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Programming of Influenza Vaccine Broadness and Persistence by Mucoadhesive Polymer-Based Adjuvant Systems

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The development of an anti-influenza vaccine with the potential for cross-protection against seasonal drift variants as well as occasionally emerging reassortant viruses is essential. In this study, we successfully generated a novel anti-influenza vaccine system combining conserved matrix protein 2 (sM2) and stalk domain of hemagglutinin (HA2) fusion protein (sM2HA2) and poly-γ-glutamic acid (γ-PGA)–based vaccine adjuvant systems that can act as a mucoadhesive delivery vehicle of sM2HA2 as well as a robust strategy for the incorporation of hydrophobic immunostimulatory 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) and QS21. Intranasal coadministration of sM2HA2 and the combination adjuvant γ-PGA/MPL/QS21 (CA-PMQ) was able to induce a high degree of protective mucosal, systemic, and cell-mediated immune responses. The sM2HA2/CA-PMQ immunization was able to prevent disease symptoms, confering complete protection against lethal infection with divergent influenza subtypes (H5N1, H1N1, H5N2, H7N3, and H9N2) that lasted for at least 6 mo. Therefore, our data suggest that mucosal administration of sM2HA2 in combination with CA-PMQ could be a potent strategy for a broad cross-protective influenza vaccine, and CA-PMQ as a mucosal adjuvant could be used for effective mucosal vaccines. ***The Journal of Immunology*****, 2015, 195: 2472–2482.**

Although vaccination is the most cost-effective biomedical approach for preventing influenza and its associated complications, current vaccine manufacturing procedures are inefficient, and the administration of these vaccines is still inconvenient (1–5). Additionally, vaccine strains need to be regularly updated to match the circulating viruses due to repeated antigenic drift (6). Influenza matrix protein 2 (M2), particularly the ectodomain (M2e), is well conserved across influenza (7) strains and is considered an attractive target for inducing cross-protection against influenza A subtypes. Similarly, the stalk domain, especially the fusion peptide (HA2) of hemagglutinin (HA), is another potent conserved region (8) that can induce a specific Ab response with cross-protection within a given subtype and even among various subtypes of influenza A virus (9). However, the drawback of these M2- and HA2-based vaccines is their low immunogenicity, which can reduce mortality but not morbidity in animal tests. Therefore, many studies have focused on increasing the immunogenicity of M2- and HA2-based vaccines with various vaccine adjuvants (10). Although several hundred different adjuvants have been tested for use in novel vaccine design during the last few decades, the vast majority have not been successfully approved for human use, with limitations including lack of efficacy, unacceptable local or systemic toxicity, difficulty of manufacturing, poor stability, and prohibitive cost (10–13). For this reason, until recently, aluminum-based mineral salts have been the most widely used adjuvant in human vaccines. However, this adjuvant is not strong enough to induce sufficient Ab-specific humoral immunity against subunit protein Ags or to stimulate cell-mediated immunity. Moreover, it can induce IgE Ab responses, which are associated with allergic reactions in some human subjects (10, 11).

Because the mucosal surface, particularly the respiratory mucosa, is the main entry point of many pathogens and serves as a first line of defense against infection, mucosal vaccines have emerged as a potent next-generation vaccination strategy that can induce protective immune responses in both the mucosal and systemic immune compartments (14). Mucosal vaccines offer advantages over systemic vaccines from a production and regulatory perspective. Specifically, mucosal vaccines are practical for mass vaccination and do not involve the risk of blood-borne infections, which may occur due to contaminated injection needles. Ease of

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Abbreviations used in this article: CA-PMQ, combination adjuvant of poly-γ-glutamic acid/3-*O*-desacyl-4′-monophosphoryl lipid A/QS21; CT, cholera toxin; dpi, day postinfection; FTIR, Fourier transformation infrared spectroscopy; HA, hemagglutinin; HA2, stalk domain of hemagglutinin; i.n., intranasally; M2, matrix protein 2; MALLS, multiangle laser light-scattering; M2e, matrix protein 2 ectodomain; MPL, 3-*O*-desacyl-4′-monophosphoryl lipid A; γ-PGA, poly-γ-glutamic acid; RI, refractive index; SEC, size-exclusion chromatography; sM2, conserved matrix protein 2; sM2HA2, fusion protein of conserved matrix protein 2 and the stalk domain of hemagglutinin; SPECT, single positron emission computed tomography; $TCID_{50}$, 50% tissue culture–infective dose.

administration, improved compliance, and possibility of delivery by personnel without medical training are also viewed as benefits of mucosal vaccine strategies, especially for preventing the pandemic spread of infections, including influenza virus infections (15–17). However, only a few commercially available mucosal vaccines currently exist because of the challenges of achieving effective mucosal vaccination, such as difficulties in generating effective mucosal immunity and the lack of safe and effective mucosal adjuvants and delivery systems (18, 19).

In this study, we suggest a poly-γ-glutamic acid (γ-PGA)–based novel complex as an efficient mucosal adjuvant system for an influenza vaccine based on the recombinant fusion protein sM2HA2, which contains the consensus matrix protein 2 (sM2) and the stalk domain of HA (HA2). The γ-PGA synthesized naturally by microbial species (bacilli) is a highly anionic polymer that is used in a variety of applications (e.g., food products, cosmetics, and medicines) and has been shown to have excellent biocompatibility and noncytotoxicity (20, 21). It can act as a mucoadhesive delivery vehicle for recombinant protein Ags and also provide an easy and robust strategy for the incorporation of hydrophobic immunostimulatory compounds such as 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) and QS21. MPL is a detoxified derivative of a LPS from the Re595 strain of *Salmonella minnesota* and was demonstrated to act through a member of the TLR family, TLR4 (22, 23). MPL-stimulated dendritic cells, acting as APCs, have been shown to express increased levels of costimulatory molecules and to secrete cytokines that are crucial for the activation and differentiation of B and T cells (22, 23). However, MPL is a poorly soluble compound that is difficult to disperse in an aqueous solution. Various solubility-enhancing approaches are therefore used, including formulation in emulsions, formulation in aqueous dispersions containing low amounts of surfactants or helper lipids, and inclusion in liposomes (23–25). QS21 is a highly purified saponin extracted from the bark of a South American tree, *Quillaja saponaria* Molina, and has been evaluated extensively as an adjuvant for human vaccines with clinical trials ongoing or completed for a number of diseases, including melanoma, influenza, HSV, and HIV-1 (26, 27). Research on the optimal formulation of amphiphilic QS21 has also been undertaken by combining QS21 with emulsions or liposomes (25–27). Nonetheless, the difficulty of manufacturing and poor stability of nanoemulsion and liposomes remain a problem for the mass production of MPL and QS21-based vaccine adjuvants. In this study, we developed a novel mucosal vaccine system based on the recombinant fusion protein sM2HA2 and the mucoadhesive γ-PGA/MPL/QS21 adjuvant that can be manufactured by an easy and robust strategy, enhances immune responses, and ensures broad protection against divergent influenza subtypes. The immunogenicity and efficacy of the novel mucosal vaccine system were evaluated in a murine model.

Materials and Methods

Ethics statement

Animal experiments were performed in strict accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All mouse procedures were approved by the Institutional Animal Care and Use Committee of Bioleaders Corporation (Daejeon, South Korea, protocol no. BLS-ABSL-13-010). The Institutional Animal Care and Use Committee of Bioleaders Corporation is registered with the Animal and Plant Quarantine Agency of Korea. All efforts were made to minimize animal suffering.

Fabrication of the γ-PGA/MPLA/QS21 complex

The γ-PGA (molecular mass of 5 kDa) was obtained from Bioleaders Corporation (Daejeon, South Korea). MPL, QS21, and OVA were purchased from Avanti Polar Lipids, Desert King Chile, and Boryung Pharmaceutical (Ansan, South Korea), respectively. MPLA (1 mg, 0.57 mmol) and QS21 (1 mg, 0.50 mmol) were dissolved in DMSO and mixed solvent of 20% tetrahydrofuran, respectively. Both solvents were mixed with γ-PGA (13.3 mg) and dissolved in 10 ml distilled water with vigorous stirring at room temperature for 5 min. After the reaction, the solution was incubated at 25˚C for 24 h while shaking. The solution was lyophilized and redissolved in 10 ml distilled water. The solution was dialyzed with a cellulose membrane tube (molecular mass cut-off of 12–14 kDa) in deionized water for 3 d. The solution was lyophilized to obtain the final product (γ-PGA/MPLA/QS21 complex) as a powder.

