Inactivated whole influenza virus particle vaccines induce neutralizing antibodies with an increase in immunoglobulin gene subclones of B-lymphocytes in cynomolgus macaques

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Abstract

The All-Japan Influenza Vaccine Study Group has been developing a more effective vaccine than the current split vaccines for seasonal influenza virus infection. In the present study, the efficacy of formalin- and/or β-propiolactone-inactivated whole virus particle vaccines for seasonal influenza was compared to that of the current ether-treated split vaccines in a nonhuman primate model. The monovalent whole virus particle vaccines or split vaccines of influenza A virus (H1N1) and influenza B virus (Victoria lineage) were injected subcutaneously into naïve cynomolgus macaques twice. The whole virus particle vaccines induced higher titers of neutralizing antibodies against H1N1 influenza A virus and influenza B virus in the plasma of macaques than did the split vaccines. At challenge with H1N1 influenza A virus or influenza B virus, the virus titers in nasal swabs and the increases in body temperatures were lower in the macaques immunized with the whole virus particle vaccine than in those immunized with the split vaccine. Repertoire analyses of immunoglobulin heavy chain genes demonstrated that the number of B-lymphocyte subclones was increased in macaques after the 1st vaccination with the whole virus particle vaccine, but not with the split vaccine, indicating that the whole virus particle vaccine induced the activation of vaccine antigen-specific B-lymphocytes more vigorously than did the split vaccine at priming. Thus, the present findings suggest that the superior antibody induction ability of the whole virus particle vaccine as compared to the split vaccine is attributable to its stimulatory properties on the subclonal differentiation of antigen-specific B-lymphocytes.

Keywords: influenza, vaccine, nonhuman primate model, immunoglobulin repertoire, somatic hypermutation

1. Introduction

The influenza virus causes seasonal epidemic and occasional pandemic infections in humans worldwide. In addition, severe infection with influenza viruses results in hundreds of thousands of deaths every year [1,2]. Prevention of influenza virus infection by vaccination is the best way to minimize severe cases. However, the current influenza split vaccines (SVs) do not induce effective immunity in naïve individuals due to low immunogenicity, which is a concern with SVs, especially for future pandemics caused by new strains in humans. Therefore, inactivated vaccines that are more immunogenic than SVs are required.

To develop a more potent vaccine than SVs, we have evaluated the efficacy of whole virus particle vaccines (WPVs) that are inactivated by formalin and/or β-propiolactone for seasonal, pandemic, and highly pathogenic avian influenza viruses using mouse and nonhuman primate models [3-6]. In the previous studies, antibody and T-lymphocyte responses were more vigorously induced in animals vaccinated with WPVs than in those vaccinated with SVs. Furthermore, we revealed that vaccination with WPVs induced the upregulation of inflammatory cytokines, including interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and interferon-inducible protein of 10 kD (IP-10), as part of the innate immune responses since RNA in WPVs that is effectively incorporated by antigen-presenting cells activates RNA-sensing pathways and induces an inflammatory cytokine response [6,7]. However, the process between the early production of inflammatory cytokines and effective antibody and T-lymphocyte responses due to WPVs has not been clarified.

To examine the process of effective induction of acquired immune responses, including the induction of neutralizing antibodies by WPVs, we compared the efficacy of vaccines and the changes in the immunoglobulin (Ig) gene repertoire after vaccination with WPVs to those

after vaccination with SVs in cynomolgus macaques. WPVs for H1N1 influenza A virus and influenza B virus induced neutralizing antibodies in the plasma of macaques, and showed more effective protection against the propagation of seasonal influenza viruses than did SVs. Furthermore, an increase of subclones in responding B-lymphocytes designated by the analysis of sequences and the frequency of Ig heavy chain genes, which suggests somatic hypermutation and the production of high affinity antibodies against the antigen, was found in macaques vaccinated with WPVs, but not in those vaccinated with SVs, especially after the 1st vaccination. These results are concordant with the higher priming efficacy and earlier induction of neutralizing antibodies by WPVs than by SVs [6-9].

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the Guidelines for the Husbandry and Management of Laboratory Animals of the Research Center for Animal Life Science at Shiga University of Medical Science, and the Standards Relating to the Care and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. The protocols were approved by the Shiga University of Medical Science Animal Experiment Committee (Permit numbers: 2015-6-3HH and 2016-6-3(H2)). The Research Center for Animal Life Science at the Shiga University of Medical Science has a permit for the importation of cynomolgus macaques. Regular veterinary care and monitoring, balanced nutrition and environmental enrichment were provided by the Research Center for Animal Life Science at the Shiga University of Medical Science. The macaques were euthanized at the endpoint (7 days after virus inoculation) using ketamine/xylazine followed by intravenous injection

of pentobarbital (200 mg/kg). Animals were monitored every day during the study to undergo veterinary examinations to help alleviate suffering. Animals would be euthanized if their clinical scores reached 15, which is a humane endpoint (Supplemental Table S1), but no macaques used in the present study reached a clinical score of 15.

2.2. Animals

Healthy 4- to 11-year-old female and male cynomolgus macaques from the Philippines and Vietnam were used in the present study. All procedures were performed under intramuscular ketamine (5 mg/kg) and xylazine (1 mg/kg) anesthesia, and all efforts were made to minimize suffering. Food pellets of CMK-2 (CLEA Japan, Inc., Tokyo, Japan) were provided once a day after recovery from anesthesia and drinking water was available *ad libitum.* Animals were singly housed in cages equipped with bars to climb up and puzzle feeders for environmental enrichment under controlled conditions of humidity (39% – 61%), temperature (23.9 °C – 25.1 °C), and light (12-h light/12-h dark cycle; lights on at 8:00 a.m.). In the text and figures, individual macaques are distinguished by identification numbers (Supplemental Table S2). The absence of influenza virus-specific antibodies in their plasma was confirmed before experiments using an antigen-specific enzyme-linked immunosorbent assay (ELISA) as described below. Two weeks before virus inoculation, a telemetry probe (TA10CTA-D70, Data Sciences International, St. Paul, MN) was implanted in the peritoneal cavity of each macaque under ketamine/xylazine anesthesia followed by isoflurane inhalation to monitor the body temperature. The macaques used in the present study were free from herpes B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp., and *Entamoeba histolytica*.

Under ketamine/xylazine anesthesia, two cotton sticks (TE8201, Eiken Chemical, Ltd., Tokyo, Japan) were used to collect fluid samples in the nasal cavities, and the sticks were

subsequently immersed in 1 mL of Hanks balanced salt solution (HBSS) containing 0.1% bovine serum albumin (BSA) and antibiotics. Blood samples were collected with the addition of heparin. Peripheral blood mononuclear cells (PBMCs) and plasma were separated using Leucosep tubes (Greiner Bio-One International, Kremsmunster, Germany).

2.3. Vaccines

Monovalent WPVs and SVs were produced by Denka Seiken Co., Ltd. and Daiichi Sankyo Co., Ltd [6]. Each monovalent vaccine contained a reassortant with the HA and NA antigens of A/California/07/2009 (H1N1) or B/Texas/2/2013 (Victoria lineage). Vaccine virus strains were propagated in embryonated chicken eggs, and purified from the allantoic fluids through sucrose density gradient zonal centrifugation. Thereafter, WPVs were prepared from the purified virions by inactivation with formalin and/or β-propiolactone according to the standard methods used by each vaccine manufacturer. SVs were prepared by disrupting the purified virions with ether, according to the license for the current seasonal influenza vaccine production [6]. The vaccine (15 μg/dose of HA protein in the vaccine inactivated with formalin and β-propiolactone, and 50 μg/dose of total protein (at least 15 μg of HA protein) in the vaccine inactivated with β-propiolactone) was inoculated subcutaneously into macaques using syringes twice with a 3-week interval between injections under ketamine/xylazine anesthesia (Supplemental Fig. S1). SVs used in parallel contained the same amount of HA protein or total protein as WPVs. HA protein concentrations in WPVs and SVs were determined using a single-radial-immunodiffusion method.

2.4. Viruses

The virus strains for challenge infection and neutralization tests were A/Narita/1/2009

(H1N1) (NCBI taxonomy ID: 645520, GISAID: EPI_ISL_30176), A/Singapore/GP1908/2015 (IVR-180) (H1N1) (GISAID: EPI_ISL_236221), and B/Maryland/15/2016 (Victoria lineage) (GISAID: EPI_ISL_243867) ,which were kindly provided by Dr. Shinji Watanabe, National Institute of Infectious Disease (NIID), Japan [9], and B/Yokohama/14/2015 (Victoria lineage) (GISAID: EPI_ISL_177028), which was kindly provided by Dr. Chiharu Kawakami, Yokohama City Institute of Public Health, Japan. The viruses were propagated once in Madin-Darby canine kidney cells (MDCK cells, American Type Culture Collection, Manassas, VA) at the Shiga University of Medical Science.

