



Enhancing immunogenicity and protective efficacy of inactivated avian influenza H9N2 vaccine with recombinant chicken IFN- α in chicken

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ABSTRACT

Control of currently circulating re-assorted low-pathogenicity avian influenza (LPAI) H9N2 is a major concern for both animal and human health. Thus, an improved LPAI H9N2 vaccination strategy is needed to induce complete immunity in chickens against LPAI H9N2 virus strains. Cytokines play a crucial role in mounting both the type and extent of an immune response generated following infection with a pathogen or after vaccination. To improve the efficacy of inactivated LPAI H9N2 vaccine, prokaryotic expression recombination chicken interferon- α (rchIFN- α) was used as vaccine adjuvant. In this study chIFN- α was used as adjuvant in inactivated AI H9N2 vaccine, modulated the immune response of chickens against the vaccine antigen through enhanced humoral and Th1-biased cell-mediated immunity, compared to chickens that received single AI H9N2 vaccine. To further test the protective efficacy of this improved vaccination regimen, immunized chickens were challenged with a high dose of LPAI H9N2 virus. Combined administration rchIFN- α showed markedly enhanced protection compared to single administration of the vaccine, as determined by mortality, clinical severity, and feed and water intake. This enhancement of protective immunity was further confirmed by reduced rectal shedding and replication of AIV H9N2 in challenged chickens. Our results indicate the value of combined administration of rchIFN- α to generate an effective immunization strategy in chickens against LPAI H9N2.

1. Introduction

Cytokines are natural mediators of innate and adaptive immune responses which play a crucial role in controlling the immune response. The use of chicken cytokines is becoming more feasible with the recent cloning of a number of cytokine genes since the chicken's immune system is similar to that of mammals (Kaiser, 2010). Recent studies of avian cytokines identified a number of cytokines having immunomodulatory and antiviral properties against several viral infections. Additionally, some chicken cytokines have already been proven to have potent adjuvant activities (Asif et al., 2004). In fact, the use of recombinant chicken cytokines as adjuvants is attracting extensive attention over existing oil-based or chemical adjuvants, because they promote better protection without causing any adverse site reactions or distress to chickens when administered with a vaccine. Chicken interferon- α (chIFN- α) belongs to type I IFNs and plays an essential role in the host antiviral response by stimulating the T-dependent lymphocyte

system and induction of numerous IFN stimulated genes (ISGs) (Samuel, 2001). Recent studies showed that recombinant chIFN- α has immunomodulatory and anti-viral properties against several viral infections, such as Avian influenza viruses (AIV).

AIV of the H9N2 subtype are classified as low-pathogenicity viruses both by molecular characterization and pathotyping. Among low pathogenicity avian influenza (LPAI) viruses, this particular subtype has attracted great concern due to its wide host range (Alexander, 2000), chance of genetic reassortment (Butt et al., 2005) and possible avian-to-human transmission (Li et al., 2003). Thus, circulation of H9N2 viruses in poultry not only causes industrial losses, but also poses a potential threat to human health. Although the vaccine has been proved to be highly immunogenic in laboratory trials and can prevent clinical disease and reduce viral shedding in field conditions, it cannot prevent vaccinated poultry from becoming infected and from shedding wild viruses in farm settings (Choi et al., 2008; Swayne and Halvorson, 2003). Therefore, an improved vaccination strategy is urgently required

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to control H9N2 outbreaks in poultry farms.

In this study, we investigated the adjuvant activities of chIFN- α by using prokaryotic expression recombination chIFN- α (rchIFN- α) modulated immune responses of chickens against inactivated LPAI H9N2 vaccine through enhanced humoral and Th1-biased cell-mediated immunity. Our results indicate the useful value of combined administration of chIFN- α in inactivated H9N2 LPAI vaccination.

2. Materials and methods

2.1. Animals and ethics statement

Specific pathogen free (SPF) White Leghorn layer chickens were obtained from Merial Vital Laboratory Animal Technology Co., Ltd. (Beijing, China), and reared with formulated commercial feed and water provided ad libitum throughout the experimental period. All experimental and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committees of Anhui Medical University. The animal facility of Anhui Medical University is fully accredited by the National Association of Laboratory Animal Care.

