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Inactivated influenza vaccine formulated with single-stranded RNA-based adjuvant confers mucosal immunity and cross-protection against influenza virus infection

Yun-Hee Kim [a,b,1], Yoo-Jin Bang [a,1], Hyo-Jung Park [a,1], Hae Li Ko [a,c,1], Sang-In Park [c], Kyung-Ah Hwang [d], Hun Kim [b], Jae-Hwan Nam [a,*]

[a] Department of Biotechnology, The Catholic University of Korea, Bucheon 14662, Republic of Korea
[b] Department of R&D, SK bioscience, Pangyoro 332, Bundang-gu, Republic of Korea
[c] Scripps Korea Antibody Institute, Chuncheon, Kangwon-Do 24341, Republic of Korea
[d] SML Genetree Research Department of Research and Development, SML Genetree, Seoul, Baumero 225, Seocho-gu 06740, Republic of Korea

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ABSTRACT

Influenza vaccination is considered the most valuable means to prevent and control seasonal influenza infections, which causes various clinical symptoms, ranging from mild cough and fever to even death. Among various influenza vaccine types, the inactivated subunit type is known to provide improved safety with reduced reactogenicity. However, there are some drawbacks associated with inactivated subunit type vaccines, with the main ones being its low immunogenicity and the induction of Th2-biased immune responses. In this study, we investigated the role of a single-stranded RNA (ssRNA) derived from the intergenic region in the internal ribosome entry site of the Cricket paralysis virus as an adjuvant rather than the universal vaccine for a seasonal inactivated subunit influenza vaccine. The ssRNA adjuvant stimulated not only well-balanced cellular (indicated by IgG2a, IFN-γ, IL-2, and TNF-α) and humoral (indicated by IgG1 and IL-4) immune responses but also a mucosal immune response (indicated by IgA), a key protector against respiratory virus infections. It also increases the HI titer, the surrogate marker of influenza vaccine efficacy. Furthermore, ssRNA adjuvant confers cross-protective immune responses against heterologous influenza virus infection while promoting enhanced viral clearance. Moreover, ssRNA adjuvant increases the number of memory $CD4^+$ and $CD8^+$ T cells, which can be expected to induce long-term immune responses. Therefore, this ssRNA-adjuvanted seasonal inactivated subunit influenza vaccine might be the best influenza vaccine generating robust humoral and cellular immune responses and conferring cross-protective and long-term immunity.

1. Introduction

Seasonal influenza viruses cause respiratory diseases that present with clinical symptoms such as cough, fever, and headache. According to the WHO's 2017 report, globally between 290,000 and 650,000 people lose their lives each year due to respiratory diseases related to seasonal influenza [1]. Currently, annual vaccination with the inactivated influenza vaccine (IIV) is regarded as one of the most effective approaches to prevent and control seasonal influenza. However, there are several inherent limitations associated with the current vaccine strategy including, i) exclusive protection against the corresponding virus strain of immunization, ii) short-lived protection period, and iii) low mucosal immune response, which is important to protect against respiratory virus infections [2].

Abbreviations: Alum, aluminum salt; ANOVA, analysis of variance; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CrPV, Cricket paralysis virus; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot; GMT, geometric mean titer; GOI, gene of interest; HA, hemagglutinin; HI, hemagglutination inhibition; IEDB, Immune Epitope Database and Analysis Resource; IGR, intergenic region; IIV, inactivated influenza vaccine; IRES, internal ribosome entry site; LAIV, live attenuated influenza vaccine; LP, cationic peptide-linked lipid; MERS-CoV, Middle East respiratory syndrome coronavirus; OD, optical density; P, protamine; PBS-T, PBS containing 0.05% Tween 20; PR8, A/Puerto Rico/8/34 virus; RT, room temperature; S, spike; SD, standard deviation; ssRNA, single-stranded RNA; TLR, Toll-like receptor.

* Corresponding author at: The Catholic University of Korea, 43-1 Yeokgok-dong, Wonmi-gu, Bucheon 14662, Republic of Korea.
E-mail address: jhnam@catholic.ac.kr (J.-H. Nam).
[1] These authors contributed equally.

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To improve the immune response elicited by IIVs, vaccination combined with an adjuvant is encouraged, and numerous studies regarding adjuvants are in progress. For influenza vaccines, aluminum salt (alum), MF59, and AS03 are currently used as adjuvants in humans [2,3]. However, they do not sufficiently enhance the balanced Th1/Th2 lymphocyte response [4,5]. To promote Th1 and Th2 responses by influenza vaccines with reduced reactogenicity, various adjuvants such as Toll-like receptor (TLR) ligands, cytokines, and immunostimulators are being tested [2]. Of the adjuvants in the development stage, RNA-based adjuvants acting through TLR3 and TLR7/8 have also been reported to induce augmented Th1 responses [6].

We previously developed several novel single-stranded RNA (ssRNA) expression platforms, which originated from several viral internal ribosome entry sites (IRESs) [7]. Among them, we also reported that the Cricket paralysis virus (CrPV) intergenic region (IGR) IRES-derived ssRNA (termed ssRNA adjuvant) demonstrated adjuvant effects against the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) spike (S) protein [8]. In this study, we tried to confirm the function of ssRNA as an adjuvant rather than the universal vaccine. We investigated whether the ssRNA adjuvant can increase the efficacy of the current seasonal inactivated subunit influenza vaccine (IIV). Our results demonstrate that the ssRNA adjuvant confers seasonal IIV with the ability to induce balanced Th1/Th2 responses as well as innate immunity and mucosal immune responses. Finally, we demonstrated that the ssRNA adjuvant-formulated seasonal IIV enhanced cellular immune responses and antigen-specific neutralizing antibodies with cross-protective immunity.

2. Methods

2.1. Supporting information (SI) for Materials and methods

Refer to the Supplemental data for Materials and Methods for details regarding *in vitro* transcription and RNA purification, RNA stabilizer, cell culture, western blot, bronchoalveolar lavage (BAL), histological analysis, enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI) assay, enzyme-linked immunospot (ELISPOT), flow cytometry, serum and T cell adoptive transfer, real-time polymerase chain reaction for virus titration, assessment of influenza antigenicity and statistical analyses.

2.2. Mice

For immunization with the seasonal IIV with/without ssRNA adjuvant, 5-weeks-old female BALB/c mice were purchased from Samtako Biokorea (Osan-si, Gyeonggi-do), housed at the QuBEST BIO under specific-pathogen-free conditions with 12 h light/dark cycles, and handled according to protocols approved by QuBEST BIO. All BALB/c mice experimental procedures related to immunization conducted in this study followed the guidelines of the Institutional Animal Care and Use Committee of QuBEST BIO (Approval No. QBSIACUC-A18107). For influenza virus challenge experiments, 6-weeks-old female BALB/c mice were purchased from Dae-Han Bio-Link (Seoul, Korea), and all BALB/c mice experimental procedures conducted in this study followed the guidelines of the Institutional Animal Care and Use Committee of the Catholic University of Korea (Approval No. CUK-IACUC-2019-004).

2.3. Vaccines and viruses

The SKYCellflu® (SK bioscience) of the 2018–2019 Northern hemisphere season, which is a cell culture-based quadrivalent influenza vaccine consisting of A/Michigan/45/2015(NYMC X-275) for A/H1N1, A/Singapore/INFIMH-16-0019/2016 (IVR-186) for A/H3N2, B/Phuket/3073/2013 for B/Yamagata, and B/Maryland/15/2016 for B/Victoria, was used as the seasonal IIV and was obtained from SK Bioscience (Seoul, Korea). The A/H1N1 viruses, A/California/04/2009 and A/Puerto Rico/8/34 (PR8), and H5N2 virus, A/Aquatic bird/Korea/w81/05, used in the viral infection challenges were kindly provided by Dr. Seong BL working at Yonsei University (Seoul, Korea).

2.4. In vitro transcription and RNA purification

A DNA platform was designed using IGR IRES and SV40 late polyadenylation signal sequences (Fig. 1) [7,8]. DNA templates were linearized with NotI. The detailed protocols for *in vitro* transcription and RNA purification are provided in SI.

