Vaccine 37 (2019) 839–847

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Penton-dodecahedron of fowl adenovirus serotype 4 as a vaccine candidate for the control of related diseases

Xinglong Wang *[,1], Qiuxia Tang [1], Li Qiu, Zengqi Yang *

College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China

ARTICLE INFO

Article history:
Received 21 August 2018
Received in revised form 10 December 2018
Accepted 21 December 2018
Available online 11 January 2019

Keywords:
Adenovirus
Penton-dodecahedron
Immunization
Protection

ABSTRACT

In some serotypes of adenovirus (Ad), the penton base and attached trimeric fiber assemble into dodecameric virus-like particles called penton-dodecahedron (Pt-Dd), which can be internalized and used to deliver the vaccine antigens and drugs. Fowl adenovirus serotype 4 (FAdV-4) is an important pathogen, causing seriously economic loss to poultry industry in China and other counties. Pt-Dd particles from FAdV-4 infected cells, as well as in those infected with recombinant human Ad expressing fiber-1, fiber-2, and penton base of FAdV-4, were visualized by transmission electron microscopy. For the first time, we proved that FAdV-4 produced Pt-Dd in infected cells. Pt-Dd can also be assembled by the over-expressed recombinant proteins fiber-1, fiber-2, and penton base. Pt-Dd, as well as the recombinant proteins fiber-1, fiber-2, and penton base, were then used to immunize chickens. The humoral immune response, expression of selected immune molecules and challenge results were used to evaluate the immune efficacy of the vaccine candidates. Pt-Dd induced the highest level of enzyme-linked immunosorbent assay antibodies and significant high levels ($p < 0.05$) of interferonγ, interleukin-4, and major histocompatibility complex II expression in peripheral blood mononuclear cells at 48 h post-infection. The challenge results showed that Pt-Dd, inactivated FAdV-4 vaccine, and fiber-1 induced the best protection (100%), followed by fiber-2 (80%) and penton base (67%). The present study showed that FAdV-4 -Pt-Dd and recombinant fiber-1 are promising FAdV-4 vaccine candidates and could be used to replace the tissue-sourced inactivated FAdV-4 vaccine.

1. Introduction

Adenoviruses (Ad) are large, non-enveloped viruses with an icosahedral nucleocapsid containing a double-stranded DNA genome of approximately 36 KD [1]. They have a broad range of vertebrate hosts and, in humans, more than 50 distinct adenoviral serotypes have been found to cause a wide range of illnesses, from mild respiratory infections in children to life-threatening multi-organ diseases in people with a weakened immune system [2,3]. The capsid of the Ad is composed of three major capsid proteins (hexon, penton base, and fiber) and four minor capsid proteins (IIIa, VI, VIII, and IX; also known as cement proteins) [4,5]. The pseudo-T = 25 icosahedral capsid has 12 pentons, each with trimeric fiber proteins, and 240 trimeric hexons [6].

In cells infected with some adenovirus serotypes, penton base, with their attached trimeric fibers, assemble into dodecameric virus-like particles (VLP) called Ad penton-dodecahedron (Pt-Dd) [14]. Trimeric fiber and penton base are responsible for adenovirus attachment and endocytosis, respectively [7,8]. Native Pt-Dd can be observed in HAd-B3, E4, D9-, -B11, and -D15 infected cells [9–11]. Pt-Dd can also be assembled spontaneously in the baculoviral system [8,12] and in *Escherichia coli* [13] upon expression of the penton base and fiber genes of HAd-B3. No such particles have been found for HAd -C2 or -C5 [14–16]. Pt-Dd show a remarkable cell penetration ability with 200–300 thousand VLPs per cultured cell [17,18]; thus, it can be engineered to deliver several millions of foreign cargo molecules into a single target cell [17–20].

Pt-Dd has been exploited to deliver antigen proteins [21] or small molecule drugs [22] linked by an additional domain to the penton base protein. In a previous study, Pt-Dd was used as a cancer vaccine vehicle to deliver model antigen ovalbumin (OVA) fused with the linker WW domain from the ubiquitin ligase Nedd4 [21]. Pt-Dd can efficiently deliver WW-OVA into cultured cells and the Pt-Dd/WW complex can be readily internalized by dendritic cells. Immunization with WW-OVA/Pt-Dd induced an OVA-specific CD8 + T cells immune response and robust humoral responses in mice resulting in 90% protection against B16-OVA

* Corresponding authors.
E-mail addresses: wxlong@nwsauf.edu.cn (X. Wang), yzq1106@nwsuaf.edu.cn (Z. Yang).
[1] Equal contribution.

https://doi.org/10.1016/j.vaccine.2018.12.041
0264-410X/

melanoma implantation in syngeneic mice [21]. Pt-Dd was also used to carry multiple influenza virus epitopes, as an influenza vaccine. With this platform, the immunodominant epitopes of the influenza M1 were properly presented by human dendritic cells, thus triggering the efficient activation of antigen-specific T cell responses and inducing cellular immunity in vivo in chickens [20].

Fowl adenoviruses (FAdVs) are classified into five species (A–E) [23] and twelve serotypes (FAdV 1–7, 8a, 8b, 9–11) [24]. FAdV-4 is the causative agent of the hydropericardium hepatitis syndrome (HHS) that is characterized by hydropericardium, hepatitis, and nephritis [24–26]. FAdV-4 is the predominant serotype of FAdVs in southwestern China. As reported, about 86.4% (19/22) isolates were FAdV-4 [27]. FAdVs have been found to infect 2- to 5-week-old neonatal chicks [27], causing a mortality of approximately 30% to 70% [28,29]. Inactivated vaccines [30–32], attenuated live vaccines [33,34], and recombinant vaccines have been used to control HHS and these types of vaccines have been proven to be effective in protecting against FAdV infection. In a previous study, scientists purified Pt-Dd produced by FAdV-8b via density gradient centrifugation; this purified Pt-Dd induced neutralizing antibodies and cytotoxic T-cell responses in breeders, and successfully prevented clinical disease in progeny via maternal antibody transfer [35]. FAdV-4 might also produce Pt-Dd during infection; therefore, the present study utilized HAdV-C5 to produce recombinant FAdV–4–Pt-Dd and used these recombinant VLPs as a vaccine to prevent FAdV-4 infection.

2. Materials and methods

2.1. Viruses and cells

The replication-defective HAdV-C5 with E1 and E3 deletion was purchased from TaKaRa (Dalian, China) and HAdV-C5-based recombinants were propagated and titered in HEK-293 A (ATCC® CRL-1573D) and HEK-293T (ATCC® CRL-3216™) cells as reported [36]. HK-2 cells (ATCC® CRL-2190™) were used to produce recombinant proteins by infected with recombinant HAdV. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The fowl adenovirus strain SX15 was grown and titered in LMH cells (Chicken Liver Hepatocellular carcinoma cell line, ATCC® CRL-2117™) and stored at −80 °C.

2.2. Construction of rAds (rAd-f1, rAd-f2, and rAd-p)

The respective open reading frames of fiber-1, fiber-2, and penton base were amplified by polymerase chain reaction (PCR) from viral DNA (FAdV-4 isolate SXD15, refer to KU569296.1) using the primers listed in Table 1. The PCR amplicons of fiber-1, fiber-2, and penton base were cloned into a pAd-shuttle-CMV vector. The recombinant adenoviral vectors were generated by homologous recombination of linearized transfer vectors with pAdEasy-1 in *E. coli* BJ5183 and confirmed by restriction enzyme digestion (New England Biolabs) [37]. The rAds were generated by transfection of HEK293 A cells at 80% confluence in 24-well plate with 1 μg plasmids (PacI linearized) using 3 μL of Trans Fast TM Transfection Reagent (Promega, Madison, USA). When 90% of the cells showed cytopathic effect (CPE, at about 10 days post transfection), rAds were released by three cycles of rapid freezing and thawing and stored at −80 °C after the addition of 10% glycerol.

