. The NA and NI assays described by Aymard-Henry *et al.* (28) were performed with modifications as follows. For the NA assay, 10 µL of virus stock solution [A/California (H1N1), A/Singapore (H1N1), A/Brisbane (H1N1), or A/Hokkaido (H5N1)] was serially diluted two-fold with PBS in Eppendorf tubes and 20 µL of fetuin substrate [containing 2.5% (w/v) fetuin; Sigma Aldrich Co., and phosphate buffer (0.4 M Na_2HPO_4 and 0.4 M NaH_2PO_4); Kanto Chemical Co., Inc.] was added to all tubes. Following incubation at 37°C with 5% CO_2 for 16 hours, 20 µL of periodate reagent [comprising 4.48% (w/v) $NaIO_4$ and 62% 14 M H_3PO_4; Kanto Chemical Co., Inc.] was added. The tubes were incubated at room temperature for 20 minutes followed by the addition of arsenite reagent [comprising 10% (w/v) $NaAsO_2$, 7.1% (w/v) Na_2SO_4, and 0.3% concentrated H_2SO_4; Sigma Aldrich] at ten times the volume of periodate reagent, and the tubes were vortexed thoroughly until the brown color disappeared. Then, 500 µL of thiobarbituric acid reagent [comprising 0.6% (w/v) $C_4H_4N_2O_2S$ and 7.1% (w/v) Na_2SO_4; Kanto Chemical Co., Inc.] was added. Reaction tubes were incubated in boiling water (100°C) for 15 minutes. After cooling at room temperature, 600 µL of butanol reagent (containing 95% $C_4H_{10}OH$ and 5% 12 M HCl; Sigma Aldrich Co.) was added. The tubes were centrifuged for 10 minutes at 1500 × *g*, after which 100 µL of the pink chromophore in the butanol phase from each tube was transferred to a transparent flat-bottomed 96-well plate, and the optical density (OD) was measured at 540 nm using an iMark microplate reader (Bio-Rad, Hercules, CA, USA). The virus dilution giving an OD reading of 0.5 was used for the subsequent NI assay.

To perform the NI assay, 10 µL serum samples were serially diluted two-fold with PBS in tubes and equal volumes of diluted virus solutions (as determined by NA assay) were added to each tube. The tubes were incubated at room temperature for 60 minutes, after which 20 µL of fetuin substrate was added, followed by incubation at 37°C with 5% CO_2 for 16 hours. The NA assay protocol was followed for the remaining NI assay steps. The OD

was plotted against the sample dilution, and the NI antibody titer was determined as the reciprocal of the highest dilution with ≤ 50% OD obtained in the control tubes containing PBS instead of serum.

Virus neutralization assay

Neutralization assay was performed to measure neutralizing antibody titers in the serum. A monolayer of MDCK cells was incubated at 37°C and 5% CO_2 overnight in six-well plates at a density of 4.75×10^6 in 3 mL of RP10. The RDE-treated sera prepared as described above were serially diluted two-fold in PBS in a 96-well plate and incubated with 100 PFU of the virus antigens [A/California (H1N1) or A/Singapore (H1N1)] for antigen–antibody reaction. After 60 minutes incubation at room temperature, the mixture of virus and serum was added to MDCK cells and incubated at 37°C and 5% CO_2 for 45 minutes with shaking every 15 minutes to allow the non-neutralized virus to adsorb to the cells. Warmed L15 overlay medium [consisting of Leibovitz L-15 (Life Technologies Corp., Carlsbad, NY, USA) with glutamine at pH 6.8 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 0.028% (w/v) $NaHCO_3$, 1 mg/mL N-tosyl-L-phenylalanine chloromethylketone (TPCK)-treated trypsin (Merck), and 0.9% (w/v) agarose (Merck)] was added to the six-well plates. The plates were incubated at 37°C with 5% CO_2 for 3 days. Following manual counting of the plaques, the neutralizing antibody titer was determined as the reciprocal of the highest dilution that prevented the growth of plaques to 50% of that obtained in the control wells.

Plaque assay

A monolayer of MDCK cells was incubated at 37°C with 5% CO_2 overnight in six-well plates at a density of 4.75×10^6 in 3 mL of RP10. The RP10 was aspirated, the cell monolayers were washed with $RPMI_{anti}$, and 125 µL of diluted lung homogenate (10^{-1} to 10^{-3}) in $RPMI_{anti}$ was added to each well, except for the control plate. The plates were placed in an incubator at 37°C with 5% CO_2 for 45 minutes with shaking every 15 minutes to allow virus attachment, followed by the addition of warmed L15 overlay medium to each well of the six-well plates. The plates were incubated at 37°C and 5% CO_2 for 3 days. The plaques were manually counted following plaque formation and the lung virus titers were determined.

Ethics statement

All mice experiments were performed under the approval (approval numbers: 17-0003 and 21-0020) of the Animal Care and Use Committee of Hokkaido University following Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Significant difference was denoted by p values < 0.05 using one-way analysis of variance (ANOVA) with multiple comparisons.

Results

Mice vaccinated with WPV exhibit superior cross-protection compared to those vaccinated with SV

The protective potency between WPV and SV in mice against heterologous influenza virus was compared. Three weeks after two subcutaneous vaccinations with either WPV or SV of A/California (H1N1) or PBS as a control, mice were challenged with 3,000 PFU of A/Singapore (H1N1) (**Figure 1a**) and monitored daily for changes in body weight (**Figure 1b**). Mice in the PBS group showed signs of disease including ruffled fur, hunched back, and lost weight of up to 25% of the initial measurement before the challenge, reaching the humane end point by 5 dpi. Those inoculated with SV showed similar symptoms, but the body weight loss was milder than in the control group. By contrast, those vaccinated with WPV did not lose weight or exhibit any clinical symptoms compared to the SV and PBS groups. At 3 and 5 dpi, lung tissues were harvested from mice for measurement of virus titers by plaque assay (**Figure 1c** and **d**). At 3 dpi, mice in the control group had the highest virus titers in the lungs of over 10^6 PFU/lung, and SV-vaccinated mice had virus titers of around 10^5 PFU/lung. Meanwhile, 3 out of 5 WPV-vaccinated mice had undetectable virus titers, and 2 other mice also had significantly low virus titers in the lungs compared with mice inoculated with PBS or SV (**Figure 1c**). At 5 dpi, the virus titers in the lungs of SV- and WPV-vaccinated mice were even lower than at 3 dpi (**Figure 1d**). Although the difference did not reach significance, virus titers in the lungs at 5 dpi tended to be lower in the WPV group than in the SV group. Based on these results, WPV appears to be more effective than SV in protecting against heterologous virus infection.

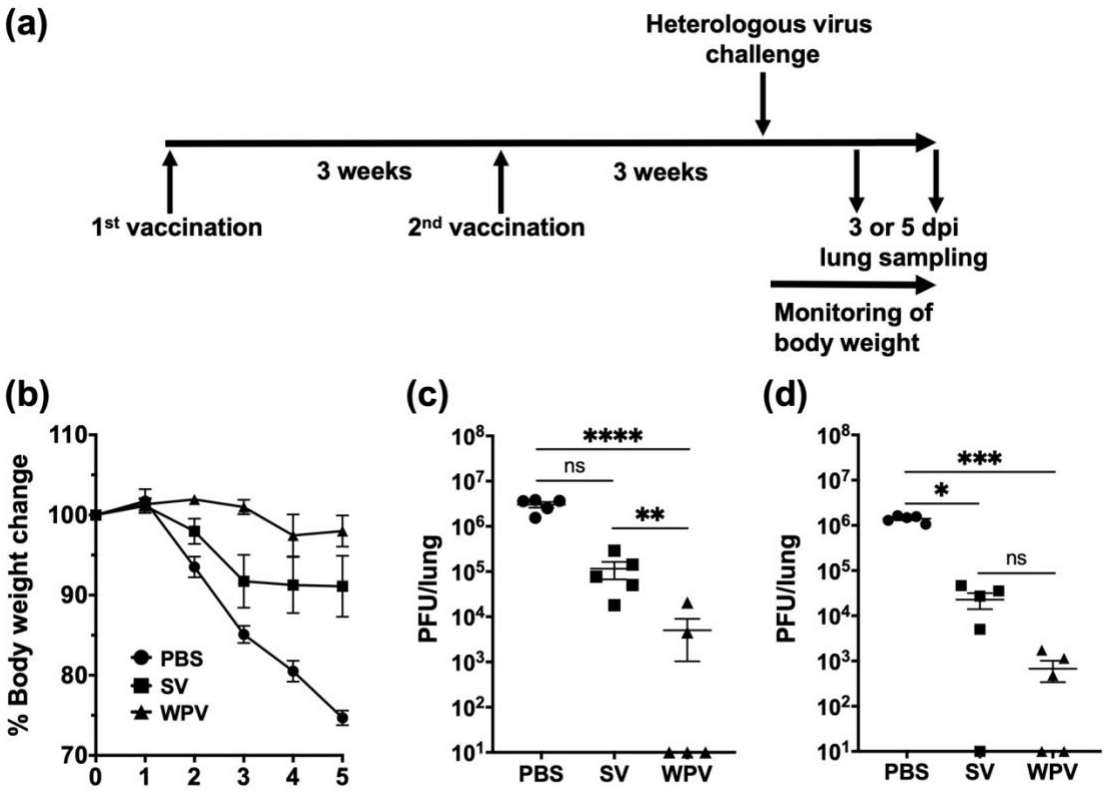

Figure 1. Protectivity of WPV and SV against heterologous virus challenge

(**a**) Schematic representation of the experimental schedule. C57BL/6 mice were subcutaneously inoculated with PBS or 9 μg of monovalent A/California (H1N1) WPV or SV twice at three-week intervals (n = 10 per group). Following the second vaccination, mice were challenged intranasally with 3,000 PFU of heterologous strain A/Singapore (H1N1). (**b**) The change in body weight of mice was calculated as a percentage of the original weight at 0 days post-infection (dpi). (**c** and **d**) Five mice in each group were euthanized for the collection of lung samples at 3 and 5 dpi. Plaque assays were performed to calculate the lung virus titers at 3 (**c**) and 5 dpi (**d**). Samples that had undetectable plaques were assigned a value of 10 for graphing purposes. Dots represent individual values, and lines indicate the mean values with standard error of the mean (SEM). (**b**–**d**) The circles, squares, and triangles indicate data from mice inoculated with PBS, SV, and WPV, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.0005$, $****p < 0.0001$, one-way ANOVA with Tukey's multiple comparison correction; ns indicates not significant ($p > 0.05$).

After multiple vaccinations, mice inoculated with SV induced HI antibodies comparable to those of mice vaccinated with WPV

Since the superior protective potency of WPV over SV against heterologous virus challenge was observed, possible driving factors were investigated. Serum samples were serially collected from the same mice after 1, 2, and 3 doses of WPV or SV and were evaluated for the induction of HI antibody titers against homologous strain A/California (H1N1) and heterologous strain A/Singapore (H1N1) (**Figure 2a**). Although HI antibody titers against homologous strain A/California (H1N1) were induced to a greater extent by WPV than SV after the first dose (**Figure 2b**), the difference was reduced after the second dose (**Figure 2c**), and no significant difference was observed between the antibody titers induced by WPV and SV after three doses (**Figure 2d**). On the other hand, the HI antibody titers against heterologous strain A/Singapore (H1N1) was significantly induced by WPV, but not by SV after the first dose (**Figure 2e**) but the titers were comparable between WPV- and SV-vaccinated mice after two or three doses (**Figure 2f** and **g**).These results imply that both WPV and SV have the potential to induce HI antibodies against the homologous and heterologous strains tested here. Furthermore, given similar HI antibody titers against A/Singapore (H1N1) in both vaccine groups after the second vaccination, it indicated that there was no significant contribution of HI antibodies to better cross-protection against the heterologous virus challenge in WPV-vaccinated mice shown in **Figure 1**.