Characterization of the γ-PGA/MPLA/QS21 complex

For Fourier transformation infrared spectroscopy (FTIR)-attenuated total reflectance (Cary 630 FTIR spectrometer, Agilent Technologies) analysis, the γ-PGA/MPLA complex was also dissolved in mixed solution, DMSO-d_6/D_2O (8:2 [v/v]), γ-PGA/MPLA (1:5 [w/w]), and the γ-PGA/QS21 complex was also dissolved in mixed solution, DMSO-d_6/D_2O (8:2 [v/v]), γ-PGA/QS21 (1:5 [w/w]). The molecular mass and the molar radius of the complex of γ-PGA samples were determined by size-exclusion chromatography (SEC) coupled with a refractive index (RI) and a multi-angle laser light-scattering (MALLS) detector. The static laser light-scattering detectors measure each retention time of the SEC chromatogram. The scattering intensity is related to the concentration at each retention time, which is measured by a separate concentration-sensitive lined refractive index detector. The system was eluted at a flow rate of 0.8 ml/min when the viscosity detector was attached. Three serially connected HPLC-SEC columns TSKgel GMPWXL (Tosoh Bioscience) were used. This characterization was performed at 40˚C in a column oven. The γ-PGA and γ-PGA complexes (γ-PGA/MPLA/QS21 and γ-PGA/MPLA/QS21 plus OVA) were dissolved in 0.1 M $NaNO_3$, which was also the mobile phase in the SEC analysis, filtered (pore size, 220 nm; Millex-GV), and injected (200 μl). Light scattering was monitored by a DAWN HELEOS-II multiangle laser light-scattering detector. The RI detector used was a Viscotek 3580 RI. The excitation and fluorescence emission spectra of the γ-PGA/MPLA/QS21 plus OVA resuspended in PBS were obtained using a fluorescence spectrophotometer (FS-2, Scinco, Seoul, South Korea). The antifreeze activities of γ-PGA were measured by differential scanning calorimetry and calculated as shown in Supplemental Table I.

Storage stability and adjuvant effect of the PGA/MPLA/QS21

To test the storage stability and adjuvant effect of the γ-PGA/MPLA/QS21 (CA-PMQ) complex, the OVA Ag-specific IgG response in serum was analyzed after 4 mo of shelf-life storage (22–29˚C). The Ab titers were determined by ELISA using the serum samples from each mouse ($n = 5$). First, 96-well immunosorbent plates (Nunc, Roskilde, Denmark) were sensitized with 100 μl of each Ag (1 μg/ml) overnight at 4˚C. After blocking the plate (2% BSA in PBS containing 0.05% Tween 20, 1 h at 37˚C) for nonspecific binding, 100 μl 2-fold serially diluted samples, diluted in blocking buffer, was added to the plate (1 h at 37˚C), followed by the addition of 1:4000 diluted HRP-conjugated goat anti-mouse IgG (Southern-Biotech) Abs. After incubation at room temperature for 1 h, 100 μl peroxidase substrate tetramethylbenzidine (BD Pharmingen) was added to each well. The reaction was stopped by adding 2 N H_2SO_4. The absorbance was recorded by an ELISA reader at a wavelength of 450 nm (Molecular Devices, Sunnyvale, CA). The endpoint titer was determined by an OD cut-off value of 0.2.

In vivo single positron emission computed tomography/computed tomography imaging

Radiolabeling of OVA with [^{123}I] was performed using the iodogen tube method. In brief, Na[^{123}I] was added to the iodogen tube (Pierce, Rockford, IL) and gently stirred for 5 min. To this solution, 0.4 mg OVA dissolved in 1 ml PBS (pH 7.4) was added and allowed to react with Na[^{123}I] for 1 h at room temperature. The labeled OVA ([^{123}I]) was purified using a PD-10 column (Pierce) and the product was concentrated with a centrifugal filter (cut-off of 10 kDa; Millipore, Billerica, MA) (Supplemental Fig. 1). The OVA ([^{123}I]) was mixed with PBS (for a control) or CA-PMQ dissolved in PBS and allowed to react for 30 min prior to use. The mass ratio of OVA ([^{123}I]) to CA-PMQ was 1:5. Approximately 40 μl sample was intranasally administered to BALB/c mice (6 wk old, –20 g). Animal single positron emission computed tomography (SPECT) and computed tomography were performed on an Inveon PET/CT/SPECT (Siemens, Knoxville, TN) at Korea Basic Science Institute Ochang Center (Cheongwon, South Korea). The mice were scanned at 6 and 12 h after administration.

Preparation of the recombinant sM2HA2 fusion protein

The consensus sM2 spanning of extracellular and cytoplasmic residues without its transmembrane domain was generated from the analysis of sequences of H5N1, H1N1, and H9N2 subtypes in the database. Previous studies showed that the extracellular domain in particular (amino acids 1–13) is well conserved among the influenza virus subtypes and is recognized as an epitope for the induction of mAbs, which could protect influenza virus infection (8, 28). Considering the role of the conserved epitope (amino acids 1–13), we modified the residues at positions 14 (E→G) and 18 (R→K) of H5N1 to ensure optimal homology with the other subtypes used in this study. The consensus template was amplified by PCR using specific primers that included restriction endonuclease sites at the terminals (SecI and EcoRI). The gene was then cloned into the pGEM-T Easy vector using the TA cloning method (Promega, Madison, WI). The gene fragment was excised and inserted in-frame into the multicloning site of the pRSET-A vector (Invitrogen, Carlsbad, CA) using corresponding sites to form the plasmid pRSET-A–sM2. Similarly, HA2 genes (residues 15–137) from A/EM/Korea/W149/06 (H5N1) were modified by adding an EcoRI and HindIII site at the 5′ and 3′ terminal sites, respectively, to form the plasmid pRSET-A–sM2HA2 for prokaryotic expression of the target proteins. The plasmid construct was verified using restriction enzyme digestion and confirmed by sequencing (Solgent, Daejeon, South Korea). *Escherichia coli* BL21 (DE3) cells were transformed with the pRSET-A–sM2HA2 plasmid using the heat shock method. Colonies were seeded in 5 ml Luria–Bertani broth supplemented with 100 μg/ml ampicillin (96.9% purity by HPLC; Calbiochem, La Jolla, CA) and 35 mg/ml chloramphenicol (97% purity by HPLC; Calbiochem) and were grown at 37˚C with shaking. Cells were then cultured in a large volume of Luria–Bertani media at 37˚C with shaking at 200 rpm overnight. When the culture reached an OD_{600} of 0.6, expression of the target proteins was induced by adding 2 mM isopropyl-β-D-thiogalactopyranoside (99% purity; Bio Basic, Markham, ON, Canada) and incubating for another 12 h at 30˚C. Cultures were harvested by centrifugation at 6000 × *g* for 20 min at 4˚C. Cell pellets were resuspended in 20 ml cold buffer containing 20 mM Tris-HCl, 0.5 M NaCl, 10% glycerol, and protease inhibitor (1 mM PMSF, Sigma-Aldrich, Seoul, South Korea). Bacterial lysis was performed by sonication for 3 min with intervals of 2-s pulses and 1-s resting at 25% amplitude and then centrifuged (12,000 × *g*, 20 min) at 4˚C. The supernatant was removed and the remaining pellet was retained as inclusion bodies. The inclusion bodies were resuspended in denaturing buffer (20 mM Tris-HCl, 0.5 M NaCl, 10% glycerol, and 6 M urea) followed by sonication for 1 min and centrifuged as before. The debris supernatant was discarded, and the remaining pellet was resuspended in denaturing buffer II (20 mM Tris-HCl, 0.5 M NaCl, 8 M urea [pH 8.0]) and shaken at 4˚C overnight. The pellet was sonicated and centrifuged as before. The supernatant was separated as rescued protein from the inclusion bodies and purified by His-tag affinity chromatography (Qiagen, Valencia, CA). The purified proteins were dialyzed using a permeable cellulose membrane (molecular mass cut-off, 12–14 kDa; Spectrum Laboratories, Auckland, New Zealand) in PBS at 4˚C. The target proteins were confirmed by SDS-PAGE and the concentration was measured using Bradford assays (Bio-Rad, Hercules, CA). For immune detection, Western blot analysis was performed using mouse anti-histidine Abs (1:500, Invitrogen) and rabbit anti-M2 and anti-HA2 Abs (1:1000). The rabbit anti-M2 and anti-HA2 Abs used in this experiment were generated by i.m. inoculation of keyhole limpet hemocyanin–conjugated M2 and HA2 peptide into the rabbit, twice at 2-wk intervals. The purified proteins were used as vaccine Ags.

Animal grouping and vaccination

Specific pathogen-free BALB/c (5-wk-old) mice ($n = 380$) were purchased from Samtako (Seoul, South Korea). The mice were maintained in a specific pathogen-free environment following approval from the Institutional Animal Care and Use Committee (Daejeon, South Korea). The mice were divided into five experimental sets containing five groups each. Of five sets, three sets had 14 and two sets had 17 mice per group. Three groups of mice in each set were vaccinated intranasally (i.n.) with 20 μl sM2HA2 (2 μg) alone or with CA-PMQ or cholera toxin (CT) adjuvant three times at 1-wk intervals. As a control, two groups from each set were similarly treated with PBS or CA-PMQ alone. All mice were anesthetized with ether before inoculation, and the study was conducted in biosafety level 2 laboratory facilities. At day −1 (pre), 7 (first), 21 (second), 35 (third), and 180 (long-lasting), blood samples were collected through the retro-orbital plexus and the sera were separated by centrifugation and stored at −20˚C for further analysis. One and 24 wk after the last vaccination, three mice in each group were randomly sacrificed to collect samples from the lungs and intestine, which were stored at −70˚C for the investigation of mucosal IgA. Spleens were collected aseptically at day 35 to analyze the CTL response randomly from three mice of one set.

