Chapter 6

Influenza Inactive Virus Vaccine with the Fusion Peptide (rTα1-BP5) Enhances Protection Against Influenza Through Humoral and Cell-Mediated Immunity

Chen Wang, Chengshui Liao, Wufan Zhang,
Deyuan Li and Puyan Chen

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64403

Abstract

Thymosin α1 (Tα1) and Bursopentin (BP5) are both immunopotentiators. To explore whether the thymosin α1-Bursopentin (rTα1-BP5) is an adjuvant or not, we cloned the gene of Tα1-BP5 and provided evidence that the gene of Tα1-BP5 in a recombinant prokaryotic expression plasmid was successfully expressed in *Escherichia coli* BL21. To evaluate the immune adjuvant properties of rTα1-BP5, chickens were immunized with rTα1-BP5 combined with H9N2 avian influenza whole-inactivated virus (WIV). The titers of HI antibody, antigen-specific antibodies, Avian influenza virus (AIV)-neutralizing antibodies, levels of Th1-type cytokines (gamma interferon (IFN-γ)) and Th2-type cytokines (interleukin 4 (IL-4)), and lymphocyte proliferation responses were determined. We found that rTα1-BP5 enhanced HI antibody and antigen-specific immunoglobulin G (IgG) antibodies titers, increased the level of AIV-neutralizing antibodies, induced the secretion of Th1- and Th2-type cytokines, and promoted the proliferation of T and B lymphocyte. Furthermore, virus challenge experiments confirmed that rTα1-BP5 contributed to the inhibition replication of the virus (H9N2 AIV (A/chicken/Jiangsu/NJ07/05) from chicken lungs. Altogether, these findings suggest that rTα1-BP5 is a novel adjuvant suitable for H9N2 avian influenza vaccine.

Keywords: thymosin α1 (Tα1), Bursopentin (BP5), fusion peptide, avian influenza vaccine, adjuvant

1. Introduction

Avian influenza virus (AIV) is an enveloped virus that belongs to the *Orthomyxoviridae* family and has an eight-segmented, single-stranded, negative-sense RNA genome. Among the proteins encoded by the genome, there are two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [1]. AIVs are classified into subtypes according to the combination of 16 HA and nine NA molecules. Among the many subtypes of AIV, H9N2 is thought to have originated from shorebirds and gulls, and has rapidly spread to become one of the most prevalent diseases in domestic poultry worldwide. It also causes serious economic loss in the poultry industry [2] (see **Table 1**).

Abbreviation	Full name
Tα1	Thymosin α1
BP5	Bursopentin
rTα1-BP5	Thymosin α1-Bursopentin
WIV	Whole-inactivated virus
AIV	Avian influenza virus
HA	Hemagglutinin
NA	Neuraminidase
BRM	Biological response modifier
MIF	Macrophage migration inhibitory factor
BF	Bursa of Fabricius
BLP	Bursin-like epitope peptide
HI	Hemagglutination inhibition assay
IFN-γ	Interferon-γ
IL-4	Interleukin-4
TNF-α	Tumor necrosis factor-α
Th	T-helper type
Th1	T-helper type 1
Th2	T-helper type 2
MDCK	Madin-Darby canine kidney
FBS	Fetal bovine serum
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
IPTG	Isopropyl-β-D-thiogalactoside
ConA	Concanavalin A
PMA	Phorbol-12-myristate-13-acetate

Abbreviation	Full name
TMB	Tetramethyl benzidine
HRP	Horseradish peroxidase
SPF	Specific pathogen-free
SOE-PCR	Splicing overlap extension PCR method
TRX	Thioredoxin
$TCID_{50}$	50% tissue culture infective dose
PBS	phosphate-buffered saline
pfu	Plaque-forming unit
OD	Optical density
IgG	Immunoglobulin G
ELISA	Enzyme-linked immunosorbent assay
$PRNT_{50}$	50% plaque-reducing neutralizing titer

Table 1. Abbreviations for full name.

In domestic avian species in North America, H9N2 influenza viruses occur primarily in turkeys, occasionally in quail, and rarely if ever in chickens. The H9N2 virus subtype was first isolated from turkeys in 1966 [3], when the virus was associated with mild respiratory disease. In Asia, long-term surveillance in live poultry markets in Hong Kong from 1975 to 1985 detected H9N2 influenza viruses in apparently healthy ducks but not in chickens [4]. Since the early 1990s, H9N2 influenza viruses have become widespread in domestic chickens in Asia [5]. Among the avian influenza A virus subtypes, H9N2 viruses have the potential to cause an influenza pandemic because they are widely prevalent in avian species in Asia and have demonstrated the ability to infect humans [6]. In April 1999, two World Health Organization reference laboratories independently confirmed the isolation of avian influenza A (H9N2) viruses for the first time in humans [7].

The best protection against influenza virus infection remains effective vaccination [8]. Inactivated vaccines have been undergoing clinical trials as pandemic vaccine candidates, and it has been shown that inactivated vaccines elicit strong humoral responses; however, it is commonly accepted that no adequate mucosal or cellular immunity is achieved [9]. Adjuvants are able to improve the quantity and quality of innate immune responses by enhancing their speed and duration, and by inducing adequate adaptive immunity [10]. To improve methods for influenza vaccine production, the current strategy of many investigators is to increase the efficacy of pandemic influenza vaccines by the addition of adjuvants to boost immune responses, such as aluminum salts, MF59, IC31®, and chitosan [11–14].

A defined peptide sequence able to stimulate specific immune cell subsets has the potential to act as an adjuvant for a variety of immunogens. The thymus is an important central immune organ for T-lymphocyte differentiation and maturation [15]. It is capable of secreting many peptides with the functions of regulating the development of different phenotypic markers

and lymphocyte [16]. Thymosin alpha 1 (Tα1), an immunomodulatory peptide consisting of 28 amino acid residues, was isolated originally from calf thymus [17]. As a biological response modifier (BRM), Tα1 has multiple biological activities in the immune system. It can promote specific lymphocyte functions, stimulate the production of lymphokines such as gamma interferon (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin 2 (IL-2), macrophage migration inhibitory factor (MIF), and precursor stem cell into the CD4+/CD8+ T cells, increase T-cell proliferation, differentiation and maturation, and so on [18, 19]. Furthermore, it has the activities of antitumor and protection against oxidative damage [20]. Consequently, Tα1 is widely used in clinic treating various diseases including immunodeficiency diseases, severe sepsis, and systemic infectious disorder [21].

The bursa of Fabricius (BF) is a primary humoral immune organ unique to birds and is the site of B-lymphocyte development and differentiation. The tripeptidebursin (LysHisGlyNH2) has been described as an endogenous B-cell stimulant or differentiation factor [22]. BS and bursin-like peptide T-X-N-L-K-H-G significantly enhance the JEV subtype vaccine-induced immune response in immunized mice [23]. Bursin-like epitope peptide (BLP) is one of bursin-like peptides and enhances immune responses in mice immunized with inactivated H9N2 avian influenza vaccine [24]. Our previous study has been reported that Bursopentin (BP5) is a small peptide separated from BF, which amino acid sequence is CKDVY. We found that BP5 not only promotes T-cell and B-cell proliferation, enhances humoral immunity and cellular immunity but also balances Th1 and Th2 immune responses [25, 26].