2.2. Cells and viruses

LPAIV H9N2 strain, was provided by Professor Peng Daxin at Veterinary College of Yangzhou University and used for the challenge experiments. AIV H9N2 was propagated by inoculating the allantoic cavity of 10-day old embryonated eggs. Allantoic fluid was harvested 96 h after inoculation and the infectious viral titer was determined using 10-day-old embryonated eggs.

2.3. Preparation of rchIFN- α

The recombinant rchIFN- α protein was prepared from *Escherichia coli*. Briefly, the gene of rchIFN- α was amplified by polymerase chain reaction (PCR) from chicken liver (F:50-GAATTCATGTGCAACCACCTT CGCCCCA-30; R:50-AGATCTTTAAGTGC GCGTGTGCC-30), cloned into the vector pET-30a, and then induced expression in *E. coli* strain BL21 (DE3) at 32°C for 5 h. To purify the chIFN- α protein, the cells were harvested and treated using an ultrasonic cell disruptor. After that, the inclusion body was separated by centrifugation and washed with PBST (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, and 1% triton), 2 M urea, and 1 M NaCl in turn. Then the inclusion body was dissolved in 8 M urea. The denatured protein was refolded in the refolding buffer [50 mM Tris (pH 8.0), 0.5 M Arg, 2 mM EDTA, 0.5 mM glutathione (oxidized), and 20% glycerol] at 48°C for 48 h. After refolding, the chIFN- α was added to cation exchange chromatography at the rate of 0.3 ml/min and then eluted using PBS (pH 7.4) and 1 M NaCl at the rate of 1 ml/min. The purified chIFN- α were then detected by SDS-PAGE analysis with Coomassie brilliant blue staining. The antiviral titer of chIFN- α was performed using vesicular stomatitis virus/DF-1 cells according to previously described protocols (Kaiser, 2010).

2.4. Animal experiment designs for AIV H9N2 vaccination

A total of 20 chickens (14-days-old) were divided randomly into 4 groups. The first group (n = 5) was a negative control subcutaneous administered 0.3 ml vehicle (0.01 M PBS). The second group (n = 5) was subcutaneous administered 1000 IU rchIFN- α (dissolved in 0.3 ml PBS). The third group (n = 5) was subcutaneous administered AIV H9N2 inactivated vaccine (contains oil adjuvants) (Yangzhou UNI-BIO pharmaceutical CO., LTD, Yangzhou, China) combined with 1000 IU rchIFN- α (The final volume is 0.3 ml). The fourth group (n = 5) was subcutaneous administered AIV H9N2 inactivated vaccine combined with 0.01 M PBS (The final volume is 0.3 ml).

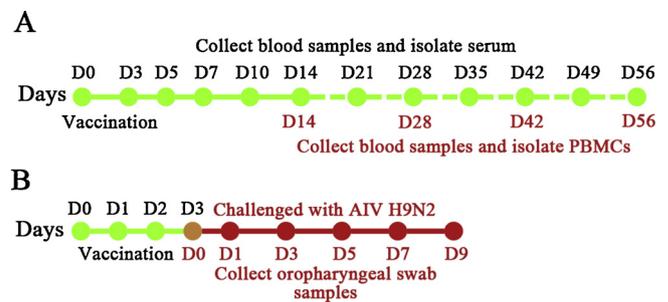


Fig. 1. Diagram illustrating the experimental design of the chicken vaccination and AIV H9N2 challenge experiment. (A) Point of swabs and blood collection after vaccination. (B) Point of swabs collection after vaccination and AIV H9N2 challenged.

Blood samples were collected 0, 3, 5, 7, 10, 14, 21, 28, 35, 42, 49 and 56 day after vaccinations followed by sera separation (Fig. 1A). Peripheral blood mononuclear cells (PBMCs) were enriched from the blood of vaccinated chickens using OptiPrep™ (13.8% iodixanol) 14, 28, 42 and 56 days post vaccination (Fig. 1A), according to the manufacturer's instructions (Axis-Shield, Oslo, Norway).

