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Immune responses of chickens inoculated with a recombinant fowlpox vaccine coexpressing HA of H9N2 avain influenza virus and chicken IL-18

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ABSTRACT

Control of the circulation of H9N2 avian influenza virus (AIV) is a major concern for both animal and public health, and H9N2 AIV poses a major threat to the chicken industry worldwide. Here, we developed a recombinant fowlpox virus (rFPV-HA) expressing the haemagglutinin (HA) gene of the A/CH/JY/1/05 (H9N2) influenza virus and a recombinant fowlpox virus (rFPV-HA/IL18) expressing the HA gene and chicken interleukin-18 (IL-18) gene. Recombinant plasmid pSY-HA/IL18 was constructed by cloning chicken IL-18 expression cassette into recombinant plasmid pSY-HA containing the HA gene. Two rFPVs were generated by transfecting two recombinant plasmids into the chicken embryo fibroblast cells pre-infected with S-FPV-017, and assessed for their immunological efficacy on one-day-old White Leghorn specific-pathogen-free chickens challenged with the A/CH/JY/1/05 (H9N2) strain. There was a significant difference in HI antibody levels ($P < 0.05$) elicited by either rFPV-HA or rFPV-HA/IL18. The level of splenocyte proliferation response in the rFPV-HA/IL18-vaccinated group was significantly higher ($P < 0.05$) than that in the rFPV-HA group. After challenge with $10^{6.5}$ ELD_{50} H9N2 AIV 43 days after immunization, rFPVs vaccinated groups could prevent virus shedding and replication in multiple organs in response to H9N2 AIV infection, and rFPV-HA/IL18 vaccinated group had better inhibition of viruses than rFPV-HA vaccinated group. Our results show that the protective efficacy of the rFPV-HA vaccine could be enhanced significantly by simultaneous expression of IL-18.

1. Introduction

Avian influenza (AI) is a contagious viral disease of animals caused by avian influenza virus (AIV). AIV is classified into a number of subtypes based on antigenic differences in their two surface glycoproteins, hemagglutinin (HA; 16 subtypes) and neuraminidase (NA; nine subtypes) (Fouchier et al., 2005). Among the many subtypes, the H9N2 AIV has been detected in many countries since its first isolation in the United States in 1966 (Homme and Easterday, 1970; Alexander, 2007). Infection with H9N2 virus often decreases the laying rate of hens, and coinfection with other viruses or bacteria can cause severe morbidity and high mortality in chickens (Kishida et al., 2004; Kim et al., 2006). The frequent heavy losses incurred with H9N2 infection have raised serious concerns for the poultry industry in many countries. H9N2 AIV normally infects only birds, but human infection with H9N2 viruses was reported in Hong Kong in 1999 as the first avian-to-human transmission, and another case was reported in 2003 (Peiris et al., 1999; Butt et al., 2005), and H9N2 AIV infection in pig farms has also been confirmed in several provinces in China (Xu et al., 2004), suggesting that H9N2 AIV has crossed the species barrier to infect other animals including humans.

In mainland China, H9N2 AIV was first isolated in 1994 from chickens in Guangdong province (Li et al., 2005), and subsequently spreads to other southern provinces, resulting in severe economic losses for the poultry industry, suggesting that urgent attention should be paid to the control of H9N2 AI in chickens. Vaccination is one of the most promising control measures for H9N2 AI to date. Inactivated whole virus vaccines in oil emulsion have been developed and used since 1998 on layer and breeder chickens in farms in China (unpublished data). Although inactivated whole AIV virus vaccines have been proven to be safe and efficacious against H9N2 AIV, the cost of production, laborious administration of this vaccine, lack of long-term immunity of inactivated vaccines, and interference of routine surveillance by serological test, are limitations for their wide application in the field. Thus, it is highly desirable for developing a vaccine to control this disease.

HA on the viral surface is the major protective antigen of AIV and HA antibodies against it are critical for virus neutralization

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and protection against infection (Stevens et al., 2006). Thus HA was a logical choice for inclusion in a recombinant candidate vaccine vector. The basis of protective humoral immunity is the development of neutralizing antibody against HA and a variety of vaccines derived from HA gene of AIV, including recombinant virus vaccines (Chambers et al., 1988; Hunt et al., 1988; Qiao et al., 2009; Ge et al., 2010), subunit hemagglutinin protein (Lin et al., 2008; Oh et al., 2010), reassortant influenza vaccines (Song et al., 2008), and DNA vaccines (Pan et al., 2009) that could induce subtype-specific immunity and have shown efficacy against challenge with homologous virus are under development.

