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Sublingual administration of a helper-dependent adenoviral vector expressing the codon-optimized soluble fusion glycoprotein of human respiratory syncytial virus elicits protective immunity in mice

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ABSTRACT

Sublingual (s.l.) immunization has been described as a convenient and safe way to induce mucosal immune responses in the respiratory and genital tracts. We constructed a helper-dependent adenoviral (HDAd) vector expressing a condon-optimized soluble fusion glycoprotein (sFsyn) of respiratory syncytial virus (HDAd-sFsyn) and explored the potential of s.l. immunization with HDAd-sFsyn to stimulate immune responses in the respiratory mucosa. The RSV specific systemic and mucosal immune responses were generated in BALB/c mice, and the serum IgG with neutralizing activity was significantly elevated after homologous boost with s.l. application of HDAd-sFsyn. Humoral immune responses could be measured even 14 weeks after a single immunization. Upon challenge, s.l. immunization with HDAd-sFsyn displayed an effective protection against RSV infection. These findings suggest that s.l. administration of HDAd-sFsyn acts as an effective and safe mucosal vaccine against RSV infection, and may be a useful tool in the prevention of RSV infection.

1. Introduction

As human respiratory syncytial virus (RSV) is a serious pediatric pathogen of the lower respiratory tract worldwide (Graham, 2011; Hall, 2010; Hall et al., 2009; Nair et al., 2010). The generation of an effective RSV vaccine remains a global priority (Collins and Crowe, 2007; Karron, 2008; Power, 2008). RSV target the respiratory mucosa as the portal of entry, and mucosal SIgA antibodies can preclude RSV entry into endothelial cells in the upper respiratory tract and inhibit its cell–cell syncytial spread. A critical goal of vaccine development is the induction of RSV-specific mucosal antibodies to reduce or prevent the respiratory transmission of RSV at mucosal surfaces. Recently, many studies shown that intranasal (i.n.) immunization with adenovirus vectors based RSV vaccine can elicit robust protective immune responses against RSV infection and adenovirus vectors remain a promising platform for the development of effective vaccines against RSV (Fu et al., 2009, 2010a,b; Kim et al., 2010; Kohlmann et al., 2009; Shao et al., 2009; Yu et al., 2008), indicating i.n. vaccine delivery is a promise approach to generate mucosal antibodies.

Another promising approach is sublingual (s.l.) vaccine delivery. S.l. immunization has been described as an effective novel way to induce mucosal immune responses in the respiratory and genital tracts (Appledorn et al., 2011; Cuburu et al., 2007; Domm et al., 2011; Raghavan et al., 2010; Zhang et al., 2009). S.l. immunization offers several important conceptual advantages over other vaccine delivery methods. First, it is needle-free. Second, it has a strong safety profile associated with minimal side-effects (Dahl et al., 2008, 2006; Didier et al., 2007). Third, it provides an attractive approach to mucosal immunization.

One study recently showed that the s.l. epithelium harbors a dense lattice of DCs (Cuburu et al., 2007). Another study showed that s.l. administration of live influenza virus protected mice against influenza virus challenge without redirecting the

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immunizing virus to the central nervous system (Song et al., 2008). Recently, some study reported that s.l. vaccination with Ad-based vectors vaccines induced strong antigen-specific cellular immune responses and humoral immune responses (Appledorn et al., 2011; Choi et al., 2012; Domm et al., 2011; Jun et al., 2011) without redirection of the virus to the brain (Shim et al., 2012), which were not affected by pre-existing Ad5 immunity (Choi et al., 2012; Domm et al., 2011), indicating s.l. vaccination with Ad-based vectors is a promising platform for the development of effective vaccines.

In contrast to the FGAd vector, the helper-dependent adenoviral (HDAd) vector has all the adenovirus (Ad) coding regions deleted and exhibits lower Ad-specific cellular immunity and stronger longer-term gene expression in vivo (Harui et al., 2004; Weaver et al., 2009a,b). In the present study, we explored the suitability of the s.l. route for administering HDAd vector expressing the codon-optimized soluble fusion protein (sFsyn) of RSV (HDAd-sFsyn) on specific mucosal and systemic immunity in mice. We found that s.l. delivery of HDAd-sFsyn in mice can effectively induce protection against RSV infection in association with antigen-specific cellular immune responses and humoral immune responses.

