



Title	Potent priming by inactivated whole influenza virus particle vaccines is linked to viral RNA uptake into antigen presenting cells
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Potent priming by inactivated whole influenza virus particle vaccines is linked to viral RNA uptake into antigen presenting cells.

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Running title

Superior priming potency of inactivated whole influenza virus particle vaccine to current ether-split vaccine

Key words

Inactivated whole influenza virus particle vaccine; seasonal and pandemic influenza.

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1. Introduction

Influenza is a highly contagious respiratory illness that is responsible for significant morbidity and mortality. Over 1 billion cases are reported each year, 3 to 5 million of which are severe and result in 290,000 to 650,000 deaths annually worldwide [1-3]. Adults 65 years and older are at particular risk of severe disease, hospitalization, and mortality due to seasonal influenza [4]. Young children are also at risk due to higher attack rates [5, 6]. Children are key virus spreaders in a community because they are more susceptible to influenza virus infection than adults and frequently make contact with friends and family members, resulting in shedding larger amounts of virus in the community over a long period of time [7, 8]. In fact, the best predictor for influenza occurring in a household is the presence of children [9, 10]. Thus, children should be prioritized for prevention of influenza through vaccination.

Annual vaccination is the most effective way to prevent influenza and to reduce an individual's risk of severe influenza-related disease. The first inactivated seasonal influenza vaccine was developed as a whole virus particle vaccine (WPV) in the 1940s and was used for 30 years [11]. However, the administration of the WPV vaccine to children occasionally caused side effects such as fever, pain, and fatigue [12, 13], which led many people to mistakenly assume that the vaccine might cause flu-like symptoms. One of the reasons for this reactogenicity was thought to be due to impurities, such as egg-derived contaminants, in the vaccine [13]. Therefore, the manufacturing process for the newly developed seasonal influenza vaccines had an additional purification step to remove the impurities using zonal centrifugation. Moreover, most current influenza vaccines are disrupted using ether and/or detergents to decrease febrile reactions, hence the name "split" vaccine (SV). Several clinical trials demonstrated that SV was indeed less pyrogenic than WPV but was also less immunogenic, especially in young children [14-16]. Despite some disagreements, SV was approved in the United States as a standard influenza vaccine and replaced WPV in 1968 [11], and in Japan in 1972. A report of a clinical trial by Gross *et al.* [15, 16] highlighted three interesting findings: 1) in young children WPV causes fever more frequently than does SV, 2) young children previously vaccinated with influenza virus vaccine are unlikely to experience fever subsequent to immunization with a related antigen and 3) SV induces less vaccine strain-specific antibodies than does WPV in immunologically unprimed young children. The fact that WPV but not SV induces immunity in "naïve" children with a febrile response implies that the fever caused by WPV accompanies immune priming responses.

To re-evaluate WPV, we established the All Japan Influenza Vaccine Study Group and requested that member vaccine manufacturers in Japan prepare WPV and SV from the same purified virus batches from which egg-derived contaminants had been removed. Our previous study utilizing such matched sets of WPV and SV preparations demonstrated the superiority of WPV over SV in terms of the induction of neutralizing antibodies and CD8⁺ T cell responses in naïve mice as well as protection against severe disease following homologous virus challenge [17]. These results not only encouraged us to advance this project to preclinical study, but also raised a new question as to why WPV, rather than SV, elicits an effective priming response. To address this question, we compared priming and boosting effects of WPV and SV in cynomolgus macaques. The present study provides data

45 supporting the strong priming effect of WPV compared to SV and provides evidence for the
46 mechanistic basis that underpins this phenomenon.

47

48 **2. Material and methods**

49 **2.1 Cells and viruses**

50 Madin-Darby canine kidney (MDCK) cells were grown in RP10 (RPMI 1640;
51 Thermo Fisher Scientific, MA, USA) supplemented with 10% inactivated fetal calf serum
52 (FCS) (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK), 1 mM of sodium
53 pyruvate (Thermo Fisher Scientific), 50 μ M of 2-mercaptoethanol (Merck, Darmstadt,
54 Germany), 100 U/ml of penicillin (Thermo Fisher Scientific), 100 μ g/ml of streptomycin
55 (Thermo Fisher Scientific), and 20 μ g/ml of gentamicin (Thermo Fisher Scientific). These
56 were used for neutralization assays and plaque assays. Influenza viruses
57 A/Singapore/GP1908/2015 (IVR-180) (H1N1), A/California/7/2009 (X-179A) (H1N1)
58 pdm09, A/Hong Kong/4801/2014 (X-263) (H3N2), B/Phuket /3073/2013 (Yamagata lineage),
59 and B/Texas/2/2013 (Victoria lineage) were kindly provided by the National Institute of
60 Infectious Diseases in Japan. Viruses were propagated in 10-day-old embryonated chicken
61 eggs. The collected allantoic fluids were stored at -80°C until use.

62

63 **2.2 Vaccines**

64 WPV and SV used in this study were prepared by vaccine manufacturers in Japan
65 [17]. Quadrivalent vaccine contained antigens from influenza virus vaccine strains used in
66 2017-2018, namely A/Singapore/GP1908/2015 (IVR-180) (H1N1), A/Hong Kong/4801/2014
67 (X-263) (H3N2), B/Phuket /3073/2013 (Yamagata lineage), and B/Texas/2/2013 (Victoria
68 lineage). The monovalent vaccine contained antigens from A/California/7/2009 (X-179A)
69 (H1N1) pdm09. Vaccine virus strains were propagated in embryonated chicken eggs and
70 highly purified from the allantoic fluids through sucrose density gradient centrifugation [18].
71 In the present study, the highly purified virions were inactivated with formalin and/or β -
72 propiolactone to prepare WPVs according to the standard methods used by vaccine
73 manufacturers [17]. SVs were prepared by disrupting the purified virions with ether,
74 according to the license for current seasonal influenza vaccine production. HA protein
75 concentrations of WPV and SV were measured using a single-radial-immunodiffusion
76 method. In addition, WPVs in this study showed almost equivalent chicken cell agglutination
77 (CCA) values to the classical WPVs. Influenza vaccines were subcutaneously injected to
78 animals as in our previous report.

79

80 **2.3 Animals**

81 Female C57BL/6 mice were purchased from Hokudo Co., Ltd. (Sapporo, Japan) and
82 kept in a BSL-2 laboratory at the Research Center for Zoonosis Control, Hokkaido
83 University. Either quadrivalent WPV (3, 1.2, 0.6, and 0.3 μ g HA protein per each vaccine
84 strain) or SV (3 μ g HA protein per each vaccine strain) was injected subcutaneously into 7-
85 week-old female C57BL/6 mice under inhalation anesthesia with isoflurane. Serum samples
86 were collected on day 24 after injection to estimate neutralizing antibody titers. On day 28
87 after injection, mice were challenged with an intranasal infection of 3,000 plaque forming
88 units (PFUs) of A/Singapore/GP1908/2015 (IVR-180) (H1N1) in 40 μ l of phosphate buffered

89 saline (PBS) as a 100% lethal dose under inhalation anesthesia with isoflurane. Body weight
90 was monitored daily after infection. Lungs were harvested 5 days post infection and
91 homogenized in RPMI_{anti} (RPMI 1640 medium supplemented with 100 U/ml penicillin, 100
92 µg/ml streptomycin and 20 µg/ml gentamicin) and centrifuged at 2,000rpm to obtain
93 supernatant, and stored at -80°C until use.

94 A total of 61 healthy female and male cynomolgus macaques (*Macaca fascicularis*)
95 imported from Cambodia, aged around 3 years and weighing 2-5 kg, were used for the
96 experiments. Monkeys were singly housed in steel cages measuring 68-cm-deep by 62-cm-
97 wide by 77-cm-high in a ventilated animal room with controlled temperature (23 to 29°C),
98 relative humidity (30 to 70%), and 12 hr of light (7:00 to 19:00) at Shin Nippon Biomedical
99 Laboratories, Ltd (Kagoshima, Japan). Macaques were vaccinated subcutaneously with
100 quadrivalent WPV or SV. Blood samples were collected in heparin tubes at indicated
101 timepoints, and plasma for the measurement of neutralizing antibody titers was separated and
102 stored at -80°C until use. Throughout the experiments, all efforts were made to minimize the
103 suffering of animals.

104 Current SV for human use contains 15 µg HA for each strain. In this study macaques
105 and mice received WPV containing HA of 1/8 to equivalent amount and 1/50 to 1/5 amount,
106 respectively, compared with a dose applied in humans.

107

108 **2.4 Ethics statement**

109 All mouse experiments were performed with approval (Approval No. 17-0003) from
110 the Animal Care and Use Committee of Hokkaido University following Fundamental
111 Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic
112 Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports,
113 Science and Technology in Japan.

114 All monkey experiments had been approved by the Institutional Animal Care and Use
115 Committee (Approval No. IACUC718-007, 008, and 009) and carried out in strict accordance
116 with animal welfare regulations of Shin Nippon Biomedical Laboratories, Ltd., Drug Safety
117 Research Laboratories, which is accredited by the Association for Assessment and
118 Accreditation of Laboratory Animal Care (AAALAC) International.

119

120 **2.5 Measurement of virus titers in the lungs of mice by plaque assay**

121 Virus titers in the lung supernatants of infected mice were determined by a plaque
122 assay. MDCK cells were seeded into 6-well tissue culture plates at a density of 1.2×10^6 cells
123 per well and incubated overnight at 37°C in 5% CO₂. Cell monolayers were washed with
124 RPMI_{anti} and 125 µl of lung supernatant, diluted from 10⁻¹ to 10⁻³ in RPMI_{anti}, was added to
125 each well. After incubation of the plate for 45 min at 37°C in 5% CO₂, 3 ml of warmed
126 (45°C) overlay medium consisting of Leibovitz L-15 with glutamine at pH 6.8 (Thermo
127 Fisher Scientific) supplemented with 0.028% (w/v) NaHCO₃ (Merck), 100 IU/ml penicillin,
128 100 mg/ml streptomycin, 0.1% (w/v) TPCK-treated trypsin (Merck), and 0.9% (w/v) agarose
129 (Merck) was then added to each well. The plates were incubated for 3 days at 37°C in 5%
130 CO₂. Plaques formed on monolayers were counted and the number of plaque-forming units
131 (PFU) in the original lung sample was calculated.

132

133 **2.6 Receptor-destroying enzyme (RDE) treatment of the sera of mice and monkeys and**
134 **hemagglutination inhibition (HI) assay**

135 To reduce inhibition of hemagglutination by non-specific inhibitors, the sera were
136 treated with RDE II (Denka, Tokyo, Japan) at a ratio of 1:3 (serum: RDE) and then incubated
137 overnight at 37°C. After inactivation of RDE by incubation at 56°C for 1 hr, phosphate
138 buffered saline (PBS) was added at 4 times the volume of the serum (the final ratio of
139 serum:RDE:PBS is 1:3:6) to give a starting dilution of 1:10. Serial two-fold dilutions of the
140 RDE-treated sera in PBS were carried out in Round bottom 96-well microplates. The diluted
141 sera were mixed with 8 hemagglutinin units of virus antigen [A/Singapore/GP1908/2015
142 (IVR-180) (H1N1), A/California/7/2009 (X-179A) (H1N1) pdm09, A/Hong Kong/4801/2014
143 (X-263) (H3N2), B/Phuket /3073/2013 (Yamagata lineage), or B/Texas/2/2013 (Victoria
144 lineage)] and incubated at room temperature (RT) for 30 min. An equal volume of 0.5%
145 suspension of chicken red blood cells were added to the antigen-serum dilution mixtures and
146 incubated at RT for 30 min. HI titers were expressed as reciprocals of the highest serum
147 dilutions that showed complete inhibition of hemagglutination.

