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Inactivated whole virus particle vaccine with potent immunogenicity and limited IL-6 induction is ideal for influenza

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ABSTRACT

In contrast to current ether- or detergent-disrupted "split" vaccines (SVs) for influenza, inactivated whole influenza virus particle vaccines (WPVs) retain the original virus structure and components and as such may confer similar immunity to natural infection. In a collaboration between academia and industry, the potential of WPV as a new seasonal influenza vaccine was investigated. Each of the four seasonal influenza vaccine manufacturers in Japan prepared WPVs and SVs from the same batches of purified influenza virus. Both mice and monkeys vaccinated with the WPVs exhibited superior immune responses to those vaccinated with the corresponding SVs. Vaccination with A/California/07/2009 (H1N1) WPV enabled mice to survive a lethal challenge dose of homologous virus whereas those vaccinated with SV succumbed to infection within 6 days. Furthermore, mice vaccinated with WPV induced substantial numbers of multifunctional $CD8^+$ T cells, important for control of antigenically drifted influenza virus strains. In addition, cytokines and chemokines were detected at early time points in the sera of mice vaccinated with WPV but not in those animals vaccinated with SV. These results indicate that WPVs induce enhanced innate and adaptive immune responses compared to equivalent doses of SVs. Notably, WPV at one fifth of the dose of SV was able to induce potent immunity with limited production of IL-6, one of the pyrogenic cytokines. We thus propose that WPVs with balanced immunogenicity and safety may set a new global standard for seasonal influenza vaccines.

1. Introduction

Influenza remains a major public health concern with more than 5 million severe infections and 500,000 deaths per year worldwide (WHO) [1,2]. Children and the elderly are at particular risk of severe disease, hospitalization, and mortality due to seasonal influenza virus infection. In fact, over 90% of influenza-

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related deaths occur in the elderly population [3]. Furthermore, children play an important role in transmission of influenza viruses within the community and, as such, limiting infection in this group is key to reducing spread of the disease in the wider population. Annual vaccination is the best strategy to prevent influenza epidemics and to reduce an individual's risk of severe disease caused by influenza virus infection.

There are three types of inactivated vaccines available for influenza: whole virus particle vaccine (WPV), split virus vaccine (SV), and purified subunit vaccine. The classical WPV production starts with propagation of influenza virus in embryonated chicken eggs followed by inactivation with formalin and/or β-propiolactone. SV is prepared by treating purified virus with ether or detergents to disrupt the viral envelope. Subunit vaccines have an additional step to isolate the haemagglutinin (HA) and neuraminidase proteins [4–6]. Based on these differences in preparation, WPV is the only form of vaccine that retains the original structure of influenza virus including the entire set of structural proteins and genomic RNAs.

Although WPV was used as the original seasonal influenza vaccine, these were later replaced with SV due to concerns about pyrogenicity and side reactions [7–10]. However, SV boosts immunity to only a small extent in primed populations and does not efficiently induce immunity in unprimed populations such as young children, or in the elderly and immunocompromised individuals [3,11–15]. Thus, the current vaccine program, in which SV has been used for more than 45 years, needs to be updated to provide enhanced protective efficacy, particularly for high risk individuals. In contrast to SV, many studies have shown that WPV effectively induces humoral and cellular immune responses, even in unprimed populations [16–19]. However, in theory, the immune processes that provide WPV with enhanced immunogenicity also have the potential to induce undesired side reactions [7–9]. An influenza vaccine that is highly immunogenic but does not result in excess pyrogenic response would be a significant improvement for the control of influenza epidemics.

To develop a WPV with balanced safety and immunogenicity, especially for unprimed young children and the elderly, we have established the All-Japan Influenza Vaccine Study Group, which includes all four seasonal influenza vaccine manufacturers in Japan. To compare the immunological potency of WPV to that of SV in animals, each manufacturer prepared highly purified and inactivated influenza virus preparations to reduce undesired reactions and used these to produce both WPV and SV. We found that the WPV prepared by each of the four vaccine manufacturers induced higher neutralizing antibodies and innate immune responses in mice than did the corresponding SV and had other features suggestive of improved potency.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby canine kidney (MDCK) cells were grown in RP10 (RPMI 1640; Thermo Fisher Scientific, MA, USA) supplemented with 10% inactivated fetal bovine serum (FBS; GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK), 1 mM of sodium pyruvate (Thermo Fisher Scientific), 50 μM of 2-mercaptoethanol (Merck, Darmstadt, Germany), 100 μg/ml of penicillin (Thermo Fisher Scientific), 100 μg/ml of streptomycin (Thermo Fisher Scientific), and 20 μg/ml of gentamicin (Thermo Fisher Scientific). These were used for the neutralization assays. Influenza viruses A/California/07/2009 (H1N1) and B/Texas/2/2013 (Victoria lineage) were kindly provided by the National Institute of Infectious Diseases (NIID) in Japan. Viruses were propagated in 10-day embryonated chicken eggs. The collected allantoic fluids were stored at −80 °C until use.

2.2. Vaccines

WPVs and SVs used in this study were provided by each of the four vaccine manufacturers (KM Biologics Co. Ltd., Denka Seiken Co., Ltd., The Research Foundation for Microbial Diseases of Osaka University, and Kitasato Daiichi Sankyo Vaccine Co. Ltd.) in Japan. Monovalent vaccines contained a reassortant with the surface antigens of A/California/07/2009 (H1N1) or B/Texas/2/2013 (Victoria). One vaccine manufacturer also produced a quadrivalent vaccine containing viruses with the surface antigens of A/California/07/2009 (H1N1), A/Hong Kong/4801/2014 (H3N2), B/Phuket/3073/2013 (Yamagata), and B/Texas/2/2013 (Victoria). Vaccine virus strains were propagated in embryonated chicken eggs and highly purified from the allantoic fluids through sucrose density gradient centrifugation [20] that had not being applied for the preparation of the old WPVs until the time when influenza vaccine for human use was changed to ether-split one in 1972 in Japan. In the present study, WPVs were prepared from the highly 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 current seasonal influenza vaccine production. HA protein concentrations of WPV and SV were calculated using the single-radial-immunodiffusion method. In addition, WPVs in this study showed almost equivalent chicken cell agglutination (CCA) values with the old WPVs.