2.3. Western blot

The proteins in cell lysates or in cell culture media derived from HEK293 A cells or HK-2 cells infected with rAds were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Pall Corporation). The membrane was incubated overnight in blocking solution (10% fat-free milk in phosphate buffered saline (PBS), PBS-M) at room temperature and incubated with FAdV-4 fiber-1, fiber-2, and penton base polyclonal mouse antiserum for 2 h. The membrane was subsequently reacted for 1 h with goat anti-mouse IgG conjugated with horseradish peroxidase (AS003) at a dilution of 1/2000 in PBS-M. Detection was performed using chemiluminescence luminol reagents (Super Signal West PicoTrial Kit, Pierce). Utilizing gray scan with the software ImageJ and comparison with the known concentration of marker bands, the concentrations of proteins were calculated based on the obtained gray value of the bands.

2.4. Observation of Ad virion and Pt-Dd by electron microscopy (EM)

Virus samples derived from HEK293 A cells or HK-2 cells infected with rAds were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 1% sodium silicotungstate, pH 7.0. Micrographs were taken with a transmission electron microscope (TECNAI G2 SPIRIT BIO), under low-dose conditions with the microscope (FEI, America) at 100 kV and a maximum magnification of 300,000.

2.5. Observation of Pt-Dd internalization into cells by confocal microscopy

HeLa cells (ATCC® CCL-2™) were grown overnight on glass coverslips (approximately 10^5 cells/cm^2) in DMEM supplemented with 10% FBS at 37 °C under 5% CO_2 atmosphere. Cells were incubated for different periods with a 0.6 nM concentration of the FAdV-4 VLPs. After a given period of entry, cells were washed with PBS, fixed, and permeabilized with cold methanol for 10 min. Cells were then incubated with anti-fiber-1 mouse polyclonal serum at 1/200 in 50 μL 3% bull serum albumin and subsequently detected in red by Cy3-conjugated goat anti-mouse antibody (BA1035, Bosterbio, Wuhan) that was diluted 1/200 in the same buffer. Cell nuclei were counterstained in blue with DAPI (5 μg/mL). Laser scanning confocal microscopy was performed on an MRC600 (Andorra, England).

2.6. Immunization and challenge

Day-old Specific Pathogen Free chickens were randomly divided into 7 groups of 15. The chickens in the first 5 groups received 0.5 μg of FAdV-4Pt-Dd VLPs, fiber-1, fiber-2, penton base, and inactivated vaccine of FAdV-4 by intramuscular injection on day 7 and again on day 14. The targeting proteins were in infected HK-2 cell lysates and the concentrations of the proteins were calculated as described in 2.3. The other two groups received PBS.

At day 21, all chickens except those in the negative control group (PBS), were challenged with $10^{5.5}$ $TCID_{50}$ of FAdV-4 SX15 in 200 μL cell culture media by intramuscular injection. Sera (n = 3 per group) were collected on days 14, 21, 28, and 35 to determine serum antibodies. Additionally, blood was collected at 24 h post-challenge (hpc) to measure transcript levels encoding IL-4, IFN gamma and MHC-II in PBMCs.

Viral loads in sera collected at day 28 and 35 were detected by real-time PCR using the reported probe and primers as reported [38]. A standard curve was produced by using serial dilutions of the standard plasmid (pMD-18 T vector, Takara) harboring a 341 bp fragment of the L1 region of Hexon from 1×10^1 to 1×10^{11} copies/μl. Viral loads were calculated as reported [38].

Table 1
Primers used for recombinant adenoviruses construction.

Primer name	Sequence (5′–3′)*	Restriction site	Product
fiber-1: forward	5′-cgGGATCCATGTCGGCCCTAATCGCCTCCGCAG-3′	*Bam*H I	1295 bp
fiber-1: reverse	5′-ccgCTCGAGTTAGGGGCCCGGAGCATTGTTCCCG-3′	*Xho* I	
fiber-2: forward	5′-cgGATATCATGCTCCGGGCCCCTAAAAGAAGAC-3′	*Eco*R V	1440 bp
fiber-2: reverse	5′-agaatGCGGCCGCTTACGGGAGGGAGGCCGCTGG-3′	*Not* I	
Penton base: forward	5′-cgGGATCCATGTGGGGGTTGCAGCCGCCGACGT-3′	*Bam*H I	1577 bp
Penton base: reverse	5′-ccgCTCGAGCTACTGCAAGGTCGCGGAACTCAGA-3′	*Eco*R I	
hexon loop1: forward	5′-cgGGATCCATGGATAACACGGGCACCAATTACC-3′	*Bam*H I	1272 bp
hexon loop1: reverse	5′-gGAATTCTTAGTGGTGGTTGAAGGGGTTGACGTT-3′	*Xho* I	

* The underlined sequences represent the restriction site.

2.7. Histopathology and immunohistochemistry analyses

Three surviving chickens from each group were randomly selected and euthanized by CO_2 asphyxiation at 6-day post-challenge (dpc). Liver samples were fixed in 10% neutral formalin for 52 h at room temperature. After routine treatment, liver tissues were embedded in paraffin wax and cut into 4 to 5 μm slices. For micro lesion observation, the slices were stained with hematoxylin and eosin. In addition, immunohistochemical staining was conducted to detect viral antigens in the livers. Briefly, slices were incubated with mouse anti-FAdV polyclonal antibody (diluted 1:200) for 1 h and then reacted with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Solarbio, Beijing, China) for 1 h. Diaminobenzidine was used as the chromogenic substrate. After counterstaining with hematoxylin and eosin, the slides were dehydrated and mounted. Slides without the primary antibody were used as negative controls.

2.8. Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Guangzhou Jet Bio-Filtration Co., Ltd., China) were coated with 0.2 μg (each)/well of mixed purified fiber-1, fiber-2, and penton base (expressed in *E. coli* and stored by our laboratory as reported in our previous study [39]). Then, the plates were reacted with sera from chickens immunized with the corresponding proteins. Commercial peroxidase-conjugated rabbit anti-chicken IgG (Sigma) was used as the secondary antibody. Following incubation with tetra-methylbenzidine substrate (Tiangen Biotech (Beijing) Co., ltd., China), the reaction was stopped with 0.5 M sulfuric acid and the optical density of each well was measured at a wavelength of 450 nm.

To calculate antibody titer, serum sample containing FAdV-4 antibody and negative sera (SPF chicken sera) were diluted in serial ratios (1:100, 1:200, 1:400, 1:800... and so on). Three replications were used for each test. A positive or negative antibody determination is derived by comparing the test samples to Negative Controls. Where (Test Sample OD450) < 2.1 × (Negative Control OD450) = Negative. Where (Test Sample OD450) ≥ 2.1 × (Negative Control OD450) = Positive. The assigned titer value is indicative of the last dilution in which the antibody was detected.

2.9. Statistical analysis

One-way ANOVA, followed by a Tukey post-test, was performed for multiple comparisons. Differences were considered significant at $p \leq 0.05$. Statistical analysis was run using SPSS (version 22) software.