148

149 **2.7 Neutralization assay of the sera of mice**

150 Neutralizing antibody titers of RDE-treated mouse sera were measured in a plaque
151 reduction assay. Monolayers of MDCK cells were prepared by seeding 1.2×10^6 cells in 3 ml
152 of RP10 medium in each well of a 6-well tissue culture plate and incubated overnight at 37°C
153 in 5% CO₂. Serial ten-fold dilutions of the RDE-treated sera in PBS were carried out in 96-
154 well microplates. An equal volume of influenza virus was then added to each serum dilution
155 to give a final concentration of 100 PFU/100 µl and the mixtures incubated for 1 hr at RT.
156 Cell monolayers were washed with RPMI_{anti} then 100 µl of virus-serum mixtures added. Any
157 non-neutralized virus was allowed to adsorb for 45 min during which time the plates were
158 shaken gently at 15 min intervals. Warmed (45°C) overlay medium (3 ml/well) was then
159 added to each well. The plates were incubated at 37°C in 5% CO₂ for 3 days and plaques on
160 the monolayers were counted without staining. The virus neutralizing antibody titers were
161 expressed as the reciprocal of the highest serum dilutions that reduced the number of plaques
162 to 50% of that obtained in control wells that had no serum.

163

164 **2.8 Micro-neutralization Enzyme-Linked Immunosorbent Assay (ELISA) of macaque**
165 **sera**

166 RDE-treated macaque sera were serially 2-fold diluted with PBS in 96-well
167 microplates. The diluted sera were mixed with an equal volume of virus (100 TCID₅₀) and
168 the virus-serum mixtures were incubated at 37°C for 1 hr. MDCK cell suspensions containing
169 1×10^6 cells in 100 µl were added to 50 µl of virus-serum mixture and incubated in 96-well
170 plates in the presence of 1 µg/ml TPCK-trypsin at 37°C for 24 hr. Cell monolayers were
171 washed with PBS and fixed in cold 80% acetone for 15 min. The presence of nucleoprotein
172 (NP) of influenza virus was detected by ELISA with a specific monoclonal antibody
173 (HB65/H16-L10-4RS: BioXCell, Lebanon, NH, USA).

174 The ELISA was performed at RT. The fixed monolayers were washed three times
175 with PBS containing 0.05% Tween 20 (wash buffer). After incubation for 1hr with a blocking
176 buffer containing 1% of bovine serum albumin (BSA) in PBS, the anti-NP antibody diluted to

177 1/6,000 in blocking buffer was added to each well. The plates were incubated at RT for 1 hr.
178 The plates were washed three times with wash buffer, and 100 µl of horseradish peroxidase-
179 labeled goat anti-mouse immunoglobulin G (IgG) (Thermo Fisher Scientific) diluted to
180 1/6,000 in blocking buffer was added to each well. The plates were incubated for 1 hr at RT
181 and then washed three times with wash buffer. One hundred microliters of freshly prepared
182 substrate using SIGMAFAST™ OPD (Thermo Fisher Scientific) was added to each well, and
183 the plates were incubated at RT for 15 min. The reaction was stopped by the addition of 50 µl
184 of 2N sulfuric acid. The absorbance at 490 nm (A490) was measured with a microplate
185 reader. The intermediate OD value was determined from quadruplicate wells of virus-
186 infected and uninfected control wells, and the neutralization titer was determined as the
187 maximum dilution below the intermediate OD value.

188

189 **2.9 Cytokine detection in CD14 positive cells and CD14 depleted cells from cynomolgus** 190 **macaques**

191 Splens were collected from unimmunized macaques at autopsy, homogenized, and
192 stored at -80°C until use. CD14⁺ splenocytes were isolated using CD14 microbeads for
193 magnetic cell sorting (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The
194 remaining splenocytes were used as CD14-depleted splenocytes. The cells (5×10^5 of CD14-
195 depleted splenocytes or 1×10^5 of CD14⁺ splenocytes) were cultured in the presence of the
196 vaccine preparations (3 µg/ml of HA proteins) or lipopolysaccharide (LPS, 1 mg/ml) for 6 hr.
197 Levels of cytokines in supernatants were measured using a MAGPIX Milliplex MAP kit for
198 nonhuman primate (Merck) and a Luminex 200 system (Millipore Corp., Billerica, MA).

199

200 **2.10 Cytokine detection in dendritic cells (DCs) from mice**

201 Mouse DCs were isolated from single cell suspensions of spleen, using CD11c micro
202 beads (Miltenyi Biotec GmbH) according to the manufacturer's instructions. One million
203 DCs were suspended in 100 µl of RP10 in 96-well round-bottom tissue culture plates and
204 immediately stimulated with WPV or SV preparations containing 0.5 µg HA. PBS was used
205 as a non-stimulation control. Total RNA was extracted from the cells at 6 hr post-stimulation
206 for mRNA analysis as described below. Culture supernatants were collected at 24 hr post-
207 stimulation for cytokine analysis. Concentrations of cytokines and chemokines (IFN-γ, IL-6,
208 TNF-α, IP-10 and MCP-1) were determined using a MAGPIX Milliplex kit (Merck)
209 according to instructions of the manufacturer. Briefly, 50 µl of culture supernatants, positive
210 control samples, and standards for calibration curve were added to a 96-well plate. Magnetic
211 beads coated with the antibodies against the target cytokines and chemokines were added into
212 each well and the plate was incubated on a plate shaker overnight at 4°C. After washing with
213 wash buffer provided in the kit, the samples were reacted with biotinylated detection
214 antibodies for 1 hr and then streptavidin-phycoerythrin for 30 min. After washing with the
215 wash buffer and addition of loading buffer from the kit, the samples were analyzed by a
216 MAGPIX system (Luminex, Austin, TX).

217

218 **2.11 RNA extraction and Quantitative-PCR (qPCR)**

219 Total RNA was extracted from cells or vaccine solutions using Trizol LS reagent
220 (Thermo Scientific) according to the manufacturer's instructions. The concentration of
221 extracted RNA in each vaccine was estimated using a Nanodrop spectrophotometer (Thermo
222 Scientific, Wilmington, DE, USA). Complementary DNA was synthesized from the
223 extracted total RNA using random hexamer and ReverTra Ace® qPCR RT Master Mix
224 (Toyobo, Osaka, Japan) according to the manufacture's instructions. To determine the RNA
225 copy number, real time qPCR was performed using a SYBR Premix Ex Taq II kit (Tli
226 RNaseH Plus, Takara Bio, Otsu, Japan) in a CFX96 system (BioRad, Hercules, CA). The
227 primer sequences for target genes were as follows: For mouse IP10 (CXCL10) gene, forward
228 primer is 5'-GGATCCCTCTCGCAA-3' and reverse primer is 5'-ATCGTGGCAATGATC-
229 3'. For mouse IFN- α gene, forward primer is 5'-TCTGATGCAGCAGGTGGG-3' and
230 reverse primer is 5'-AGGGCTCTCCAGACTTCTGCTCTG-3'. For mouse IFN- β gene,
231 forward primer is 5'-CAGCTCCAAGAAAGGACGAAC-3' and reverse primer is 5'-
232 GGCAGTGTA ACTCTTCTGCAT-3'. For influenza A virus NP segment, forward primer is
233 5'-GATTGGTGG AATTGGACGAT-3' and reverse primer is 5'-
234 AGAGCACCATTCTCTCTATT-3'. To determine the RNA copy number incorporated into
235 DCs, 1×10^5 CD11c-positive DCs from mouse spleens were inoculated with WPV or SV
236 containing 5 μ g of HA. After an hr incubation, the DCs were washed twice with ice-cold PBS
237 and total RNA was extracted for reverse transcription and qPCR as described earlier.

238

239 **2.12 Fluorescent staining and *in situ* hybridization**

240 Mouse splenic CD11c⁺ DCs were cultured overnight on Labtek chamber slides II
241 (Nalge *Nunc* Int., Wiesbaden, Germany) and the cells were exposed to WPV or SV, the
242 amounts of which had been adjusted to provide equivalent copy numbers of the viral NP gene
243 segment (5×10^5 copies). Live virus, at multiplicity of infection (MOI) of 10, was used as a
244 positive control. After 3 hr incubation, viral NP segments on the slides were stained using a
245 ViewRNA ISH Cell Assay Kit (Affymetrix, Santa Clara, CA, USA) with a fluorescein-
246 conjugated NP segment specific probe (Affymetrix) and DAPI according to the
247 manufacturer's instructions. The fluorescent signals on the slides were detected using a Zeiss
248 780 LSM confocal microscope (Carl Zeiss, Jena, Germany), and colocalization was analyzed
249 using ZEN 2011 software (Carl Zeiss).

250

251 **2.13 Evaluation of CD86 expression on DCs by flow cytometry**

252 Lymph nodes were collected from mice 6-48 hr after vaccination with WPV or SV
253 and single cell suspensions prepared in RP10. Lymph node cells were stained with PE-
254 conjugated anti-CD86 (clone; GL-1), fluorescein-conjugated anti-CD11c (clone; HL3),
255 PerCP5.5-conjugated anti-CD19 (clone; 6D5), Alexa Fluor 700-conjugated anti-B220 (clone;
256 RA3-6B2) and BV421-conjugated anti-CD11b (clone; M1/70) antibodies, following Fc-block
257 with anti-CD16/32 (clone; 93) antibody. After washing twice with ice-cold PBS containing
258 0.5% BSA and 0.1% NaN₃, the cells were further stained with 7AAD (BioLegend, San
259 Diego, CA). Fluorescent intensities of the cells were detected on an LSR Fortessa system
260 (BD Biosciences, San Jose, CA). Conventional dendritic cells (cDCs) were determined as
261 7AAD negative, CD19 negative, CD11b positive, CD11c positive and B220 negative.
262 Plasmacytoid dendritic cells (pDCs) were determined as 7AAD negative, CD19 negative,

263 CD11b negative, CD11c positive and B220 positive. The CD86 expression on cDCs and
264 pDCs were analyzed using FlowJo software (Treestar, Ashland, OR). All antibodies were
265 purchased from Biolegend.

266

267 **2.14 Statistical analysis**

268 Prism 7 (GraphPad Software, San Diego, CA, USA) was used to perform statistical
269 analyses. *P* values were obtained using one-way ANOVA or two-way ANOVA with multiple
270 comparisons or unpaired *t*-test. A *p* value less than 0.05 was considered statistically
271 significant.

272

273 **3. Results**

274 **3.1 The priming potency of quadrivalent WPV is greater than that of the corresponding** 275 **SV.**

276 Our previous study demonstrated that a single inoculation of monovalent WPV
277 induced protective immunity in naïve mice, whereas monovalent SV did not, indicating that
278 WPV but not SV has the capacity to prime an immune response in naïve animals [17]. Since
279 quadrivalent not monovalent vaccines are routinely used in humans, we conducted similar
280 experiments in a mouse model utilizing quadrivalent WPV and SV prepared by the same
281 manufacturer. Mice were vaccinated with different amounts of WPV (0.3, 0.6, 1.2, or 3 µg
282 HA/strain), SV (3 µg HA/strain) or PBS, and then infected with 3,000 PFU of homologous
283 influenza virus A/Singapore/2015 (H1N1) on day 28 after vaccination. To evaluate the ability
284 of vaccine-induced immunity to reduce virus replication, virus titers in the lungs on day 5
285 after virus challenge were examined. Pulmonary virus titers of mice inoculated with PBS
286 were approximately 10⁶ PFU (Figure 1a). When comparing SV and WPV containing the
287 same amount of HA (3 µg) to the PBS group, the virus titers in the lungs were reduced
288 approximately ten-fold on average in SV vaccinated mice, whereas the virus was barely
289 detectable (< 10² PFU) in WPV vaccinated mice. In addition, suppression of the virus titers in
290 the lungs observed in WPV-vaccinated groups showed a dose-dependency. Body weight
291 changes, one of the indicators for disease severity, were also monitored daily until 5 days
292 post-infection (dpi). Body weight of mice inoculated with SV or PBS decreased to around
293 80% of starting weight at 5 dpi (Figure 1b). In contrast, mice vaccinated with WPV
294 containing 3 µg HA showed little body weight loss after virus challenge. Body weight loss of
295 mice vaccinated even with diluted WPV only reached low levels, significantly less than the
296 SV group, which received 10-times the vaccine dose. The severity of weight loss appeared to
297 correlate with virus titers in the lungs of mice. To evaluate potency of WPV and SV to induce
298 protective antibodies, neutralizing antibody titers in the sera of mice sampled on day 24 were
299 measured against the homologous virus, A/Singapore/2015 (H1N1). In PBS-treated mice,
300 serum neutralizing antibody titers were under the detection limit (Figure 1c). There was no
301 significant difference in the titer between PBS- and SV-treated groups. In contrast,
302 neutralizing antibody levels were significantly greater in mice immunized with the same HA
303 dose of WPV than of SV. Furthermore, data of diluted WPVs demonstrated that the
304 neutralizing antibody response induced by WPV was dose-dependent and negatively
305 correlated with virus titers in the lungs. These results confirmed strong priming potency of
306 the quadrivalent WPV as observed for a monovalent WPV previously [17].