Fowlpox virus (FPV) has a large double strand of DNA genome and a host range limited to avian species (Bolte et al., 1999). FPV has been developed as an effective live viral vector, successfully expressing protective foreign genes from several avian pathogens, including Newcastle disease virus, infectious bronchitis virus, infectious laryngotracheitis virus, H5N1 AIV, and Marek's disease virus. Vaccination with the recombinant fowlpox viruses was able to protect chickens from challenge with the corresponding viruses (Butter et al., 2003; Sun et al., 2008; Wang et al., 2009). The immune efficacy of a recombinant FPV co-expressing H5N1 AIV HA and NA in SPF and commercial chickens has been reported previously (Qiao et al., 2009). However, the use of fowlpox virus as a vaccine vector for recombinant H9N2 AIV vaccine has not been previously investigated. Interleukin-18 (IL-18) is an important cytokine with multiple functions in innate and acquired immunity (Dinarello and Fantuzzi, 2003; Gracie et al., 2003). Previous research indicated that recombinant chicken IL-18 has the positive adjuvant effect for the recombinant fowlpox virus (Mingxiao et al., 2006). Therefore, a recombinant fowlpox virus expressing the H9N2 AIV HA gene (rFPV-HA) and a recombinant fowlpox virus co-expressing the H9N2 AIV HA gene and the chicken IL-18 gene (rFPV-HA/IL18) were constructed and their immunological efficacy investigated by immunizing SPF chickens.

2. Materials and methods

2.1. Virus, experimental animals and plasmids

Fertilized White Leghorn specific-pathogen-free (SPF) eggs were purchased from Beijing Merial Vital Laboratory Animal Technology Co. Ltd., Beijing, PR China. Chickens were hatched and housed in SPF isolators with negative pressure. The H9N2 influenza virus A/chicken/Jiyuan/1/2005 (abbreviated for A/CH/JY/1/05) was isolated from a group of approximately 30-week-old layers in Henan Province suffering from severe drop in egg production and propagated in the allantoic cavities of 10-day old SPF embryonated chicken eggs for 72 h at 37 °C. The 50% embryo lethal dose (ELD_{50}) was determined by inoculating serial 10-fold dilutions of virus into 10-day-old SPF embryonated chicken eggs. All operations with A/CH/JY/1/05 virus were performed in a biosafety level 3 laboratory.

The parental fowlpox virus, S-FPV-017, was a less attenuated FPV strain (a kind gift from Dr. Hua-Lan Chen, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences) and propagated in monolayers of SPF chicken embryo fibroblast (CEF) cells in DMEM supplemented with 5% fetal calf serum.

Plasmid pSY538 had the early-late LP_2EP_2 promoters of FPV, while plasmid pSC11 had a LacZ gene fragment with the P11 late promoter of vaccinia virus and pSY681 had two FPV DNA regions (Fig. 1A). These FPV DNA regions were the recombinant arms of FPV that allowed crossing over to occur when the plasmids were co-infected with FPV in CEF cells.

2.2. Homologous recombination and screening of the recombinant fowlpox virus

cDNA encoding the HA gene of A/CH/JY/1/05 virus was amplified by RT-PCR using the primers HAfs and HArs (Table 1), and inserted into the *Bam*H I site of plasmid pSY538 under the control of the early-late LP_2EP_2 promoter of FPV. The LacZ gene fragment with the P11 late promoter of vaccinia virus from the plasmid pSC11 was digested with *Pst* I and *Xba* I, and cloned into the *Sma* I site downstream of the HA gene in the pSY538 plasmid. The DNA fragment containing the HA expression cassette and LacZ expression cassette was cloned into the *Not* I site between the homologous arms of the poxvirus gene in the FPV transfer vector pSY681, resulting in plasmid pSY-HA (Fig. 1B). The chicken IL-18 gene was amplified by PCR reported previously (GenBank accession No. AY775782) using the primers ChIL18fs and ChIL18rs (Table 1), and cloned into the *Eco*R I site of plasmid pSY538. Then the chicken IL-18 expression cassette in pSY538 was ligated into a *Not* I site of the pSY-HA plasmid cut partially with *Not* I to generate the transfer vector pSY-HA/IL18 (Fig. 1C). The presence of all genes in recombinant plasmids was confirmed by restriction endonuclease digestion and DNA sequencing.

Table 1
Primers and probe used in this study.

