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A prime–boost vaccination strategy using attenuated *Salmonella typhimurium* and a replication-deficient recombinant adenovirus vector elicits protective immunity against human respiratory syncytial virus

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ABSTRACT

Human respiratory syncytial virus (RSV), for which no clinically approved vaccine is available yet, is globally a serious pediatric pathogen of the lower respiratory tract. Several approaches have been used to develop vaccines against RSV, but none of these have been approved for use in humans. An efficient vaccine-enhancing strategy for RSV is still urgently needed. We found previously that oral SL7207/pcDNA3.1/F and intranasal FGAd/F were able to induce an effective protective immune response against RSV. The heterologous prime–boost immunization regime has been reported recently to be an efficient vaccine-enhancing strategy. Therefore, we investigated the ability of an oral SL7207/pcDNA3.1/F prime and intranasal (i.n.) FGAd/F boost regimen to generate immune responses to RSV. The SL7207/pcDNA3.1/F prime–FGAd/F boost regimen generated stronger RSV-specific humoral and mucosal immune responses in BALB/c mice than the oral SL7207/pcDNA3.1/F regimen alone, and stronger specific cellular immune responses than the i.n. FGAd/F regimen alone. Histopathological analysis showed an increased efficacy against RSV challenge by the heterologous prime–boost regimen. These results suggest that such a heterologous prime–boost strategy can enhance the efficacy of either the SL7207 or the FGAd vector regimen in generating immune responses in BALB/c mice.

1. Introduction

Human respiratory syncytial virus (RSV) is globally a serious pediatric pathogen of the lower respiratory tract. In the developed world, RSV causes serious lower respiratory tract disease, necessitating hospitalization in 0.5–1% of the population ⩽ 1 year of age [1]. RSV infection is also an important illness in elderly and high-risk adults, with a disease burden similar to that of non-pandemic influenza A in a population in which the prevalence of vaccination for influenza is high [2].

The importance of RSV as a respiratory pathogen has been recognized for a long time. Several approaches have been used to develop vaccines against RSV; but none of these has been approved for use in humans. An efficient vaccine-enhancing strategy for RSV is still needed urgently.

Because of the availability of the virulence-attenuating mutation, the feasibility to be genetically manipulated and the ability to stimulate the immune system, *Salmonella typhimurium* has been used as an efficient mucosal delivery vehicle for DNA vaccines against a variety of infectious diseases [3,4]. A study has shown that *S. typhimurium* aroA strain SL7207 carrying pcDNA3.1/F (SL7207/pcDNA3.1/F) induces strong Th1 and CTL responses following oral immunization [4]. However, the strength of the antibody responses induced by this kind of vector vaccine was relatively weak [4]. In addition, studies have also shown that prior exposure to *Salmonella* diminishes the immune responses toward the vaccine antigen [5,6].

As viruses are being able to infect and deliver genes into cells, live viruses have been used as vaccine vectors. Among these, a replication-deficient first generation adenoviral vector (FGAd) is an attractive vaccine vector [7,8]. Some studies have reported recently that a serotype 5 FGAd vector encoding the RSV fusion (F) glycoprotein (FGAd/F) effectively induces protective immunity and is a promising candidate vaccine against RSV infection [9,10]. Nevertheless, the immunity to Ad5, either pre-existing or elevated after

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repeated administration, will reduce the immune response to the transgene product [11,12].

Recently, the heterologous prime–boost immunization regime has been reported to be an efficient vaccine-enhancing strategy. Many studies have shown that vaccines applying such a strategy are able to generate high levels of immune responses in animal models [13–15]. One of the most successful heterologous prime–boost immunization strategies involves the FGAd vector, which is a very efficient vaccination approach in different animal models [16,17]. Until now, however, information has been limited regarding the efficacy of live attenuated bacterial vector prime–live virus vector boost regimen in vaccine development.

In the current study, we have constructed SL7207/pcDNA3.1/F and FGAd/F to inoculate BALB/c mice with an oral SL7207/pcDNA3.1/F prime–i.n. FGAd/F boost regimen. We report here for the first time that such a heterologous prime–boost regimen is effective in protecting against RSV infection.