Although both Tα1 and BP5 have the potent adjuvant effects, this study designed and synthesized Tα1-BP5 fusion gene according to the preferential codons of *Escherichia coli*, fused with prokaryotic expression vector pET-32a, and then transferred into *E. coli* BL21 to induce its expression. Then, we tested whether rTα1-BP5 could enhance immune responses in chicken upon vaccination with H9N2 avian influenza whole-inactivated virus (WIV).

2. Materials and methods

2.1. Plasmid, viruses, and reagents

pET-32a (+), *E. coli* DH5α, *E. coli* BL21 (DE3), pET32a (+)-BP5, and avian influenza virus A/Chicken/Jiangsu/JS-1/2002(H9N2) were maintained in our laboratory. Avian influenza virus A/Chicken/Jiangsu/NJ08/05(H9N2) was kindly provided by Dr. Qi-Sheng Zheng. Virus titers were determined in MDCK cells. H9N2 avian influenza whole-inactivated virus (WIV) was prepared by diluting the virus 1:4000 (v/v) in formalin [27, 28]. All restriction enzymes and *Taq* polymerase were purchased from TakaRa Biotechnology (Dalian, China). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (New York, NY, USA). Isopropyl-β-D-thiogalactoside (IPTG), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), TEMED, dNTP, Concanavalin A (ConA), Phorbol-12-myristate-13-acetate (PMA), and tetramethylbenzidine (TMB) were purchased from Jiu Shi Corporation (Zhengzhou, China). Horseradish peroxidase (HRP)-conjugated goat anti-mice IgG was obtained from Boshide Corporation (Wuhan, China). Control Standard Tα1 and BP5 peptides were synthesized by

Shanghai Science Peptide Biological Technology Co., Ltd. (Shanghai, China), and the purity was over 95%.

2.2. Chicken embryos, animals, and vaccines

Specific pathogen-free (SPF) Roman chicken and chicken embryos were obtained from the Henan Experimental Animal Research Center. Avian influenza virus A/Chicken/Jiangsu/NJ08/05(H9N2) (10^7 $TCID_{50}$/0.1 mL) was inoculated into the allantoic cavities of 10-day-old SPF chicken embryos; the embryos that died within 24 h were discarded, and the allantoic fluids were harvested from the infected embryos at 48 h postinfection and inactivated by treatment with 0.2% formalin. The inactivated virus was emulsified with mineral oil to make an oil-formulated inactivated H9N2 AIV vaccine. One dose of the vaccine contained 10^7 $TCID_{50}$/0.1 mL, which was equal to it before inactivation. Procedure and test of inactivated vaccine were described according to OIE Terrestrial Manual 2012 [29].

2.3. Gene cloning and expression of the recombinant fusion peptide Tα1-BP5

Gene of the recombinant fusion peptide thymosin α1-Bursopentin (Tα1-BP5) was designed according to the preferential codons of *E. coli* and amplified by splicing overlap extension polymerase chain reaction (SOE-PCR) method [30]. Sequences of the primers used for the synthetic Tα1-BP5 are as follows: F1: 5′-CCG GAA TTC AGC GAC GCT GCT GTT GAC ACT AGC AGC GAA ATC ACT ACTA AAG ACT TG-3′; F2: 5′-GTT CGG GGT GCTG CCG CCG CCG CCG TTT TCA GCT TCT TCA ACA ACT TCT TTT TTT TCT TTC AAG TCT TTA GTA GT-3′; and F3: 5′-GGC GGC GGC GGC AGC TGC AAA AAT GTG TAT TAA GTC GAC TCG-3′, with *Eco*R I and *SaI* I site (underlined). The genes of Tα1 and BP5 were connected with the gene of the GGGGS linker to form the Tα1-BP5 fusion gene. And then, the amplified DNA fragment was digested by *Eco*R I and *SaI* I, and then ligated into the expression vector pET32a. The ligation mix was transformed into competent DH5α cells and the single bacterial colony was selected by overnight growth on Luria broth (LB) agar plates containing 100-μg/mL ampicillin. The obtained recombinant plasmid pET32a-Tα1-BP5 was confirmed by restriction endonuclease digestion and DNA sequencing. The pET32a-Tα1-BP5 plasmid was transformed into *E. coli* BL21 (DE3) for inducing expression. The expression products were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). And the recombinant proteins were purified on a Ni-affinity chromatography column (Amersham Bioscience HiTrap chelating HP 5 mL × one column) following the manufacturer's instructions.

2.4. Activity testing of fusion peptide Tα1-BP5 (rTα1-BP5) in vitro

Thymus and spleens from 4 to 6-week-BALB/c mice with (20 ± 2) g were collected aseptically, put them at 200-mesh stainless screen mesh cells, and gently minced into single cell suspension with a syringe followed by adding Hank's solution. The red blood cells were removed by centrifugation at 500 rpm for 5 min. The supernatant was centrifuged at 500 rpm for 5 min. The obtained pellet was washed with Hank's solution twice. The density of lymphocytes was adjusted to around 5×10^6 cells/mL using RPMI-1640 medium containing 10% FBS. ConA and PMA were added into thymic lymphocytes and splenic lymphocytes to make the concentra-

tions reach 5 μg/mL and 300 ng/mL, respectively. The two kinds of solutions were subpackaged into a 96-well plate with 100 μL/well, respectively, and three parallel samples were set for each well. The plates were incubated in CO_2 incubator at 37°C for 6 h, followed by adding 100-μL/well rTα1-BP5 (affinity chromatography purified through Ni column) with different concentrations (1.25, 2.5, 5.0, 10.0, and 20.0 μg/mL) and continued culturing for 72 h. Control groups (phosphate-buffered saline (PBS), 10.0 μg/mL thioredoxin, 10.0 μg/mL Tα1, and 10.0 μg/mL BP5) were used following the same procedures. MTT method was used to test the effect of rTα1-BP5 effect on thymic and splenic lymphocytes proliferation. Relative ratio of cell proliferation (%) = (experimental group OD_{570}/control group OD_{570}) × 100% [31, 32].

2.5. Immunization of chickens

All animal experiments were approved by the Henan University of Science and Technology Animal Care and Use Committee. Twenty-one-day-old SPF Roman chickens were randomly divided into six experimental groups of 25 chickens each and intramuscularly immunized two times on days 0 and 14 with (i) 100 μL PBS as a negative control, (ii) 100 μL H9N2 WIV (A/Chicken/Jiangsu/NJ08/05, 10^7 $TCID_{50}$/0.1 mL), (iii) a mixture of 100 μL H9N2 WIV and Tα1 (50 μg), (iv) a mixture of 100 μL H9N2 WIV and BP5 (50 μg), (v) a mixture of 100 μL H9N2 WIV and rTα1-BP5 (50 μg), and (vi) 100 μL oil-formulated inactivated H9N2 AIV vaccine (A/Chicken/Jiangsu/NJ08/05, 10^7 $TCID_{50}$/0.1 mL) as a positive control (**Table 2**).

