Enhancing mucosal immunity in mice by recombinant adenovirus expressing major epitopes of porcine circovirus-2 capsid protein delivered with cytosine-phosphate-guanosine oligodeoxynucleotides

Hong-tao Chang†, Xiu-yuan He†, Yu-Feng Liu, Lu Chen*, Quan-hai Guo, Qiu-ying Yu, Jun Zhao, Xin-wei Wang, Xia Yang, Chuan-qing Wang

Animal Infectious Disease Lab, College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, China

Introduction

Porcine circovirus-2 (PCV-2) is a small, non-enveloped DNA virus that is a member of the Circoviridae family. To date, four main PCV-2 open reading frames (ORFs) have been identified that encode five viral proteins. Rep and Rep’, both encoded by ORF1, are considered essential for viral replication [4]. ORF2 encodes a 27.8 kDa immunogenic capsid protein (Cap) that is the only structural protein of PCV2 [32]. The ORF3 and ORF4 genes produce an 11.9 kDa protein [27] and a 6.5 kDa protein [15], respectively. PCV-2 is horizontally transmitted via the mucosal route [12,33], and is the causative pathogen associated with post-weaning multisystemic wasting syndrome (PMWS) that mainly affects 7- to 15-week-old piglets with a mortality of up to 50% [12]. PMWS was first described in the 1990s and has since been responsible for dramatic economic losses to the swine industry worldwide [1,8].

Mucosa is the major portal of entry for a great majority of pathogens, and thus vaccines capable of eliciting mucosal immunity can fortify disease resistance at the mucosal frontlines [34]. However, most licensed vaccines administered via the parenteral route fail to induce protective mucosal immunity, which may only confer protection from clinical disease, and cannot eliminate infection at local mucosal invasion sites [34]. In this sense, the current vaccination strategy is not optimal since vaccinated animals remain susceptible to infection and serve as carriers of infectious viruses capable of transmission to other animals. In contrast, mucosal
vaccines are designed to induce broad protective immunity at the mucosa, which will be more efficacious in providing protection against pathogen entry at mucosal sites. However, targeting all mucosal compartments to generate both local and systemic protective immunity is still a considerable challenge. Efforts should focus on screening for antigens with high immunogenicity, developing efficient mucosa-targeting delivery vectors and routes, and selecting a potent immunostimulatory adjuvant [37].

Adenovirus is a promising vaccine vector even in the presence of preexisting anti-vector immunity [2,5,6,17,38]. Based on previously published epitope mapping of the PCV-2 capsid protein [24], we constructed a recombinant replication-defective adenovirus expressing the major epitopes of the PCV-2 capsid protein (rAd/Cap/518) [11] and evaluated its efficacy in mice. The results showed that rAd/Cap/518 can induce specific mucosal, humoral, and Th1-type cellular immunity following intranasal delivery, which was determined to be the optimal mucosal immunization route [28]. However, mucosal immunity induced by a simple antigen is generally less efficacious and persists for only a short time [26,34,37]. Therefore, delivering an adenoviral vector with mucosal adjuvant might enhance mucosal immunity.

Unmethylated cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN) can interact with and stimulate cells that express Toll-like receptor-9 (TLR-9), thereby initiating an immunomodulatory cascade that culminates in the production of Th1 and proinflammatory cytokines or chemokines [16,20,22]. Co-administration of CpG ODN with different types of antigen and vaccine vector along with various delivery routes have been shown to improve humoral and/or cellular immune responses, resulting in enhanced host immunity [3,10,14]. In the current study, CpG ODN was administered together with rAd/Cap/518 using an intranasal immunization protocol to evaluate the potential immune-enhancing effects in mice and provide insights into the development of novel mucosal vaccines against PCV-2.

### Materials and Methods

#### Materials and reagents

rAd/Cap/518 was previously constructed [11] and preserved by Henan Agricultural University (China). Cap protein used for in vitro stimulation during flow cytometry [24,25,26] was provided by Dr. Ping Jiang (Nanjing Agricultural University, China). ODN 1826 with unmethylated CpG (CpG ODN): 5'-TCCATGACGTTCC TGACGTT-3' and ODN without CpG (non-CpG ODN): 5'-TCCAGGACTTTCTCAGGTT-3' [4,15] were synthesized by Sangon Biotech with all bases phosphorothioate-modified.