To evaluate the protective immunity of AI H9N2 vaccine in chicken coadministered rchIFN- α , 14-days-old chickens were vaccinated according to the same protocol. Three days after treatment, all groups were intravenous challenged with AIV H9N2 ($10^{10.5}$ EID₅₀/chicken) (Fig. 1B). Following challenge, chickens were observed daily for clinical signs and mortality throughout the duration of the experiment. Clinical signs were scored as follows: 0, no sign; 1, slight depression; 2, moderate depression + reduced movement + reduced food/water intake (anorexia); 3, moderate respiratory distress (sinusitis, cough); 4, severe respiratory distress (sinusitis, severe cough) + diarrhea; 5, death. Average feed and water intake was determined daily for 9 days after challenge. Oropharyngeal swab samples were collected at 0, 1, 3, 5, 7, and 9 days post-infection (p.i.) for virus isolation and AIV H9N2 RNA test.

2.5. Hemagglutination inhibition (HI) assay

To determine the HI titers of the sera samples collected from vaccinated chickens at 0, 1, 3, 5, 7, and 9 days post-infection (p.i.), HI tests were performed with AIV H9N2 using a standard method. The geometric means of serum HI titers obtained from each group were defined as the reciprocal logarithm in a base of 2 of the highest serum dilution completely inhibiting agglutination.

2.6. Virus isolation

The H9N2 viruses were isolated and identified by using standard laboratory procedures. Briefly, the Throat swabs were processed and inoculated into 10-days-old-embryonated chicken eggs via allantoic route. The embryos were incubated at 37 °C till death or maximum of 5 days post inoculation. Amnioallantoic fluid (AAF) was harvested and subjected to hemagglutination assay (HA). Three blind passages were carried out before deciding the negativity of the samples.

2.7. AIV H9N2 antigen-specific proliferation of PBMCs

AIV H9N2 antigen-specific proliferation of PBMCs was assessed by measuring viable cell ATP bioluminescence. Briefly, PBMCs (responder) were prepared from vaccinated chickens as previously described (Geiss et al., 2002), and cultured together with stimulator cells at three different ratios. Autologous PBMCs (106 cells/ml), which were isolated from corresponding chickens before vaccination and kept at liquid nitrogen tank, had been pulsed with ultraviolet (UV)-inactivated AIV H9N2 antigen (2.5×10^2 HA units/ml) for 3 h followed by treatment

with mitomycin C (25 µg/ml) for 5 min, and were employed as stimulator cells. Following 72 h incubation, the proliferated cells were evaluated using a VialightW Cell proliferation assay kit (Cambrex Bio Science, Rockland, ME, USA) according to the manufacturer's instructions. PBMC stimulators that were not pulsed with UV-inactivated AIVH9N2 antigen were used for negative control. PBMCs pulsed with ConA (400 ng/ml) were used for positive control.

Enzyme-linked immunosorbent assay (ELISA) for detecting IFN-γ and IL-4 secreted by AIV H9N2 antigen pulsed PBMCs

The secretion levels of IFN-γ and IL-4 in PBMCs supernatant were detected by using Chicken IFN-γ ELISA Kit or Chicken IL-4 ELISA Kit (LifeSpan Biosciences, USA) according to the manufacturer's instructions.

2.8. Real-time quantitative RT-PCR (qRT-PCR) analysis

Real-time quantitative RT-PCR (qRT-PCR) analysis was used for determining the mRNA expression levels of IFN-γ and IL-4 in PBMCs and the amount of AIV H9N2 in oropharyngeal swab samples. Briefly, total RNA were extracted from collected samples using viral and total RNA extraction kits (iNtRON), according to the manufacturer's instructions, and then subjected to real-time qRT-PCR using a One-Step SYBRW qRT-PCR reagent kit (Takara, Dalian, China) and primers specific for the IFN-γ, IL-4, and AIV H9 gene (Table 1). Real-time PCR amplification of targeted genes were carried out under the same reaction conditions. After the reaction cycle was completed the temperature was increased from 50 °C to 95 °C at a rate of 0.2 °C/15 s and fluorescence was measured every 5 s to construct a melting curve that was used to confirm the authenticity of the amplified products. A control sample that contained no template RNA was run with each assay, and qRT-PCR data was normalized using the commonly used reference gene, GAPDH (Table 1). The copy number of the experimental samples was determined by interpolating the threshold cycle values using the standard curve. All data were analyzed using LC480 manager software version 1.6 (Roche).