Group	Vaccination on days 0 and 14[a]
1	100 μL PBS
2	10^7 $TCID_{50}$ H9N2 WIV
3	10^7 $TCID_{50}$ H9N2 WIV + 50 μg Tα1
4	10^7 $TCID_{50}$ H9N2 WIV + 50 μg BP5
5	10^7 $TCID_{50}$ H9N2 WIV + 50 μg rTα1-BP5
6	10^7 $TCID_{50}$ H9N2 AIV vaccine

[a] H9N2 WIV, inactivated H9N2 avian influenza whole-inactivated virus; H9N2 AIV vaccine, H9N2 avian influenza virus vaccine prepared with oil/water as an adjuvant.

Table 2. Animal groups and the experimental design.

The details of the animal experiment time points are shown in **Figure 1**.

2.6. Detection of antibodies in serum

Chicken (n = 5 per group) sera were collected on 7 and 21 days after the first immunization. Serum antibody (HI and antigen-specific antibodies) titers were determined using standard HI microliter and enzyme-linked immunosorbent assay (ELISA) as described [25, 33]. Briefly, to detect the HI titers in chicken serum, sera were inactivated by incubation for 30 min at 56°C and serially diluted twofold in PBS, then transferred in duplicate to 96-well round-bottomed

http://dx.doi.org/10.5772/64403

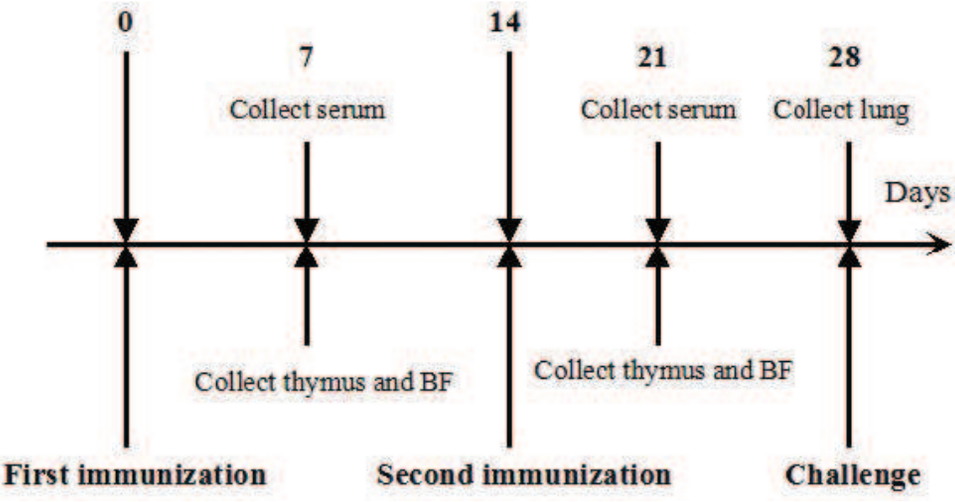

Figure 1. Experimental scheme of immunization, sample collection, and challenge.

plates. Standard avian influenza virus (A/Chicken/Shandong/6/96(H9N2)) antigen with four units was then added to each diluted serum samples in a volume of 50 μL, and followed by an equal volume of 0.5% chicken erythrocyte suspensions. The mixture was incubated for 1 h at room temperature before the results were read. The HI titers were defined as the highest serum dilution capable of preventing hemagglutination.

To evaluate the antigen-specific antibodies titers, ELISA plates were coated with 10-μg/mL recombinant influenza HA protein (expressed in *E. coli* BL21) and blocked with 1% bovine serum albumin (BSA) for 2 h at 37°C. Aliquots of diluted chicken sera were added to the plates, which were then incubated overnight, washed, and incubated with HRP-conjugated goat anti-chicken IgG. Finally, TMB was added and the reaction was stopped by the addition of 2N H_2SO_4 and the absorbance was read at OD_{450}. Each serum sample was repeated in quintuplicate. The results were plotted as OD versus dilution (log scale). Titers at half maximal OD were determined by linear interpolation [34].

2.7. Determination of AIV-neutralizing antibodies

Inactivated sera were incubated with 100 plaque-forming unit (pfu) of avian influenza virus (A/Chicken/Jiangsu/JS-1/2002(H9N2), and the titers of AIV-neutralizing antibodies determined as described [35].

2.8. Cytokine assays

On 7 and 21 days after the first immunization, the serum levels of Th1-type cytokine (IFN-γ) in chickens were determined using commercial Chicken cytokines gamma interferon ELISA kits (Cusabio Biotech, MD, USA), whereas Th2-type cytokine (IL-4) was determined with another commercial Chicken cytokines interleukin 4 ELISA kits (Cusabio Biotech, USA). The procedure followed the manufacturer's instructions.

2.9. Lymphocyte proliferation response

To detect changes in cellular immunity, lymphocyte proliferation response was performed. Thymus and bursa of Fabricius were collected from immunized chickens at 7 and 21 days after the first immunization. The thymus and BF lymphocytes were isolated and maintained in 1640 medium supplemented with 10% FBS at 37°C with 5% CO_2. The thymus lymphocytes (5×10^6 cells/mL) were seeded in a 96-well plate and incubated with 50 μL of ConA (40 μg/mL) at 40°C/5% CO_2 for 48 h, whereas the BF lymphocytes (5×10^6 cells/mL) were treated with 50 μL of PMA (1 μg/mL) in a 96-well plate at 40°C/5% CO_2 for 24 h. Then, the lymphocyte proliferation assay was performed using a standard MTT method as described previously [36, 37]. Then, the plate was incubated with 10 μL of 5 mg/mL MTT for 3 h. Finally, 100 μL of 10% (w/v) SDS in 0.01 M HCl was added into the plate and allowed to incubate for 2 h. A spectrophotometric measurement was taken at A_{570}.

2.10. Virus challenge experiment

Two weeks after the second vaccination, chickens (n = 15 per group) were intranasally challenged with 2.5×10^6 $TCID_{50}$ avian influenza virus A/chicken/Jiangsu/JS-1/2002(H9N2) in 100 μL PBS. Five chickens per group were humanely sacrificed at 3, 5, and 7 days after virus challenge and the viral titers in their lungs assessed by plaque formation assays using MDCK cells as described [35].

2.11. Statistical analysis

Statistical analyses were performed using unpaired *t*-tests or one-way analysis of variance (ANOVA) *F*-statistics followed by GraphPad Prism 6 software. Data are presented as the mean ± standard deviation (SD). Turkey multiple comparison tests were used to assess differences among the five experimental groups, with differences being considered significant at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Expression of the recombinant fusion peptide Tα1-BP5

The gene of Tα1-BP5 was amplified by SOE-PCR with the primers F1, F2, and F3. The PCR products were identified by electrophoresis, and then about 114bp strip was observed. The recombinant plasmid was extracted and identified with *Hind* III enzyme. The results showed that the recombinant plasmid was not digested by *Hind* III, indicating that the recombinant plasmid had deleted the *Hind* III restriction site (**Figure 2A**). Sequencing result showed that the gene of the recombinant fusion peptide Tα1-BP5 was inserted into pET32a vector, and it was consistent to the expected size (**Table 3**), which was suggested that the recombinant expression vector was constructed successfully, and it was named pET32a-Tα1-BP5. Then, pET32a-Tα1-BP5 was transferred into *E. coli* BL21 (DE3) for its expression, and the expressed products were detected by using SDS-PAGE. The result showed that Tα1-BP5 was expressed

and purified with the molecular weight of 31.4 kDa (**Figure 2B**), which is consistent with its predicted molecular weight.