Target gene	Primer or probe	Sequence (5′–3′)[a]	Expected product (bp)
HA	HAfs	CGGGATCCATGGAAGTAGTATCACTA	1683
	HArs	CCCGGATCCTTATATACAA ATGTTGCAT	
Chicken IL-18	ChIL18fs	CCCGAATTCATGAGCTGTGAAGAGATC	597
	ChIL18rs	CGGGGAATTCTCATAGGTTGTGCCTTT	
NP	NPfs	ACGGAAGTTATAAGGATGA	151
	NPrs	TGTCTCCGAAGAAATAAGA	

[a] Underlined nucleotides indicate restriction endonuclease cleavage sites used for cloning.

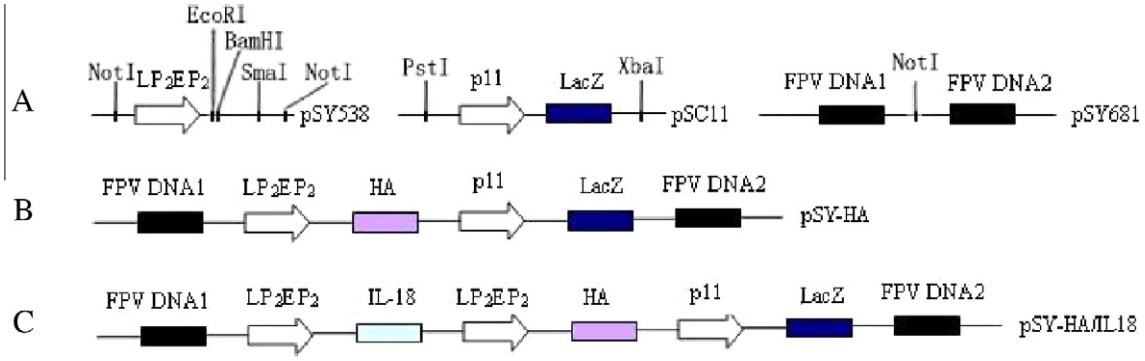

Fig. 1. Schematic representations of fowlpox virus expression plasmids (pSY538, P11 and pSY681) and recombinant plasmids pSY-HA and pSY-HA/IL18.

Plasmid DNA was purified with a Wizard PureFection Plasmid DNA purification system (Promega, Madison, WI), and transfected into 80% confluent CEF that had already been infected with the parental fowlpox virus of S-FPV-017 strain via Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA). rFPVs were generated by DNA homologous recombination of recombinant plasmids and FPV genome. Based on the expression of reporter gene for beta-galactosidase activity in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) (TaKaRa, Dalian, China), rFPVs were selected by blue plaque after cytopathic effect (CPE) appeared. Purified by several successive rounds, cloning of blue plaque, the two rFPVs were obtained and cultured in CEF cells.

The genomic DNA of rFPVs, extracted using a commercial test kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) according to the Manufacturer's instructions, were used as PCR template. PCR was carried out with *Taq* DNA polymerase (TaKaRa, Dalian, China) and the primers HAfs and HArs to confirm insertion of the recombinant gene into the FPV genome.

2.3. Expression of HA and IL-18 proteins in rFPVs

The CEF cells were harvested at 48 h after infection. The total cellular RNA, was prepared from the cells using the HIGH PURE®4 viral RNA kit (Roche, Vilvoorde, Belgium) according to the Manufacturer's instructions. The reverse transcription (RT) reaction was performed using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). PCR was then amplified with specific primer sets for the HA gene and IL-18 as described above.

Western blot analysis was carried out as described previously (Chen et al., 2008). Briefly, after infection for 48–72 h, the total cell lysates were prepared with lysis buffer (10 mM Tris–HCl, pH7.4, 1 mM $MgCl_2$, 0.5% NP40, 20 μg/mlDNase|) and subjected to SDS–PAGE using 12% gel, and then the expressed proteins were blotted to a nitrocellulose membrane (Amersham Pharmacia Biotech, New Jersey). The membrane was blocked in TBS-T (25 mM Tris–HCl, 125 mM NaCl, 0.1% Tween-20, pH 8.0) containing 5% skimmed milk for 2 h at 37 °C, and probed with a dilution of 1:500. After washing three times with TBS-T, bound antibody was detected by incubation of the filter with Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:4000, Southern Biotechnology Associates Inc., Birmingham, AL) in TBS-T containing 5% skimmed milk. DAB (3,3′-diaminobenzidine) was added for color development.

2.4. Immunization and serologic testing

One-day-old White Leghorn SPF chickens were used. Two groups of 16 chickens each were immunized with rFPV-HA/IL18 or rFPV-HA, respectively. Chickens which served as negative controls were immunized with the same amount of S-FPV-017. Mock-infected control chickens were inoculated with sterile PBS. All groups were vaccinated by wing-web puncture with a double needle used for commercial vaccination of poultry with FPV. Approximately 50 μl of inoculum containing 10^4 plaque-forming units (PFU) of FPV were given to each chicken.

