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ORIGINAL ARTICLE

# Mucosal Immunization with Recombinant Adenovirus Encoding Soluble Globular Head of Hemagglutinin Protects Mice Against Lethal Influenza Virus Infection

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Influenza virus is one of the major sources of respiratory tract infection. Due to antigenic drift in surface glycoproteins the virus causes annual epidemics with severe morbidity and mortality. Although hemagglutinin (HA) is one of the highly variable surface glycoproteins of the influenza virus, it remains the most attractive target for vaccine development against seasonal influenza infection because antibodies generated against HA provide virus neutralization and subsequent protection against the virus infection. Combination of recombinant adenovirus (rAd) vector-based vaccine and mucosal administration is a promising regimen for safe and effective vaccination against influenza. In this study, we constructed rAd encoding the globular head region of HA from A/Puerto Rico/8/34 virus as vaccine candidate. The rAd vaccine was engineered to express high level of the protein in secreted form. Intranasal or sublingual immunization of mice with the rAd-based vaccine candidates induced significant levels of sustained HA-specific mucosal IgA and IgG. When challenged with lethal dose of homologous virus, the vaccinated mice were completely protected from the infection. The results demonstrate that intranasal or sublingual vaccination with HA-encoding rAd elicits protective immunity against infection with homologous influenza virus. This finding underlines the potential of our recombinant adenovirus-based influenza vaccine candidate for both efficacy and rapid production.

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## INTRODUCTION

Influenza virus is an important cause of respiratory infections. According to the World Health Organization, global seasonal influenza epidemic accounts for 3～5 million infections and is responsible for 250,000～500,000 deaths annually. In 2009, a new swine/human/avian-origin influenza A (H1N1) virus emerged in Mexico and caused the most recent influenza pandemic underscoring the necessity for better preparedness against future pandemics. Currently, inactivated and live-attenuated influenza vaccines are widely used for vaccination in humans. The current common vaccine production method that has been utilized for past decades is the cultivation of vaccine viruses in embryonated chicken eggs. Although the egg-based system has been well established for production of seasonal influenza vaccines, it evidently failed to produce sufficient amount of influenza vaccine during the 2009 pandemic mainly as a result of the lack of availability of embryo-

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Abbreviations: BAL, bronchoalveolar lavage; Balb/c, a mouse strain; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; HI, hemagglutination inhibition; HRP, horseradish peroxidase; Ig, immunoglobulin; i.n., intranasal; LD50, 50% lethal dose; PFU, plaque-forming unit; PR8, A/Puerto Rico/8/34; rAd, recombinant adenovirus; s.l., sublingual; TMB, 3,3',5,5'-tetramethylbenzidine

nated chicken eggs and appropriate vaccine production facilities (1). Such inability to produce sufficient amount of influenza vaccines in timely manner poses a significant concern.

Adenovirus is a non-enveloped virus with linear, double-stranded DNA genome. There are several benefits of using adenovirus as the vector for influenza vaccine delivery. First, adenoviral vectors infect wide range of dividing and non-dividing cells. Adenoviral vectors also share the route of infection with influenza virus by infecting epithelial cells of respiratory tract. There is no integration of viral vector genome into the host genome while yielding high transduction efficiency. Moreover, adenoviral vectors have been shown to be safe for use in humans as it was confirmed in over 150 clinical trials (2). Importantly, adenovirus-base influenza vaccines can be manufactured in large quantities at a short notice using cell-culture based technology. Moreover, adenoviral vectors can inherently stimulate innate immune responses via Toll-like receptor-dependent and independent pathways (3-5). Activation of innate immune responses by the adenoviral vectors can exerts adjuvant-like effect resulting in the induction of immunogen-specific humoral and cell-mediated immune responses.

Mucosal surface is the main entry way for invading pathogens, and serves as the first line of defense against infection. The mucosal immune system is functionally distinct from the systemic immune system in that it possesses its own highly organized immunological tissues which function to maintain homeostasis within the mucosa (6,7). Currently established parenteral route of administering influenza vaccines targets systemic induction of virus-specific IgG antibodies. However, previous studies have shown that influenza vaccination efficacy is closely correlated to the induction of appropriate immune responses in the respiratory mucosa, and parenteral vaccines are inefficient in stimulating immune responses of mucosal tissues (8). As such, vaccination schemes that specifically target the respiratory mucosa could provide better protection characterized by induction of antigen-specific IgA in the respiratory mucosa as wells as systemic antigen-specific IgG. Hence, intranasal (i.n.) immunization is a promising method for mucosal vaccination. Intranasal delivery of antigens has shown to induce secretory antibodies in the airway and in the genital track mucosa as well as strong systemic immune responses including IgG and cytotoxic T cell responses (9,10). However, there is an evidence of potential retrograde passage of vaccine components through the olfactory epithelium to the central nerve system posing a serious safety concern (11,12).