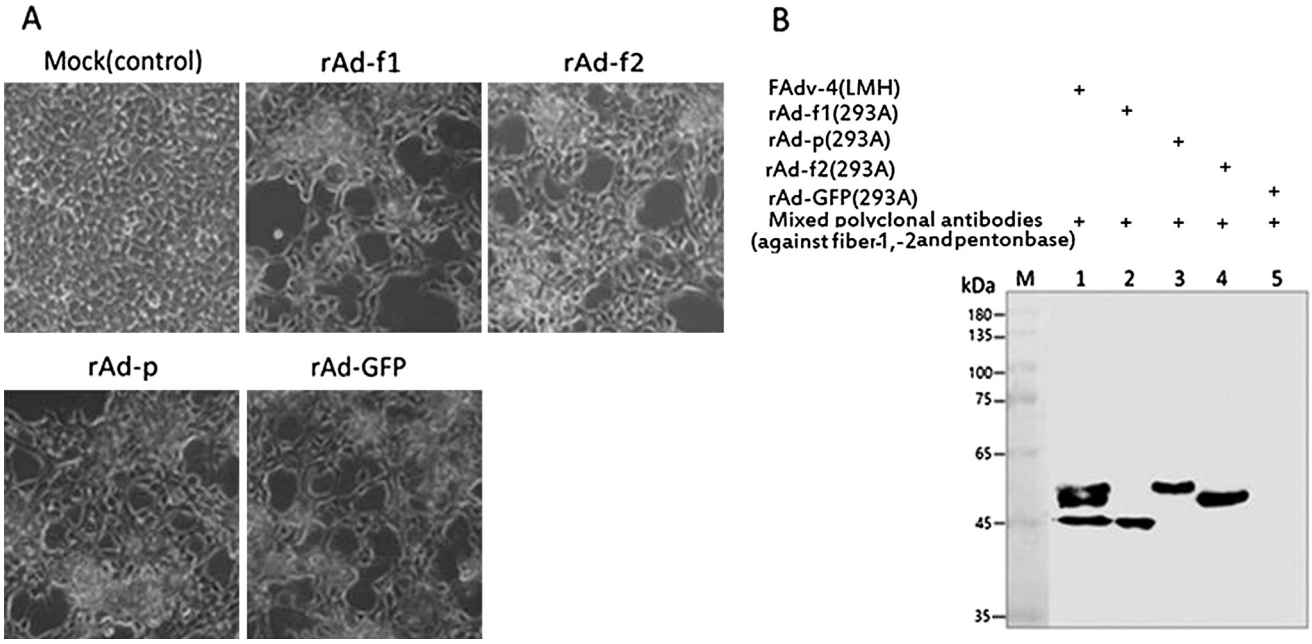

Fig. 1. Production of rAd in HEK293 A cells and identification of recombinant protein expression by western blot. (A) Micrographs of HEK293 A cells at 10 days post-transfection with non-plasmid (mock) or linearized pAd-fiber-1, pAd-fiber-2, and pAd-penton base. rAd-GFP was used as the control (2 days post-viral inoculation); (B) Western blot identification of fiber-1, fiber-2, and penton base protein expression in transfected HEK293 A cells with a mixture of polyclonal antibodies against fiber-1, fiber-2 and penton base of FAdV-4. Lane 1 was the positive control with lysate of LMH cells infected with FAdV-4. Lane 5 contained lysate of HEK293 A cells infected with rAd-GFP. Lanes 2,3, and 4 contained lysate of HEK293 A cells infected with rAd-f1, -p, and -f2, respectively.

3. Results

3.1. Construction of recombinant viruses

Shuttle vectors containing expression cassettes for fiber-1, fiber-2, and penton base of FAdV-4 under the control of the cytomegalovirus immediate early (CMV) promoter were constructed and verified by sequencing (data not shown). By recombination with Ad backbone vector pAdEasy-1 in BJ5183 cells, recombinant adenoviral plasmids, pAd-fiber-1, pAd-fiber-2, and pAd-penton base were obtained. Then, the recombinant plasmids were linearized with endonuclease *Pac* I and used to transfect HEK293 A cells to generate recombinant adenovirus (rAd), including rAd-f1 (expressing fiber-1), rAd-f2 (expressing fiber-2), and rAd-p (expressing penton base). After approximately 10 days incubation, cells transfected with recombinant plasmids showed CPE characteristic of adenovirus infection while the mock-transfected cells (control samples) retained their singularity (Fig. 1A).

The obtained rAds, rAd-f1, rAd-f2, and rAd-p were plaque-purified three times and titered in HEK293 A cells and HEK293 T cells. The titers of these three rAds that were tested in HEK293 A and HEK293 T cells were all 4.1×10^8 plaque forming units (pfu) per ml. Expression of the proteins, fiber-1, fiber-2, and penton base with predicted sizes of 45.0, 49.3, and 57.4 kDa was confirmed by western blotting. As shown in Fig. 1B, the protein bands corresponding to fiber-1, penton base, and fiber-2 are found in Lane 2, 3, and 4, respectively. Three protein bands were observed in Lane 1 with proteins from LMH cells infected with FAdV-4. No band was observed in Lane 5, in which the proteins from HEK293 A cells infected with rAd-GFP (expressing green fluorescent protein) were added.

3.2. Fiber-1, fiber-2, and penton base derived from FAdV-4 or rAds formed Pt-Dd

Virions of FAdV-4 approximately 100 nm in size and particles 50 nm in diameter (Pt-Dd) were observed in lysates of FAdV-4-infected LMH cells by TEM (Fig. 2). The virions showed the characteristic icosahedral symmetry of adenovirus and the Pt-Dds showed an ordered structure, characteristic of dodecahedra.

To check whether Pt-Dd could also assemble by the expressed recombinant proteins. HEK293 A cells, as well as HK-2 cells, were infected with rAd-f1, rAd-f2, and/or rAd-p. Pt-Dds were observed in HEK293 A cells and HK-2 cells co-infected with rAd-f1, rAd-f2, and rAd-p (Fig. 2E and 2F). In addition, HAdV-C5 virions were found in infected HEK293 A cells (Fig. 2B), but not in HK-2 cells. Dodecahedrons formed in HEK293 A cells infected with rAd-p, expressing FAdV-4 penton base alone (Fig. 2D), but not in cells