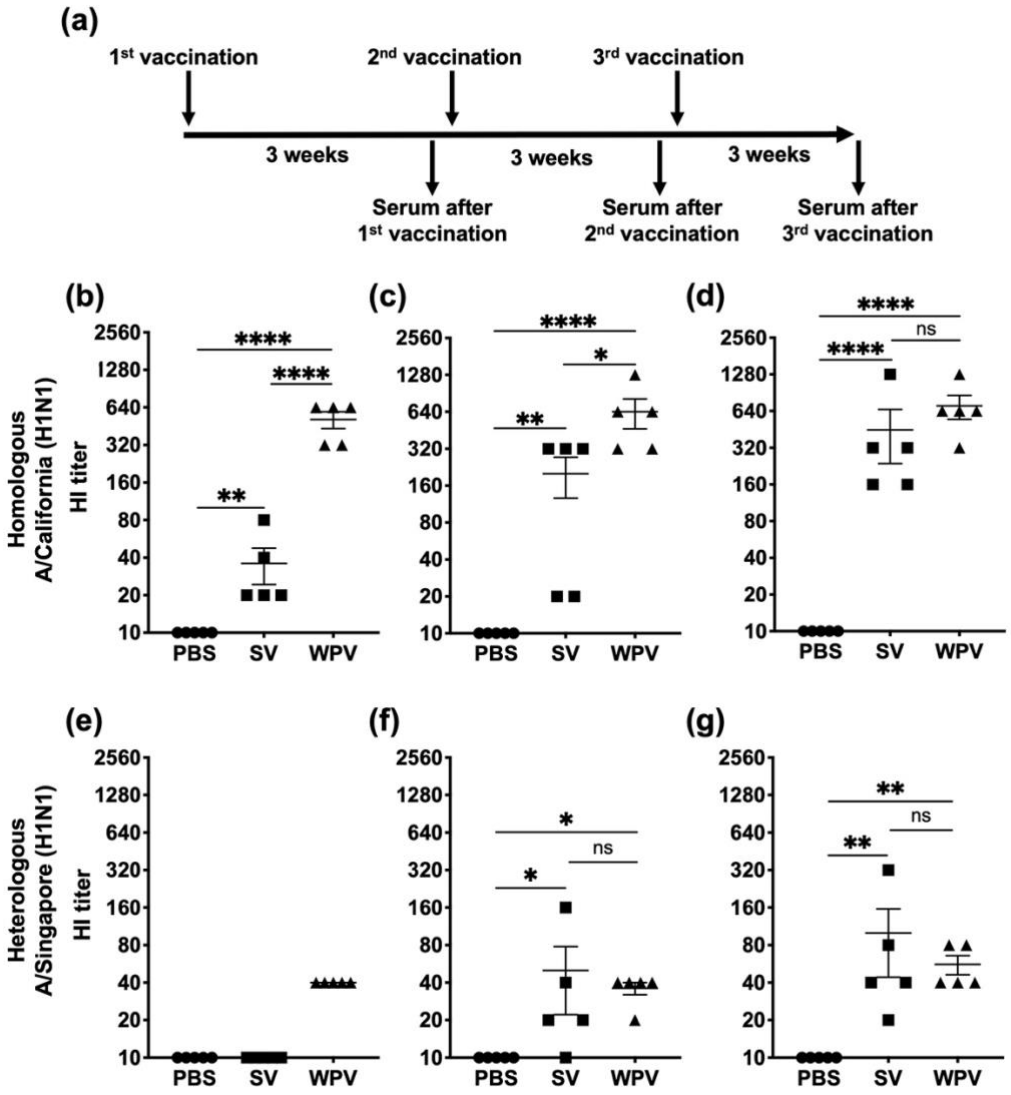

Figure 2. HI antibody titers after vaccination with WPV or SV

(**a**) Schematic representation of the experimental schedule. C57BL/6 mice were subcutaneously vaccinated three times at three-week intervals with 9 μg of monovalent A/California (H1N1) WPV or SV (n = 5 per group). HI antibody titers against homologous strain A/California (H1N1) (**b**–**d**) and heterologous strain A/Singapore (H1N1) (**e**–**g**) were evaluated in serum samples collected on days 19 (one dose; **b** and **e**), 41 (two doses; **c** and **f**), and 52 (three doses; **d** and **g**). Dots represent individual values, and the mean values with SEM are indicated by lines. The circles, squares, and triangles indicate data from mice inoculated with PBS, SV, and WPV, respectively. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, one-way ANOVA using Tukey's multiple comparison correction; ns indicates not significant ($p > 0.05$).

WPV induced better NI antibody titers than SV in vaccinated mice

In addition to anti-HA antibodies, WPV and SV were evaluated for the induction of antibodies against NA, the second major surface glycoprotein of influenza viruses. Serum samples collected from mice vaccinated with either WPV or SV as described above were subjected to the NI assay. NI antibody titers against homologous strain A/California (H1N1) in the WPV-vaccinated group were markedly increased after the first vaccination, and the values were further elevated by additional vaccinations (**Figure 3a**–**c**). On the other hand, NI antibody titers against A/California (H1N1) in SV-vaccinated mice ranged from undetectable to very low at all time points. Even after the third vaccination, 2 out of 5 mice produced no detectable NI antibody titers (**Figure 3c**).

NI antibody titers against heterologous strain A/Singapore (H1N1) were also evaluated. Although a single inoculation of WPV only slightly increased the NI antibody titers against A/Singapore (H1N1) (**Figure 3d**), the titers were significantly elevated by the second and third shots of WPV (**Figure 3e** and **f**). On the other hand, the NI antibody titers against A/Singapore (H1N1) in SV-vaccinated mice were below the detectable limit at almost all the time points, as in the PBS control group (**Figure 3d**–**f**). Since A/Singapore (H1N1) was replaced by A/Brisbane (H1N1) as an H1N1 vaccine strain during the 2019/2020 influenza season, I sought to find out if the breadth of NI antibodies induced by WPV cross-reacted with A/Brisbane (H1N1). Fortuitously, three doses of WPV engaged NI antibody titers against A/Brisbane (H1N1) in almost all mice (4/5), ranging from 22–40 while none were detected in SV-vaccinated or PBS control animals.

Furthermore, the induction of NI antibodies against an avian influenza virus strain A/Hokkaido (H5N1) belonging to a different subtype was examined. Mice vaccinated with SV did not produce detectable NI antibody titers after one or multiple vaccinations (**Figure 3g**–**i**). Interestingly, NI antibody titers against A/Hokkaido (H5N1) were detectable in some WPV-vaccinated mice even after the first inoculation and significantly increased with subsequent doses, reaching an average titer of 71 ± 14 after the third shot. These findings suggest that WPV has an excellent ability to induce effective NI antibodies which work against a wide range of virus strains.

In addition to HI and NI antibody titers, neutralizing antibody titers were also evaluated in serum samples collected 3 weeks after the second inoculation with WPV or SV.

Neutralizing antibody titers against A/California (H1N1) or A/Singapore (H1N1) were induced by both WPV and SV, but the titers against both strains were significantly higher in WPV-vaccinated mice than those induced by SV (**Figure 4a** and **b**). Taken together with the results of HI and NI antibody titers, the higher titer of neutralizing antibodies observed in WPV-vaccinated mice may be attributed to the superior inducibility of NI antibodies by WPV. Collectively, the findings of the present study suggest that WPV confers better cross-protective immunity against heterologous virus infection than SV.

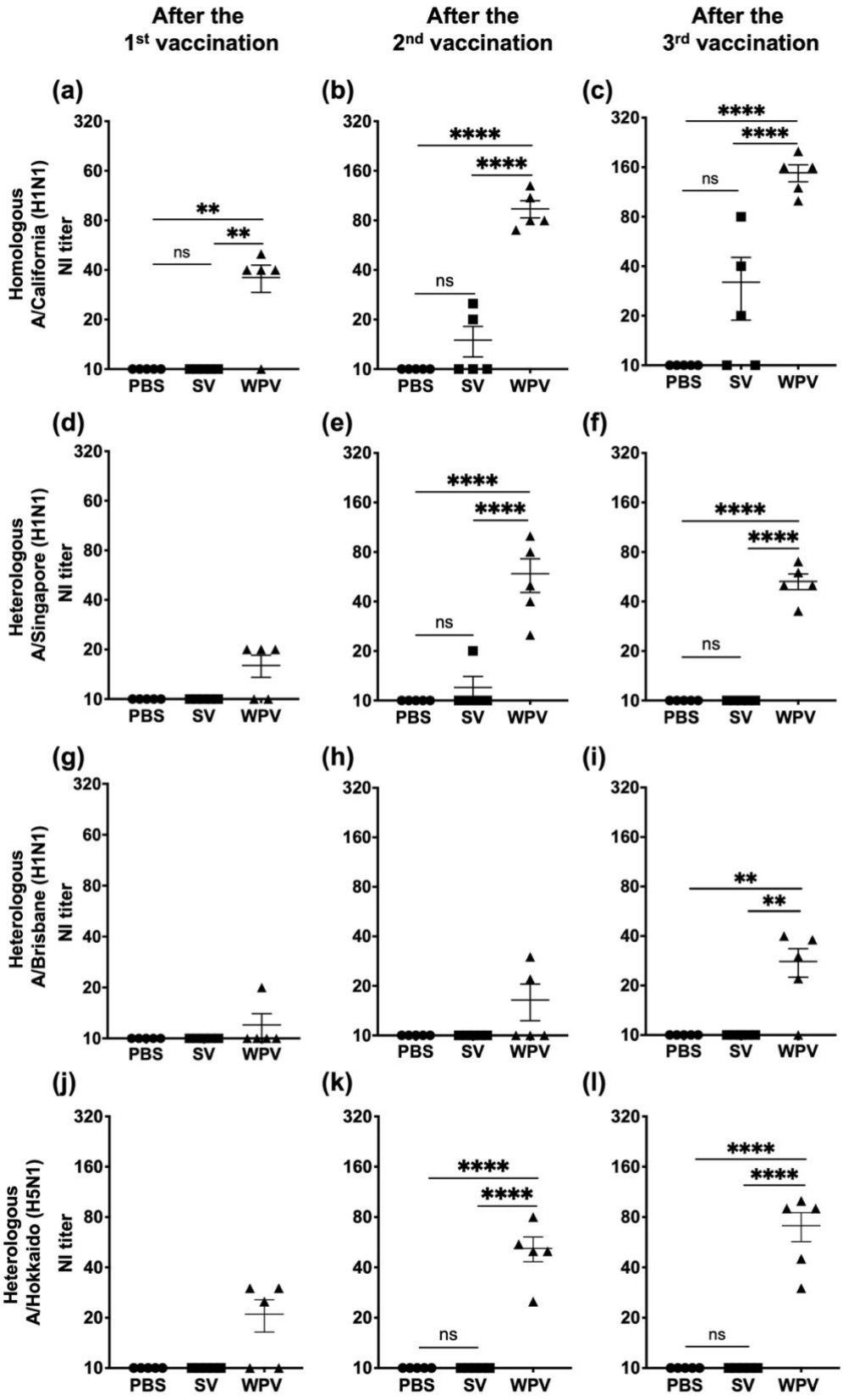

Figure 3. NI antibody titers after vaccination with WPV or SV

(**Figure 3** continued)

C57BL/6 mice were subcutaneously vaccinated three times at three-week intervals with 9 μg of monovalent A/California (H1N1) WPV or SV, as shown in **Figure 2a** (n = 5 per group). NI antibody titers against homologous strain A/California (H1N1) (**a**–**c**) and heterologous strains A/Singapore (H1N1) (**d**–**f**), A/Brisbane (H1N1) (**g–i**), and A/Hokkaido (H5N1) (**j**–**l**) were evaluated in serum samples collected on days 19 (one dose; **a**, **d**, **g** and **j**), 41 (two doses; **b**, **e**, **h**, and **k**), and 52 (three doses; **c**, **f**, **i**, and **l**). Dots represent individual values, and the mean values with SEM are indicated by lines. The circles, squares, and triangles indicate data from mice inoculated with PBS, SV, and WPV, respectively. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, one-way ANOVA with Tukey's multiple comparison correction; ns indicates not significant ($p > 0.05$).

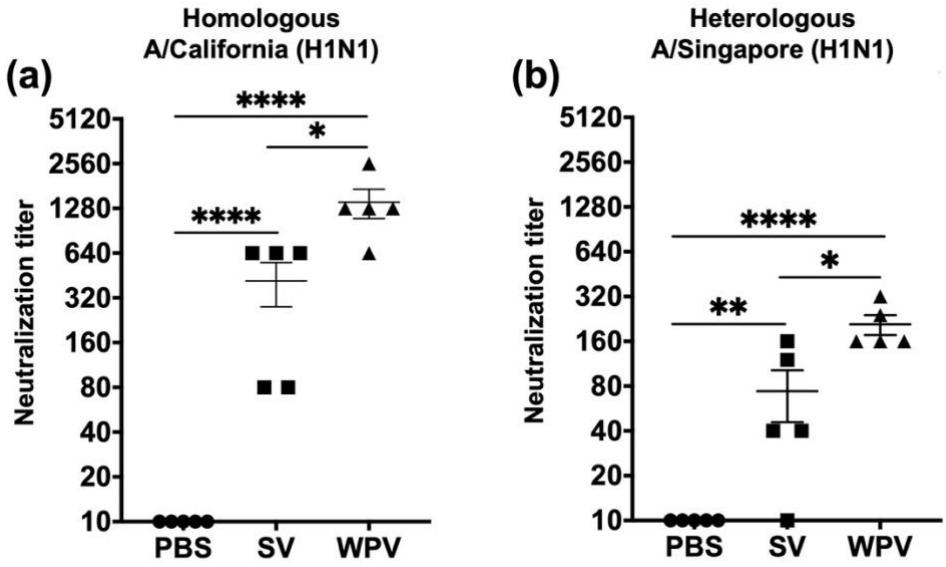

Figure 4. Neutralizing antibody titers after vaccination with WPV and SV

C57BL/6 mice were subcutaneously vaccinated twice at three-week intervals with 9 μg of monovalent A/California (H1N1) WPV or SV, as shown in **Figure 1a** (n=5 per group). Serum samples collected on day 41 were evaluated for neutralizing antibody titers against A/California (H1N1) (**a**) and A/Singapore (H1N1) (**b**). The circles, squares, and triangles indicate data from mice inoculated with PBS, SV, and WPV, respectively. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, one-way ANOVA with Tukey's multiple comparison correction.

Discussion

In this study, the immunogenicity and protective efficacy of WPV and SV against heterologous influenza virus infection in mice are reported. Although mice in this study received equal amounts of WPV and SV protein prior to challenge infection, those vaccinated with WPV had superior protection in terms of prevention of body weight loss and efficient suppression of virus replication in lungs, suggesting that WPV has more protective potency than SV (**Figure 1**). Indeed, other groups have documented the superior immunogenicity and protection conferred by WPV over SVs (18,29,30) and it has been suggested that the differences in antigen preservation in the vaccines which inherently influence immune response are responsible for the differences in protective efficacy.