2. Materials and methods

2.1. Preparation of RSV stock

Subgroup A RSV Long strain (kindly provided by Prof. Y. Qian, Capital Institute of Pediatrics, Beijing, China) was propagated in HEp-2 cells (ATCC, Rockefeller, MD, USA) in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 2% fetal calf serum (Invitrogen), L-glutamine (2 mol/L), penicillin G (40 U/mL), streptomycin (100 μg/mL) and 0.2% sodium bicarbonate. The infectivity of the resulting RSV was titrated using the immunoenzyme assay described by Wang et al. (2010) with slight modifications. Briefly, serial 10-fold dilutions of RSV were prepared in virus diluents (EMEM with L-glutamine containing 2% FBS, 2.5% HEPES (1 mol/L) and 1% antibiotic/antimycotic, 100×). Then, 100 μl of the RSV dilutions were absorbed onto 85% confluency of HEp-2 cells in 96 well plate. Following incubation for 45 min at room temperature (RT), the medium was removed and the cells were washed with DMEM without serum. Finally, DMEM containing 1.0% carboxy methyl cellulose (Sigma, St. Louis, MO, USA) was added. Each dilution was tested in triplicate. After 3 days of incubation, the monolayers were fixed in 95% cold alcohol and viral replication on the monolayer was revealed by RSV F-specific mouse monoclonal antibody (Novocastra, Newcastle, UK), developed by incubation with horseradish peroxidase goat anti-mouse IgG (Santa Cruz, California, CA, USA), and visualized after adding TMB (Promega, Madison, WI, USA). The resultant RSV titers were expressed as plaque-forming units (pfu).

2.2. Construction of HDAd-sFsyn

For construction of HDAd-sFsyn, the coding sequence for the soluble form of RSV F (sFsyn) (amino acids 1–524 of full-length RSV F, according to GenBank database entry EF566942) was synthesized by Geneart (Regensburg, Germany). The sFsyn gene expression cassette with the cytomegalovirus (CMV) enhancer/promoter and the bovine growth hormone (BGH) polyA was cloned into the HDAd shuttle plasmid pSC11. The expression cassette was further cloned into the HDAd backbone plasmid pSC15B to produce pSC15B-sFsyn. This resulting plasmid was linearized using the restriction enzyme *Pme I* and transfected into 293Cre4 cells (293 cells expressing Cre recombinase, Microbix, Toronto, ON, Canada) using the calcium phosphate transfection method. The cells were infected with E1-E3-deleted helper virus H14 (Microbix) 16 h after transfection. HDAd-sFsyn was amplified by serial coinfection of 293Cre4 cells with helper virus and crude lysates from the previous passage. Large-scale production of HDAd-sFsyn was achieved by infecting 293Cre4 in 150-mm dishes with crude HDAd-sFsyn and helper virus, purified by CsCl banding. The viral particle number of purified HDAd-sFsyn was determined by the nucleic acid content (Ng et al., 2002). HDAd vector encoding enhance green fluorescent protein (HDAd-EGFP) was constructed as described previously and used as a control vector (Fu et al., 2010a,b).

2.3. Western blot analysis

Vero cells were lysed after 48 h following infection with HDAd-sFsyn. Protein separation was performed under reducing conditions (with 100 mmol/L 2-mercaptoethanol and boiling) on sodium dodecyl sulfate 10% polyacrylamide gels. After blotting onto nitrocellulose membranes, proteins were incubated at 4 °C over night with polyclonal antibody against RSV (AB1128) (Chemicon, Temecula, CA, USA). After washing, the membrane was incubated with horseradish peroxidase-coupled secondary antibody (Dako, Hamburg, GER) and visualized by enhanced chemiluminescence (Biozym, Hamburg, GER).

2.4. Animals

Specific pathogen-free female BALB/c mice, aged between 6 and 8 weeks, were purchased from Vital River Laboratories (Beijing, China) and kept under specific pathogen-free conditions. All animal studies were performed according to the guidelines of our Institutional Animal Care and Use Committee. The protocol was approved by the Experimental Animal Ethics Committee of Beijing jiaotong University (Permit No. 2011-0003). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.5. Immunization and challenge

BALB/c mice were lightly anesthetized with pentobarbital sodium (4 μg/kg weight), and a dosage of 1×10^8 viral particles (in 5 μl) of HDAd-sFsyn or HDAd-EGFP was delivered with a micropipette applied against the ventral side of the tongue and directed toward the floor of the mouth on weeks 0 and 4. Animals were maintained with heads placed in ante flexion for 120 s during each delivery. Three weeks after the final immunization, the mice were challenged intranasally with 50 μl of subgroup A RSV Long strain (5×10^6 PFU/ml).

2.6. Collection of splenocytes

Seven days after the final immunization, spleens from vaccinated mice were harvested and placed in mouse lymphocyte separation medium. The spleens were triturated and ground gently through cell strainers (Becton–Dickinson, San Jose, CA, USA) to obtain single-cell suspensions. The single-cell suspensions were centrifuged at 800*g* for 30 min. The splenocytes were collected and washed with complete RPMI 1640 medium (Invitrogen).

2.7. Isolation of lung mononuclear cells

Seven days after the final immunization, mice were euthanized. Lungs were perfused with 20 ml Hanks by injecting into the right ventricle of the heart, and removed and cut up into ~1 mm pieces in Hanks in sterile dish. Transfer the lung pieces into a 50 ml tube and add 10 ml of 150 U/ml Collagenase suspended in HANKS to

each lung. After incubating lung pieces in collagenase for 1 h in a 37 °C shaker incubator at 200 rpm, the lung pieces were triturated and ground gently through cell strainers (Becton–Dickinson, San Jose, CA, USA) to obtain single-cell suspensions. The single-cell suspensions were centrifuged at 1400*g* for 5 min. The lung mononuclear cells were collected and washed with complete RPMI 1640 medium (Invitrogen).