#### Immunization and challenge protocols

All animal experiments were approved by the Ethics Committee for Animal Experimentation of the provincial Bureau of Health of Henan (China). BALB/c mice were used as an animal model as previously described [6,7,18,19]. A total of 175 specific pathogen-free (SPF) female BALB/c mice 6 to 8 weeks old were obtained from the Experimental Animal Center of Henan Province (China). The animals were kept under SPF conditions in individually ventilated cages (IVC) with unlimited access to rat food and water, and were housed under controlled temperature (around 22°C) and relative humidity (40% ~ 50%) conditions with a 12 h light/dark cycle. The mice were randomly divided equally into seven groups. The experimental groups and immunization scheme are shown in Table 1.

A total volume of 100 μL rAd/Cap/518 mixed with adjuvant or placebo (Sigma, USA) was delivered to each animal into the nostrils for intranasal immunization (three times, 15 ~ 20 μL in each nostril every time). The animals were boosted 2 weeks after priming immunization (Table 1).

### Table 1. Experimental groups and vaccination strategies

| Group                  | Vaccine reagents | Dosage (TCID50/mouse) |
|------------------------|------------------|------------------------|
| 1 PBS                  | PBS              | 100 μL                 |
| 2 wild-type rAd        | wild-type rAd    | 10^10 TCID50           |
| 3 rAd/Cap/518          | rAd/Cap/518      | 10^10 TCID50           |
| 4 CpG ODN              | CpG1826          | 10 μg                  |
| 5 non-CpG ODN          | non-CpG          | 10 μg                  |
| 6 CpG ODN + rAd/Cap/518| CpG1826 + rAd/Cap/518 | 10 μg + 10^10 TCID50 |
| 7 non-CpG ODN + rAd/Cap/518 | non-CpG + rAd/Cap/518 | 10 μg + 10^10 TCID50 |

PBS: phosphate-buffered saline, CpG ODN: cytosine-phosphate-guanosine oligodeoxynucleotides, TCID50: tissue culture infective dose 50. rAd/Cap/518: replication-defective adenovirus expressing the major epitopes of porcine circovirus-2 (PCV-2) capsid protein.
14 days after boosting immunization, five mice out of each group were challenged intranasally with $3 \times 10^6$ tissue culture infective dose (TCID$_{50}$) of wild-type PCV-2 YY virus (genotype PCV2b), which was preserved by Animal Infectious Diseases Laboratory, Henan Agricultural University.

**Sample collection**

Serum, saliva, and lung and intestinal lavage samples were collected from five mice in each group to detect PCV-2-specific IgG and IgA 14, 21, 28, and 35 days after priming immunization as previously described [5,17]. Blood was collected through 20-gauge needle (Nipro, Japan) intravenous catheter from the lateral saphenous vein and allowed to clot overnight at 4°C. Serum was recovered from the clotted blood by centrifugation at 15,000 × g for 5 min, 4°C. Salivation was induced by administering 2 mg of carbamylcholine (Sigma) intraperitoneally to collect saliva with swab.

The mice were euthanized with ketamine (93 mg/kg body weight; Sigma) before lung and intestinal lavage. The airways were washed with 1 mL of phosphate-buffered saline (PBS) to harvest lung lavage fluids. The small intestines of the mice were removed and clamped with string at each end. Next, 1 mL of PBS containing 1% fetal bovine serum (Gibco; Life Technologies, USA) was injected into the intestines using a 27-gauge needle (Nipro) and vigorously vortexed to harvest the intestinal fluids. The lung and intestinal lavage fluids were clarified by centrifugation at 4°C 12,000 × g for 5 min. The supernatants were then collected and stored at −70°C. Splenic lymphocytes were harvested to evaluate cell-mediated immune responses using a nylon mesh screen (Becton, Dickinson and Company, USA) as previously described [40]. Briefly, lymphocytes were isolated from spleen 14 days after boosting immunization. Single cell suspensions were adjusted to a final concentration of 1 × 10$^6$ cells/mL in PBS containing 2% FBS. The cells were stained with 100-fold diluted fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3, phycoerythrin (PE)-conjugated anti-mouse CD4, and peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD8a antibody (BioLegend, USA) in the dark at room temperature for 30 min according to standard flow cytometry procedures [19]. The cells were washed with PBS and approximately 20,000 cells were analyzed with a FACSscalibur flow cytometer (BD Biosciences, USA) to identify CD3$^+$, CD3$^+$CD4$^+$CD8$^-$, and CD3$^+$CD4$^-$CD8$^+$ T cell subpopulations.