Name	Amino acid sequence
Tα	Ser Asp Ala Ala Val Asp Thr Ser Ser Glu Ile Thr Thr Lys Asp Leu Lys Glu Lys Lys Glu Val Val Glu Glu Ala Glu
1-BP5	Asn Gly Gly Gly Gly Ser Cys Lys Asp Val Tyr

Table 3. Amino acid sequence of recombinant fusion peptide Tα1-BP5.

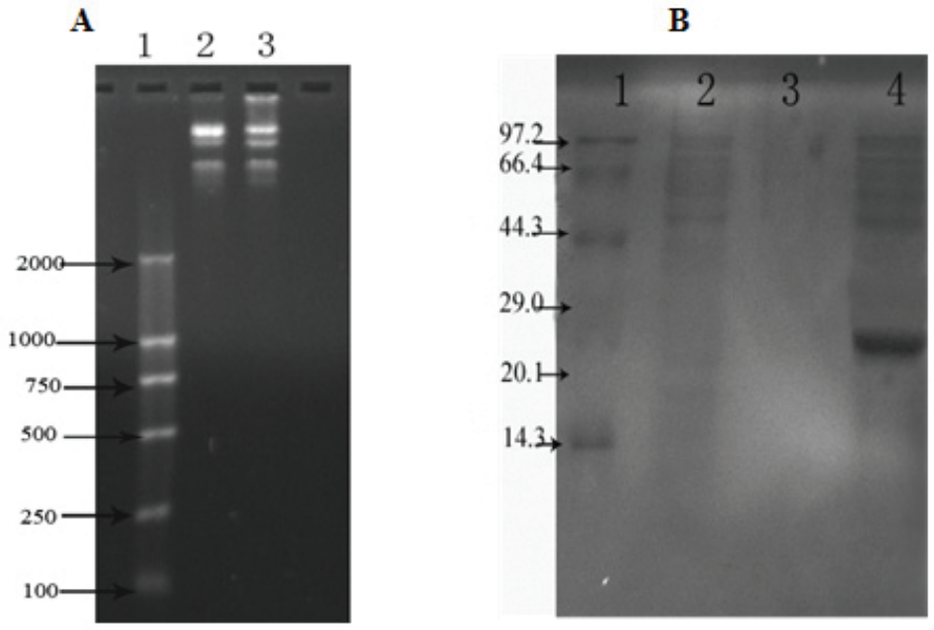

Figure 2. Identification of the recombinant plasmid pET32a-Tα1-BP5 and the expression of fusion peptide Tα1-BP5 in *E. coli*. (A) 1: DL2000 marker; 2: the recombinant plasmid pET32a-Tα1-BP5 after *Hind* III enzyme digestion; and 3: the recombinant plasmid pET32a-Tα1-BP5. (B) 1: low molecular weight protein marker; 2: not induced *E. coli* BL21 (DE3); 3: Not induced recombinant *E. coli* BL21 (DE3)/pET32a-Tα1-BP5; 4: induced recombinant *E. coli* BL21 (DE3)/pET32a-Tα1-BP5.

3.2. Activity of rTα1-BP5 in vitro

The expressed product of TBP5 recombinant bacteria was affinity chromatography purified through protein Ni column and quantified through spectrophotometer. MTT method was used to test the effect of rTα1-BP5 on the proliferation of mouse thymic and splenic lymphocytes. The results showed that all rTα1-BP5 with different concentrations (1.25, 2.5, 5.0, 10.0, and 20.0 μg/mL) could promote the proliferation of thymic and splenic lymphocytes compared to PBS group. rTα1-BP5 could stimulate thymic and splenic lymphocytes proliferation stronger than TP5 and BP5. The differences were significant ($P < 0.05$) on the concentrations of 5.0 and 20.0 μg/mL, and the differences were more significant ($P < 0.01$) on the concentrations of 10.0 μg/mL (**Figure 3A** and **B**). All these data demonstrated that rTα1-BP5 could promote the proliferation of mouse thymic T lymphocytes and splenic B lymphocytes.

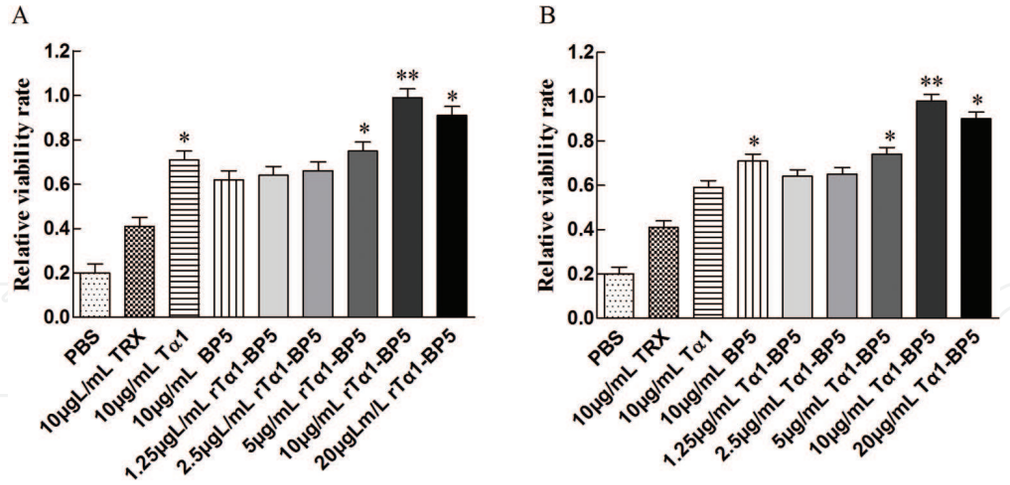

Figure 3. The effects of rTα1-BP5 on the proliferation of thymic lymphocytes (A) or splenic lymphocytes (B) from immunized mice. The data presented are of five replicates. *, $P < 0.05$, compared with mice immunized with PBS, and **, $P < 0.01$, compared with mice immunized with BP5 or Tα1.

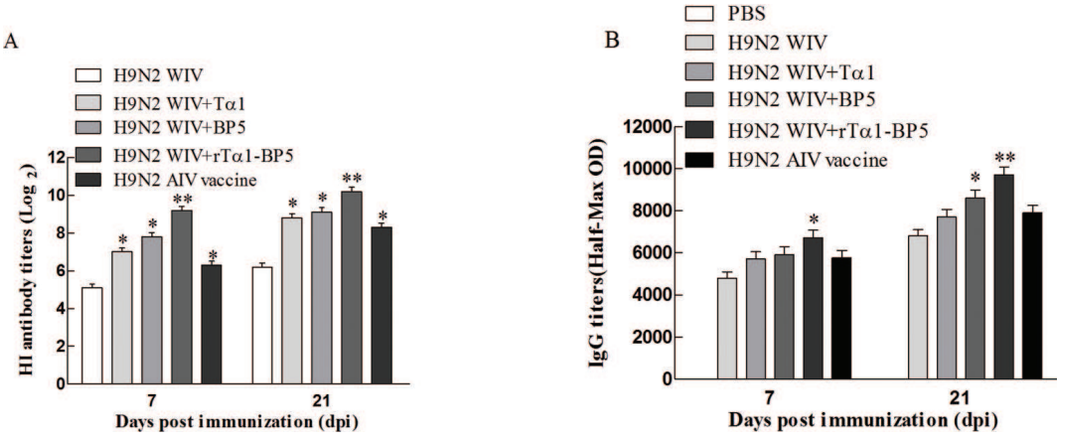

Figure 4. Effect of rTα1-BP5 to H9N2 AIV vaccination on antigen-specific HI titers and anti-HA IgG antibodies. Chickens were immunized two times, and chicken sera were collected on days 7 and 21 after the first immunization, and the serum HI titers (A) and IgG titers (B) were analyzed by HI assay and ELISA, respectively. The data presented are means ± SD of results from five replicates. *, $P < 0.05$, and **, $P < 0.01$, compared with chickens immunized with H9N2 WIV alone.