The macaques were challenged with the virus (4×10^5 $TCID_{50}$) by inoculation into the nostrils with pipettes 4 weeks after the 2^{nd} vaccination under ketamine/xylazine anesthesia. Experimental infection was performed in the biosafety level 3 facility of the Research Center for Animal Life Science, Shiga University of Medical Science.

To assess virus propagation, serial dilutions of swab samples were inoculated onto confluent MDCK cells as described previously [3]. Cytopathic effects were examined under a microscope 72 h later, and the virus titers were calculated. The sum of the virus titers for 7 days (virus titer areas under the virus titer time curves (virus titer AUCs) were calculated as described previously [10].

2.5. Cells

The MDCK cells were grown in Eagle's minimal essential medium (EMEM, Nichirei Biosciences, Tokyo, Japan) supplemented with 10% inactivated fetal bovine serum (Capricorn Scientific GmbH, Ebsdorfergrund, Germany), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 20 μg/mL of gentamicin (Nacalai Tesque, Kyoto, Japan). The cells were used for the propagation of viruses and for neutralization assays.

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179 **2.6. Detection of antibodies specific for vaccine antigen by ELISA**

180 The antibody titers of plasma samples against split antigens were determined by ELISA.
181 Ninety-six-well plates were coated with 50 μL of purified virus antigen (20 μg/mL). After
182 blocking with phosphate-buffered saline (PBS) containing 0.1% BSA, serially diluted
183 samples (50 μL) were incubated overnight in the coated plates. After washing five times,
184 horseradish peroxidase-conjugated anti-monkey IgG antibody (MP Biomedicals,
185 Inc./Cappel, Aurora, OH; 1:2000 × 50 μL) was added and incubated for 1 h at room
186 temperature. After washing six times, the horseradish peroxidase activity was assessed using
187 3,3',5,5'-tetramethyl benzidine substrate (100 μL). The reaction was stopped by the addition
188 of 1 M hydrogen chloride (100 μL). The optical density (OD) was measured at 450 nm and
189 620 nm. Results are shown after subtraction of the OD at 620 nm from the OD at 450 nm.

190

191 **2.7. Virus neutralization assay**

192 Plasma samples were pretreated with a receptor-destroying enzyme (RDEII, Denka
193 Seiken, Tokyo, Japan) at 37 °C overnight, then inactivated at 56 °C for 1 h. The diluted
194 samples were mixed with 100 $TCID_{50}$ of A/Narita/1/2009 (H1N1),
195 A/Singapore/GP1908/2015 (IVR-180) (H1N1), B/Yokohama/14/2015 and
196 B/Maryland/15/2016 viruses for 30 min. Then the mixture was added onto an MDCK
197 monolayer. After 1 h of incubation, the cells were cultured in EMEM containing 0.1% BSA
198 and 5 μg/mL trypsin. After incubation at 35 °C for 3 days, the number of wells with
199 cytopathic effects was counted in quadruplicate cultures. Neutralization titers were
200 determined as the dilution at which cytopathic effects were observed in 50% of the wells.

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202 **2.8. Histopathological examination**

Lungs obtained at autopsy were immersed in 10% neutral buffered formalin for fixation, embedded in paraffin, and cut into 3-μm-thick sections on glass slides. The sections were stained with hematoxylin and eosin (H & E), and observed under a light microscope. In each macaque, 6 to 8 lung sections were scored independently by three pathologists to calculate the average scores in each section as a following scoring; 0: normal lung, 1: mild destruction of epithelium in trachea and bronchus, 2: mild infiltration of inflammatory cells around the periphery of bronchioles, 3: moderate infiltration of inflammatory cells around the alveolar walls, resulting in alveolar thickening, 4: mild alveolar injury accompanied by vascular damage of ≤ 10%, 5: moderate alveolar and vascular injury (11% to approximately 30%), 6: severe alveolar injury with hyaline membrane–associated alveolar hemorrhage of 31% to approximately 50%, 7: severe alveolar injury with hyaline membrane–associated alveolar hemorrhage of ≥ 51% [11].

2.9. RNA preparation, cDNA synthesis, 5′-rapid amplification of cDNA ends (RACE) PCR amplification, and next-generation sequencing for immunoglobulin gene repertoire analysis

These processes were conducted by the method of Kono et al. [12] with modifications. Total RNA was extracted from the PBMCs recovered from heparinized blood by using a TRIzol Plus RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA), then the extracted RNA was used for first-strand cDNA synthesis by using a SMARTer RACE cDNA Amplification Kit (Clontech, San Jose, CA) with oligo-dT-containing 5′-RACE CDS Primer A and SMARTer II A Oligonucleotide. Next, cDNAs were amplified by PCR in a 20-μL reaction mixture containing 0.5 μL of unpurified cDNA, 0.5 U of Ex Taq HS DNA Polymerase, 200 μM of each dNTP, and 250 nM of primers in 1 × Ex Taq buffer (Takara Bio Inc., Shiga, Japan) with primers containing an

adaptor sequence for next-generation sequencing, the universal forward primers of 5′-RACE (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGCAGTGGTATCAACGCAGAGT-3′), and reverse primers specific for Ig-constant-region-1 of IgM (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTCACAGGAGACGAGGGGGAAAAGGGTTG -3′), IgGs (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAGGGGGAAGACBGATGGGCCCTTGGTGG -3′), IgAs (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGYTCAGCGGGAAGACCTTGGGKYTGGTC -3′), or IgE (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAGCGGGTCAAGGGGAAGACGGATGGGCTC -3′) to amplify the repertoires of each isotype through thermal cycling (94 °C for 2 min, 30 cycles of 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min). Each PCR product was tagged with an index sequence for next-generation sequencing and 8 bases of a sample identifier sequence (5′-AATGATACGGCGACCACCGAGATCTACAC-[sample identifier sequence]-ACACTCTTTCCCTACACGACGCTCTTCCGATCT -3′ for the forward primer, and 5′-CAAGCAGAAGACGGCATACGAGA-[sample identifier sequence]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT -3′ for the reverse primer) using a minimal cycle for PCR cycling (94 °C for 2 min, 15 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min). All amplicons were mixed, and the 600–800-bp PCR products were gel-purified using NucleoSpin Gel and PCR Clean-up kits (Macherey-Nagel GmbH & Co., Düren, Germany). Next-generation sequencing was performed using the Miseq reagent kit v3 (Illumina, Inc., San Diego, CA) according to the manufacturer's protocol for the kit and a Miseq sequencer (Illumina).

253

254 **2.10. Immunoglobulin gene repertoire analysis**

255 The Ig gene sequences were analyzed according to a method described previously
256 [13]. The Ig heavy chain gene sequences of macaques were processed using IMGT/HighV-
257 Quest [14,15] to annotate V and J germline gene segment usage, and the locations of the
258 complementarity determining regions (CDRs) and frame work regions were determined.
259 The output data were parsed using Change-O [16]. Germline sequences were then
260 reconstructed for each clonal cluster (VH) with D segment and N/P regions masked
261 (replaced with Ns) using the CreateGermlines.py function within Change-O. The V and J
262 gene usage frequency in each sample of macaques was calculated using countGenes in the
263 Alakazam (version 1.1.0) package [14,15].

264 To identify B-lymphocyte clones (sequences derived from cells descended from a
265 common ancestor), sequences belonging to the same clonal B-lymphocyte lineage were
266 identified by a spectral hierarchical clustering method using the SCOPer Package (version
267 1.1.0) [16,17]. Briefly, sequences sharing the same Ig heavy chain variable (IGHV) and Ig
268 heavy chain joining (IGHJ) genes and junction lengths were grouped. Within groups,
269 sequences differing by a length-normalized Hamming distance were defined as clones.
270 This distance threshold was determined using a kernel density estimate with a cutoff of
271 90% nucleotide sequence identity in the CDR3 and the rest of the variable region.

272 To track the dynamics of each B-lymphocyte clone, the frequencies of each clone were
273 defined as the number of sequences within the clone divided by the total number of total
274 sequences in each sample. Vaccine-responsive lineages and challenge infection-responsive
275 lineages were identified based on the fold-change (> 2-fold increase) in their frequencies
276 between week 0 (the day of the 1st vaccination) and week 2, and between week 7 (the day of
277 challenge infection) and week 8, respectively [17]. Subclones were categorized as identical

amino acid sequences in the junctional regions (CDR3). Statistical analyses were performed using R [18]. The ggplot2 (5 Wickham) package for R was used for constructing graphs.

