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Intranasal vaccination with a helper-dependent adenoviral vector enhances transgene-specific immune responses in BALB/c mice

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ABSTRACT

Helper-dependent adenoviral (HDAd) vectors were developed primarily for genetic disease therapy by deleting all coding regions for attenuating the host cellular immune response to adenovirus (Ad) and long-lasting gene expression. Recently Harui et al. reported that HDAd vaccine could stimulate superior transgene-specific cytotoxic T lymphocyte (CTL) and antibody responses via the intraperitoneal route, compared to first-generation adenoviral (FGAd) vaccine. This prompted us to explore the potential of HDAd as a vaccine vector administrated intranasally. In this study, we prepared HDAd and FGAd vectors expressing enhanced green fluorescent protein (EGFP), respectively, and compared their efficacy in mice. Mice were immunized intranasally with 5×10^9 vp HDAd or FGAd vector particles. Despite stimulating similar anti-Ad antibody responses with FGAd vaccine in the prime/boost strategy, HDAd vector expressing EGFP displayed superior transgene-specific serum IgG, mucosal IgA and cellular immune response, with the characterization of balanced or mixed Th1/Th2 CD4+ T-cell responses. Meanwhile, a single dose of intranasal (i.n.) vaccine of HDAd–EGFP induced a serum IgG response with more efficacy than FGAd–EGFP. In addition, i.n. boost immunization enhanced transgene-specific humoral and cellular responses, compared to single i.n. HDAd–EGFP immunization. Our results suggest that HDAd has potential for a mucosal vaccine vector via i.n. route, which will be useful for the development of vaccines against respiratory viruses, such as respiratory syncytial virus and influenza virus.

Introduction

Vaccines remain the most efficacious medical intervention to reduce mortality and morbidity due to viral infections. As viruses possess the ability to deliver genes into cells, live viruses have been reasonably used as vaccine vectors. Among them, a replication-deficient first-generation adenoviral (FGAd) vector is considered to be an effective vaccine vector. Many studies have shown that FGAd vaccines can elicit both specific systemic and mucosal immune responses against viral pathogens such as influenza virus, avian influenza virus and human respiratory syncytial virus (RSV) [1–4]. It has also been shown that intranasal (i.n.) FGAd vaccines are highly effective at inducing neutralizing antibodies and protective immunity despite the presence of preexisting anti-adenoviral antibodies [2,3,5]. However, the weak immunogenicity of FGAd vaccines is still a major confronted problem. Therefore, developing safer, more efficient vaccine vectors remains a priority worldwide.

In contrast to FGAd vector, helper-dependent adenoviral (HDAd) vector has all adenovirus (Ad) coding regions deleted, and displays much more reduced Ad-specific cellular immunity [6–8] and stronger, longer-term gene expression *in vivo* [9,10]. Over the last decade, HDAd has been extensively used as a vector for gene therapy. However, it was only successfully reported as a vaccine vector in 2004 by Harui et al. [11] who found that HDAd vaccine could stimulate superior transgene-specific cytotoxic T lymphocyte (CTL) and antibody responses via the intraperitoneal route, compared to FGAd vaccine. More recently, it was also demonstrated that HDAd vaccine administered via the intramuscular or intravenous routes generated more potent transgene immune responses and lower side effects than FGAd vaccine [12]. Therefore, HDAd may represent a potent and safe vaccine vector. Because most viral pathogens target the respiratory mucosa as the portal of entry, a critical goal of vaccine development is the induction of both serum IgG and cellular responses and strong, lasting mucosal immunity. It is well known that mucosal FGAd vaccines

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encoding viral antigens can induce strong serum IgG and mucosal IgA responses [2,13]. In contrast, little is known about the potential for HDAd as an i.n. vaccine vector to stimulate transgene-specific immune responses. Therefore, the immunogenicity and efficacy of i.n. HDAd vaccine were evaluated in this study.

Enhanced green fluorescent protein (EGFP) was used in this study as a transgene to clarify the real efficacy of HDAd as a mucosal vaccine vector. It was ideal for this role as it is not only an attractive reporter protein, but it is also a model antigen possessing B lymphocyte, CD4+ Th lymphocyte and CD8+ CTL epitopes. It also displays no side effects of toxicity or immune inhibition [12,14,15].