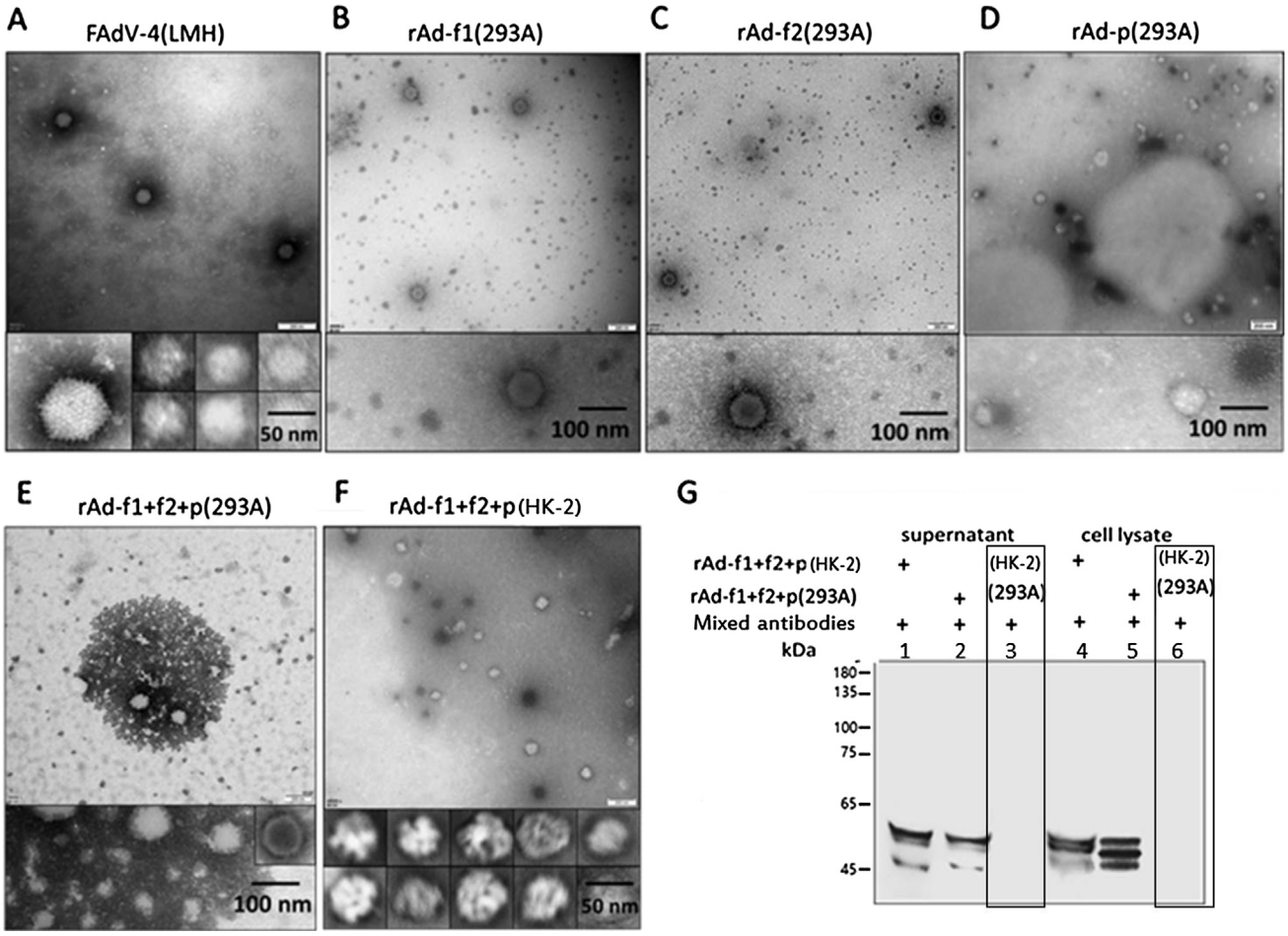

Fig. 2. Transmission electron micrographs of Ad virions and/or Pt-Dds in cell culture media. (A) FAdv-4 virions (100 nm) and Pt-Dds (50 nm) in cell culture media of LMH cells infected with FAdV-4; (B, C) HAdV-C5 viral particles (100 nm) in cell culture media of HEK293 A cells infected with rAd-f1 and rAd-f2 respectively; (D) HAdV-C5 virions and penton base Dds (50 nm) in cell culture media of HEK293 A cells infected with rAd-p; (E) HAdV-C5 viral particles and Pt-Dds in cell culture media of HEK293 A cells co-infected with rAd-f1, rAd-f2, and rAd-p; (F) Pt-Dds in cell culture media of HK-2 cells co-infected with rAd-f1, rAd-f2, and rAd-p; (G) Western blot detection of fiber-1, fiber-2, and penton base in supernatant and cell lysate fraction of HEK293 A cells (lane 1,4) or HK-2 (lane 2,5) co infected with rAd-f1, rAd-f2, and rAd-p or control samples (non-infected cell sample, lane 3 and 5).

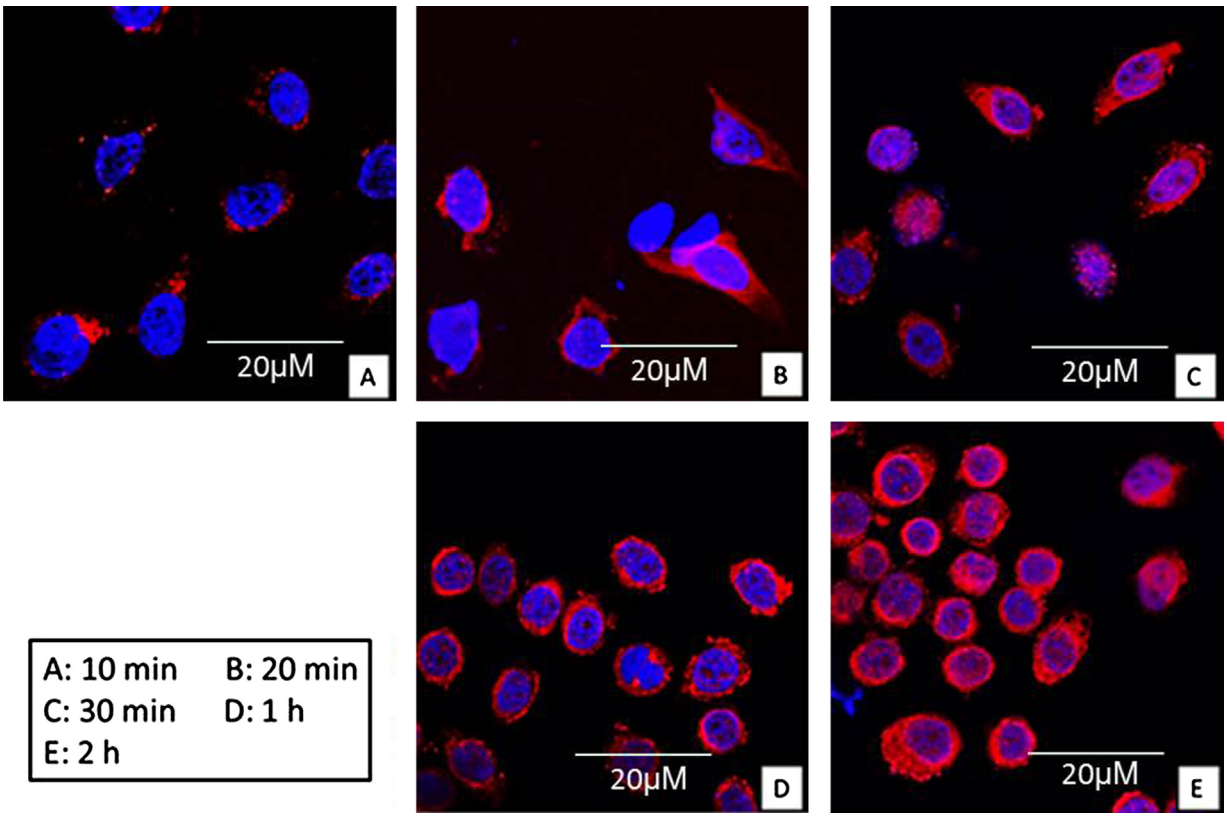

Fig. 3. FAdV-4–Pt-Dd internalized into HeLa cells as determined by confocal microscopy. FAdV-4– Pt-Dd are shown in red with a Cy3-conjugated mouse antibody against fiber-1, while the nuclei are counterstained in blue with DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

infected with rAd-f1 or -f2, expressing only one of the FAdV-4 fibers (Fig. 2B and 2C). As HAdV-C5 virions would not assemble in non-complementing HK-2 cells and HAdV-C5 has no Pt-Dd product, the obtained Pt-Dd in Fig. 2F could only be derived from the overexpressed fiber-1, fiber-2, and penton base of FAdV-4. In addition, Pt-Dd in cell culture supernatants and cell lysates were detected with western blot (Fig. 2G) and the concentration of the Pt-Dd in cell supernatants was approximately 50 μg/mL.

3.3. Internalization of FAdV-4 VLPs

To generate Pt-Dd without HAdV-C5 virions, rAd-f1 and rAd-f2 together with rAd-p were used to infect HK-2 cells. Culture media were collected at 48 h post-infection. The cell culture media from HK-2 cells infected with rAd-GFP were used as the control.

The internalization of Pt-Dds into HeLa cells was analyzed by confocal microscopy after staining with polyclonal antibodies against fiber-1, −2 and Cy3-labeled secondary antibodies. Pt-Dd was observed to gather around cells as early as 10 min after inoculation (Fig. 3A) and the internalization was finished within 2 h, as all the fluorescence was in the cells by that time (Fig. 3E) and no Pt-Dds were detected in cell culture media with WB at that time (data not shown). The internalization process was clearly related to the time of incubation (Fig. 3A–3E). Each cell that had been incubated with the collected supernatant contained thousands of Pt-Dds. Fiber-1, -2 alone and GFP could not be internalized into cells (data not shown).