2.8. Analysis of RSV F-specific antibody production

Blood was obtained from the retro-orbital plexus with a capillary tube, and collected in an Eppendorf tube. After centrifugation (5000*g* for 15 min), sera were stored at 4 °C. The bronchoalveolar lavage (BAL) fluid was obtained by lavage with three successive 1 ml volumes of PBS from cannulation of the trachea. After centrifugation (1000*g* for 5 min), BAL were stored at 4 °C. The RSV-specific antibody responses in immunized mice were measured by ELISA, as described previously. Briefly, RSV was adsorbed onto ELISA plates overnight in carbonate buffer (pH 9.8) at 4 °C. The plates were blocked with 5% FBS in PBS for 2 h at 37 °C. After thorough washing with 1% BSA in PBS, the sera or BAL fluid samples were added to the plate and incubated for 1 h at 37 °C. The plates were washed again, and HRP-conjugated anti-mouse IgA (1:500 dilution) or IgG (1:5000 dilution) antibodies were added (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and allowed to incubate for another 1 h. The plates were developed with 100 μL of tetramethyl benzidine (TMB; Sigma, St. Louis, MO, USA) substrate solution, stopped with 50 μL of 2 mol/L H_2SO_4, and analyzed at 450 nm using an ELISA plate reader (Tecan, Grödig, Austria).

2.9. RSV-specific neutralizing antibody assay

In order to analyze RSV-specific neutralizing antibody titer, serum samples were heat-inactivated at 56 °C for 30 min. Serial two-fold dilutions of mouse sera or BAL were prepared in virus diluent (EMEM with L-glutamine containing 2% FBS, 2.5% HEPES (1 mol/L) and 1% antibiotic/antimycotic). To each serial diluted sample, 50 pfu of RSV virus suspension was added and incubated at 37 °C for 1 h. RSV-specific neutralizing antibody titers were analyzed using immunoenzyme assay described above. Neutralization titers were expressed as the reciprocal of the serum or BAL dilution giving a 50% reduction in the number of PFU number in control wells.

2.10. CD8+ T cell responses to RSV F

To enumerate the number of cytokine-producing cells, IFN-γ was performed using an ELISPOT Set (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. In brief, 2×10^5 freshly isolated lung mononuclear cells or splenocytes were added to each of three replicate wells coated with purified anti-mouse IFN-γ monoclonal antibody and stimulated with peptides F85–93, which corresponded to a known H-2 K^d-restricted RSV F protein epitope [KYKNAVTEL (Chang et al., 2001), purity ⩾ 95%], at 10 μg/ml for 24 h. Unstimulated splenocytes were used to measure background cytokine production. The cells were then lysed with deionized (DI) water, and the plates were incubated at RT with biotinylated IFN-γ antibody for 2 h and peroxidase-labeled streptavidin for 1 h. After washing with PBS, 100 μl of the final substrate solution (BD Biosciences) was added to each well and spot development was monitored. The plates were washed with distilled water to stop the reaction. IFN-γ spot-forming cells (SFC) were counted automatically using a CTL ELISPOT reader (BD Biosciences) and analyzed using ImmunoSpot image analyzer software v4.0 (BD Biosciences).

2.11. RSV titer in lungs

Mice were sacrificed on day 5 after challenge. The left lung was harvested from mice in each group, weighed, placed in sterile MEM (1 mL/0.1 g lung), and homogenized with a glass tissue grinder. The homogenates were centrifuged (10,000*g* for 1 min) and RSV titers in the supernatants were measured by immunoenzyme assay as mentioned earlier.

2.12. Statistical analyses

Statistical analyses were performed with SPSS 11.5 software (SPSS, Chicago, IL, USA). Antibody titers and viral loads are converted to log2 values. Differences were compared using an independent, two-sided Student's *t*-test, if the data meets the normal distribution. Differences were compared using a Mann–Whitney *U*, if the data do not meet the normal distribution. $P < 0.05$ was considered statistically significant.

3. Results

3.1. In vitro characterization of HDAd-sFsyn

To analyze the effect of s.l. administration of HDAd vector expressing the soluble form of RSV F on specific mucosal and systemic immunity in mice, we inserted the sFsyn gene to HDAd vector. After Vero cells were infected with HDAd-sFsyn, the expressed F protein from the infected cells was deleted by Western blot analysis under reducing conditions (with 2-mercaptoethanol and boiling), and the RSV sF protein could be detected obviously (Fig. 1).