307 We further investigated the potency of WPV and SV to prime and boost protective
308 immune responses in cynomolgus macaques, which are biologically more closely related to
309 humans than are mice (Figure 2). Naïve cynomolgus macaques were vaccinated on day 0 and
310 28 with either WPV or SV as shown in Figure 2. Plasma samples were collected on day 0, 28,
311 and 49 to evaluate antibody titers against the vaccine strains. On day 28 after the first
312 vaccination, HI antibodies against influenza A virus vaccine strains, A/Singapore/2015
313 (H1N1) and A/Hong Kong/2014 (H3N2), were induced only in macaques vaccinated with
314 WPV (left and middle data in Figure 2a and b). HI titers against influenza B virus vaccine
315 strains, B/Phuket/2013 (Yamagata lineage) and B/Texas/2013 (Victoria lineage), showed a
316 similar tendency (Figure 2c and 2d). In contrast, one inoculation of SV did not induce HI
317 antibodies against any of the virus strains. These data from the cynomolgus macaque model
318 mirrors the ineffective and effective priming by SV and WPV, respectively, that has been
319 observed in young children.

320 Next, to evaluate boosting potency of WPV and SV, we focused on HI antibody titers
321 in the sera obtained on day 49 (Figure 2). Notably, both WPV and SV were able to boost the
322 levels of HI antibodies in WPV-primed macaques after the second vaccination. The HI
323 antibody titers against influenza B virus vaccine strains also showed a similar tendency, but
324 the titers were lower than those against influenza A virus vaccine strains. One of the reasons
325 may be that the inactivation method applied to all four strains of viruses in the vaccine under
326 the current regulations may be suitable for influenza A viruses but not for influenza B viruses
327 in terms of maintaining antigenicity. Importantly, even after the second SV vaccination of
328 macaques previously immunized with SV, no increase of HI antibody titers was observed,
329 again indicating that the first SV vaccination had not primed the HI antibody response in the
330 macaques. The results suggest that SV can only induce HI antibody responses in primed
331 animals.

332 To confirm the boosting potency of SV, neutralizing antibody titers against influenza
333 A virus vaccine strains were examined by micro-neutralization ELISA assays (Supplemental
334 Figure 1). As expected, SV could induce neutralizing antibody response in animals
335 previously inoculated with WPV but not with SV. Consistent with the HI antibody responses,
336 even one inoculation of WPV induced substantial levels of neutralizing antibody and the
337 titers were further elevated by the second inoculation with either WPV or SV. These results
338 indicate that SV cannot prime naïve macaques effectively but only boost primed macaques,
339 which is consistent with the observation from the previously conducted human clinical trial
340 [16]. On the other hand, WPV can prime naïve macaques and boost primed macaques. Since
341 the potency of vaccines to induce priming responses is indispensable when naïve individuals
342 acquire protective immunity, these data indicate that WPV would be more suitable for naïve
343 populations, such as young children.

344

345 **3.2 Viral RNA in WPV effectively induces dendritic cell maturation.**

346 To investigate the mechanistic basis for the differences in priming potency between
347 WPV and SV, we next carried out *in vitro* experiments to determine whether differences in
348 the handling of the vaccine antigens by antigen presenting cells (APCs) could account for this
349 observation. Activation of APCs, such as macrophages and DC, in response to foreign
350 antigens is triggered by the engagement of APC-expressed pattern recognition receptors

351 (PRRs) and results in secretion of proinflammatory cytokines and chemokines. To investigate
352 whether WPV and SV could activate APC, key proinflammatory cytokines IL-6, TNF- α , and
353 IFN- γ and the chemokine MIP-1 α were evaluated in *in vitro* cultures of macaque splenic
354 APC exposed to the vaccines (Figure 3). Since the isolation of cynomolgus macaque DCs
355 was not reproducible in our system, CD14⁺ monocytes from spleen were used as APCs. The
356 remaining CD14-depleted splenocytes were also used as a DC-containing mixed cell
357 population. Both populations were incubated for 6 hr with or without stimulants (either 1
358 mg/ml of LPS as positive control or the vaccine preparations containing 3 μ g/ml HA), then
359 culture supernatants were harvested and the concentrations of cytokines measured. In CD14-
360 depleted splenocytes, concentrations of IL-6, TNF- α , MIP-1 α , and IFN- γ were higher in
361 WPV-treated cells than those in untreated and SV-treated cells. CD14⁺ splenocytes
362 responded strongly to LPS and produced IL-6, TNF- α , and MIP-1 α , but WPV induced only
363 IL-6. In contrast, SV failed to stimulate either CD14-depleted splenocytes or CD14⁺
364 monocytes. IFN- γ was greatly induced by WPV in CD14-depleted splenocytes which
365 contained DCs. These results suggest that WPV is a much more powerful activator of APC
366 than is SV.

367 DC are the APC involved in presenting processed antigen to naïve T helper cells for
368 the initial priming of these T cells to endow them with the capacity to provide “help” to B
369 cells for the production of antibodies [19]. To study DC in more detail, analyses were
370 performed using a single population of mouse splenic DCs. These were exposed to
371 monovalent vaccines of A/California/07/2009 (H1N1) to simplify the interpretation. The DCs
372 were stimulated with WPV or SV for 24 hr and induction of cytokines IL-6, TNF- α , IFN- γ ,
373 and chemokines MCP-1 and IP-10 was evaluated (Figure 4). As expected, WPV induced
374 significantly higher levels of those cytokines than did SV and a PBS control. Since type-I
375 IFNs are also typical innate immune products induced by activated DC, the mRNA levels of
376 IFN- α and IFN- β as well as IP-10 in the DCs at 6 hr post-stimulation were also evaluated
377 using quantitative-PCR (Figure 5). Compared with PBS, WPV induced almost 40-fold higher
378 expression level of IP-10 mRNA, whereas SV induced barely detectable levels. In addition to
379 mRNA of IP-10, mRNA levels of IFN- α and IFN- β were drastically elevated approximately
380 170- and 160-fold in DCs stimulated with WPV, respectively. SV did not transcribe mRNA
381 of type-I IFNs in the DCs at all. Together these data indicate that WPV, but not SV, induced
382 type-I IFNs as well as inflammatory cytokine and chemokine production from DC indicative
383 of a much more potent activation and maturation of DC by WPV than SV.

384 Influenza viral RNA is a potent innate immune activator that can interact with the
385 PRRs of DC, including Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I)
386 like receptors (RLRs) [20-22] resulting in DC maturation and production of inflammatory
387 cytokines and chemokines, especially type-I IFNs. Due to the different composition of WPV
388 and SV it is likely that the characteristics of viral RNA contained in vaccines may differ. To
389 investigate this, the RNA content in WPV and SV was compared by quantifying the RNA
390 concentrations of each vaccine, which were calculated by the absorbance at 260 nm. Both
391 vaccines contained equivalent amounts of viral RNA (Figure 6a), which is not surprising
392 because they were prepared from the same virus suspension with or without an ether-
393 disruption step. However, although these vaccines contain viral RNA at similar levels, the
394 absorbance measurement does not distinguish viral RNA with the lengths or base-paired

395 structures that stimulate PRRs from degraded nucleotide fragments [23-27]. To detect viral
396 RNA of a length recognizable by PRRs, quantitative-PCR was performed, targeting an 110
397 bp sequence in the influenza NP gene segment (Figure 6b). The copy number of the NP gene
398 segment in WPV was 6.28×10^7 RNA copies in 1 ng total RNA, while that in SV was only
399 5.34×10^5 RNA copies in 1 ng total RNA. Therefore, WPV was considered to be over 100-
400 fold richer in viral RNA with the length longer than 110 bp, compared with that in SV.
401 Furthermore, as the PRRs that recognize viral RNA are intracellular, it was examined
402 whether viral RNA in the vaccine preparations is actually taken up into DCs (Figure 6c).
403 After the DCs were incubated with WPV or SV for an hr, unincorporated free RNA was
404 removed by washing with PBS extensively, then total RNA was extracted from the cells for
405 quantitative-PCR. The copy number of NP segments incorporated into 10^5 DCs was $5.77 \times$
406 10^5 after the treatment with WPV but only 634 RNA copies after treatment with SV. Since
407 the amount of viral RNA of appropriate length contained in WPV was approximately 100
408 times more than that in SV, it is considered that the uptake of viral RNA contained in WPV is
409 10 times more efficient even if input RNA amount was the same. *In situ* hybridization of the
410 NP gene segment further confirmed that viral RNA from WPV was actually incorporated into
411 DCs (Supplemental Figure 2). While viral RNA signals in DCs incubated with SV randomly
412 diffused through a viewing field, the signals in DCs incubated with WPV were localized in
413 areas close to the nuclei. It is noted that input copy number of viral RNA in SV was adjusted
414 to the same level as that in WPV treatment in this experiment. Similar perinuclear
415 localization of the viral RNA signals was observed in DCs infected with influenza virus.
416 Although this *in situ* hybridization data support the difference in RNA uptake efficiency,
417 higher resolution analysis is needed to clarify the exact localization of RNA. Taken together,
418 this effective delivery of viral RNA into DCs may explain a reason why WPV but not SV
419 greatly stimulates innate responses in spleen-derived immune cells, which lead to the
420 activation and maturation of DCs.

421 To examine whether WPV stimulates DCs *in vivo*, inguinal lymph nodes were
422 collected from vaccinated mice at 6, 12, 24, and 48 hr post-vaccination and the surface
423 expression level of CD86, a marker for mature DC, was analyzed using a flow cytometer
424 (Figure 7 and Supplemental Figure 3). In addition to conventional DCs (cDCs), plasmacytoid
425 DCs (pDCs) were analyzed. This subset of DC is known to be a potent inducer of IFN- α in
426 response to viral RNA recognition by TLR7 [28-30]. The CD86 expression of cDCs in lymph
427 nodes sampled at 24 and 48 hr after vaccination with WPV, was much greater than after
428 vaccination with SV. The pDC from WPV-injected mice also expressed higher levels of
429 CD86 than those from SV-inoculated mice at 12 hr post-vaccination. Greater expression of
430 CD86 on DCs from WPV-vaccinated mice implied that WPV activates innate immunity and
431 promotes DC maturation to enhance the antigen presentation potency for effective priming *in*
432 *vivo*. Given that WPV delivered greater amounts of viral RNA into DCs as demonstrated by
433 *in vitro* experiments, the activation of APCs by the viral RNA contained in WPV provides an
434 explanation for its effective priming potency in mice and macaques.

435

436 **3.3 WPV induced a significant antibody response at low doses.**

437 A dose-sparing experiment using macaques was conducted to investigate any
438 advantage of WPV in addition to the priming potency (Figure 8). Eight naïve cynomolgus

439 macaques were vaccinated twice with the various doses (full, 1/2, 1/4, or 1/8 doses) of
440 quadrivalent WPV with a 4-week interval between injections. The full dose contained 15 μ g
441 HA per strain, the same amount as the commercially available seasonal influenza split
442 vaccines. Plasma samples were periodically collected on day 0, 28, and 49 to evaluate
443 antibody responses against the vaccine strains. HI antibody titers against influenza A virus
444 vaccine strains on day 49 were significantly increased from day 0 or day 28 in all groups of
445 monkeys. HI antibody titers against influenza B virus vaccine strains on day 49 increased
446 significantly from day 0 or day 28 in macaques vaccinated with full or 1/2 doses of WPV, but
447 not in monkeys vaccinated with 1/4 and 1/8 doses. The titres of HI antibody responses to
448 influenza B virus vaccine strains were modest compared to those to influenza A virus vaccine
449 strains, consistent with the data shown in Figure 2. Plasma samples were also tested in micro-
450 neutralization ELISA assays and the results were in accord with the data of HI assay
451 (Supplemental Figure 4). The boosting effect of the second vaccination seems to be fully
452 achieved by even half the dose of WPV. The present results suggest that the full dose of
453 WPV is not necessary for the boosting effect, thus the amount of vaccine could be reduced to
454 half that of current seasonal influenza SVs.