3. Results

3.1. Prevention of virus propagation in the nasal swab samples of macaques vaccinated with the whole virus particle vaccines after virus infection

To compare the efficacy of WPVs and SVs, cynomolgus macaques were immunized with two subcutaneous vaccinations of the monovalent formalin- and β-propiolactone-inactivated WPV or ether-disrupted SV, which were prepared from vaccine strain A/California/07/2009 (H1N1) (Supplemental Fig. S1, Fig. 1A-D). Four weeks after the 2nd vaccination, challenge virus A/Narita/1/2009 (H1N1) was inoculated into the nasal cavities of the macaques. The viruses were detected in the nasal swab samples of the control macaques until day 6 or 7 after virus infection (Fig. 1A). In the macaques immunized with SV, the virus was detected until day 6, and the virus titers in their nasal swab samples were lower than those in nasal swab samples of macaques inoculated with saline, but the difference was not statistically significant (Fig. 1B). In the macaques vaccinated with WPV, the virus was detected until day 5 in one of the macaques (H1W2) and only on day 5 in another macaque (H1W1) (Fig. 1C). No virus was detected in one macaque (H1W3). The virus titer AUCs of the macaques immunized with WPV was lower than that of macaques inoculated with saline (Fig. 1D).

Protective effects of vaccination with monovalent WPVs were observed with the A (H1N1) and B (Victoria) vaccines inactivated by β-propiolactone (Fig. 1E-K). Four weeks after the 2nd vaccination, challenge virus strain A/Narita/1/2009 (H1N1) or B/Yokohama/14/2015 (Victoria lineage) was inoculated into the nasal cavities of the macaques. In the macaques immunized with SV, virus was detected until day 6 in the A

(H1N1) virus-challenged group and B (Victoria) virus-challenged group (Fig. 1E, I). In the macaques vaccinated with WPV, the A (H1N1) virus and B (Victoria) virus were detected until day 5 and day 4, respectively (Fig. 1F, J). No virus was detected in two macaques infected with B (Victoria) virus (BW2 and BW3). The virus titer AUCs of the macaques immunized with WPV were significantly lower than those of the macaques inoculated with saline in the A (H1N1) virus-challenge group and that of macaques without vaccination or immunized with SV in the B (Victoria) virus-challenge group (Fig. 1G, K). Thus, WPVs inactivated by formalin/β-propiolactone were more effective than SVs for preventing the propagation of the A (H1N1) and B (Victoria) viruses.

3.2. Prevention of body temperature changes and pneumonia in the challenge infection of macaques vaccinated with WPV

The suppressive effects of vaccination on the increase in body temperature due to virus infection were compared among the WPV, SV, and control groups. The average temperatures from 8 p.m. to 8 a.m. the next day were calculated on the basis of data for individual macaques, then the average temperatures on each day were compared to those from 8 p.m. the day before virus inoculation (day -1) to 8 a.m. on the day of virus inoculation (day 0). With A (H1N1) virus-challenge infection, body temperature increases were detected after challenge infection in macaques inoculated with WPV or SV, whereas in macaques inoculated with WPV, the average temperatures on days 1, 2, 3, and 4 were significantly lower than the average temperatures in macaques inoculated with saline (Fig. 2A). Similar tendencies were seen in macaques vaccinated with WPVs inactivated with β-propiolactone, although no statistically significant difference was detected (Fig. 2B, C). Thus, immunization with WPV ameliorated the body temperature changes at challenge infection.

Since the pandemic H1N1 virus caused viral pneumonia in cynomolgus macaques

as previously reported [19], we examined the efficacy of vaccination with WPV on pneumonia. Intranasal challenge of A/Narita/1/2009 (H1N1) in unvaccinated macaques (saline) caused pneumonia with the infiltration of lymphocytes and macrophages in the alveoli and the alveolar wall (organizing pneumonia) on day 7 after infection (Fig. 3A). Severe pneumonia was seen in two macaques vaccinated with SV, and they showed inflammation comparable to that in macaques inoculated with saline (Fig. 3B, D). In contrast, very mild pneumonia with thin alveolar walls and abundant air space was observed in macaques immunized with WPV (Fig. 3C). Focal lymphoid infiltration and bronchus-associated lymphoid tissues (BALTs) were detected in the macaques vaccinated with WPV. Histological scores revealed significantly mild inflammation in the lungs of macaques vaccinated with WPV compared to the control macaques (Fig. 3D). These results indicate that vaccination with WPV is effective for the prevention of pneumonia.

3.3. Antibody responses after vaccination with whole virus particle vaccines in macaques

To understand the immune responses that were associated with the preventative effects of WPVs on virus propagation and pneumonia, we examined the antibody titers in plasma by ELISA and a neutralization assay. Two weeks after the 1st vaccination, IgG specific for H1N1 vaccine antigen was detected in all macaques vaccinated with WPVs and in two and one of three macaques vaccinated with SVs in the experiments shown in Fig. 4A and B. IgG specific for vaccine antigen was detected in all macaques after the 2nd vaccination (4 weeks after the 1st vaccination). Neutralizing antibodies against the challenge strain A/Narita/1/2009 (H1N1) were detectable in the plasma samples of all of the macaques immunized with the WPVs 2 to 3 weeks after the 1st immunization, and the titers were increased after the 2nd vaccination (Fig. 4D, E). On the other hand, neutralizing antibodies

were detected only after the 2nd vaccination or challenge infection in macaques vaccinated with SVs. Neutralizing antibodies against the more recent strain A/Singapore/GP1908/2015 (IVR-180) (H1N1) were detected in the plasma samples of the macaques immunized with the WPVs, but in those of the macaques immunized with the SVs (Fig. 4G, H).

In influenza B virus vaccination, IgG specific for the Victoria lineage vaccine antigen was detected in all macaques vaccinated with WPV and in one of three macaques vaccinated with the SV 3 weeks after the 1st vaccination and 2nd vaccination, respectively (Fig. 4C). In two of three macaques vaccinated with SV, neutralizing antibody was detected after the 2nd vaccination, and it was not induced in the other macaque even after the challenge infection (Fig. 4F). The level of neutralizing antibody against the challenge strain B/Yokohama/14/2015 after the 1st vaccination increased more in the three macaques vaccinated with WPV than that in the macaques vaccinated with SV. Neutralizing antibody against a more recent strain B/Maryland/15/2016 was detected two weeks after the 2nd vaccination in one and three macaques vaccinated with SV and WPV, respectively (Fig. 4I).

A relationship between neutralization titers against the challenge strains and virus titer AUCs were examined. There is a tendency of an inverse correlation between neutralization titers and virus titer AUCs in the macaques vaccinated and infected with the H1N1 virus (Fig. 4J, K), whereas a significant correlation between neutralization titers and virus titer AUCs was seen in the macaques vaccinated and infected with the influenza B virus (Fig. 4L). Thus, the WPVs against influenza A and B viruses induced antibody responses more effectively than did the SVs in the macaque model, which contributed to prevention and inhibition of virus propagation.

3.4. Immunoglobulin repertoires in macaques vaccinated with the whole virus particle vaccine

We compared the Ig repertoire in macaques vaccinated with the H1N1 WPV to that in macaques vaccinated with the H1N1 SV to examine the characteristics of the antibodies induced in macaques vaccinated with the WPV (Fig. 4). Peripheral blood cells were collected before vaccination, at 2 weeks, 5 weeks, and 7 weeks after the 1st vaccination, and at 1 week after the challenge infection (8 weeks after the 1st vaccination) (Supplemental Fig. S1). Ig heavy chain genes were amplified by PCR using Igμ-, Igα-, and Igγ-specific primers after the extraction of RNA and reverse transcription. Thereafter, the nucleotide sequences were analyzed with a next-generation sequencer. At least 11,000 Ig sequence reads were obtained in each sample of individual macaques (Supplemental Table S3). Among the samples analyzed, 76 VH genes and 7 JH genes were identified (Supplemental Figs. S2 and S3). There was no significant difference in the percentages of VH and JH gene usage among the groups. IGHV4S7 gene and IGHJ4 gene were the most frequently detected in all of the macaques.