2.3. Animals

Healthy female and male cynomolgus macaques (*Macaca fascicularis*) aged 5–9 years old were imported from the Philippines and kept in a BSL-2 and -3 laboratories at Shiga University of Medical Science. Macaques were singly housed under the proper conditions. Macaques (3 animals/group) were vaccinated subcutaneously with A/California/07/2009 (H1N1) or B/Texas/2/2013 (Victoria) monovalent WPV or SV containing 15 μg HA under anesthesia with ketamine (5 mg/kg) and xylazine (1 mg/kg). Three weeks after vaccination, blood samples were collected in heparin tubes under the same anesthesia conditions as described above, and plasma for the measurement of neutralizing antibody titers was separated and stored at −80 °C until use. Throughout the experiments, all efforts were made to minimize suffering of animals.

Female C57BL/6 mice were purchased from Hokudo Co. Ltd, Sapporo, Japan and kept in a BSL-2 laboratory at the Research Center for Zoonosis Control, Hokkaido University. Either A/California/07/2009 (H1N1) monovalent WPV (3, 0.6, 0.3, 0.12, and 0.06 μg HA protein) or SV (3 μg HA protein) was injected subcutaneously into 7-week-old female C57BL/6 mice under inhalation anesthesia with isoflurane. Serum samples were collected on day 24 after injection to estimate neutralizing antibody titers. On day 28 after injection, mice were challenged intranasally with 3000 plaque forming unit (PFU) of A/California/07/2009 (H1N1) in 40 μl of PBS under inhalation anesthesia with isoflurane. Body weight loss was monitored daily after infection with a maximum weight loss limit of 20%. Mice meeting this criterion were humanely euthanized. Current SVs for human use contain 15 μg HA for each strain. In this study, macaques were vaccinated with equivalent HA amount of WPV and mice were vaccinated with 1/5 to 1/250 amount of the HA in WPV compared with a dose applied in humans.

2.4. Serological tests

To measure neutralizing antibody titers in the sera of macaques and mice, monolayers of MDCK cells were prepared by seeding 1.2×10^6 cells in 3 ml of RP10 medium in each well of a 6-well tissue culture plate and incubated overnight at 37 °C in 5% CO_2. The monolayers were then washed with RPMI 1640 medium with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 20 μg/ml of gentamicin before addition of 100 μl of virus-serum mixtures to each well. The virus-serum mixtures comprised serial dilutions of sera in 50 μl mixed with an equal volume of influenza virus to give a final concentration of 100 PFU/100 μl and incubated together for 1 h at RT. After washing with PBS, 100 μl of virus-serum mixture was added to each well and allowed to adsorb to the monolayers for 45 min at 37 °C. The plates were shaken gently at 15 min intervals. Warmed (45 °C) overlay medium (3 ml/well), consisting of Leibovitz L-15 with glutamine at pH 6.8 (Thermo Fisher Scientific) supplemented with 0.028% (w/v) $NaHCO_3$ (Merck), 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.1% (w/v) TPCK-treated trypsin (Merck), and 0.9% (w/v) agarose (Merck) was then added. The plates were incubated at 37 °C in 5% CO_2 for 3 days and plaques on the monolayers counted without staining. The virus neutralizing antibody titer was expressed as the reciprocal of the highest dilution of serum that reduced the number of plaques to 50% of the value of the control that had no serum.

2.5. Intracellular cytokine staining

For the cytotoxic T cell analysis, 7-week-old female mice were inoculated subcutaneously with WPVs or SVs (containing 3 μg HA for monovalent vaccines or 3 μg HA of each strain for quadrivalent vaccines) or with PBS. Spleens were collected on day 10 after injection. Cell suspensions (1×10^6 splenocytes) from the spleen were cultured in the presence of the H-2D^b-restricted influenza virus nucleoprotein (NP)-derived immunodominant epitope (ASNENMETM; 2 μg/ml) in a mixture with 200 μl of RP10 containing BD GolgiPlug (1 mg/ml) from a Cytofix/Cytoperm Plus kit (BD Biosciences, CA, USA) and recombinant IL-2 (10 U/ml; Roche, Mannheim, Germany) at 37 °C in 5% CO_2. After 12 h, splenocytes were washed with fluorescence-activated cell sorter (FACS) wash buffer (1% FBS–5 mM EDTA–PBS) and stained with a PerCP Cy5.5-conjugated rat anti-mouse CD8 antibody (Clone: 53–6.7, BioLegend, CA, USA) for 30 min at 4 °C. Fixation and permeabilization were then performed for 20 min at 4 °C using Cytofix/Cytoperm solution (BD Biosciences) according to the manufacturer's instructions. Cells were washed once and stained for intracellular IFN-γ (FITC; Clone: XMG1.2, BioLegend), IL-2 (APC; Clone: JES6-5H4, BioLegend), or TNF-α (PE/Cy7; Clone: MP6-XT22, BioLegend) for 30 min at 4 °C. Flow cytometric analysis was performed with FACS Fortessa system (BD Biosciences) and data analysis was performed using FlowJo software (FlowJo Llc., OR, USA).