In this study, mice were immunized intranasally with either HDAd or FGAd vaccine, both containing the same transgene of EGFP, and monitored for the induction of EGFP-specific serum IgG, mucosal IgA, cellular immune responses and anti-Ad immunity. This is the first report that i.n. HDAd vaccine is highly effective at stimulating highly effective transgene-specific systemic and mucosal immunity, which will be useful for the development of vaccines against respiratory viruses, such as respiratory syncytial virus and influenza.

Materials and methods

Construction of FGAd–EGFP and HDAd–EGFP. FGAd–EGFP was constructed by co-transforming adenoviral shuttle plasmid pAdTrack and backbone plasmid AdEasy-1 into BJ5183 cells (pAdTrack, pShuttle-CMV, AdEasy-1 and BJ5183 are gifts from Prof. Bert Vogelstein, John Hopkins Oncology Center, Baltimore, MD, USA). After homologous recombination between pAdTrack and AdEasy-1 in BJ5183 cells, a pre-adenoviral plasmid encoding EGFP was produced. The resulting plasmid of pFGAd–EGFP was linearized by digestion with restriction enzyme PacI and transfected into 293 packaging cells to generate FGAd–EGFP. For the construction of HDAd–EGFP, the EGFP open reading frame (ORF) was amplified by PCR from pAdTrack and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). After sequence analysis, the EGFP gene expressing cassette with the cytomegalovirus (CMV) enhancer/promoter and the bovine growth hormone (BGH) polyA was cloned into the HDAd shuttle plasmid, pSC11 [16]. Then, the expressing cassette was cloned further into pSC15B [16] to produce pSC15B–EGFP. This resultant plasmid was linearized using restriction enzyme PmeI, and transfected into 293Cre4 cells (293 cells expressing Cre recombinase, Microbix, Canada) using the calcium phosphate transfection method [17]. The cells were infected by helper virus H14 (Microbix, Canada) 16 h after transfection. HDAd–EGFP was amplified by serial co-infecting 293Cre4 cells by helper virus and the crude lysates from the previous passage until it reached a plateau of amplification by monitoring EGFP expression. The Cre/loxP method to generate HDAd was developed by Graham and co-workers [18]. The HDAd–EGFP and FGAd–EGFP were purified by CsCl ultracentrifugation. The viral particle number (vp) of purified HDAd–EGFP and FGAd–EGFP was determined by the nucleic acid content [19].

Animals. Specific pathogen-free female BALB/c mice, aged between 6 and 8 weeks, were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, 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.

Immunization. Twenty-four BALB/c mice were used for immunization experiments and divided into four groups of HDAd–EGFP (once), FGAd–EGFP (once), HDAd–EGFP (prime/boost) and FGAd–EGFP (prime/boost). 5×10^9 vp of either HDAd–EGFP or FGAd–EGFP were delivered via the i.n. route either once (at week 0) or twice (at weeks 0 and 3).

Collection of splenocytes. Spleens were harvested from vaccinated mice 3 weeks after primary immunization 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. Then, splenocytes were collected and washed with complete RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA).

Collection of mucosal secretions. Mucosal secretions were harvested from vaccinated mice on day 28 as previously described [20] with minor modifications. Three successive 20-μl samples of PBS containing 0.1% BSA were instilled into the vagina and absorbed secretions from both the exocervix and vagina were collected. The secretions were centrifuged at 10,000*g* for 5 min, and the supernatant was analyzed for secretary IgA (sIgA) by ELISA.

Preparation of lung and intestinal homogenates. The vaccinated mice were sacrificed on day 28. Lung and intestinal homogenates were prepared and used to measure mucosal immune responses as previously described [21] with minor modifications. The left lungs and 20-cm sections of the small intestines from vaccinated mice in each group were harvested, washed in PBS three times, weighed, placed in a PBS solution (0.1 ml per 0.1 g tissue) containing 0.1% BSA, and homogenized with a glass tissue grinder. The homogenates were centrifuged (10,000*g* for 5 min) and the supernatant was analyzed for sIgA by ELISA.