While protective efficacy differed between mice receiving two doses of either vaccine, the HI antibody titers induced by WPV and SV were comparable, which suggests that the HI antibody titers were not responsible for the differences observed in protective efficacy. It also suggests that other aspects of the immune system, including antibodies against other surface proteins like NA, may have contributed to better protection from heterologous virus challenge. This notion is strongly supported by previous studies that showed an association between virus growth suppression and the induction of anti-NA antibodies (31–33), whose functionality is similar to NA inhibitors, the main antivirals for the therapeutic treatment of influenza. Moreover another group recently showed that antigenically mismatched HA in vaccine strains can be partially compensated by a matched NA subtype (25). In that study, ferrets vaccinated with adjuvanted SV of heterologous H1N2 limited weight loss and suppressed the virus replication in the lower respiratory tract when challenged with influenza virus H3N2, similar to those animals vaccinated with homologous H3N2 SV. Altogether, these previous studies and the current study suggest the importance of the NA in vaccine formulations for improving vaccine performance.

Several factors could account for the low or negligible induction of NA antibodies by SV. First, the HA which binds to sialic acid receptors, is preferentially endocytosed by APCs over NA or other viral proteins, subsequently inducing antibodies mainly against HA. It could be the case if viral proteins are contained in vaccines in a discrete state like SV. It is suggested that the intact virion in WPV provides an advantage during antigen presentation

since the entire virion can be internalized and processed by APCs thus producing broader immunity against all types of viral proteins, not limited to HA. Second, ether- or detergent-disrupted SVs are poor immunogens in mice and humans (34,35) because of the degradation of viral RNA which is required for the efficient activation of APCs. Indeed, although a group reported the induction of anti-NA antibodies by SV, in ferret models, the use of adjuvants were needed to improve the immunogenicity of SV (36). On the other hand, viral RNA which serves as an adjuvant and is linked to improved priming potency was preserved in WPV as previously demonstrated (17). Therefore, the ability of WPV to induce both anti-HA and -NA antibodies not solely against the homologous virus but also against the heterologous viruses makes its use advantageous for controlling seasonal influenza caused by antigenic variant viruses.

The eminent threat of zoonotic H5N1 infections in humans has led to the development of vaccines for pandemic preparedness (37–39) as it is associated with high mortality rates due to its ability to cause respiratory and systemic infection in humans (40). Since humans do not possess immunity to H5N1 viruses and given the broad cross-reactivity of NA antibodies induced by WPV in this study, the use of WPV as a seasonal influenza vaccine strain may partially contribute to the control of pandemic influenza caused by the H5N1 strain via anti-NA antibodies. Moreover, it was previously reported that vaccination with WPVs of a variety of subtypes including seasonal influenza H1N1 and H3N2 viruses protected mice from lethal H5N1 infection (26) recapitulating the advantageous use of WPV for seasonal influenza and during pandemics.

Vaccination for the control of seasonal influenza is clearly fundamental for reducing severe morbidity and mortality. A vast number of studies on the development of immunogenic, cross-reactive, and safe vaccines are ongoing. The results of this study have shown that WPV is not only an excellent vaccine for naïve populations because of efficient priming but also has the remarkable advantage of protecting against heterologous virus strains. It also induces a broad range of anti-NA antibodies that inhibit neuraminidase activity of heterologous strains, which may protect against infection with divergent strains. WPV is, therefore, ideal not only for the control of seasonal influenza but also for establishing preparedness for future pandemics.

Summary

Despite decades of coordinated vaccination campaigns, there is evidence that current seasonal influenza vaccines do not confer desired protection, particularly high-risk groups such as small children and the elderly. Previous reports from my laboratory showed the superiority of WPV in terms of immunogenicity and protectivity in comparison to widely used SV and recommended its use as an alternative seasonal influenza vaccine. In the current study, the immunogenicity of monovalent WPV and SV of influenza virus A/California (H1N1) against heterologous influenza virus strains including A/Singapore (H1N1), A/Brisbane (H1N1), and A/Hokkaido (H5N1) in mice was examined. Although two doses of either WPV or SV protected mice from challenge with a lethal dose of A/Singapore (H1N1), the weight loss was milder, and the virus growth was more suppressed in WPV-vaccinated mice than in SV-vaccinated counterparts. To investigate factors responsible for the differences in protective efficacy, serum HI and NI antibodies against the main virus surface proteins HA and NA respectively were measured. While both vaccines elicited high HI antibody titers against A/Singapore (H1N1) after three vaccinations, NA antibodies against A/Singapore (H1N1) and A/Brisbane (H1N1) were only detected in WPV-vaccinated animals. Furthermore, given that WPV-inoculated animals mounted NI antibodies against an avian influenza virus A/Hokkaido (H5N1), WPV demonstrated the potential to induce anti-NA antibodies against divergent influenza virus strains with the same NA subtypes. Taken together, the superiority in protectivity against A/Singapore (H1N1) in WPV-vaccinated animals over those vaccinated with SV may have been contributed by immunity against viral proteins other than HA, particularly anti-NA antibodies. The present results indicate that WPV effectively mounts antibodies against both HA and NA of heterologous viruses and may be a useful vaccine to conquer vaccine strain mismatch.

Chapter II: Immunogenicity and protective efficacy of a co-formulated two-in-one inactivated whole virus particle influenza/COVID-19 vaccine

Introduction

Prior to the emergence of the novel SARS-CoV-2 and the subsequent declaration of the COVID-19 pandemic, seasonal influenza viruses, including influenza A and B, accounted for the majority of respiratory infections. After the outbreak of the COVID-19 pandemic, morbidities and mortalities resulting from seasonal influenza viruses declined steeply (41–43) owing to non-pharmaceutical interventions for COVID-19 control, such as global travel restrictions, pro-social behavioral changes as well as good hygiene practices (44–46). However, with the opening of countries to international travel and the recovery of economies along with the lifting of strict control measures, the number of cases of seasonal influenza is projected to increase (47). Given that the COVID-19 pandemic is still ongoing, both diseases pose a serious public health threat, especially the risk of co-infection, which is associated with the exacerbation of clinical outcomes (48–50). Seasonal influenza and COVID-19 share several similarities including mode of transmission, susceptibility, clinical manifestations, and the constant threat of the emergence of new variants/subtypes, which necessitates preventive strategies such as annual vaccination against both diseases (51–53).

To date, vaccination is the most effective and economical method for controlling seasonal influenza and COVID-19. Annual vaccination against seasonal influenza has been in effect for decades, providing varying levels of protection to individuals, depending on the degree to which the antigenicity of vaccine strains match to that of predominant strains circulating during the influenza season. Since SARS-CoV-2 is expected to be a recurrent epidemic or endemic with antigenic changes, annual vaccination may be advisable. Several vaccine formulations have been developed to protect against COVID-19, leading to global mass vaccination (54–56). Mass vaccination efforts against both influenza and COVID-19 may cause an overlap that would overwhelm healthcare systems, and delays would increase the risk of infection with either virus. For instance, vaccination rates against seasonal influenza were significantly lower in post-COVID-19 years (57,58), thus there is a need for

efficient vaccination strategies to alleviate the impact of both diseases. Moreover, making appointments for separate vaccinations may be inconvenient for many people thus affecting the rate of vaccine uptake in the community. Accordingly, the WHO now recommends the concomitant administration of licensed seasonal influenza and COVID-19 vaccines (59).

Concomitant or combined vaccination can be in different forms including co-formulation and co-administration (60). In the context of vaccines, the former implies a single vaccine containing antigens from different microbes administered as a single dose, while the latter refers to the inoculation of different vaccine formulations at the same time, usually on different body parts (60,61). Currently, there is limited evidence regarding the safety and protectivity of co-formulated seasonal influenza and COVID-19 vaccines. The risk of reduced immunogenicity of one or more antigens in the vaccine and the possible exacerbation of side effects needs to be addressed.

Various techniques have been employed in developing COVID-19 vaccines including the mRNA vaccine which has drawn much attention due to its great advantages such as short development cycle, ease of scale up, and potent immunogenicity and other application prospects. Recently, a novel co-formulated mRNA vaccine encoding the SARS-CoV-2 spike protein and HA of influenza protected mice against influenza and COVID-19 (62). While mRNA vaccine has several advantages, the difficulty in handling, particularly the requirement of maintenance of a cold chain during transportation in addition to the unignorable adverse effects (56–58), may make their use undesirable for annual vaccination. Moreover, although an mRNA vaccine with improved stability was developed it demonstrated low efficacy of less than 50% in clinical trials indicating that producing thermal stable and cheap mRNA vaccines suitable for global use including resource-limited countries is still a challenge (63–65).

Apart from mRNA vaccines, traditional COVID-19 vaccines such as the WPV were developed and approved for use in humans, with relatively mild adverse effects compared to mRNA or vectored vaccines (66). Improved production technologies have alleviated WPV-associated side effects historically linked to contaminants during vaccine preparation. Coupled with the low cost of production, ease of handling and good immunogenicity, WPVs may be ideal for annual influenza and COVID-19 vaccination. Furthermore, a study by Bao et al. showed successful protection from seasonal influenza and COVID-19 in an animal

model after vaccination with a co-formulated vaccine containing adjuvanted COVID-19 WPV and influenza SV while another group reported the safety and immunogenicity of the same adjuvanted COVID-19 vaccine co-administered with a seasonal quadrivalent influenza SV in adults (49,67). However, the use of influenza SV in the Bao et al. study may pose a challenge since it has modest immunogenicity in high-risk groups thus an alternative such as WPV would be appropriate.

Here, the development of a two-in-one influenza/COVID-19 WPV containing antigens of seasonal influenza virus strains and a SARS-CoV-2 (Ancestral strain) derived from embryonated chicken eggs and cell culture, respectively is reported for induction of humoral immunity and protection against seasonal influenza viruses and SARS-CoV-2. It is shown that a single inoculation with the unadjuvanted vaccine elicits an antigen-specific immune response and protects BALB/c mice from influenza virus or SARS-CoV-2 infection.

Materials and methods

Cells

RPMI 1640 and Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) were used to grow MDCK cells and transmembrane serine protease 2 (TMPRSS2)-expressing Vero E6 (Vero TMPRSS2) cells, respectively. Both media were supplemented with 10% heat-inactivated FBS, 1 mM of sodium pyruvate, 50 mM of 2-mercaptoethanol, 100 mg/mL of penicillin, 100 mg/mL of streptomycin, and 20 mg/mL of gentamicin. These cells were used for the neutralization and plaque assays.

Viruses

Influenza viruses A/California (H1N1), A/Singapore (H1N1), A/Singapore/INFIMH-16-0019/2016 (IVR-186) (H3N2) [A/Singapore (H3N2)], B/Phuket/3073/2013 (Yamagata lineage) [B/Phuket (Yamagata)], B/Maryland/15/2016 (NYMC BX-69A) (Victoria lineage) [B/Maryland (Victoria)], and SARS-CoV-2 WK-521 (Ancestral strain), Alpha (B.1.1.7, QHN002), Delta (B.1.617.2, TY11-927), and Omicron (BA.5, TY41-702) variants were kindly provided by the NIID in Japan. Mouse-adapted SARS-CoV-2 (SARS-CoV-2 MA-P10) , was kindly provided by Dr. Sato (Shionogi & Co., Ltd, Osaka, Japan) and Dr. Sawa (Hokkaido University) (68). Influenza viruses were propagated in 10-day embryonated chicken eggs at 35°C for 48 hours and chilled at 4°C overnight, and the allantoic fluids were harvested and stored at –80°C until use. SARS-CoV-2 was propagated in Vero TMPRSS2 cells for 2 days at 37°C, 5% CO2, and the culture medium was centrifuged at 1750 × *g*, 4°C for 30 minutes to separate the virus from Vero TMPRSS2 cells and then supernatants were stored at –80°C until use.

Vaccines

COVID-19 WPV (Co WPV) was prepared from the SARS-CoV-2 WK-521 (Ancestral strain). The virus was propagated in Vero TMPRSS2 and after 2 days of culture at 37°C, 5% CO_2, centrifugation was performed at 1690 × *g*, 4°C for 15 minutes for debris clarification. Then 10% formaldehyde (Kanto Chemical Co., Inc.) was added to the supernatant (final concentration of 0.1%) and incubated for one week at 4°C for virus

inactivation. Following inactivation, the virus was concentrated by centrifugation at 53,900 × *g*, 4°C for 2 hours and resuspended in PBS containing 0.08% (w/v) sodium azide (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan) then purified by sucrose density ultracentrifugation at 107,000 × *g*, 4°C for 1.5 hours. The concentration of total protein was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Influenza WPV (Flu WPV) was prepared from the A/California (H1N1) virus as described in Chapter I. Quadrivalent influenza WPV (qFlu WPV) containing the 2018/2019 virus strains [A/Singapore (H1N1), A/Singapore (H3N2), B/Phuket (Yamagata), B/Maryland (Victoria)] was kindly provided by KM biologics Co. Ltd, Kumamoto, Japan. To prepare the two-in-one monovalent or quadrivalent influenza vaccine/COVID-19 (Flu/Co WPV and qFlu/Co WPV), respectively, equal protein amounts of the and the Flu WPV or qFlu WPV and Co WPV were combined in a single vial before use. The vaccines were diluted with PBS to adjust the protein concentrations to 90 µg/each viral antigen/mL, and 100 µL of each vaccine was inoculated into mice. The total amount of protein contained in a single dose is summarized in **Table 1**.