2.6. Multiplex cytokine detection assay

WPVs or SVs containing 3 μg HA, or PBS were injected subcutaneously into 7-week-old female mice and blood samples were collected at 3, 6, 12, 24, or 48 h post injection. In addition, to examine dose-responses of WPV for cytokine responses, WPVs (containing 3, 0.6, 0.3, or 0.12 μg HA), SV (3 μg HA), or PBS were injected subcutaneously into mice and blood samples collected at 3 h post injection. The blood samples were allowed to clot at RT for 1–2 h followed by centrifugation at 800 × *g* for 10 min to collect the sera, which were stored at −80 °C until use. Concentrations of cytokines and chemokines (IFN-γ, IL-1β, IL-6, IL-12(p70), IL-17, TNF-α, IP-10, and MCP-1) were determined using a MAGPIX Milliplex kit (Merck) according to the instruction of the manufacturer. Briefly, 25 μl of serum samples, standards, and controls were added to a 96-well plate containing an equal amount of assay buffer in the kit. Magnetic beads coated with the antibodies against the target cytokines were added into each well and the plates were incubated on a plate shaker overnight at 4 °C. After washing with wash buffer in the kit, the samples were reacted with biotinylated detection antibodies for 1 h at RT and then streptavidin-phycoerythrin for 30 min at RT. After washing and addition of loading buffer from the kit, the samples were analyzed by the MGPIX system (Luminex, TX, USA).

2.7. Statistical analysis

Prism 7 (GraphPad Software, CA, USA) or Excel (Microsoft Corporation, WA, USA) was used to perform statistical analysis. *P* values were obtained using unpaired *t*-test or one-way ANOVA non-parametric analysis. The $p < 0.05$ values were considered to be statistically significant.

2.8. Ethics statement

All monkey experiments were 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 Standards Relating to the Care and 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, Japan. The protocols were approved by the Shiga University of Medical Science Animal Experiment Committee (permit number 2015-6-3HH). The Research Center for Animal Life Science at Shiga University of Medical Science has a permit for 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 Shiga University of Medical Science.

All mouse experiments were performed with approval from 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.

3. Results

3.1. WPVs induced higher neutralizing antibody responses than SVs in both macaques and mice

The immunogenicity of A/California/07/2009 (H1N1) or B/Texas/2/2013 (Victoria) monovalent WPV and SV in macaques was evaluated by comparing serum neutralizing antibody titers against the respective homologous influenza viruses (Fig. 1). The serum neutralizing antibody titers against A/California/07/2009 (H1N1) in macaques vaccinated with A/H1N1 WPV reached 40–160 whereas only one of the animals vaccinated with the corresponding SV exhibited a detectable neutralizing antibody, which was 10 (Fig. 1A). In macaques vaccinated with B/Texas/2/2013 (Victoria) WPV, all 3 produced neutralizing antibodies against the homologous influenza virus and two achieved titers of 80 (Fig. 1B). In contrast, none of the animals immunized with SV exhibited detectable responses against the B virus. These results indicate that, for an equivalent HA dose, WPV induces superior neutralizing antibody immunity over SV in primates. After vaccination with WPV or SV, local reactions were not found in mice nor macaques, and maximum body temperature of macaques was 38.0 °C in the present study.

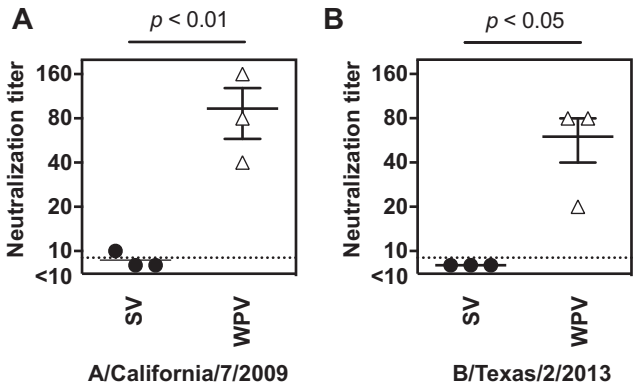

Fig. 1. WPV induced higher neutralizing antibodies than did SV in cynomolgus macaques. Healthy cynomolgus macaques (n = 3 per group) were vaccinated subcutaneously with A/California/07/2009 (H1N1) WPV or SV (monovalent A/H1N1) or B/Texas/2/2013 (Victoria lineage) WPV or SV (B/Victoria monovalent), each containing 15 μg HA. Plasma was sampled from each animal at week 3 to examine neutralizing antibody titers. Neutralizing antibody titers were assessed against A/California/07/2009 (H1N1) virus (A) and B/Texas/2/2013 (Victoria) virus (B). Symbols represent the titers obtained from individual macaques and horizontal lines indicate the mean ±SEM for the group. Statistical analysis was performed using a Student *t*-test.

More detailed analyses were performed in a mouse model using A/California/07/2009 (H1N1) monovalent vaccines from each vaccine manufacturer. Neutralizing antibody titers in the sera of mice against homologous influenza virus on day 24 after vaccination were compared for WPVs (3–0.06 μg HA) and SVs (3 μg HA) (Fig. 2). SVs from all the vaccine manufacturers scarcely induced detectable neutralizing antibodies. In contrast, neutralizing antibody levels were significantly greater (16–64-fold) in mice immunized with WPV containing the same amount of HA, reaching 1280 on average in mice vaccinated with WPV prepared by manufacturer A. With the exception of manufacturer B, WPV containing as little as 0.3 μg HA induced significantly higher neutralizing antibody titers than did SV. For all preparations, WPV provided a significantly greater neutralizing antibody response at one fifth of the dose of SV ($p < 0.05$).