3.2. Sublingual administration of HDAd-sFsyn induces specific systemic and mucosal antibody responses

To determine the efficacy of s.l. HDAd-sFsyn for inducing systemic and mucosal antibody responses, BALB/c mice were immunized twice at weeks 0 and 4 by the s.l. route with HDAd-sFsyn. As shown in Fig. 2A, s.l. immunization with HDAd-sFsyn stimulated strong serum IgG responses ($p < 0.05$). To confirm this result, the pooled sera from the same group of animals were diluted (1:100) and tested by Western blot analysis under reducing conditions (with 2-mercaptoethanol and boiling) (Fig. 2B). Consistent with the ELISA result, serum antibodies induced via HDAd-Fsyn could specifically recognize RSV F protein. As major component of mucosal immunity, SIgA is the first line of host defense against RSV infection and protects against infection of upper respiratory tract. Thus, we next examined SIgA responses in the respiratory tract following HDAd-sFsyn vaccination. RSV F-specific SIgA levels in BAL fluid were analyzed by ELISA. As shown in Fig. 2C, s.l.

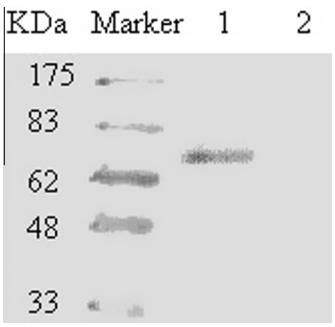

Fig. 1. Analysis of the expressed RSV F. Western blot analysis of lysates of Vero cells infected with HDAd-sFsyn (panel 1) using RSV F-specific polyclonal antibody. Vero cells infected with HDAd-EGFP (panel 2) served as negative control.