3.3. rTα1-BP5 stimulates significant antigen-specific immune responses

To determine antigen-specific immune responses to immunization, chickens were immunized two times, then sera were taken on days 7 and 21 after the first immunization and detected for HI and anti-HA antibody titers. HI antibody titers of chickens immunized with inactivated vaccine, Tα1 combined with H9N2 WIV and BP5 combined with H9N2 WIV increased significantly compared with chickens immunized with the H9N2 WIV alone at days 7 and 21 ($P < 0.05$). However, HI antibody titers in chickens immunized with rTα1-BP5 combined with H9N2 WIV were significantly higher than in chicken immunized with BP5 combined with

H9N2 WIV at days 7 and 21 ($P < 0.05$) (**Figure 4A**). Anti-HA IgG antibody was observed in immunized chickens on days 7 and 21 after the first immunization. rTα1-BP5 enhanced the secretion of IgG antibody on day 7 after the first immunization, and the effect was greater than that induced by H9N2 AIV vaccine, Tα1 combined with H9N2 WIV and BP5 combined with H9N2 WIV. On day 21 after the first immunization, BP5 significantly enhanced IgG antibody secretion levels compared with that induced by H9N2 vaccine, Tα1 combined with H9N2 WIV ($P < 0.05$), while the effect of rTα1-BP5 was the greatest than the other groups ($P < 0.01$) (**Figure 4B**). These results suggested that rTα1-BP5 stimulates significant antigen-specific immune responses.

3.4. rTα1-BP5 promoted the production of AIV-neutralizing antibody

To assess whether rTα1-BP5 can effectively enhance virus-neutralizing antibodies, chicken sera were collected on days 7 and 21 after the first immunization and the titers of AIV-neutralizing antibody were assessed. The result showed that the titers of neutralizing antibody of chickens immunized with Tα1 plus H9N2 WIV, BP5 plus H9N2 WIV, and H9N2 AIV vaccine were higher than that in chickens immunized with H9N2 WIV alone on day 7, while it was higher in chickens immunized with Tα1-BP5 plus H9N2 WIV than that of other groups. Consistent with this, AIV-neutralizing antibody titers of chicken injected with rTα1-BP5 plus H9N2 WIV were the highest on day 21 (**Table 4**). These results indicated that rTα1-BP5 significantly stimulates the production of AIV-neutralizing antibodies.

Treatment	PRNT50[a]	
	First boost	Second boost
PBS	–	–
H9N2 WIV	10 ± 0.38	19 ± 0.21
H9N2 WIV + Tα1	15 ± 0.30*	22 ± 0.25*
H9N2 WIV + BP5	18 ± 0.32*	28 ± 0.43*
H9N2 WIV + rTα1-BP5	22 ± 0.24**	32 ± 0.19**
Inactivated H9N2 AIV vaccine	16 ± 0.15*	25 ± 0.54**

a Chickens were vaccinated on days 0 (first boost) and 14 (second boost). Chicken sera ($n = 5$) were collected on days 7 and 21, and plaque-reducing neutralizing antibody titers were determined. The 50% plaque-reducing neutralizing titer ($PRNT_{50}$) was reported as the geometrical reciprocal of the serum dilution resulting in a 50% reduction in plaques. The data presented are means ± SD of results from five replicates.
*, $P < 0.05$, and
**, $P < 0.01$, compared with chickens immunized with H9N2 WIV alone.

Table 4. Titers of plaque-reducing neutralizing antibody in groups of chicken.

3.5. rTα1-BP5 increases the production of both Th1- and Th2-type cytokines

We then examined the levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines from immunized chickens. Compared with stimulation with H9N2 WIV alone, both IFN-γ and IL-4 secretion were remarkably increased after immunization with inactivated H9N2 AIV vaccine, Tα1 plus

H9N2 WIV and BP5 plus H9N2 WIV at days 7 and 21, and the highest level of IFN-γ secretion was observed in the vaccination group with rTα1-BP5 plus H9N2 WIV ($P < 0.01$) (**Figure 5A** and **B**). Taken together, the results suggested that rTα1-BP5 promoted the secretion of both Th1 and Th2 cytokines.

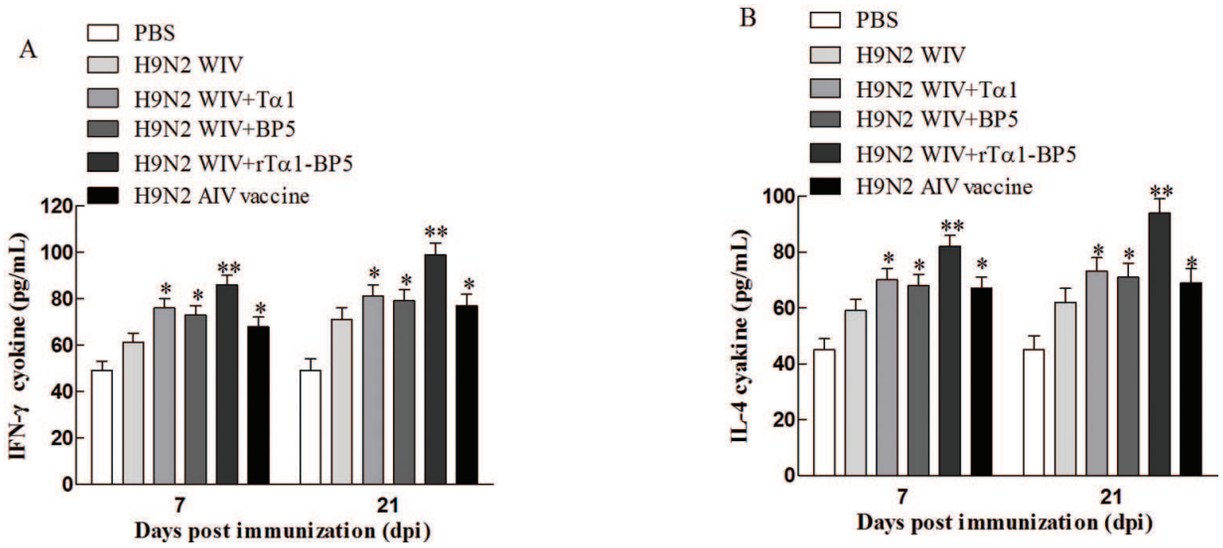

Figure 5. Effect of rTα1-BP5 to H9N2 AIV vaccination on cytokine production in chicken sera. Chickens were immunized two times, and chicken sera were collected on days 7 and 21 after the first immunization. Cytokine release was measured by using commercial chicken cytokines gamma interferon (IFN-γ) and interleukin 4 (IL-4) ELISA kits. The data presented are means ± SD of results from five replicates. *, $P < 0.05$, and **, $P < 0.01$, compared with chickens immunized with H9N2 WIV alone.