Analysis of EGFP-specific antibody production. Blood was obtained from the retro-orbital plexus with a capillary tube at days 0, 14 and 28 as previously described [4], After centrifugation (5000*g* for 15 min), serum was stored at −20 °C. EGFP-specific antibodies were analyzed by ELISA. Then, 70 ng of purified EGFP (purity of the protein ⩾95%) were adsorbed onto ELISA plates overnight in carbonate buffer (pH 9.8) at 4 °C. The plates were blocked with 1% (w/v) BSA in PBS at 37 °C for 2 h. Samples were added to the wells and incubated for 1 h at 37 °C. The plates were washed again, and HRP-conjugated anti-mouse IgA or IgG antibodies were added (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and allowed to incubate for 1 h. Finally, the plates were washed and developed with 100 μl 3,3,5,5-tetramethylbenzidine (TMB; Sigma, St. Louis, MO, USA) substrate solution. The reaction was stopped with 50 μl of 2 M H_2SO_4 and analyzed at 450 nm with a Thermo ELISA plate reader (Bio-Rad 550, Hercules, CA, USA).

For the analysis of IgG1 and IgG2a, standard curves were first determined using standard preparations of purified IgG1 or IgG2a (Sigma, St. Louis, MO) of known concentration. The amounts of IgG1 and IgG2a were calculated by OD450 readings onto standard curves, and the ratio of IgG2a/IgG1 isotype response was calculated according to the amounts of IgG2a and IgG1.

Analysis of anti-adenoviral serotype 5 (Ad5) immunity. The anti-Ad5 serum IgG antibody was analyzed by indirect ELISA as described in the previous section (analysis of EGFP-specific antibody). The microtiter plates were coated 100 μl of 1×10^8 vp/ml purified Ad5 (propagated in 293 cells and purified by CsCl ultracentrifugation) at 4 °C overnight.

Lymphocyte proliferation test. Lymphocyte proliferation was measured with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) as previously described [22]. Triplicate cultures of 5×10^4 spleen cells/well were incubated in complete RPMI 1640 medium with EGFP (0.1 μg/well) for 72 h. At the same time, an un-stimulated culture was set up as a control. Ten microliters of CCK-8 solution was added to each well of the plate. The plate was incubated for 3 h. Absorbance at 450 nm was measured with a microplate reader (Bio-Rad 550). The stimulation index of each culture was calculated as follows: SI = OD of stimulated culture/OD of un-stimulated culture.

Statistical analysis. Statistical analysis was performed using SPSS 11.5 software (SPSS, Chicago, IL, USA). Comparison of differences

was conducted using an unpaired, two-tailed Student's *t*-test. $P < 0.05$ was considered significant.

Results

HDAd–EGFP induces higher EGFP-specific humoral immunity than FGAd–EGFP

To assess serum and mucosal immune efficiency induced by two different Ad vaccine vectors, EGFP-specific serum IgG and mucosal sIgA were measured by ELISA (Fig. 1). EGFP-specific serum IgG responses were higher in mice vaccinated with HDAd–EGFP than with FGAd–EGFP in a homologous boost regime, 1 week after the final immunization ($P < 0.05$) (Fig. 1A). Intranasal HDAd–EGFP homologous boost immunization enhanced EGFP-specific serum antibody response, compared to single immunization at 1 week after the final immunization ($P < 0.05$) (Fig. 1A). Additionally, as shown in Fig. 1A, the transgene-specific serum IgG induced by single HDAd–EGFP vaccination continually increased, at least until 4 weeks post-immunization, in contrast to the antibody level induced by single FGAd–EGFP vaccination. Mucosal EGFP-specific sIgA levels in vagina, lung and intestinal secretions were also analyzed at week 4. As shown in Fig. 1B, i.n. HDAd–EGFP homologous boost immunization enhanced mucosal antibody responses, compared to both FGAd–EGFP homologous boost immunization and HDAd–EGFP single immunization.

HDAd–EGFP induces higher EGFP-specific cell-mediated immunity than FGAd–EGFP

To assess transgene-specific cellular immune responses, BALB/c mice were immunized intranasally by FGAd–EGFP or HDAd–EGFP at weeks 0 and 3. Spleen cells were harvested from vaccinated mice 1 week after the final immunization and incubated in complete RPMI 1640 medium with purified EGFP (0.1 μg/well) for 72 h, and lymphocyte proliferation was measured with CCK-8. HDAd–EGFP stimulated stronger specific lymphocyte proliferation than FGAd–EGFP ($P < 0.05$) (Table 1).