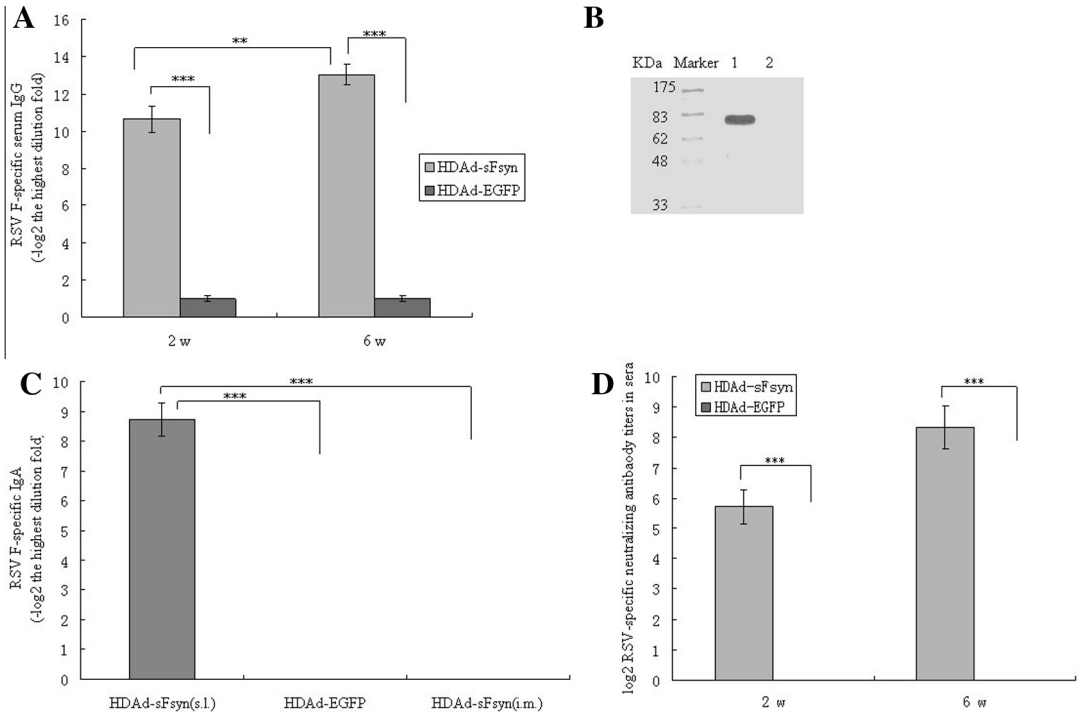

Fig. 2. Characterization of antibody responses following sublingual administration of HDAd-sFsyn in BALB/c mice. $^{***}P < 0.0001$. $^{**}P < 0.01$. $^{*}P > 0.05$. Value represents means ± SD. (A) Serum anti-RSV F IgG responses induced by HDAd-sFsyn. Six BALB/c mice were immunized sublingually with HDAd-sFsyn at weeks 0 and 4. RSV F-specific antibody titers were measured at weeks 2 and 6 after primary immunization by ELISA. The results represent $\log_2$ endpoint values for six individual mice. (B) RSV F-specific serum IgG confirmed by Western blot. Six BALB/c mice were immunized sublingually with HDAd-sFsyn at weeks 0 and 4. RSV F was performed under reducing conditions (with 100 mmol/L 2-mercaptoethanol and boiling) on sodium dodecyl sulfate 10% polyacrylamide gels. After blotting onto nitrocellulose membranes, RSV F was incubated at 4 °C over night with sera from vaccinee with HDAd-sFsyn (panel 1) or HDAd-EGFP (panel 2). Molecular weight standards were shown on the left. (C) The anti-RSV F SIgA levels in BAL fluid induced by HDAd-sFsyn. Six BALB/c mice were immunized sublingually or intramuscularly with HDAd-sFsyn at weeks 0 and 4. RSV F-specific secretary IgA antibody titers were measured on day 7 after the secondary immunization by ELISA. The results represent $\log_2$ endpoint values for six individual mice. (D) Virus-neutralizing activity of sera from vaccinee with HDAd-sFsyn. Virus-neutralizing antibody titers were analyzed on week 6 after primary immunization by immunoenzyme assay. Results were expressed as neutralizing titers that corresponded to the dilution of immune sera giving 50% inhibition of plaque formation. (E) Virus-neutralizing activity of IgA in BAL from vaccinee with HDAd-sFsyn. BALB/c mice were immunized sublingually or intramuscularly with HDAd-sFsyn at weeks 0 and 4. Virus-neutralizing antibody titers of IgA were analyzed on day 7 after the secondary immunization by immunoenzyme assay. Results were expressed as neutralizing titers that corresponded to the dilution of immune sera giving 50% inhibition of plaque formation. (F) Serum anti-Adenovirus IgG responses induced by HDAd-sFsyn. Six BALB/c mice were immunized sublingually with HDAd-sFsyn at weeks 0 and 4. Adenovirus-specific antibody titers were measured at weeks 2 and 6 after primary immunization by ELISA. The results represent $\log_2$ endpoint values for six individual mice. (G) Serum anti-RSV F IgG responses induced by HDAd-sFsyn. BALB/c mice were divided into HDAd-sFsyn (once) group and HDAd-sFsyn (prime/boost) group. HDAd-sFsyn (once) group of BALB/c mice were vaccinated with HDAd-sFsyn only once at week 0. HDAd-sFsyn (prime/boost) group of BALB/c mice were vaccinated with HDAd-sFsyn at weeks 0 and 4. RSV F-specific antibody titers were measured at weeks 2 and 6 by ELISA. The results represent $\log_2$ endpoint values for six individual mice.

immunization with HDAd-sFsyn generated strong mucosal SIgA responses ($p < 0.05$), but intramuscular immunization with HDAd-sFsyn didn't generate mucosal SIgA responses ($p > 0.05$). The neutralizing antibodies in the serum are highly related to protective role on the lower respiratory tract against RSV infection. We therefore measured neutralizing activity of the sera from mice vaccinated with HDAd-sFsyn by immunoenzyme assay. As shown in Fig. 2D, sera from mice s.l. immunized with HDAd-sFsyn displayed RSV-specific neutralizing activity ($p < 0.05$). We also measured neutralizing activity of the BAL from mice vaccinated with HDAd-sFsyn by immunoenzyme assay. As shown in Fig. 2E, BAL from mice s.l. immunized with HDAd-sFsyn displayed RSV-specific neutralizing activity ($p < 0.05$), but BAL from mice intramuscularly immunized with HDAd-sFsyn did not display RSV-specific neutralizing activity ($p > 0.05$). To evaluate the immune response against the vector itself, the levels of Ad-specific antibody were analyzed at weeks 2 and 6 after BALB/c mice had been immunized sublingually. We found that HDAd-sFsyn or HDAd-EGFP induced similar anti-Ad immune responses after prime/boost immunization (Fig. 2F) ($p > 0.05$). To test whether a second dose of an HDAd vector of the RSV F protein enhanced antibody titers with s.l. immunization, groups of BALB/c mice were vaccinated with a dosage of 1×10^8 viral particles (in 5 μl) of HDAd-sFsyn. Mice were given s.l. booster immunizations 4 weeks later with HDAd-sFsyn at the same dose used for priming. Serum antibody responses to RSV were analyzed 2 and 6 weeks later. As shown in Fig. 2G, the s.l. booster immunization enhanced serum antibody responses ($p > 0.05$).suggested an advantage for the homologous s.l. booster immunization.

3.3. Induction of cellular immune responses

The RSV-specific CD8+ T-cell responses are universally recognized as playing a major role in virus clearance. Therefore, we investigated whether the HDAd-sFsyn could generate IFN-γ producing CD8+ T cells after *in vitro* stimulation with a known H-2 K^d-restricted RSV F protein epitope. As shown in Fig. 3, the HDAd-sFsyn indued CD8+ T-cell immune responses were evident, as compared with the control group ($p < 0.05$), indicating sublingually administered HDAd-sFsyn is competent in producing cellular immune responses against transgene.