2.5. RNA stabilizer

Protamine (P; Sigma Aldrich, St. Louis, MO, USA) was used, as previously reported [9]. The cationic peptide carrier with lipid chain (termed LP) was designed based on the cationic peptide that was previously reported [10]. The detailed preparation protocol is in SI.

2.6. Immunization

For ssRNA-adjuvanted seasonal IIV studies, BALB/c mice aged 5 weeks were inoculated by intramuscular injections into the upper thigh, two times at an interval of 2 weeks, with the following formulations: inactivated influenza virus vaccine (0.6 μg hemagglutinin [HA] antigen/mouse) with/without RNA stabilizer (19 μg LP or 19.4 μg P)-formulated ssRNA adjuvant (20 μg). For studies on influenza virus challenges, 6-week-old BALB/c mice were intramuscularly challenged in the upper thigh, twice at 2-week intervals, with the following formulations: inactivated influenza virus vaccine (0.6 μg hemagglutinin [HA] antigen/mouse) with/without ssRNA adjuvant (20 μg).

2.7. Virus challenge

The mice were challenged with 1×10^3 plaque forming units (PFU) of A/California/04/2009 virus, 50 PFU of PR8 virus, and 8.6×10^3 tissue culture infectious dose$_{50}$ (ID_{50}) of A/Aquatic bird/Korea/w81/05. The final inoculum volume (50 μL) was administered per animal via the intranasal route using a pipette. The mice were evaluated post-challenge to assess body weight, survival, and clinical illness. The clinical illness was scored using the following scale: 0 = no visible signs of disease; −1 = slight ruffling of fur; −2 = ruffled fur, reduced mobility; −3 = ruffled fur, reduced mobility, rapid breathing; −4 = ruffled fur, minimal mobility, huddled appearance, rapid and/or labored breathing [11]. Animals that lost >25% of their original body weight were sacrificed [11].

3. Results

3.1. ssRNA adjuvant preparation for seasonal IIV

Herein, an ssRNA adjuvant based on CrPV IGR IRES was prepared by screening a previously developed RNA expression platform [7], as described in Fig. 1. Although we desired to confirm the function of ssRNA focusing on the adjuvant, not the universal vaccine, this platform was designed to contain subunit genes coding for type A and B influenza HA2 subunit as a gene of interest (GOI) (containing substantial conserved domains among different influenza strains as compared to HA1, globular head region of

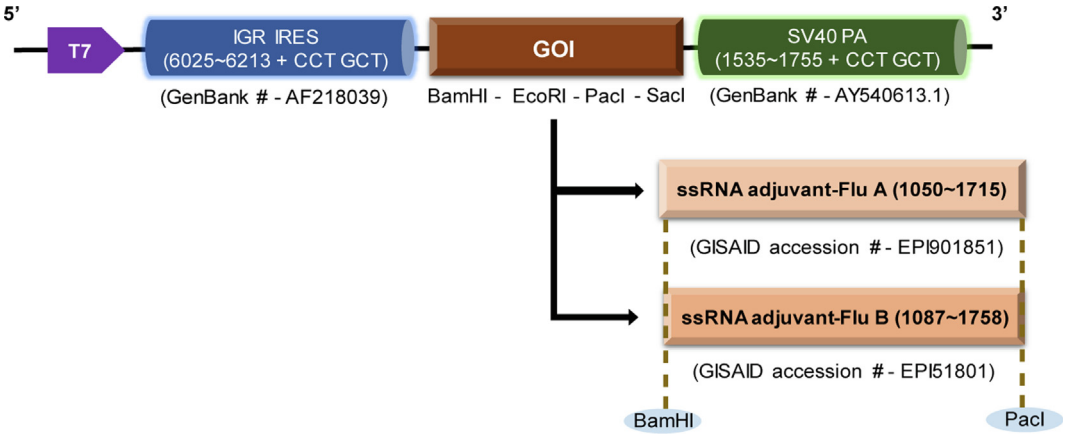

Fig. 1. Schematic diagram of the ssRNA adjuvant containing type A and B influenza HA2 genes. The numbers below the figure indicate the gene sequence number of Genbank or the accession number of GISAID (Global Initiative on Sharing All Influenza Data).

HA). The HA2 subunit largely belongs to influenza A and influenza B, and the HA2 subunit of influenza A is further divided into two groups: group 1 includes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18; group 2 includes H3, H4, H7, H10, H14, and H15 of influenza A [12–14]. Firstly, we searched for a common epitope on the HA2 domain which encompasses the three groups of the HA protein. However, there was no epitope known to contain common regions of HA2 in the three groups. Consequently, we decided to prepare ssRNA adjuvants derived from type A and type B influenza virus (Fig. 1).

Wang et al. revealed that the most conserved epitopes of HA2 from H3N2 (IEDB ID: 30386 and 97636) among influenza vaccine strains studied from 1968 to 2010 were located in a domain from HA2 of the A/Hong Kong/1/1968 strain [15]. Accordingly, we selected the HA2 full sequence of A/Hong Kong/1/1968 as GOI for the preparation of the type A ssRNA adjuvant (Table 1; Fig. 1). We then confirmed that HA2 protein was expressed following transfection into HEK293 cells by western blot (Supplementary Fig. 1).

Type B influenza viruses are split into two lineages, the Yamagata and Victoria lineages, by an antigenic and genetic distinction, and their prototype viruses are B/Yamagata/16/1998 and B/Victoria/2/1987, respectively [16]. We could identify a difference of a single amino acid residue at position 142 (142D for B/Yamagata and 142E for B/Victoria) when comparing the amino acid sequences of HA2 from both viruses. Additionally, we compared the HA2 of various type B influenza viruses to find the highly conserved epitope [17]. Even though there were differences in other positions of HA2 amino acids among the compared viruses, B/Florida/4/2006, one of the viruses of the Yamagata lineage, showed 142E, which was mainly found in the Victoria lineage viruses. Consequently, we chose the HA2 full sequence of B/Victoria/2/1987 as GOI for the preparation of the type B ssRNA adjuvant (Table 1; Fig. 1).

3.2. Seasonal IIV formulated with ssRNA adjuvant stimulates cellular and mucosal immune response as well as humoral immune responses

We investigated the effects of the ssRNA adjuvant with the inactivated seasonal IIV. For this experiment, a commercially available cell-derived quadrivalent IIV (SKYCellflu®) for the 2018–2019 Northern hemisphere influenza season was 100-fold diluted with PBS to evaluate the effect of the ssRNA adjuvant encoding HA2 of influenza type A or B (termed ssRNA adjuvant-Flu A or ssRNA adjuvant-Flu B), which is described in Fig. 1. The ssRNA adjuvant used in this study was prepared by mixing it with the same amount of two types of ssRNA adjuvants, type A and B, and was then injected into BALB/c mice intramuscularly, with or without the respective RNA stabilizers (LP and P), at 0 and 2 weeks (Fig. 2A and B). RNA stabilizers protect RNA from degradation [9,10]. LP is a derivate of CR12C conjugated with a palmitoyl group [10], and P is a basic arginine-rich small nuclear protein [18]. LP and P were previously reported as carriers/stabilizers of mRNA vaccines [10,19]. In this study, we used LP and P as RNA stabilizers to compare their effects with those of ssRNA adjuvant alone (Fig. 2A).

For serological studies, bleeding was conducted at 0, 2, and 4 weeks after the 1st vaccination (Fig. 2B). At 2 weeks after the 1st vaccination, the antigen-specific IgG1 (indicating Th2 responses) and IgG2a (indicating Th1 responses) titers of mice in groups G3–G5 (treated with ssRNA adjuvant) were higher than those observed in mice from G2 (not treated with ssRNA adjuvant) (Fig. 2C). At 2 weeks after the 2nd vaccination, the IgG1 titers in mice from G2 were induced to similar levels as those observed in mice from G3–G5. However, the IgG2a titers in mice from G2 were still low. In contrast, mice in all groups (G3–G5) administered with ssRNA adjuvant-Flu had high levels of both IgG1 and IgG2a titers with a strong boosting effect (Fig. 2C).