Abs specific to sM2 and HA2

Serum IgG values, including isotypes (IgG1 and IgG2a), and mucosal IgA (lungs and intestinal), were determined by ELISA using sM2 and HA2 protein as a coating Ag as previously described (29). The coating Ags used in this study were generated by *E. coli* expression of the plasmids pRSET-A–sM2 and pRSET-A–HA2 and were purified by His-tag affinity chromatography (Qiagen) as previously described (30, 31). The 96-well ELISA plates (Nunc) were coated with 300 ng/well sM2 or HA2 protein in coating buffer (pH 9.6) overnight at 4˚C. After washing with PBS containing 0.05% Tween 20, the plates were blocked with 300 μl 10% skim milk. Mouse sera diluted 1:200 in PBS containing 2% skim milk for the detection of IgG, IgG1, IgG2a, or undiluted supernatant from homogenized tissue for the detection of local IgA (lungs and intestine) were dispensed into designated wells and incubated at 37˚C for 2 h, followed by the addition of 1:3000 diluted HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, or IgA (Sigma-Aldrich, St. Louis, MO). After incubation for 2 h at 37˚C, 100 μl substrate solution containing tetramethylbenzidine (Millipore, Bedford, MA) was added to the plates and the reaction proceeded for 10 min. After adding the stop solution (2 N H_2SO_4), the OD was measured at 450 nm using an ELISA autoreader (Molecular Devices).

T cell ELISPOT specific to sM2 and HA2

IFN-γ and IL-4 ELISPOT assays were performed on splenocytes using mouse IFN- γ and IL-4 ELISPOT kits (BD Biosciences, San Jose, CA) as previously described (32). Briefly, ELISPOT 96-well plates were coated with anti-mouse IFN-γ and IL-4 capture Abs (5 μg/ml) in PBS at 4˚C overnight. After discarding the Abs, the plates were blocked with solution containing complete RPMI 1640 medium with 10% FBS (Invitrogen) and incubated for 2 h at room temperature. Splenocytes from the vaccinated mice were isolated aseptically and added at 5×10^5 cells/well in media containing sM2 or HA2 protein (1 μg/well), medium only (negative control), adjuvant only, or 5 μg/ml PHA (positive control, Invitrogen). After 24 h of incubation at 37˚C in 5% CO_2, the plates were sequentially treated with biotinylated anti-mouse IFN-γ and IL-4 Abs, streptavidin-HRP, and substrate solution. Finally, the spots were counted using an ImmunoScan Entry analyzer (Cellular Technology, Shaker Heights, OH).

Viruses and challenge test

To investigate and compare the protective efficacy of sM2HA2 vaccines adjuvanted with CA-PMQ against divergent influenza viruses, mice were infected with pathogenic A/EM/Korea/W149/06 (H5N1), A/Puerto Rico/8/34 (H1N1), A/aquatic bird/Korea/W81/2005 (H5N2), A/aquatic bird/Korea/W44/2005 (H7N3), or A/chicken/Korea/116/2004 (H9N2) influenza subtypes. All viruses used in this study were a gift of Dr. Young-Ki Choi (College of Medicine and Medical Research Institute, Chungbuk National University, Cheongju, South Korea). Mice were anesthetized and infected i.n. with 10-fold the mouse LD_{50} of the mouse-adapted viruses in 20 μl PBS 1 wk after the final vaccination. The mice were monitored for 13 d at fixed time points for measuring weight loss and survival. Six mice in each group were sacrificed at 3 and 5 d postinfection (dpi) to check viral titers in the lungs, and five mice remained in each group to be used for the survival test. The survival rate was determined by death or a cut-off of 25% lost body weight, at which point the animals were euthanized. All efforts were made to minimize suffering, and all of the surviving mice were humanely euthanized using CO_2 inhalation for 5 min after final monitoring.

Viral titers and histopathological analysis of lungs

Fifty percent tissue culture–infective dose ($TCID_{50}$) assays were performed to determine virus titers in the lungs as previously described (33). Briefly, lung tissues were homogenized in PBS containing antibiotic and antimycotic agents (Life Technologies, Grand Island, NY) and centrifuged at 12,000 × *g* to remove the cellular debris. All samples were immediately serially diluted 10-fold and incubated with confluent Madin–Darby canine kidney cells at 37˚C in a humid atmosphere for 1 h. An overlay medium containing L-1-tosylamide-2-phenylethyl chloromethyl ketone trypsin (Thermo Fisher Scientific, Rockford, IL) was added to the infected cells, which were incubated for 72 h. Virus titers were determined by an HA test, after observing the cytopathic effect, and were calculated by the Reed and Muench method, expressed as $\log_{10}$ $TCID_{50}$/lung tissues. For histopathology, lung tissues were collected at 5 dpi from ether narcosis–anesthetized mice. Tissues were fixed immediately in 10% neutral buffered formalin, embedded in paraffin wax, sectioned, mounted onto slides, and

stained with eosin stain. Histopathological changes were examined by light microscopy as previously described (34).

Statistical analyses

Data are presented as the means ± SD and represent at least three independent experiments. Differences between groups were analyzed by ANOVA, and means were compared by a Student *t* test. The *p* values <0.05 and <0.01 were regarded as significant and highly significant, respectively. Comparison of survival was performed by a log-rank test using GraphPad Prism 6.0.

Results

Preparation of sM2HA2 recombinant protein and γ-PGA/MPL/QS21 adjuvant

Fig. 1 depicts universal vaccine systems that can be constructed by the combination of the recombinant fusion protein sM2HA2, containing the sM2 and HA2, with the mucoadhesive CA-PMQ. Fig. 1A suggests an easy and robust strategy for the simultaneous incorporation of hydrophobic MPL, QS21, and recombinant proteins using anionic γ-PGA. The vaccine system can be fabricated by hydrogen bonding and hydrophobic interaction–induced complex formation between γ-PGA and MPL/QS21 and by ionic and hydrophobic interaction–induced complex formation between γ-PGA and the protein Ag (Fig. 1A, *upper* and *middle panels*). To improve the breadth of protection against influenza A viruses, a well-conserved sM2 and HA2 fusion construct was generated (Fig. 1A, *lower panel*). The recombinant proteins (sM2HA2) were refolded from inclusion bodies of *E. coli*, purified by His-tag affinity chromatography and dialyzed using permeable cellulose membranes. Purified proteins were confirmed by SDS-PAGE and Western blot using rabbit anti-M2 and anti-HA2 Abs. Through Coomassie staining and immunoblotting, proteins were observed at the expected molecular mass of 25 kDa (Fig. 1A, *lower panel*). The presence of carboxyl groups within γ-PGA can provide a strong interaction moiety with the mucus layer. These interactions were thought to be a result of the hydrogen bonds between γ-PGA and the proton-accepting groups within the mucous glycoprotein. Thus, CA-PMQ can act as a mucoadhesive delivery vehicle of sM2HA2 and induce strong mucosal and systemic antivirus immunity (Fig. 1B). When γ-PGA was mixed with MPL and QS21, the water-insoluble MPL and amphiphilic QS21 could be easily dispersed in aqueous solution. The expanded uncoiled state of γ-PGA at pH 5.5–7.4 due to the electrostatic repulsion arising from the ionized carboxyl groups could make it more susceptible to hydrogen bonding and secondary interactions with MPL and QS21.

The FTIR-attenuated total reflectance spectra results (Fig. 2A) supported the hydrogen bonding interaction of γ-PGA with MPL and QS21. The interaction of γ-PGA with MPL and QS21 was characterized, focusing on two parts (i.e., carboxylic acid peak at 2509.11 cm^{-1}, hydrogen and carboxylate combination at 3416.31 cm^{-1}). The hydrogen bond indicating point at 3416.31 cm^{-1} was increased due to the intermolecular hydrogen bond of γ-PGA with MPL and QS21. The carboxylic acid peak (at 2509.11 cm^{-1}) was also decreased after complex formulation, as the carboxylic acid groups of γ-PGA were used for the formation of hydrogen bonds with MPL and QS21. The hydrophobic main chain of γ-PGA can also interact with the hydrophobic alkyl chains of MPL and QS21 by hydrophobic interactions. To investigate the Ag-specific immune