Five chickens from each group were bled on a weekly basis after immunization. Sera were treated with receptor destroying enzyme (RDE) to remove non-specific hemagglutination inhibitors, as described previously (Ninomiya et al., 2002), before a hemagglutination inhibition (HI) test was performed. HI assays were performed by following the Office International des Epizooties (OIE) standard (Edwards, 2006). Formalin-inactivated A/CH/JY/1/05 virus was used as antigen in the HI test.

2.5. Peripheral blood lymphocyte proliferation assay

Five blood samples (2.5 ml/chicken) from each group were collected via wing vene puncture in 2.5-ml syringes pre-loaded with 0.2 ml of sodium heparin on day 28 after immunization. Peripheral blood mononuclear cells (PBMCs) were isolated and adjusted to 3×10^6/ml. PBMCs (3×10^5 cells/well) were seeded in a 96-well plate in triplicate. Cultures were stimulated under various conditions at 37 °C for 60 h in a humid atmosphere with 5% CO_2; these conditions included treatment with 5 μg/ml concanavalin A (Con A; positive control), 5 μg/ml purified HA antigen (specific antigen), 5 μg/ml bovine serum albumin (BSA; irrelevant antigen), or medium alone (negative control). A 20-μl aliquot of CellTiter 96 Aqueous One Solution Reagent (Promega) was added into each well according to the Manufacturer's protocol. After a 4-h incubation at 37 °C, the absorbance was read at 490 nm. Proliferative activity was estimated using the stimulation indexes (SI) that were defined as the mean OD 490 of the antigen-containing wells divided by the mean OD 490 of the wells without antigen.

2.6. Virus challenge experiment

On day 43 after immunization, all of the chickens were challenged by intranasal instillation of 0.1 ml containing $10^{6.5}$ ELD_{50} of A/CH/JY/1/05 viruses. Cloacal and oropharyngeal swabs were taken at 3 and 5 days post challenge for further detection of virus. Cotton swabs were immersed in 1 ml sampling buffer (OPTI-MEM®3; Invitrogen Corp., Merelbeke, Belgium) supplemented with antibiotics (gentamicin 0.5% [v/v]) and glutamine (1% [v/v]). Virus shedding was confirmed by RT-PCR as described below.

On day 5 post challenge, the chickens were sacrificed and the various tissues were taken as described previously (Choi et al., 2008). All tissue specimens were inoculated into SPF embryonated eggs for virus isolation. Ten-day-old embryonated chicken eggs were inoculated with 0.2 ml of 10-fold dilutions of 20% tissue suspensions by the allantoic route. After 72 h incubation at 37 °C, the allantoic fluid was tested for hemagglutinating activity, using 0.5% chicken erythrocyte suspensions.

2.7. Detection of virus by RT-PCR

RNA was extracted from swab specimens using the HIGH PURE®4 viral RNA kit according to the Manufacturer's protocol. The RT reaction was performed using Revert Aid™ First Strand cDNA Synthesis Kit. A pair of primers NPfs and NPrs (Table 1) was selected using the Primer Premier (version 5.0) software and designed to detect the available AIV sequences based on a highly conserved sequence within the nucleoprotein (NP) region of the AIV genome. PCR assays were performed in a total reaction volume of 25 μl. The reaction mixtures contained the following reagents: 3.0 mM $MgCl_2$, 1× PCR Buffer II (500 mM KCl and 100 mM Tris–HCl, pH 8.3), 200 μM of each dNTP, 0.5 μl of 50 μM each primer, 2 μl cDNA, and 2.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The thermal profile for the PCR was 94 °C for 5 min, followed by 36 cycles of 94 °C for 5 s, 50 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. Total protection was defined as the absence of detectable virus.

2.8. Statistical analysis

Differences between groups were analyzed by analysis of variance (ANOVA) and Student's *t*-test using SPSS11.5 biostatistics software. *P* values less than 0.05 were regarded as significant and those less than 0.01 were regarded as highly significant.

3. Results

3.1. Screening of recombinant fowlpox viruses

As the rFPVs have LacZ genes, blue plaques were observed in the cells infected by the rFPVs in the presence of X-gal, but not in the cells infected by S-FPV-017. rFPVs were purified by eight rounds of blue plaque selection.