In addition, to evaluate the mucosal immune responses, which are key players in the protection against influenza virus infection [20], antigen-specific secretory IgA titers in BALF were measured by ELISA. IgA titers in mice from G3–G5 appeared to be increased, whereas those in mice from G2, which was immunized with the seasonal IIV alone, did not (Fig. 2D). This indicated that the ssRNA adjuvant can trigger mucosal immune responses even through intramuscular administration of the seasonal IIV.

Furthermore, HI titers, a gold standard for protection against influenza virus infection, were determined. At 2 weeks after the 1st vaccination, all mice in G2 were seronegative for the four strains (HI GMT < 1:10). However, HI titers against A/H1N1 and A/H3N2 were measured within a range of 1:10–1:80 in mice from G3–G5. In the case of the B type, the HI titers for the B/Yamagata lineage were measured at a low level (approximately 1:10), and the HI titers for the B/Victoria lineage were measured at 1:5 even in the groups administered with ssRNA adjuvant at 2 weeks after the 1st immunization (Fig. 2E). However, in accordance with previous studies, in which the sensitivity of the HI assay for influenza type B was found to be relatively low as compared to influenza type A [21,22], these results were expected. At 2 weeks after the 2nd immunization, although the HI titers of all groups were increased with the boosting effect, those of mice in G3–G5 were 9–14-fold higher for A/H1N1, 3–11-fold higher for A/H3N2, 3-

Table 1
Influenza strains referenced for ssRNA adjuvants.

Influenza Type		Strain	Sequence	GISAID isolate ID/Accession No.
Type A		**A/Hong Kong/1/1968 (H3N2)**[a]	HA2 full sequence	EPI_ISL_246097/EPI901851
Type B	Victoria lineage	B/Lee/1940	HA2 full sequence	EPI_ISL_70140/EPI243269
		B/Victoria/02/1987[b]	HA2 full sequence	EPI_ISL_6612/EPI51801
		B/Malaysia/2506/2004	HA2 full sequence	EPI_ISL_29398/EPI175755
		B/Ohio/01/2005	HA2 full sequence	EPI_ISL_99804/EPI346478
		B/Mississippi/07/2008	HA2 full sequence	EPI_ISL_33119/EPI187575
		B/Brisbane/33/2008	HA2 full sequence	EPI_ISL_24366/EPI163726
		B/Brisbane/60/2008	HA2 full sequence	EPI_ISL_80394/EPI283743
		B/Brisbane/63/2014	HA2 full sequence	EPI_ISL_171789/EPI561876
	Yamagata lineage	B/Yamagata/16/1988	HA2 full sequence	EPI_ISL_20958/EPI51820
		B/Nashville/45/1991	HA2 full sequence	EPI_ISL_3858/EPI20716
		B/Harbin/7/1994	HA2 full sequence	EPI_ISL_30252/EPI179997
		B/Houston/B60/1997	HA2 full sequence	EPI_ISL_6806/EPI57075
		B/Jilin/20/2003	HA2 full sequence	EPI_ISL_22803/EPI159930
		B/Florida/04/2006	HA2 full sequence	EPI_ISL_142731/EPI457185
		B/Mississippi/04/2008	HA2 full sequence	EPI_ISL_28256/EPI172539
		B/Wisconsin/01/2010	HA2 full sequence	EPI_ISL_112325/EPI363743
		B/Massachusetts/02/2012	HA2 full sequence	EPI_ISL_246884/EPI904337
		B/Phuket/3073/2013	HA2 full sequence	EPI_ISL_166958/EPI544267
		B/Brisbane/9/2014	HA2 full sequence	EPI_ISL_165595/EPI539769

[a, b] Selected strain for manufacturing ssRNA adjuvant-Flu A and ssRNA adjuvant-Flu B.

fold higher for B/Yamagata, and 5–9-fold higher for B/Victoria than those in mice from G2 (Fig. 2E).

3.3. Seasonal IIV formulated with ssRNA adjuvant induces a balanced Th1/Th2 immune response

In addition to antibody-mediated responses, the number of influenza virus-specific cytokine (IFN-γ and IL-4, which are Th1 and Th2 cytokines, respectively)-releasing cells was also measured by ELISPOT assay on splenocytes harvested from immunized mice at 2 weeks after the 2nd vaccination (Fig. 3A). As expected, similar to the results shown in Fig. 2C, the numbers of IFN-γ-secreting immune cells in splenocytes obtained from mice in G3–G5 were approximately 2–4 fold increased compared to mice in G2 that were not stimulated by the influenza vaccine antigen and about 3-fold increased compared to mice from G2 that were stimulated (Fig. 3B). The number of IL-4-secreting immune cells was similar across all immunized groups (G2–G5) with and without stimulation with the influenza vaccine antigen (Fig. 3C), indicating that, although the seasonal IIV usually induces Th2 responses in a biased manner [23], the ssRNA adjuvant can overcome this by inducing balanced Th1/Th2 responses.

3.4. Seasonal IIV formulated with ssRNA adjuvant elicits protective immunity against infection of influenza virus with similar antigenicity

To investigate the protective immunity induced by the seasonal IIV formulated with ssRNA adjuvant against influenza virus possessing similar antigenicity, we challenged the immunized mice with the A/California/04/2009 virus because the A/Michigan/45/2015 H1N1 component of the seasonal IIV used in this study belongs to the A(H1N1)pdm09-like virus as A/California/04/2009 does (Supplementary Fig. 2). Based on the results described above, we decided to use only the ssRNA adjuvant without any RNA stabilizers for the immunization of mice as RNA stabilizers were not significantly effective in inducing immune responses when conjugated with the ssRNA-adjuvanted seasonal IIV. The seasonal IIV was 100-fold diluted with PBS and then formulated with or without ssRNA adjuvants (Fig. 4A). The antigens prepared as described above were injected into BALB/c mice intramuscularly without RNA stabilizers at 0 and 2 weeks (Fig. 4B). For serological studies, bleeding was conducted at 0, 2, and 5 weeks after the 1st immunization and 1 week after challenge. Body weight, survival rate, and clinical illness were measured daily for 26 days after the challenge (Fig. 4B). We confirmed that the balanced Th1/Th2 responses were more significantly induced in mice from G3 compared to those in G2 before the challenge infection. This was confirmed by analyzing antigen-specific IgG1 and IgG2a titers as well as HI titers against H1N1 (Supplementary Fig. 3).

Immunized mice were challenged with the A/California/04/2009 virus at 3 weeks after the 2nd immunization. Mice from G2 and G3 showed a slight body weight decrease, about 10%, till 5–6 days after challenge (Fig. 4C) and 100% survival (Fig. 4D). Contrarily, mice from G1 showed a significant body weight decrease of more than 25% compared to those from G2 and G3 (Fig. 4C). These mice were therefore sacrificed. Similar to the results of the body weight, the clinical score of mice from G2 and G3 was found to be −1 at 4 and 5 days after the challenge. These mice showed only mild symptoms with slight ruffling fur, whereas those in G1 scored −4 showed rapid and/or labored breathing (Fig. 4E). Moreover, at 1 week after challenging with A/California/04/2009, the influenza virus titers (copy number) in lung tissue and BALF were significantly decreased in mice from G2 and G3 compared to those from G1 (Fig. 4F). Especially, the virus titers of mice from G3 were found to be lower than those of mice from G2, suggesting that the ssRNA adjuvant causes enhanced viral clearance. Histopathological analysis was performed 1 week after a challenge with A/California/04/2009. The results of hematoxylin and eosin (H&E) staining were consistent with the viral titers in the lung and BALF tissues. Lung congestion and severe vascular cuffs (including bronchitis, perivascular inflammation, and interstitial inflammation) were observed in G1 (Fig. 4G upper). Moreover, cytoplasmic vacuolation in some pneumocytes, shrunken alveoli, and thickening of the alveolar walls and necrotic and inflammatory cells were predominantly observed in G1 (Fig. 4H upper). In contrast, in G2 and G3, the reduction in peribronchiolar and perivascular inflammation, thickness of alveolar wall, and frequency of inflammatory cells were investigated. In particular, in G3, few inflammatory cells and nearly normal alveolar architecture were observed (Fig. 4G and H, middle and lower). These results indicate that interstitial pneumonia is milder in G3 mice than in G2 mice, consistent with viral titers.