Table 1: Vaccine composition and doses used in this study

Vaccine name	Virus	Amount of protein in a single dose
Flu WPV	A/California (H1N1)	9 µg
Co WPV	SARS-CoV-2 (Ancestral strain)	9 µg
Flu/Co WPV	SARS-CoV-2 (Ancestral strain) A/California (H1N1)	18 µg
qFlu WPV	A/Singapore (H1N1) A/Singapore (H3N2) B/Phuket (Yamagata) B/Maryland (Victoria)	36 µg
qFlu/Co WPV	SARS-CoV-2 (Ancestral strain) A/Singapore (H1N1) A/Singapore (H3N2) B/Phuket (Yamagata) B/Maryland (Victoria)	45 µg

Animals

Six-week-old female BALB/c mice were purchased from Hokudo, Co., Ltd., and housed in BSL-2 and BSL-3 animal rooms at the International Institute for Zoonosis Control, Hokkaido University under standard laboratory conditions (temperature of 22°C ± 2°C, humidity of 50% ± 10%, and a 12 hours/12 hours light/dark cycle). All mice were given food pellets (CE-2; CLEA Japan) and water *ad libitum*.

Vaccination and serum collection

Seven-week-old BALB/c mice were immunized once or twice subcutaneously with PBS as a control or either WPV containing 9 μg each viral antigen; Flu WPV, Co WPV, qFlu WPV, Flu/Co WPV, or qFlu/Co WPV (Table 1). Sera were collected at 19 days (for one dose) or 36 days (for two doses) post-first vaccination from the caudal vena cava after euthanasia for measuring neutralizing antibody titers.

Virus neutralization assay

The neutralization assay for influenza viruses was conducted as described in Chapter I to measure neutralizing antibody titers of serum collected from vaccinated mice. For measurement of neutralizing antibodies against SARS-CoV-2, monolayers of Vero TMPRSS2 cells were prepared by seeding 4.75×10^5 cells in 3 mL DMEM and incubated overnight at 37 °C in 5% CO_2. Mouse sera were inactivated at 56°C for 30 minutes and serially diluted two-fold in 96-well microplates to which 100 PFU of SARS-CoV-2 was added and incubated for 1 hour at room temperature. Following washing of Vero TMPRSS2 cells with $DMEM_{anti}$ (DMEM supplemented with 20 μg/mL gentamicin, 100 U/mL penicillin, and 100 μg/mL streptomycin), the virus-serum mixture was transferred to six-well plates containing the Vero TMPRSS2 cells and incubated at 37°C in 5% CO_2, with shaking every 15 minutes. Finally, warmed L15 overlay medium was added to the six-well plates. The plates were incubated for 3 days at 37°C in 5% CO_2 and the neutralizing antibody titer was determined as the reciprocal of the highest dilution that prevented the growth of plaques to 50% of that obtained in the control wells.

Virus challenge and measurement of lung viral titer

Vaccinated mice were inoculated with either 3,000 PFU of A/California (H1N1) or 10^5 PFU of SARS-CoV-2 MA-P10 intranasally 22 days after vaccination and monitored daily for body weight changes. At 3 and 5 dpi, some mice were humanely euthanized, and lung tissues (right lobes) were harvested for measurement of virus titers and gene expression of proinflammatory cytokines and chemokines. Influenza virus titer in the lungs were determined by plaque assays in MDCK cells as described in Chapter I. To measure the SARS-CoV-2 virus titer, monolayers of Vero TMPRSS2 were prepared by seeding 4.75 × 10^5 cells in 3 mL DMEM and incubated overnight at 37°C in 5% CO_2. Homogenized lung samples were diluted 10-fold (10^{-1} to 10^{-3}) and then transferred to six-well plates containing a monolayer of Vero TMPRSS2 cells. The neutralization assay protocol outlined above was followed for the remaining plaque assay. Following incubation for three days at 37°C, 5% CO_2, visible plaques were counted manually, and the PFU was determined.

Measurement of gene expression levels using real-time RT-PCR

Fresh lung tissues (right lobes) were collected at necropsy, placed in TRIzol (ZYMO Research, Orange, CA, USA) homogenized using a Micro Smash, (MS-100/100R; Wakenyaku Co., ltd., Kyoto, Japan), and stored at –80°C until use. Total RNA was extracted according to the manufacturer's instruction using the classical chloroform/isopropanol precipitation method and quantified by a NanoDrop One spectrophotometer. The extracted RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) to prepare cDNA. The synthesized cDNA was subjected to real-time PCR reactions for quantification of gene expression levels of *colony stimulating factor 2* (*Csf2), Csf3, interleukin- 1 beta* (*Il1b*), *Il6, Il10, interferon-gamma* (*Ifng*), *tumor necrosis factor* (*Tnf*), *C-X-C motif chemokine ligand 10* (*Cxcl10*), *C-C motif chemokine ligand 2* (*Ccl2*), and *intracellular adhesion molecule 1* (*Icam1*) by using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and QIAGEN Quantinova SYBR green PCR kit (QIAGEN, Hilden, Germany). Relative expression was normalized by expression levels of the ribosomal *18S* gene and calculated as the fold change ($2^{-\Delta\Delta Ct}$) relative to the non-infected controls. The used primers are described in **Table 2**.

Table 2: Primers used in this study

Gene name	Primer sequence	
Csf2 (69)	Forward	CGGCCGGGGAAGCATGTAGA
	Reverse	GCTTGTGTTTCACAGTCCGT
Csf3 (69)	Forward	CATGAAGCTAATGGCCTGC
	Reverse	CTGACAGTGACCAGGGGAAC
Il1b (69)	Forward	TGCCACCTTTTGACAGTGATG
	Reverse	CAAAGGTTTGGAAGCAGCCC
Il10 (69)	Forward	CCAGCTGGACAACATACTGCTA
	Reverse	GAGAAATCGATGACAGCGCC
Il6	Forward	TACCCCAATTTCCAATGCTCTCC
	Reverse	GGATGGTCTTGGTCCTTAGCCA
Ifng (69)	Forward	AGGAACTGGCAAAAGGATGGT
	Reverse	CTGGTGGACCACTCGGATG
Tnf (69)	Forward	AGGCACTCCCCCAAAAGATG
	Reverse	CTTGGTGGTTTGCTACGACG
Cxcl10 (70)	Forward	GCCGTCATTTTCTGCCTCAT
	Reverse	GCTTCCCTATGGCCCTCATT
Ccl2	Forward	TAACGCCCCACTCACCTGCT
	Reverse	TCCTTCTTGGGGTCAGCACA
Icam (71)	Forward	CCGCAGGTCCAATTCACCACT
	Reverse	TCCAGCCGAGGACCATACAG
18S (72)	Forward	GCCGCTAGAGGTGAAATTCTTG
	Reverse	CTTTCGCTCTGGTCCGTCTT

Histopathology and immunohistochemistry

Lung tissue samples for histopathology and immunohistochemistry were harvested from non-infected control mice and infected mice at 3, 5, and 10 dpi. The left lobe from each mouse was excised and placed in masked formalin (Japan Tanner, Osaka, Japan) for 7 or 10 days at room temperature. For histopathological analyses, the tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Immunostaining was performed for detection of viral antigens and inflammatory cell markers (macrophages and neutrophils). Unstained paraffin-embedded lung sections were immersed in Trilogy (Cell Marque, Sierra College Boulevard, Rockline, California) for deparaffinization and antigen retrieval at 121°C for 45 minutes in an antigen retriever (2100 Antigen Retriever; Aptum Biologics, Southampton, UK). To block internal peroxidase activity, sections were placed in 30% hydrogen peroxide (H_2O_2) in methanol (final concentration of 0.03%; Fujifilm Wako Pure Chemical Corp.) for 20 minutes at room temperature after which they were probed with either antibodies, anti-influenza A, B (1:2,000, rabbit polyclonal antibody, TaKaRa Bio inc., Shiga, Japan), anti-nucleocapsid protein of SARS-CoV-2 (1:1,000, HL344, rabbit monoclonal antibody; GeneTex, Irvine, CA, USA), anti-F4/80 (1:500, F4/80 rabbit monoclonal antibody; Cell Signaling Technology, Danvers, Massachusetts, USA), or anti-Ly-6G (1:500, Ly-6G rabbit monoclonal antibody; Cell Signaling Technology), and incubated overnight at 4°C. Following washing with 0.01 M PBS (Fujifilm Wako Pure Chemical Corp.), sections were incubated with TaKaRa POD conjugate (Anti Rabbit Ig-Fab-peroxidase conjugate, MK205; TAKARA Bio. Inc.) for 30 minutes at room temperature. After washing with 0.01 M PBS, the sections were incubated with TaKaRa DAB substrate (TaKaRa Bio. Inc.) for 5 minutes at room temperature and then placed in distilled water to stop the reaction. Counterstaining was performed using hematoxylin (Mayer's Hematoxylin Solution; Fujifilm Wako Pure Chemical Corp.) for 30 minutes. Finally, the sections were washed with running water, dehydrated using isopropanol (Fujifilm Wako Pure Chemical Corp.), and mounted using a permanent mounting medium (VectaMount Express, Vector Laboratories, Inc., Burlingame, CA, USA).

Ethics statement

All mice experiments were performed under the approval (approval number:20-0070) of the Animal Care and Use Committee of Hokkaido University following Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 software. Significant difference was denoted by p values < 0.05 using one-way or two-way ANOVA with multiple comparisons correction.

Results

Co-formulation does not interfere with inducing vaccine-specific antibody responses

Individual influenza and COVID-19 WPVs (Flu WPV and Co WPV, respectively) and influenza/COVID-19 two-in-one WPV (Flu/Co WPV) were prepared as described in the Materials and methods section. To examine whether the co-formulation of Flu WPV and Co WPV negatively affects the induction of virus-specific immunity, mice were subcutaneously inoculated with a single dose of either WPV for assessing serum antibody titers and protectivity from virus challenges (**Figure 5a**). Mice that received Flu WPV or Flu/Co WPV mounted significantly higher levels of HI and neutralizing antibodies titers against the influenza A/California (H1N1) virus 19 days after vaccination with a single dose of either vaccine, compared to mice inoculated with PBS (**Figure 5b** and **c**). Neutralizing antibody titers against the influenza virus ranged from 160 to 640 in mice vaccinated with Flu WPV or Flu/Co WPV with no statistically significant differences between the groups. As expected, HI or neutralizing antibody titers against the influenza virus were not detected in animals inoculated with either PBS or Co WPV. As for investigating induction of neutralizing antibody titers against SARS-CoV-2, it ranged from 80 to 320 in animals receiving Co WPV or Flu/Co WPV which were significantly higher than those that received PBS (**Figure 5d**). These results reveal that co-formulation of Flu WPV and Co WPV has no apparent interfering effect on antibody induction against respective antigens.

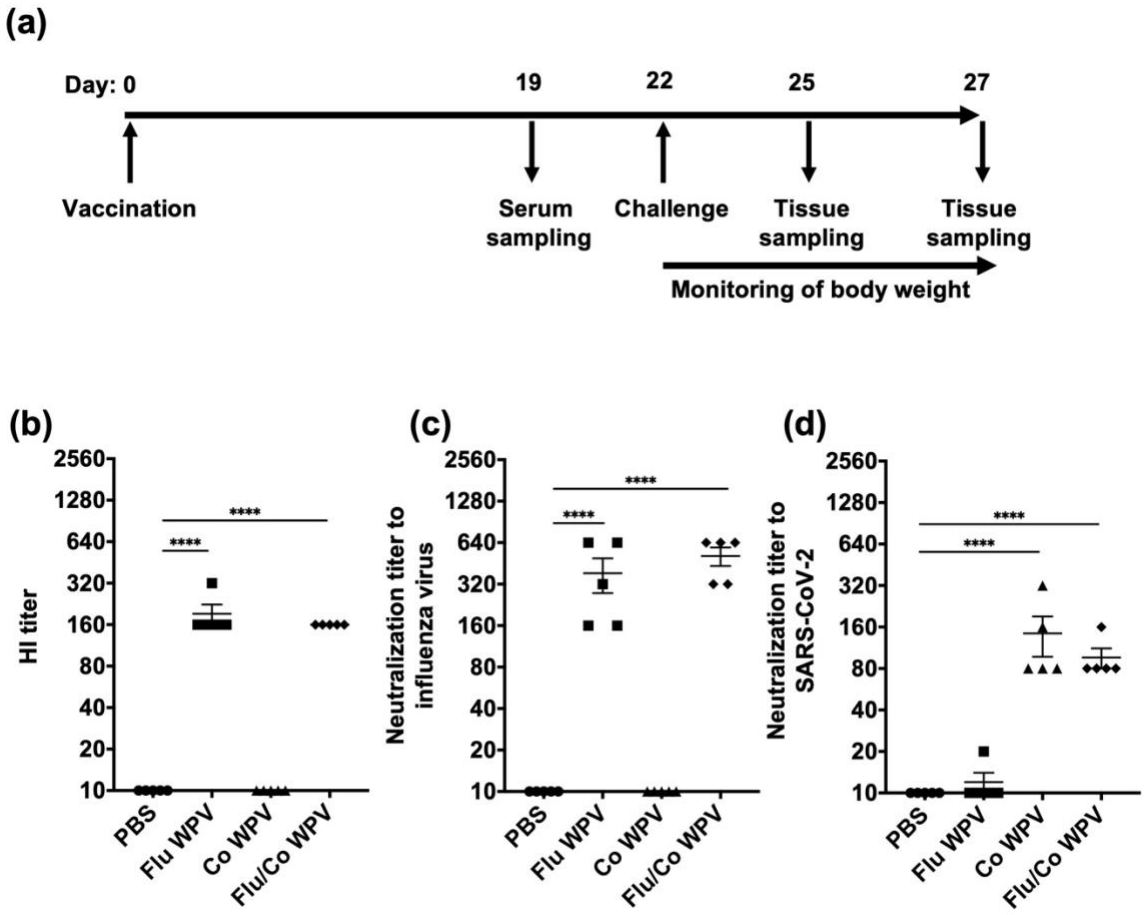

Figure 5: Antibodies against influenza virus and SARS-CoV-2

(a) Schematic representation of the experimental schedule. Female BALB/c mice were subcutaneously inoculated with Flu WPV, Co WPV, Flu/Co WPV, or with PBS as a control (n=5 per group). Sera were collected at 19 days post-vaccination for measurement of HI antibody titers (**b**) and neutralizing antibodies against influenza A/California (H1N1) virus (**c**) and SARS-CoV-2 Ancestral strain (**d**). The circles, squares, triangles, and diamonds indicate data from mice inoculated with PBS, Flu WPV, Co WPV, and Flu/Co WPV, respectively, and lines indicate mean with SEM. ****$p < 0.0001$, one-way ANOVA with Tukey's multiple comparison correction.