2. Materials and methods

2.1. Preparation of RSV stock

Subgroup A RSV Long strain (kindly provided by Y. Qian, Capital Institute of Pediatrics, Beijing, China) was propagated in HEp-2 cells in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum, L-glutamine (2 mol/l), penicillin G (40 U/ml), streptomycin (100 μg/ml) and 0.2% sodium bicarbonate, and titrated for infectivity by measuring the 50% tissue culture infective dose ($TCID_{50}$) per milliliter.

2.2. Construction of SL7207/pcDNA3.1/F and FGAd/F

Plasmid DNA encoding the RSV F gene (pcDNA3.1/F) and FGAd-F were constructed previously [18]. The pcDNA3.1/F was transformed by electroporation into a live attenuated *aroA*-auxotrophic mutant of *S. typhimurium* (SL7207) (kindly provided by Z.T. Qi, Secondary Military Medical University, Shanghai, China).

2.3. 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.

2.4. Immunization and challenge

Forty-eight BALB/c mice were used for immunization experiments and divided into four groups. On week 0, the mice in FGAd/F group were intranasally inoculated with 1×10^{11} viral particles (vp) of FGAd/F, and the mice in SL7207/pcDNA3.1/F group and SL7207/pcDNA3.1/F-FGAd-F group were immunized orally with 100 μl of SL7207/pcDNA3.1/F (Bacteria were re-suspended at 1×10^{10} cells/ml in PBS). On week 3, mice in FGAd/F group and SL7207/pcDNA3.1/F-FGAd-F group were inoculated intranasally with 1×10^{11} vp of FGAd/F, and the mice in SL7207/pcDNA3.1/F group were immunized orally with the same amount of SL7207/pcDNA3.1/F as the first immunization. Sodium bicarbonate (10%) was used to neutralize the gastric acid before oral immunization. On weeks 0 and 3, the mice in placebo group were inoculated intranasally with 50 μl PBS. Three weeks after the final immunization, mice were challenged intranasally with 100 μl of subgroup A RSV strain Long (log10 $TCID_{50}$, 10^6/ml).

2.5. Collection of splenocytes

Spleens were harvested from vaccinated and control mice 7 days after the final 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 1640 medium (Invitrogen, Carlsbad, CA, USA).

2.6. 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), serum was stored at −20 °C. The bronchoalveolar lavage (BAL) fluid was obtained by lavage with three successive 1 ml volumes of PBS from cannulation of the trachea. The RSV F-specific antibody response in immunized mice was measured on weeks 2 and 4 by ELISA, as described previously [19]. Briefly, 80 ng purified RSV (Hytest, Turku, Finland) were adsorbed onto ELISA plates overnight in carbonate buffer (pH 9.8) at 4 °C. The plates were blocked with 1% BSA in PBS for 2 h at 37 °C. After thorough washing with PBS–1% BSA, the serum or BAL fluid samples were added to the plate and allowed to incubate 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. Finally, the plates were washed and developed with 100 μl of 3,3,5,5-tetramethylbenzidine (Sigma, St. Louis, MO, USA) substrate solution. The reaction was stopped with 50 μl 2 M H_2SO_4 and analyzed at 450 nm with a Thermo ELISA plate reader (Bio-Rad 550, Hercules, CA, USA).

2.7. Analysis of anti-adenoviral serotype 5 (Ad5) immunity

The anti-Ad5 serum IgG antibody was analyzed by ELISA, as described in Section 2.6. The microtiter plates were coated overnight at 4 °C with 100 μl of 1×10^8 vp/ml purified Ad5 (propagated in 293 cells and purified by cesium chloride gradient centrifugation).

2.8. Cytotoxic T lymphocyte assay

Spleen cells from BALB/c mice were removed at 7 days after the final immunization, stimulated with RSV (Hytest, Turku, Finland) at 0.5 μg/ml for 5 days, and then added to assay plates at the specified effector-to-target ratios (*E:T* ratios). RSV-specific cytotoxicity of splenocytes was determined by the CytoTox 96 non-radioactive assay (Promega, Madison, WI, USA), as described previously [9]. BCH4 cells (kindly provided by Professor B.S. Graham, National Institutes of Health, Bethesda, MD, USA) were used as targets in the CTL assays.