The neutralizing antibody titers induced by the WPVs containing 3 μg HA ranged on average from approximately 160–1280 depending on the vaccine manufacturer. This difference in potency was likely due to the different preparation processes used by each manufacturer, especially in the method of virus inactivation. Of note, WPV prepared by manufacturer A was inactivated only with β-propiolactone whereas other manufacturers used β-propiolactone and formalin or only formalin. Since β-propiolactone primarily interacts with RNA in the inactivation step, it is assumed that the structure of immunogenic epitopes on the viral proteins remains intact. On the other hand, formalin-inactivation causes conformational change of the structural proteins of virus particles by cross-linking of amino acids. WPV inactivated only with β-propiolactone, could thus be potentially more immunogenic. Paradoxically, our study using 4 different pairs of products indicates that superiority of WPV over SV in immunogenicity was confirmed regardless of the vaccine preparation methods.

3.2. WPVs induced superior protective immune responses in mice compared to SVs

In order to evaluate protective immunity induced by WPV and SV, the vaccinated mice were subjected to lethal challenge with 3000 PFU of A/California/07/2009 (H1N1) virus. Body weight was monitored daily after challenge (Fig. 3A–D) as a measure of well-being. Those that reached the pre-determined humane endpoint were euthanized and the numbers of surviving mice are shown in Fig. 3E–H. Mice vaccinated with WPVs containing 3 μg HA from any of the manufacturers showed minimal weight loss after challenge whereas mice inoculated with SV or PBS reached the humane endpoint by day 6 post challenge. Although a slight body weight loss was observed in the groups vaccinated with WPVs containing 0.6 μg HA from manufacturers C and D, and WPVs containing 0.3 μg HA from manufacturers A and C, all animals started to regain the lost weight during the course of the experiment. In contrast, mice vaccinated with WPV containing 0.6 μg HA from manufacturer B and WPV containing 0.3 μg HA from manufacturer D showed more substantial weight loss with 2 of 5 mice and 4 of 5 mice, respectively as shown by the fact that the animals met humane endpoint criteria. The surviving mice were those with pre-challenge neutralizing antibody titers of over 40, whereas those needing to be euthanized had titers of only 20 or less (Figs. 2B, D, 3B, and D). All the mice vaccinated with WPV containing 0.12 or 0.06 μg HA lost more than 20% of their original body weight by day 7 post challenge. WPV containing 0.12 μg HA from manufacturer A induced neutralizing antibodies with titers of 40 as shown in Fig. 2A. Although the mice vaccinated with WPV containing 0.12 μg HA from manufacturer A did not survive, disease signs such as body weight loss and ruffled fur were limited compared to those injected with PBS or SV (Fig. 3E). It suggests that this dose of vaccine induces immunity at the limits of mortality prevention. Overall, these results indicate that WPVs containing more than 0.6 μg HA induce immunity capable of protection against severe morbidity following lethal dose challenge with homologous influenza virus. In contrast, SV was incapable of inducing such responses even at 3 μg HA. The protective effect is likely to be associated with the induction of neutralizing antibodies and the titers greater than 40 seemed to correlate with protection against severe disease.

3.3. Cytokine induction by WPV

To compare innate immune responses induced by monovalent A/California/07/2009 (H1N1) WPV and SV containing 3 μg HA, we examined cytokine production in the sera collected at early time points (3, 6, 12, 24, and 48 h) after vaccination (Fig. 4). Among 8 cytokines/chemokines investigated in the present study, IP-10, IL-6, and MCP-1 were significantly increased in mice vaccinated with WPV prepared by each of the manufacturers. No significant induction or reduction of IFN-γ, IL-1β, IL-12(p70), IL-17, or TNF-α was found in the sera of WPV- or SV-injected mice (Supplemental Fig. S1). The levels of IP-10 were highest at 6 h post vaccination with WPVs from 3 out of 4 manufacturers and lasted as long as 24 or 48 h (Fig. 4A–D). In contrast, the highest concentration of IL-6 was detected 3 h post vaccination then quickly dropped to the normal levels (Fig. 4E–H). In both cases, the peak levels were significantly increased compared to those induced by SV. MCP-1 levels were also higher in mice vaccinated with WPV than those with SV at several time points (Fig. 4I–L). These results indicate that WPV stimulates innate immunity at early time points whereas SV does not.

Since sufficient neutralizing antibody induction (Fig. 2) and protective effects (Fig. 3) were observed even with a one in five dilution of WPV (0.6 μg HA), we examined the cytokine response (IL-6, IP-10, and MCP-1) in mice vaccinated with diluted WPV from manufacturer A at 3 h after vaccination (Fig. 5), because WPV from manufacturer A consistently exhibited high level of cytokine responses. Although mice vaccinated with WPV containing 0.6 μg HA induced higher levels of IP-10 than those injected with SV and PBS, the animals showed only basal levels of IL-6. Even a ten-fold dilution of WPV, represented as WPV containing 0.3 μg