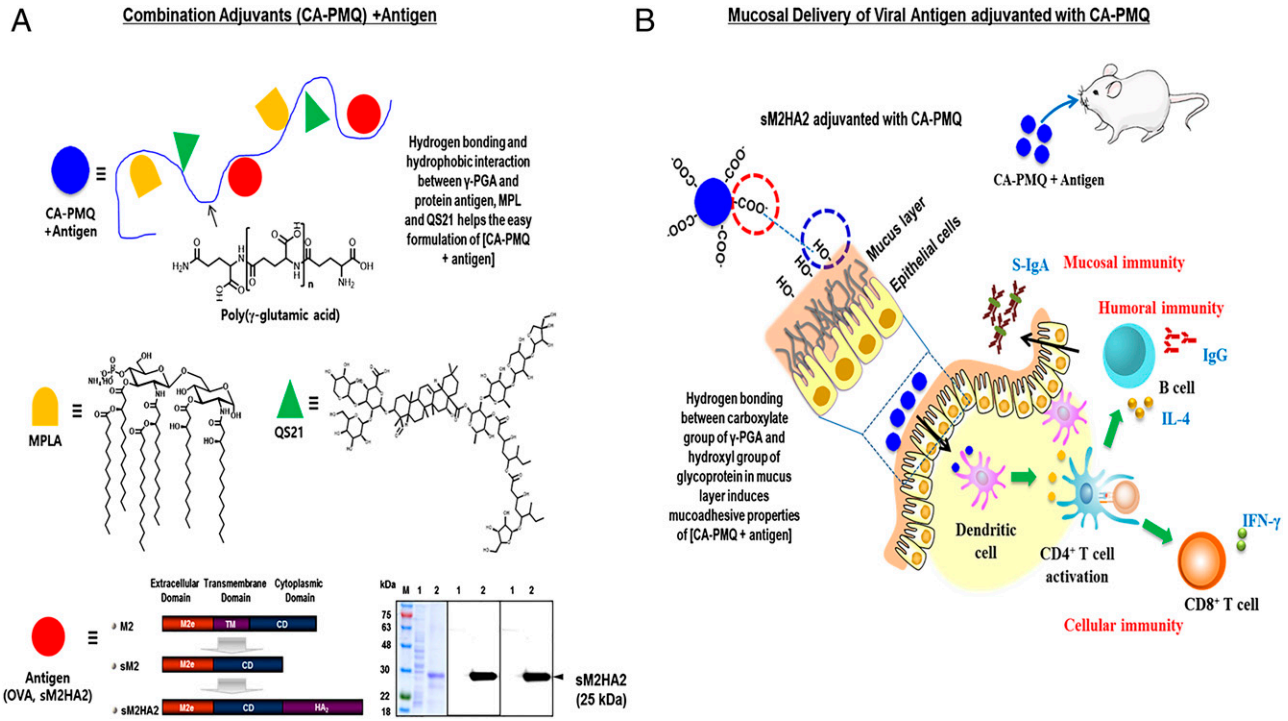

FIGURE 1. Schematic illustration of the vaccine system for cross-protection against divergent influenza virus using the combination of recombinant protein Ags (sM2HA2) and mucoadhesive polymer (γ-PGA)–based combination adjuvants (CA-PMQ). (**A**) Chemical and molecular structure of γ-PGA, MPL, QS21, and sM2HA2. γ-PGA provides an easy and robust strategy for the incorporation of hydrophobic MPL, QS21, and protein Ags. Diagram of the consensus sequence sM2 spanning the residues of the extracellular and cytoplasmic domains without its transmembrane domain, conjugated with fusion peptide HA2 (residues 15–137) from A/EM/Korea/W149/06 (H5N1) in the pRSET-A vector and the sM2HA2 protein. Purified sM2HA2 protein from a prokaryotic expression system was detected by SDS-PAGE. Coomassie brilliant blue staining (*left panel*) confirmed the protein to have a molecular mass of 25 kDa. Proteins were further verified by Western blot analysis using anti-M2 (*middle panel*) and anti-HA2 Abs (*right panel*). *Lane 1*: Whole-cell lysates of *E. coli* bearing pRSET-A vector without sM2HA2 gene. *Lane 2*: Purified sM2HA2 protein. (**B**) CA-PMQ–based vaccination for antiviral mucosal and systemic immunity: γ-PGA can act as a mucoadhesive delivery vehicle of sM2HA2 vaccine.

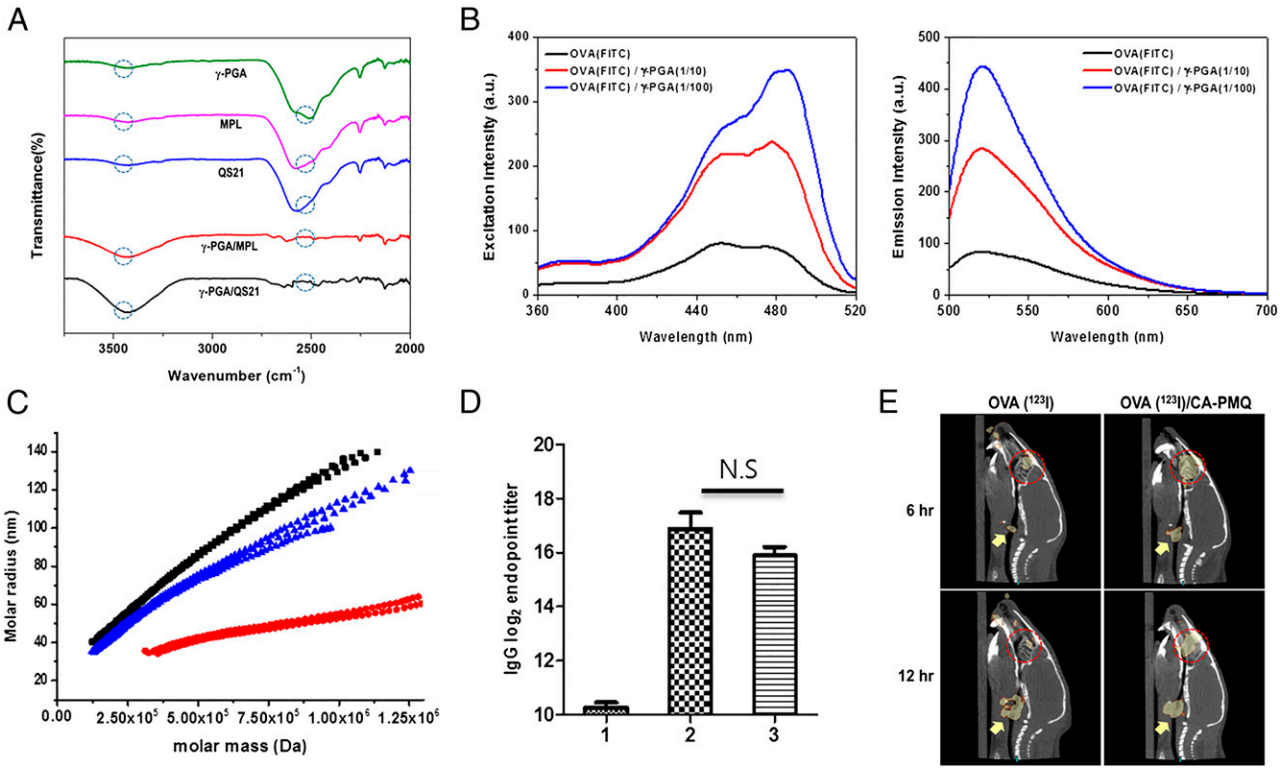

FIGURE 2. Characterization and adjuvant properties of CA-PMQ. (**A**) FTIR spectra of γ-PGA, MPLA, QS21, γ-PGA/MPLA, and γ-PGA/QS21. The inset dotted circles indicate the chemical shift of decreased carboxylic acid protonation of γ-PGA (2509.11 cm^{-1}) and increased hydrogen bonding (3435.31 cm^{-1}). (**B**) Excitation and emission spectra of the γ-PGA/OVA (FITC) complex. (**C**) Gel permeation chromatography–MALLS analysis of molar radius per molar mass ($\triangle R/\triangle Da \times 10^5$), for γ-PGA (black square, 11), γ-PGA/MPLA/QS21 complex (blue triangle, 8), and γ-PGA/MPLA/QS21-OVA complex (red circle, 2). (**D**) OVA-specific Ab titer tested with 1) OVA only, 2) OVA adjuvanted with CA-PMQ right after formulation, and 3) 4 mo after formulation. (**E**) OVA Ag residence time in the nasal cavity determined using SPECT/CT. SPECT images were obtained at 6 and 12 h following nasal administration of [^{123}I]-labeled OVA (OVA ([^{123}I])) in the presence and absence of CA-PMQ (dotted red circle, nasal cavity; yellow arrow, thyroid).

response, OVA, a model protein Ag, could also be incorporated into γ-PGA. The efficient dispersion of OVA in the γ-PGA matrix through ionic and hydrophobic interactions was studied using fluorescence spectrophotometry. Both the intensities of excitation and the emission peak of OVA (FITC) increased when OVA (FITC) was mixed with γ-PGA (Fig. 2B). Furthermore, the excitation band of OVA (FITC) was red-shifted, whereas the emission peak showed a blue shift, suggesting that γ-PGA acted as a dispersion agent, formed hydrophobic interactions with OVA (FITC), and resulted in the inhibition of the aggregation of OVA (FITC). The gel permeation chromatography–MALLS data also showed that the slope (molar radius divided by molar mass) was decreased after γ-PGA was mixed with MPLA, QS21, and OVA, which suggested the complex formation of γ-PGA, MPL, QS21, and OVA (Fig. 2C). After incorporation of MPL and QS21, the CA-PMQ complex can be stored in a powdered form that can be used as an intermediate material for further formulation of different types of vaccine adjuvants (particle-based or hydrogel-type vaccine adjuvants) and combinations of various viral Ags before administration. The lyophilized CA-PMQ complexes have an advantage in storage stability compared with conventional adjuvants, which are liquid formulations and require cold storage. Furthermore, during the lyophilization process of the CA-PMQ complex, γ-PGA can act as a cryoprotectant for MPL and QS21. During sample preparation by freeze-drying, the freezing and thawing process frequently impairs the biologically active substances (35). The γ-PGA produced by bacilli was anticipated to have high antifreeze activities because of its highly acidic amino acid composition (36). The antifreeze activities of γ-PGA were measured by differential scanning calorimetry and calculated as shown in Supplemental Table I. The antifreeze activity of γ-PGA was significant (Supplemental Table I). When the immunostimulatory activity of the lyophilized CA-PMQ complex was checked at 4 mo after fabrication, the Ab titer value was nearly same (Fig. 2D). The experimental results suggest that γ-PGA–based materials can be used for the incorporation of hydrophobic immunostimulatory materials by an easy and robust fabrication strategy. We also investigated the mucoadhesive properties of combination vaccine systems composed of OVA (as a model Ag) and the CA-PMQ. To investigate the mucoadhesive properties of γ-PGA, OVA was added to CA-PMQ and the mixture was administered i.n. To track the in vivo conditions of the administered dose, OVA was labeled with iodine ([^{123}I]), and micro–single positron emission computed tomography/computed tomography (SPECT/CT) imaging was performed (Supplemental Fig. 1) (37). By performing micro-SPECT/CT whole-body imaging, we observed a strong SPECT signal from the administered OVA ([^{123}I]) with CA-PMQ (Fig. 2E). The signal remained strong in the nasal cavity even after 12 h. In contrast, the signal decreased drastically via the mucociliary clearance mechanism within 6 h when OVA ([^{123}I]) was administered by itself. These experimental results suggest that γ-PGA increased the residence time of the codelivered OVA Ags in the mucus layer and acted as a controlled-release reservoir for the nasal epithelial cells. We speculated that γ-PGA could interact with mucin molecules through physical chain entanglements and form hydrogen bonds with the sugar residues on the oligosaccharide chains (38, 39). Because γ-PGA is in the expanded uncoiled state at the pH of the nasal secretions (pH 6.0–6.5), the electrostatic repulsion arising from the ionized functional groups renders γ-PGA more susceptible to mechanical chain entanglement and secondary interactions with