2.9. Statistical analysis

All data were expressed as the average ± standard error. Differences between the two groups were compared using an unpaired two-tailed Student's t-test. Differences between multiple groups were compared using two-way analysis of variance (ANOVA) using SAS version 9.1(SAS Institute Inc., Cary, NS, USA). A p-value < 0.05 was considered to indicate a statistically significant difference between the groups.

3. Results

3.1. Preparation of recombinant rchIFN-α

In this study, the rchIFN-α gene was cloned from chicken liver and the deduced amino acid sequence without signal sequence (32–193 aa) exhibited 100% identity with IFN-α of Gallus (GenBank accession No.

Table 1
PCR primers for amplification of AIV HA, IFN-γ, IL-4, and GAPDH.

Target gene		Primer sequence (5'-3')
AIV HA	F	CTACTGTTGGGAGGAAGAGAATGGT
	R	TGGGCGTCTTGAATAGGGTAA
IFN-γ	F	CAAAGCCGCACATCAAACA
	R	TTTCACCTTCTCAGGCCATC
IL-4	F	GAGAGGTTTCCTCGCTCAAG
	R	TGGTGGAAGAAGGTACGTAGG
GAPDH	F	AGAACATCATCCAGCGTCC
	R	CGGCAGGTCAGGTCAACA

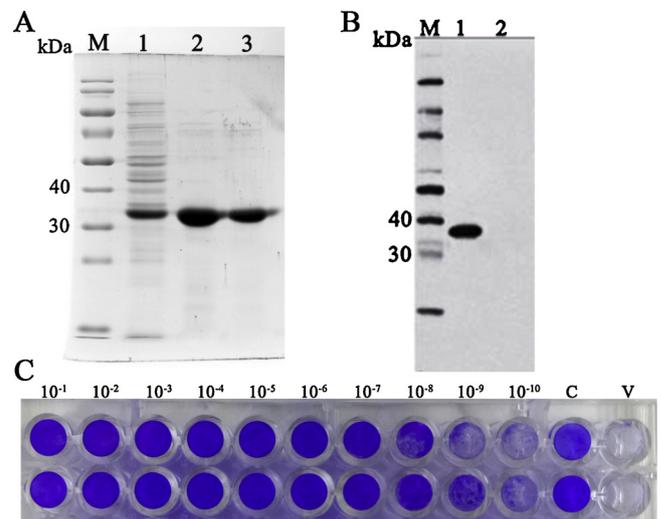


Fig. 2. Preparation of recombinant rchIFN-α. (A) The result of rchIFN-α Purification (M: Molecular-weight marker; 1: Supernatant after ultrasonic crash; 2: Purified rchIFN-α). (B) The results of western blot identify the rchIFN-α (M: Molecular-weight marker; 1: BL21 (DE3) harboring pET-30a vector; 2: BL21 (DE3) harboring pET-30a-chIFN-α vector). (C) The anti-vesicular stomatitis virus activity of chIFN-α (C: Cell control; V: Virus control).

ABB05335). The recombinant chIFN-α protein was expressed in *E. coli* strain BL21 (DE3) and purified as previously described. The anti-vesicular stomatitis virus activity of chIFN-α is 1×10^7 U/mg, and the chIFN-α was used in the subsequent experiments (Fig. 2).