3.4. Immune responses against FAdV-4 following chicken vaccination

Chickens were immunized on day 7 with approximately 0.5 μg (0.1 mL) of recombinant proteins (in cell lysates) obtained by transduction of HK-2 cells with the corresponding rAd and the chickens were challenged with FAdV-4 on day 21 by intramuscular injection. The chicken sera were collected on days 14, 21, and 35 post-immunization and antibodies were detected by enzyme-linked immunosorbent assay (ELISA) with the mixed proteins (*E. coli* expressed fiber-1, fiber-2, and penton base; 0.2 μg each in one well) as reported previously [39]. Antibodies reached maximal titer on day 28. The average antibody titers (log10) in chickens immunized with Pt-Dd, fiber-1, fiber-2, penton base, or inactivated vaccine were 4.56 ± 0.67, 3.54 ± 0.20, 3.82 ± 0.21, 1.46 ± 0.46, and 3.82 ± 0.00, and the chickens immunized with Pt-Dd gained significantly higher ($p < 0.05$) levels of antibodies than the other immunized groups at day 21 (Fig. 4A).

Host responses in PBMCs collected at 24 hpc were evaluated by quantitation of interferonγ (IFN-γ), interleukin-4 (IL-4), and major histocompatibility complex II (MHC-II) expression using RT-qPCR as reported previously [40]. As shown in Fig. 4B, 4C and 4D, vaccination with Pt-Dd significantly increased the expression of MHC-II, IFN-γ, and IL-4 in PBMC compared to vaccination with fiber-2 or penton base. Fiber-1 also induced high levels of IL-4 expression (Fig. 4D).

3.5. Challenge protection test

All chickens except those in the non-infection control group were challenged with $10^{5.5}$ $TCID_{50}$ of FAdV-4 SX15 by intramuscular injection. The FAdV-4 SX15 challenge resulted in 73.3% (11/15) mortality in the challenge control group (GFP). Immunization with Pt-Dd, fiber-1, fiber-2, penton base or inactivated vaccine reduced the mortality from 73.3% to 0%, 0%, 20%, 33.3%, and 0%, respectively, and the corresponding survival ratios were 26.7%, 100%, 80%, 67.7%, and 100%, respectively (Fig. 5).

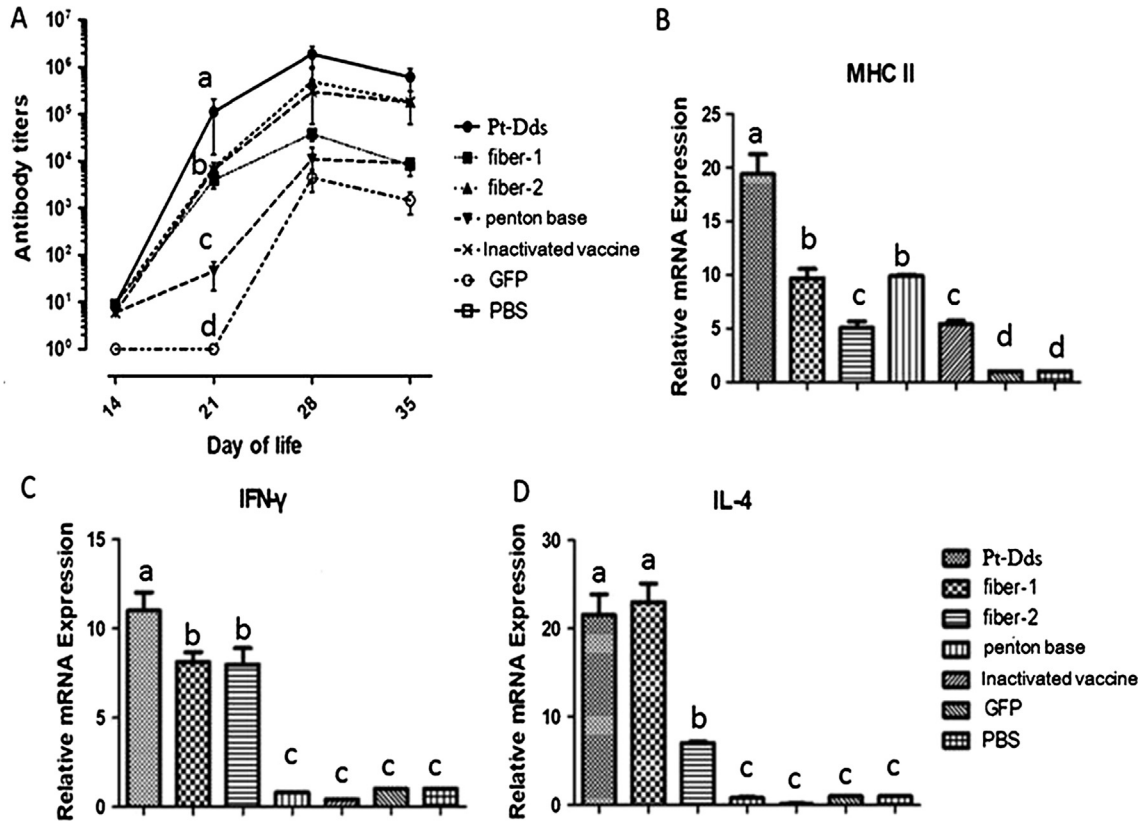

Fig. 4. Humoral response and immune molecules expression induced by immunization. (A) Detection of FAdV-4 antibodies with ELISA; (B, C, D) Expression of MHC-II, IFN-γ and IL-4 in PBMCs, as determined by RT-qPCR. Data are presented as mean ± standard deviation and significant differences are marked with different letters ($p \leq 0.05$).

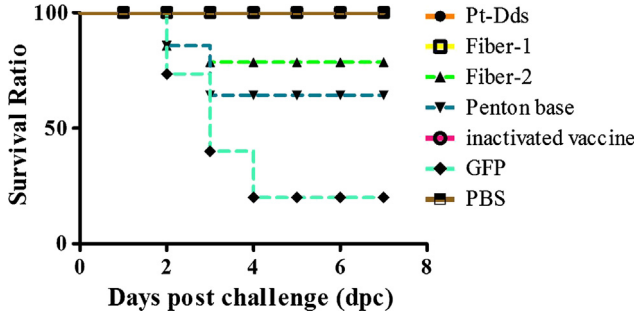

Fig. 5. Survival ratios of chickens to the challenge of virulent FAdV-4. Chickens were challenged with $10^{5.5}$ $TCID_{50}$ of FAdV-4 SX15 strain by intramuscular injection and the survival ratios were recorded for 7 days.

3.6. Histology and immunohistochemistry

Histology and immunohistochemistry analyses showed that the most serious micro lesions were observed in the livers of chickens in the control group given GFP. The hepatic lobule structures were completely missing. The hepatocyte tubes were arranged haphazardly with severe congestion and inflammatory cell infiltration. Liver cells appeared necrotic with nuclear fragmentation (Fig. 6 GFP). Immunization with penton base reduced the pathological changes mentioned above (Fig. 6 Penton base). However, the hepatic lobule structures were still not very clear and parts of the liver cells appeared necrotic with nuclear fragmentation (Fig. 6 Penton base). Fiber-2 also did not protect liver microstructures well; however, the lesions were only slight and necrotic liver cells were even fewer (Fig. 6 Fiber-2). Comparatively, Pt-Dd, fiber-1, and the inactivated vaccine immune group provided better protection against FAdV-4 infection based on observations of the pathological tissue slices. The hepatic lobules had a clear structure and the hepatocyte tubes were arranged orderly and closely in the livers from these groups. Meanwhile, ecchymosis in the hepatic lobules was observed in the livers from both groups, and immunohistochemical analysis indicated that serious infection occurred in chickens immunized with GFP and penton base. Fewer FAdV-4-positive signals were detected in the liver sections of chickens from groups immunized with FAdV-4 VLPs and inactivated vaccine group, although the difference was not significant among the VLP, fiber-1, fiber-2, and inactivated vaccine (InV) immune groups (Fig. 7A).

3.7. Testing viral loads in sera

Viral DNA copies in sera collected at day 28 and 35 were detected using quantitative PCR (qPCR). The highest viral loads were detected at day 28 i.e 7 dpc in sham immunized chickens (in group rAd-GFP). Viral DNA copies in sera collected from chickens in group rAd-GFP were about $10^{6.81}$ copies/100 μL on day 28 and $10^{2.95}$ copies/100 μL at day 35 i.e 14 dpc. In sera of immunized chickens, viral load ranges from 800 to 11,500 copies/ 100 μL at day 28 and reduced to 70 to 700 copies/100 μL at day 35 (Fig. 7B).