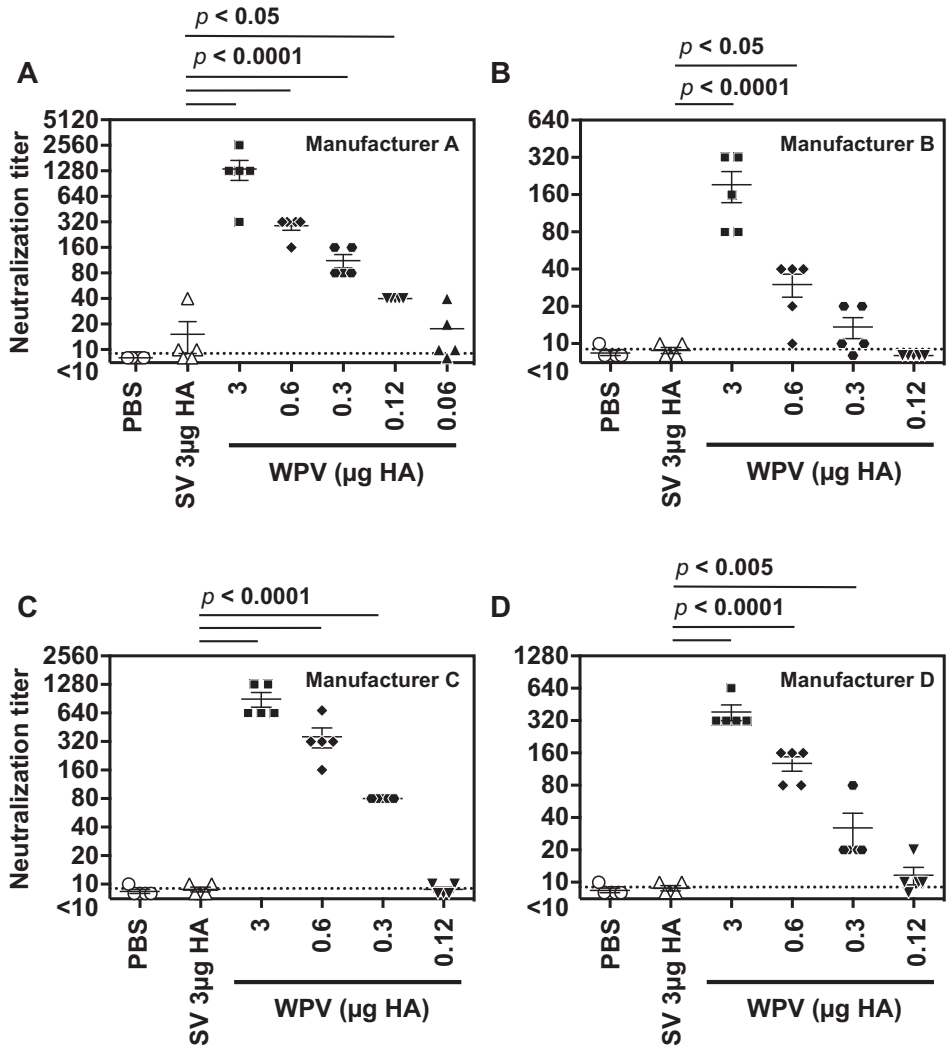

Fig. 2. Mice vaccinated with WPV induced strong neutralizing antibody responses. C57BL/6 mice (n = 5 per group) were inoculated via the subcutaneous route with PBS, SV (3 μg HA) or various amount of WPV (3–0.06 μg HA) from Manufacturer A (A), Manufacturer B (B), Manufacturer C (C), or Manufacturer D (D). The serum collected on day 24 after vaccination was used in a plaque neutralization assay. Symbols represent the titers obtained from individual mice, and horizontal lines indicate the mean ±SEM for the group. Statistical analysis was performed using a one-way ANOVA with Tukey post hoc test.

HA, induced higher IP-10 level than the SV containing 3 μg HA. These results suggest that the production of these cytokines may be independently regulated. Considering the results of experiments with different doses of WPV (Figs. 2, 3, and 6), serum IL-6 levels were not necessarily associated with effective acquired immune responses. Since IL-6 is known to be one of the important pyrogenic cytokines and to mediate synthesis of prostaglandins (especially prostaglandin E2) in the hypothalamus and fever [9,21–24], vaccination without excess IL-6 induction is preferable. Taking all the results together, the present study implies that proper dilution of WPV should achieve adequate immunogenicity with limited IL-6 production. Although further investigation is needed to determine the best dilution ratio, one-fifth of the current SV dose seems to be a good starting point.

3.4. Mice immunized with WPVs induced stronger CD8⁺ T cell responses than those with SVs

Although protection against influenza virus infection is largely based on antibody immunity, $CD8^+$ T cell responses also have an important role in viral clearance and can be particularly important when the challenge virus is serologically distinct from the vaccine strain. We therefore compared the ability of WPVs and SVs to induce NP-specific cytokine-secreting $CD8^+$ T cells in the spleen at day 10 post vaccination. A quadrivalent WPV vaccine based on strains used for the 2016–2017 season was prepared by one of the manufacturers and assessed in parallel with the corresponding quadrivalent SV and also monovalent A/California/07/2009 (H1N1) WPV and SV from the same manufacturer.

The numbers of cytokine-secreting $CD8^+$ T cells was approximately four-fold greater in the spleens of mice vaccinated with monovalent WPV compared to mice vaccinated with monovalent SV (Fig. 6A). Furthermore, WPV induced more $CD8^+$ T cells secreting multiple cytokines, so called "multi-functional" $CD8^+$ T cells, than did SV. These results indicate that WPV induces quantitatively and qualitatively stronger $CD8^+$ T cell immunity than does SV.

Current seasonal influenza vaccine is quadrivalent containing two influenza A strains (H1N1 and H3N2 subtypes) and two influenza B strains (Victoria and Yamagata lineages). Evaluation of the $CD8^+$ T cell responses induced by quadrivalent WPV and SV influenza vaccines (Fig. 6B) revealed that WPV induced almost two-fold more cytokine-secreting $CD8^+$ T cells than did SV and these reached almost 10,000/spleen. As observed for monovalent WPV, quadrivalent WPV induced more multi-functional $CD8^+$ T cells than