Both FGAd–EGFP and HDAd–EGFP stimulate potent anti-Ad immune responses

To evaluate the immune response against the vector itself, the levels of Ad-specific antibody were analyzed at weeks 0 and 3 after BALB/c mice had been immunized intranasally with FGAd–EGFP or HDAd–EGFP. We found that HDAd–EGFP and FGAd–EGFP induced similar anti-Ad immune responses after prime/boost immunization (Fig. 2).

Table 1
The specific lymphocyte proliferation responses of HDAd–EGFP or FGAd–EGFP immunized mice stimulated with EGFP *in vitro*.

	Immunogens	
	FGAd–EGFP	HDAd–EGFP
SI	1.12 ± 0.11	1.33 ± 0.12

Abbreviation: SI, stimulation index: OD of stimulated culture/OD of un-stimulated culture. Results are expressed as means ± SD. Each group contained six mice. $P < 0.05$, compared with FGAd–EGFP.

Intranasal HDAd–EGFP boost immunization enhances cellular responses to EGFP

To test the ability of i.n. HDAd–EGFP homologous boost immunization to enhance cellular immunity, 5×10^9 vp of either HDAd–EGFP were delivered to mice via the i.n. route either once (at week 0) or twice (at weeks 0 and 3). We found that boost immunization with HDAd–EGFP induced stronger EGFP-specific cellular immune responses (Table 2).

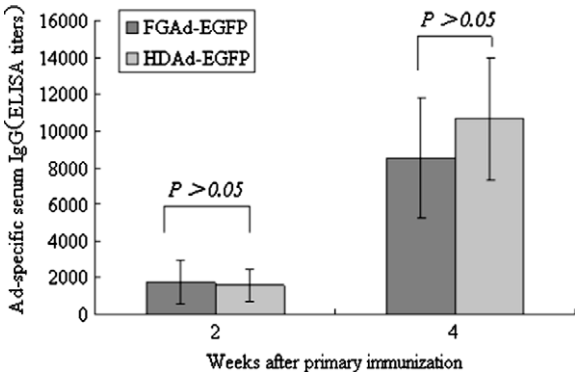

Fig. 2. Anti-adenovirus antibody responses induced by intranasal administration of HDAd–EGFP and FGAd–EGFP. BALB/c mice were intranasally immunized by FGAd–EGFP or HDAd–EGFP at weeks 0 and 3, and serum adenovirus-specific IgG was analyzed at weeks 2 and 4 by ELISA. ELISA titers were expressed as the reciprocal of the final dilution.