Recently, sublingual (s.l.) route gained considerable attention due to the facts that it induced both mucosal and systemic immune responses. For example, it has been shown that s.l. immunization induced antigen-specific IgG antibodies in plasma and IgA antibodies in mucosal secretions including saliva, nasal wash, and bronchoalveolar lavage (BAL) (13). Moreover, there is evidence supporting s.l. immunization as an effective method for influenza vaccine delivery. Mice received formalin-inactivated A/PR/8/34 virus via sublingual route induced considerable levels of A/PR/8/34 -specific IgG and IgA antibodies in plasma, BAL, and nasal wash (14). Overall, a growing body of evidences suggests that s.l. immunization induces immune responses that are comparable in magnitude and anatomical dissemination to those induced by nasal immunization (15). Also, unlike nasal immunization, s.l. immunization does not cause retrograde passage of vaccine antigens to the central nerve system, rendering it the safer immunization route to induce mucosal immune responses (14,16).

In this study, we designed novel recombinant adenovirus-based vaccines encoding secreted forms of globular head domains of Hemagglutinin (HA) and evaluated their immunogenicity and efficacy of intranasal and sublingual administration in murine model.

## MATERIALS AND METHODS

### Viruses and Cell-line

A/Puerto Rico/8/1934 (PR8; a gift from Baik L. Seong, Department of Biotechnology, Yonsei University, Korea) influenza virus was amplified in embryonated-chicken eggs, and the titers were quantified by plaque-forming assay.

### Preparation of recombinant adenoviruses

A coding sequence of HA1 protein consisting of amino acid residues 62-284 of PR8 was synthesized and HA-tag was attached (TOP Gene Technologies, Montreal, Canada). This synthetic DNA was sequenced and then subcloned into pGEM-t-EASY vector (Promega, Madison, WI). The region of HA1 protein was amplified and the signal sequence of human tissue plasminogen activator was inserted into N-terminus of the fragments. The entire open reading frame was excised and inserted into pShuttle-CMV vector. Replication-defective adenoviruses (serotype 5) were generated by insertion of foreign sequences by homologous recombination and sub-

sequent purification of recombinant adenovirus (rAd) as described previously (17). HA1 fragment was separated by 4x Gly linker and HA-tag was attached at the end of the third fragment. The expression and secretion of HA1 protein fragments by rAd-infected HEK293 cells were verified by immunoblotting using mouse anti-HA-tag antibody (Roche Applied Science, Indianapolis, IN) and HRP-conjugated goat anti-mouse Ig antibody (BD Pharmingen, San Diego, CA).

### Immunization and challenge

Female Balb/c mice were purchased from Charles River Laboratories (Yokohama, Japan) and kept under specific pathogen-free conditions. For immunization, 6-week old mice were administrated with rAd ($1\times10^8$ PFU) via i.n. or s.l. route. For i.n. immunization, mice were lightly anesthetized by isoflurane inhalation, and vaccine (50 $\mu$l) was inoculated to the left nostril. For s.l. immunization, mice were anesthetized with a ketamine/rompun mixture, and vaccine (10 $\mu$l) was inoculated under the tongue. Blood was collected from the retro-orbital plexus and sera were stored at $-20^{\circ}$C. Mice were challenged intranasally with mouse-adapted influenza virus (10 LD50). Morbidity (body weight loss) and mortality were monitored daily after the challenge. Five days after the challenge a subset of mice from each group were sacrificed and tracheostomized for BAL fluid. All animal studies were approved by institutional animal care and use committee (IACUC, Approval No. 2011-01-032) in Ewha Womans University.

### ELISA

Titers of HA-specific immunoglobulin (HA-Ig) G in the sera and HA-IgA in BAL fluids were determined by direct-ELISA. Briefly, 96-well plates were coated with recombinant HA1 protein of A/PR/8/34 (Immune Technology Corp., New York, NY) in PBS, and blocked with PBS containing 1% skim milk. Sera or BAL fluids were added in serial dilutions and incubated. The plates were washed with PBS containing 0.05% Tween 20 and incubated with a HRP-conjugated goat anti-mouse total Ig (BD Pharmingen), IgG, or IgA (Zymed Laboratories, San Francisco, CA) as a secondary antibody. The plates were washed, developed with TMB peroxidase substrate (KPL, Gaithersburg, MD), stopped with 1 M $H_3PO_4$, and analyzed at 450 nm by a Thermo ELISA plate reader.