3.4. Protective efficacy of HDAd-sFsyn against RSV challenge

In order to protective efficacy of HDAd-sFsyn vaccines in vivo, the vaccinated mice were challenged with RSV intranasally 3 weeks after boost immunization. Five days after challenge, mice were sacrificed and lung homogenates were prepared. As shown in

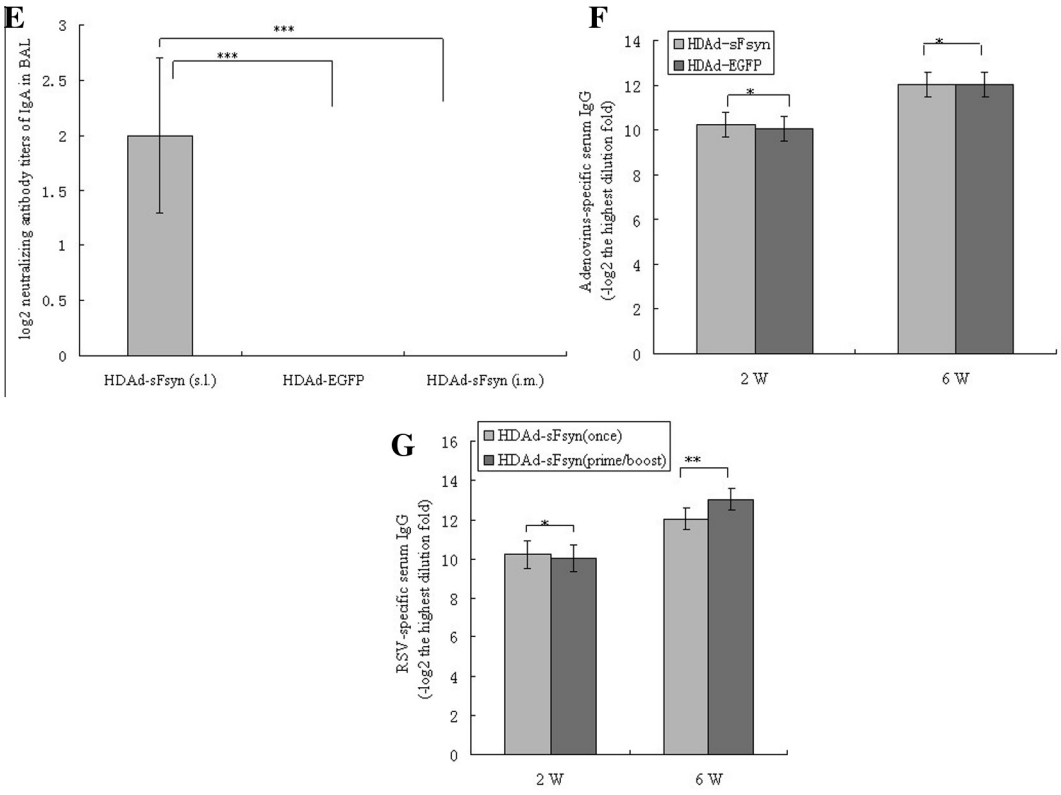

Fig. 2 (*continued*)

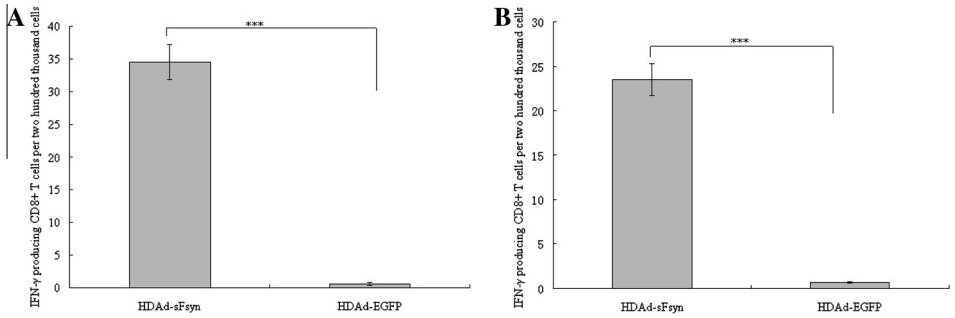

Fig. 3. CD8+ T-cell responses after immunization with HDAd-sFsyn. Six BALB/c mice were immunized sublingually with HDAd-sFsyn at weeks 0 and 4. RSV F-specific CD8+ T-cell responses were assessed on day 7 after the secondary immunization by ELISPOT. (A) Results were expressed as the average number of IFN-γ producing CD8 + T cells two hundred thousand cells input splenocytes. ***$P < 0.0001$. (B) Results were expressed as the average number of IFN-γ producing CD8+ T cells two hundred thousand cells input lung mononuclear cells. ***$P < 0.0001$.

Fig. 4, the lung viral titer of the HDAd-sFsyn-immunized group was significantly reduced compared with the control group ($p < 0.05$).

3.5. Durability of serum antibody responses after immunization

The strength of an efficient vaccine is based also on the persistence of immune responses able to block the infection. To determine the duration of immune response induced by HDAd-sFsyn, we examined the humoral immune responses at 2-week intervals up to 14 weeks following single vaccination with HDAd-sFsyn. As shown in Fig. 5, specific IgG titers are long-lasting ($p < 0.05$).