Based on the results described above, we could conclude that the seasonal IIV formulated with the ssRNA adjuvant induces more

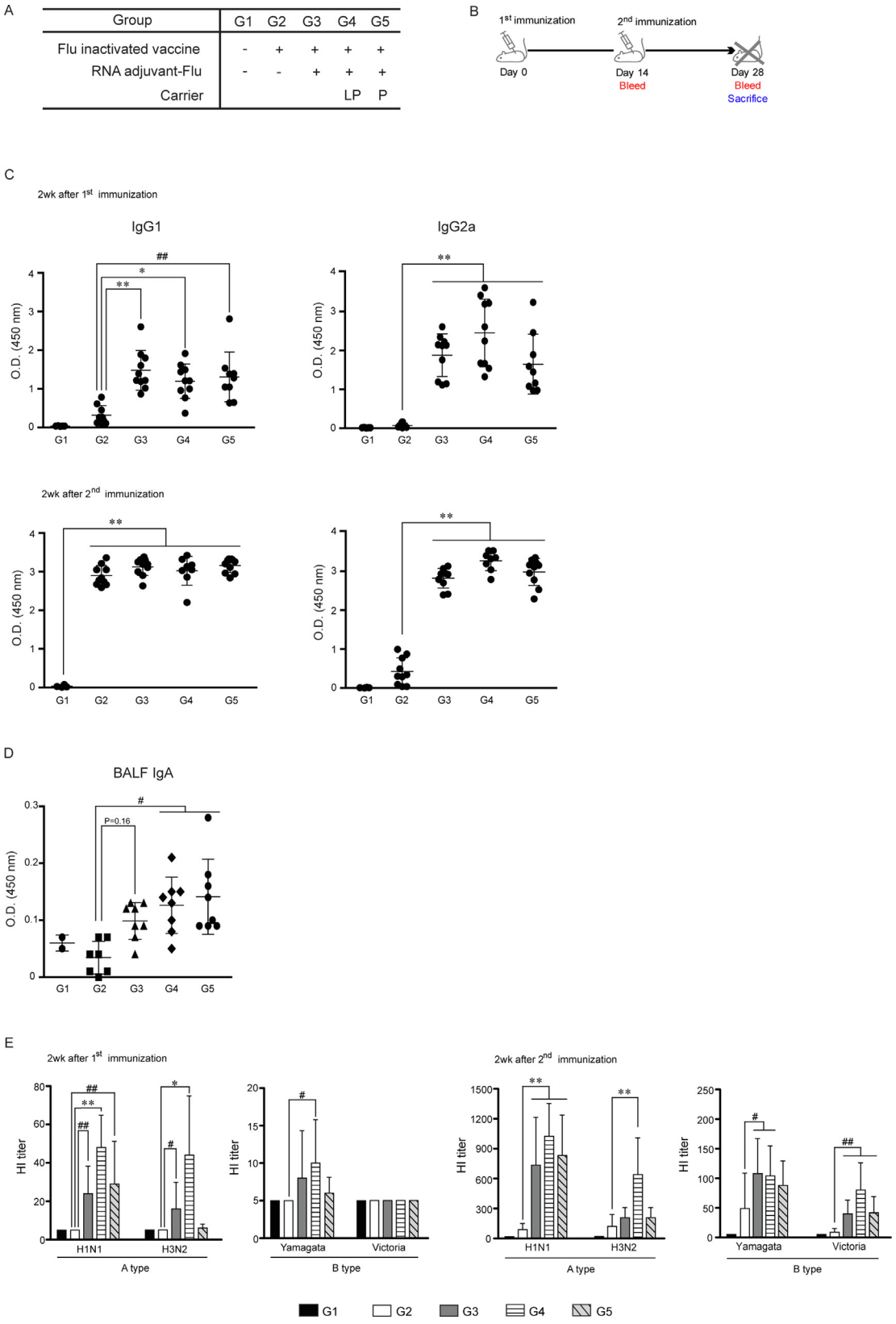

Fig. 2. ssRNA-adjuvanted seasonal IIV promotes mucosal immune responses as well as Th1 and Th2 immune responses. BALB/c mice were intramuscularly immunized at an interval of 2 weeks with two doses of the seasonal IIV (0.6 μg HA antigen/mouse) with or without ssRNA adjuvants. ssRNA adjuvants are LP (19 μg) and P (10 μg) formulated with RNA (20 μg) encoding HA2 of A/Hong Kong/1/1968 (H3N2) and B/Victoria/02/1987. The data were statistically analyzed by ANOVA. The significance of differences between groups are indicated by bars and symbols as follows: #, $p < 0.05$; *, $p < 0.01$; ##, $p < 0.005$; **, $p < 0.001$. **(A)** Overall study design. **(B)** Mouse experiment schedule for immunization and bleeding. **(C)** Influenza-specific IgG1 and IgG2a responses were measured by ELISA at 2 weeks after the 1st and 2nd immunization. Data represent mean ± SD. $n = 10$ mice for G2 to G5, and $n = 5$ mice for G1. **(D)** Mucosal IgA titer in bronchoalveolar lavage fluid (BALF) of immunized mice was measured by ELISA. Data represent mean ± SD. $n = 8$ mice for G2 to G5, and $n = 2$ mice for G1. **(E)** HA inhibition titers were determined by HI assay using 0.5% chicken RBC at 2 weeks after the 1st and 2nd immunization. Error bars indicate the SD of duplicate samples acquired in 4 or 10 independent experiments ($n = 4$ mice for G1, and $n = 10$ mice for G2 to G5).

A

Group	G1	G2	G3	G4	G5
Flu inactivated vaccine	-	+	+	+	+
RNA adjuvant-Flu	-	-	+	+	+
Carrier				LP	P

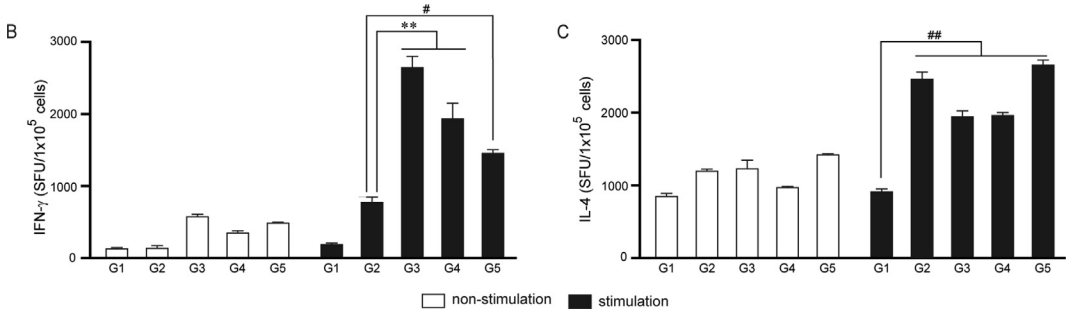

Fig. 3. ssRNA-adjuvanted seasonal IIV stimulates cellular immune responses. Splenocytes were harvested at 2 weeks after the 2nd immunization (refer to Fig. 2B) and stimulated for 2 days with or without the vaccine protein. Data were statistically analyzed by ANOVA. The significance of differences between groups are indicated by bars and symbols as follows: #, $p < 0.05$; ##, $p < 0.005$; **, $p < 0.001$. **(A)** Overall study design. The frequency of influenza-specific IFN-γ-producing cells **(B)** and influenza-specific IL-4-producing cells **(C)** was measured by ELISPOT assay. Data represent mean ± SD. $n = 4$ mice for G1, and $n = 10$ mice for G2 to G5 (pooling).

effective protection against infection of influenza virus possessing similar antigenicity compared to the seasonal IIV without the ssRNA adjuvant.