### Hemagglutination inhibition assay

HA titer for PR8 was determined, and added a fixed amount of viruses to every well of a 96-well plate, respectively. Sera from immunized mice were prepared as two-fold dilutions of each serum, and added to a 96-well plate. Then red blood cells were added and incubated for 30 minutes. The highest dilution-fold of each serum to prevent hemagglutination was measured and determined as the hemagglutination inhibition (HI) titer of the serum.

### Plaque-forming assay

Five days after challenge, mice from each group were sacrificed and the supernatants were collected from the lung tissues. Virus titers in the supernatants were determined by plaque-forming assay using Madin-Darby canine kidney cells. The data were expressed as the PFU per gram of lung tissue and the limit of detection is 200 PFU/g.

### Data analysis

All data were expressed as the mean$\pm$SD and compared by an unpaired, two-tailed Student's *t*-test. The difference was considered statistically significant when p-value$<$0.05.

## RESULTS

### Construction of rAd/HA(PR8) and expression of PR8 HA1 by the recombinant adenovirus

Influenza virus HA binds to sialic acids expressed on host cell surface, and its binding subsequently facilitates the entry of viral genome into the host cell cytoplasm via fusion of host and viral membranes. Previous studies have identified HA1 as the predominant target of neutralizing antibody responses against influenza virus infection (18). Moreover, HA-specific antibody levels have been considered to be the best correlate of protection against influenza virus infection and, hence, the primary standard for vaccine efficacy. Accordingly, as the target of our influenza vaccine development strategy, we chose HA1 of PR8 influenza virus and used rAd as the vector carrying gene encoding our target immunogen. Given the natural tropism, replication-defective rAd can be an ideal vector for delivery of vaccine antigens to the respiratory mucosa. The adenoviral DNA was engineered to express the secreted form of recombinant PR8 HA1 protein by addition of signal sequence derived from t-PA and subsequent codon optimization, resulting in the generation of rAd/HA(PR8) (Fig. 1A). In order to evaluate the expression of recombinant HA1 protein fragment in the culture supernatant of rAd/HA(PR8)-infected 293T cells, we performed immunoblotting using a

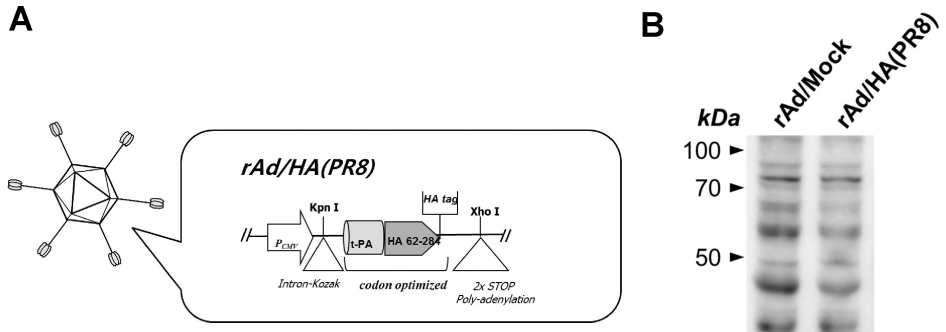


**Figure 1.** Construction of rAd/HA (PR8). (A) The adenoviral DNA was engineered to express globular head domain of HA (amino acids 62-284) using codon-optimized sequences of A/PR/8/34 virus, and contain the signal sequence derived from t-PA for efficient secretion. (B) Expression of the recombinant HA1 protein fragment in culture supernatant of rAd/HA(PR8)-infected 293T cells was detected by immunoblotting as described in the Materials and Methods.