455

456 **4. Discussion**

457 In this study we demonstrated the relative inefficiency of SV compared to WPV in
458 priming a protective antibody response and provide evidence that this difference is due to
459 inferior induction of innate immune mechanisms, most importantly the maturation of DCs. In
460 contrast, SV exhibits comparative effectiveness to WPV in boosting immunity in macaques
461 previously primed by WPV. These data are consistent with the observation in humans that
462 current SV only provides adequate immunity in primed populations such as most adults but
463 does not induce sufficient immune responses in unprimed populations [14, 16].

464 Historically, when SV were developed, these replaced WPV in spite of the impaired
465 immunogenicity in young children because of the emphasis on WPV-associated adverse
466 reactions such as fever [12, 13]. However, these side reactions, with the exception of those
467 caused by egg-derived contaminants and residual formalin, can be indicative of well-
468 functioning host immune responses, particularly the innate immune system [31, 32]. This is
469 consistent with the present results where WPV, but not SV at a comparable antigen dose,
470 induced innate immune responses as shown by the induction of cytokines and chemokines.
471 Among them, inflammatory cytokines such as IL-6, IL-1, and TNF- α , as well as type-I IFNs,
472 trigger febrile responses [31, 32]. Concurrently, the cytokines induced by signaling cascades
473 of PRRs, such as TLRs and RLRs, play important roles in activation and maturation of DCs,
474 leading to the induction of effective adaptive immunity. Thus, the innate immune system,
475 including inflammatory cytokines, is tightly linked to the formation of robust adaptive
476 immunity. This double-edged sword action of cytokines was only revealed in the 1990s and
477 later, so at the time when WPV was replaced by SV because of the emphasis on the undesired
478 pyrogenic reactions, the accompanying benefits from WPV-induced innate immunity were
479 not understood nor properly evaluated.

480 Here we showed that recognition of viral RNA by PRRs on DC may be responsible
481 for the differences in the ability of the vaccines to mount a primary response. The RNA

482 recognized by PRRs includes replication products and viral RNA structure; e.g. double- and
483 single-stranded RNA, 5'-ppp RNA, and virus mRNA cap [33]. Given that activation of DCs
484 through PRRs is necessary for DC maturation and subsequent adaptive immune responses
485 and effective priming [34, 35], characteristics of viral RNA in vaccines are particularly
486 important. Inactivated WPV does not replicate but viral RNA is retained within a virion and
487 is able to efficiently trigger innate immune responses. Although SV also contains viral RNA,
488 the amount of viral RNA detectable by qPCR in SV was only 1/100 of that in WPV as shown
489 in Figure 6b. It was probably because the viral RNA in SV was degraded during storage
490 and/or the manufacturing process involving disruption of the virion structure by ether and/or
491 detergent. More importantly, the viral RNA is taken up by APCs such as DCs, to induce
492 innate immune responses via PRRs located in the endosome or cytoplasm, not on the cell
493 surface [36]. When the DCs were stimulated with WPV or SV containing the same HA
494 protein concentration, the amount of viral RNA internalized to the cells was more than 1,000
495 times higher in WPV-stimulated cells compared with SV-stimulated ones (Figure 6c). Thus,
496 this is the most likely reason for superiority of WPV both in innate and adaptive immunity in
497 naïve animals, compared to SV. The differences in the potency to induce the “priming”
498 between WPV and SV are schematically summarized in Supplemental Figure 5. After a WPV
499 particle is captured, the entire component of the virion, including viral RNA, is incorporated
500 into a DC. The virion structure is degraded in endosomes and released viral RNA is
501 recognized by PRRs. Activation of DC through PRRs by the viral RNA induces DC
502 maturation with up-regulation of MHC class I and II and costimulatory molecule CD80 and
503 CD86 to enhance antigen presentation. After the WPV is endocytosed, digested and
504 processed, antigenic epitopes are loaded onto MHC II molecules, which subsequently
505 activate CD4⁺ helper T cells. In addition, some antigens are directed to endolysosomes where
506 they may gain access to the MHC I antigen processing pathway called the “cross-presentation
507 pathway” in which, subsequently, antigenic epitopes are loaded onto MHC I molecules, and
508 to activate CD8⁺ cytotoxic T cells [37-40]. Importantly, WPV allows all epitopes of the
509 structural proteins of a virus particle to be presented on MHC molecules. In fact, our previous
510 study utilizing WPV and SV demonstrated the superiority of WPV over SV in terms of the
511 induction of neutralizing antibodies and CD8⁺ T cell responses in naïve mice [17]. Therefore,
512 WPV induce stronger and broader humoral and cellular adaptive immunity in vaccinated
513 individuals. On the other hand, in SV, the HA proteins that bind cellular sialic acid receptor
514 are incorporated into DC, and the other viral proteins, particularly internal proteins and viral
515 RNA, are not preferably taken up by APCs including DCs, resulting in inadequate DC
516 maturation. As a result, SV does not effectively prime naïve populations. However, the
517 addition of adjuvant to SV is one way to compensate for an ability to effectively activate
518 immune response in naïve populations [41, 42]. In contrast to priming responses, DCs are not
519 necessary for boosting responses, but rather the only interaction between memory B cells and
520 memory T follicular helper cells is sufficient [43, 44]. As shown in Figure 2, SV boosted the
521 primed macaques. However, it has been reported that WPV, but not SV, induces T cell-
522 independent antibody responses in the boosting responses, resulting in faster recall of the
523 memory response [45]. Although the present study did not confirm the superiority of WPV
524 over SV in the boosting responses, it is now clear that WPV has advantages in the boosting as
525 well as the priming response.

526 Since school-age children are one of the key spreaders for influenza in a community,
527 immunization of children is essential to achieve “herd immunity” [7-10]. Herd immunity is a
528 basic concept for epidemic control and an effective vaccine is the key to achieving this state
529 of population immunity. With our findings, it seems difficult that herd immunity could be
530 achieved using current split vaccines due to lack of priming potency. SV may still effectively
531 work for people who are primed to influenza virus, but development of other types of
532 vaccines to induce enough protective immune responses against influenza in children is an
533 urgent task. Our new WPV is expected to immunize young children effectively and not to
534 cause undesired reactions, at least those caused by egg-derived contaminants, due to the
535 contribution of improved virus purification methods. Therefore, the present results prompt
536 this project to clinical trials especially with young children. Comparing vaccine efficacy of
537 WPV to SV in adults and children will provide valuable and new insights into the benefits of
538 WPV. In addition, the dose-sparing experiments using macaques indicate that vaccination
539 with even half dose of WPV is sufficient to induce protective immunity, probably with
540 limited induction of inflammatory responses as reported previously [17]. This dose reduction
541 is also an additional advantage for raising production capacity as well as lowering costs.
542 Thus, this new WPV should be an alternative option for seasonal influenza vaccines,
543 especially for children, because of effective immunogenicity in naïve individuals. In addition,
544 WPV should be ideal to control pandemic influenza, a situation where all individuals are
545 naïve to the causative agent. In both these contexts, we envisage that use of WPV would
546 contribute significantly to global health.

547

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553 A/Singapore/GP1908/2015 (IVR-180) (H1N1), A/Hong Kong/4801/2014 (X-263) (H3N2),
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568

569 **Figure legends**

570

571 **Figure 1**

572 **Protective immune responses induced in mice vaccinated with WPV.**

573 C57BL/6 mice (n =5 per group) were inoculated subcutaneously with PBS, SV (3 µg
574 HA), or various doses of WPV (3–0.12 µg HA). On day 28 after vaccination, the mice were
575 challenged intranasally with a lethal dose (3,000 PFU in 40 µl PBS) of A/Singapore/2015
576 (H1N1) virus. Mice were euthanized at 5 days post-infection (dpi) and lungs harvested for
577 plaque assay (a). The body weight of the mice was monitored daily until 5 dpi (b). Data are
578 expressed as the percentage of the starting weight on day 0 and symbols represent the mean
579 and SEM of the group. To measure the neutralizing antibody titers induced by vaccines, sera
580 were collected on day 24 after vaccination prior to challenge (c). Box plots in (a) and (c)
581 indicate the median (line in box), the interquartile range (ends of box), and whiskers from
582 min to max. Statistical analysis was performed using two-way ANOVA with multiple
583 comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

584

585 **Figure 2**

586 **Hemagglutination inhibiting antibodies induced after vaccination of naïve macaques**

587 Cynomolgus macaques (n=4 per group) were vaccinated twice by the subcutaneous
588 route with either WPV or SV (prime and boost) as indicated. Serum samples were collected
589 from each animal on day 0, 28, and 49 and assayed for hemagglutination inhibiting
590 antibodies against vaccine strains, A/Singapore/ 2015 (H1N1) (a), A/Hong Kong/2014
591 (H3N2) (b), B/Phuket /2013 (Yamagata lineage) (c), or B/Texas/2013 (Victoria lineage) (d).
592 Box plots indicate the median (line in box), the interquartile range (ends of box), and
593 whiskers from min to max. Statistical analysis was performed using two-way ANOVA with
594 multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

595

596 **Figure 3**

597 **Cytokine production in macaque CD14⁺ and CD14⁻ splenocytes cultured with the**
598 **vaccines**

599 CD14⁺ and CD14-depleted splenocytes collected from unimmunized cynomolgus
600 macaques were cultured with WPV, SP, or LPS for 6 hr. Concentrations of IL-6 (a), TNF- α
601 (b), MIP-1 α (c), and INF- γ (d) in the supernatants were measured. The results shown are
602 representative of two independent experiments.

603

604 **Figure 4**

605 **Vaccine-induced cytokine production in mouse DCs**

606 DCs collected from mice were incubated in the presence of PBS, WPV (5µg of HA),
607 or SV (5µg of HA) for 24 hr and culture supernatants were collected for measurement of the
608 cytokines and chemokines IL-6 (a), TNF- α (b), MCP-1 (c), IFN- γ (d), and IP-10 (e). Each
609 bar represents the mean \pm SEM of 3 replicates. In each panel, black bars indicate the amount
610 of cytokine induced from cells treated with PBS, light gray bars indicate the amount of
611 cytokine induced from WPV-stimulated cells, and dark gray bars indicate the amount of

612 cytokine induced from SV-stimulated cells. Statistical analysis was performed using one-way
613 ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$.

614

615 **Figure 5**

616 **Type-I IFN induction in mouse DCs stimulated with the vaccines**

617 One hundred thousand DCs from mouse spleen were cultured with WPV, SV, or PBS
618 for 6 hr. The mRNA expression levels of IP-10 (a), IFN- α (b) and IFN- β (c) in DCs were
619 analyzed using qPCR. Each bar represents the mean \pm SEM of triplicate samples and the data
620 shown are representative of three independent experiments. Statistical analysis was
621 performed using one-way ANOVA with multiple comparisons. *** $p < 0.001$.

622

623 **Figure 6**

624 **Levels of viral RNA in vaccine preparations and its uptake into DCs**

625 The concentration of RNA extracted from 5 μ g HA of WPV or SV was calculated
626 from the absorbance at 260 nm (a). The copy number of 110 bp sequence of the influenza
627 virus NP segment in the extracted RNA was determined using qPCR (b). One hundred
628 thousand DCs were incubated with WPV or SV for an hr. After removal of residual RNA by
629 extensive washing with PBS, intracellular RNA was extracted and the copy number of the NP
630 segment in DCs determined using qPCR (c). Each bar represents the mean \pm SEM of
631 triplicate samples and the data shown are representative of three independent experiments.
632 Statistical analysis was performed using unpaired t -test. *** $p < 0.001$.