the mucous glycoprotein (40). To determine the effect of CA-PMQ on inducing immune responses, OVA proteins, as model protein Ags, were administered through the i.n. route with CA-PMQ. The level of OVA-specific serum IgG induced by the CA-PMQ/OVA group was significantly higher than the level induced by the OVA only group (Fig. 3A). Similarly, high levels of OVA-specific IgA titers were detected at the site of inoculation (Fig. 3B). Additionally, OVA-specific IgG1 and IgG2a levels were also tested, and both IgG1 and IgG2a increased significantly in the groups that received OVA with CA-PMQ or CT compared with OVA only (Fig. 3C). Mice vaccinated with CA-PMQ showed highly significant numbers of IL-4 and IFN-γ–secreting cell populations in the spleens stimulated with OVA protein compared with mice immunized with OVA only or control groups (Fig. 3D, 3E). These results suggest that our CA-PMQ system can function as an effective adjuvant that potently induces both humoral and cellular immune responses as well as strong mucosal immunity.

Mucosal vaccination of sM2HA2 with CA-PMQ induces sM2- and HA2-specific immune responses

To determine the ability of CA-PMQ to induce immune responses, groups of mice were immunized (i.n.) with 2 μg sM2HA2, alone or in combination with CA-PMQ. In fact, the components of CA-PMQ are generally recognized as safe materials and the safety profile was already evaluated (20, 21, 25, 26). After administration of CA-PMQ into mice, no weight loss and respiratory distress was observed. To compare the effect of CA-PMQ with the well-known mucosal adjuvant CT, groups of mice were similarly immunized using sM2HA2 with CT. Serum IgG responses specific to sM2 (Fig. 4A) and HA2 (Fig. 4C) were induced in the sM2HA2 group with the CA-PMQ adjuvant at levels that were significantly higher than those in the sM2HA2 alone group. The control, vaccination with PBS and CA-PMQ alone, did not induce sM2- or HA2-specific immune responses above the levels seen in the sM2HA2 groups without CA-PMQ and CT, indicating that the increased responses were the result of CA-PMQ or CT. Similarly, high IgA titers specific to sM2 and HA2 were detected both at the site of inoculation and at remote areas. As shown in Fig. 4B and 4D (*left panel*), mice immunized using sM2HA2 with CA-PMQ or CT adjuvant showed significantly higher levels of mucosal IgA in the lungs than did the mice immunized using sM2HA2 alone or the control groups. Interestingly, vaccination with sM2HA2/CA-PMQ or sM2HA2/CT induced significant levels of sM2- and HA2-specific IgA in the intestine (Fig. 4B and D, *right panel*) as well. Additionally, sM2- and HA2-specific IgG1 and IgG2a levels were also tested, and both IgG1 and IgG2a increased to significantly higher levels in the groups that received sM2HA2 with CA-PMQ or CT compared with sM2HA2 only and the controls after the booster immunization (Supplemental Fig. 2). IgG1 was more predominant than IgG2a. Taken together, these results indicated that vaccination with sM2HA2/CA-PMQ through the i.n. route more effectively induced sM2- and HA2-specific IgG and IgA responses than sM2HA2 alone or CA-PMQ, which was similar to or somewhat improved over the strong mucosal adjuvant CT. Additionally, because sM2HA2/CA-PMQ was able to induce sufficient levels of IgG1 and IgG2a in mice and Th1 and Th2 cells to improve the production of IgG2a and IgG1 Abs, respectively, we therefore suggest that the newly introduced vaccine approach was able to induce both humoral and cell-mediated immunity effectively.

CA-PMQ enhances sM2- and HA2-specific T cell responses induced by the sM2HA2 vaccine

T cell responses are important for the regulation of an effective immune response and are known to contribute to broad cross-protective immunity (41). To investigate this protective immune mechanism, the induction of IFN-γ– and IL-4–secreting cells in the splenocytes were determined by ELISPOT at 1 wk after the

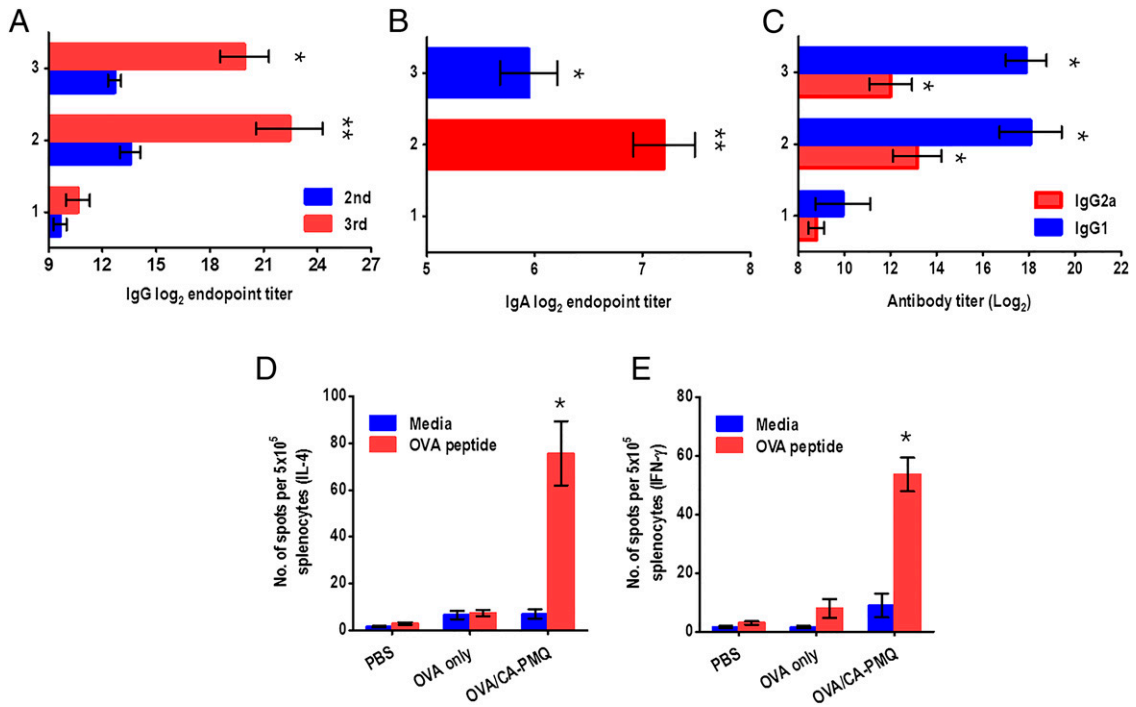

FIGURE 3. Induction of Ab-specific to (**A**) serum IgG, (**B**) nasal wash IgA, and (**C**) serum IgG isotypes in mice vaccinated with 1) OVA only (20 μg), 2) OVA plus γ-PGA/MPL/QS21 (PMQ), and 3) OVA plus CT (1 μg). Ab titers were determined using ELISA analysis. The cellular immune response was analyzed via (**D**) IL-4 and (**E**) IFN-γ cytokine production in splenocytes from vaccinated mice. OVA peptide (SIINFEKL, 5 μg/ml) stimulated splenocytes of vaccinated mice ($n = 5$) were determined using the ELISPOT assay. All data were obtained in triplicate and are presented as a mean ± SD. *$p < 0.05$, **$p < 0.01$ between OVA/PMQ, OVA/CT, and control groups.