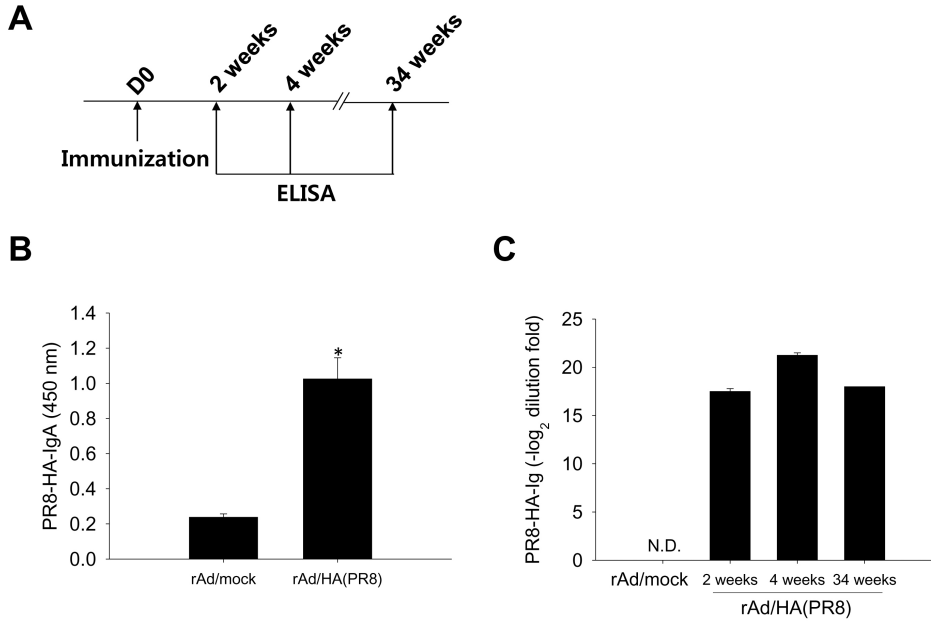


**Figure 2.** Humoral immune responses induced in rAd/HA(PR8)-immunized mice. (A) Experimental scheme. Balb/c mice were immunized once via intranasal route with rAd/HA (PR8) or control adenovirus (rAd/Mock). (B) Anti-HA IgA titer in BAL fluid was measured at 4 weeks after immunization. (C) Anti-HA antibody titers in the immune sera were determined at 2, 4 and 34 weeks after immunization. *Indicates statistical significance to "rAd/mock".

HA tag-specific antibody, and single ~25 kDa band was detected in the culture supernatant (Fig. 1B). Our results indicate that DNA sequence encoding PR8 HA1 fragment was correctly expressed and the protein was secreted from vector-infected cells.

## Induction of humoral immune responses in rAd/HA(PR8)-immunized mice

In order to determine whether our recombinant adenoviral influenza vaccine is capable of inducing antigen-specific immune response *in vivo*, Balb/c mice were given primary immunization via i.n. route with rAd/HA(PR8) or rAd/mock control adenovirus ($1\times10^8$ PFU/mouse) (Fig. 2A). Previous studies have shown that secretory IgA in the respiratory mucosa plays a critical role in the defense against incoming pathogens during respiratory tract infections (19). Therefore, we determined the levels of H1N1 HA-specific secreted IgAs in the BAL following immunization. As determined by ELISA, immunization with rAd/HA(PR8) induced significant PR8 HA-specific IgA levels in the BAL at four weeks post-prime compared to those induced by rAd/mock-immunized group (Fig. 2B). Moreover, when PR8 HA-specific total Ig levels in