3.5. Seasonal IIV formulated with ssRNA adjuvant not only enhances Th1 immune responses but also induces memory T cell responses

To investigate the protection mechanisms induced by the seasonal IIV formulated with the ssRNA adjuvant (Fig. 5A), serological analyses were performed at 1 week after challenging with A/California/04/2009. The IgG1 and IgG2a titers of mice from G3 were higher than those of mice from G2. Especially, IgG2a showed a greater increase (about 2-fold) in mice from G3 compared to those in G2 (Supplementary Fig. 4). In addition, the antigen-specific secretory IgA titer in BALF was measured by ELISA to evaluate the mucosal immune responses. IgA titers in mice from G3 showed a significant increase compared to mice from G2 (Supplementary Fig. 5).

Furthermore, to gain insight into the effect of the ssRNA adjuvant on Th1 immune response induction, the number of IFN-γ and IL-2-secreting cells was measured by ELISPOT assay at 1 week after challenge using splenocytes. Compared to mice from G1, the number of cells secreting IFN-γ and IL-2 in the splenocytes of mice from G2 and G3 increased more than about 22-fold and 4-fold, respectively, under stimulation with the influenza HA-specific T cell epitope peptide (detailed in Materials and Methods). In particular, when comparing mice from G2 and G3, the number of cells secreting IFN-γ and IL-2 in mice from G3 was 1.2-fold higher than those in mice from G2 (Fig. 5B and C). The expression levels of IFN-γ, IL-2, and TNF-α (known as Th1 cytokines) [24] was also measured by ELISA using the supernatant of unstimulated splenocytes or those stimulated with influenza HA-specific T cell epitope peptide. At 1 week after challenge, IFN-γ and IL-2 titers of mice from G3 were 167-fold and 2.5-fold higher, respectively, than those of mice from G2 (without stimulation with the influenza HA-specific T cell epitope peptide). Under stimulatory conditions, they showed a 1.5-fold and 1.7-fold increase, respectively (Fig. 5D and E). The TNF-α titer of mice from G3 also showed an increase compared to those in G2 (10.5-fold without stimulation and 1.2-fold under stimulation) (Fig. 5F).

Vaccines that trigger long-term immunogenicity are considered as a very protective means against infectious pathogens. In order to determine whether the ssRNA adjuvant has the ability to induce long-term immunity, we measured the number of effector memory $CD4^+$ and $CD8^+$ T cells from the lungs and spleen at 1 week after challenging with influenza infection. $CD62L^-CD44^{+hi}$ cells were considered effector memory T cell markers in mice [25, 26]. $CD4^+$ and $CD8^+$ T cells with a $CD62L^-CD44^{+hi}$ phenotype after stimulation with the influenza vaccine and the influenza HA-specific T cell epitope peptide, respectively, were assessed via FACS. The number of $CD4^+$ and $CD8^+$ T cells from lungs with a $CD62L^-CD44^{+hi}$ phenotype increased 1.6-fold and 1.4-fold, respectively, in mice from G3 compared to those from G2 (Fig. 5G). Similar to the results obtained for the lungs, the number of $CD4^+$ and $CD8^+$ T cells from the spleen with a $CD62L^-CD44^{+hi}$ phenotype increased 1.9-fold and 1.6-fold, respectively, in mice from G3 compared to those from G2 (Fig. 5G). These results suggest that the ssRNA-adjuvanted seasonal IIV is able to induce long-term immunity.

Taken together, the data suggest that the seasonal IIV formulated with the ssRNA adjuvant confers effective protection against influenza virus infection due to the induction of Th1 immune responses as well as memory T cell responses.

3.6. Seasonal IIV formulated with ssRNA adjuvant provides protective immunity against heterologous influenza virus infection

To investigate the protective immunity triggered by the seasonal IIV formulated with the ssRNA adjuvant against heterologous influenza virus of the same subtype, we challenged the immunized mice with the PR8 virus (Supplementary Fig. 2). As in the A/California/04/2009 challenge studies, antigens were prepared (Fig. 6A), and BALB/c mice were immunized with each antigen at 0 and 2 weeks (Fig. 6B). For serological studies, bleeding was conducted at 0, 2, and 4 weeks after the 1st immunization. One week after the challenge, body weight, survival rate, and clinical illness were measured daily for 27 days after challenge (Fig. 6B). Before the PR8 challenge, we measured the HI titer against A/Michigan/45/2015, which is the vaccine strain, and PR8. HI titers against A/Michigan/45/2015 of mice from G3 were higher than those of mice from G2 (Fig. 6C). This was similar to the results obtained