Protection from influenza virus challenge

Upon intranasal infection with 3,000 PFU of the influenza A/California (H1N1) virus on day 22, mice in the PBS group and those vaccinated with Co WPV showed a sharp decrease of their body weight up to 20% of the initial body weight while the weight of those vaccinated with Flu WPV or Flu/Co WPV fluctuated within the normal ranges (**Figure 6a**). To evaluate the efficacy of the vaccines in suppressing virus replication, virus titers in the right lobes of the lungs at 3 and 5 dpi were measured. Virus titers in the lung homogenates of mice in the PBS and Co WPV groups were comparable at approximately 10^5 and 10^4 PFU/right lobe at 3 and 5 dpi, respectively (**Figure 6b** and **c**). By contrast, the virus titers were under the detection limit in the lung homogenates from mice inoculated with Flu WPV or Flu/Co WPV (**Figure 6b** and **c**). These observations were consistent with the results of immunohistochemistry staining for the influenza virus, where viral antigens were not detected in the lungs of mice inoculated with either Flu WPV or Flu/Co WPV at 5 dpi, suggesting efficient protection by the vaccines (**Figure 6d**).

The protective efficacy of Flu/Co WPV from influenza-associated pathological changes was also examined in the lungs using H&E-stained sections (**Figure 6d**). Five days after influenza virus challenge, the mice in the PBS and Co WPV groups showed diffuse pneumonia with increased alveolar wall thickness, infiltration of inflammatory cells, denudation of bronchial epithelium, and deposition of necrotic debris in the bronchial lumen. In addition, perivascular and peribronchiolar cuffing was observed, which indicates virus-induced vascular and bronchial inflammation. On the other hand, mice that received the Flu WPV or the Flu/Co WPV showed normal lung histology that was comparable to the non-infected control group (**Figure 7**), suggesting that these animals were sufficiently protected from influenza virus-induced pneumonia. Furthermore, infiltrating pro-inflammatory cells, particularly macrophages and neutrophils, in the lung parenchyma were examined by immunohistochemistry with anti-F4/80 and anti-Ly-6G antibodies, respectively. Mice inoculated with PBS or Co WPV showed a higher frequency of both F4/80 and Ly-6G positive cells in the alveolar wall and peribronchial region, compared to the non-infected control animals, suggesting the infiltration of macrophages and neutrophils into the infected lung. On the other hand, animals vaccinated with Flu WPV or Flu/Co WPV after infection showed only sporadic staining for both F4/80 and Ly-6G antigens similar to non-infected

control animals, which is consistent with the observation in H&E stained sections indicating the attenuation of lung lesion associated with influenza virus infection by Flu WPV and Flu/Co WPV vaccination (**Figure 6d** and **7**).

To further investigate protection from pulmonary pathology, gene expression of inflammatory cytokines and chemokines in lung tissue collected at 3 and 5 dpi was measured (**Figure 6e**). After the influenza virus challenge, expression levels of most genes investigated here were significantly elevated by 4 to over 200-fold in unvaccinated mice (PBS control) as well as Co WPV-vaccinated animals. On the other hand, only slight increases in the levels of the genes were observed also in animals vaccinated with Flu WPV or Flu/Co WPV at both sampling time points. Except for *Csf3, Cxcl10* (both at 3 dpi), and *Il10* (at 3 and 5 dpi), there was no statistical difference between gene expression levels in mice vaccinated with Flu WPV, those vaccinated with Flu/Co WPV, and the non-infected control group. Collectively, these results suggest that the co-formulated Flu/Co WPV profoundly protects mice from influenza virus challenge in inhibiting virus replication and infection-associated pneumonia.

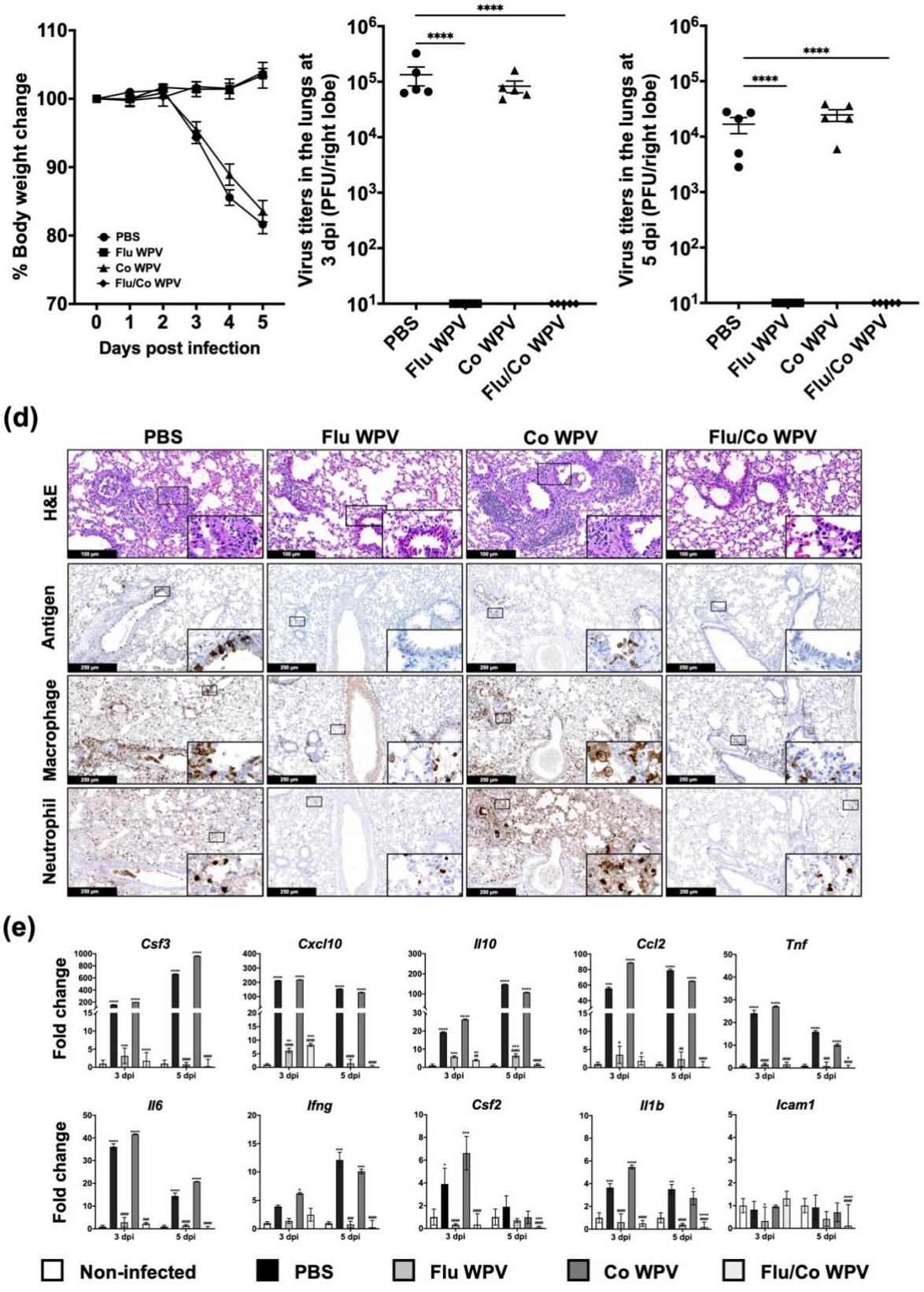

Figure 6: Protection from influenza virus challenge

(**Figure 6** continued)

Female BALB/c mice were subcutaneously immunized with Flu WPV, Co WPV, or Flu/Co WPV or inoculated with PBS as a control as in **Figure 5a** (n=10 per group). At 22 days post-vaccination, the animals were challenged with 3,000 PFU of the influenza A/California (H1N1) virus. Following the influenza virus challenge, body weight change was monitored until 5 dpi (**a**), and lung tissue samples were collected at 3 or 5 dpi for further analyses. Virus titer in the right lobes of the lungs collected at 3 and 5 dpi were measured by plaque assay (**b** and **c**). For histopathological analysis, lung sections prepared from left lobes collected at 5 dpi were stained with H&E, and immunohistochemistry was performed to detect influenza A virus antigens and macrophage (F4/80) and neutrophil (Ly-6G) markers (**d**). Gene expression levels of pro-inflammatory cytokines and chemokines were measured in the right lung lobes and are presented as fold changes relative to the non-infected control group (**e**). (**a**–**c**) The circles, squares, triangles, and diamonds indicate data from mice inoculated with PBS, Flu WPV, Co WPV, and Flu/Co WPV, respectively, and lines indicate mean with SEM. ****$p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons tests. (**e**) In each panel, bars denote mean values with SEM, and white, black, and gray colors indicate data from non-infected mice, infected animals inoculated with PBS, and infected animals inoculated with either WPV, respectively. The * and # symbols indicate statistical significance between the non-infected group and infected groups and between PBS and vaccinated groups, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$ and #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ ####$p < 0.0001$, two-way ANOVA with Sidak's multiple comparisons test.

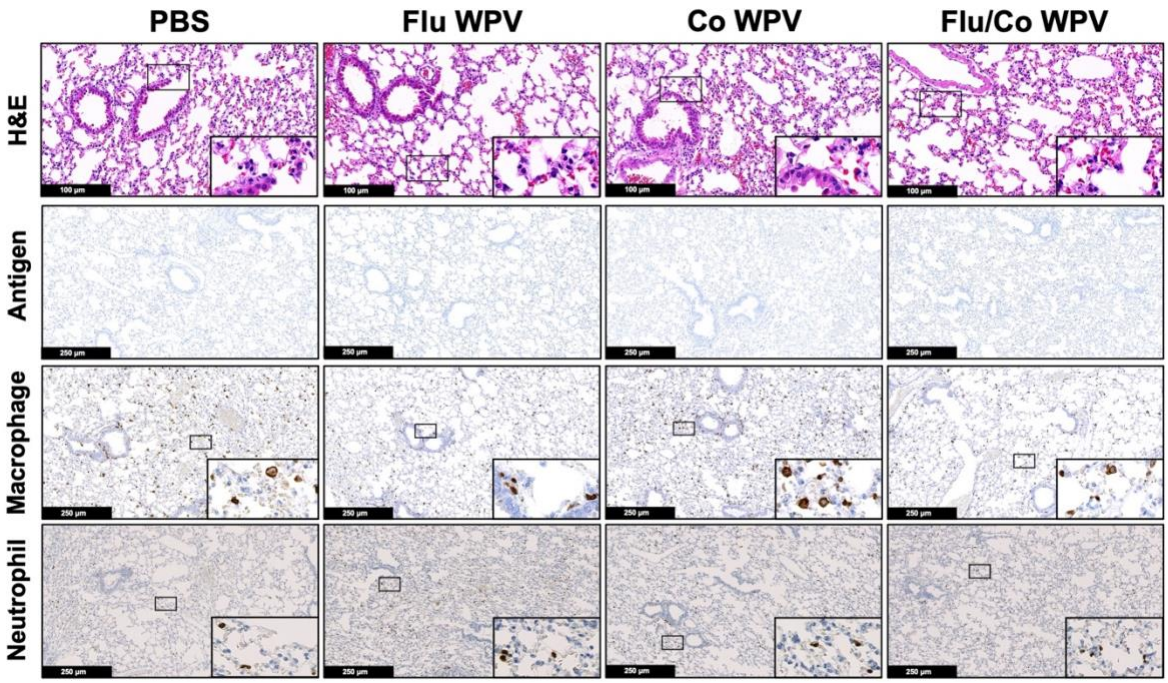

Figure 7: Histopathology and immunohistochemistry of lung sections from non-infected control mice

Female BALB/c mice were subcutaneously immunized with Flu WPV, Co WPV, or Flu/Co WPV or inoculated with PBS as a control, and lung tissues were harvested on day 19 post-vaccination (n=3 per group). For histopathological analysis, lung sections prepared from left lobes were stained with H&E, and immunohistochemistry was performed to detect SARS-CoV-2 nucleocapsid protein and macrophage (F4/80) and neutrophil (Ly-6G) markers.