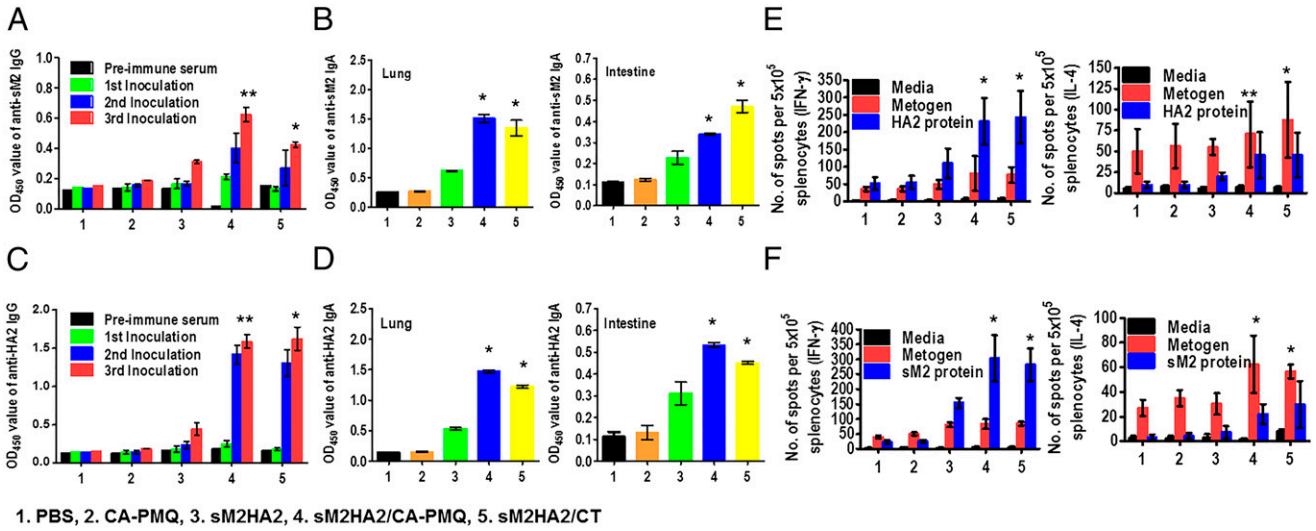

FIGURE 4. CA-PMQ enhances immune responses to sM2 and HA2 when delivered with the recombinant sM2HA2 vaccine. BALB/c mice were immunized i.n. with sM2HA2 alone or with CA-PMQ or CT adjuvant, CA-PMQ only, or PBS at weeks 0, 2, and 4. Serum samples were taken at day −1 (pre), 7 (first), 21 (second), and 35 (third). Serum IgG and lung and intestinal IgA were investigated by ELISA and measured at an absorbance of 450 nm. ELISA plates were coated with purified sM2 or HA2 proteins (3 μg/ml) for the determination of sM2- or HA2-specific systemic and local Abs. (**A**) Serum IgG Abs specific to sM2 and (**B**) mucosal IgA specific to sM2 in the lungs (*left panel*) and intestine (*right panel*). (**C**) Serum IgG Abs specific to HA2 and (**D**) mucosal IgA specific to HA2 in the lungs (*left panel*) and intestine (*right panel*). Splenocytes were harvested 10 d after the last immunization. Cells were restimulated in vitro with the sM2 or HA2 protein, and cytokine-forming cell spots were determined by ELISPOT assay. IFN-γ and IL-4 spot-forming cells per 5×10^5 splenocytes were determined. Results from plenocytes producing IFN-γ and IL-4 stimulated with (**E**) sM2 and (**F**) HA2 proteins are shown. Bars denote mean ± SD for the group ($n = 5$). The data are representative of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ between sM2HA2/CA-PMQ, sM2HA2/CT, and control groups.

final boosting. Mice vaccinated with CA-PMQ showed highly significant numbers of IFN-γ–secreting cell populations in the spleens stimulated with both sM2 and HA2 protein compared with mice immunized with sM2HA2 alone or the control groups (Fig. 4E, 4F, *left panel*). A significantly increased number of IL-4–secreting cells was also detected in the spleens of mice that received sM2HA2 with CA-PMQ compared with mice vaccinated with sM2HA2 only or control groups (Fig. 4E, 4F, *right panel*). However, sM2HA2 recombinant protein without any adjuvant could also induce detectable levels of both IFN-γ– and IL-4–secreting cells in the spleen after stimulation with sM2 and HA2 protein, whereas only background levels of cytokine-secreting cells were detected in the control groups (Fig. 4E, 4F), indicating that the fusion protein sM2HA2 itself is capable of inducing cellular immunity and that CA-PMQ significantly enhances this immunity. Importantly, mice vaccinated with sM2HA2 along with CT showed similar levels of IFN-γ– and IL-4-secreting cells in the spleen after stimulation with both proteins. These results provided evidence that i.n. immunization of sM2HA2 with CA-PMQ enhanced sM2- and HA2-specific T cell immune responses to a similar degree to use of the well-known potent mucosal adjuvant CT.

The CA-PMQ–adjuvanted sM2HA2 vaccine ensures protection and improves lung features against lethal infection with divergent influenza subtypes

To evaluate the potency of sM2HA2-induced immune responses against lethal infection with influenza virus, vaccinated mice were challenged with 10 LD_{50} of mouse-adapted H5N1, H1N1, H5N2, H7N3, or H9N2 influenza A subtypes. Protective efficacies were measured by survival rates and weight loss, monitored every other day for 13 dpi. As expected, none of the mice in the control unimmunized group survived the lethal influenza infection. Significant body weight losses were observed in the mice vaccinated with sM2HA2 without CA-PMQ or CT (Fig. 5A–E, *upper panel*), and 0–40% survived the lethal infection with divergent subtypes (Fig. 5A–E, *middle panel*). In contrast, 100% of mice that received sM2HA2 with CA-PMQ survived against H1N1, H5N1, H5N2, and H9N2 (Fig. 5A–C, 5E, *middle panel*), and 80% survived against H7N3 (Fig. 5D, *middle panel*) lethal infection. Of the mice vaccinated with sM2HA2/CT, 100% survived against lethal infection with H1N1, H7N3, and H9N2 (Fig. 5B, 5D, 5E, *middle panel*) and 60 and 80% against H5N1 (Fig. 5A, *middle panel*) and H5N2 (Fig. 5C, *middle panel*) influenza subtypes, respectively. Although mice immunized with sM2HA2/CT exhibited similar levels of protection (60–100%) to sM2HA2/CA-PMQ, significant weight loss was observed in the case of sM2HA2/CT-vaccinated mice (Fig. 5A–E, *upper panel*), indicating a lack of effective protection against lethal infection in the CT-adjuvanted group. These results are consistent with previous studies that demonstrated that i.n. vaccination with 3M2eC plus CT induced Abs that could improve mortality but not morbidity (7). In contrast, mice that received sM2HA2 with CA-PMQ showed negligible signs of disease and only a slight and transient loss of weight.

To better understand the protective efficacy observed by the sM2HA2/CA-PMQ vaccine, virus titers in the lungs of challenged mice were measured to estimate viral clearance at 3 and 5 dpi. The sM2HA2/CA-PMQ–immunized mice showed reduced lung viral titers at day 3 and had completely cleared the infection by day 5 in the case of H5N2, H7N3, and H9N2 virus challenge (Fig. 5C–E, *lower panel*). Likewise, in the case of H5N1 and H1N1 lethal infection, CA-PMQ–adjuvanted sM2HA2-immunized mice exhibited a significant reduction in lung viral titers compared with sM2HA2 only or control mice by 5 dpi (Fig. 5A, 5B, *lower panel*). The clearance of virus from the lungs of sM2HA2/CT-immunized mice was also observed to correlate with the survival results against infections with divergent influenza subtypes. A histopathological examination was also determined to correlate with viral clearance in the lungs. Representative lungs samples were collected after challenge with H5N2 virus and processed for examination with light microscopy. Clear signs of profound pulmonary inflammation were