633

634 **Figure 7**

635 **Surface expression of the maturation marker CD86 on DCs from vaccinated mice**

636 Mice were vaccinated with WPV or SV and lymph nodes were collected at 6, 12, 24,
637 and 48 hr post vaccination. The inguinal lymph nodes collected from five mice were
638 combined and used for data analysis at each point. CD86 expression on conventional DCs
639 (cDC) (a) and plasmacytoid DCs (pDCs) (b) in the lymph nodes was analyzed by flow
640 cytometry. Expression levels of CD86 are shown as mean fluorescence intensity (MFI). cDCs
641 were identified as the CD19 negative, CD11b positive, CD11c positive, and B220 negative
642 population, while pDCs were CD19 negative, CD11b negative, CD11c positive, and B220
643 positive.

644

645 **Figure 8**

646 **The relationship between WPV dose and hemagglutination inhibition titers in macaques**

647 Cynomolgus macaques (n=8 per group) were vaccinated twice, 4 weeks apart, with
648 the indicated doses of WPV delivered subcutaneously. Serum samples were collected on day
649 0, 28, and 49 and hemagglutination inhibition titers against vaccine strains, A/Singapore/
650 2015 (H1N1) (a), A/Hong Kong/2014 (H3N2) (b), B/Phuket /2013 (Yamagata lineage) (c), or
651 B/Texas/2013 (Victoria lineage) (d), were measured. Box plots indicate the median (line in
652 box), the interquartile range (ends of box), and whiskers from min to max. Statistical analysis
653 was performed using two-way ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$,
654 *** $p < 0.001$.

655

656 **Supplemental Figure 1**

657 **Neutralization titers in the serum of vaccinated macaques**

658 Cynomolgus macaques (n=4 per group) were vaccinated twice, 4 weeks apart, by the
659 subcutaneous route with either WPV or SV (prime and boost) as indicated. Serum samples
660 were collected on day 0, 28, and 49 and serum neutralizing antibody titers against
661 A/Singapore/2015 (H1N1) (a) or A/Hong Kong/2014 (H3N2) (b) were measured. Box plots
662 indicate the median (line in box), the interquartile range (ends of box), and whiskers from
663 min to max. Statistical analysis was performed using two-way ANOVA with multiple
664 comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

665

666 **Supplemental Figure 2**

667 **Incorporated viral RNA in DCs detected using *in situ* hybridization**

668 The mouse DCs were cultured overnight on tissue culture slides. The attached DCs on
669 slide were incubated for 3 hr with WPV or SV, each of which had been adjusted to 5×10^5 ,
670 copies of the NP segment. As a positive control, live virus at MOI = 10 was used. The NP
671 segment incorporated into the DCs was detected by *in situ* hybridization. In these
672 representative images, green or blue color indicate specific signal to NP segment or cell
673 nuclei, respectively. Long arrows indicate NP segments in the DCs. Short arrow indicates
674 aggregated NP segments.

675

676 **Supplemental Figure 3**

677 **Gating Strategy and CD86 expression on cDCs and pDCs from vaccinated mice**

678 Mice were vaccinated with WPV or SV and lymph nodes were collected at 6, 12, 24,
679 and 48 hr post vaccination. The inguinal lymph nodes collected from five mice were
680 combined and used for data analysis at each point. Representative gating strategy for FACS
681 analysis is shown in (a); CD11b-positive/B220-negative and CD11b-negative/B220-positive
682 populations in CD19-negative/CD11c-positive populations are determined as cDCs and
683 pDCs, respectively. The CD86 expression on cDCs and pDCs at each time point was shown
684 in (b); Surface expression of CD86 on cDCs and pDCs was compared between WPV
685 vaccinated (red-lined histograms) and SV vaccinated mice (blue-filled histograms). Gray-
686 filled and black-lined histograms represent matched isotype controls for cDCs and pDCs in
687 mice vaccinated with WPV and SV vaccines.

688

689 **Supplemental Figure 4**

690 **Neutralization titers after two inoculations of reduced doses of WPV in macaques**

691 Cynomolgus macaques (n=8 per group) were vaccinated twice, 4 weeks apart, with
692 the indicated doses of WPV. Serum samples were collected on day 0, 28, and 49 and
693 neutralization titers against A/Singapore/ 2015 (H1N1) (a) or A/Hong Kong/2014 (H3N2) (b)
694 were measured. Box plots indicate the median (line in box), the interquartile range (ends of
695 box), and whiskers from min to max. Statistical analysis was performed using two-way
696 ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

697

698 **Supplemental Figure 5**

699 **The differences in the potency to induce DC maturation between WPV and SV**

700 Schematic representation of the expected mechanism of DC maturation and antigen
701 presentation by WPV and SV is shown. Upon capturing a WPV particle, the entire
702 component of the virion, including viral RNA, is incorporated into a DC. Viral RNA is
703 released from the virion and stimulates a PRR signaling cascade, leading to the DC
704 maturation with up-regulation of MHC class I/II and CD80/86. In contrast, although HA
705 proteins of SV bind cellular sialic acid and are incorporated into a DC, other viral
706 components, particularly viral RNA, are not effectively taken into the cell, resulting in
707 inadequate DC maturation. MHC, Major Histocompatibility Complex; TCR, T Cell Receptor.

708

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710

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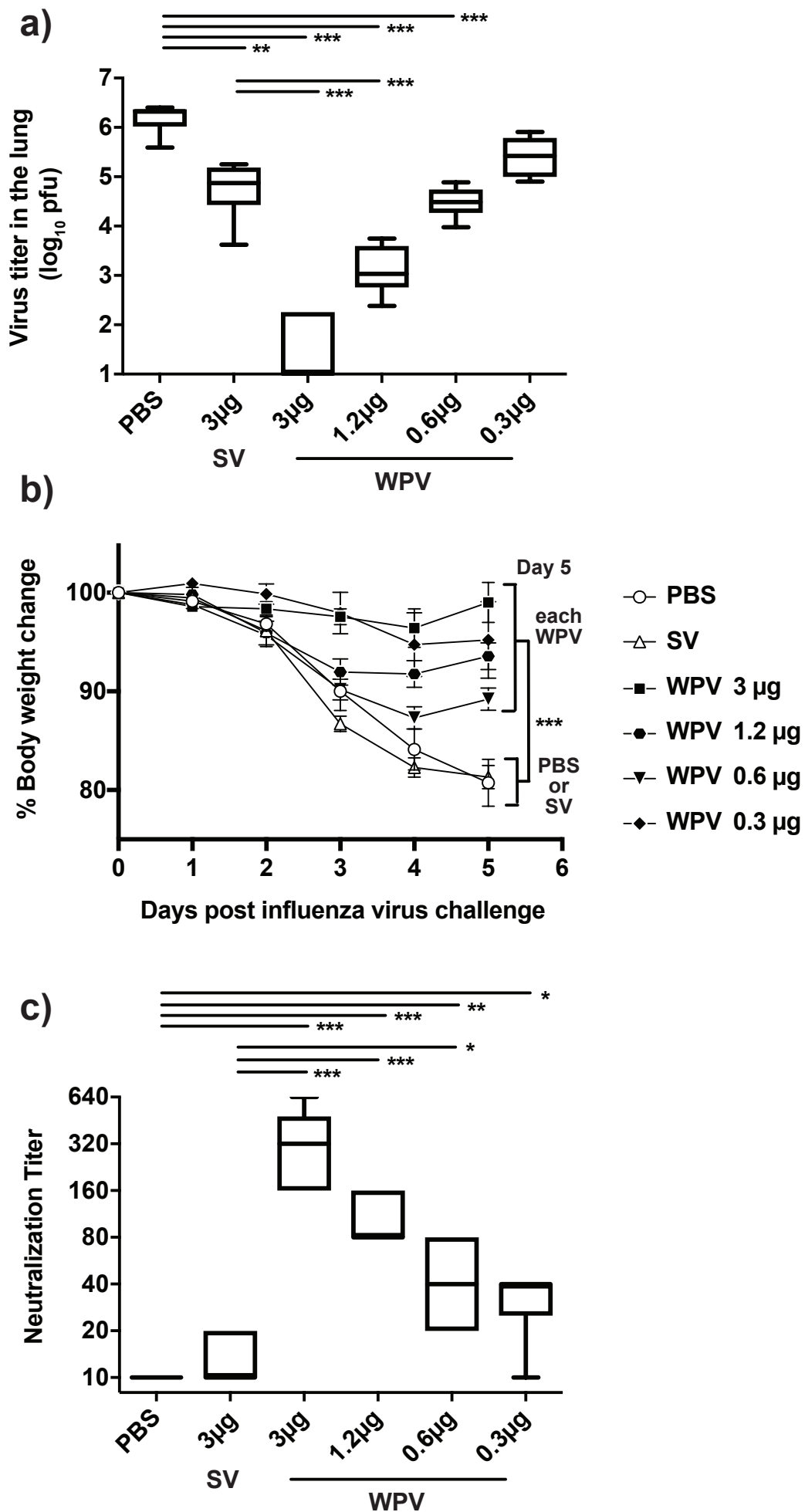