Next, to examine the sequences at the clonal level, we grouped sequences originating from identical V and J germline genes and had identical junctional lengths. Thereafter, clonal lineages were identified by single-linkage clustering on the CDR3 sequence using a cutoff of 90% sequence identity (Table 1). The percentages of sequence reads for each clone were calculated as the clone frequency, and clones with an increase of more than 2-fold were assumed to respond to the stimulation (vaccination or challenge infection) as previously reported [17]. The average numbers of clones that responded to the 1st vaccination (increased by more than 2-fold in sequence reads) with SV and WPV were 85.7 (3.23%) and 18.7 (1.49%), respectively. In macaques inoculated with saline, 98.7 clones (2.16%) increased from day 0 to day 14, and they were thought to indicate a background response under the specific pathogen-free (SPF) condition (Table 2).

To examine the difference in clonal diversity among the groups, we analyzed the

amino acid length in CDR3 of clones with an increase of more than 2-fold after the 1st vaccination (Fig. 5). In comparison between week 0 and week 2, the average and range of the amino acid length did not differ between the SV group and WPV groups after the 1st vaccination. On the other hand, the frequency of clones that increased by more than 10-fold was higher in the WPV group than in the SV group, although no statistically significant difference was detected (Table 3). These results suggest that the Ig clone diversity in the WPV group seems to be smaller than that in the SV group in Table 2 since the percentage of highly-responding clones in the WPV group was higher and the frequency of low-responding clones in the WPV group was lower than that in the SV group.

Since the levels of IgG specific for the vaccine antigen and the neutralization titers in the WPV group were higher than those in the SV group, we hypothesized that B-lymphocytes were activated to increase subclones more effectively in macaques vaccinated with WPV than in macaques vaccinated with SV. To examine this hypothesis, we compared the number of subclones in each clonal population at each time point (Supplemental Fig. S4A). In the macaques vaccinated with WPV, the average number of subclones in each clone was significantly increased by 8.3-fold as compared to that of subclones before vaccination ($P = 0.01$; Supplemental Table S4; Fig. 6A), whereas the average fold increases in the numbers of subclones in each clone were 3.0 and 5.8 in macaques inoculated with saline and SV, respectively. On the other hand, the increase in the number of subclones in B-lymphocyte clones was not remarkable after the 2nd vaccination with WPV (from week 2 to week 5; data not shown).

Since IgG responses specific for vaccine antigens and the neutralization titers were increased 1 week after the challenge infection, we examined the number of clones with an increase in frequency from week 7 to week 8 and the numbers of their subclones. The average numbers of clones with an increase in frequency of more than 2-fold 1 week after

the challenge infection were 28.0, 126.3, and 40.3 in macaques inoculated with saline, SV, and WPV, respectively (Table 4). The numbers of subclones 1 week after the challenge infection were increased in 88%, 56%, and 83% of responding clones in macaques inoculated with saline, SV, and WPV, respectively (Supplemental Fig. S4B). However, the average numbers of subclones in vaccinated macaques did not increase (Fig. 6B).

4. Discussion

We examined the protective efficacy of WPVs and SVs against seasonal influenza A and B viruses using cynomolgus macaques in the present study. The influenza A virus (H1N1) and influenza B virus titers in the nasal samples of macaques vaccinated with WPVs were lower than those in the nasal samples of macaques vaccinated with SVs. Inflammation in the lungs on day 7 after H1N1 virus infection was milder in the macaques immunized with WPVs than that in the macaques immunized with SVs. Neutralizing antibodies against the challenge viruses were more effectively induced in the macaques vaccinated with WPVs than in those vaccinated with SVs. In addition, in the analysis of the Ig heavy chain gene repertoire in macaques vaccinated with WPV, the number of subclones in clones responding 2 weeks after the 1st vaccination was increased.

The efficacy of WPVs was confirmed against the propagation of H1N1 influenza A virus and influenza B virus in the Victoria lineage. In the present study, SVs for H1N1 virus prepared by two manufacturers showed some effectiveness in preventing the propagation of the A/Narita/1/2009 (H1N1) strain. However, the SV for influenza B virus in the Victoria lineage did not prevent the propagation of the challenge strain B/Yokohama/14/2005 strain. From the comparison of database sequences, two amino acids differ between the HA protein in the H1N1 vaccine strain A/California/07/2009 and the HA protein in the challenge strain A/Narita/1/2009 (GISAID EPI176504 vs. EPI179437). On the other hand, there are

differences in four amino acids of the HA protein between the vaccine strain B/Texas/2/2013 and the challenge strain B/Yokohama/14/2015 (GISAID EPI443690 vs. EPI581800). Among them, two amino acid substitutions at positions 194 and 196 are located in the 190-helix around the receptor-binding site [20]. Since WPV showed a protective effect against the challenge with B/Yokohama/14/2015, it was thought that the antibodies induced by SV were affected by the amino acid substitutions in the HA protein, and that the antibodies induced by WPV were thus not induced by SV. We thought of two reasons to explain this difference between the antibodies induced by WPV and the antibodies induced by SV. Firstly, it is possible that the antibodies induced by WPV bound not only to the 190-helix and the other substituted sites, but also to the conserved residues between the vaccine strain and the challenge strain, whereas the antibodies induced by SV did mainly bound to the 190-helix and the other substituted sites (epitope spread) [21,22]. Secondly, it is possible that the antibodies induced by WPV bound more strongly to the HA protein than did those induced by SV; as a result, the amino acid substitutions had a small effect on the binding of the antibodies induced by WPV (affinity difference) [23]. The latter possibility could be supported by the increase of subclone numbers in B-lymphocytes that was shown in the present study, suggesting the induction of somatic hypermutation and affinity maturation.

We have previously reported the efficacy of WPVs against various influenza virus infections in animal models including mice and cynomolgus macaques [3-7,9]. In the previous studies, neutralizing antibody and T-lymphocyte responses were more effectively induced by vaccination with WPVs than by vaccination with SVs. One of the mechanisms for the effective induction of immune responses by WPVs is thought to be an increase of inflammatory cytokines after vaccination, which has been reported as an increase in the serum levels of IL-6, MCP-1, and IP-10 in mice 1 to 2 days after WPV vaccination [6]. Early upregulation of IL-6 in plasma after viral infection was also related to an increase in

478 neutralizing antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-
479 CoV-2) in cynomolgus macaques [24]. It is thought that the upregulation of inflammatory
480 cytokines, including IL-6, was caused by the stimulation of pattern-recognition receptors,
481 such as toll-like receptor (TLR)-7 and retinoic acid-inducible gene I (RIG-I)/melanoma
482 differentiation-associated gene 5 (MDA-5) in antigen-presenting cells [25-30]. Since it was
483 reported that the co-stimulation of B-cell receptor and TLR-7 increased somatic
484 hypermutation and memory B-cell responses [31], we examined the Ig repertoire, including
485 the clonal frequency and the number of subclones, the expansion of which indicates somatic
486 hypermutation, before and after vaccination/challenge infection. The analysis of B-
487 lymphocyte clones that responded after the 1st vaccination showed that the B-lymphocyte
488 clones in macaques immunized with WPV included a larger number of subclones than did
489 those in macaques immunized with SV. This suggests that WPV induces B-lymphocyte
490 activation including somatic hypermutation and affinity maturation, especially at the
491 priming stage of the 1st vaccination [7]. However, the average number of subclones in the
492 WVP group was not necessarily related to the neutralization titers at week 2.

493 The antibody titers of IgG specific for vaccine antigens and neutralizing antibodies
494 against the challenge strains were increased after challenge infection in macaques vaccinated
495 with WPV and SV. However, the average number of subclones in responding clones did not
496 increase in the vaccinated macaques (Fig. 6B) since the number of subclones did not increase
497 in all of the responding clones after challenge infection (Supplemental Fig. S4B). These
498 results suggest that the increase in Ig gene transcripts (presumably the increase in the amount
499 of Ig proteins) rather than affinity maturation contributes to the increase in the neutralization
500 titers at the challenge infection. In addition, when the subclone numbers in the B-lymphocyte
501 clones responding to the challenge infection (week 8) were tracked back to weeks 0 and 2 in
502 Supplemental Fig. S4B, the percentage of clones responding to the 1st vaccination with WPV

503 (31.2% on average) was larger than that of clones responding to the 1st vaccination with SV
504 (9.09% on average). This means that the B-lymphocytes activated by the 1st vaccination with
505 WPV responded at the challenge infection, indicating that WPV induces memory B-
506 lymphocytes that are reactivated at the challenge infection. Furthermore, since virus
507 propagation was very limited in macaques vaccinated with WPVs, a small amount of viral
508 antigen is thought to effectively activate memory B-lymphocytes in macaques vaccinated
509 with WPVs, suggesting that WPVs have the advantage of inducing memory B-lymphocytes
510 that are sensitive to a small amount of antigen entering through a different site (airway) from
511 the site of the priming (subcutaneous tissue).