For intracellular cytokine-specific staining, approximately 2 × 10$^6$ lymphocytes were stimulated with 1 μg of PCV-2 Cap protein at 37°C in 5% CO$_2$. After 4 h of stimulation, brefeldin A (10 mg/mL) was added and cells were cultured for an additional 4 h at 37°C. The cells were then washed with PBS containing 2% FBS, fixed with Cytofix/Cytoperm (Sigma), and permeabilized with 1× Perm/Wash (Sigma). Next, the cells were incubated with a cocktail of 100-fold diluted PE-conjugated anti-mouse CD4, PerCP-conjugated anti-mouse CD8a, Alexa Fluor 700-labeled anti-mouse IFN-γ, and allophycocyanin-labeled anti-mouse IL-2 antibody (BioLegend). Approximately 500,000 lymphocytes were analyzed with a FACSscalibur flow cytometer to measure the secretion of IL-2 by CD4$^+$ T cells and IFN-γ production by CD8$^+$ T cells.

**Detection of PCV-2-specific IgG and IgA by iELISA**

iELISA was performed to measure PCV-2-specific IgG levels in serum and IgA in saliva as well as lung and intestinal lavage (mucosal) samples [29]. In brief, 96-well ELISA plates (Corning, USA) were coated with 0.2 μg PCV-2 antigen in 0.05 M carbonate buffer (pH 9.6) per well and incubated overnight at 4°C. After blocking with 5% skimmed milk (Bio-Rad, USA) in PBS plus 0.5% Tween 20 (PBST) at 37°C for 1 h, 100 μL of serum sample diluted 200-fold or undiluted mucosal samples were added to each well and the plates were incubated at 37°C for 1 h. The plates were then washed three times with PBST to remove unbound antibodies. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (1 : 2,000; Baosen Biotechnology, China) or HRP-conjugated goat anti-mouse IgA (1 : 10,000; Bethyl Laboratories, USA) was subsequently added to each well. After incubating at 37°C for 1 h, the plates were washed three times and developed with 3,3’-5,5’-tetramethylbenzidine (TMB; Sigma, USA) for 15 min. The reaction was stopped by adding 1 M H2SO4, and the optical density (OD) was measured at a wavelength of 450 nm using a Thermo plate reader (Thermo Scientific, USA).

**Analysis of T cell sub-populations and intracellular cytokine concentrations**

Flow cytometry was used to analyze T cell sub-populations and staining for intracellular cytokines was performed to measure the frequency of IL-2-secreting CD4$^+$ T cells as well as IFN-γ-producing CD8$^+$ T cells as previously described [40]. Briefly, lymphocytes were isolated from spleen 14 days after boosting immunization. Single cell suspensions were adjusted to a final concentration of 1 × 10$^6$ cells/mL in PBS containing 2% FBS. The cells were stained with 100-fold diluted fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3, phycoerythrin (PE)-conjugated anti-mouse CD4, and peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD8a antibody (BioLegend, USA) in the dark at room temperature for 30 min according to standard flow cytometry procedures [19]. The cells were washed with PBS and approximately 20,000 cells were analyzed with a FACScalibur flow cytometer (BD Biosciences, USA) to identify CD3$^+$, CD3$^+$CD4$^+$CD8$^-$, and CD3$^+$CD4$^-$CD8$^+$ T cell subpopulations.

For intracellular cytokine-specific staining, approximately 2 × 10$^6$ lymphocytes were stimulated with 1 μg of PCV-2 Cap protein at 37°C in 5% CO$_2$. After 4 h of stimulation, brefeldin A (10 mg/mL) was added and cells were cultured for an additional 4 h at 37°C. The cells were then washed with PBS containing 2% FBS, fixed with Cytofix/Cytoperm (Sigma), and permeabilized with 1× Perm/Wash (Sigma). Next, the cells were incubated with a cocktail of 100-fold diluted PE-conjugated anti-mouse CD4, PerCP-conjugated anti-mouse CD8a, Alexa Fluor 700-labeled anti-mouse IFN-γ, and allophycocyanin-labeled anti-mouse IL-2 antibody (BioLegend). Approximately 500,000 lymphocytes were analyzed with a FACSscalibur flow cytometer to measure the secretion of IL-2 by CD4$^+$ T cells and IFN-γ production by CD8$^+$ T cells.