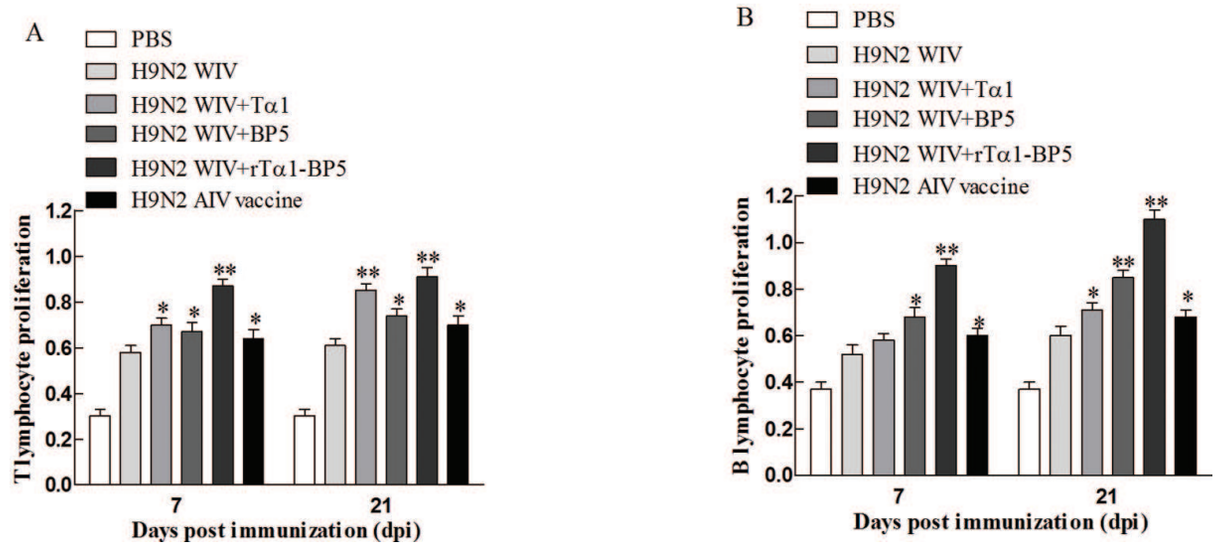

Figure 6. rTα1-BP5 significantly stimulates chicken T- and B-lymphocyte proliferation. Chickens were immunized two times, and chicken thymus and bursa of Fabricius were collected on days 7 and 21 after the first immunization. T- (A) and B (B)-lymphocyte proliferation assays were evaluated by MTT method. The data presented are means ± SD of results from five replicates. *, $P < 0.05$, and **, $P < 0.01$, compared with chickens immunized with H9N2 WIV alone.

http://dx.doi.org/10.5772/64403

3.6. rTα1-BP5 significantly enhances T- and B-lymphocyte proliferation

To investigate the effects of rTα1-BP5 on T- and B-lymphocyte proliferation, thymus and BF were collected from chickens immunized with rTα1-BP5 plus H9N2 WIV. T-lymphocyte proliferation responses of chickens immunized with Tα1 plus H9N2 WIV, BP5 plus H9N2 WIV, and H9N2 AIV vaccine were enhanced at 7 days compared with chickens immunized with H9N2 WIV alone ($P < 0.05$), whereas it was higher for that immunized with rTα1-BP5 plus H9N2 WIV ($P < 0.01$). On day 21, T-lymphocyte proliferation responses of chickens immunized with Tα1 plus H9N2 WIV and H9N2 AIV vaccine were higher than chickens immunized with H9N2 WIV alone ($P < 0.05$), while it was highest for that immunized with rTα1-BP5 plus H9N2 WIV ($P < 0.01$) (**Figure 6A**). Similarly, B-lymphocyte proliferation responses of chickens immunized with BP5 plus H9N2 WIV and H9N2 AIV vaccine were enhanced at 7 and 21 days compared with chickens immunized with H9N2 WIV alone ($P < 0.05$), whereas, it was highest for that immunized with rTα1-BP5 plus H9N2 WIV ($P < 0.01$) (**Figure 6B**). The data indicated that rTα1-BP5 promoted T- and B-lymphocyte proliferative responses.

3.7. rTα1-BP5 significantly promotes immune protection against H9N2 AIV challenge

To evaluate whether rTα1-BP5 promotes immune protection against H9N2 AIV infection, viral titers in chicken lungs were evaluated at 3, 5, and 7 days after viral challenge by plaque formation assays. Chickens immunized with Tα1 plus H9N2 WIV, BP5 plus H9N2 WIV, and H9N2 AIV vaccine showed significant virus removal from the lungs at 3, 5, and 7 days after challenge compared with H9N2 WIV groups ($P < 0.05$), while the viral titers of lungs from chicken immunized with rTα1-BP5 plus H9N2 WIV were significantly lower than that in the H9N2 AIV vaccine group ($P < 0.01$). Moreover, chickens immunized with rTα1-BP5 plus H9N2 WIV had almost no detectable virus particles in the lungs 7 days after challenge (**Figure 7A–C**). The data indicated that rTα1-BP5 significantly promoted immune protection against H9N2 AIV challenge.

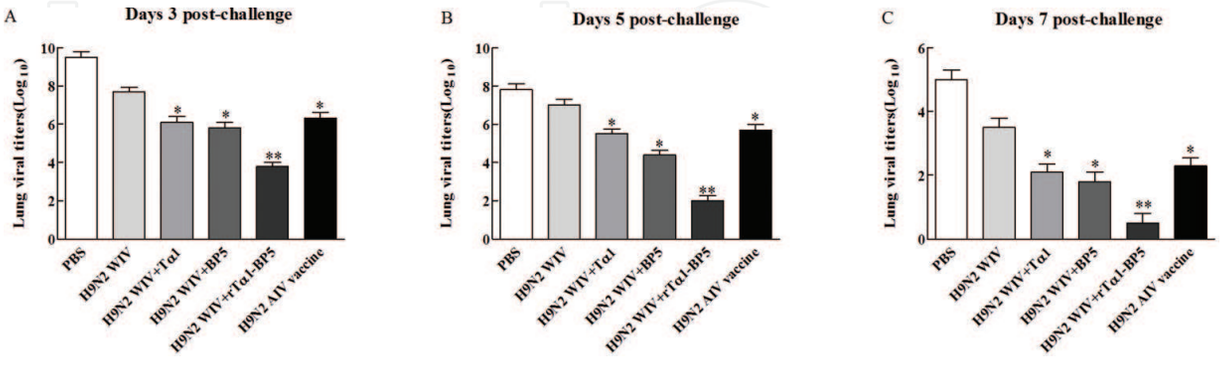

Figure 7. AIV-viral titers of lung in chickens. Lung samples from individual chicken in each group ($n = 5$) were collected on days 3, 5, and 7 post challenge with 2.5 × 106 $TCID_{50}$ avian influenza virus (A/chicken/Jiangsu/JS-1/2002(H9N2)). Each lung sample was diluted to 1 mL with 1640 media. The titers are presented as pfu per mL. The data presented are means ± SD of results from five replicates. *, $P < 0.05$, and **, $P < 0.01$, compared with chickens immunized with H9N2 WIV alone.