To confirm that the genes of interest were successfully inserted into the fowlpox genome via homologous recombination, PCR was performed using the genomic DNA of rFPVs as PCR template. As shown in Fig. 2, the expected products of 1.7 kb (HA) and 0.6 kb (chicken IL-18) were amplified from cells infected with rFPV-HA/IL18, no product was amplified from cells infected with the parental fowlpox virus S-FPV-017 (data not shown). The expected product of the 1.7 kb (HA) was amplified from cells infected with rFPV-HA (Fig. 2). The sequencing results showed the nucleotide sequence of HA (1683 bp) and chicken IL-18 (597 bp) was the same as HA (GU471795) and chicken IL-18 (AY775782) published, respectively. Both PCR and sequencing results proved that the target genes had been successfully recombined into the rFPVs.

3.2. Expression of HA and IL-18 proteins in rFPVs

To confirm the expression of HA and IL-18 in CEF cells, total RNA from the rFPV infected cells was analyzed by RT-PCR for the presence of each corresponding mRNA. The predicted RT-PCR products were 1.7 kb in size for the HA gene and 0.6 kb for the chicken IL-18 gene, all of which were confirmed by gel electrophoresis. No specific band of a similar size was seen in any of the mRNA samples in the absence of reverse transcription (data not shown). The sequencing results showed the nucleotide sequences of HA and chicken IL-18 were identical as sequenced above, respectively. Both RT-PCR and sequencing results showed that HA and IL-18 could be successfully expressed in CEF cells infected with rFPV-HA/IL18.

H9N2 AIV HA proteins expressed by rFPV-HA/IL18 and rFPV-HA, and chicken IL-18 expressed by rFPV-HA/IL18 were revealed by Western blot analysis (Fig. 3). The rFPVs infected cells were collected after CPE appeared. The lysates were sampled into SDS–PAGE and transferred into nitrocellulose membrane. The proteins were probed by anti-H9N2 AIV and anti-chicken IL-18 antibodies, respectively, and bound antibody was detected by HRP-conjugated rabbit anti-chicken IgG and visualized with DAB. As shown in Fig. 3, two bands with a molecular weight of 56 kDa were detected in the lysates of CEF infected by rFPV-HA/IL18 and rFPV-HA (Fig. 3, Lane 2–3), and a band with a molecular weight of 22.9 kDa was detected in the lysates of CEF infected by rFPV-HA/IL18 (Fig. 3, Lane 5), but not cells infected with S-FPV-017 alone (Fig. 3, Lane 4), demonstrating that H9N2 AIV HA protein and chicken IL-18 could be expressed correctly in CEF cells.

3.3. Antibody responses to AIV in chickens immunized with rFPVs

As shown in Table 2, HI antibodies were detected 1 week later after vaccination with rFPV, the level was lower. No detectable HI antibody was elicited from chickens vaccinated with either PBS or S-FPV-017 before or after immunization, whereas HI antibody titers remained above 6 $\log_2$ until 42 days after vaccination with rFPV ($P < 0.05$). However, the anti-AIV antibody level of chickens immunized with rFPV-HA/IL18 was slightly higher but not significantly different ($P > 0.05$) from that of chickens immunized with rFPV-HA alone from the first week after vaccination.

3.4. HA-protein-specific T-cell proliferation

To determine whether T-cell proliferation response to the rFPV vaccine encoding the HA gene could be boosted by chicken IL-18, we examined the PBMCs from the vaccinated chickens for antigen-specific T-cell proliferation. As shown in Fig. 4, an enhanced T-cell proliferative response to HA was clearly observed in the groups immunized with rFPVs when stimulated with purified AIV HA protein, whereas the chickens vaccinated with S-FPV-017 or PBS did not respond to the HA protein ($P < 0.05$). The level of T-cell proliferative response in the group immunized with rFPV-HA/IL18 was significantly higher than that in the group immunized with rFPV-HA alone ($P < 0.05$). The Con A control sample showed a stimulation index of 4–5. This result indicates that higher levels of antigen-specific T-cell proliferative responses can be elicited by immunization with rFPV-HA/IL18 than by immunization with rFPV-HA alone.