3.2. Enhancement of humoral immune responses against AI vaccine by rchIFN-α

In order to examine the humoral immune responses in AIV H9N2-vaccinated chickens with or without rchIFN-α, groups of chickens (n = 5) vaccinated with AIV H9N2 inactivated vaccine or H9N2 inactivated vaccine combined with rchIFN-α/rchIFN-α. Sera samples were collected at several time points after vaccination and used for the determination of HI antibody titers. Significantly enhanced HI antibody levels were observed at all-time points in the sera of H9N2 inactivated vaccine combined with rchIFN-α chickens compared to that of H9N2 inactivated vaccine chickens. Therefore, these results indicate that rchIFN-α produces enhanced humoral immune responses against AI vaccine (Fig. 3).

3.3. Enhanced Th1-biased immunity against AI vaccine by rchIFN-α

To evaluate cellular immune responses, PBMCs were prepared from vaccinated chickens, and subsequently subjected to stimulation with auto logous PBMCs that had been previously pulsed with UV-

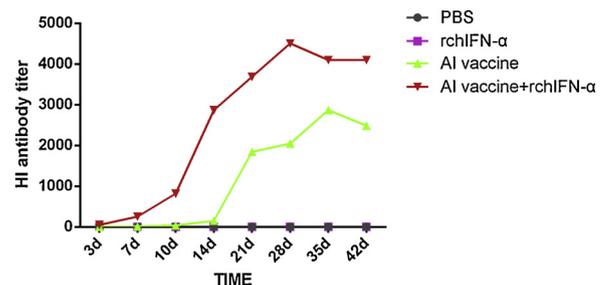


Fig. 3. Serum HI antibody titers of inactivated AIV-vaccinated chickens. Data are expressed as reciprocal log₂ of the geometric average and SEM of HI titers obtained from five chickens per group. P < 0.001 compared to chIFN-α-treated chickens.