4. Discussion

In the event of an influenza pandemic, vaccination is one of the most effective ways of intervention in terms of reducing cost, disease, and even death. Appropriate adjuvant can enhance the immunogenicity of the vaccine and improve the immune responses [38, 39]. However, most of the adjuvants used in conjugation with antigen have unacceptable levels of side effects, only a few of them are used clinically [40]. Thus, we need to find new and optimal adjuvant candidates for vaccine. In recent years, some small peptide immunostimulants were reported in use for vaccine adjuvants [41–43]. Both Tα1 and BP5 are associated with immune regulation. Previous studies showed that both Tα1 and BP5 had high potential as an adjuvant for vaccines [26, 44].

In this study, the fusion peptide of rTα1-BP5 was designed and synthesized, and to investigate it as an adjuvant for inducing immune responses in chickens upon vaccination with inactivated H9N2 avian influenza virus (WIV). An effective adjuvant should be able to enhance the levels of both humoral and cell-mediated immunity. To investigate the effect of rTα1-BP5 on humoral responses, chickens were immunized with H9N2 WIV combined with Tα1-BP5, and then titers of HI antibody, antigen-specific antibodies, and AIV-neutralizing antibodies were assessed. Then, we found that rTα1-BP5 significantly enhanced HI antibody and antigen-specific IgG antibodies titers, promoted the secretion of AIV-neutralizing antibodies, which suggested that rTα1-BP5 enhanced the levels of humoral immune responses in chickens when it was co-immunized with H9N2 WIV.

In addition to humoral immune responses, cellular immunity also plays an important role in fighting influenza virus infections [45]. The levels of Th1- and Th2-type cytokines are important references to measure cellular immunity. And lymphocyte homeostasis is required for the maintenance of normal immune function [46]. Th1-type cytokines mainly include IL-2, TNF-α, and IFN-γ, whereas Th2-type cytokines include IL-4, IL-5, and IL-10 [47]. Our study though analyzed the production of Th1 (IFN-γ)- and Th2 (IL-4)-type cytokines, and T- and B-lymphocytes proliferation in vaccinated chickens post immunization to evaluate the cell-mediated immunity. The results suggested that rTα1-BP5 promoted the secretion of both Th1 and Th2 cytokines and T- and B-lymphocyte proliferative responses. Overall, this study found that rTα1-BP5 not only enhanced the humoral immune responses but also promoted the cell-mediated immune responses, and it had the potential to use as an adjuvant.

To further evaluate the influence of rTα1-BP5 as an adjuvant on the immunity protection provided by H9N2 AIV vaccine against AIV infection, chickens were intramuscularly challenged with H9N2 AIV (A/chicken/Jiangsu/JS-1/2002) on day 28 post immunization. After 3 days post challenge, the PBS group chickens that received the challenge virus were mildly depressed. No other clinical signs were observed in that group or any of the other groups, which is typical of low-pathogenicity AIV in chickens [48, 49]. At 7 days post challenge, only the PBS-challenged group had mild, grossly detectable lesions in both the respiratory and gastrointestinal tract. And we found that the viral titers of lungs from chicken immunized with rTα1-BP5 plus H9N2 WIV were significantly lower than all the other groups at 3 days. Chickens immunized with rTα1-BP5 plus H9N2 WIV had almost no detectable virus particles in the

lungs at 7 days after challenge. Our data indicated that rTα1-BP5 could effectively inhibit the replication of H9N2 AIV in chickens and promote virus clearance in the lungs of chickens. Thus, rTα1-BP5 had the potential to be used in vaccine formulations to provide improved protection against H9N2 AIV infection in poultry.

In summary, this study demonstrated that inactivated H9N2 AIV vaccine with Tα1-BP5 as an adjuvant enhanced strong immune responses at both humoral and cellular levels against AIV infection in chickens. These data may provide a novel insight to find new adjuvant in vaccines.

Acknowledgements

This work was supported by Grant no. 31101792 from the National Natural Science Foundation of China and no. 2012GGJS-077 from the Foundation for University Key Teacher by Higher Education of Henan Province.

Author details

Chen Wang[1*], Chengshui Liao[1], Wufan Zhang[1], Deyuan Li[2] and Puyan Chen[2]

*Address all correspondence to: wangchen2001@126.com

1 Key Laboratory of Veterinary Oncological Immunology, Henan University of Science and Technology, Luoyang, China

2 Key Laboratory of Animal Bacteriology of China's Department of Agriculture, Nanjing Agricultural University, Nanjing, China

#C.WANG and C.S.LIAO equally contributed to this work.

References

[1] Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal of Virology. 2005; 79: 2814–2822. DOI: 10.1128/JVI.79.5.2814-2822.2005

[2] Banet-Noach C, Perk S, Simanov L, Grebenyuk N, Rozenblut E, Pokamunski S, Pirak M, Tendler Y, Panshin A. H9N2 influenza viruses from Israeli poultry: a five-year outbreak. Avian Diseases. 2007; 51: 290–296. DOI: 10.1637/7590-040206R1.1

[3] Homme PJ, Easterday BC. Avian influenza virus infections. IV. Response of pheasants, ducks, and geese to influenza A-turkey-Wisconsin-1966 virus. Avian Diseases. 1970; 14: 285–290. DOI: 10.2307/1588473

[4] Shortridge KF. Pandemic influenza: a zoonosis? Seminars in Respiratory Infections. 1992; 7: 11–25.

[5] Sun Y. Proceedings of the Fourth International Symposium on Avian Influenza: avian influenza, a global problem: Georgia Center for Continuing Education, the University of Georgia, Athens, Georgia, USA, May 28–31, 1997. pp. 47–49.