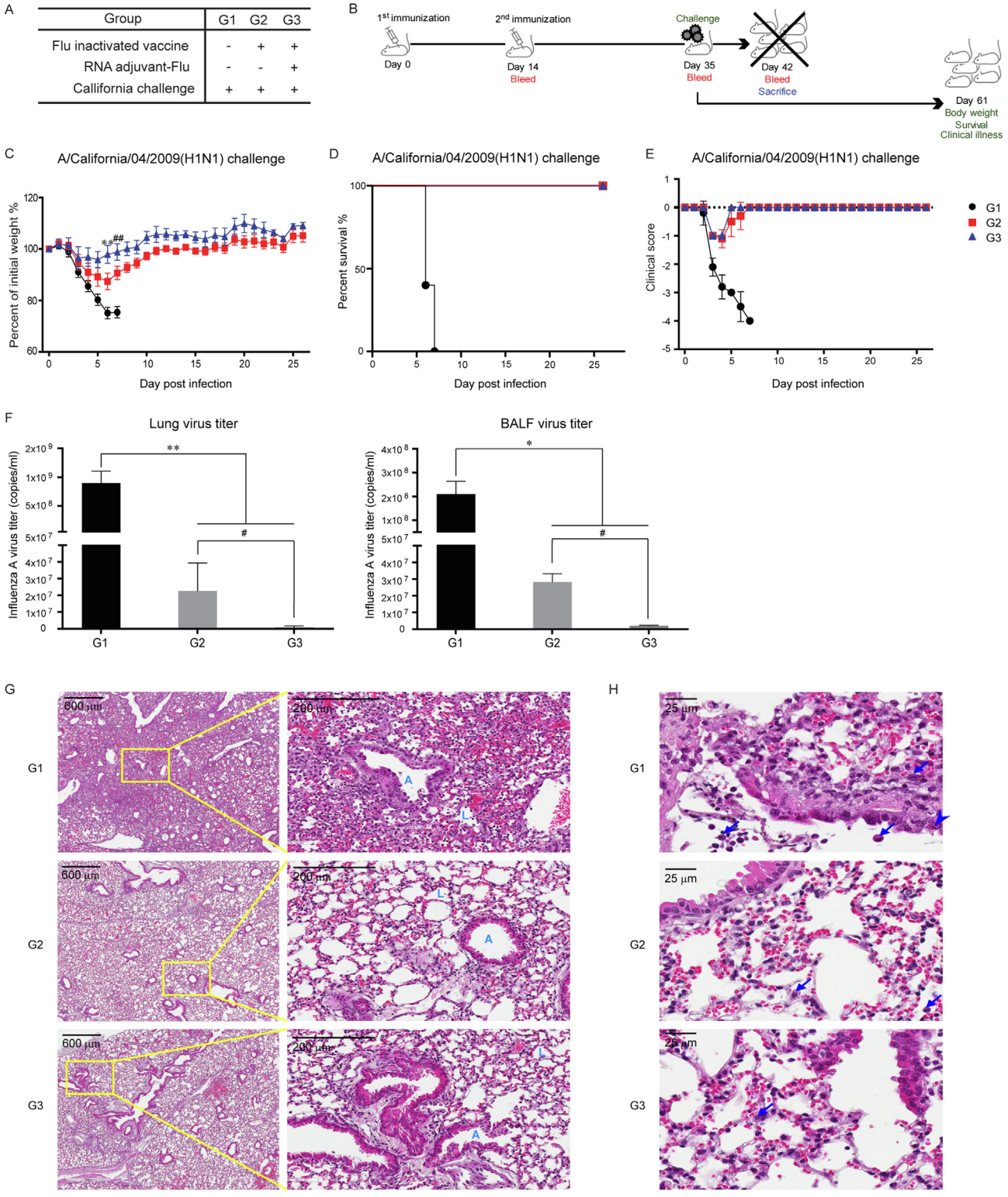

Fig. 4. ssRNA-adjuvanted seasonal IIV induces protection and enhanced viral clearance against influenza virus possessing similar antigenicity. BALB/c mice were intramuscularly immunized at an interval of 2 weeks with two doses of seasonal IIV (0.6 μg HA antigen/mouse) with or without ssRNA adjuvant (without RNA stabilizer) followed by challenging with A/California/04/2009 virus at 3 weeks after the 2nd immunization. Data were statistically analyzed by ANOVA. The significance of differences between groups are indicated by bars and symbols as follows: #, $p < 0.05$; *, $p < 0.01$; **, $p < 0.001$. **(A)** Overall study design. **(B)** Mouse experiment schedule for immunization and challenge. **(C)** Percentage body weight studied for 26 days after challenge with A/California/04/2009. **(D)** Survival rate of BALB/c mice studied for 26 days after challenge. **(E)** Clinical score studied for 26 days after challenge. **(F)** The viral titer of A/California/04/2009 in lungs and bronchoalveolar lavage fluid (BALF) was measured by real-time PCR at 1 week after challenge with A/California/04/2009. Data represent mean ± SD. $n = 5$ mice for lungs (individuals), and $n = 5$ mice for BALF (pooling). **(G and H)** Intact lungs were harvested at 1 week after the viral challenge, fixed, processed, and stained with hematoxylin and eosin (H&E) for lung histopathological evaluation. A; Airway lumen, L; Alveolar lumen, Arrow; Inflammatory cells (eosinophils and macrophage), Arrowhead; Necrotic cells.

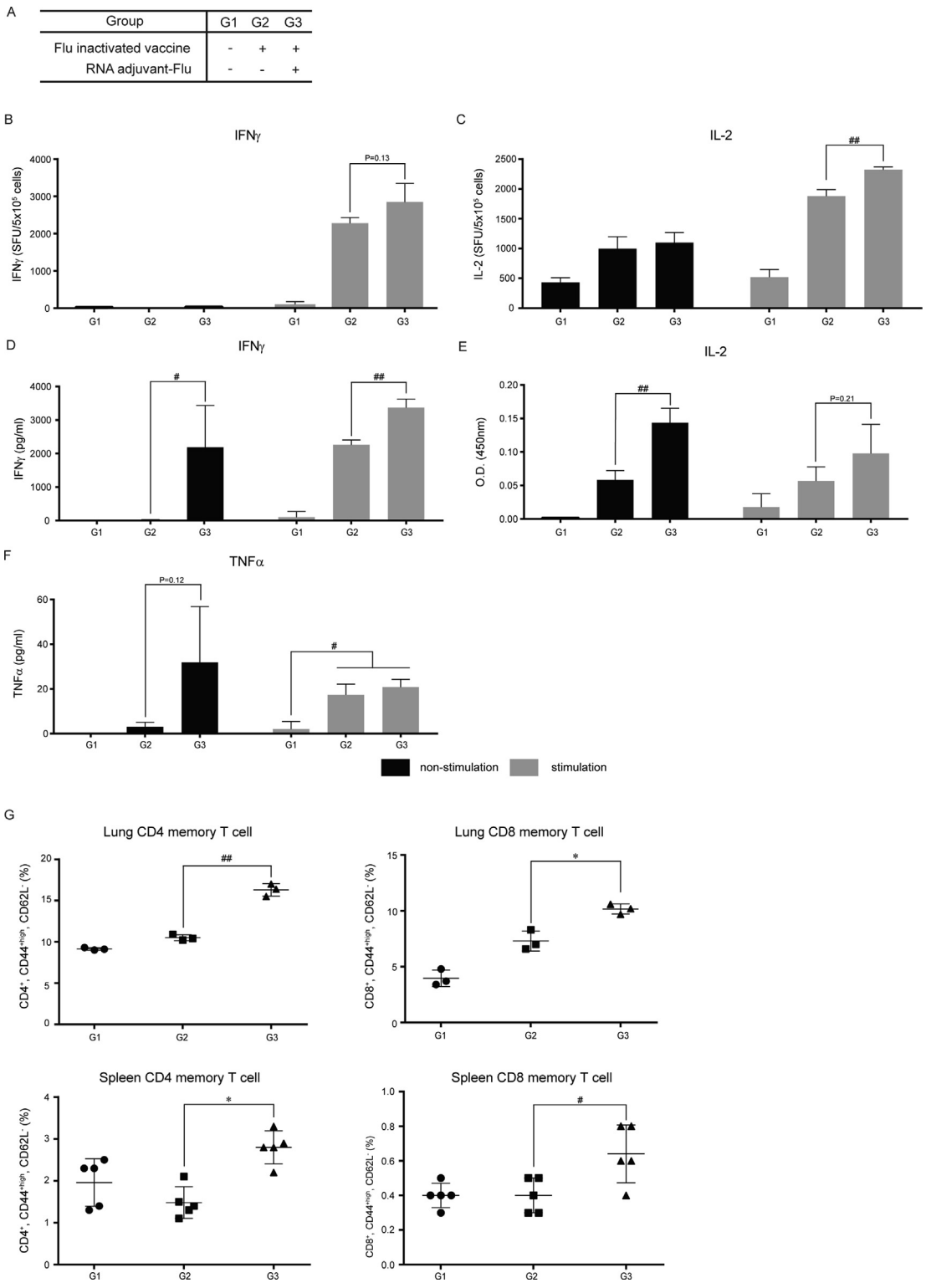

Fig. 5. ssRNA-adjuvanted seasonal IIV promotes Th1 immune responses and memory T cell responses even against influenza virus possessing similar antigenicity. Splenocytes were harvested at 1 week after challenging with A/California/04/2009 virus (refer to Fig. 4B) and stimulated for 2 days with or without influenza HA-specific T cell epitope peptide (detailed in Materials and Methods). The data were statistically analyzed by ANOVA. The significance of differences between groups are indicated by bars and symbols as follows: #, $p < 0.05$; *, $p < 0.01$; ##, $p < 0.005$. **(A)** Overall study design. The frequency of influenza-specific IFN-γ-producing cells **(B)** and influenza-specific IL-2-producing cells **(C)** was measured by ELISPOT assay. Data represent mean ± SD. $n = 5$ mice (pooling). In addition, the concentration of IFN-γ **(D)**, IL-2 **(E),** and TNF-α **(F)** was also measured by ELISA in the supernatant of splenocytes after stimulation with or without influenza HA-specific T cell epitope peptide for 2 days. Data represent mean ± SD. $n = 5$ mice (pooling). **(G)** Memory $CD4^+$ and $CD8^+$ T cells from the lungs (pooling) and spleen (individual) were measured by FACS. Data represent mean ± SD. $n = 5$ mice.

in the A/California/04/2009 challenge study. However, HI titers against PR8 were not detected in all groups (Fig. 6C). Moreover, to confirm the antigen-specific immune response in each group, we determined the titers of antigen-specific IgG1 and IgG2a at 2 and 4 weeks after initial immunization. Consequently, mice from G3 displayed balanced Th1/Th2 responses, similar to the results obtained with the challenge with A/California/04/2009 (Supplementary Fig. 5).