**Determination of viral DNA loads by quantitative real-time PCR (qPCR)**

Two weeks after PCV-2 challenge, lymph node, spleen, and lung samples were collected and total DNA was extracted using UNIQ-10 virus DNA mini-kit (Sangon). PCV-2 viral loads were determined by qPCR as previously described [25,28]. The amplification protocol was as follows: the sense primer (5´-tataactataaccaacagt-3´) and the antisense primer (5´-atggcgggaggagtagt-3´) were
used to amplify a 276 bp fragment of PCV-2 ORF2 gene. The RT-qPCR system contained 10 pmol each primer, 10 μL SYBR Premix Ex Taq, 500 ng sample DNA as template, and ddH2O to make a final volume of 20 μL. The RT-qPCR parameters consisted of 40 cycles of denaturation at 94°C for 10 sec, annealing at 55°C for 5 sec, and extension at 72°C for 10 sec. A standard curve was prepared from serial dilutions of plasmid pORF2 containing the PCV-2 ORF2 gene for quantification of the virus copy number. The correlation coefficient and slope of standard curve were −1.00 and −3.345 respectively. The amplification efficiency was 99.0%. All reactions were performed in duplicate on a LightCycler 2.0 (Roche, Swiss).

**Statistical analysis**

Data obtained from individual mice in all groups were analyzed by GraphPad Prism for Windows (ver. 5.01; GraphPad Software, USA). Differences in mean serum titers measured by iELISA were assessed by ANOVA and Duncan’s multiple range test. Data are presented as the mean ± standard error of the mean (SEM). \( p \) values < 0.05 were considered statistically significant.

**Results**

**IgG serum titers**

PCV-2-specific IgG titers in the serum were determined (Fig. 1A). From 21 to 35 days after priming immunization, significantly higher levels of IgG were detected in the group immunized with rAd/Cap/518 combined with CpG ODN compared to the animals immunized with rAd/Cap/518 alone (\( p < 0.05 \)). No significant difference in IgG titers was observed between the group immunized with rAd/Cap/518 along with non-CpG ODN and the mice immunized with rAd/Cap/518 alone (\( p > 0.05 \)).

**Fig. 1.** Levels of PCV-2-specific IgG in serum (A) or IgA in saliva (B) along with lung (C) and intestinal (D) lavage fluid from mice (n = 5) immunized with rAd/Cap/518 plus CpG ODN through an intranasal route on the indicated sampling day. Identical letters on top of the bars at each time point indicate no significant difference (\( p > 0.05 \)). Different letters on top of the bars indicate a significant difference (\( p < 0.05 \)).
Enhancement of mucosal immunity with rAd/Cap/518 of PCV-2 delivered with CpG ODN

**IgA titers in saliva, lung, and intestinal lavage fluid**

PCV-2-specific IgA titers in the saliva, lung, and intestinal lavage fluid were measured. From 21 to 35 days after priming immunization, significantly higher levels of IgA were detected in saliva from the group immunized with rAd/Cap/518 combined with CpG ODN compared to mice immunized with rAd/Cap/518 alone (p < 0.05). No significant differences in antibody responses were found between the group immunized with rAd/Cap/518 along with non-CpG ODN and the group immunized with rAd/Cap/518 alone (p > 0.05, Fig. 1B).

Between 14 and 28 days after priming immunization, significantly higher IgA levels were detected in lung lavage fluid from the group immunized with rAd/Cap/518 combined with CpG ODN relative to animals immunized with rAd/Cap/518 alone (p < 0.05). Significant differences were no longer detected on day 35 (p > 0.05). Except for 21 days following priming immunization, no significant differences in antibody responses were found between the mice immunized with rAd/Cap/518 as well as non-CpG ODN and the group immunized with rAd/Cap/518 alone (p > 0.05, Fig. 1C). From 14 to 28 days post-priming immunization, significantly higher levels of IgA were detected in the intestinal lavage fluid from the animals immunized with rAd/Cap/518 and CpG ODN compared to the group immunized with rAd/Cap/518 alone (p < 0.05). Significant differences were no longer detected on day 35 (p > 0.05). From 14 to 21 days after priming immunization, no significant differences in antibody response were observed between the group immunized with rAd/Cap/518 combined with non-CpG ODN and mice that received rAd/Cap/518 alone (p > 0.05, Fig. 1D).