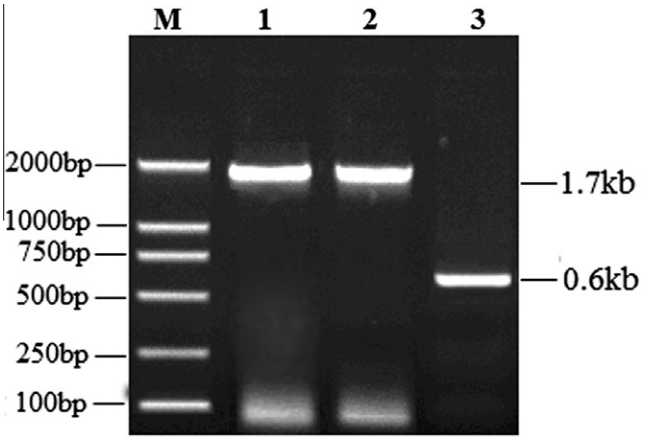

Fig. 2. PCR confirmation of presence of HA and chicken IL-18 genes in rFPVs. M, DNA molecular Marker (DL2000); 1, HA gene in rFPV-HA; 2, HA gene in rFPV-HA/IL18; 3, chicken IL-18 in rFPV-HA/IL18. Also, the results were double-checked by DNA sequencing done on the PCR products. DNA sequencing results showed that HA and chicken IL-18 genes are 1683 and 597 bp, respectively, and consistent with predicted results.

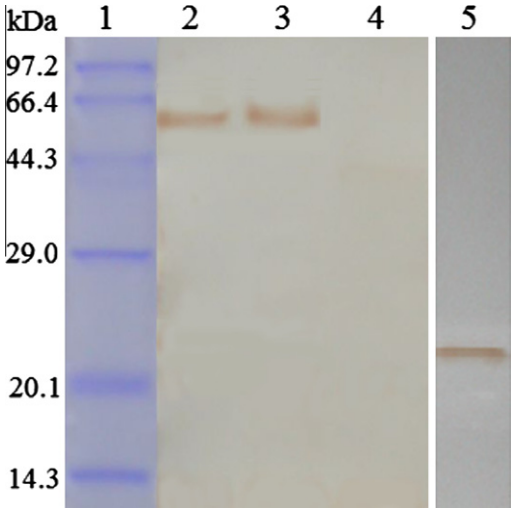

Fig. 3. Western blot analysis. Lane 1 represents the molecular mass marker; lane 2 reveals that the HA protein was detected in CEFs infected by rFPV-HA/IL18; lane 3 reveals that the HA protein was detected in CEFs infected by rFPV-HA; lane 4 reveals that negative control was infected with S-FPV-017; lane 5 reveals that the chicken protein was detected in CEFs infected by rFPV-HA/IL18.

Table 2
Detection of antibodies in different vaccine inoculated groups by ELISA[a].

Group	Animal no.	Serum HI titer(Log_2)[b]						
		0 day	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
rFPV-HA/IL18	16	0	3.6 ± 0.3*,c	7.8 ± 0.2*	7.5 ± 0.3*	7.3 ± 0.3*	7.2 ± 0.2*	7.0 ± 0.3*
rFPV-HA	16	0	3.0 ± 0.3*	7.1 ± 0.2*	6.8 ± 0.3*	6.7 ± 0.3*	6.4 ± 0.3*	6.3 ± 0.2*
S-FPV-017	16	0	0	0	0	0	0	0
PBS	16	0	0	0	0	0	0	0

[a] One-day-old White Leghorn SPF chickens were vaccinated with 10^4 PFU FPV via wing web puncture. Five sera from each group were collected on a weekly basis to detect HI antibody.
[b] Values are expressed as mean HI antibody titer ± standard error.
[c] Statistically significant differences ($P < 0.05$) are indicated by * (compared with compared with S-FPV-017 or PBS).
* Indicates statistically significant differences $P < 0.05$.

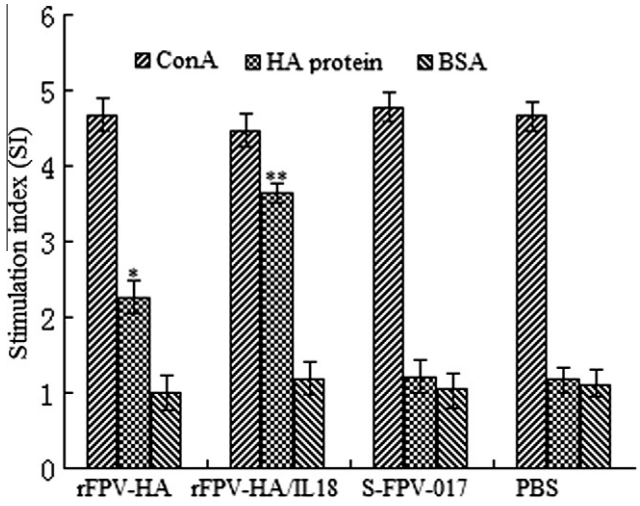

Fig. 4. Peripheral blood T lymphocyte proliferation assay ($n = 5$, i.e. number of times the test was repeated). Values are expressed as mean counts ± standard error. Statistically significant differences ($P < 0.05$) are indicated by * (compared with S-FPV-017 or PBS) or ** (compared with rFPV-HA alone).