2.9. Lung histopathology study

Mice were sacrificed on day 5 after challenge. The right lungs from the above section were harvested and fixed in PBS-buffered formalin. Four-micrometer-thick sections were stained with hematoxylin and eosin. Lung pathology was assessed on the basis of the infiltration of inflammatory cells and pulmonary alveolar wall thickness. Infiltration of inflammatory cells was assessed by counting the cells, and pulmonary alveolar wall thicknesses were measured in randomly selected visual fields of each slide at a magnification of 400×. Images were captured with a Nikon DXM1200F (Nikon Instruments, Melville, NY) with ACT-1 software

and analyzed with Image-Pro Plus 6.0 (MediaCybernetics, Silver Spring, MD, USA).

2.10. Statistical analyses

Statistical analyses were performed with 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.

3. Results

3.1. Antibody responses

The strength of the antibody responses induced by the SL7207/pcDNA3.1/F regimen alone was relatively weak. Therefore we investigated whether the SL7207/pcDNA3.1/F prime–i.n. FGAd/F boost regimen induced higher serum antibody responses than the SL7207/pcDNA3.1/F regimen alone. RSV F-specific antibody responses were assessed by ELISA at 2 and 4 weeks after the first immunization. No differences in the serum IgG levels were observed among the three vaccinated groups at 2 weeks after the first immunization. The SL7207/pcDNA3.1/F-FGAd/F group induced a little higher serum anti-RSV IgG responses than the SL7207/pcDNA3.1/F regimen alone at 4 weeks after the first immunization (Fig. 1A). BAL fluid RSV-specific secretory IgA (sIgA) levels were measured at 1 week after the final immunization. The sIgA level in the SL7207/pcDNA3.1/F-FGAd/F group was higher than that in the SL7207/pcDNA3.1/F group (Fig. 1B).

To examine the potential advantage of the SL7207/pcDNA3.1/F-FGAd/F group in overcoming the drawback of the Ad5-specific immune response, Ad-specific antibody responses were also assessed