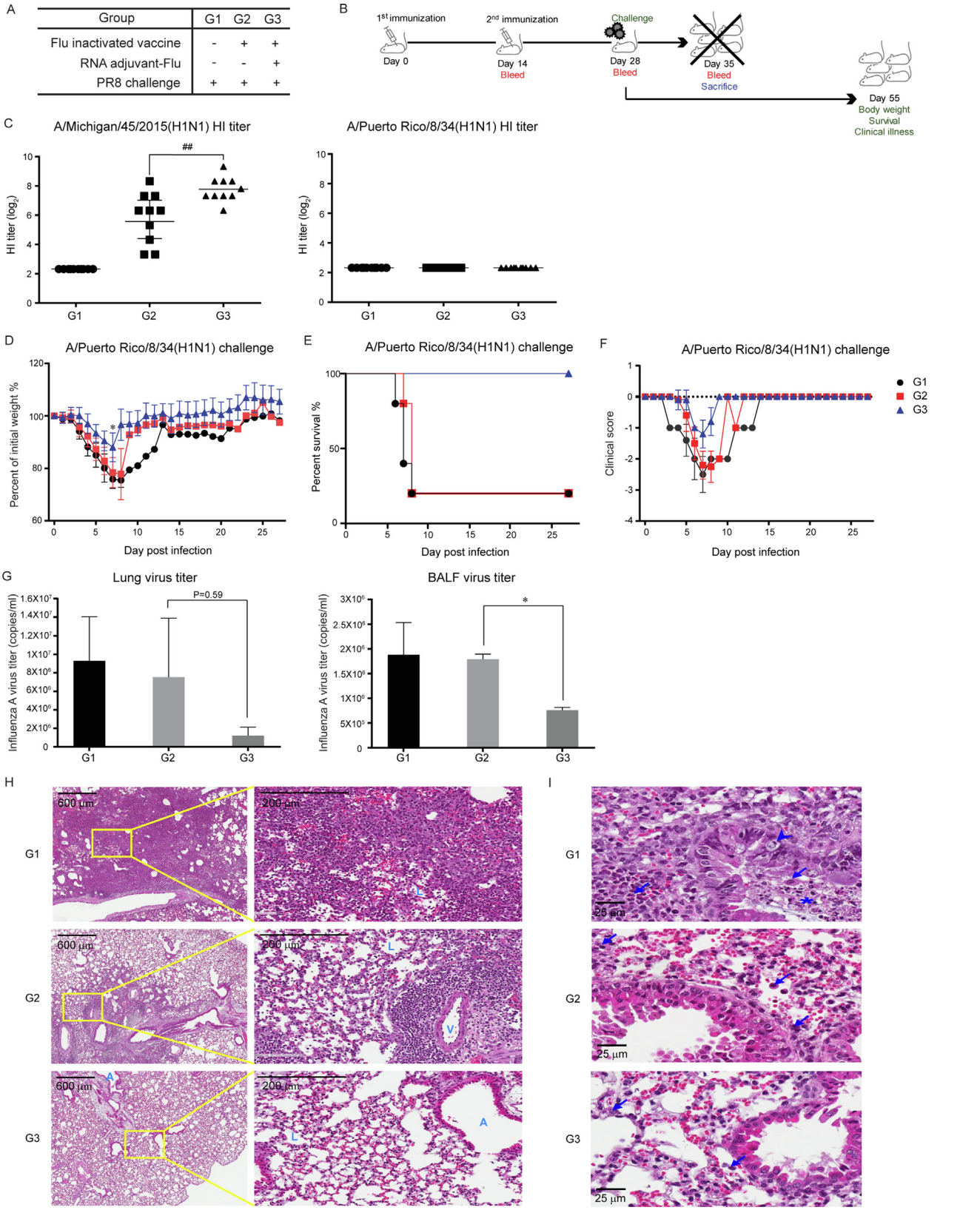

Fig. 6. ssRNA adjuvant triggers cross-protective immune responses against heterologous influenza virus of the same subtype. BALB/c mice were intramuscularly immunized at 2-week intervals with two doses of seasonal IIV (0.6 μg HA antigen/mouse) with or without ssRNA adjuvant (without RNA stabilizer) and then challenged with A/Puerto Rico/8/34 (PR8) virus at 2 weeks after the 2nd immunization. Data were statistically analyzed by ANOVA. The significance of differences between groups are indicated by bars and symbols as follows: *, $p < 0.01$; ##, $p < 0.005$. **(A)** Overall study design. **(B)** Mouse experiment schedule for immunization and challenge. **(C)** HA inhibition titers were determined by HI assay using 0.5% chicken RBC at 2 weeks after the 2nd immunization. Data represent geomean of duplicated results from one sample ($n = 10$ mice for G1 to G3). **(D)** Percentage body weight during 27 days after challenging with PR8. **(E)** Survival rate of BALB/c mice during 27 days after challenging. **(F)** Clinical score during 27 days after challenging. **(G)** The viral titer of PR8 in the lungs and bronchoalveolar lavage fluid (BALF) was measured by real-time PCR at 1 week after challenge with PR8. Data represent mean ± SD. $n = 5$ mice (individual) for lungs, and $n = 5$ mice (pooling) for BALF. **(H and I)** Intact lungs were harvested at 1 week after challenge, fixed, processed, and stained with hematoxylin and eosin (H&E) for lung histopathological evaluation. A; Airway lumen, L; Alveolar lumen, V; Blood vessel, Arrow; Inflammatory cells (eosinophils and macrophage), Arrowhead; Necrotic cells, Asterisk; Exudate.

After PR8 challenge, unlike the results from the A/California/04/2009 challenge study, we observed that mice in G2 showed severe body weight decrease (more than 25%) until 8 days after challenge, similar to those in G1 (Fig. 6D), and the survival rate was the same for mice in G1 and G2 (20%) (Fig. 6E). The clinical illness of mice from G1 and G2 was scored between −2 and −3 at 6–10 days after challenge (Fig. 6F). On the other hand, mice in G3 that were immunized with the ssRNA adjuvant showed a body weight decrease, about 16%, till 7 days after challenge and then recovered gradually (Fig. 6D), showing 100% survival unlike those in G1 and G2 (Fig. 6E). Mice in G3 also showed mild clinical symptoms compared to those in G1 and G2, with a clinical score of about −1 at 6–8 days after challenge (Fig. 6F). Moreover, the influenza virus titers in the lung tissue and BALF were about 6–8-fold and 2–3-fold significantly decreased, respectively, in mice from G3 compared to those from G1 and G2 at 1 week after challenging with PR8 (Fig. 6G). In addition, IgG1 and IgG2a titers of sera and the antigen-specific secretory IgA titer in BALF were measured by ELISA at 1 week after challenge. IgG1, IgG2a, and IgA titers in mice from G2 and G3 were higher than those in mice from G1. The IgG1 titer was found to be similar between mice from G2 and G3, and both IgG2a and IgA titers in mice from G3 were 1.7-fold higher than those in mice from G2 (Supplementary Fig. 5A–D). Moreover, the numbers of IFN-γ secreting immune cells in the splenocytes obtained from mice in G2 and G3 were increased by approximately 4-fold and 9-fold, respectively, as compared to those obtained from mice in G1 upon stimulation with influenza HA-specific T cell epitope peptide (Supplementary Fig. 5E). G1 in the PR8 challenge (Fig. 6H upper) is similar to G1 in the A/California/04/2009 challenge (Fig. 4G and H upper), indicating damage to lung tissue after both viral challenges. Interestingly, no significant difference between G1 (Fig. 6H upper) and G2 (Fig. 6H middle) were observed; however, levels of inflammatory cells and exudate decreased in G2 compared to G1 in the PR8 challenge (Fig. 6I upper and middle). However, although G3 did not completely protect against the PR8 challenge, G3 displayed not only a reduction in the levels of inflammatory cells and exudate, but also fewer pathological changes such as thickening of the alveolar wall and mucosal epithelium in bronchioles in comparison with G1 and G2 (Fig. 6H and I lower). All the above results indicate that the ssRNA adjuvant confers cross-protective immunity against heterologous influenza virus infection.