Figure 1

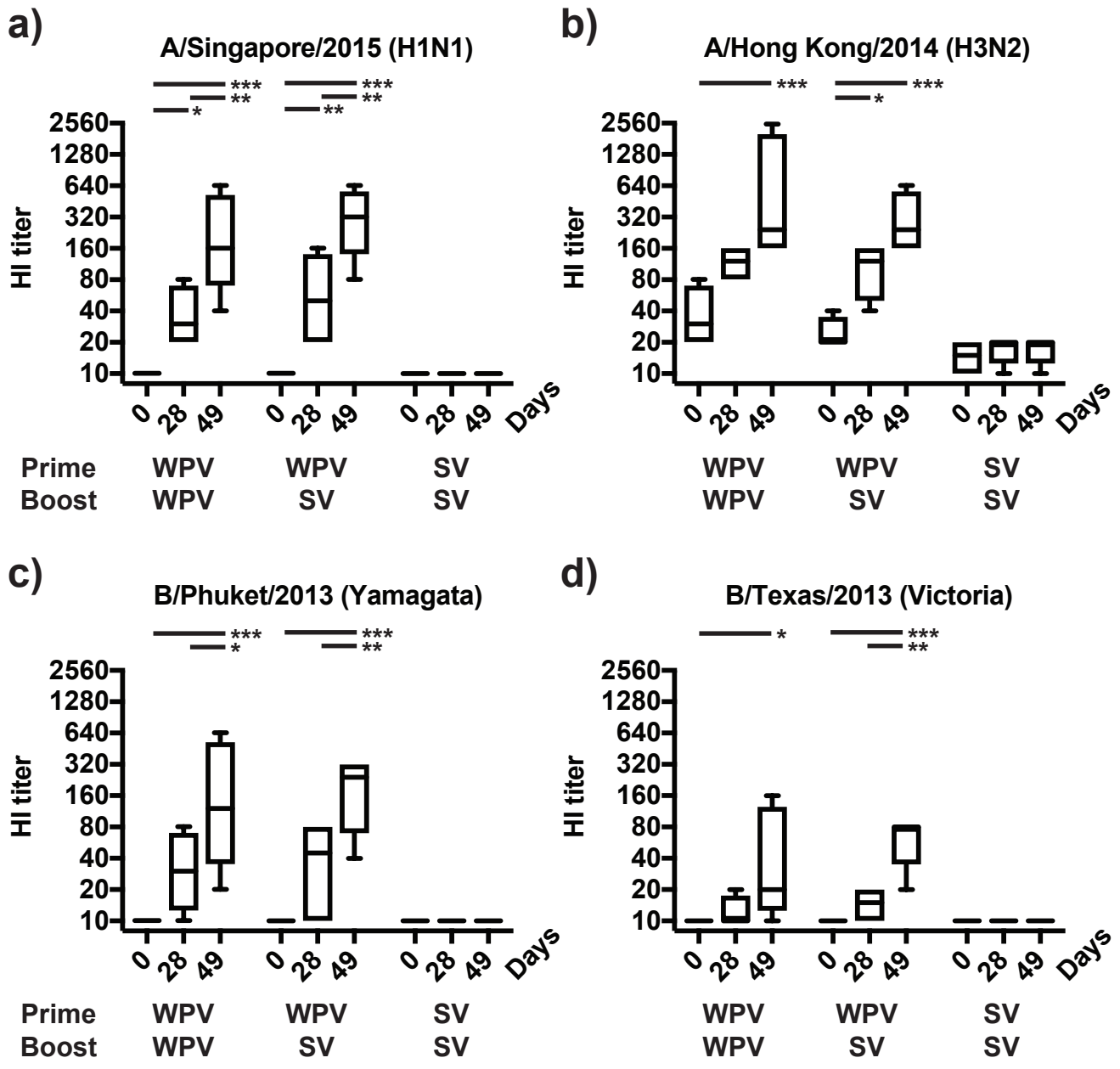


Figure 2

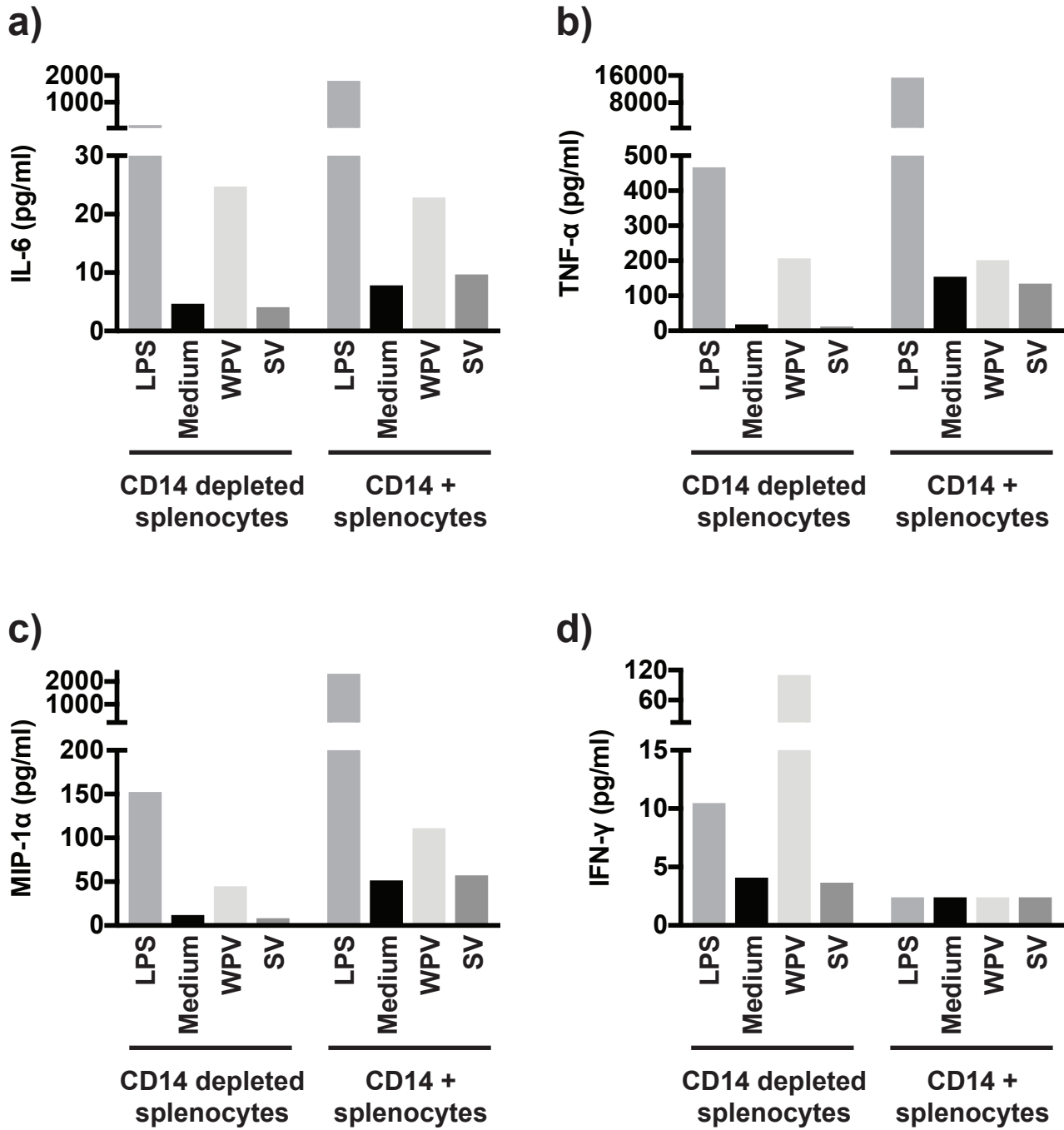


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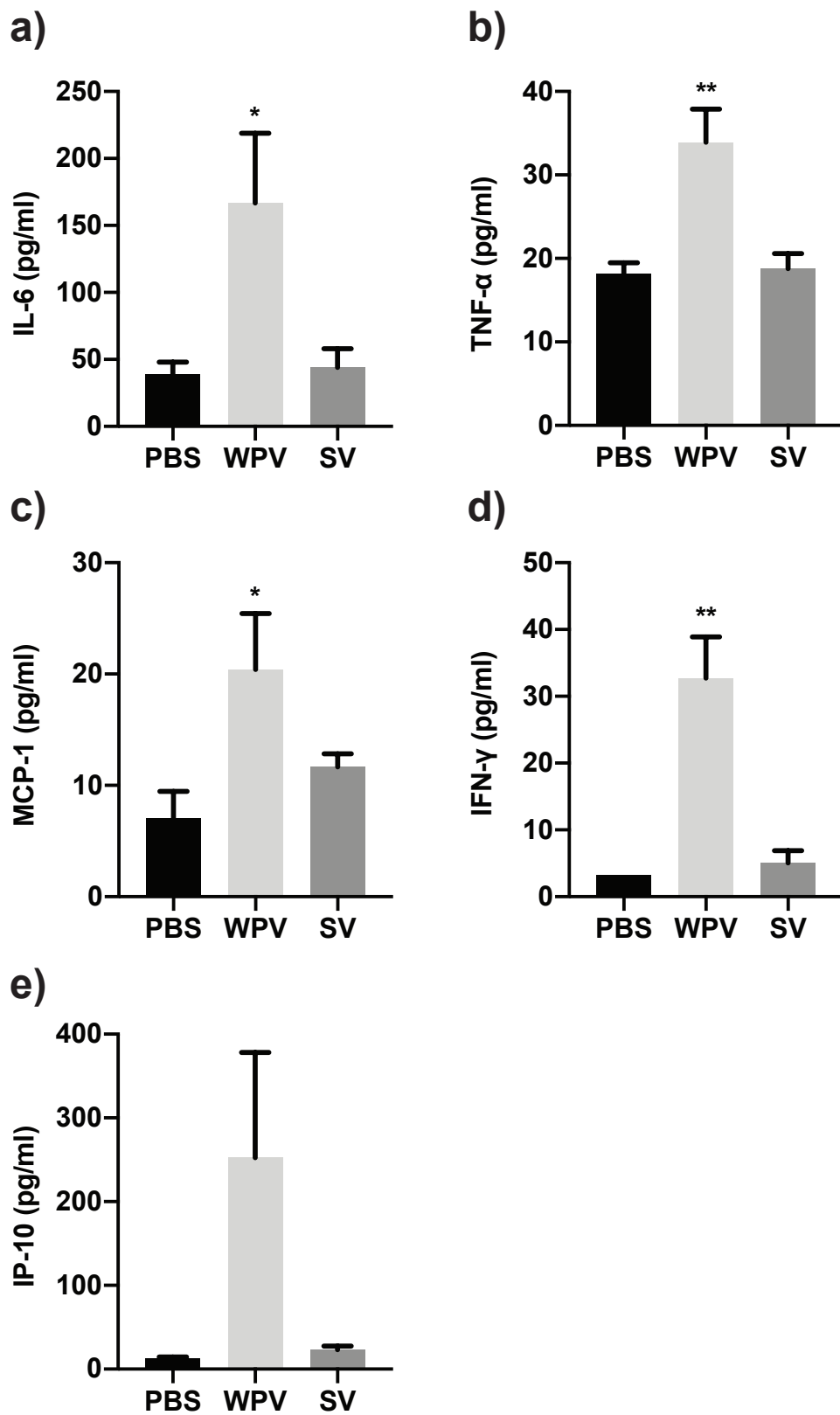


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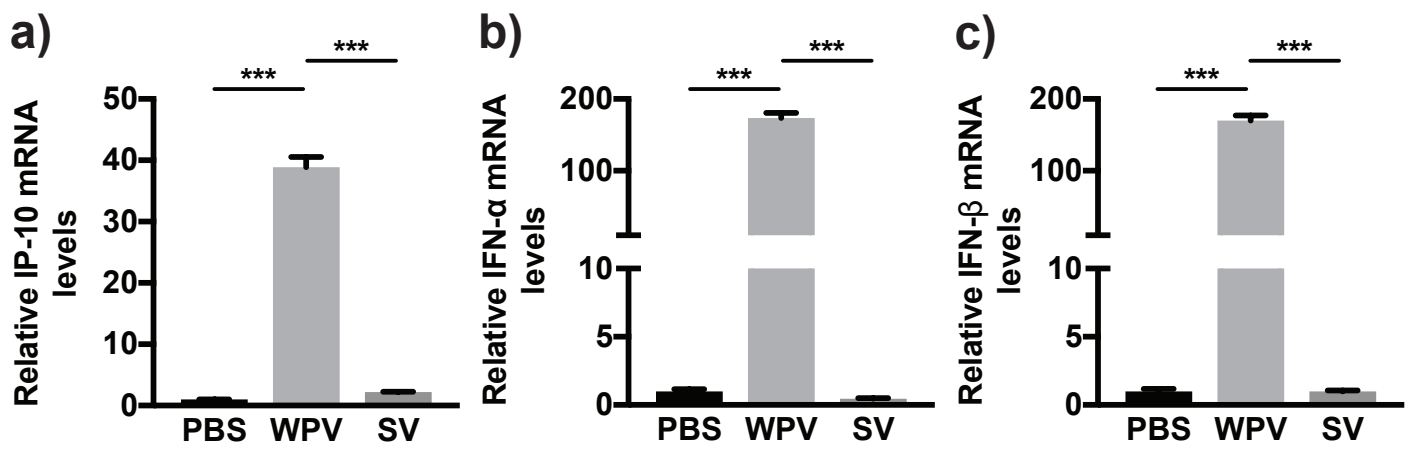


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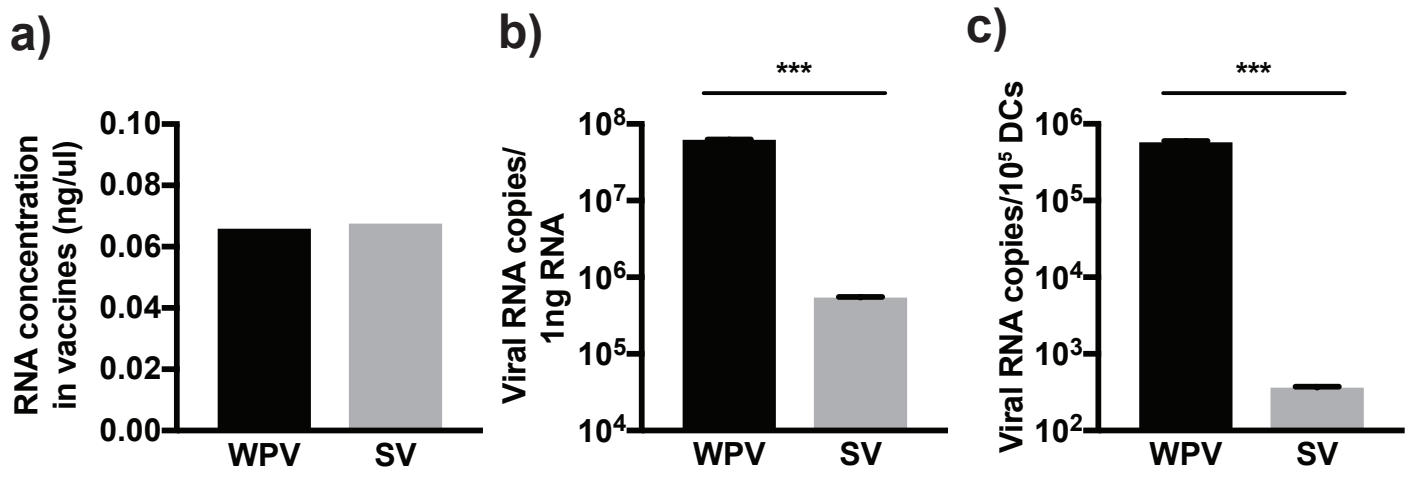