**Analysis of T cell subsets**

The frequencies of various T cell subsets within spleen lymphocyte populations were analyzed at 14 days after boosting immunization (Fig. 2). The frequency of CD3+CD8+ T cells in the group immunized with rAd/Cap/518 and CpG ODN was significantly higher than that of the group immunized with rAd/Cap/518 alone (p < 0.05). In contrast, no significant difference was detected between the group that received rAd/Cap/518 with non-CpG ODN and the group immunized with rAd/Cap/518 alone (p < 0.05). Increased frequencies of CD3+CD4+ T cells and CD3+CD8+ T cells were similar to that of CD3+ T cells. No significant difference in the frequency of CD3+ T cells or CD3+CD4+ T cells was observed between the group immunized with CpG ODN alone and the mice that received rAd/Cap/518 with CpG ODN (p > 0.05). Furthermore, both groups showed significant differences in the frequencies of CD3+ T cells and CD3+CD4+ T cells compared to the other groups that were not treated with CpG ODN (p < 0.05). Surprisingly, a higher proportion of CD3+CD8+ T cells was detected in the group treated with CpG ODN alone than the mice treated with rAd/Cap/518 and CpG ODN (p < 0.05). These observations suggested CpG ODN can stimulate the propagation of CD3+, CD3+CD4+, and CD3+CD8+ T cells in a non-specific manner, and may serve as a suitable adjuvant that efficiently promotes cellular immunity.

**Intracellular cytokine analysis**

The percent of IL-2-secreting CD4+ T cells (IL-2+/CD4+ I) and IFN-γ-secreting CD8+ T cells (IFN-γ+/CD8+ T) was measured (Fig. 3). The percent of IL-2+/CD4+ T cells in the group immunized with rAd/Cap/518 and CpG ODN was 0.55%. In contrast, this percent was only 0.2% in the animals immunized with rAd/Cap/518 alone and 0.1% in the CpG ODN-treated group. Similarly, the percent of IFN-γ+/CD8+ T cells was 4.07% in the group immunized with rAd/Cap/518 and CpG ODN whereas this percent was only 2.79% and 0.54% in the groups treated only with rAd/Cap/518 or CpG ODN, respectively.

**Evaluation of protection against PCV-2 challenge**

Two weeks following viral challenge, PCV-2 loads were measured by qPCR (Fig. 4). The viral load in the mice intranasally immunized with rAd/Cap/518 and CpG ODN rAd-Cap was 7-fold lower than that in animals immunized with rAd/Cap/518 alone although the difference was not statistically significant (p > 0.05). However, the viral loads in these two groups were significantly lower than those found in any of the other groups (p < 0.05).

**Discussion**

Approximately 70% of pathogens infect humans or...
Fig. 3. Representative FACS results showing the percentages of IL-2-secreting CD4+ T cells and IFN-γ-secreting CD8+ T cells among spleen lymphocytes measured 14 days after boosting immunization.

Fig. 4. qPCR result showing the PCV-2 viral genomic copy loads in spleen, lung, and lymph node samples collected from mice 14 days after viral challenge. Identical letters on top of the bars indicate no significant difference ($p > 0.05$). Different letters on top of the bars indicate significant differences ($p < 0.05$).

animals via the mucosal route [37]. Mucosal immunity therefore represents the frontline of host immune defense. Most commercially available vaccines are administered through the parenteral route, which can induce the production of high serum IgG levels. This may provide protection against clinical symptoms but cannot completely prevent local infection at mucosal entry sites or subsequent virus replication and shedding. For example, classical swine fever virus can chronically propagate in the tonsils [30] while pseudorabies virus can establish latent infection in the trigeminal ganglion after entry via the nasal mucosa [39] even in immunized animals possessing high levels of serum antibodies. Similarly, influenza virus [31] and Newcastle disease virus [35] can cause local infection in the respiratory or genital tract, leading to mild respiratory symptoms and continuous virus shedding in poultry that have been vaccinated intramuscularly. It is therefore imperative to develop effective mucosal vaccines that can induce protective immunity against mucosal invasion. However, the host usually strives to maintain mucosal homeostasis by responding to mucosal antigens through a tolerogenic mechanism as opposed to primary immune reactivity [9]. In this sense, induction of mucosal immunity still poses a considerable challenge.