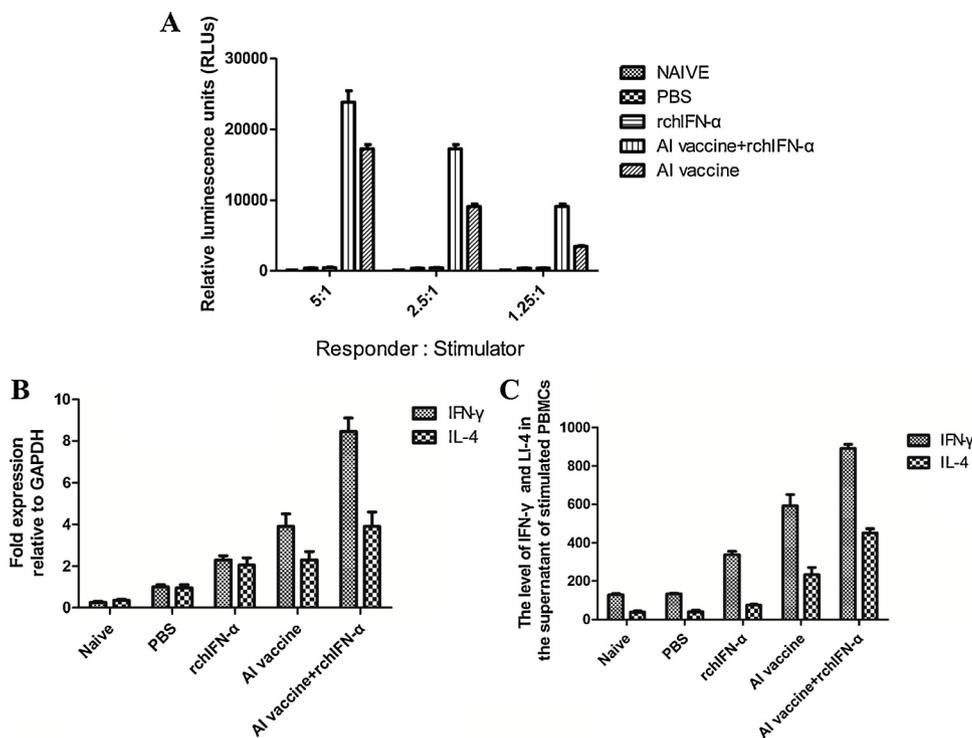


Fig. 4. Enhanced Th1-biased immunity against AI vaccine by rchIFN- α . (A) AIV H9N2 antigen-specific proliferation of PBMCs was assessed by measuring viable cell ATP bioluminescence following incubation for 72 h. (B) The expression of IFN- γ and IL-4 mRNA by PBMCs following stimulation with AIV H9N2 antigen. Total RNA was extracted from PBMCs stimulated with AIV H9N2 antigen for 72 h, and subjected to real-time qRT-PCR to determine the expression of IFN- γ and IL-4. Data show the average and SEM of IFN- γ and IL-4 mRNA expression normalized to GAPDH (n = 5). (C) The level of IFN- γ and IL-4 in the supernatant of the PBMCs culture were determined by ELISA.

inactivated AIV H9N2 antigen. PBMCs of chickens that received H9N2 inactivated vaccine combined with rchIFN- α were found to have significantly enhanced proliferation upon AIV H9N2 antigen-specific stimulation, compared to PBMCs from chickens that received H9N2 inactivated vaccine (Fig. 4A). In addition, the mRNA expression levels of IFN- γ and IL-4 in PBMCs were determined by real-time qRT-PCR following stimulation with AIV H9N2 antigen. Both IFN- γ and IL-4 mRNA levels in PBMCs prepared from chickens that received a single administration of H9N2 inactivated vaccine combined with rchIFN- α were significantly enhanced, compared to chickens that received H9N2 inactivated vaccine. More importantly, the expression of IFN- γ mRNA was more significantly up-regulated than IL-4 mRNA with a single administration of H9N2 inactivated vaccine, and co-administration of H9N2 inactivated vaccine and rchIFN- α induced more enhanced upregulation of IFN- γ mRNA than single administration of the constructs (Fig. 4B). The ELISA showed that the level of IFN- γ and IL-4 in the supernatant of the cell culture were correlated with the mRNA expression levels of IFN- γ and IL-4 in PBMCs. Taken together, our results indicate that rchIFN- α enhances Th1-biased immunity against an AI vaccine in chickens (Fig. 4C).

3.4. Enhanced protective immunity of AI vaccine by co-administration of rchIFN- α

To evaluate the protective immunity of AI H9N2 vaccine in chickens co-administered rchIFN- α , chickens (14-days-old) co-administered rchIFN- α were vaccinated and then challenged with AIV H9N2 ($10^{10.5}$ EID₅₀/chicken). Following challenge, chickens were observed daily to record mortality and clinical severity signs throughout the duration of the experiment. Mortality occurred between 4 and 6 days p.i., and the chickens that received PBS and rchIFN- α without AI vaccine had the highest mortality (50%). Vaccination with inactivated AI vaccine reduced the mortality to 25% and co-administered rchIFN- α with AI vaccination reduced it further to 0% (Fig. 5A). Also, when the severity of clinical signs caused by AIV H9N2 challenge infection was scored, clinical signs appeared 2 days p.i., and the severity of clinical signs peaked at 4–7 days p.i. (Fig. 5B). Chickens that received combined administration of AI vaccination and rchIFN- α showed significant

alleviation of clinical severity during the entire course of clinical infection, when compared to the group that administration of AI vaccination and control. Furthermore, average feed and water intake improved in chickens that received the AI vaccine, compared to chickens that received PBS or rchIFN- α . In particular, average feed and water intake improved more when the chickens received rchIFN- α either singly or in combination (Fig. 5C and D). Overall, these results indicate that oral rchIFN- α could markedly reduce mortality and alleviate clinical signs induced by infection with AIV H9N2.

3.5. Reduction of AIV H9N2 shedding and replication in vaccinated chickens

To evaluate the effect of co-administration of rchIFN- α on virus shedding from AIV H9N2-infected chickens that received AI vaccine, the amount of virus in oropharyngeal swabs were determined by real-time qRT-PCR at 0, 1, 3, 5, 7 and 9 days post-challenge. Virus shedding was detected from 2 days after AIV H9N2 infection and peaked at 5 days p.i. (Fig. 5A). However, the chickens that received AI vaccine combined with rchIFN- α had significantly lower peak levels of virus shedding at 3, 5, 7 and 9 days p.i., compared to the chickens that received only the AI vaccine (Fig. 6A).