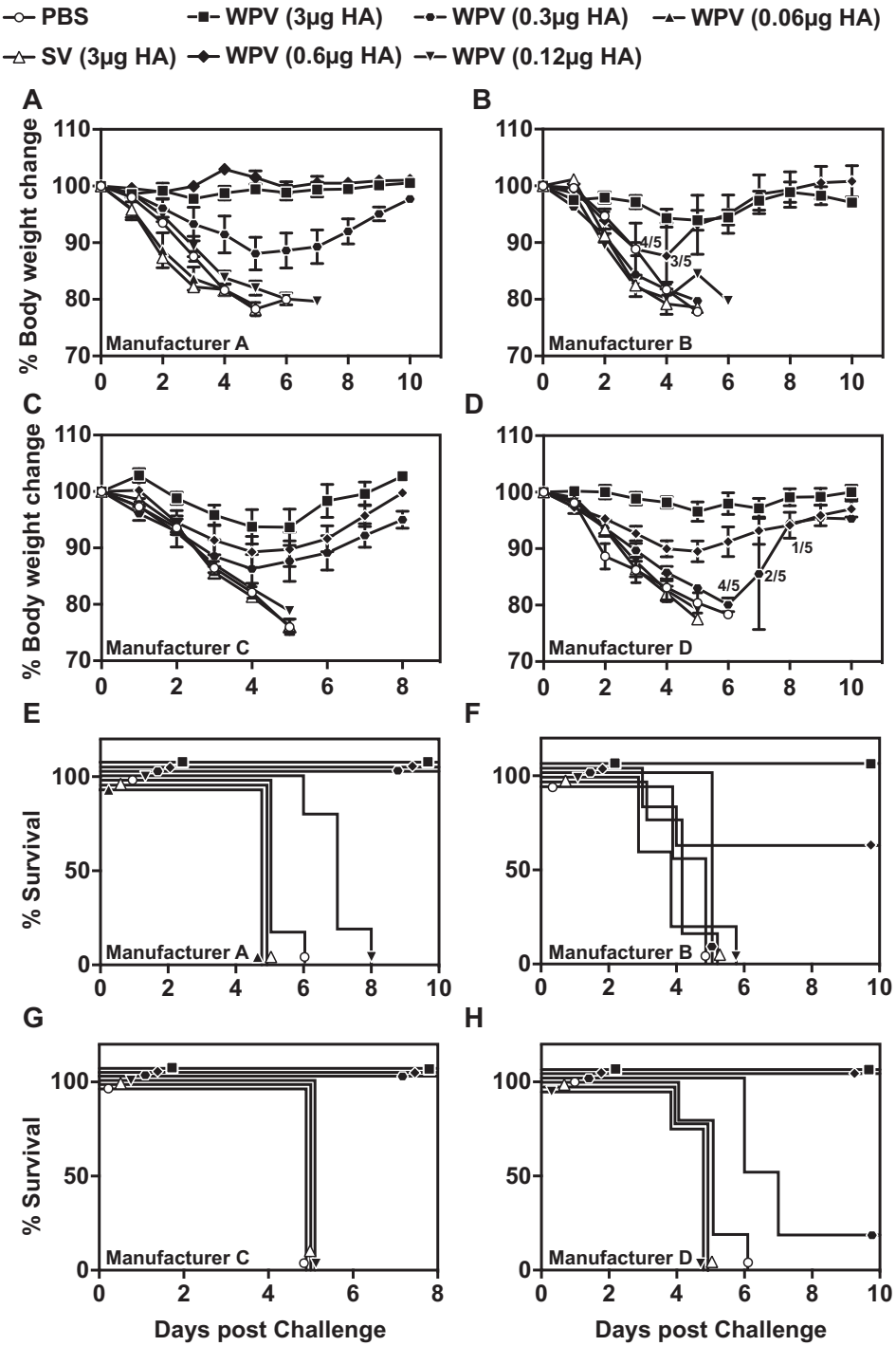

Fig. 3. Mice vaccinated with WPV but not SV survived challenge with a lethal dose of homologous virus. On day 28 after vaccination, the mice described in Fig. 2 were challenged with a lethal dose (3000 PFU in 40 μl) of A/California/07/2009 (H1N1) virus. The mice were monitored daily for up to 14 days and euthanized at the humane endpoint, defined by>20% weight loss, where necessary. The mean body weight of mice is represented as a percentage of the original weight as depicted in panel A, B, C, and D and the corresponding Kaplan-Meier survival curves are shown in panel E, F, G, and H.

did the corresponding SV. These results suggest that both monovalent and quadrivalent WPVs induce strong antigen-specific $CD8^+$ T cells responses in naïve animals.

4. Discussion

The current seasonal SV ensures safety as a vaccine and gives a booster effect to primed populations. However, SV gives no or low effect for children and elderly who belong to the high risk group as it does not prime naïve children nor induce enough immunity to protect elderly [3,11–15]. In order to solve this problem, we established the All Japan Influenza Vaccine Study Group comprising all four seasonal influenza vaccine manufacturers in Japan. The group was supported by a grant from the government in Japan. Each manufacturer modified their previous WPV preparation protocol, especially purification and inactivation steps to reduce undesired reactions. In the collaborative study, we evaluated the immunogenicity of WPV and SV with equivalent quality to commercial