To increase mucosal immunity and develop an effective PCV-2 vaccine, we have sought to improve the expression vector, delivery method, and immune adjuvant. In a previous study, we showed that mucosal immunization with recombinant adenoviral vectors expressing the major epitopes of the PCV-2 Cap protein (rAd/Cap/518) induce a PCV-2-specific humoral immune response and a Th1-type immune response at the mucosal site as well as a systemic
level in mice [28]. To further improve the efficacy, the use of CpG ODN as an adjuvant was explored.

In the present study, the levels of serum IgG and mucosal IgA in the group intranasally immunized with rAd/Cap/518 together with 10 μg CpG ODN were significantly higher than those of the group immunized with rAd/Cap/518 alone. Frequencies of CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells in the group immunized with rAd/Cap/518 and CpG ODN were significantly higher than those for the group treated with rAd/cap/518 alone. However, CpG ODN alone significantly increased the frequencies of CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells. This observation indicated that CpG ODN is an effective adjuvant capable of augmenting humoral and cellular immunity induced by rAd/Cap/518. Nevertheless, the impact of CpG ODN on the production of PCV2-specific neutralizing antibodies that were shown by others to prevent PMWS is still unclear [20].

T helper cells can be classified into two groups based on their function, responses to different cytokines, and ability to secrete various cytokines. IFN-γ and IL-2 contribute to the differentiation of Th1 cell types whereas IL-4 and IL-10 induce the differentiation of Th2 cells. Th1-type cellular immunity is considered to be more important for antiviral immune defense. Production of cytokines such as IL-2 and IFN-γ by splenic lymphocytes was enhanced by immunization with rAd/Cap/518 and CpG ODN, suggesting that mucosal delivery of CpG ODN induces high levels of Th1-type cytokines. In addition, the viral load after challenge in mice immunized with rAd/Cap/518 and CpG ODN was 7-fold lower than that in animals treated with rAd/Cap/518 alone. This difference wasn’t statistically significant (p > 0.05). However, the viral load after challenge in mice immunized with rAd/Cap/518 and CpG ODN was significantly lower relative to that in the PBS placebo group. Many studies have shown that commercially available and experimental vaccines against PCV-2 can significantly reduce viremia, systemic viral loads, and shedding but cannot prevent or clear viral infection [28,29]. Much still has to be done before a more efficacious vaccine against PCV-2 can be developed.

The immunostimulatory activity of CpG ODN is determined by the structure of the CpG motif and other related factors. Active CpG ODN must contain unmethylated CpG dinucleotides flanked by two purines and two pyrimidines [22]. Other factors, including CpG dose, delivery systems such as the utilization of conjugate nanoparticles, liposomes, or biodegradable microparticles, and delivery route can also affect immunostimulatory activities [13,23]. Considering the characteristics of CpG and specificity of different mucosal administration routes, these variables should be further investigated to optimize the immunization regime and improve the adjuvant effect as well as vaccine potency.

In summary, we demonstrated that intranasal immunization with CpG ODN and rAd/Cap/518 markedly enhanced humoral immunity at both a systemic level and the mucosal sites compared to treatment with rAd/Cap/518 alone. In addition, cell-mediated immune responses were significantly increased and resulted in reduced viral loads. Thus, CpG ODN was found to be an effective immune adjuvant that can be intranasally delivered with rAd/Cap/518 to enhance host immunity.

Acknowledgments

We thank Dr. Ping Jiang of Nanjing Agriculture University for providing the wild-type rAd plasmid. The authors also thank Dr. Xia Liu of Henan Province People’s Hospital for providing instructions to perform flow cytometry. This work was supported by the Nature Science Foundation of China (No. 31272567) and the young core teacher’s project of higher education in Henan Province (2010GGJS-045).

Conflict of Interest

There is no conflict of interest.