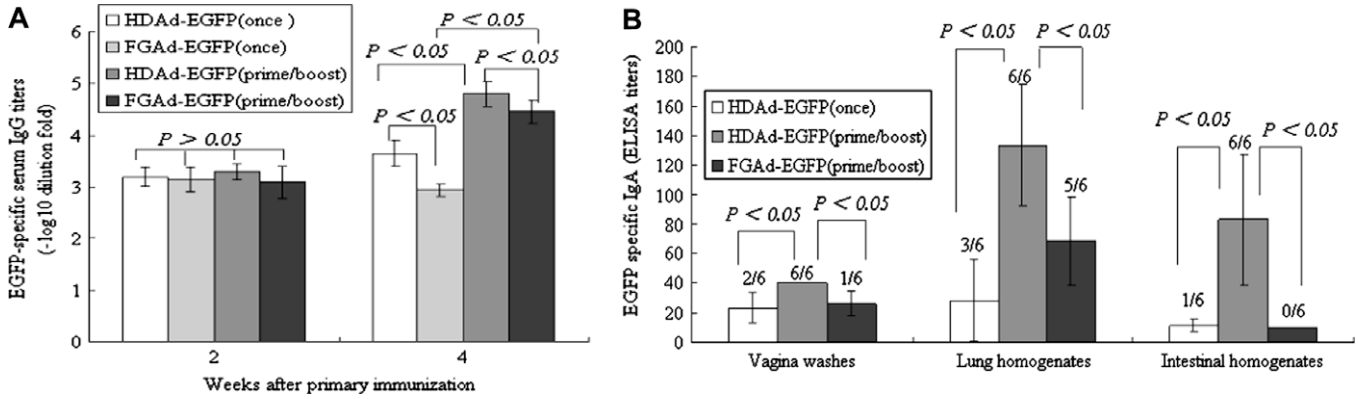

Fig. 1. Antibody responses induced by intranasal administration of HDAd–EGFP and FGAd–EGFP vaccines in BALB/c mice. BALB/c mice were immunized intranasally with FGAd–EGFP (once) or HDAd–EGFP (once) at week 0 and FGAd–EGFP (prime/boost) or HDAd–EGFP (prime/boost) at weeks 0 and 3. (A) The serum anti-EGFP IgG induced by HDAd–EGFP and FGAd–EGFP. EGFP-specific antibody titers were measured at weeks 2 and 4 after primary immunization by ELISA. The results represent log 10 end point values from six individual mice. (B) The anti-EGFP secretary IgA levels in vagina washes, lung homogenates and intestinal homogenates induced by HDAd–EGFP (once), HDAd–EGFP (prime/boost) and FGAd–EGFP (prime/boost). EGFP-specific antibody titers were measured 1 week after the final immunization by ELISA and expressed as the reciprocal of the final dilution.

Table 2
The specific lymphocyte proliferation responses to EGFP by single or prime/boost intranasal HDAd immunization.

	HDAd–EGFP	
	Once	Prime/boost
SI	1.06 ± 0.07	1.33 ± 0.02

Abbreviation: SI, stimulation index: OD of stimulated culture/OD of un-stimulated culture. Results are expressed as means ± SD. Each group contained six mice. $P < 0.05$, compared to single intranasal HDAd immunization group.

Serum IgG subclass analysis

Since the ratio of IgG2a/IgG1 isotypes is an indicator of Th1 versus Th2 cellular responses, the EGFP-specific IgG subclass was analyzed further by indirect ELISA. Significantly high titers of both IgG1 and IgG2a were observed following administration of FGAd–EGFP and HDAd–EGFP. The resulting ratios of IgG2a/IgG1 from FGAd–EGFP and HDAd–EGFP immunized mice were very close to 1 (Table 3), which showed balanced or mixed Th1 and Th2 cellular responses.

Discussion

HDAd vectors have the advantages of improved safety, stable and prolonged transgene expression, which makes them suitable candidates as vectors for gene therapy [12,18,23]. Recently, HDAd vaccines have been reported that are capable of stimulating superior transgene-specific immune responses via the intraperitoneal, intramuscular and intravenous routes, compared to FGAd vaccines [11,12]. The ability of mucosally applied HDAd vaccines to induce systemic and local immunity against transgenes has not yet been examined. We have been focusing on the roles played by mucosally delivered vaccines such as FGAd vaccine and attenuated *Salmonella* SL7207-based DNA vaccine in inducing protective immunity against RSV [4,22]. Therefore, in this study, we investigated whether HDAd vectors are suitable mucosal vaccine vectors to be used in the development of vaccines against a range of mucosal pathogens by comparing FGAd vaccine. We observed that in BALB/c mice administered with an HDAd vaccine encoding EGFP, stronger EGFP-specific serum and mucosal antibody responses and lymphocyte proliferation responses were generated than with an i.n. FGAd vaccine. Similar Ad-specific antibody responses were observed between these two vectors. These data indicate that the i.n. HDAd vaccine generates a strong immune response against an immunogen. Our results are consistent with previous reports [11,12].

The conventional two doses of i.n. HDAd–EGFP vaccine stimulated much higher EGFP-specific serum IgG, mucosal sIgA and cellular immune responses than the same amount of FGAd–EGFP vaccine, confirming that HDAd vaccine produces higher transgene-specific immune responses than FGAd. In addition, we found that the i.n. HDAd–EGFP vaccine was superior to the FGAd–EGFP vaccine at inducing both local and atopic mucosal immune responses. We also compared the magnitude of EGFP-specific immune responses by single vaccination and homologous prime–boost regimes. We demonstrate that the serum IgG is elevated significantly by the prime–boost regime with both the i.n. HDAd–EGFP and i.n. FGADd–EGFP vaccines, compared with single vaccination. Additionally, these two vectors induced similar serum Ad-specific immune responses. We speculate that the underlying mechanisms responsible for the above transgene-specific immunity observed were (i) HDAd vaccine induces longer-term transgene expression *in vivo*, a higher level of transgene protein in dendritical cells (DC) [11,23,24] and lower anti-Ad T-cell responses as reported previously [12]; (ii) mucosal vaccinations, including i.n. immunization, are able to avoid interference from serum anti-Ad antibodies [2,13,25] and (iii) the viral capsid (e.g., fiber knob, penton base and hexon components) is recognized as a potent adjuvant for eliciting transgene-specific immune responses [19,26,27].