4. Discussion

Various forms of the seasonal IIVs are being produced and marketed by numerous manufacturers globally. On the assumption that IIVs elicit a balanced Th1 and Th2 response, it might be a powerful effective vaccine with significant memory response induction. To adjust a biased immune response toward the Th2 type and to further strengthen the immunomodulatory activity of IIVs, numerous adjuvants are being studied, especially RNA-based adjuvants, and are in the spotlight because of their advantages related to safety resulting from rapid degradation and non-chromosomal integration, ease and flexibility of production, Th1 response induction caused by TLR stimulation, among others [3,27].

Herein, we investigated the immune responses induced by the seasonal IIV formulated with the ssRNA adjuvant in BALB/c mice. The ssRNA adjuvant increases both IgG2a (Th1 response) and IgG1 (Th2 response) titers (Fig. 2C; Supplementary Fig. 3B and 4B). Consistent with this, the numbers of IFN-γ-secreting splenocytes in the ssRNA adjuvant-treated group were found to be higher than those in the non-adjuvant-treated group (Fig. 3B). Furthermore, surprisingly, we could confirm that the ssRNA adjuvant induces mucosal immunity after immunization with the IIV (Fig. 2D) and more effective mucosal immune responses after virus infection by measuring the IgA titer in BALF (Supplementary Fig. 5D). Although it is known that mucosal immune responses involving IgA secretion play an important role in influenza virus infections [28], IIVs generally induce poor mucosal immunity [29]. From this point of view, it is promising that the ssRNA adjuvant induces mucosal immunity. Furthermore, the RNA-adjuvanted seasonal IIV increased the HI titer, which is a surrogate marker of immune response for influenza vaccine (Fig. 2E and 6C; Supplementary Fig. 3C). Since RNAs are extremely unstable under natural conditions, we also investigated the formulation of the ssRNA adjuvant with two kinds of cationic peptides: P (a small, arginine-rich nuclear protein) and LP (a cationic peptide-linked lipid). Generally, these cationic peptides confer stability to RNAs (negatively charged) by binding to the RNA molecule [30]. However, in this study, the effect of the cationic peptides could not be confirmed with the seasonal IIV. Further studies are needed to clarify this phenomenon.

To determine the cross-protective immunity induced by the ssRNA-adjuvanted seasonal IIV, we performed experiments in which immunized mice were challenged with influenza virus. We selected two H1N1 viruses, PR8 (heterologous virus) and A/California/04/2009 (homologous virus), as the infective virus due to differences in their antigenicity (Supplementary Fig. 2) [31,32]. The ssRNA-adjuvanted seasonal IIV elicited increased IgG1, IgG2a, and secretory IgA titers in the serum and lung mucosal surface, respectively, after challenge infection compared to the non-adjuvanted seasonal IIV. This suggests that the ssRNA-adjuvanted seasonal IIV can lead to a balanced Th1/Th2 immune response including mucosal immune response (Supplementary Fig. 5B–E). Although histopathological analysis did not reveal a complete protective effect, other findings including body weight, survival rate, and clinical score indicated that ssRNA-adjuvanted seasonal IIV exerted sufficient protection against two types of challenged viruses (PR8 virus and A/California/04/2009), indicating that the ssRNA adjuvant provides cross-protective immunity (Figs. 4 and 6). Furthermore, we performed additional challenge testing with a low pathogenic avian influenza H5N2 subtype, A/Aquatic bird/Korea/w81/05, which is also a heterologous strain. Results show that the ssRNA adjuvant significantly decreased the viral titer in lungs of H5N2 challenged animals (Supplementary Fig. 5F), indicating that it may confer cross-protective immunity against heterologous influenza viruses belong to different subtypes. These results have significant implications for the development of seasonal influenza vaccines. Sometimes, seasonal influenza vaccines show low effectiveness due to a virus mismatch [33]. The ssRNA-adjuvanted seasonal IIV holds promise for addressing this problem.

Herein, the ssRNA adjuvant was further evaluated for its ability to trigger Th1 immune responses. This is because IIVs do not sufficiently induce Th1 immune responses. After challenging with A (H1N1)pdm09 virus, splenocytes from mice immunized with the ssRNA-adjuvanted seasonal IIV secreted the Th1-related cytokines, IFN-γ, IL-2, and TNF-α at a higher level than those from mice vaccinated with the seasonal IIV without the ssRNA adjuvant (Fig. 5). Several studies demonstrated that Th1-related cytokines limit the influenza virus replication and promote viral clearance by activating $CD4^+$ and $CD8^+$ T cell responses [34–36]. Moreover, the ssRNA (CrPV IRES-derived RNA) used as an adjuvant system in this study has previously been shown to induce the expression of genes related to type 1 and 2 IFNs and T cell activation, which are important factors for limiting virus replication [8]. Moreover, in agreement with results from previous reports, here we show that the influenza virus titer in the lung tissue and BALF of the ssRNA-adjuvanted seasonal IIV-treated group was lower than that of the seasonal IIV-treated group (Fig. 4F and 6G). Similar results were confirmed via adoptive transfer, in which serum and T cells col-

lected from mice immunized with the ssRNA-adjuvanted seasonal vaccine were passively transferred to nude mice prior to PR8 virus challenge, after which the lung viral titer was quantified. The groups receiving transferred serum and T cells exhibited lower levels of virus in the lung compared to the saline immunized group (Supplementary Fig. 6). Since HI titers against PR8 were not detected (Fig. 6C), these low viral titers in the lung following serum transfer, can be interpreted as a protective effect elicited by type 1 and 2 IFNs in the transferred serum (Supplementary Fig. 6).

Generally, vaccines that elicit long-term immunity are considered optimal. Here, we measured the number of effector memory T cells in the lungs (target organ of influenza virus) and spleen to investigate the longevity of the ssRNA-adjuvanted seasonal IIV-induced protection. As expected, effector memory $CD4^+$ and $CD8^+$ T cells in the lungs and spleen of mice in the ssRNA-adjuvanted seasonal IIV-treated group increased to a higher extent compared to those of mice in the seasonal IIV-treated group, indicating that the ssRNA-adjuvanted seasonal IIV elicits immune memory (Fig. 5G). As IL-2 is important in $CD8^+$ T cell functioning (activation, expansion, and differentiation into terminal effector cells and memory cells) [37], our results showing an increased IL-2 level in ssRNA-adjuvanted seasonal IIV-treated mice (Fig. 5) also support the hypothesis that the ssRNA-adjuvanted seasonal IIV treatment induces immune memory.

The live attenuated influenza vaccine (LAIV) provides certain advantages including humoral and cellular immune responses as well as mucosal immunity that IIVs do not [38,39]. However, LAIVs are not recommended for immunosuppressed individuals and young children due to of safety concerns [3]. In this study, we confirmed that the seasonal IIV formulated with the ssRNA adjuvant-Flu overcame the drawbacks of LAIVs while possessing all the advantages associated with LAIVs.

In conclusion, the CrPV IGR IRES-derived ssRNA adjuvant offers numerous advantages as an immune stimulator and has the potential to overcome the drawbacks associated with inactivated vaccines, such as weak T cell activation and poor mucosal immunity. Furthermore, previous studies have demonstrated that *in vivo,* its short half-life improves its safety [40,41]. We also confirmed that the seasonal IIV formulated with CrPV IGR IRES-derived ssRNA can induce balanced Th1 and Th2 responses as well as mucosal immune responses. These immune responses consequently result in the production of neutralizing antibodies and strong cellular immune responses, conferring cross-protective and long-term immunity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

J.-H.N. designed the majority of the experiments and evaluated and interpreted all data. Y.-H.K., Y.-J.B., H.-J.P., and H.-L.K. equally and mostly performed the experiments. K.-A.H. performed real-time PCR experiments. S.-I. P. performed histology experiment. Y.-H.K. mainly wrote the manuscript. Y.-J.B., H.-J.P., and H.-L.K. wrote a part of the manuscript. J.-H.N. revised the manuscript. H. K. advised on experimental design and data. J.-H.N. as the principal investigator conceived the study and edited the manuscript. All authors have approved the final article.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.07.022.

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