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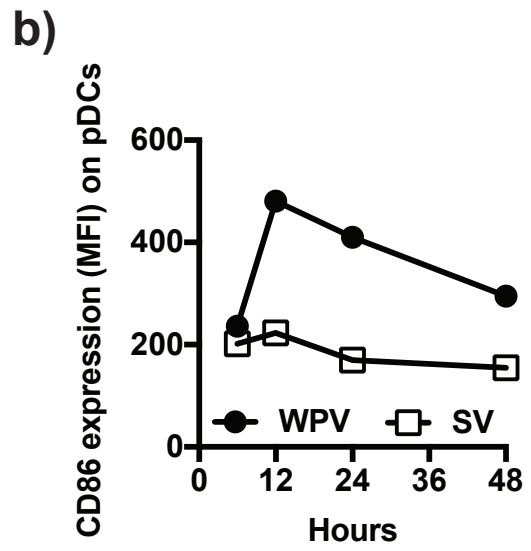
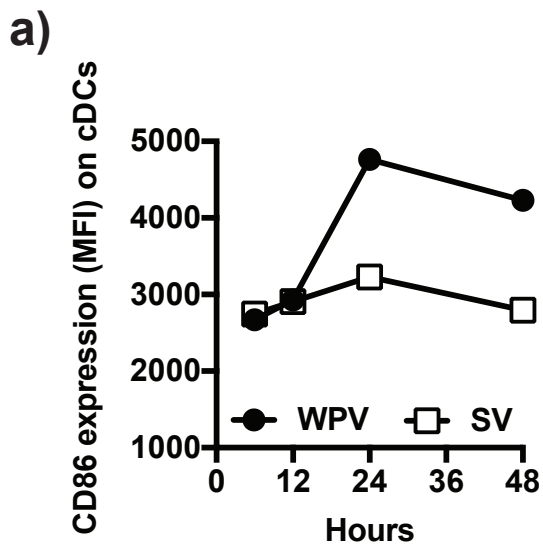


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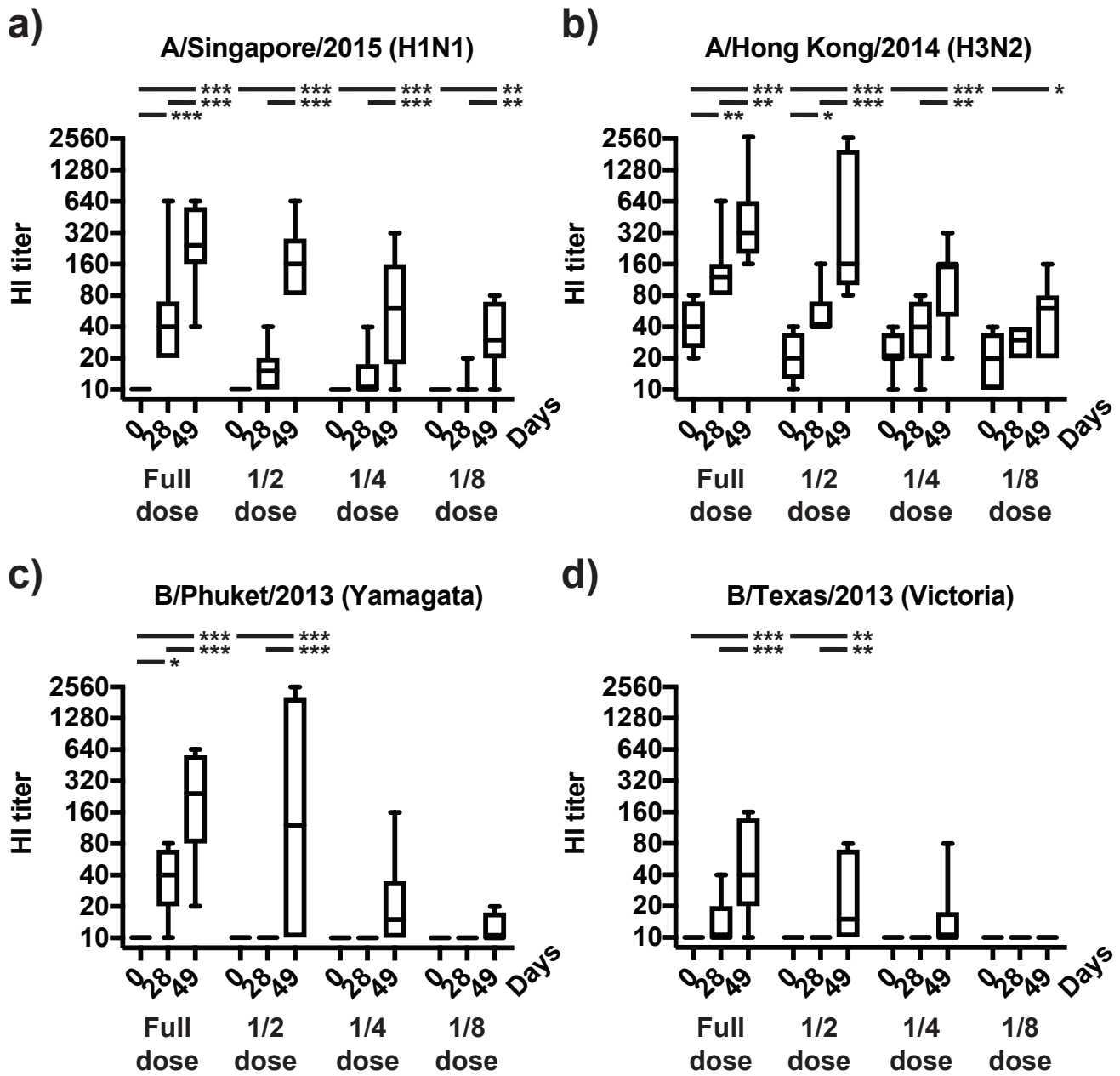
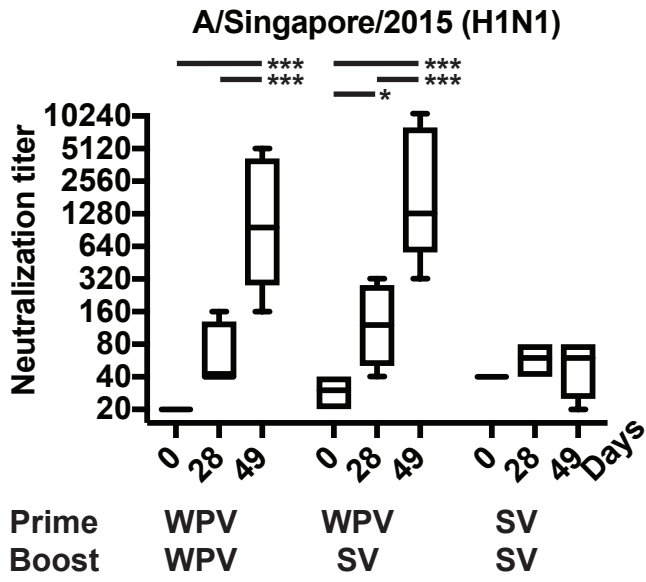
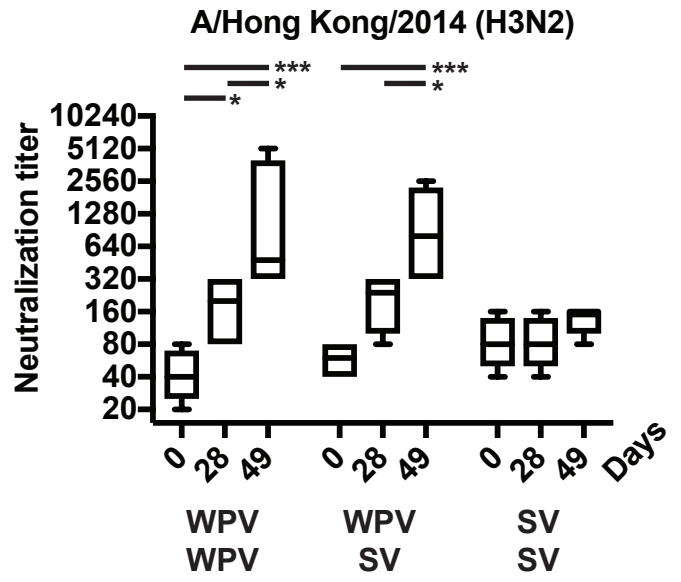


Figure 8

a)

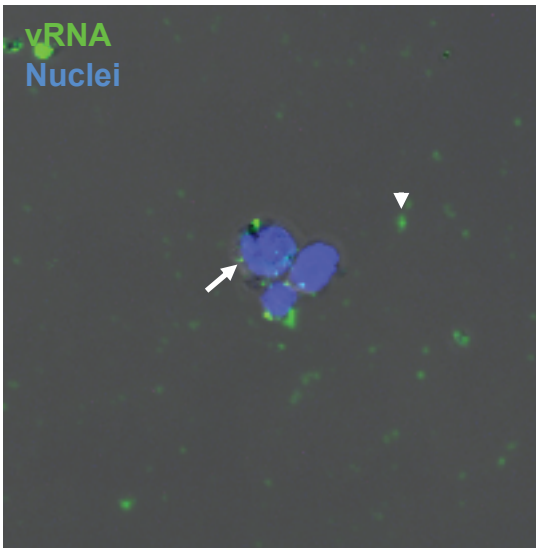


b)

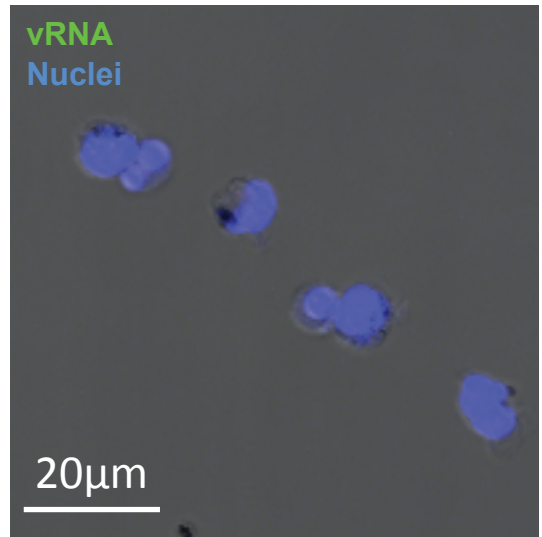
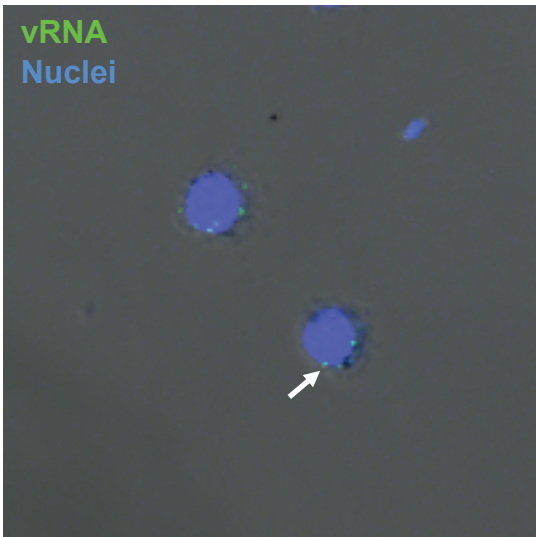
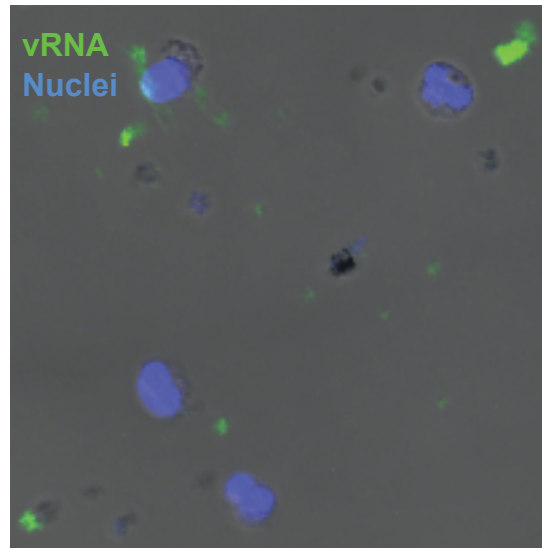