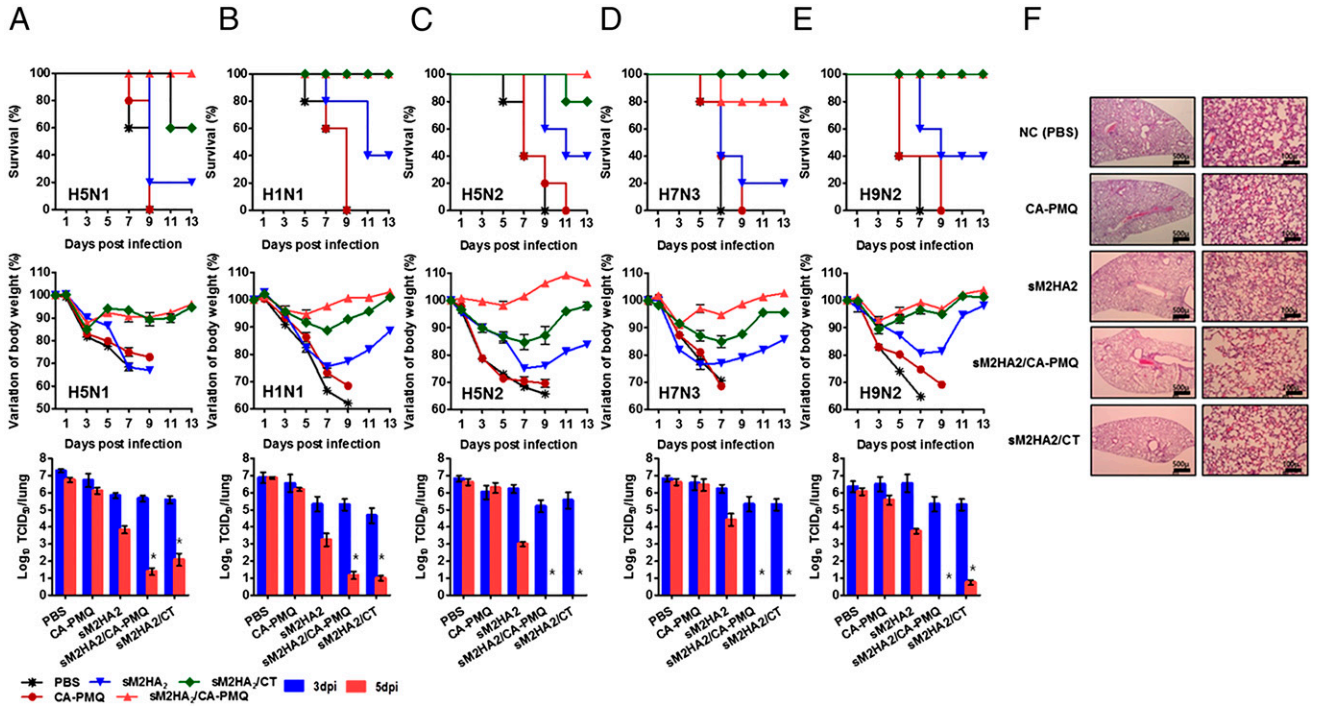

FIGURE 5. The CA-PMQ–adjuvanted sM2HA2 vaccine confers improved cross-protection against divergent influenza subtypes. BALB/c mice were immunized i.n. with sM2HA2 alone or with CA-PMQ or CT adjuvant, CA-PMQ only, or PBS at weeks 0, 2, and 4. Mice were infected i.n. with 10 LD_{50} of mouse-adapted influenza virus subtypes at 2 wk after the last boosting. Viral titers in the lung tissues were determined by $TCID_{50}$ in the Madin–Darby canine kidney cell line at 3 and 5 dpi. At 5 dpi, lungs were randomly collected from each group of one set, fixed, and stained with eosin before being examined under a light microscope. (**A**) Variation of body weight (*upper panel*), survival (*middle panel*, log-rank test, $p = 0.00063$), and lung viral titers (*lower panel*) after infection with A/EM/Korea/W149/06(H5N1). (**B**) Variation of body weight (*upper panel*), survival (*middle panel*, log-rank test, $p = 0.00063$), and lung viral titers (*lower panel*) after infection with A/Puerto Rico/8/34 (H1N1). (**C**) Variation of body weight (*upper panel*), survival (*middle panel*, log-rank test, $p = 0.00063$), and lung viral titers (*lower panel*) after infection with A/aquatic bird/Korea/W81/2005 (H5N2). (**D**) Variation of body weight (*upper panel*), survival (*middle panel*, log-rank test, $p = 0.00063$), and lung viral titers (*lower panel*) after infection with A/aquatic bird/Korea/W44/2005(H7N3). (**E**) Variation of body weight (*upper panel*), survival (*middle panel*, log-rank test, $p = 0.00063$), and lung viral titers (*lower panel*) after infection with A/chicken/Korea/116/2004 (H9N2). (**F**) The lungs of the mice vaccinated with sM2HA2/CA-PMQ showed clear alveoli without inflammatory cell infiltration, in contrast to the lungs of the mice vaccinated with sM2HA2 or the control groups. Error bars depict the mean ± SD in body weight. Asterisks indicate a significant difference between groups ($n = 5$) in body weight. The data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$ between sM2HA2/CA-PMQ, sM2HA2/CT, and control groups at 5 dpi in the lung viral titers.

observed in the lungs of mice immunized with sM2HA2 or the control, whereas the mice immunized with sM2HA2/CA-PMQ exhibited reduced or absent pulmonary inflammation (Fig. 5F). Taken together, these results indicate that CA-PMQ in combination with the conserved fusion protein sM2HA2 significantly improves protective efficacy against divergent influenza subtypes, and it is likely that sM2- and HA2-specific immune responses induced by CA-PMQ can efficiently contribute to controlling viral replication.

CA-PMQ–adjuvanted sM2HA2 induces long-lasting immune response and protection

The durations of immune response and protection are important criteria for an effective vaccine. Therefore, the longevity of the immunity induced by sM2HA2/CA-PMQ was investigated by detecting serum IgG and mucosal IgA by ELISA at 6 mo after immunization. Serum IgG as well as lung and intestinal IgA specific to sM2 and HA2 were maintained at similar levels in mice immunized with sM2HA2/CA-PMQ even at 6 mo after boosting (Fig. 6A, 6B). Likewise, to determine whether the Th cell subsets were functional 6 mo after the final vaccination with sM2HA2/CA-PMQ, IgG subclasses, such as IgG1 involved with Th2 and IgG2a involved with Th1, were examined using ELISA. Interestingly, mice vaccinated with sM2HA2/CA-PMQ showed a significant level of IgG1 and IgG2a specific to both sM2 and HA2 at almost the same level detected immediately after vaccination (Fig. 6A). Taken together, these results indicate that sM2HA2/CA-PMQ induces both humoral and cell-mediated immune responses that last at least 6 mo after final vaccination. To determine the duration of protection, immunized mice were challenged with the influenza A/Puerto Rico/8/34 (H1N1) heterosubtype. Body weight changes and survival were monitored until 13 dpi. The control mice showed >30% body weight loss and died by day 9 after infection (Fig. 6C, *left panel*). In contrast, although the sM2HA2/CA-PMQ–vaccinated mice showed a certain percentage of body weight loss that was not unexpected, it recovered by 13 dpi, and 100% of mice survived infection, which was similar to the efficacy immediately after vaccination (Fig. 6C, *right panel*). Furthermore, the mice immunized with sM2HA2 alone showed a dramatic loss of body weight, and 100% succumbed by 11 dpi, suggesting that CA-PMQ contributes to the long-lasting immunity. Collectively, these results indicate that CA-PMQ not only improves the immune response and protective efficacy of the recombinant sM2HA2 vaccine against divergent influenza subtypes but also ensures a long duration of immunity.

Discussion

Current influenza vaccines based on variable surface Ags are not suitable for providing effective protection against unpredictable influenza pandemic outbreaks. During recent decades, many approaches have been investigated to develop new vaccines that