4. Discussion

HAd vectors are commonly applied viral vectors for gene therapy and delivery of vaccine antigens. The HAd vectors that are used as vaccines are mostly replication-defective with certain essential viral genes deleted and replaced by cassette expressing a foreign gene [41]. In the present study, replication-defective HAdV-C5

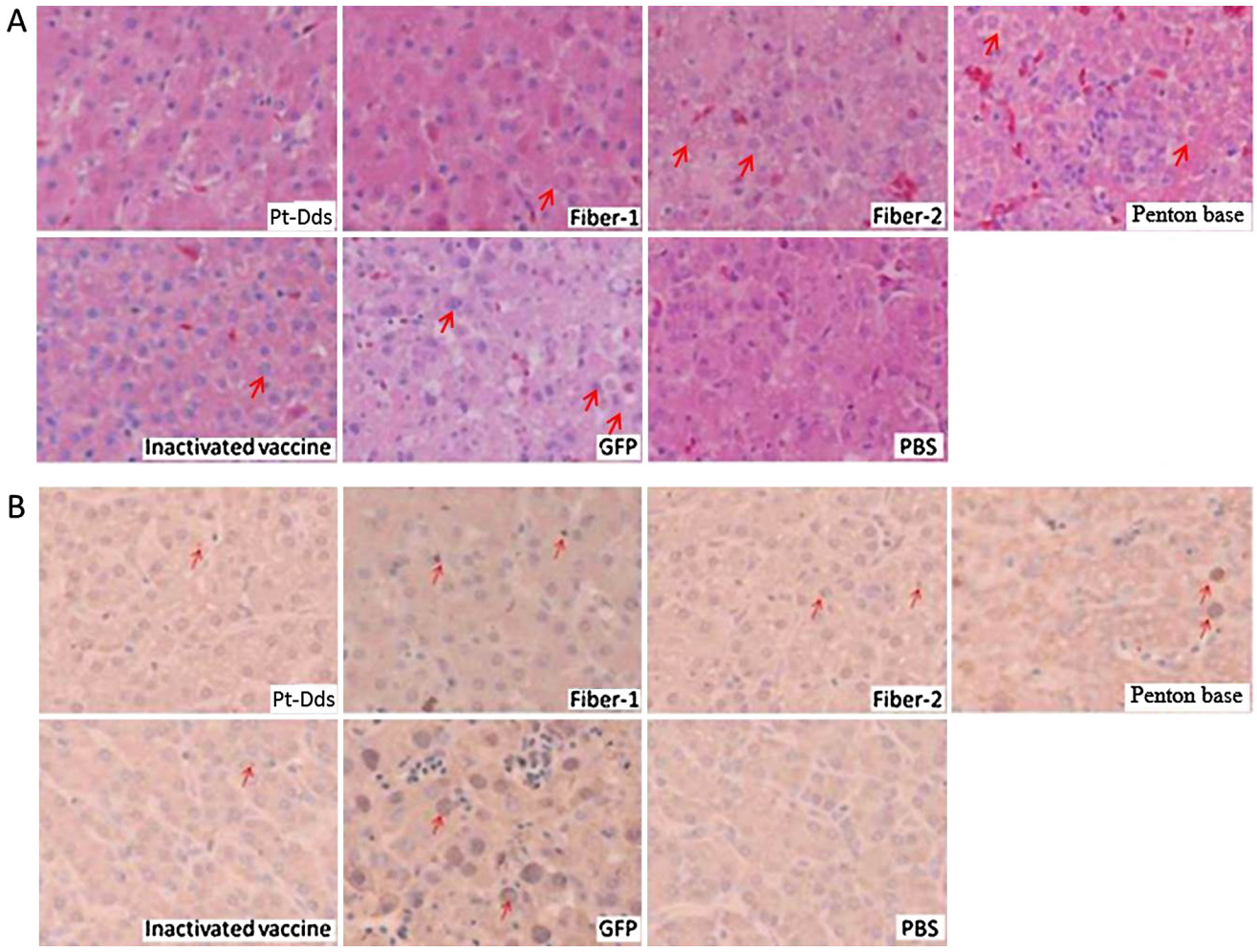

Fig. 6. Observation of micro-lesions and detection of viral antigens in the liver (A) Histological analysis of the lesions in liver (hematoxylin and eosin). The lesions are shown as cell body boggy, structure blurring, nuclear boggy (marked with arrow), even shattering, melting and so on. The most clear lesions are observed in the tissues from the non-immunized chickens and then from the penton base and fiber-2 groups; (B) Detection of viral antigens using histology and immunohistochemistry analyses. Viral positive signals (dark brown, partial of viral positive signals were marked with arrow) were detected in cells or hepatic sinusoid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

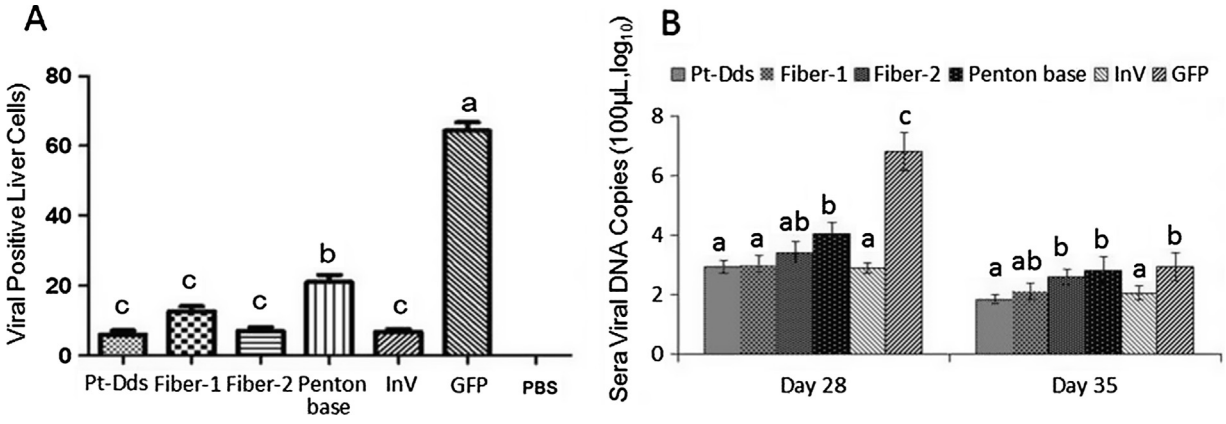

Fig. 7. Calculated viral positive cells in liver sections (A) and detected viremia with RT-qPCR (B). (A) Positive cells (stained by brown) were counted in five random fields per slice and three slices were used for each sample. Average number of positive cells per fields was calculated and the mean values were used to show the viral loads in livers. Fig. 6 represented a portion of the field used for the counts. (B) Viral DNA copies in sera were calculated according to the obtained standard curve. Viral DNA copies (100 μL serum, Log_{10}) were used to represent viremia. Data are presented as mean ± standard deviation and significant differences are marked with different letters ($p \leq 0.05$).

was used to express the fibers and penton base of FAdV-4. The HAdV-C5 vector has essential genes, E1A and E1B, deleted and replaced by a cassette for expression foreign genes under the control of immediate early CMV promoter. The E1A region encodes immediate early proteins, which play a role in initiating expression of the ~20 delayed early genes in the E1B, E2, E3, and E4 transcription units that are essential for Ad replication [41]. E1B proteins protect the infected cell from apoptosis induced by E1A expression. In a productive infection, E1B 55 K protein also mediates efficient transport of late viral mRNA transcripts to the cytoplasm [42]. These deleted HAdV-C5 vectors are constructed from plasmids or Ad DNA containing the genetically modified Ad genome, and these E1-deleted HAdV-C5-based vectors are grown up on complementing cell lines such as HEK293 A, PER.C6, or N52.E6 which express the E1A and E1B genes [43]. Thus, these E1-deleted HAdV-C5 vectors would undergo continuous passage in HEK293 A cells. In HK-2 cells without E1A and E1B genes, HAdV-C5 would not replicate though the foreign genes, under control of the IE CMV promoter, would be expressed independently of adenovirus gene expression. In the present study, HAdV-C5 virions, as well as Pt-Dds of FAdV-4 (HAdV-C5 has no Pt-Dd) were observed in HEK293 A cells that had been infected by rAd-f1, rAd-f2, and rAd-p; however, no HAdV-C5 virions, only Pt-Dds, were found in infected HK-2 cells. Accordingly, it would be interesting to produce Pt-Dds in cells which do not express adenovirus E1.