Results of virus isolation show that administration of only AI vaccine could significantly reduce the virus shedding, compared to controls but significant difference existed when control and AI vaccine were compared to AI vaccine combined with rchIFN- α groups, which indicates that use rchIFN- α as an adjuvant provided better protection than vaccination alone (Fig. 6B). Taken altogether, these results indicate that rchIFN- α along with AI vaccine could alleviate clinical signs induced by AIV H9N2 infection through reduction of virus shedding.

4. Discussion

In the present study, we demonstrate that rchIFN- α modulated the immuneresponses of chickens against inactivated LPAI H9N2 vaccine antigen by enhancing both humoral and Th1-biased cell mediated immunity, thereby conferring better protection against homologous virus challenge. Therefore, we propose that modulation of the immune

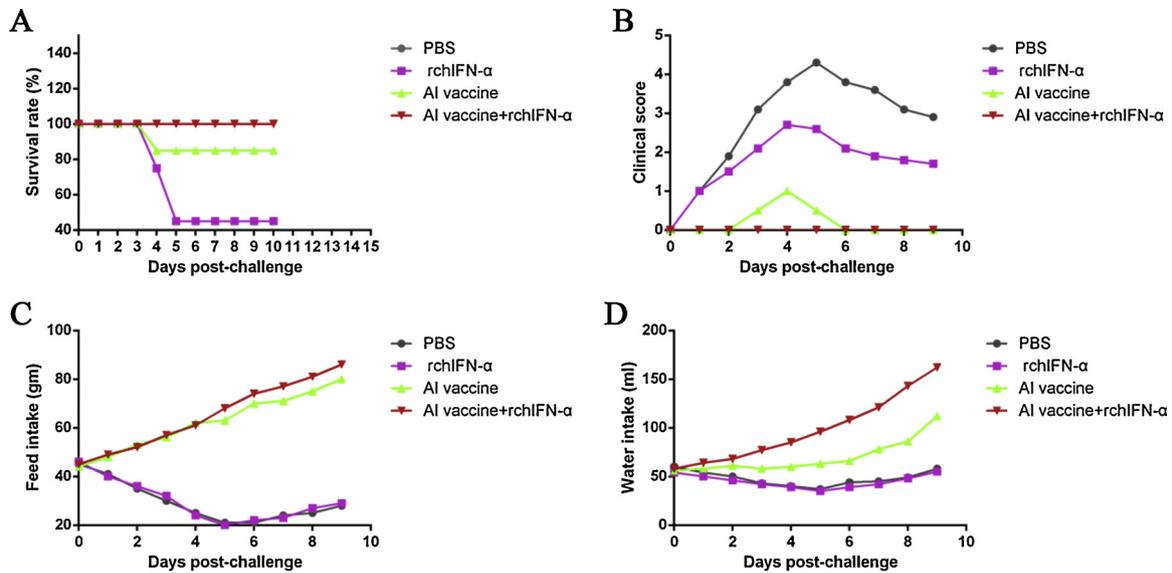


Fig. 5. Enhanced protective immunity of AI vaccine by co-administration of rchIFN- α . (A) Mortality of AIV H9N2-challenged chickens. (B) Clinical severity of AIV H9N2-challenged chickens. (C) Data show the average feed intake obtained from eight chickens per group. (D) Data show the average water intake obtained from eight chickens per group.