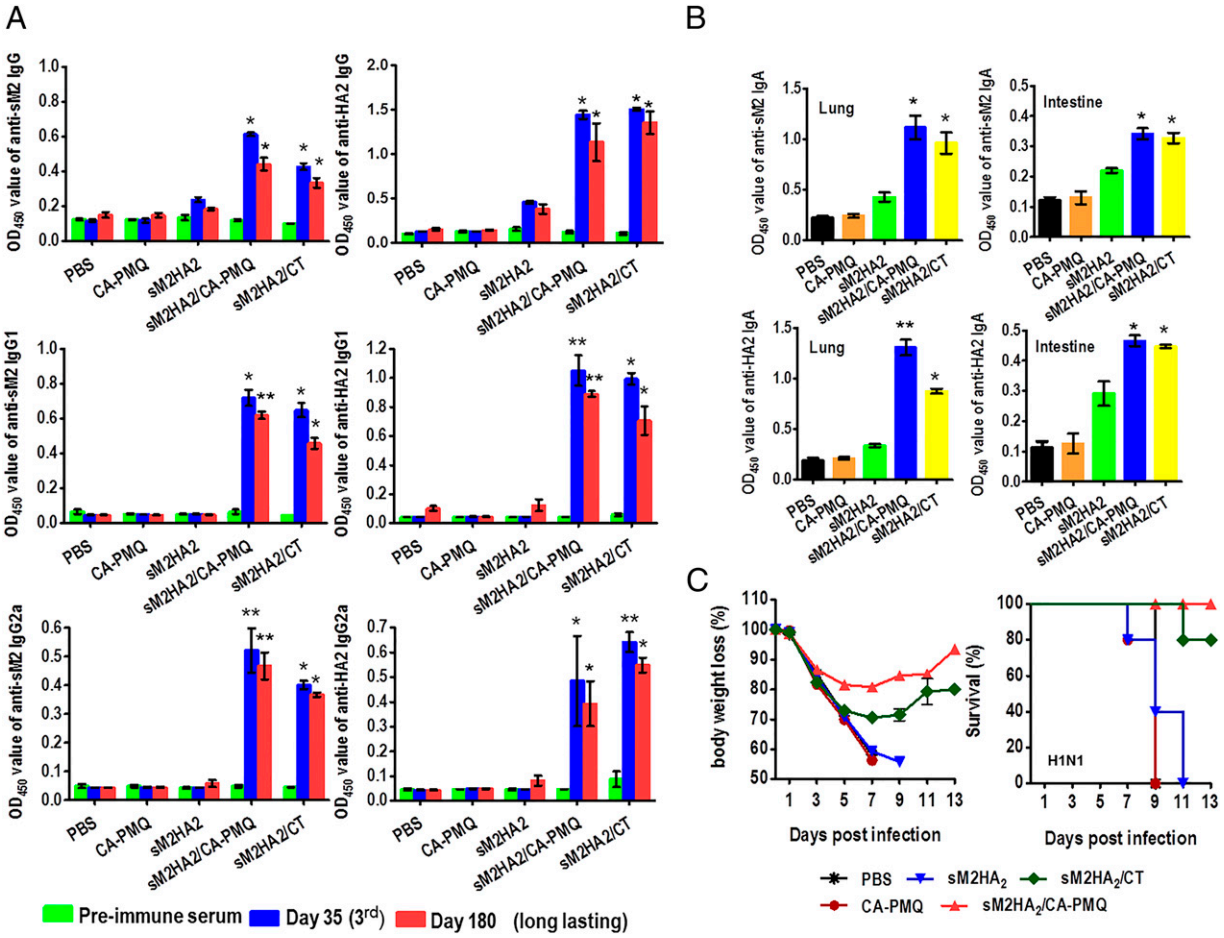

FIGURE 6. CA-PMQ–adjuvanted sM2HA2 induces long-lasting immune responses and protective efficacy against lethal infection by various influenza subtypes. Sets of mice were vaccinated i.n. according to the schedule with PBS, CA-PMQ, sM2HA2, sM2HA2/CA-PMQ, or sM2HA2/CT. Sera were collected on days 0, 35, and 180 after the final vaccination. Lung and intestine samples were collected at day 180. ELISAs were performed in triplicate using sM2 or HA2 protein as a coating Ag to confirm the long-lasting Ab levels of IgG, IgG1, IgG2a, and IgA. Mice were challenged with lethal doses of A/Puerto Rico/8/34 (H1N1) virus (10 mouse LD_{50}) 6 mo after the final vaccination. (**A**) IgG, IgG1, and IgG2a Ab levels specific to sM2 (*left*) and HA2 (*right*), respectively. (**B**) IgA Ab levels specific to sM2 (*upper panels*) and HA2 (*lower panel*) in the lungs (*left*) and intestine (*right*). (**C**) The percentage changes of initial body weight (*left*) and survival (*right*) after infection with H1N1 virus. Bars denote mean ± SD. $*p < 0.05$, $**p < 0.01$ between sM2HA2/CA-PMQ, sM2HA2/CT and control groups.

offer broad cross-protection as well as convenient preparation and administration. In particular, studies have been conducted to induce cross-protection using M2e (42–45) and HA2 (9, 30, 46, 47). Considering these observations, we hypothesized that a fusion vaccine based on sM2 and HA2 (sM2HA2) would confer significant cross-protection. Thus, we constructed a fusion plasmid of these two promising targets, expressed it in a highly efficient microbial expression system (*E. coli*), and tested it for broad cross-protective immunity. Selection of an appropriate route and adjuvant with the ability to stimulate functional immune responses is critical to the success of a subunit vaccine. Previous studies have shown that the i.n. mucosal route is superior to systemic administration for inducing cross-protective immunity in mice (7, 30, 48). Other studies have focused on chemical and genetic conjugates (carrier molecules or virus particles) or a combination of DNA and recombinant or live influenza vaccines to improve cross-protection (49–53). Unfortunately, these vaccines were not completely protective, as vaccinated animals showed considerable weight loss, even when administered with an adjuvant. In this study, a novel combination adjuvant, CA-PMQ, was prepared and administered for the first time to improve cross-protection. Importantly, we also demonstrated that vaccination with only 2 μg of two well-conserved fusion proteins (sM2 and HA2) with CA-PMQ conferred broad protection against challenge with relatively high doses of lethal influenza A subtypes.

A preliminary experiment was conducted to determine the dose and efficacy of sM2HA2 on influenza virus infection, and we found that three doses (2 μg/dose) of sM2HA2 via the i.n. route without adjuvant resulted in 40% survival and drastic body weight loss against lethal infection with H1N1 (Supplemental Fig. 3). Based on the results, CA-PMQ–adjuvanted sM2HA2 was administered and resulted in enhanced levels of sM2HA2-specific Abs. Because the vaccine was delivered by the mucosal route, a significant level of mucosal IgA was observed both in the lungs and intestine. Furthermore, significant levels of serum IgG, particularly IgG1 and IgG2a, were also induced by the CA-PMQ–adjuvanted sM2HA2 vaccine. Generally, Th1 cells improve the production of IgG2a Abs, whereas Th2 cells induce IgG1 Abs in mice (54). Although both IgG subclasses can have strong neutralizing capacities, IgG2a is known to interact efficiently with complement and Fc receptors and might contribute to viral clearance (55). Thus, the reduction of viral titers in the lungs of sM2HA2/CA-PMQ–vaccinated mice after lethal infection indicated a contribution of CA-PMQ to the induction of sM2- and HA2-specific IgG1 or IgG2a for viral clearance. The cellular immune response plays an important role in vaccination (30, 45, 56). We examined

Th1-type (IFN-γ) and Th2-type (IL-4) cytokine responses by ELISPOT, and significant levels of IFN-γ were detected in response to stimulation by both the sM2 and HA2 protein in mice immunized with the adjuvanted sM2HA2 but not in sM2HA2 only or control mice. IL-4 in CA-PMQ–adjuvanted sM2HA2-immunized mice was also observed at significantly higher levels than in unimmunized mice. These results indicate that the mucosal vaccine system of CA-PMQ–adjuvanted sM2HA2 induces a strong cellular immune response and protects mice from widely divergent influenza subtypes from phylogenetic group 1 (H1, H5, H9) and group 2 (H7). Additionally, our study demonstrated that CA-PMQ–adjuvanted recombinant sM2HA2 induced long-lasting immunity and conferred protection against lethal infection by divergent influenza subtypes, even at 6 mo after the final vaccination (Fig. 6), which is important for any successful vaccine. Immune adjuvants are a large and heterogeneous group that can be divided into two subgroups: immunostimulants and delivery systems. The first category of adjuvants stimulates the immune system by interacting with specific receptors, whereas the second type can increase the immune response by overcoming mucosal barriers and enhancing Ag uptake (10, 11). In the pharmaceutical industry, it is recognized that a new formulation of the same substance is a new drug, as the effect and performance might be significantly influenced. The ease of use and production of an adjuvant formulation are also indispensable for improved bioavailability. In this study, we developed a mucoadhesive polymer-based (i.e., γ-PGA) Ag delivery system and an easy and robust formulation strategy for the incorporation of poorly soluble immunostimulant compounds (i.e., MPL and QS21). In this regard, the novel combination of the adjuvants of the delivery system and immunostimulatory compounds based on the γ-PGA/MPL/QS21 complex is a potent candidate for the induction of a strong immune response.

The nasal route is a promising alternative form of needle-free administration owing to its particular physiological characteristics and immunological features (57). Indeed, major entities in global health, such as the World Health Organization and the Center for Disease Control and Prevention, have advocated the necessity of investing research efforts into developing advanced delivery technologies, which could simplify immunization schedules. The accomplishment of these objectives would improve vaccination coverage for several infectious diseases, especially in developing countries. Although the mucus vaccine delivery strategy is regarded as a potent needle-free vaccine delivery method, the limitations associated with the mucociliary clearance mechanism inhibit the delivery of Ags to the immune system (58). Accordingly, new approaches for mucus vaccine delivery systems that can improve Ag passage through biological barriers, such as the intestinal and nasal mucosa, are in high demand. Challenges to effective mucosal vaccination include difficulties in generating effective mucosal immunity and the lack of safe and effective mucosal adjuvants and delivery systems. The CA-PMQ system used in this research consists of all natural ingredients that enhance both humoral and cellular immunity. The γ-PGA, synthesized naturally by microbial species (bacilli), can act as a mucoadhesive delivery vehicle for recombinant protein Ags and also provide an easy and robust strategy for the incorporation of hydrophobic MPL and QS21. Our findings suggested that the presence of a unionized carboxyl group within γ-PGA was critical for strong interaction with mucus. These interactions are thought to be a result of the hydrogen bonds between γ-PGA and the proton-accepting groups within the mucus glycoprotein. It was concluded that the mucoadhesive polymer could interact with the mucin molecule by physical chain entanglements followed by hydrogen bond formation with the sugar residues on the oligosaccharide chains, forming a strengthened gel network, thus allowing the polymer to remain adhesive for extended periods of time (38, 39). As the polymer would be in an expanded uncoiled state at the pH of the medium (6.2) due to the electrostatic repulsion arising from the ionized functional groups, the polymer would be more susceptible to mechanical chain entanglement and secondary interactions with the mucus glycoprotein. Owing to the presence of numerous carboxyl groups in γ-PGA, the polymer is likely to be able to adopt a more favorable macromolecular conformation with increased accessibility of its hydrogen-bonding groups when compared with other polymers (40).

Until now, most researchers working on the development of vaccines have focused on the discovery and production of Ags that are specific to target disease or pathogens. They also tested the antigenicity of Ags with a few adjuvants available that use only a single immunostimulant. To generate optimal prophylactic or therapeutic immunity, the magnitude and persistence of induced immunity should be controlled according to the type of disease. However, the lack of either the knowledge or capacity to formulate complex adjuvant systems comprising immunostimulant and delivery vehicle limited the development of effective vaccines. The CA-PMQ adjuvant revealed in this study was formulated by the combinations of adjuvants and is capable of inducing strong, long-lasting humoral and cellular immune responses. In this respect, CA-PMQ can be a good candidate adjuvant for researchers working in vaccine developments for infectious disease as well as cancer immunotherapy.

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Disclosures

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