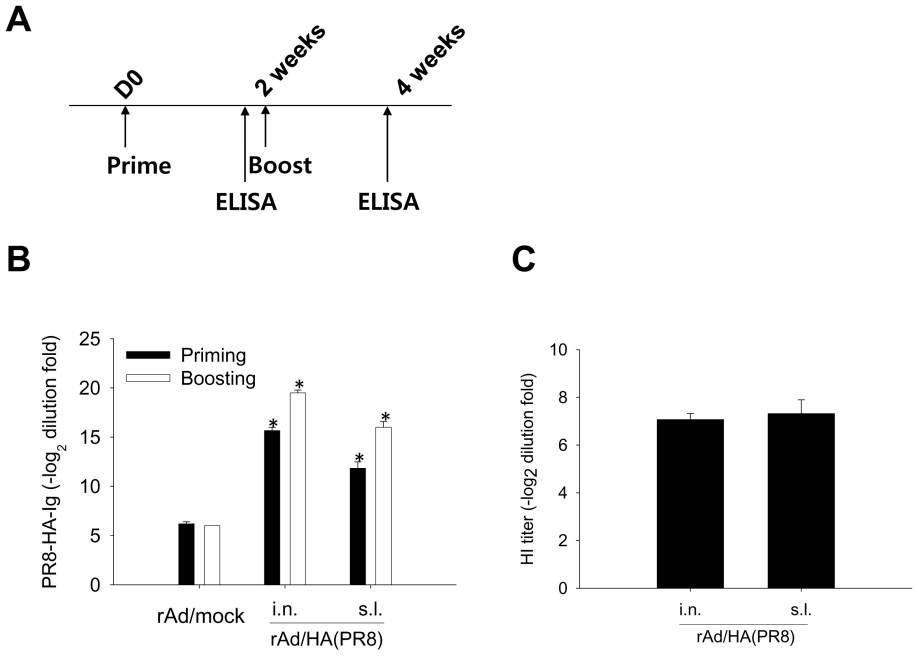


**Figure 3.** Comparison of antibody responses induced by different mucosal immunization routes. (A) Balb/c mice were immunized twice via intranasal or sublingual route with rAd/HA(PR8). (B) Anti-HA Ig titers were determined in the sera obtained from primed and boosted mice, respectively. (C) HI titers were measured in the sera obtained from boosted mice. *Indicates statistical significance to "rAd/mock".

the sera were evaluated at 2, 4, and 32 weeks post-priming, rAd/HA(PR8) immunization induced significant HA-specific systemic antibody responses (measured by serum total Ig titers) as compared to that of rAd/mock immunization at all indicated time points (Fig. 2C). Overall, our results suggest that a single immunization of rAd/HA(PR8) via i.n. route may be sufficient to elicit HA-specific mucosal IgA as well as HA-specific systemic antibody responses.

## Induction of specific immune responses by different mucosal administration routes

A previous study has shown that administration of a protein antigen via s.l. route effectively induced broad-ranged systemic and mucosal antibody responses (13). Hence, in order to compare the magnitudes of humoral responses induced by the different routes of vaccine administration, mice were given primary immunization with rAd/HA(PR8) via either i.n. or s.l. route. Further, at 2 weeks post-prime, mice were boosted accordingly the same vaccination scheme used for primary immunization (Fig. 3A). In the sera obtained from mice immunized via i.n. route, the levels of total PR8-specific Ig were significantly induced after primary as well as secondary immunization (Fig. 3B). Similarly, in the sera derived from mice that received s.l. administration of rAd/HA(PR8), considerably high levels of PR8-specific Ig were detected after primary and secondary immunization (Fig. 3B). Increased HI titers were also detected in the sera of the secondary immunized-mice via i.n. and s.l. route (Fig. 3C). These results indicate that the s.l. immunization of rAd/HA(PR8) elicits specific humoral responses at a comparable level to that induced by i.n. immunization.

## Protection against lethal influenza infection upon immunization with rAd/HA(PR8)

In order to determine the protective efficacy of the mucosal immunization with rAd/HA(PR8) against homosubtypic influenza virus infection, we determined virus titers in the lungs and weight loss of immunized mice following challenge with 10 LD50 of mouse-adapted PR8 virus. Four weeks after boosting immunization, mice were challenged intranasally with PR8 virus, and the virus titers in the lungs were determined 5 dpi by plaque assay. No detectable virus replication in the lungs of mice immunized intranasally with rAd/HA(PR8) (Fig. 4A). In contrast, high virus titers were observed in the lungs of rAd/mock-immunized mice.

Thirty-four weeks after boosting immunization, mice were challenged intranasally with PR8 virus, and weight loss of the mice was measured. Accordingly, we found no significant reduction in body weights in both groups of i.n. and s.l. immunized mice after the challenge (Fig. 4B). The results indicate