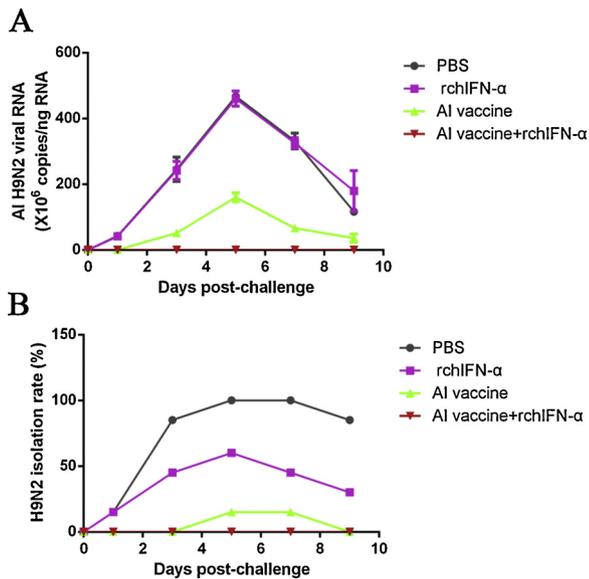


Fig. 6. Reduction of AIV H9N2 shedding and replication in vaccinated chickens. (A) H9N2 shedding were determined by real-time qRT-PCR using primers specific for hemagglutinin protein of AIV H9N2. (B) H9N2 shedding were determined by virus isolation.

response elicited by commercially available, inactivated LPAI H9N2 vaccine through combined use of chIFN- α may provide a novel approach to induce complete immunity in chickens against H9N2 LPAI virus strains.

The enhanced effect of cytokine combinations has been shown empirically, based on their biological mechanisms. IFN- α and β (type I IFNs) rapidly induced by viral infection and/or a series of events have well defined strong antiviral activity along with immunoregulatory functions. The binding of type I IFNs to type I IFN receptor complexes results in the rapid phosphorylation and activation of receptor-associated JAKs, Tyk2, and Jak1, and subsequent transcription factor STAT1/2, which induces the expression of OAS, RNase L, Mx1, and PKR genes that confer the antiviral state in cells (Samuel, 2001). Alternatively, IFN- γ , the only type II IFN, is a multifunctional cytokine produced primarily by T lymphocytes (Th1) and natural killer (NK)

cells. It has been confirmed that after infection of macrophages with influenza virus, cells produce IFN- α and enhances IFN- γ synthesis (Nakanishi et al., 2001). IFN- γ plays a vital role in macrophage activation and modulation of the immune system, in addition to its antiviral activity (Julkunen et al., 2000). Similar to mammalian IFNs, chicken type I and type II IFNs act synergistically (Moraes et al., 2007), both in terms of antiviral activity and in their ability to activate macrophages. Therefore, it is possible that chIFN- α might have enhanced immunomodulatory functions. It is conceivable that type II IFN- γ produced by IFN- α exposure might induce enhanced alleviation of the clinical signs of AIV H9N2 infection and modulate immunity. Therefore, the present data provide valuable insight into the use of combined administration of type I IFN in controlling viral infection in the poultry industry.

The primary target cells for AIV infection and replication are ciliated epithelial cells. However, AIV can also infect macrophages and dendritic cells (Kaufmann et al., 2001). In avian species, intestinal epithelia are also targets of infection and, in the later stage of infection, mononuclear cells become involved (Geiss et al., 2002). Influenza A virus causes NS1-mediated suppression of selected genes involved in IFN and IFN inducible gene expression, and induction of a weak chemokine expression in human lung epithelial cells (Veckman et al., 2006), which enables the virus to replicate before the host inflammatory and antiviral responses are activated. Thus, complete protection of chickens from AIV H9N2 requires early stimulation of the immune system by immunomodulatory cytokines like chIFN- α . Therefore, it is possible that chIFN- α combined vaccination with inactivated H9N2 LPAI could effectively modulate host innate and adaptive immune responses, thereby providing complete protection against AIV H9N2 challenge.

We have demonstrated that enhanced modulation of immune responses elicited by commercially available, inactivated H9N2 LPAI vaccine through combined administration of rchIFN- α can protect immunized chickens from high-dose challenge of homologous virus. The results suggest that naturally occurring immunomodulatory cytokines like rchIFN- α can be combined with commercially available inactivated vaccines to generate an effective immunization strategy in chickens. It will be interesting to assess the protective efficacy of this immunization strategy against challenge with currently circulating heterologous virus strains in future studies.

Disclosures

The authors have no financial conflicts of interest.

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