References

1. Alarcon P, Rushton J, Wieland B. Cost of post-weaning multi-systemic wasting syndrome and porcine circovirus type-2 subclinical infection in England - an economic disease model. Prev Vet Med 2013, 110, 88-102.
2. Appledorn DM, Aldhamen YA, Godhehere S, Seregin SS, Amalfitano A. Sublingual administration of an adenovirus serotype 5 (Ad5)-based vaccine confirms Toll-like receptor agonist activity in the oral cavity and elicits improved mucosal and systemic cell-mediated responses against HIV antigens despite preexisting Ad5 immunity. Clin Vaccine Immunol 2011, 18, 150-160.
3. Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 2011, 10, 499-511.
4. Cheung AK. Transcriptional analysis of porcine circovirus type 2. Virology 2003, 305, 168-180.
5. Croyle MA, Patel A, Tran KN, Gray M, Zhang Y, Strong JE, Feldmann H, Kobinger GP. Nasal delivery of an adenovirus-based vaccine bypasses pre-existing immunity to the vaccine carrier and improves the immune response in mice. PLoS One 2008, 3, e3548.
6. Csáigola A, CADAR D, Tuboly Replication and transmission of porcine circovirus type 2 in mice. Acta Vet Hung 2008, 56, 421-427.
7. Deng ZB, Wang ND, Xu DJ, Yuan AW, Ge M, Luo W, Xue LQ, Yu XL. Viral distribution and lesions in Kunming mice experimentally infected with porcine circovirus type 2. Vet Res Commun 2011, 35, 181-192.
8. Ellis J, Clark E, Haines D, West K, Krakowka S, Kennedy S, Allan GM. Porcine circovirus-2 and concurrent
infections in the field. Vet Microbiol 2004, 98, 159-163.
9. Fukuyama Y, Tokuhara D, Kataoka K, Gilbert RS, McGhee JR, Yuki Y, Kiyono H, Fujihashi K. Novel vaccine development strategies for inducing mucosal immunity. Expert Rev Vaccines 2012, 11, 367-379.
10. Gallichan WS, Woolstencroft RN, Guarasci T, McCluskie MJ, Davis HL, Rosenthal KL. Intra nasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. J Immunol 2001, 166, 3451-3457.
11. Guo QH, Chen L, Wang CQ, Wu DF, Wang YS, Li ZJ, Wang Y. Construction and identification of recombinant adenoviruses containing the ORF2 gene of porcine circovirus 2. Chin J Vet Sci 2011, 31, 1099-1102, 1110.
12. Grau-Roma L, Hjulsager CK, Sibila M, Kristensen CS, Lopez-Soria S, Enoe C, Casal J, Botner A, Nofrarias M, Bille-Hansen V, Fraile L, Baekbo P, Segales J, Larsen LE. Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark. Vet Microbiol 2009, 135, 272-282.
13. Hanagata N. Structure-dependent immunostimulatory effect of CpG oligodeoxynucleotides and their delivery system. Int J Nanomedicine 2012, 7, 2181-2195.
14. Harandi AM, Eriksson K, Holmgren J. A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection. J Virol 2003, 77, 953-962.
15. He J, Cao J, Zhou N, Jin Y, Wu J, Zhou J. Identification and functional analysis of the novel ORF4 protein encoded by porcine circovirus type 2. J Virol 2013, 87, 1420-1429.
16. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. Nature 2000, 408, 740-745.
17. Kim S, Jung JE, Yu JR, Chang J. Single mucosal immunization of recombinant adenovirus-based vaccine expressing F1 protein fragment induces protective mucosal immunity against respiratory syncytial virus infection. Vaccine 2010, 28, 3801-3808.
18. Kiepel M, Stevenson GW, Choi J, Latimer KS, Kanitz CL, Mittal SK. Viral replication and lesions in BALB/c mice experimentally inoculated with porcine circovirus isolated from a pig with postweaning multisystemic wasting disease. Vet Pathol 2001, 38, 74-82.
19. Kiepel M, Stevenson GW, Galbreath EJ, North A, HogenEsch H, Mittal SK. Porcine circovirus type 2 (PCV2) causes apoptosis in experimentally inoculated BALB/c mice. BMC Vet Res 2005, 31, 7.
20. Klinman DM, Klaschik S, Sato T, Tross D. CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. Adv Drug Deliv Rev 2009, 61, 248-255.