3.5. Protection induced by immunization with rFPV-HA and rFPV-HA/IL18

To evaluate the level of protective responses after challenge, oropharyngeal and cloacal swab samples were analyzed by RT-PCR. As shown in Table 3, all of the chickens immunized with rFPV-HA/IL18 were completely protected from both oropharyngeal and cloacal virus shedding; whereas 14 of 16 of the chickens immunized with rFPV-HA were protected. All chickens inoculated with either S-FPV-017 or PBS had detectable virus in their both oropharyngeal and cloacal swab samples on day 5. These results showed rFPVs vaccinated groups could inhibit H9N2 AIV shedding, and rFPV-HA/IL18 vaccinated group had better inhibition of viruses than rFPV-HA vaccinated group.

We tested the protective efficacy of rFPVs by measuring inhibition of challenge-virus replication in multiple organs. In the tissue samples taken on day 5 after challenge, no viral replication was detected from rFPV-HA/IL18 vaccinated chickens, whereas for rFPV-HA vaccinated chickens, the A/CH/JY/1/05 virus was isolated from trachea (1/16) and cecal tonsil (2/16), but not from the lung, kidney or spleen (Table 3). In contrast, challenging virus was recovered from all the organs from chickens inoculated with either S-FPV-017 or PBS.

A bird which was a negative result for RT-PCR was defined as a protected one. SPF chickens immunized with 10^4 PFU rFPV-HA/IL18 were completely protected (100%); whereas 14 of 16 of the chickens immunized with rFPV-HA were protected (87.5%). None of chickens inoculated with either S-FPV-017 or PBS was protected. Thus, there was a significant difference in protection rates between the rFPV-HA/IL18 and rFPV-HA groups ($P < 0.05$) suggesting that the protective efficacy of the rFPV-HA vaccine could be enhanced significantly by simultaneous expression of IL-18.

4. Discussion

It is worth noting that AIV in its natural hosts, which include wild waterfowl, gulls, and shorebirds, has shown a high rate of genetic conservation (Choi et al., 2008). Transmission to other species such as poultry may cause significant amounts of genetic and antigenic changes (Liu et al., 2004; Park et al., 2011). For a vaccine to be effective, it is necessary that the strain has genetic and antigenic traits similar to those of the currently circulating field viruses. Therefore, it is very important to choose the most effective vaccine strain to prevent the current circulation of viruses. Two distinct lineages of H9N2 AIV, represented by the two prototype viruses of A/Chicken/Beijing/1/94 in chickens and A/Quail/Hong Kong/G1/98 in quail, have been circulating in terrestrial poultry in Asia since the mid-1990s. In spite of mutations, the HA gene of A/CH/

Table 3
H9N2 influenza virus shedding and replication in vaccinated and control chicken[a].

Group	Oropharyngeal[c]		Cloacal		Tissue homogenate (20% w/v)[d]				
	3 dpc[b]	5 dpc	3 dpc	5 dpc	Trachea	Lung	Kidney	Spleen	Cecal tonsil
rFPV-HA/IL18	0/16[e]	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16
rFPV-HA	1/16	2/16	0/16	2/16	1/16	0/16	0/16	0/16	2/16
S-FPV-017	15/16	16/16	15/16	16/16	8/16	6/16	4/16	7/16	14/16
PBS	16/16	16/16	15/16	16/16	9/16	7/16	5/16	6/16	15/16

[a] All of the chickens were challenged by intranasal instillation of 0.1 ml containing $10^{6.5}$ ELD_{50} of A/CH/JY/1/05 viruses at day 43 after immunization.
[b] Days post challenge.
[c] Detection of challenge virus in oropharyngeal and cloacal swabs by RT-PCR.
[d] Tissues were taken at 5 dpc. All tissue specimens were inoculated into SPF embryonated eggs for virus isolation.
[e] Number shedding/number tested.

JY/1/05 (H9N2) influenza virus was still similar to the sublineage of A/Chicken/Beijing/1/94. Because of the above mentioned reasons, we developed a recombinant anti-H9N2 AIV vaccine based on this virus.

Vaccination remains the most important and efficient tool in the prevention and control of H9N2 AI in poultry. Although conventional inactivated AI vaccines have shown efficacy against H9N2 AI, they induce immune responses to the group-specific NP antigen of influenza A and thus render differentiation of vaccination from natural infection impossible (Boyle and Coupar, 1988). Many studies have been conducted that were aimed at the development of a recombinant virus vaccine strategy (Chambers et al., 1988; Ge et al., 2010). FPV vectors expressing foreign genes have become widely used in vaccine development. Several strains of FPV expressing major avian immunogenic genes have been successfully constructed, some of which are commercially available (Qiao et al., 2009). And fowlpox-based vaccines are also easy to produce and technologies for large-scale production are available, offering an attractive alternative to inactivated vaccines.