Generally, single dose vaccination avoids repeated administration and is more easily to be accepted by vaccinated subjects. Hence, EGFP-specific immunity stimulated by a single dose of i.n. HDAd–EGFP and FGAd–EGFP vaccine was also evaluated. As expected, the HDAd–EGFP vaccine evoked a more potent EGFP-specific antibody response than an equivalent dose of FGAd–EGFP, indicating that HDAd is a more attractive mucosal vaccine vector than FGAd.

Natural immunity to Ad5 and enhancement of this immunity after repeated administration of an Ad5-based FGAd vaccine could potentially reduce vaccine efficacy [1,13,28]. Although HDAd vector has all of its gene coding regions deleted, it has been packaged by the same viral capsid of Ad5 as FGAd. Therefore, the induction of Ad-specific antibodies was closely monitored in this study. Both single and double dose immunization by i.n. HDAd–EGFP or FGAd–EGFP vaccines resulted in similar high-titer anti-Ad responses (Fig. 2) even at lower doses (5×10^9 vp) which is consistent with the previous report [11] but inconsistent with another [5]. It showed that viral capsid antigens, which are shared by the two vectors and used to package HDAd, acted as the major antigens inducing serum Ad-specific antibodies via the mucosal route. Some studies have demonstrated that this kind of antiviral response could improve Ag-specific responses [19,24]. Taken together, we speculated that the anti-Ad antibodies contributed partially to the enhanced transgene-specific immune responses evoked by i.n. HDAd–EGFP vaccine.

Balanced or mixed Th1/Th2 cellular immune responses avoid the immunopathological effects of the vaccine [29]. We found that the ratio of IgG2a/IgG1 in sera from mice immunized with either HDAd–EGFP or FGAd–EGFP were close to one. This indicates that a balanced or mixed Th1/Th2 immune response occurs in both groups of immunized mice. Additionally, we observed only minimal liver edema in both groups of vaccinated mice (data not shown). Therefore, HDAd is at least as safe a mucosal vaccine vector as FGAd.

Table 3
Serum anti-EGFP isotype responses following immunization of BALB/c mice with FGAd–EGFP or HDAd–EGFP.

Immunogens	ELISA titer[a] (Means ± SD)		Amounts of IgG2a and IgG1[b] (ng/ml)		IgG2a/IgG1
	IgG2a	IgG1	IgG2a	IgG1	
FGAd-F (once)	800.00 ± 438.18	1333.33 ± 413.12	58.29 ± 13.89	47.71 ± 12.95	1.24
HDAd-F (once)	1066.67 ± 413.12	1466.67 ± 326.60	73.83 ± 19.41	62.16 ± 12.52	1.19
FGAd-F (prime/boost)	14933.33 ± 5225.58	29866.67 ± 17488.13	881.76 ± 47.37	778.46 ± 116.42	1.15
HDAd-F (prime/boost)	21333.33 ± 15501.57	44800.00 ± 32127.75	1143.29 ± 201.18	939.45 ± 169.13	1.22

[a] IgG subclass titers were determined 1 week after the final immunization. ELISA titers were expressed as the reciprocal of the final dilution. There was no significant difference between IgG subclass ($P > 0.05$).
[b] Ratio of IgG2a/IgG1 isotype response was calculated according to the amounts of EGFP-specific IgG2a and IgG1 determined 1 week after the final immunization.

In conclusion, both single and double i.n. HDAd–EGFP vaccination stimulated much higher EGFP-specific immunity than an equivalent dose of FGAd–EGFP vaccine. The mucosal immunity induced by the i.n. HDAd–EGFP vaccine includes local and atopic mucosal immune responses. The EGFP-specific immune response displayed the characteristics of a balanced or mixed Th1/Th2 cellular immune response. Although HDAd–EGFP induces the same amount of anti-Ad antibodies as FGAd–EGFP, homologous boosting immunization with HDAd–EGFP still achieved a more efficacious EGFP-specific immune response. Therefore, our results suggest that HDAd is a promising candidate as an i.n. vaccine vector for the development of vaccines against mucosal tract pathogens such as RSV and influenza virus.

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