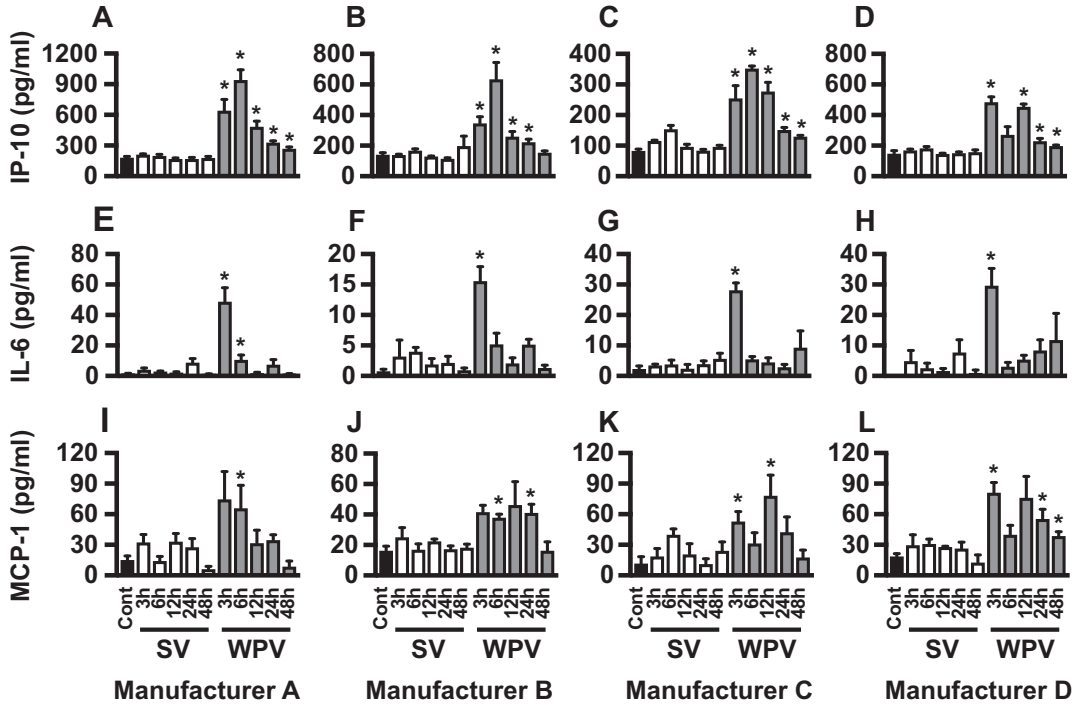

Fig. 4. Cytokine production at early time points after vaccination of mice. Mice were inoculated subcutaneously with WPV or SV and serum samples were collected 3, 6, 12, 24, and 48 h later. IP-10 (A-D), IL-6 (E-H), and MCP-1 (I-L) in sera were measured by multiplex assay. The results from each manufacturer were shown in panels A, E, and I for manufacturer A, panels B, F, and J for manufacturer B, panels C, G, and K for manufacturer C, and panels D, H, and L for manufacturer D. Each bar represents the mean ± SEM of 3–5 animals. In each panel, black, white, and gray bars indicate data from unvaccinated mice, SV immunized mice, and WPV immunized mice respectively. *$p < 0.05$, unpaired *t*-test, SV vs. WPV at each time point.

products. Similar to previous studies of seasonal and pandemic influenza vaccines [16–19,25–33], the comparison of test vaccines from each of the manufacturers indicated superiority of WPV over SV in terms of the induction of neutralizing antibody and $CD8^+$ T cell responses in naïve mice, as well as protection against severe disease following homologous virus challenge. The results suggest that WPV may minimize the risk of severe disease in children and elderly. This notion was strongly supported by the results of the study using macaques (Fig. 1).

The fact that one inoculation of WPV, but not SV, successfully induced neutralizing antibodies in mice and macaques (Figs. 1 and 2), implies that WPV is capable of effectively activating $CD4^+$ T cell responses which critically regulate humoral immunity. In fact, the priming of naïve T cells is an essential process to activate vaccine-specific acquired immunity. The difference in immunogenicity between WPV and SV observed here may be attributed to the relative antigen presentation efficiencies of antigen presenting cells (APCs) that encounter the different forms of vaccine. Since WPV retains the natural virus particle structure, the entire components of the virus particle are likely to be incorporated into APCs, including dendritic cells (DCs), and processed to peptides for presentation on the major histocompatibility complex (MHC). In contrast, with SV, engulfment of the HA after its binding to viral receptors on the APC surface would not be expected to bring the rest of the dissociated viral proteins into the cell with it. Therefore, the spectrum of viral antigens available to be processed and presented to T cells by DC may be greater from WPV than from SV. In fact, when we investigated antigen-specific $CD8^+$ T cell responses specific for an MHC class I-restricted NP peptide, these were significantly greater in the spleens of mice immunized with WPV than with SV (Fig. 6). This result clearly indicates that inoculation of WPV leads to more efficient presentation of the internal proteins of the virus. This is important because the sequence of the internal proteins is more highly conserved compared to the surface glycoproteins. Thus T cell responses are likely to be cross-reactive between virus strains and even influenza A virus subtypes.

In addition to proteins, when WPV are internalized by DC, the single-strand RNA (ssRNA) genome within the particle acts as a pathogen associated molecular pattern (PAMP) to provide the signal for DC activation and initiation of the immunological cascade for adaptive immunity via pattern recognition receptors (PRRs) [34]. It has been reported that the influenza virus genome ssRNA activates toll-like receptor 7 (TLR7) signaling cascades in plasmacytoid DCs [35–37] and is recognized by the PRR RIG-I, which plays a major role in sensing of RNA viruses to initiate and modulate antiviral immunity [38]. WPV containing ssRNA may show enhanced immunogenicity over SV by virtue of its capacity to trigger these signaling pathways. As shown in Fig. 4, WPV systemically induced IP-10, IL-6, and MCP-1, which are known to be produced during natural immune responses to influenza virus infection in humans [39,40]. The results strongly suggest that WPV induces innate immunity in a similar manner to natural influenza virus infection. Thus, it is expected that WPV provides all the immunomodulatory components to prime naïve individuals such as young children and to induce robust immunity in the elderly. The lack of such immunomodulators is a defect of the current SVs.