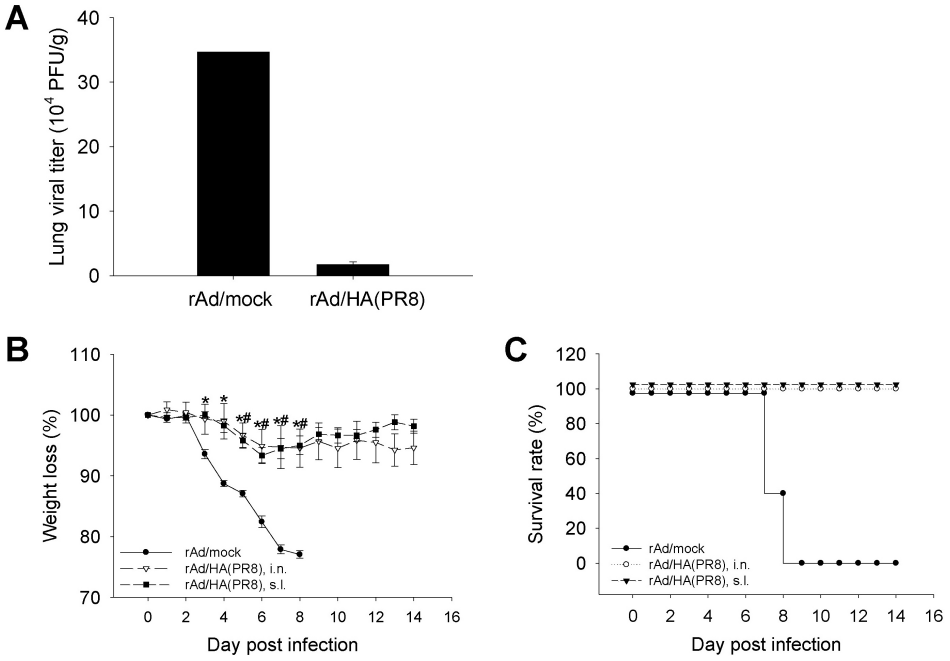


**Figure 4.** Protective efficacy of the rAd/HA(PR8) vaccine against influenza infection. Balb/c mice were immunized twice via intranasal (i.n.) or sublingual (s.l.) route with rAd/HA(PR8). (A) Virus titers in the lungs after challenge. Three weeks after last immunization, the mice immunized via i.n. route were challenged intranasally with 10 LD50 of PR8 virus and virus titers in the lung homogenates were measured by standard plaque-forming assay using MDCK cells. (B) Changes in the body weight and (C) the survival rates of the mice immunized via either i.n. or s.l. route were measured after challenge. Thirty-four weeks after last immunization, the mice were challenged intranasally with 10 LD50 of PR8 virus. *Indicates statistical significance between "rAd/mock" and "rAd/HA(PR8), i.n.". #Indicates statistical significance between "rAd/mock" and "rAd/HA (PR8), s.l.".

that immunization with rAd/HA(PR8) via i.n. or s.l. route minimized morbidity after influenza virus infection. Importantly, all mice immunized with rAd/HA(PR8) survived the lethal PR8 challenge, while all those immunized with rAd/mock did not survive the challenge (Fig. 4C).

## DISCUSSION

Our results show that mucosal (i.n. or s.l.) immunization of mice with recombinant adenovirus-based H1N1 vaccine encoding secreted forms of globular HA1 derived from PR8 influenza virus efficiently induced HA-specific antibody responses and protection against homologous virus challenge. The question as whether or not the secreted form of HA expressed in our system has similar structure of natural HA molecule is under investigation in our laboratories.

The replication-defective adenoviral vectors have been used to deliver vaccine antigen against infectious diseases and cancer, and have been proven safe in numerous clinical trials. The effectiveness of rAd-based H5N1 influenza vaccines was also demonstrated in animal models (20,21) and humans (22). However, natural pre-existing immunity against adenovirus may limit the potential of the approach. There are several options developed to circumvent this obstacle such as making modification of viral capsid proteins, use of non-human adenoviral vectors, and implementation of heterologous prime-boost vaccination scheme (23). It is also noteworthy that mucosal route has been demonstrated to be effective despite the presence of adenoviral vector immunity (22,24,25), suggesting that vector immunity could be overcome by mucosal delivery. Another virtue of replication-defective rAd-based vaccines is that they can be produced in large quantities at a relatively short time period. Thus the findings suggest that rAd-based vaccine offers an effective and timely responsive tool to combat influenza outbreaks including pandemics caused by newly emerging virus strains.

Although i.n. immunization readily induced protective immune responses, it poses a risk of directing vaccine materials into central nervous system. In this regard, s.l. delivery represents a safer alternative to the i.n. administration, while still inducing potent systemic and local mucosal immune responses. It has been shown that rAd vector efficiently transduced the s.l. epithelium (26) and s.l. delivery of rAd5-based HIV vaccines can elicit a potent antigen-specific mucosal antibody response despite preexisting vector immunity (27). Accordingly, our results demonstrate that a combination of rAd-based influenza vaccine and s.l. delivery elicited potent systemic and mucosal antibody responses and provided protection against influenza virus challenge. Thus, we propose that s.l. immunization with rAd-based influenza vaccine

promises a better future vaccination strategy to combat influenza.

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## CONFLICT OF INTEREST

The authors have no financial conflict of interest.

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