Although FPV vectors have proven to be a successful vector, there is a need to enhance immunization-induced protection by recombinant fowlpox virus vaccines (Leong et al., 1994; Swayne et al., 2000). Many studies have shown that the immunogenicity of an antigen could be enhanced by various cytokines, including IL-2, IFN-γ, IL-6, and IL-18. Among the large array of cytokines, IL-18 was initially identified as a potent IFN-γ-inducing factor, and it is an important cytokine with multiple functions in innate and acquired immunity (Dinarello and Fantuzzi, 2003; Marshall et al., 2006). As a vaccine adjuvant and an immunomodulatory molecule, IL-18 has been shown to regulate the immune response towards a Th1 type (Nakanishi et al., 2001), and chicken IL-18 enhances the immune responses in vaccines (Mingxiao et al., 2006). In our study we chose to test chicken IL-18 as an adjuvant for the HA antigen expressed from a FPV vector vaccine. rFPV-HA/IL18 and rFPV-HA were constructed, inoculated into chickens and tested in a protection-challenge experiment. The results showed that vaccination with the rFPV-HA/IL18 can induce stronger immune responses than vaccination with rFPV-HA. Thus, it seems that vaccination with rFPV-HA/IL18 expressing both HA protein and IL-18 may elicit a potent immune response.

Lymphocytes are important immune cells that play a critical role in maintaining immune functions, and optical density value is an indicator of lymphocyte proliferation. Therefore, we also evaluated whether vaccination with rFPV-HA in the presence or the absence of chicken IL-18 could influence the antigen-specific T-cell proliferation response. Our results showed that the T cells of chickens immunized with rFPV-HA alone exhibited a proliferative response. However, the level of T-cell proliferative response induced in the rFPV-HA/IL18 group was higher than that induced in the rFPV-HA group. This suggested that chicken IL-18 was able to stimulate T-cell proliferation. Similar results were also reported by Zhu et al. (2003) and Yin et al. (2009), who showed that co-delivery of an IL-18-expressing plasmid and DNA vaccine could enhance the $CD8^+$ cytotoxic T lymphocyte (CTL) response and T-cell proliferative response induced by the DNA vaccine. These data clearly show that chicken IL-18 is a strong adjuvant that enhances vaccine potency.

HI antibodies play a key role in protection against AIV by neutralizing the infectivity of challenge viruses (Brown et al., 1992). To demonstrate whether the rFPVs induce a sufficiently protective immune response, the immune responses of one-day-old SPF chickens were analyzed by HI antibody titers. All rFPV-vaccinated groups produced H9N2 AIV-specific antibodies at 7 days after vaccination, HI antibody titers of chickens inoculated with the rFPVs remained above 6 $\log_2$ until 42 days after vaccination (Table 2). The level of specific antibodies induced in the rFPV-HA/IL18 group was slightly higher but not significantly different ($P > 0.05$) from that induced in the rFPV-HA group from the first week after vaccination. However, oropharyngeal and cloacal swab samples were analyzed by RT-PCR after challenge. As shown in Table 3, the rFPV-HA/IL18 vaccinated group had better inhibition of viruses than the rFPV-HA vaccinated group. We tested the protective efficacy of rFPVs by measuring inhibition of challenge-virus replication in multiple organs. In the tissue samples taken on day 5 after challenge, no viral replication was detected from rFPV-HA/IL18 vaccinated chickens, whereas for rFPV-HA vaccinated chickens, the A/CH/JY/1/05 virus was isolated from trachea (1/16) and cecal tonsil (2/16) (Table 3). The results showed the chickens immunized by rFPV-HA/IL18 exhibit a marked inhibition of virus shedding and replication compared to rFPV-HA group, and rFPV-HA/IL18 induced better protection, demonstrating that the absolute levels of antibody cannot be used alone to evaluate the immunoprotective effects of a vaccine (Wang et al., 2009). The results suggested that the cellular immunity of AI is also very important for the protection of chicken from the challenge.

We found that all rFPV-vaccinated groups produced HI-specific antibodies, and splenocyte proliferation response induced by rFPV-HA/IL18 in chickens was significantly higher than that induced by rFPV-HA. The results proved, consistent with a previous report (Mingxiao et al., 2006). Thus rFPV-HA/IL18 may be a valuable candidate vaccine for the control of AIV and its effectiveness in field applications should be determined in the future.

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