512 In our present and previous studies, WPVs was more effective in reduction of virus
513 titers and amelioration of clinical signs of disease than SVs [3,6,7,9]. Since WPVs induced
514 more vigorous neutralizing antibody responses with the subclonal expansion in
515 immunologically naïve macaques after the 1st vaccination, one of the advantages to use
516 WPVs may be the vaccination for children. In addition, the high immunogenicity of WPVs
517 may contribute to reduction in a dose of vaccines at the 1st vaccination for priming [7]. On
518 the other hand, the second immunization with WPVs induced a further increase of antigen-
519 specific IgG and neutralization titers in blood samples, indicating that WPVs have a boost
520 effect in the human population in the presence of preexisting immunity against influenza
521 viruses. Therefore, vaccination with WPVs has advantages for naïve and immunized
522 individuals against influenza viruses over SVs.

523 In the present study, we confirmed the efficacy of WPVs against seasonal influenza
524 A and B viruses using cynomolgus macaques and revealed that WPVs induce the
525 differentiation of subclones in B-lymphocytes responding to vaccine antigens, especially at
526 the priming stage. In human studies on vaccination, it is sometimes difficult to evaluate the
527 immunogenicity of vaccines and to perform Ig repertoire analysis at the priming stage since

most human adult participants are not immunologically naïve against influenza viruses [17,29]. Thus, it is advantageous to use naïve macaques to examine the immunogenicity of vaccines and alterations in the Ig repertoire due to vaccination at the priming in addition to the challenge infection. We did not practically isolate B-lymphocyte clones reacting to vaccination. However, a comparison of Ig gene frequencies before and after vaccination/challenge infection allowed us to identify B-lymphocytes responding to stimulations. Thus, WPVs induced an increase of subclones in B-lymphocytes, resulting in affinity maturation and effective antibody responses for protection against seasonal influenza virus infection. Since the differentiation and activation of B-lymphocytes require the cooperation of helper T-cells, we will examine the characteristics of T-cell repertoires responding to vaccine antigens in the future.

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Figure legends

Figure 1. Virus titers in nasal swab samples of macaques infected with H1N1 influenza A virus or influenza B virus

Cynomolgus macaques were subcutaneously immunized twice with the whole virus particle vaccine (WPV) inactivated with formalin and β-propiolactone (C), with β-propiolactone (F, J) or with the split vaccine (SV) (B, E, I). Four weeks after the 2nd vaccination, the challenge virus A/Narita/1/2009 (H1N1) (A-G) or B/Yokohama/14/2015 (Victoria lineage) (H-K) was inoculated into the nostrils of macaques. Nasal swab samples were collected on the indicated days. (A, H) Control macaques (A: saline; H: without vaccination). (B, E, I) Macaques vaccinated with the SV. (C, F, J) Macaques vaccinated with the WPV. (D, G, K) Averages and standard deviations of virus titer areas under the virus titer time curves (virus titer AUCs) in nasal swab samples were calculated on the basis of the titers shown in Fig. 1A-C, E, F, and H-J. Virus titers under the detection limit were calculated as 0. Averages and standard deviations of three macaques are shown. Red: control; blue: SV; and orange: WPV. The P-values indicated in the graphs were calculated with one-way ANOVA with multiple comparisons (Bonferroni method), the Student's *t*-test, and the Mann-Whitney U-test, respectively.

Figure 2. Body temperatures in macaques after inoculation with the challenge viruses

Cynomolgus macaques were subcutaneously immunized with WPV inactivated with (A) formalin and β-propiolactone, (B, C) β-propiolactone or with SV (A-C). On day 0, 4 weeks after the 2nd vaccination, (A, B) A/Narita/1/2009 (H1N1) or (C) B/Yokohama/14/2015 (Victoria lineage) was inoculated into the nasal cavities of the macaques. Average body temperatures from 8 p.m. to 8 a.m. the next day were calculated on the basis of data for individual macaques since temperatures during the daytime are affected by anesthesia. For

example, the temperatures on day 0 mean the average temperatures between 8 p.m. on day 0 and 8 a.m. on day 1 after virus inoculation. The average body temperatures of each day were compared to those of day -1 (from 8 p.m. on day -1 to 8 a.m. on day 0 before virus inoculation). Asterisks indicate significant differences between the saline group and the WPV group ($P < 0.05$, one-way ANOVA with multiple comparisons (Bonferroni method)).

Figure 3. Viral pneumonia in macaques after inoculation with the challenge viruses

Cynomolgus macaques were subcutaneously immunized with WPV inactivated with formalin and β-propiolactone or with SV. On day 0, 4 weeks after the 2nd vaccination, A/Narita/1/2009 (H1N1) was inoculated into the nasal cavities of the macaques. (A-C) Lung tissues were collected on day 7 after challenge virus inoculation. Hematoxylin and eosin sections were examined. A: control; B: SV; and C: WPV. Bars indicate 100 μm. (D) Histological scores of pneumonia. Histological pneumonia was evaluated as described in the Materials and Method. Dots indicate the average histological scores of 6 to 8 lung sections in individual macaques and bars indicate averages of three macaques. A significant difference between the saline group and WPV group was calculated by one-way ANOVA with multiple comparisons (Bonferroni method).

Figure 4. Antibody responses specific for vaccine antigens and influenza A and B viruses in vaccinated cynomolgus macaques

Cynomolgus macaques were subcutaneously immunized twice (weeks 0 and 3) with SVs, with WPVs inactivated with formalin and β-propiolactone, or with β-propiolactone. Plasma was collected at the indicated weeks before and after the 1st vaccination (A-F). Plasma was collected in week 4 (G) and week 5 (H, I). (A-C) IgG antibodies specific for vaccine antigens in diluted sera were analyzed by ELISA. Optical densities at 450 nm are shown. (D-I) The

neutralization titers against (D, E) A/Narita/1/2009 (H1N1), (G, H) A/Singapore/GP1908/2015 (H1N1), (F) B/Yokohama/14/2015 (Victoria lineage), and (I) B/Maryland/15/2016 (Victoria lineage) are expressed as reciprocals of the dilution of the plasma samples that showed a cytopathic effect in 50% of the wells. Detection limits were $1:2^3$ (dotted lines). SV and WPV mean averages of three macaques. (J-L) Correlation analysis between neutralization titers and nasal virus titer AUCs. 50% neutralization titers in week 4 shown in Fig. 4D, E and F were plotted with nasal virus titer AUCs calculated based on Fig. 2B, C, E, F, I, and J.

Figure 5. The amino acid lengths of the CDR3 region of Ig clones increasing by more than 2-fold after the 1st vaccination

The amino acid lengths of CDR3 region were determined in the Ig clones that increased by more than 2-fold in the percentage from week 0 to week 2 (Table 2). Dots indicate average CDR3 lengths of Ig clones in individual macaques. Bars indicate SD. No significant differences were calculated among the groups (one-way ANOVA with multiple comparisons (Bonferroni method)).

Figure 6. Number of Ig subclones of responding B-lymphocyte clones

Peripheral blood cells of cynomolgus macaques were collected at the indicated weeks after the 1st vaccination. Ig heavy chain genes were amplified and sequenced as shown in Supplemental Table S3. After the differentiation of clones by CDR3 sequences, subclones were identified. The average numbers of subclones in clones that increased (A) from week 0 to week 2 (data of individual macaques were shown in Supplemental Table S4.) and (B) from week 7 to week 8 in individual macaques are shown.