Although activation of innate immunity is essential for the development of effective adaptive immunity, this early response sometimes causes undesired side-effects, such as febrile reactions due to over-production of inflammatory cytokines including IL-1, IL-6, and TNF-α [9,21,23]. The production of prostaglandin E2, a endogenous pyrogen, is known to be promoted by these cytokines through the induction of cyclooxyrgenase 2 [24,41–45]. Among them, IL-6 has been reported to be indispensable for systemic fever induced by either IL-1 or endotoxin injection [23]. Our present

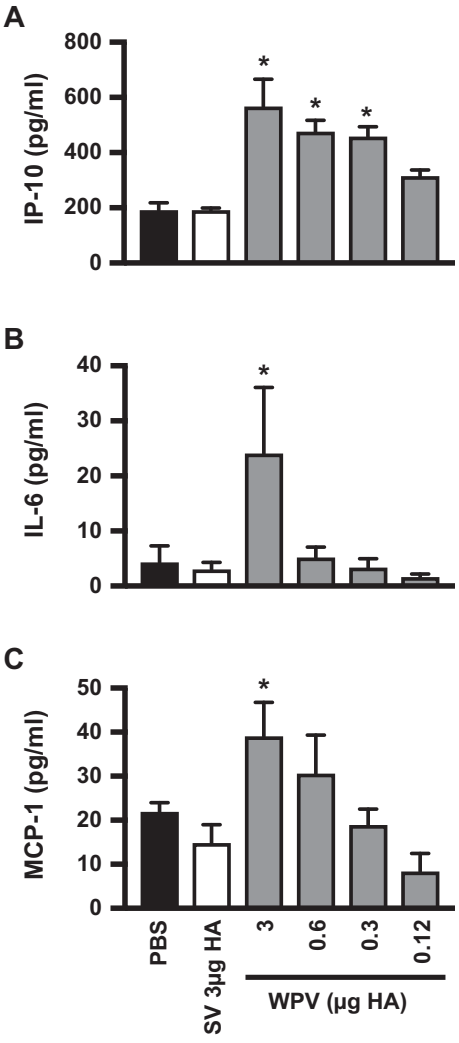

Fig. 5. WPV-induced IL-6 production was minimal at low but immunogenic vaccine doses. Mice were inoculated subcutaneously with WPV or SV and serum samples were collected 3 h later. IP-10 (A), IL-6 (B), and MCP-1 (C) in sera were measured by multiplex assay. Each bar represents the mean ± SEM of 5 animals. In each panel, black, white, and gray bars indicate data from PBS-injected mice, SV immunized mice, and WPV immunized mice, respectively. $^{*}p < 0.05$, one-way ANOVA followed by a Dunnett's multiple comparison test.

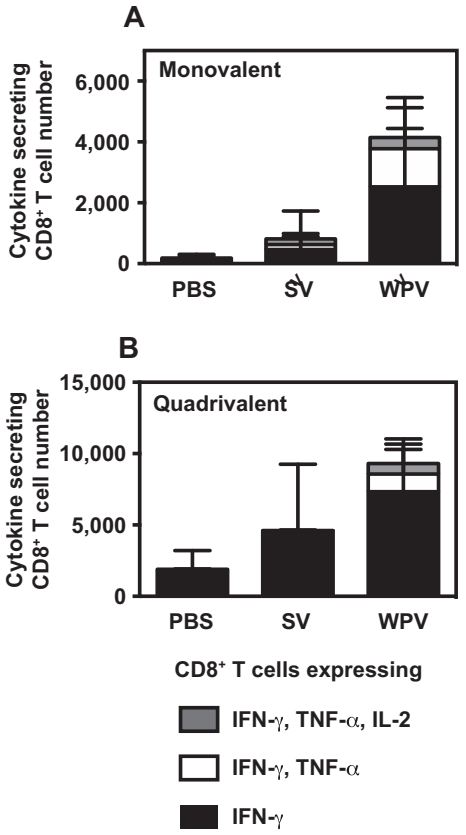

Fig. 6. WPV induced larger number of $CD8^+$ T cells secreting cytokines than SV in vaccinated mice. Mice were inoculated subcutaneously with monovalent (A) or quadrivalent (B) vaccines (WPV, SV) or PBS and splenocytes were harvested after 10 days. Cells were cultured with a peptide representing the immunodominant H-$2D^b$-restricted NP epitope and IL-2 for 12 h. $CD8^+$ T cell numbers secreting IFN-γ, TNF-α, and IL-2 were then measured by flow cytometry. Stacked bars show the number of IFN-γ secreting $CD8^+$ T cells. Black indicates $CD8^+$ T cells secreting IFN-γ, white indicates IFN-γ and TNF-α secreting cells, gray indicates IFN-γ, TNF-α, and IL-2 secreting cells.

study demonstrated that 0.6 μg HA containing WPV, equivalent to one fifth of the SV dose used, induced enough protective immunity in mice to survive a lethal challenge of homologous virus without an apparent induction of IL-6 (Figs. 3 and 5). Interestingly, since the induced level of IP-10 by the low dose WPV was comparable to that by 3 μg HA containing WPV unlike IL-6 (Fig. 5A), threshold of vaccine dose for the induction of cytokines/chemokines appeared different. Although cytokine/chemokine corresponding to undesired side-effects of vaccines is still unidentified at this point, this result implies that a proper vaccine preparation method, including purification, inactivation, and dose sparing to assure potent immunogenicity with limited pyrogenicity.

In conclusion, superiority of WPV over SV was confirmed using vaccines prepared by four vaccine manufactures in Japan. Moreover, dose sparing of WPV down to one fifth of the SV dose, was enough to induce neutralizing antibodies that protect from a lethal challenge with homologous virus. The low dose WPV could be one of the options to realize a safe and more immunogenic seasonal and pandemic influenza vaccine. Dose sparing also has additional benefits of raising production capacity as well as lowering costs. Thus, the low dose WPV is expected not only to minimize side reactions but also to contribute to the stable supply of vaccines.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.057.

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