4. Discussion

RSV is one of the most common respiratory tract pathogens. All of serum IgG, mucosal SIgA and $CD8^+$ T-cell responses play a role in protection RSV infection (Graham, 2011; Kim et al., 2010; Srikiatkhachorn and Braciale, 1997). It is known that neutralizing antibodies in serum are highly related to protection against RSV infection in the lower respiratory tract. The RSV-specific CD8+ T-cell response is universally recognized to play a major role in clearance of virus and regulating differentiation and activation of Th2 CD4+ cytokines and eosinophil recruitment into the lungs in RSV-infected mice. Mucosal SIgA is important for the prevention of RSV infection by limiting viral replication at the site of entry. So, an ideal vaccine against RSV can induce systemic and mucosal immune responses and provide protection against this mucosal pathogen. In this study, we show that s.l. immunization with HDAd-sFsyn provided substantial protection against RSV infection in the respiratory tract that is associated with RSV-specific T and B cell responses. Our results show that s.l. immunization is an efficient route of mucosal immunization that can be used to induce immune responses and protection responses against RSV infection.

Intranasal immunization with adenovirus vectors based RSV vaccine can elicit robust protective immune responses against

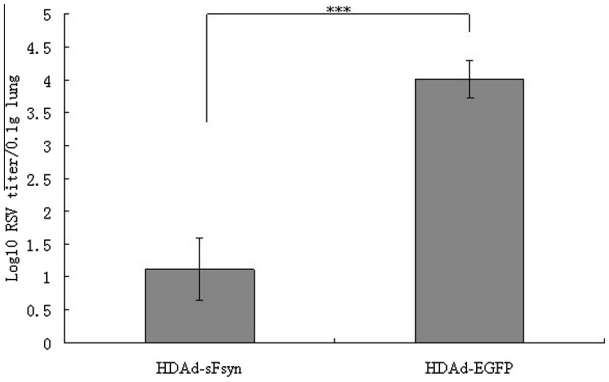

Fig. 4. Lung viral titers after RSV challenge. Six BALB/c mice were immunized sublingually with HDAd-sFsyn at weeks 0 and 4 and then challenged 3 weeks after the final immunization with intranasal administration of 50 μL of subgroup A RSV strain Long (5×10^6 pfu/mL). $^{***}P < 0.0001$. Value represents means ± SD.

RSV infection, and adenovirus vectors remain a promising platform for the development of effective vaccines against RSV (Fu et al., 2009, 2010a,b; Graham, 2011; Kohlmann et al., 2009; Shao et al., 2009; Yu et al., 2008). However, several studies have reported that intranasal immunization with adenovirus vectors diffuses through the perineural space as a result of retrograde passage through the olfactory epithelium (Damjanovic et al., 2008; Lemiale et al., 2003). In this regard, sublingual administration may be a more promising vaccination route than the intranasal route (Song et al., 2008). This situation prompts us to explore the immune responses and protective effect following s.l. vaccination with HDAd vector vaccine.

Our data indicate that s.l. vaccination with HDAd-sFsyn vaccine induces RSV-F specific cellular, humoral and mucosal immune responses. The capacity for s.l. vaccination with HDAd-sFsyn to induce such a response is most likely explained as follows. (i) Both of migratory CD8α$^-$ and resident CD8α$^+$ DCs in the s.l. mucosa after challenge of HDAd-sFsyn are responsible for the efficient priming on Ag-specific T and B cells in the cervical lymph nodes (Song et al., 2009). (ii) DCs regulated by CCR7 and its ligands, CCL19 and CCL21 play an indispensable role of induction of efficient Ag-specific systemic IgG and mucosal IgA responses by s.l. challenge (Song et al., 2009). (iii) Adenoviral capsid components such as hexon capsomers, fiber knob and RGD motif in the penton protein, are all recognized as potent adjuvants of adenovirus vectors for activating immune responses against viral and transgene proteins (Molinier-Frenkel et al., 2002, 2003; Philpott et al., 2004; Yamaguchi et al., 2007). After RSV challenge, s.l. vaccination with HDAd-sFsyn vaccine displays a protective role against RSV infection in a murine model. This situation is attributed to to RSV-specific CD8+ T-cell resonces, mucosal neutralizing antibodies and long-lasting serum neutralizing antibodies induced by s.l. vaccination with HDAd-sFsyn, indicating that the measure of s.l. vaccination with HDAd-sFsyn is very efficient and convenient way.

In contrast to the FGAd vector, the HDAd vector has all the adenovirus (Ad) coding regions deleted. We found that immunization by s.l. vaccination with HDAd-sFsyn resulted in both anti-Ad responses and immune responses against transgenes. To test whether a second dose of an HDAd vector of the RSV F protein enhanced antibody titers with s.l. immunization, BALB/c mice were given s.l. booster immunizations 4 weeks later with HDAd-sFsyn at the same dose used for priming. Although prime immunization by s.l. vaccination with HDAd-sFsyn resulted in anti-Ad responses, boost immunization by s.l. vaccination with HDAd-sFsyn still enhanced the immune responses against transgenes suggested an advantage for the homologous s.l. booster immunization. This result correspond to the i.n vaccination with adenovirus vector vaccines, suggesting mucosal vaccinations, including s.l. immunization, are able to avoid interference from serum anti-Ad antibodies (Appledorn et al., 2011; Belyakov et al., 1999).