Supplimental Figure 1

WPV



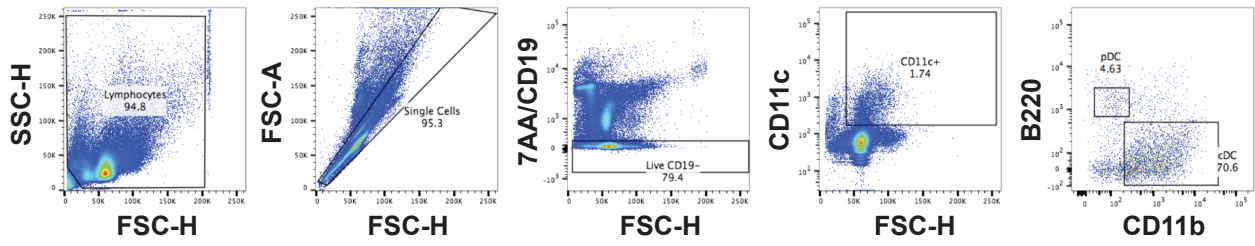
SV



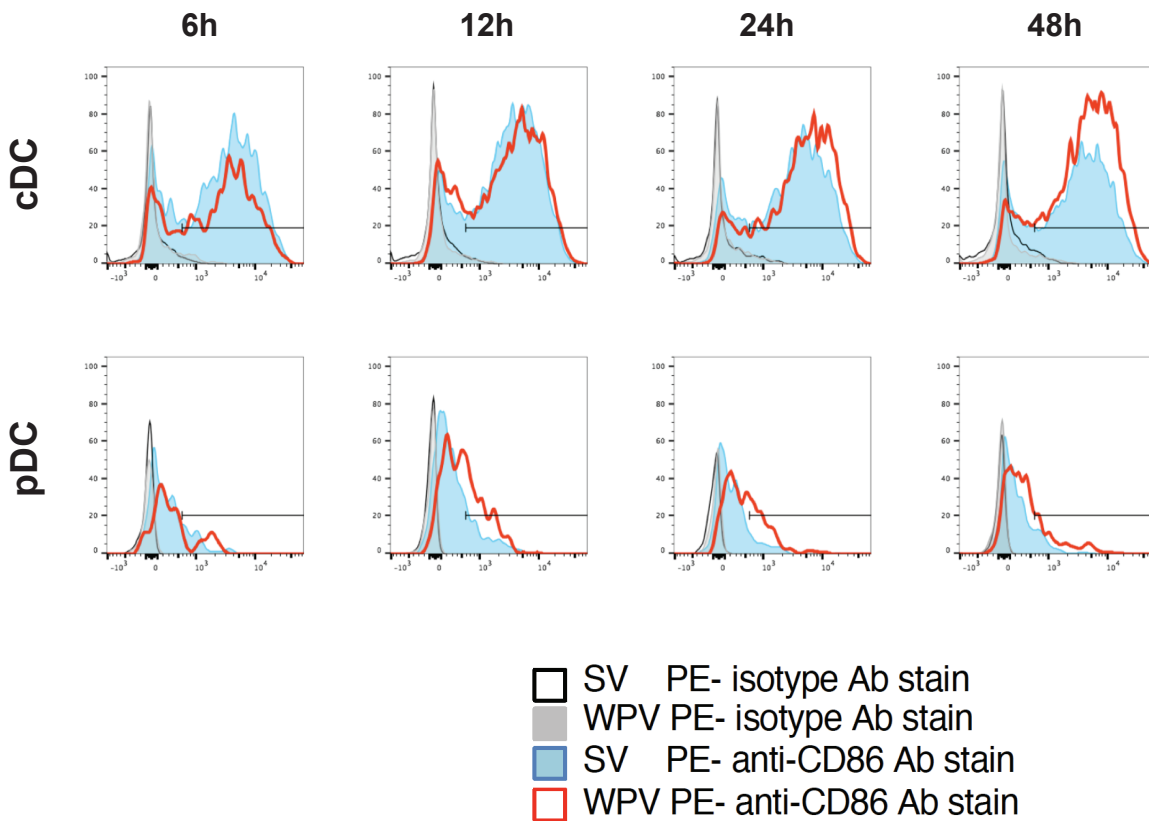
Live virus

PBS

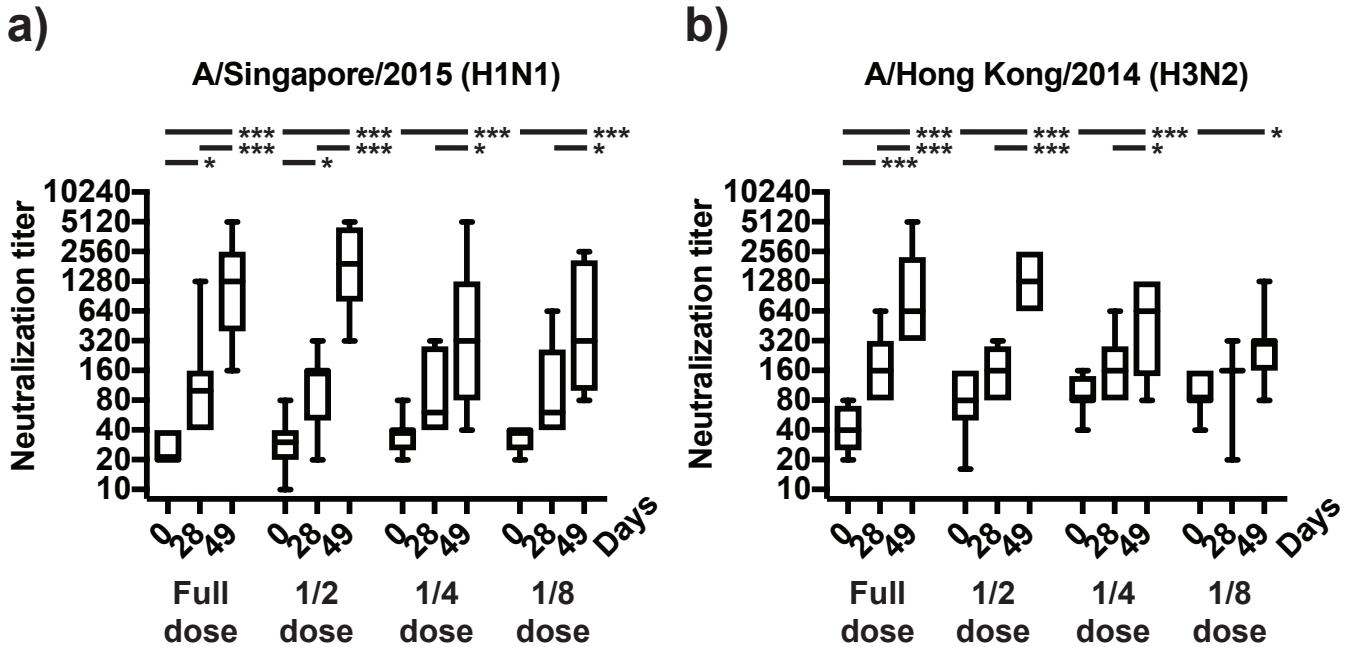
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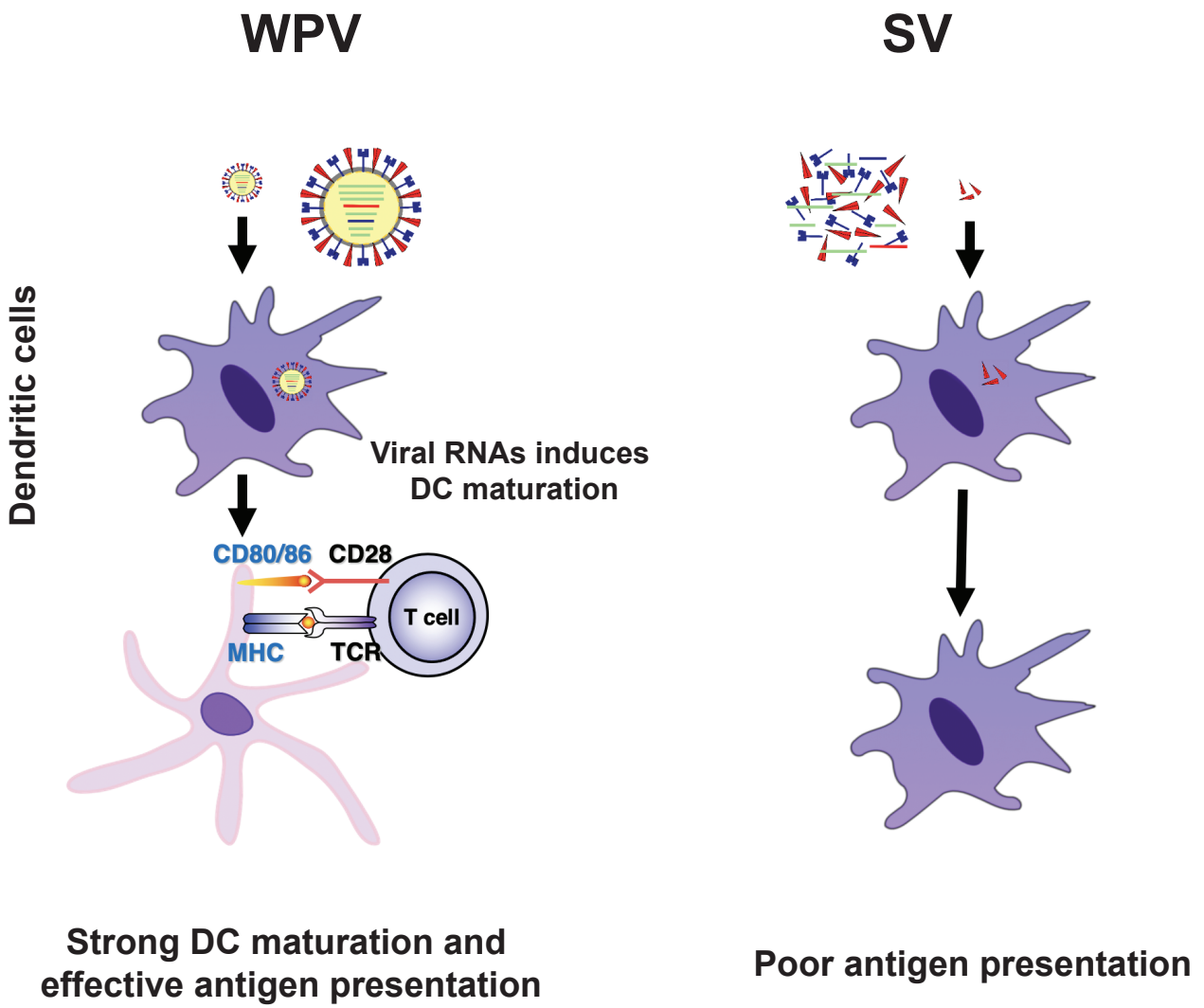
b)



Supplemental figure 3



Supplemental figure 4



Supplemental figure 5