Protection from SARS-CoV-2 infection

As in the previous section, the immunogenicity and protectivity of Flu/Co WPV against SARS-CoV-2 infection were investigated. Immunized BALB/c mice were challenged with 10^5 PFU of mouse-adapted SARS-CoV-2 MA-P10 on day 22 from vaccination and monitored for body weight changes (**Figure 5a**). At 4 dpi, animals inoculated with Flu WPV or PBS lost body weight of up to 15% of the initial measurement while those immunized with Co WPV or Flu/Co WPV did not show significant weight loss throughout the period of observation (**Figure 8a**). At 3 and 5 dpi, animals from each group were euthanized for sample collection to measure virus titers in the right lobes of the lungs by plaque assay. While the virus was detected in all mice at 3 dpi, animals vaccinated with Co WPV or Flu/Co WPV had approximately 100-fold lower virus titers than those in the PBS and Flu WPV groups (**Figure 8b**). Moreover, in the mice vaccinated with the Co WPV and Flu/Co WPV groups, one and two out of five mice, respectively did not have detectable virus at 3 dpi indicating protection by vaccine-induced neutralizing antibodies, and by 5 dpi, no infectious virus could be detected in lung homogenates from any mice (**Figure 8b** and **c)**, indicating attenuated virus replication in the groups which may be associated with vaccine-induced neutralizing antibodies. Consistently, immunostaining analyses revealed signals of SARS-CoV-2 nucleocapsid protein in the lungs of animals vaccinated with Flu WPV and PBS, but not in those vaccinated with Co WPV or Flu/Co WPV at 5 dpi (**Figure 8d**). Histopathological analyses with H&E-stained lung sections showed clear pneumonia in all immunized and non-immunized mice at 5 dpi, as indicated by increased infiltrating leukocytes in the peribronchiolar and alveolar areas, thickening of alveolar septa, perivascular cuffing, and deposition of necrotic debris in the bronchial lumen (**Figure 8d**). In line with the histopathological findings, immunostaining of immune effector cells with anti-F4/80 and anti-Ly-6G antibodies showed an increased frequency of macrophages and neutrophils (relative to the non-infected control group) in the lungs at 5 dpi in all infected animals regardless of vaccination status (**Figure 7** and **8d**). While the frequency of infiltrating macrophages seemed comparable in all animals, that of neutrophils was more pronounced in PBS- and Flu WPV-inoculated mice than in Co WPV- and Flu/Co WPV-vaccinated mice at 5 dpi (**Figure 8d**).

Contrary to the histopathological findings, the induction of pro-inflammatory cytokines at 3 and 5 dpi was suppressed by Co WPV and Flu/Co WPV (**Figure 8e**). Many of the cytokines and chemokines that have been implicated in severe COVID-19 infection including *Il6, Tnf*, and *Ilb* (72) were increased in the PBS- and Flu WPV-inoculated groups but not in mice vaccinated with Co WPV or Flu/Co WPV. Gene expression levels of cytokines and chemokines in PBS and Flu WPV groups were highest at 3 dpi and reduced at 5 dpi that may have mirrored active replication of the virus, as indicated by higher titers in the lungs at 3 dpi and then declined at 5 dpi. On the other hand, gene expression levels of inflammatory molecules tested here were not significantly elevated in the animals vaccinated with Co WPV or Flu/Co WPV except for *Il10*, *Cxcl10*, and *Ccl2* which were mildly induced at 3 dpi but much lower than the PBS and Flu WPV groups. Together, the results of the body weight change, virus titer, histopathology, and gene expression levels of pro-inflammatory cytokines and chemokines, Flu/Co WPV significantly reduced the severity of SARS-CoV-2 infection in vaccinated animals.

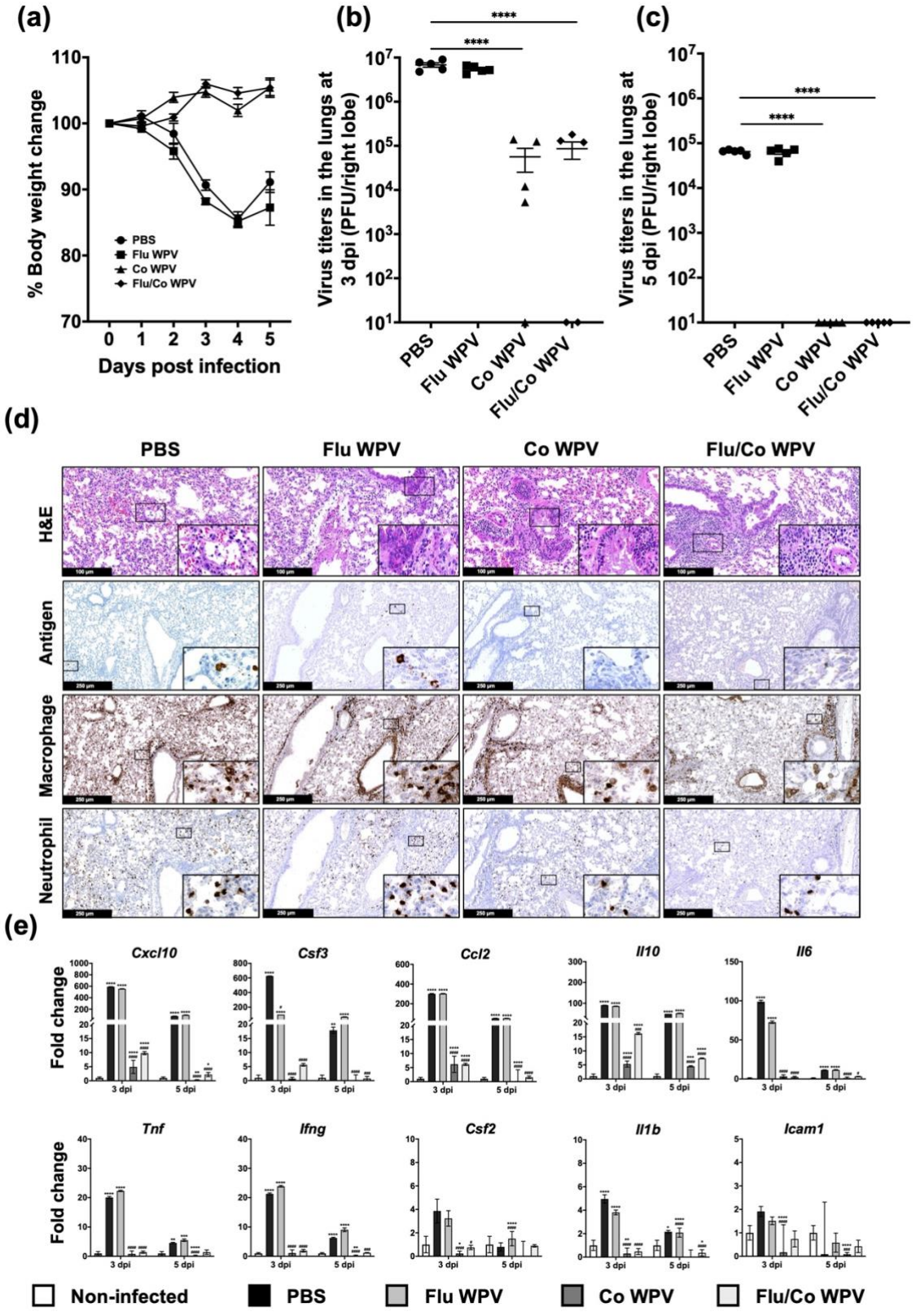

Figure 8: Protection from SARS-CoV-2

(**Figure 8** continued)

Female BALB/c mice were subcutaneously immunized with Flu WPV, Co WPV, or Flu/Co WPV or inoculated with PBS as a control as in **Figure 5a** (n=10 per group). At 22 days post-vaccination, the animals were challenged with 10^5 SARS-CoV-2 MA-P10. Following virus challenge, body weight change was monitored until 5 dpi (**a**), and lung tissues were collected at 3 and 5 dpi for further analyses. Virus titer in the right lobes of lungs collected at 3 and 5 dpi were measured by plaque assay (**b** and **c**). For histopathological analysis, lung sections prepared from left lobes collected at 5 dpi were stained with H&E, and immunohistochemistry was performed to detect SARS-CoV-2 nucleocapsid protein and macrophage (F4/80) and neutrophil (Ly-6G) markers (**d**). Gene expression levels of pro-inflammatory cytokines and chemokines and chemokines were measured in the right lobes and are presented as fold change relative to the non-infected control group (**e**). (**a**–**c**) The circles, squares, triangles, and diamonds indicate data from mice inoculated with PBS, Flu WPV, Co WPV and Flu/Co WPV, respectively and lines indicate mean with SEM. ****$p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons tests. (**e**) In each panel, bars denote mean values with SEM, and white, black, and gray colors indicate data from non-infected mice, infected animals inoculated with PBS, and infected animals inoculated with either WPV, respectively. The * and # symbols indicate statistical significance between the non-infected group and infected groups and between PBS and vaccinated groups, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$ and #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ ####$p < 0.0001$ two-way ANOVA with Sidak's multiple comparisons test.

Practical two-in-one vaccine with quadrivalent seasonal influenza vaccine

Since currently licensed influenza vaccines are available as trivalent or quadrivalent formulations, quadrivalent influenza WPV (qFlu WPV) containing antigens for the 2018/2019 flu season including A/Singapore (H1N1), A/Singapore (H3N2), B/Phuket (Yamagata) and, B/Maryland (Victoria) was co-formulated with Co WPV to prepare qFlu/Co WPV, and qFlu/Co WPV was evaluated for its immunogenicity and protective efficacy in BALB/c mice. BALB/c mice were subcutaneously immunized with, qFlu WPV, Co WPV, qFlu/Co WPV or inoculated with PBS as a control. Firstly, influenza virus- and SARS-CoV-2-specific neutralizing antibodies in the serum samples collected at 19 days post-vaccination were measured. Neutralizing antibody titers against all respective virus strains in the qFlu WPV were successfully induced to 160–320 on average with no statistical differences between the qFlu WPV and qFlu/Co WPV groups (**Figure 9a**). Neutralizing antibody titers against SARS-CoV-2 in animals vaccinated with Co WPV and qFlu/Co WPV were induced in the range of 40–80 in both groups while neutralizing antibody titers in PBS control animals were below the detection limit (**Figure 9b**). These results show that qFlu/Co WPV induces humoral immune responses to the respective viruses without apparent interference with each other.

Subsequently the protective efficacy of qFlu/Co WPV against SARS-CoV-2 infection challenge was investigated and increased the body weight monitoring up to 10 days. Following the inoculation of 10^5 PFU of SARS-CoV-2 MA-P10 at day 22 post-vaccination, body weight change was monitored. All control animals and those vaccinated with qFlu WPV showed steep weight loss until 4 dpi and then slowly recovered with a tendency of quicker weight gain in qFlu WPV-vaccinated animals than those in the PBS group (**Figure 9c**). Measurement of virus titers in the lung homogenates from the right lobes by plaque assay revealed that animals in the PBS and qFlu WPV groups had on average 2×10^7 and 1.9×10^7 PFU at 3 dpi and 5.5×10^5 and 6.2×10^5 PFU/right lung lobe at 5 dpi, respectively with no statistical difference between the groups (**Figure 9d** and **e**). By contrast, those vaccinated with Co WPV or qFlu/Co WPV showed no weight loss and only about 10^4 PFU/right lung lobe at 3 dpi and no detectable virus at 5 dpi, which were significantly lower titers compared to those in the unvaccinated group. Consistently, immunostaining analyses revealed lower signals of SARS-CoV-2 nucleoprotein in the lungs of animals vaccinated

with Co WPV or qFlu/Co WPV than in animals inoculated with PBS or qFlu WPV, at 3 and 5 dpi (**Figure 10**). Therefore, it is suggested that co-formulation of Co WPV with influenza monovalent or quadrivalent vaccines may not interfere with each other in inducing immunity against COVID-19.

Next, infection-induced lung pathology was examined by analyzing H&E-stained lung sections. Since severe lung pathology was previously reported at 9 dpi in hamsters even after beginning the recovery of body weight, in addition to lung tissues collected at 3 and 5 dpi, tissues were collected at 10 dpi to investigate vaccine effect on lung pathology (73). At 3 and 5 dpi, the lungs showed massive accumulation of immune effector cells, thickening of the alveolar wall, sloughing of the bronchial epithelium, deposition of necrotic debris, and perivascular cuffing in all infected mice regardless of vaccination status (**Figure 10**). This was consistent with the histopathological findings in the first SARS-CoV-2 challenge experiment, in which monovalent influenza WPV was used, described above (**Figure 8**). Moreover, immunostaining using anti-F4/80 and anti-Ly-6G antibodies revealed that the increased frequency of macrophages and neutrophils at 3 and 5 dpi in the lungs was not improved by Co WPV and qFlu/Co WPV, which was consistent with the results obtained in mice vaccinated with Flu/Co WPV in the first experiment of SARS-CoV-2 infection. Therefore, it was suggested that the protective effect of vaccination with Co WPV regardless of the co-formulation of influenza monovalent or quadrivalent WPVs on lung histopathology may not be apparent during the early stage of SARS-CoV-2 infection (**Figure 10**).