Dodecahedra (Dd) composed of 12 pentons are found in cells infected with HAdV-B3 [44]. These are synthesized in abundance with 5.5×10^6 Dds produced per one infectious virus in Hela cells [45]. As calculated at 24 h.p.i, approximately 200-fold more Dds than total viral particles are found in HAdV-B3-infected Hela cells determined by WB [45]. Ad Dd is different than other known VLPs of non-enveloped viruses, such as human papilloma virus or Norwalk VLPs, whose VLPs morphologically mimic native virions. Ad Dd is smaller and lacks several structural components of the Ad viral particle resulting in functional and structural properties that are different from those of the virus capsid. For example, Dd is able to attach to and penetrate cells via interaction with heparan sulfate, a pathway not used by the virus of origin, HAdV-B3 [46,47]. Owing to its high endocytosis capacity, Pt-Dd has been investigated extensively as an alternative vector for antigenic protein [48] or small molecule drugs [49]. In the present study, the characteristics of Pt-Dd from fAdV-4 were studied. By co-infection of HEK293 A or HK-2 cells with recombinant Ad5 expressing fiber-1, fiber-2, and penton base of fAdV-4, Pt-Dds were assembled, and they could be efficiently internalized into human cells, Hela cells (Fig. 3) and chicken embryo fibroblasts (data not shown). Because fAdV-4 is not from a human source, there is less chance that related antibodies would be found in human; therefore, the fAdV-4 derived Pt-Dd could be a good drug delivery system for human disease therapy.

HAdV-B3 Dds exhibit remarkable stability and can be stored for long periods at 4 °C and even at room temperature [49]. Thawing and reconstitution with water after being frozen and lyophilized did not significantly influence their integrity. As previously reported, Dds retain their particulate integrity in human serum at 37 °C for at least 2 h [49]. Accordingly, Dds can be conveniently stored and transported, and can be used as a vaccine or drug delivery system under various climates. Although we did not check the stability of Pt-Dd of FAdV-4 in multiple environments , they can penetrate HeLa cells after 4 weeks of storage at 4 °C (data not shown).

In the present study, Pt-Dds of FAdV-4 VLPs were produced using HAdV-C5, and the immune responses induced by Pt-Dd were evaluated. Pt-Dd is readily internalized by dendritic cells which are principal antigen-presenting cells. Pt-Dd, along with additional antigens, might induce strong cell mediated immune responses. Mice immunized with OVA linked to HAdV-B3 Pt-Dd by WW domain developed OVA-specific CD8 + T cells and robust humoral responses in mice [21]. Proteins or peptides alone do not easily elicit a strong immune response. Pt-Dd, by improving the antigen delivery, played the role of an adjuvant. In the present study, immunization with Pt-Dd induced humoral antibody and increased expression of mRNA encoding cytokines IL-4 and IFN-γ as well as MHC-II in PBMCs. In addition, Pt-Dd also induced higher levels of humoral immune response with significant high levels of antibodies in serum detected by ELISA. In a previous study, the purified FAdV-8b VLPs also induced neutralizing antibodies and cytotoxic T-cell responses as evidenced by a statistically significant ($p < 0.05$) CD8 + T-cell proliferative response in breeders and prevented disease in progeny via maternal antibodies transfer [35]. Accordingly, the Pt-Dd of FAdVs might be an ideal vaccine candidate for the control of related diseases.

In the current study, fiber-1 provided better protection than did fiber-2 against challenge with FAdV-4. This result is different to our previous study [39] and several other related studies [50,51], in which fiber-2 induced better protection than fiber-1 against FAdV-4 infection than fiber-1 [39,50,51]. It is interesting that, in the current study, fiber-2 induced higher antibody levels whereas fiber-1 induced higher expression of IL-4 and MHC-II by PBMCs (Fig. 4). Further studies are required to reveal the reason behind these differences. Pt-Dds of FAdV-4 induced most strong immune responses and immune protection against the challenge of FAdV-4. Since the cells and viral vector were same, we believe that the enhanced immune responses were derived from the specific function of Pt-Dds, but not the components from HK-2 cell or HAdV-C5. In addition, recombinant adenovirus expression GFP did not induced FAdV-4 specific immune response and immune protection against FAdV-4 infection.

In summary, Pt-Dds were assembled in human cells infected with E1-deleted HAdV-C5 encoding penton base and both fiber proteins of FAdV-4. These Pt-Dds could penetrate different types of cells with high efficiency indicating that they could provide a new delivery system for drugs or for antigenic molecules, in the case of vaccines. Immunization with Pt-Dd induced high levels of humoral antibody as well as IL-4, IFN-γ and MHC-II expression in PBMCs and offered complete protection against challenge with FAdV-4.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 31672581).

References

[1] Davison AJ, Benkő M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol 2003;84:2895–908.
[2] Lion T. Adenovirus infections in immunocompetent and immunocompromised patients. Clin Microbiol Rev 2014;27:441–62.
[3] Kajon AE, Ison MG. Severe infections with human adenovirus 7d in 2 adults in family, Illinois, USA, 2014. Emerg Infect Dis 2016;22:730–3.
[4] San Martín C. Latest insights on adenovirus structure and assembly. Viruses 2012;4:847–77.
[5] Reddy VS, Nemerow GR. Structures and organization of adenovirus cement proteins provide insights into the role of capsid maturation in virus entry and infection. In: Proceedings of the National Academy of Sciences of the United States of America. p. 11715–20.
[6] Zubieta C, Schoehn G, Chroboczek J, Cusack S. The structure of the human adenovirus 2 penton. Mol Cell 2005;17:121–35.