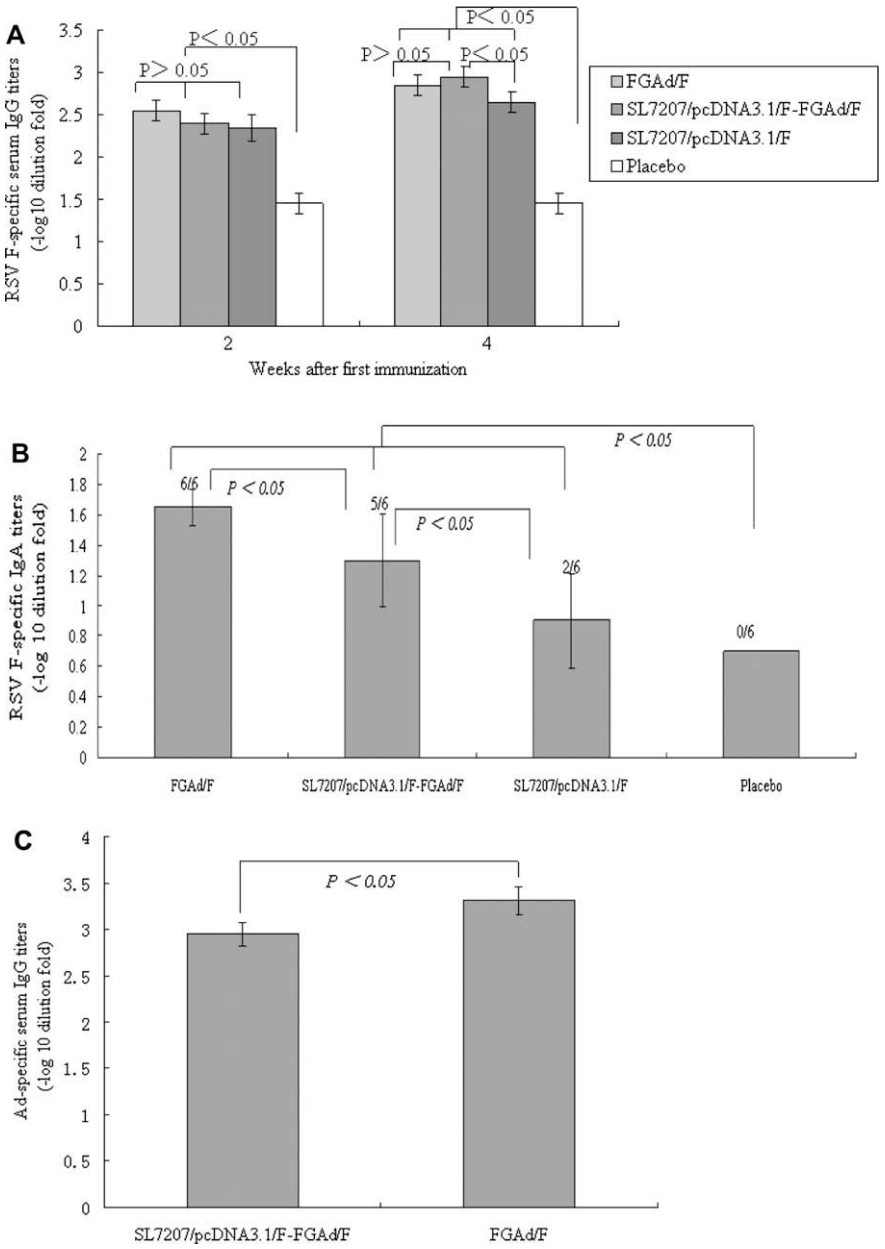

Fig. 1. Antibody levels. BALB/c mice were immunized intranasally at weeks 0 and 3, and antibody titers were measured by ELISA. (A) Serum RSV-specific IgG antibody titers were measured at weeks 2 and 4 after the first immunization. The results represent log10 end point values from six individual mice. (B) BAL fluid RSV-specific secretory IgA levels were measured at 1 week after the final immunization. The results represent log10 end point values from six individual mice. (C) Serum adenovirus-specific IgG levels were measured by ELISA at 1 week after the final immunization and expressed as the reciprocal of the final dilution.

further by ELISA. When the sera were detected 1 week after the final immunization, the reduced Ad-specific antibody responses in the SL7207/pcDNA3.1/F-FGAd/F group were observed, compared with the FGAd/F group (Fig. 1C).

3.2. Cell-mediated immune response

To compare the ability of heterologous prime–boost regimen to induce cellular immunity with homologous prime–boost regimen, we analyzed their efficacy in inducing RSV-specific CTL in mice at various effector-to-target cell ratios (*E:T*). More efficient target cell lysis was observed in the vaccinated groups than in the placebo group at *E:T* ratios of 20:1 and 50:1 ($P < 0.05$), and in the group immunized with SL7207/pcDNA3.1/F-FGAd/F than with FGAd-F alone group at the *E:T* ratio of 50:1 ($P < 0.05$) (Fig. 2).

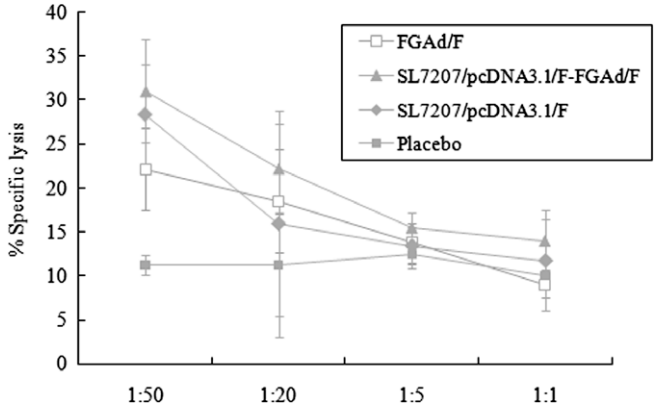

Fig. 2. Cell-mediated immune response. BALB/c mice were immunized intranasally at weeks 0 and 3. RSV-F-specific CTL activity was assessed 1 week after the final immunization.

Table 1
Infiltration of inflammatory cells and pulmonary alveolar wall thickness.