On the other hand, clear differences were observed in the lung histological changes at 10 dpi between the groups that received Co WPV or qFlu/Co WPV (**Figure 10**). PBS control animals and those vaccinated only with qFlu WPV showed more severe inflammation in the lungs in aspects of thickened alveolar walls, marked deposition of debri in alveolar spaces, vascular damage and higher frequency of macrophages than at early time points even after starting to recover the body weight. By contrast, lung tissues collected from animals vaccinated with Co WPV or qFlu/Co WPV showed much less inflammation than control animals, as indicated by marked resolution of bronchial and alveolar wall thickness, decreased deposition of debri in alveolar spaces and the reduced frequency of macrophages (**Figure 10**). Altogether, with the results of the first challenge experiment, vaccination with Flu/Co WPV and qFlu/Co WPV significantly reduced the severity of SARS-CoV-2 infection

in mice and ameliorated SARS-CoV-2-induced lung damage particularly at the later phase of the disease.

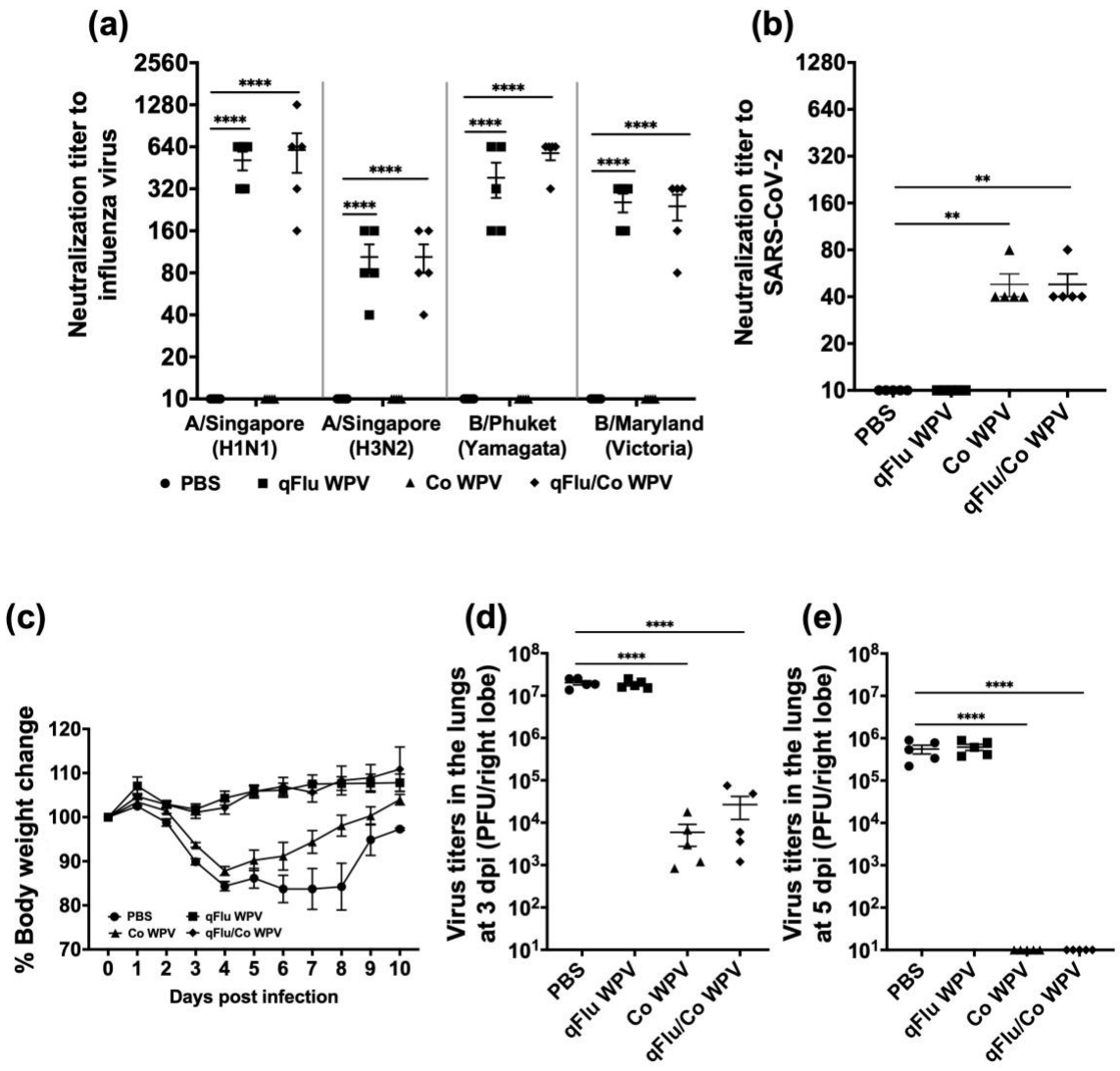

Figure 9: Practical two-in-one vaccine with quadrivalent seasonal influenza vaccine

Female BALB/c mice were subcutaneously immunized with qFlu WPV, Co WPV, or qFlu/Co WPV or inoculated with PBS as a control as in **Figure 5a** (n=18 per group). Sera were collected at 19 days post-vaccination for measurement of neutralizing antibody titers against A/Singapore (H1N1), A/Singapore (H3N2), B/Phuket (Yamagata), and B/Maryland (Victoria) (**a**), and SARS-CoV-2 (**b**). At 22 days post-vaccination, the animals were challenged with 10^5 PFU of SARS-CoV-2 MA-P10. Following virus challenge, body weight change was monitored until 10 dpi (**c**), and lung tissues were harvested at 3 and 5 dpi for further analyses. Virus titer in the right lung lobes collected at 3 and 5 dpi were determined by plaque assay (**d** and **e**).

(**Figure 9** continued)

The circles, squares, triangles, and diamonds indicate data from mice inoculated with PBS, qFlu WPV, Co WPV, and qFlu/Co WPV, respectively and lines indicate mean with SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons tests.

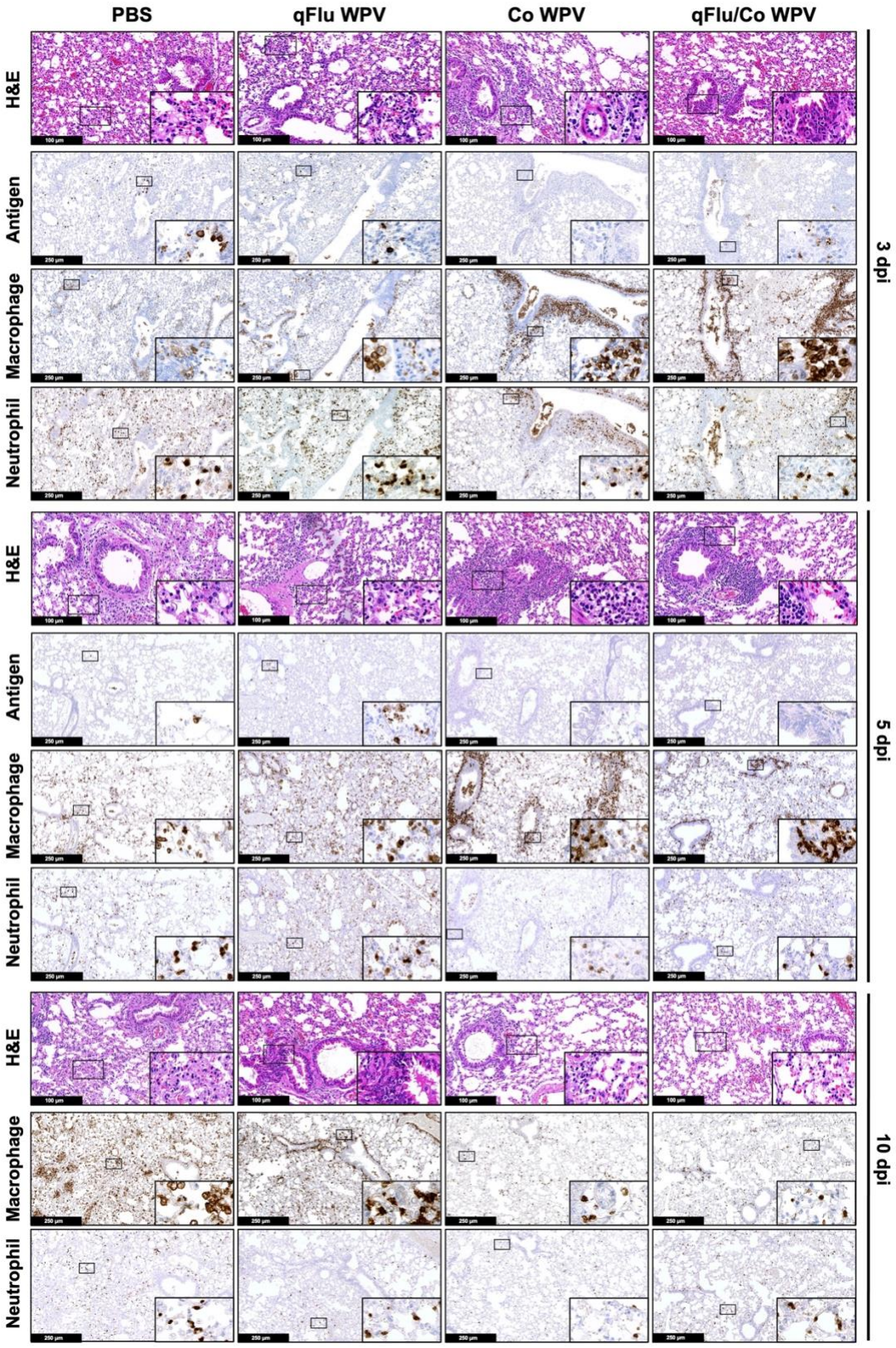

Figure 10: Histopathology and immunohistochemistry of lung sections at 3, 5, and 10 dpi

(**Figure 10** continued)

Female BALB/c mice were subcutaneously immunized with qFlu WPV, Co WPV, or qFlu/Co WPV or inoculated with PBS as a control as in **Figure 5a**. At 22 days post-vaccination, animals were infected with 10^5 PFU of SARS-CoV-2 MA-P10. Lung tissues (left lobes) from mice infected with SARS-CoV-2 MA-P10 were harvested at 3, 5, and 10 dpi for histopathology using H&E staining and immunostaining of SARS-CoV-2 nucleocapsid protein and macrophage (F4/80), and neutrophil (Ly-G6) markers.

Induction of cross-reactive neutralizing antibodies

The emergence of variants of concern (VOCs) has been a major challenge in controlling the COVID-19 pandemic as VOCs could evade infection- and vaccine-induced immunity. I sought to find out whether the co-formulated vaccine of the Ancestral strain mounts an antibody response against SARS-CoV-2 VOCs that appeared later. For this purpose, BALB/c mice were immunized twice with qFlu WPV, Co WPV, or qFlu/Co WPV at day 0 and 22. Serum samples were collected 14 days after the second vaccination to evaluate neutralizing antibodies against the Ancestral strain, Alpha (B.1.1.7), Delta (B.1.6.17.2), and Omicron (BA.5) variants (**Figure 11**). The results of neutralization assay showed effective induction of neutralizing antibody titers against the Ancestral and Alpha variants in animals vaccinated with Co WPV or qFlu/Co WPV, reaching to 2560 and 2463 on average against the Ancestral strain and 2048 and 1808 against the Alpha variant, respectively (**Figure 11a** and **b**). In addition, high neutralizing antibody titers against the Delta variant were detected with means of 304 and 256 in animals vaccinated with Co WPV or qFlu/Co WPV, respectively (**Figure 11c**). On the other hand, neutralizing antibody titers in animals inoculated with PBS or qFlu WPV were below the detection limit against all viruses tested. Neutralizing antibody titers against the recent dominant variant, Omicron were measured (**Figure 11d**). In contrast to the robust antibody induction against the Alpha and Delta variants in Co WPV- and qFlu/Co WPV-vaccinated animals, neutralizing antibody titers against the Omicron variant were very low, and, in two of five animals in each group, the levels were below the detection limit. Collectively, it was suggested that a two-dose vaccine regimen with the original Ancestral strain can effectively mount humoral immunity against the Alpha and Delta variants, however the induced immunity may not be sufficient to neutralize the Omicron variant probably due to accumulated mutations in the viral proteins. This result emphasizes the importance of annual vaccination for controlling COVID-19 in individuals to prevent prevalence.