[7] Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 1993;73:309–19.
[8] Szolajska E, Burmeister WP, Zochowska M, Nerlo B, Andreev I, Schoehn G, et al. The structural basis for the integrity of adenovirus Ad3 dodecahedron. PLoS One 2012;7:e46075.
[9] The Norrby E. Relationship between the soluble antigens and the virion of adenovirus Type 3. 1.morphological characteristics. Virology 1966;28:236–48.
[10] Gelderblom H, Bauer H, Frank H. The structure of group II adenoviruses. J Gen Virol 1967;1:553–60.
[11] Norrby E, Skaaret P. Comparison between soluble components of adenovirus types 3 and 16 and of the intermediate strain 3–16 (the San Carlos Agent) ☆. Virology 1968;36:201–11.
[12] Fender P, Ruigrok RW, Gout E, Buffet S, Chroboczek J. Adenovirus dodecahedron, a new vector for human gene transfer. Nat Biotechnol 1997;15:52–6.
[13] Liew MWO, Chuan YP, Middelberg APJ. High-yield and scalable cell-free assembly of virus-like particles by dilution. Biochem Eng J 2012;67:88–96.
[14] Boulanger PA, Puvion F. Occurrence of a peculiar type of adenovirus 2 penton oligomer. Intervirology 1976;7:126–34.
[15] Wilcox WC, Ginsberg HS. Structure of type 5 adenovirus. 1963;118:307–14.
[16] Karayan L, Gay B, Gerfaux J, Boulanger PA. Oligomerization of recombinant penton base of adenovirus type 2 and its assembly with fiber in baculovirus-infected cells. Virology 1994;202:782–95.
[17] Fender P, Schoehn G, Foucaudgamen J, Gout E, Garcel A, Drouet E, et al. Adenovirus dodecahedron allows large multimeric protein transduction in human cells. J Virol 2003;77:4960–4.
[18] Garcel A, Gout E, Timmins J, Chroboczek J, Fender P. Protein transduction into human cells by adenovirus dodecahedron using WW domains as universal adaptors. J Gene Med 2006;8:524–31.
[19] Zochowska M, Paca A, Schoehn G, Andrieu JP, Chroboczek J, Dublet B, et al. Adenovirus dodecahedron, as a drug delivery vector. PLoS One 2009;4:e5569-e.
[20] Naskalska A, Szolajska E, Chaperot L, Angel J, Plumas J, Chroboczek J. Influenza recombinant vaccine: matrix protein M1 on the platform of the adenovirus dodecahedron. Vaccine 2009;27:7385–93.
[21] Villegas-Mendez A, Garin MI, Pineda-Molina E, Veratti E, Bueren JA, Fender P, et al. In vivo delivery of antigens by adenovirus dodecahedron induces cellular and humoral immune responses to elicit antitumor immunity. Mol Ther 2010;18:1046–53.
[22] Kang X, Xiao H-H, Song H-Q, Jing X-B, Yan L-S, Qi R-G. Advances in drug delivery system for platinum agents based combination therapy. Cancer Biol Med 2015;12:362–74.
[23] Mccracken RM, Mcferran JB, Evans RT, Connor TJ. Experimental studies on the aetiology of inclusion body hepatitis. Avian Pathol J Wvpa 1976;5:325–39.
[24] Hess M. Detection and differentiation of avian adenoviruses: a review. Avian Pathol 2000;29:195–206.
[25] Wang X, Tang Q, Chu Z, Wang P, Luo C, Zhang Y, et al. Immune protection efficacy of FAdV-4 surface proteins fiber-1, fiber-2, hexon and penton base. Virus Res 2017;245:1.
[26] Schachner A, Marek A, Jaskulska B, Bilic I, Hess M. Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis-hydropericardium syndrome (HHS). Vaccine 2014;32:1086–92.
[27] You GJ, Xia J, Yao KC, Liu P, Zhao Q, Cao SJ, et al. Isolation and molecular characterization of prevalent Fowl adenovirus strains in southwestern China during 2015–2016 for the development of a control strategy. Emerg Microbes Infect 2017;6:e103.
[28] Changjing L, Haiying L, Dongdong W, Jingjing W, Youming W, Shouchun W, et al. Characterization of fowl adenoviruses isolated between 2007 and 2014 in China. Vet Microbiol 2016;197:62–7.
[29] Zhang T, Jin Q, Ding P, Wang Y, Chai Y, Li Y, et al. Molecular epidemiology of hydropericardium syndrome outbreak-associated serotype 4 fowl adenovirus isolates in central China. Virology J 2016;13:188.
[30] Kim MS, Lim TH, Lee DH, Youn HN, Yuk SS, Kim BY, et al. An inactivated oil-emulsion fowl Adenovirus serotype 4 vaccine provides broad cross-protection against various serotypes of fowl Adenovirus. Vaccine 2014;32:3564–8.
[31] Kumar R, Chandra R, Shukla SK, Agrawal DK, Kumar M. Hydropericardium syndrome (HPS) in India: a preliminary study on the causative agent and control of the disease by inactivated autogenous vaccine. Trop Anim Health Prod 1997;29:158.
[32] Anjum AD. Experimental transmission of hydropericardium syndrome and protection against it in commercial broiler chickens. Avian Pathol 1990;19:655–60.
[33] Schonewille E, Jaspers R, Paul G, Hess M. Specific-pathogen-free chickens vaccinated with a live FAdV-4 vaccine are fully protected against a severe challenge even in the absence of neutralizing antibodies. Avian Dis 2010;54:905–10.
[34] Mansoor MK, Hussain I, Arshad M, Muhammad G. Preparation and evaluation of chicken embryo-adapted fowl adenovirus serotype 4 vaccine in broiler chickens. Trop Anim Health Prod 2011;43:331–8.
[35] Gupta A, Ahmed KA, Ayalew LE, Popowich S, Kurukulasuriya S, Goonewardene K, et al. Immunogenicity and protective efficacy of virus-like particles and recombinant fiber proteins in broiler-breeder vaccination against fowl adenovirus (FAdV)-8b. Vaccine 2017;35:2716.
[36] Jia L-J, Zhang S-F, Qian N-C, Xuan X-N, Yu L-Z, Zhang X-M, et al. Generation and immunity testing of a recombinant adenovirus expressing NcSRS2-NcGRA7 fusion protein of bovine Neospora caninum. Kor J Parasitol 2013;51:247–53.
[37] Wang X, Qiu L, Hao H, Zhang W, Fu X, Zhang H, et al. Adenovirus-based oral vaccine for rabbit hemorrhagic disease. Vet Immunol Immunopathol 2012;145:277–82.
[38] Pan Q, Yang Y, Shi Z, Liu L, Gao Y, Qi X, et al. Different dynamic distribution in chickens and ducks of the hypervirulent, novel genotype fowl adenovirus Serotype 4 recently emerged in China. Front Microbiol 2017;8.
[39] Wang X, Tang Q, Chu Z, Wang P, Luo C, Zhang Y, et al. Immune protection efficacy of FAdV-4 surface proteins fiber-1, fiber-2, hexon and penton base. Virus Res 2018;245:1–6.
[40] Wang X, Wang X, Jia Y, Wang C, Tang Q, Han Q, et al. Coadministration of recombinant adenovirus expressing GM-CSF with inactivated H5N1 avian influenza vaccine increased the immune responses and protective efficacy against a wild bird source of H5N1 challenge. J Interferon Cytokine Res 2017;37:467–73.
[41] Wold WSM, Toth K. Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. Curr Gene Ther 2013;13:421–33.
[42] Turner RL, Wilkinson JC, Ornelles DA. E1B and E4 oncoproteins of adenovirus antagonize the effect of apoptosis inducing factor. Virology 2014;456–457:205–19.
[43] Silva AC, Peixoto C, Lucas T, Küppers C, Cruz PE, Alves PM, et al. Adenovirus vector production and purification. Curr Gene Ther 2010;10:-.
[44] Norrby E. Comparison of soluble components of adenovirus types 3 and 11. J Gen Virol 1968;2:135–42.
[45] Fender P, Boussaid A, Mezin P, Chroboczek J. Synthesis, cellular localization, and quantification of penton-dodecahedron in serotype 3 adenovirus-infected cells. Virology 2005;340:167–73.
[46] Vivès RR, Lortat-Jacob H, Chroboczek J, Fender P. Heparan sulfate proteoglycan mediates the selective attachment and internalization of serotype 3 human adenovirus dodecahedron. Virology 2004;321:332–40.
[47] Fender P, Schoehn G, Perron-Sierra F, Tucker GC, Lortat-Jacob H. Adenovirus dodecahedron cell attachment and entry are mediated by heparan sulfate and integrins and vary along the cell cycle. Virology 2008;371:155–64.
[48] Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, et al. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. PLoS One 2008;3:e2257.
[49] Zochowska M, Paca A, Schoehn G, Andrieu J-P, Chroboczek J, Dublet B, et al. Adenovirus dodecahedron, as a drug delivery vector. PLoS One 2009;4:e5569.
[50] Schachner A, Marek A, Jaskulska B, Bilic I, Hess M. Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis–hydropericardium syndrome (HHS). Vaccine 2014;32:1086–92.
[51] Shah M, Ashraf A, Rahman M, Khan M, Qureshi J. A subunit vaccine against hydropericardium syndrome using adenovirus penton capsid protein. Vaccine 2012;30:7153–6.