Immunogens	Infiltration of inflammatory cells (cell numbers)	Pulmonary alveolar wall thickness (μm)
FGAd/F	28.33 ± 4.05	3.12 ± 0.57[a]
SL7207/pcDNA3.1/F-FGAd/F	35.33 ± 9.58	2.11 ± 0.40
SL7207/pcDNA3.1/F	45.46 ± 3.72[a]	3.31 ± 0.99[a]

[a] Compared to SL7207/pcDNA/3.1/F-FGAd/F group, there were significant differences ($P < 0.05$). Each value represents means ± SD.

3.3. Histopathology

Histopathological changes were examined by hematoxylin and eosin staining. Infiltration of inflammatory cells and the pulmonary alveolar wall thickness were the indexes of the severity of pneumonia. Compared with the FGAd/F group and the SL7207/pcDNA3.1/F group, there was significantly less severe pneumonia in the SL7207/pcDNA3.1/F-FGAd/F group (Table 1 and Fig. 3 show the values and the representative results, respectively).

4. Discussion

Our previous research demonstrated that protective immunity against RSV is induced by SL7207/pcDNA3.1/F and FGAd/F, respectively [4,9]. However, in most cases, these protective roles are not potent and long-term enough. The weak immunogenicity is the main problem in the development of an RSV vaccine. Many strategies have been tried to improve this situation [8,20–22]. Since the heterologous prime–boost immunization strategy is efficient to improve immune response against transgenes *in vivo* [23], and little is known about the efficacy of the live, attenuated bacterial vector prime–live virus vector boost regimen in vaccine development, we wanted to investigate the potency of the oral SL7207/pcDNA3.1/F prime–i.n. FGAd/F boost regimen to strengthen the immune response against RSV.

Generally, humoral responses play more important roles in short-term and long-term protection against viral infections. The protective role of antibodies against RSV disease has been demonstrated in several animal models [24,25], and confirmed in humans [26,27]. Mucosal sIgA antibodies can preclude RSV entry into endothelial cells in the upper respiratory tract and/or inhibit its cell–cell syncytial spread, whereas protection of the lower respiratory tract may be primarily mediated by the IgG and IgM serum antibodies [1,20,28,29]. In our previous experiments, however, the capacity of SL7207 vector vaccines to induce strong antibody responses was very limited [4]. Therefore, in the current study, antibody titers against RSV F protein were evaluated, following the administration of the SL7207 vector alone or the SL7207/pcDNA3.1/F prime–FGAd/F boost regimen. SL7207/pcDNA3.1/F priming, followed by FGAd/F boosting, was more efficient in inducing RSV F-specific IgG and sIgA than the SL7207/pcDNA3.1/F homologous prime–boost regimen. The rationale behind these results is that the adenovirus vector has the ability to bypass the immune response elicited against the SL7207 vector, and also strengthen the immune response against the target antigen. Upon antigen re-exposure, memory B cells specific for RSV are believed to expand rapidly, and to mount an enhanced and broadened secondary response.

The RSV-specific T-cell response plays a major role in the clearance of virus, and in the clinical outcome of RSV infection [30].

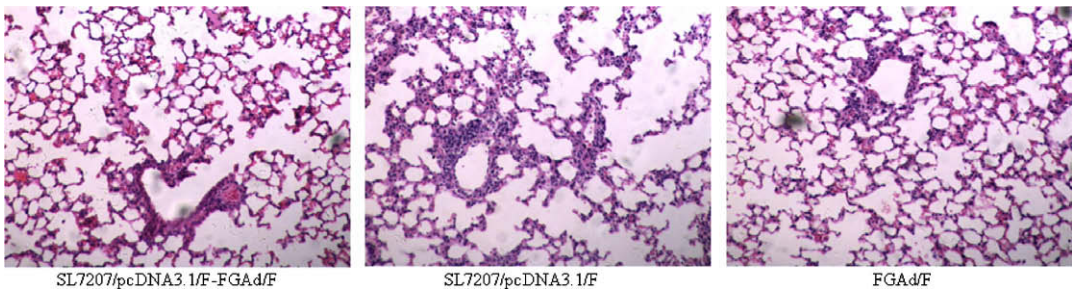

Fig. 3. Lung pathology at day 5 after RSV challenge. The immunized mice were challenged intranasally with 100 μl subgroup A RSV strain Long (log10 $TCID_{50}$, 10^6/ml) at week 3 after the final immunization, and lung pathology was analyzed at day 5 after challenge. Microscopic examination of lung tissue showed a marked difference in infiltration of inflammatory cells and pulmonary alveolar wall thickness.