Long-lasting protective immunity is indispensable for a potential vaccine. The previous study showed i.n. vaccination with HDAd vector vaccine could evoked RSV-F-specific serum neutralizing antibody responses lasting for at least 14 weeks. We also detected the duration of immune response induced by s.l. vaccination with HDAd-sFsyn, we found that RSV-F-specific IgG titers are long-lasting after s.l. vaccination with HDAd-sFsyn. Four mechanisms may be responsible for the long-lasting protective immunity: (i) HDAd vector has all adenovirus (Ad) coding regions deleted and could not produce adenoviral protein in vivo, and displays much more reduced Ad-specific T-cell responses (Weaver et al., 2009a). (ii) This reduced Ad-specific cellular immunity allows for transgene expression durable in vivo (Weaver et al., 2009a). (iii) HD-Ad vectors, all viral genes are deleted eliminating expression of potentially toxic viral protein in transduced cells (Weaver et al., 2009a). (iv) HD-Ad vectors achieve a high level of transgene protein in infected dendritic cells (DCs) (Roth et al., 2002).

Recently, Huo observed that sublingual immunization generally induced a similar pattern of immune responses to intramuscular, but at much lower magnitudes (Huo et al., 2012). This study showed that s.l. vaccine in humans are not promising. In fact, we also found intramuscular immunization in mice induced higher

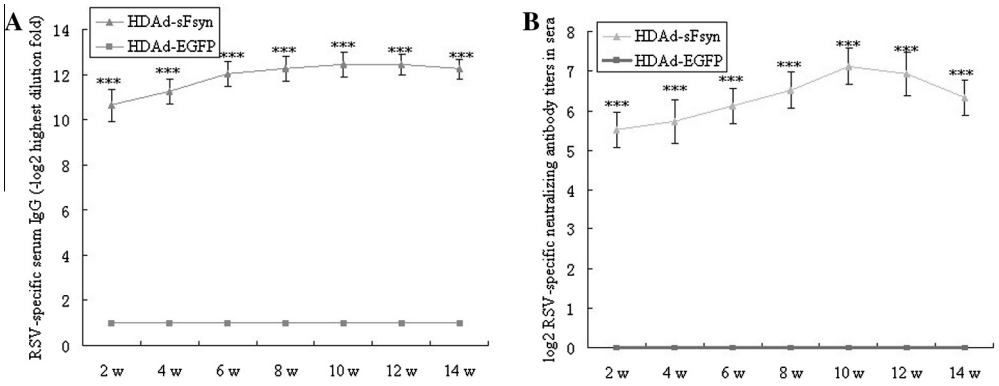

Fig. 5. Duration of the immune responses stimulated by single sublingual administration of HDAd-sFsyn in BALB/c mice. (A) Six BALB/c mice were immunized sublingually with one dose of HDAd-sFsyn in week 0. RSV F-specific antibody titers were measured by ELISA at 2-week intervals up to 14 weeks after immunization. $^{***}P < 0.0001$. Values represent mean ± SD. (B) Virus-neutralizing activity in sera from mice immunized with HDAd-sFsyn. Virus-neutralizing titers in sera were measured by immunoenzyme assay at 2-week intervals up to 14 weeks after immunization. Results are expressed as neutralizing titers corresponding to the serum dilution giving 50% inhibition of plaque formation.

serum neutralizing antibodies and CD8+ T cell responces compared with s.l. immunization at the same dosage(data not showen), but intramuscular immunization in mice could not induce mucosal SIgA in BAL compared with s.l. immunization inducing mucosal neutralizing antibodies in BAL. Intramuscular vaccination with adenovirus-based vector vaccine could provided protective immunity against RSV infection mediated through serum neutralizing antibodies and CD8+ T cell responces (Johnson et al., 2014). Though results from s.l. vaccine are not promising, s.l. vaccine still have some advantages. Firstly, sublingual route is a needle-free option. Secondly, the sublingual route may have a role in boosting pre-existing immunity induced by another or same route, and is capable of inducing functional antibody (Huo et al., 2012). Thirdly, the sublingual route induces mucosal immune protection against viral infections.

In summary, this study has provided evidence that s.l. vaccination with HDAd-sFsyn provides an effective means of inducing both mucosal as well as systemic immune responses. Furthermore, RSV F-specific immune responses induced by s.l. vaccination with HDAd-sFsyn provide protective immunity against RSV infection. These findings suggest that s.l. vaccination with HDAd-sFsyn provides a foundation for further primate testing, and may lead to the development of effective and safe vaccines against RSV infection.

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