Figure 1

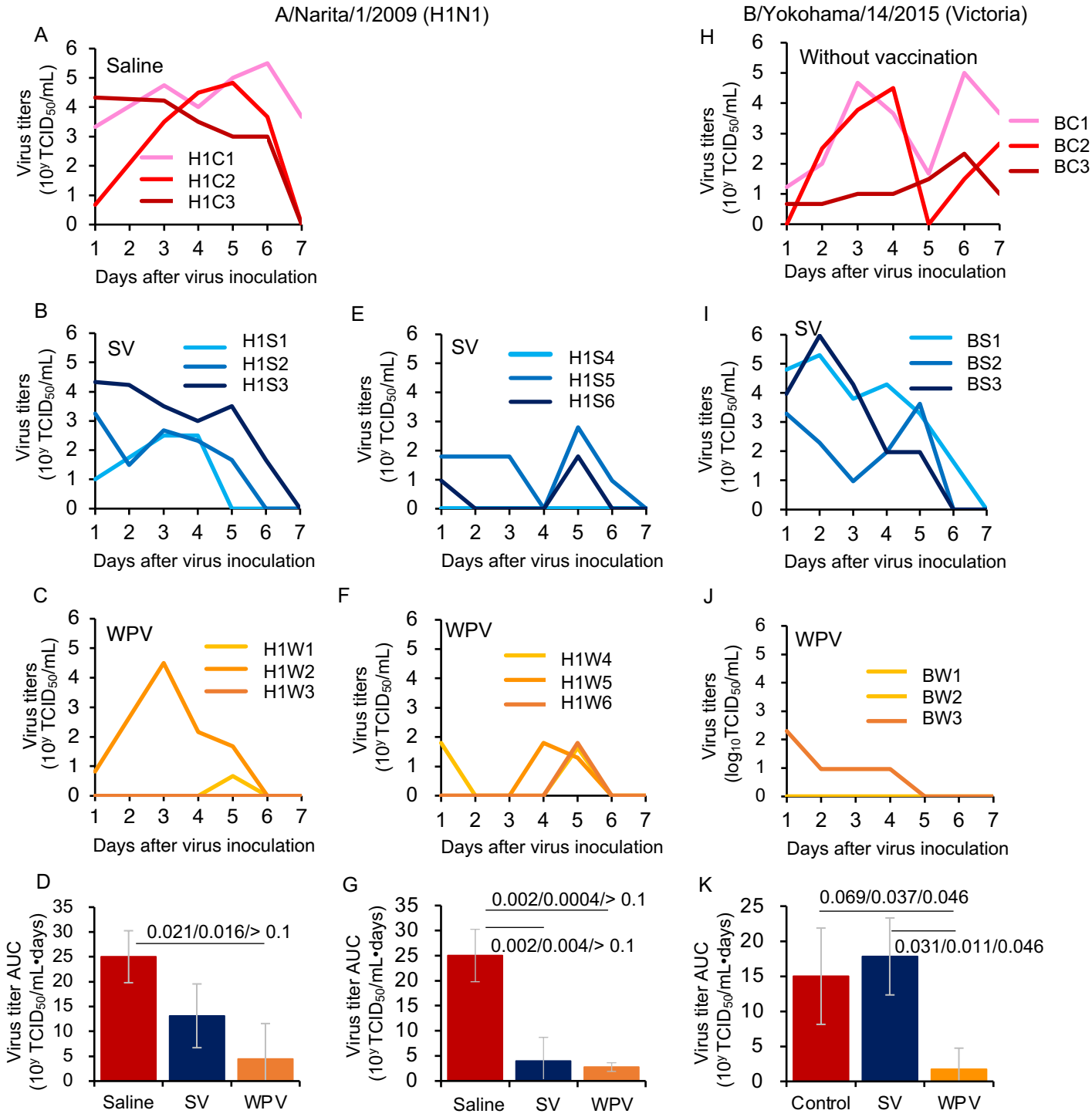

Figure 1. Virus titers in nasal swab samples of macaques infected with H1N1 influenza A virus or influenza B virus
Cynomolgus macaques were subcutaneously immunized twice with the whole virus particle vaccine (WPV) inactivated with formalin and β-propiolactone (C, D), with β-propiolactone (F, J) or with the split vaccine (SV) (B, E, I). Four weeks after the 2nd vaccination, the challenge virus A/Narita/1/2009 (H1N1) (A-G) or B/Yokohama/14/2015 (Victoria lineage) (H-K) was inoculated into the nostrils of macaques. Nasal swab samples were collected on the indicated days. (A, H) Control macaques (A: saline; H: without vaccination). (B, E, I) Macaques vaccinated with the SV. (C, F, J) Macaques vaccinated with the WPV. (D, G, K) Averages and standard deviations of virus titer areas under the virus titer time curves (virus titer AUCs) in nasal swab samples were calculated on the basis of the titers shown in Fig. 1A-C, E, F, and H-J. Virus titers under the detection limit were calculated as 0. Averages and standard deviations of three macaques are shown. Red: control; blue: SV; and orange: WPV. The P-values indicated in the graphs were calculated by one-way ANOVA with the multiple comparions (Bonferroni method), the Student's *t*-test, and the Mann-Whitney U-test, respectively.

Fig. 2

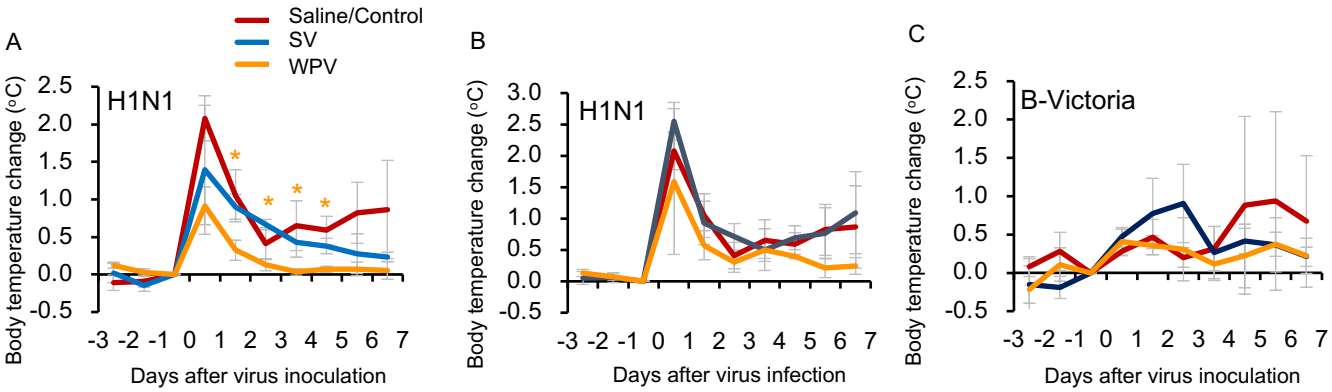

Figure 2. Body temperatures in macaques after inoculation with the challenge viruses
Cynomolgus macaques were subcutaneously immunized with WPV inactivated with (A) formalin and β-propiolactone, (B, C) β-propiolactone or with SV (A-C). On day 0, 4 weeks after the 2nd vaccination, (A, B) A/Narita/1/2009 (H1N1) or (C) B/Yokohama/14/2015 (Victoria lineage) was inoculated into the nasal cavities of the macaques. Average body temperatures from 8 p.m. to 8 a.m. the next day were calculated on the basis of data for individual macaques since temperatures during the daytime are affected by anesthesia. For example, the temperatures on day 0 mean the average temperatures between 8 p.m. on day 0 and 8 a.m. on day 1 after virus inoculation. The average body temperatures of each day were compared to those of day -1 (from 8 p.m. on day -1 to 8 a.m. on day 0 before virus inoculation). Asterisks indicate significant differences between the saline group and the WPV group ($P < 0.05$, one-way ANOVA with multiple comparisons (Bonferroni method)).