Cytotoxic T-lymphocytes are vital cells in the immune response to the majority of viral infections [31]. Our data demonstrated that the more efficient target cell lysis at the *E:T* ratio of 50:1 ($P < 0.05$) in heterologous prime–boost immunized mice, compared with the FGAd-F homologous prime–boost regimen, was stimulated by a known MHC-I restricted peptide. This result supports the notion that heterologous prime–boost immunization is very effective in boosting cell responses [32].

Because FGAd is grown readily and purified in large quantities, and able to express transgenes efficiently in dividing and non-dividing cells, it has been considered to be an attractive vaccine vector. However, a potential limitation of Ad5 vectors is that a high percentage of humans have pre-existing immunity to Ad5, particularly in the developing world [33]. Additionally, enhancement of this immunity after repeated administration of Ad vectors also limits their preclinical study and clinical application [34]. To investigate the ability of the heterologous prime–boost regimen to overcome this shortcoming, we measured the antibodies against Ad. Our results showed that this regimen reduced the adenovirus-specific antibody response; this is very encouraging because it suggests that the heterologous prime–boost regimen is an effective alternative to the homologous adenovirus vector prime–boost regimen.

Upon challenge, mice from the SL7207/pcDNA3.1/F prime–FGAd/F boost group had less severe lung damage against pathogen challenge than the other two vaccinated groups. The oral SL7207/pcDNA3.1/F homologous boost regimen had more infiltration of inflammatory cells than the other two vaccinated groups.

The central characteristics important for the above phenomenon are: (i) The oral SL7207/pcDNA3.1/F prime–i.n. FGAd-F boost regimen generated higher anti-RSV humoral and mucosal immune responses than the oral SL7207/pcDNA3.1/F homologous boost regimen, and stronger CTL responses than the i.n. FGAd/F homologous boost regimen in BALB/c mice. (ii) The heterologous prime–boost regimen generated lower levels of anti-Ad humoral immune responses than the FGAd-F homologous boost regimen in BALB/c mice. The lung damage induced by responses of Ad-specific CD8 T cells is greatly reduced in the heterologous boost regimen. (iii) Compared to oral immunization, i.n. immunization induces better nasal mucosal immunity and immune protective role [35,36]. This was one of the factors responsible for the i.n. FGAd/F homologous prime–boost group inducing the most potent mucosal immunity among all the vaccinated groups, and the oral SL7207/pcDNA3.1/F prime–i.n. FGAd/F boost group having less severe lung damage against pathogen challenge than the SL7207/pcDNA3.1/F vaccine group.

We also noticed that the lung damage in the FGAd/F homologous prime–boost group was more severe than the heterologous boost group. We infer that the underlying reasons are: (i) the lung damage, initially attributed to enhanced host cellular immunity against Ad induced by the re-administered FGAd/F, is aggravated by subsequent RSV challenge; (ii) the SL7207/pcDNA3.1/F prime–FGAd/F boost regimen generates stronger anti-RSV-specific cellular immune responses than the i.n. FGAd/F alone regimen.

In conclusion, we report here for the first time that the heterologous oral SL7207/pcDNA3.1/F prime–i.n. FGAd/F boost regimen improved the *in vivo* immune responses and protective role against RSV infection, compared to the homologous prime–boost regimen. To enhance both humoral and cellular immune responses, several other candidate vaccines, powerful in inducing antibody responses, such as subunit vaccines, and virus-like particle (VLP) vaccines are suitable to combine with mucosal vector vaccines to establish new, highly effective heterologous boosting regimens against mucosal pathogens. The potential of the optimal prime–boost strategy deserves further exploration.

Acknowledgments

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