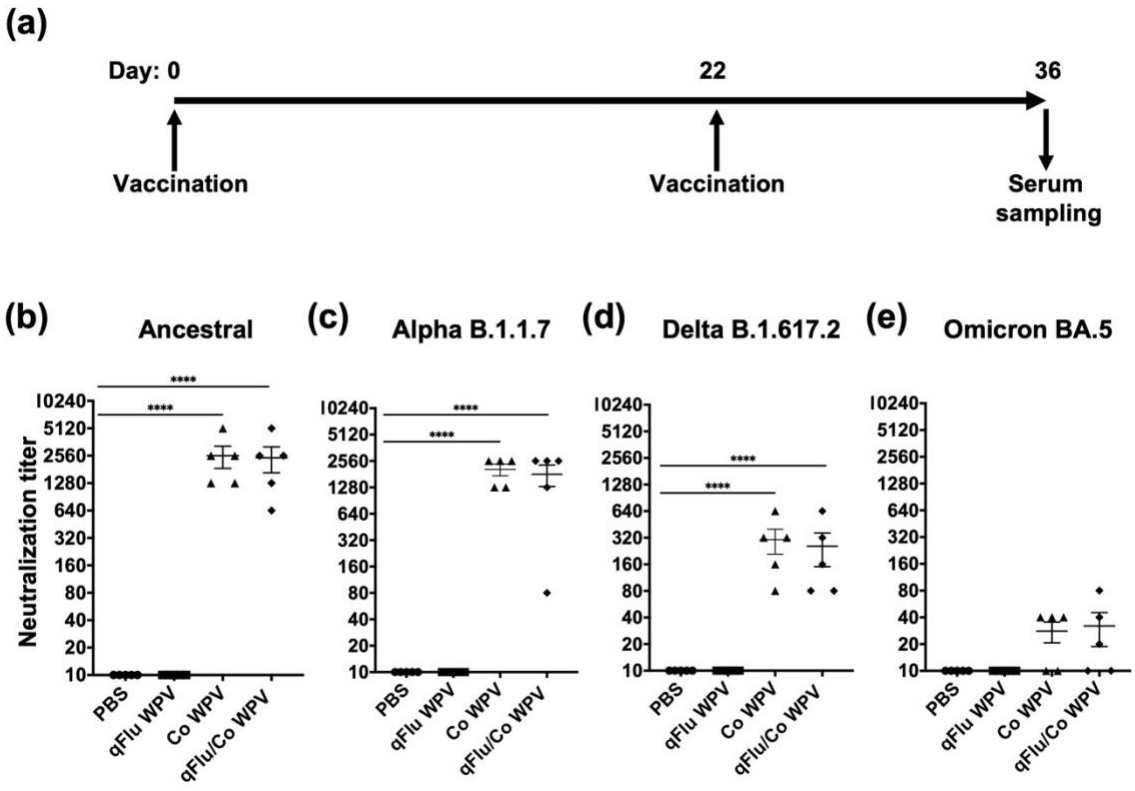

Figure 11: Cross-neutralizing antibodies against SARS-CoV-2 variants

(a) Schematic representation of the experimental schedule. Female BALB/c mice were subcutaneously immunized twice with qFlu WPV, Co WPV, or qFlu/Co WPV or inoculated with PBS as a control at days 0 and 22 (n=5 per group). Sera were collected 2 weeks after the second vaccination (36 days after initial vaccination) for measurement of neutralizing antibody titers against SARS-CoV-2 (Ancestral strain), Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (BA.5) (**a**–**d**). The circles, squares, triangles, and diamonds indicate data from mice inoculated with PBS, qFlu WPV, Co WPV, and Flu/Co WPV, respectively, and lines indicate mean with SEM. ****$p < 0.0001$, one-way ANOVA with Tukey's multiple comparison test corrections.

Discussion

Since the COVID-19 pandemic, mRNA vaccines have been used primarily in developed countries such as the United States and Europe. Like seasonal influenza, COVID-19 has spread globally, and annual vaccination is being discussed. Given the ease of mRNA vaccine production, such as *in vitro* synthetic capability and the fact that they can be rapidly produced once the genetic information is known, mRNA vaccines are very useful vaccines for early phase of pandemics. However, mRNA vaccines may be suitable for use as annual vaccines because of their strong adverse reactions as well as cold chain problems. Therefore, there is a need for safe and effective vaccine options other than mRNA vaccines.

In this study, the development of a two-in-one vaccine against seasonal influenza and COVID-19 in terms of immunogenicity and protective efficacy in BALB/c mice is reported. Co-formulation of either monovalent or quadrivalent Flu WPV with Co WPV does not seem to interfere with each other in priming immunity and enhancing antibodies against SARS-CoV-2 and seasonal influenza viruses (**Figures 5** and **9**). Although this finding cannot be directly extrapolated to the human population, it is consistent with several studies reporting the concomitant administration of licensed inactivated trivalent or quadrivalent seasonal influenza vaccines with currently used COVID-19 vaccines. Co-administration of either BNT162b2 (74), mRNA 1273 (75), ChAdOx1 nCoV-19 (74), or BBIBP-CorV (76) with quadrivalent seasonal influenza vaccines reported no safety concerns or interference in immunogenicity except for NVX-CoV2373 (77) and CoronaVac (67) study that demonstrated a mild reduction in neutralizing antibodies against SARS-CoV-2. In contrast to this study, in which influenza and COVID-19 vaccines were co-formulated and simultaneously administered, influenza and COVID-19 vaccines were administered separately in contralateral arms on the same day or on different dates for all the above studies. A vaccination strategy utilizing a single vaccine with influenza virus and SARS-CoV-2 antigens, as in this study, may be more advantageous in terms of improving the timely delivery of vaccines and minimizing stress on individuals and healthcare systems, particularly during mass campaigns, while maintaining the immunogenicity of individual vaccines.

In addition to the induction of antibodies, in the influenza challenge study, Flu/Co WPV conferred sufficient protection in mice, as indicated by the significant reduction of both severe pneumonia accompanied by infiltration of a large number of macrophages and neutrophils and increased levels of inflammatory gene expression demonstrated in unvaccinated and Co WPV-vaccinated mice, which could be associated with strong suppression of virus replication in the lungs (**Figure 7**). Furthermore, the protective effect of Flu/Co WPV did not differ from that observed in mice vaccinated with Flu WPV alone, which indicates that there was no apparent interference in the vaccine effect on inducing immunity against influenza when influenza and COVID-19 vaccines are simultaneously administered. Moreover, given that the immunogenicity and protectivity of Flu WPV were comparable to those of the seasonal influenza vaccines used in previous studies in terms of induced antibody titers and the degree of inhibition of viral growth (17,19), indirectly confirms the vaccine quality and the reliability of results in this study.

Although several inactivated COVID-19 vaccines have been developed and demonstrated for their immunogenicity and protectivity in animals and humans (78–82), none of them have been analyzed for protectivity after a single vaccination without any adjuvants like in this study. Therefore, the comparisons in terms of the protectivity between COVID-19 vaccines used in this study and others were difficult due to differences in experimental design such as the number of vaccine doses, dosage, types of animal models used, and use of adjuvants. Nonetheless, given that a single inoculation of the Flu/Co WPV and qFlu/Co WPV significantly prevented body weight loss, gene induction of pro-inflammatory molecules, and reduced virus titers in the lung upon SARS-CoV-2 infection, it is suggested that the two-in-one qFlu/Co WPV is sufficiently immunogenic and has satisfactory protective efficacy (**Figures 8, 9,** and **10**). Importantly, the results that the protective efficacy of Flu/Co WPV and qFlu/Co WPV was not different from that of Co WPV further provide evidence that co-formulation is a feasible strategy to control seasonal influenza and COVID-19.

Unlike in the influenza challenge, clear pathological changes were observed in the lungs of all mice at early time points (3 and 5 dpi) after the SARS-CoV-2 infection. This could be partly explained by the differences in virus PFU during challenge infection which was over 30 times higher for SARS-CoV-2 MA-P10 (10^5 PFU) compared to A/California

(H1N1) (3000 PFU) coupled with lower neutralizing antibodies against SARS-CoV-2 compared to those against influenza viruses. However, subsequent experiments demonstrated the beneficial effects of the vaccines at the later stage of infection. This is in view of a recent report that demonstrated severe lung inflammation in hamster models at 9 dpi even after the animals started to gain body weight and had undetectable viral antigens in the lungs. In line with the previous report (78), mice in the PBS control and Flu WPV groups showed severe signs of pneumonia with massive infiltration of macrophages at 10 dpi (Figure 10). Since virus infectivity was high in qFlu WPV and PBS control animals at 3 and 5 dpi as observed by the presence of high virus titer in the lung (**Figure 9d** and **e**), it may have caused enhanced host cell damage accompanied by increased production of chemokines and cytokines thereby triggering an aggressive and prolonged innate immune response. The efficient reduction of virus replication, owing to the presence of protective antibodies in animals vaccinated with Co WPV and qFlu/Co at early time points after SARS-CoV-2 infection could have attenuated subsequent inflammatory responses leading to lesser lung damage. Therefore, while the effect of the co-formulated vaccine on lung pathology seemed mild at early time points after SARS-CoV-2 infection, the improvement of lung lesions and reduced frequency of inflammatory cells at the later phase shows that vaccination with the co-formulated vaccine ameliorates SARS-CoV-2-induced lung pathology.

Since immune cross-reactivity is one of the key features required for successful COVID-19 vaccines, the induction of antibodies against Alpha and Delta virus strains, including recent Omicron variants by the two-in-one qFlu/Co was evaluated. As with previous groups investigating combined influenza and COVID-19 vaccines in mice models (62,83), two doses of qFlu/Co WPV were sufficient to engage antibodies against the Alpha and Delta variants (**Figure 11b** and **c**). In the first study utilizing an mRNA vaccine (62), neutralizing antibody titers against the Alpha variant tended to be higher (1:4097) than the Delta variant (1:2148), as was observed in this study, while the second study (83) measured antibodies against the Alpha and Beta variants with higher neutralizing antibody titers against the Alpha variant. Currently, the most dominant strain circulating around the world is the Omicron variant and it has the most amino acid substitutions, deletions, and insertions in the spike protein leading to reduced neutralization by ancestral antibodies (84). Indeed it is reported that two doses of the licensed COVID-19 vaccines are not sufficient to neutralize

this variant (85,86). This is similar to this study's result that the co-formulated qFlu/Co WPV induced very low–undetectable antibodies against the Omicron (**Figure 11d**). Data on the induction of cross-reactive antibodies against Omicron by co-formulated/co-administered influenza and COVID-19 vaccines are not yet reported, however, previous studies have revealed that neutralizing capacity of antibodies induced by approved COVID-19 vaccines administered alone can be improved by three-dose vaccination regimens in naïve individuals or two-dose-regimen in COVID-19 convalescent (87–89). Therefore, three doses of qFlu/Co WPV may be sufficient to improve cross-reactive immune response against the Omicron variant.

In conclusion, the results reported here support existing recommendations of co-formulation as a feasible strategy for controlling seasonal influenza and COVID-19 simultaneously. The maintenance of immunogenicity of vaccine antigens and the potent protective efficacy against seasonal influenza viruses and SARS-CoV-2 is crucial for optimum vaccine delivery thus providing timely protection against both diseases.

Summary

The synchronous circulation of seasonal influenza viruses and SARS-CoV-2, the causative agent of COVID-19, poses a remarkable threat to public health. Given the continuous and unpredictable emergence of new variants from both viruses, there is a need for routine vaccination for both influenza and COVID-19 to prevent spread of diseases. In this study, Flu WPV and Co WPV were prepared and mixed to produce a two-in-one vaccine, Flu/Co WPV. Serum analysis from the vaccinated mice revealed that a single dose of Flu/Co WPV induced high levels of neutralizing antibodies against both viruses, similar to those induced by either type of WPV alone. Moreover, upon infection with either virus, mice vaccinated with Flu/Co WPV showed no weight loss, significantly reduced lung tissue damage and virus titers, and lower proinflammatory cytokine expression as observed in those vaccinated with individual WPVs infected with homologous virus. These results suggest that a single dose of two-in-one WPV provided efficient protection against both SARS-CoV-2 and influenza virus infections without apparent interference from each other in mice. Therefore, a two-in-one influenza/COVID-19 WPV could be useful for controlling both diseases.

General conclusion

Issues of current seasonal influenza and COVID-19 vaccines need to be resolved to preserve human health. The co-circulation of influenza viruses and SARS-CoV-2 is exacerbated by the rise of immune-escape variants which either require additional vaccine doses or a complete change in the vaccine strain to achieve optimum protection. Therefore, in attempt to resolve these issues, this work explored the use of WPV for minimizing the impact of heterogenous influenza virus infection and the use of a co-formulated two-in-one influenza/COVID-19 vaccine to control both diseases simultaneously.

In Chapter I, the study compared the immunogenicity and protectivity of WPV and SV against a heterologous virus *in vivo*. The results revealed that WPV had superior protectivity than SV despite inducing similar levels of HI antibody titers. The superior protection seemed to be linked to WPVs ability to induce anti-NA antibodies against the challenge virus, A/Singapore (H1N1). Given that the anti-NA antibodies induced by WPV cross-reacted to other heterologous viruses including A/Brisbane (H1N1) and A/Hokkaido (H5N1), the use of WPV could ameliorate severe influenza due heterologous viruses through NI antibodies.

In Chapter II, a co-formulated two-in-one influenza/COVID-19 WPV was evaluated for its immunogenicity and protectivity against both diseases. The results revealed that co-formulation of vaccines does not interfere with antigen-specific antibody induction and protects mice from challenge infection with either virus in a similar fashion to when the vaccines are given individually.

Combined with the prominent advantages of WPV, including its potent immunogenicity, particularly the priming potency and induction of cross-reacting neutralization antibody titers, safety, ease of production and handling, this vaccine could provide timely protection from seasonal influenza and COVID-19. Therefore, WPV could potentially resolve issues of current seasonal influenza and COVID-19 vaccines.

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