Fig. 3

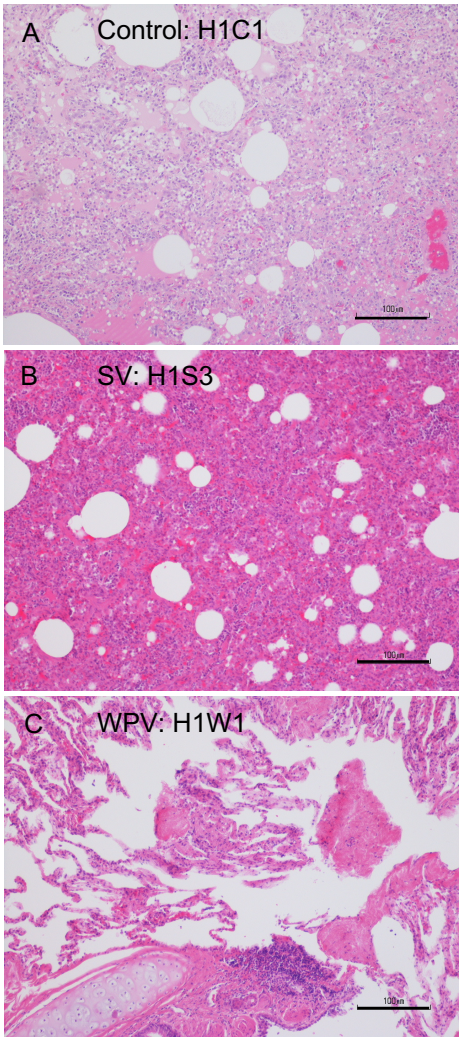

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Figure 4

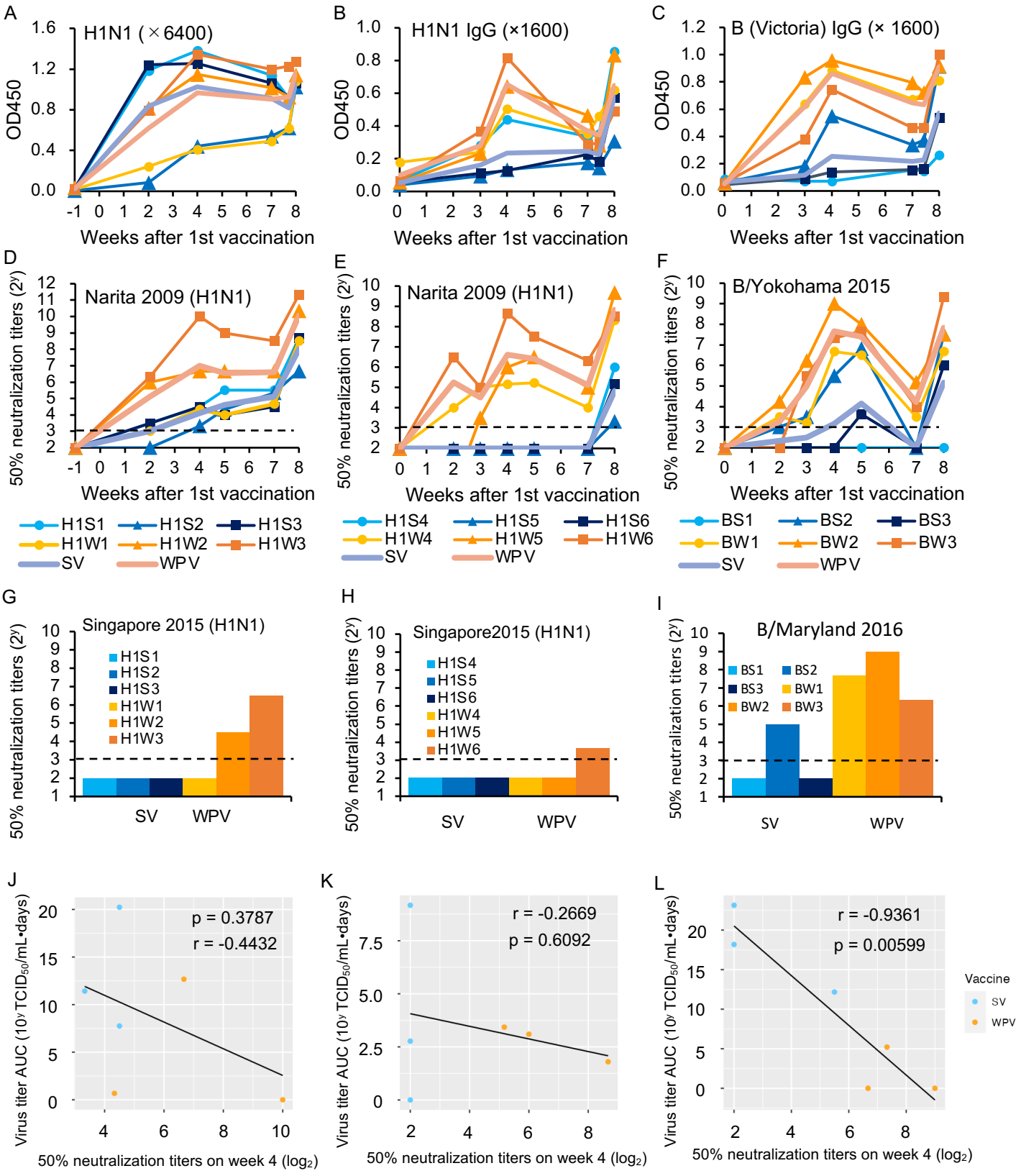
A
H1N1 (×6400)
OD450
Weeks after 1st vaccination
B
H1N1 IgG (×1600)
OD450
Weeks after 1st vaccination
C
B (Victoria) IgG (× 1600)
OD450
Weeks after 1st vaccination
D
Narita 2009 (H1N1)
50% neutralization titers (2^y)
Weeks after 1st vaccination
H1S1 H1S2 H1S3
H1W1 H1W2 H1W3
SV WPV
E
Narita 2009 (H1N1)
50% neutralization titers (2^y)
Weeks after 1st vaccination
H1S4 H1S5 H1S6
H1W4 H1W5 H1W6
SV WPV
F
B/Yokohama 2015
50% neutralization titers (2^y)
Weeks after 1st vaccination
BS1 BS2 BS3
BW1 BW2 BW3
SV WPV
G
Singapore 2015 (H1N1)
50% neutralization titers (2^y)
H1S1
H1S2
H1S3
H1W1
H1W2
H1W3
SV WPV
H
Singapore2015 (H1N1)
50% neutralization titers (2^y)
H1S4
H1S5
H1S6
H1W4
H1W5
H1W6
SV WPV
I
B/Maryland 2016
50% neutralization titers (2^y)
BS1 BS2
BS3 BW1
BW2 BW3
SV WPV
J
p = 0.3787
r = -0.4432
Virus titer AUC (10^y TCID50/mL•days)
50% neutralization titers on week 4 (log2)
K
r = -0.2669
p = 0.6092
Virus titer AUC (10^y TCID50/mL•days)
50% neutralization titers on week 4 (log2)
L
r = -0.9361
p = 0.00599
Virus titer AUC (10^y TCID50/mL•days)
50% neutralization titers on week 4 (log2)
Vaccine
SV
WPV

Figure 4. Antibody responses specific for vaccine antigens and influenza A and B viruses in vaccinated cynomolgus macaques

Cynomolgus macaques were subcutaneously immunized twice (weeks 0 and 3) with SVs, with WPVs inactivated with formalin and β-propiolactone, or with β-propiolactone. Plasma was collected at the indicated weeks before and after the 1st vaccination (A-F). Plasma was collected in week 4 (G) and week 5 (H, I). (A-C) IgG antibodies specific for vaccine antigens in diluted sera were analyzed by ELISA. Optical densities at 450 nm are shown. (D-I) The neutralization titers against (D, E) A/Narita/1/2009 (H1N1), (G, H) A/Singapore/GP1908/2015 (H1N1), (F) B/Yokohama/14/2015 (Victoria lineage), and (I) B/Maryland/15/2016 (Victoria lineage) are expressed as reciprocals of the dilution of the plasma samples that showed a cytopathic effect in 50% of the wells. Detection limits were $1:2^3$ (dotted lines). SV and WPV mean averages of three macaques. (J-L) Correlation analysis between neutralization titers and nasal virus titer AUCs. 50% neutralization titers in week 4 shown in Fig. 4D, E and F were plotted with nasal virus titer AUCs calculated based on Fig. 2B, C, E, F, I, and J.

Table 1. Number of Ig clones before and after vaccination and challenge infection

		Weeks after 1st vaccination				
Vaccine	Monkey	0	2	5	7	8
Saline	H1C1	2100[1]	3389	961	2220	3015
	H1C2	4127	4200	742	1724	2858
	H1C3	8037	5319	682	1212	1141
SV	H1S1	4850	6093	1974	3801	3246
	H1S2	3394	5572	3308	3159	1903
	H1S3	437	6331	3855	3390	899
WPV	H1W1	1361	3972	3109	1890	1426
	H1W2	1414	7357	4056	829	1351
	H1W3	894	4549	3336	1820	3002

[1] Ig clones were determined as Ig heavy chain gene sequences that had identical V and J genes and identical amino acid lengths in the CDR3 regions with more than 90% similarity in the amino acid sequences.

Table 2. Number of Ig clones increasing 2 weeks after the 1st vaccination

Vaccine	Monkey	Number of clones[1]	%clones[2]	Average Number of clones	SD	Average %	SD%
Saline	H1C1	34	1.62	98.7	56.1	2.16	0.97
	H1C2	135	3.27				
	H1C3	127	1.58				
SV	H1S1	121	2.49	85.7	60.3	3.23 [3]	0.64
	H1S2	120	3.54				
	H1S3	16	3.66				
WPV	H1W1	22	1.62	18.7	6.7	1.49 [3]	0.23
	H1W2	23	1.63				
	H1W3	11	1.23				

[1] Ig clones were identified as described in Table 1. After the calculation of the frequency of each clone in the total reads, the clones that increased by more than 2-fold in the percentage from week 0 to week 2 (before and after the 1st vaccination) were counted.