21. Ko SY, Cheng C, Kong WP, Wang L, Kanekkyo M, Einfeld D, King CR, Gall JG, Nabel GJ. Enhanced induction of intestinal cellular immunity by oral priming with enteric adenovirus 41 vectors. J Virol 2009, 83, 74-756.
22. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Kortetzky GA, Klinman DM. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995, 374, 546-549.
23. Krishnamachari Y, Salem AK. Innovative strategies for co-delivering antigens and CpG oligonucleotides. Adv Drug Deliv Rev 2009, 61, 205-217.
24. Lekeharoenkuk P, Morozov I, Paul PS, Thanghunniyom N, Wajjwalku W, Meng XJ. Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. J Virol 2004, 78, 8135-8145.
25. Li Z, Wang Y, Chen L, Yang X, Zhao J, Wang C. Study and application of SYBR green I-based fluorescent quantitative PCR assay in type 2 porcine circovirus detection. Yangzhou da xue xue bao. Nong ye yu sheng ming ke xue ban 2011, 32, 49-54.
26. Lin SW, Cun AS, Harris-McCoy K, Ertl HC. Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8+ T cell responses in the gut. Vaccine 2007, 25, 2187-2193.
27. Liu J, Chen I, Du Q, Chua H, Kwang J. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. J Virol 2006, 80, 5065-5073.
28. Liu YF, Guo QH, Chen L, Zhao J, Chang HT, Wang XW, Yang X, Wang CQ. Induction of mucosal immunity by intranasal immunization with recombinant adenovirus expressing major epitopes of Porcine circovirus-2 capsid protein. Vet Immunol Immunopathol 2013, 154, 48-53.
29. Liu Y, Ma P, Chen L, Yang X, Yao H, Wang C, Zhao J. Development and primary application of indirect ELISA method for detecting the IgA against porcine circovirus type 2. Chin J Prev Vet Med 2012, 34, 301-304.
30. Loeffen W. Persistent infection with the classical swine fever virus in vaccinated animals: a risk factor? Tijdschr Diergeneesk 2008, 133, 482-484.
31. Naeem K, Naurin M, Rashid S, Bano S. Seroprevalence of avian influenza virus and its relationship with increased mortality and decreased egg production. Avian Pathol 2003, 32, 285-289.
32. Nawagitgul P, Morozov I, Bolin SR, Harms PA, Sorden SD, Paul PS. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol 2000, 81, 2281-2287.
33. Patterson AR, Madson DM, Halbur PG, Opriessnig T. Shedding and infection dynamics of porcine circovirus type 2 (PCV2) after natural exposure. Vet Microbiol 2011, 149, 225-229.
34. Pavot V, Rochereau N, Genin C, Verrier B, Paul S. New insights in mucosal vaccine development. Vaccine 2012, 30, 142-154.
35. Takada A, Kida H. Protective immune response of chickens against Newcastle disease, induced by the intranasal vaccination with inactivated virus. Vet Microbiol 1996, 50, 17-25.
36. Tutykhina IL, Logunov DY, Shcherbinin DN, Shmarov MM, Tukhvatulin AI, Naroditsky BS, Gintsburg AL. Development of adenoviral vector-based mucosal vaccine against influenza. J Mol Med (Berl) 2011, 89, 331-341.
37. Woodrow KA, Bennett KM, Lo DD. Mucosal vaccine design and delivery. Annu Rev Biomed Eng 2012, 14, 17-46.
38. Xiang ZQ, Gao GP, Reyes-Sandoval A, Li Y, Wilson JM,
Ertl HC. Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. J Virol 2003, 77, 10780-10789.

39. Yoon HA, Aleyas AG, George JA, Park SO, Han YW, Hyun BH, Lee JH, Song HJ, Cho JG, Eo SK. Correlation between the nature of immunity induced by different immunogens and the establishment of latent infection by wild-type pseudorabies virus. Res Vet Sci 2007, 83, 73-81.

40. Zhao J, Lai L, Amara RR, Montefiori DC, Villinger F, Chennareddi L, Wyatt LS, Moss B, Robinson HL. Preclinical studies of human immunodeficiency virus/AIDS vaccines: inverse correlation between avidity of anti-Env antibodies and peak postchallenge viremia. J Virol 2009, 83, 4102-4111