[2] The percentage of clones that increased by more than 2-fold was calculated as follows: %clones = number of clones that increased by more than 2-fold in the percentage from week 0 to week 2 / the number of clones in week 0.

[3] A significant difference was detected in the percentage of clone numbers increasing after the 1st vaccination between the SV group and the WPV group (P = 0.011, Student's t-test), but not by one-way ANOVA with multiple comparisons (Bonferroni method).

Figure 5

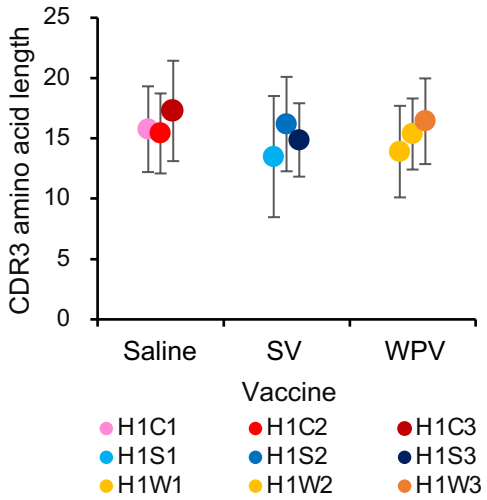

Figure 5. The amino acid lengths of the CDR3 region of Ig clones increasing by more than 2-fold after the 1st vaccination

The amino acid lengths of CDR3 region were determined in the Ig clones that increased by more than 2-fold in the percentage from week 0 to week 2 (Table 2). Dots indicate average CDR3 lengths of Ig clones in individual macaques. Bars indicate SD. No significant differences were calculated among the groups (one-way ANOVA with multiple comparisons).

Table 3. Ig clones increasing by more than 10-fold 2 weeks after the 1st vaccination

Vaccine	Monkey	Number of responding clones[1]	Number of clones with >10 fold increase[2]	%clones with >10-fold increase[3]	Average%[4]	SD%
Saline	H1C1	34	5	14.71	19.58	5.75
	H1C2	135	35	25.93		
	H1C3	127	23	18.11		
SV	H1S1	121	9	7.44	22.34	19.01
	H1S2	120	19	15.83		
	H1S3	16	7	43.75		
WPV	H1W1	22	8	36.36	37.68	25.32
	H1W2	23	3	13.04		
	H1W3	11	7	63.64		

[1] The number of clones that increased by more than 2-fold in the percentage from week 0 to week 2 as Table 2.
[2] The number of clones that increased by more than 10-fold in the percentage from week 0 to week 2.
[3] The percentage of clones that increased by more than 10-fold was calculated as follows: %clones = the number of clones that increased by more than 10-fold / the number of clones that increased by more than 2-fold.
[4] No significant difference in the percentage of clones with a more than 10-fold increase between the Saline group and the WPV group and between the SV group and the WPV group was calculated by the one-way ANOVA with multiple comparisons (Bonferroni method).

Figure 6

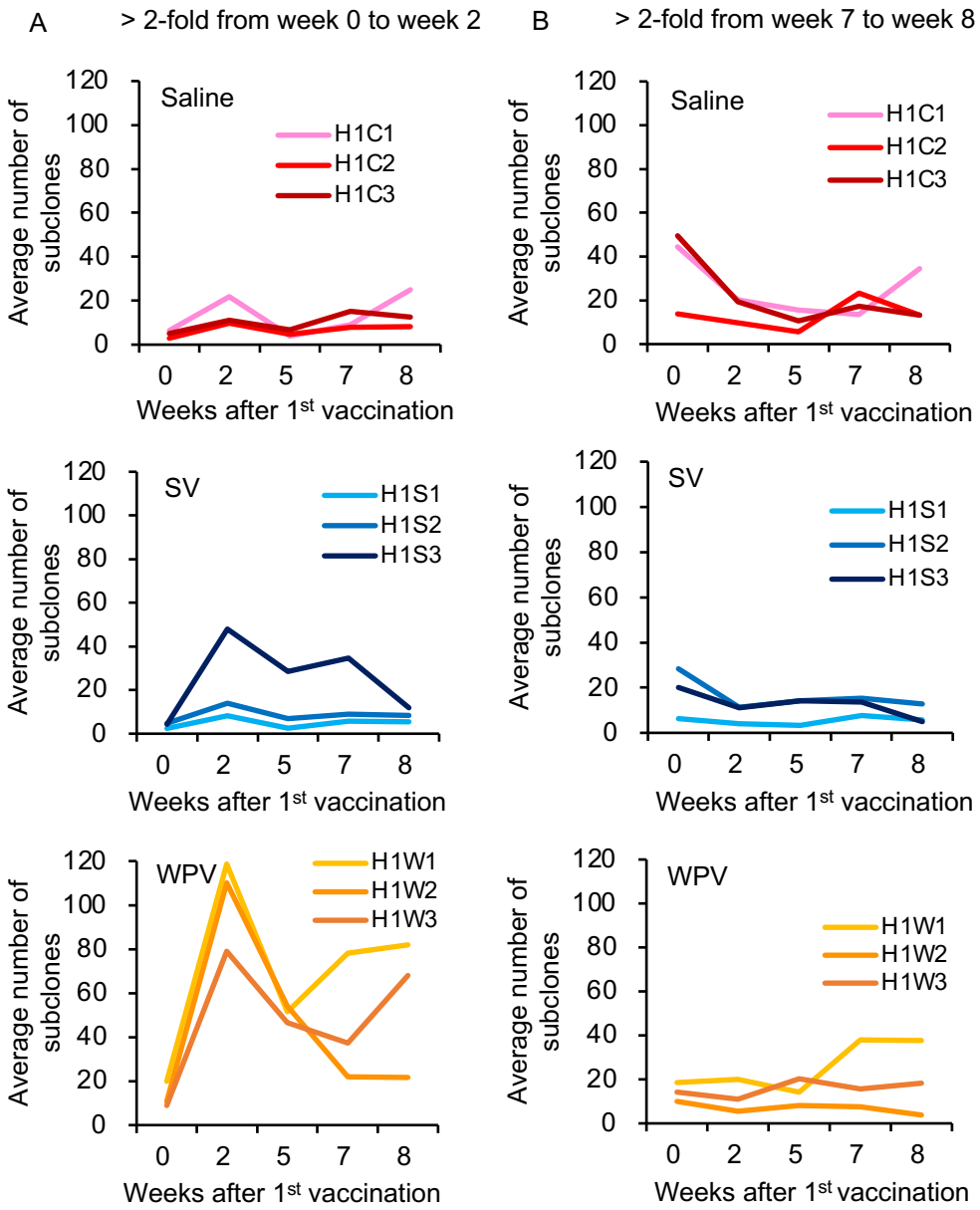

Figure 6. Number of Ig subclones of responding B-lymphocyte clones

Peripheral blood cells of cynomolgus macaques were collected at the indicated weeks after the 1st vaccination. Ig heavy chain genes were amplified and sequenced as shown in Supplemental Table S3. After the differentiation of clones by CDR3 sequences, subclones were identified. The average numbers of subclones in clones that increased (A) from week 0 to week 2 (data of individual macaques were shown in Supplemental Table S4.) and (B) from week 7 to week 8 in individual macaques are shown.

Table 4. Number of Ig clones increasing after the challenge infection

Vaccine	Monkey	Number of clones[1]	%clones[2]	Average Number of clones	SD	Average%[3]	SD%
Saline	H1C1	33	1.49	28.0	4.58	1.68	0.265
	H1C2	27	1.57				
	H1C3	24	1.98				
SV	H1S1	191	5.02	126.3	56.2	3.59	1.26
	H1S2	99	3.13				
	H1S3	89	2.63				
WPV	H1W1	57	3.02	40.3	17.6	2.66	0.354
	H1W2	22	2.65				
	H1W3	42	2.31				

[1] Ig clones were identified as described in Table 1. After the calculation of the frequency of each clone in the total reads, the clones that increased by more than 2-fold in the percentage from week 7 to week 8 (before and after the challenge infection) were counted.

[2] The percentage of clones that increased by more than 2-fold was calculated as followings: %clones = number of clones that increased by more than 2-fold in the percentage from week 7 to week 8/ the number of clones in week 7.

[3] A significant difference was detected in the percentage of clone numbers increasing after the challenge infection between the saline group and the SV group (P = 0.021, Student's t-test). The P value of a comparison between the SV group and the WPV group was 0.051. No significant difference was detected by one-way ANOVA with multiple conparisons (Bonferroni method).