[6] Alexander DJ. Proceedings of the Fourth International Symposium on Avian Influenza: Avian Influenza, A Global Problem: Georgia Center for Continuing Education, the University of Georgia, Athens, Georgia, USA, May 28–31, 1997. pp. 9–13

[7] Li C, Yu K, Tian G, Yu D, Liu L, Jing B, Ping J, Chen H. Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology. 2005; 340: 70–83. DOI: 10.1016/j.virol.2005.06.025

[8] Nicholson KG, Wood JM, Zambon M. Influenza. Lancet. 2003; 362: 1733–1745. DOI: 10.1016/S0140-6736(03)14854-4

[9] Nichol KL, Treanor JJ. Vaccines for seasonal and pandemic influenza. The Journal of Infectious Diseases. 2006; 194:S111–S118. DOI: 10.1086/507544

[10] Wareing MD, Tannock GA. Live attenuated vaccines against influenza; an historical review. Vaccine. 2001; 19: 3320–3330. DOI: 10.1016/S0264-410X(01)00045-7

[11] Singh M, O'Hagan D. Advances in vaccine adjuvants. Nature Biotechnology. 1999; 17(11): 1075–1781. DOI: 10.1038/15058

[12] Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, Huckriede A. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine. 2008; 26: 2350–2359. DOI: 10.1016/j.vaccine.2008.02.063

[13] Riedl K, Riedl R, von Gabain A, Nagy E, Lingnau K. The novel adjuvant IC31 strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice. Vaccine. 2008; 26: 3461–3468. DOI: 10.1016/j.vaccine.2008.04.029

[14] O'Hagan DT. MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection. Expert Review of Vaccines. 2007; 6: 699–710. DOI: 10.1586/14760584.6.5.699

[15] Li J, Zheng L, Li P, Wang F. Intein-mediated expression, purification, and characterization of thymosin alpha 1-thymopentin fusion peptide in *Escherichia coli*. Protein Expression and Purification. 2012; 84: 1–8. DOI: 10.1016/j.pep.2012.04.013

[16] Lunin SM, Glushkova OV, Khrenov MO, Novoselova TV, Parfenyuk SB, Fesenko EE, Novoselova EG. Thymic peptides restrain the inflammatory response in mice with

experimental autoimmune encephalomyelitis. Immunobiology. 2013; 218: 402–407. DOI: 10.1016/j.imbio.2012.05.023

[17] Goldstein AL, Low TL, McAdoo M, McClure J, Thurman GB, Rossio J, Lai CY, Chang D, Wang SS, Harvey C, Ramel AH, Meienhofer J. Thymosin alpha1: isolation and sequence analysis of an immunologically active thymic polypeptide. Proceedings of the National Academy of Sciences of the United States of America.1977; 74: 725–729. DOI: 10.1073/pnas.74.2.725

[18] Ahmed A, Wong DM, Thurman GB, Low TL, Goldstein AL, Sharkis SJ, Goldschneider I. T-lymphocyte maturation: cell surface markers and immune function induced by T-lymphocyte cell-free products and thymosin polypeptides. Annals of the New York Academy of Sciences. 1979; 332: 81–94. DOI: 10.1111/j.1749-6632.1979.tb47100.x

[19] Hadden JW, Verastegui E, Hadden E. IRX-2 and thymosin alpha 1 (zadaxin) increase T lymphocytes in T lymphocytopenic mice and humans. Thymosins in Health and Disease: First International Symposium. Annals of the New York Academy of Sciences. 2007; 1112: 245–255. DOI: 10.1196/annals.1415.032

[20] Lao X, Liu M, Chen J, Zheng H. A tumor-penetrating peptide modification enhances the antitumor activity of thymosin alpha 1. PLoS One. 2013; 8: e72242. DOI: 10.1371/journal.pone.0072242

[21] Li YM, Chen H, Li X, Zhou WC, He MY, Chiriva-Internati M, Wachtel MS, Frezza EE. A new immunomodulatory therapy for severe sepsis: Ulinastatin Plus Thymosin {alpha} 1. Journal of Intensive Care Medicine. 2009; 24: 47–53. DOI: 10.1177/0885066608326970

[22] Audhya T, Kroon D, Heavner G, Viamontes G, Goldstein G. Tripeptide structure of bursin, a selective B-cell-differentiating hormone of the bursa of fabricius. Science. 1986; 231: 997–999. DOI: 10.1126/science.3484838

[23] Wang C, Wen WY, Su CX, Ge FF, Dang ZG, Duan XG, Cao RB, Zhou B, Chen PY. Bursin as an adjuvant is a potent enhancer of immune response in mice immunized with the JEV subunit vaccine. Veterinary Immunology and Immunopathology. 2008; 122: 265–274. DOI: 10.1016/j.vetimm.2007.11.010

[24] Wang C, Li XK, Wu TC, Li DY, Niu MF, Wang Y, Zhang CJ, Cheng XC, Chen PY. Bursin-like peptide (BLP) enhances H9N2 influenza vaccine induced humoral and cell mediated immune responses. Cellular Immunology. 2014; 292: 57–64. DOI: 10.1016/j.cellimm.2014.09.003

[25] Li DY, Xue MY, Wang C, Wang JB, Chen PY. Bursopentine as a novel immunoadjuvant enhances both humoral and cell-mediated immune responses to inactivated H9N2 avian influenza virus in chickens. Clinical and Vaccine Immunology. 2011; 18(9): 1497–1502. DOI: 10.1128/CVI.05133-11

[26] Li DY, Geng ZR, Zhu HF, Wang C, Miao DN, Chen PY. Immunomodulatory activities of a new pentapeptide (Bursopentin) from the chicken bursa of Fabricius. Amino Acids. 2011; 40(2): 505–515. DOI: 10.1007/s00726-010-0663-7

[27] Novak M, Moldoveanu Z, Schafer DP, Mestecky J, Compans RW. Murine model for evaluation of protective immunity to influenza virus. Vaccine. 1993; 11: 55–60. DOI: 10.1016/0264-410X(93)90339-Y

[28] Sha Z, Compans RW. Induction of CD4 (+) T-cell-independent immunoglobulin responses by inactivated influenza virus. Journal of Virology. 2000; 74: 4999–5005. DOI: 10.1128/JVI.74.11.4999-5005.2000

[29] OIE 2012. Manual of diagnostic tests and vaccines for terrestrial animals. Paris: Office International des Epizooties. [Internet]. Available from: http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/

[30] Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene. 1989; 77: 61–68. DOI: 10.1016/0378-1119(89)90359-4

[31] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods.1983; 65: 55–63. DOI: 10.1016/0022-1759(83)90303-4

[32] Yu L, Li JR, Huang YW, Liang XY, Meng SS. Enhanced immunogenicity of plasmid encoding polyprotein gene of infectious bursal disease virus by co-administration of chicken interleukin 2 (IL-2). Sheng Wu Gong Cheng Xue Bao. 2001; 17: 652–657. DOI: 10.3321/j.issn:1000-3061.2001.06.013

[33] Haan L, Verweij WR, Holtrop M, Brands R, van Scharrenburg GJ, Palache AM, Agsteribbe E, Wilschut J. Nasal or intramuscular immunization of mice with influenza subunit antigen and the B subunit of Escherichia coli heat-labile toxin induces IgA- or IgG-mediated protective mucosal immunity. Vaccine. 2001; 19: 2898–2907. DOI: 10.1016/S0264-410X(00)00556-9

[34] Fritz JH, Brunner S, Birnstiel ML, Buschle M, Gabain AV, Mattner F, Zauner W. The artificial antimicrobial peptide KLKLLLLLKLK induces predominantly a TH2-type immune response to co-injected antigens. Vaccine. 2004; 22: 3274–3284.DOI: 10.1016/j.vaccine.2004.03.007

[35] Rimmelzwaan GF, Baars M, van Beek R, van Amerongen G, Lovgren-Bengtsson K, Claas EC, Osterhaus AD. Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. The Journal of General Virology. 1997; 78: 757–765. DOI: 10.1099/0022-1317-78-4-757

[36] Du L, Lyle CS, Obey TB, Gaarde WA, Muir JA, Bennett BL, Chambers TC. Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity:

evidence that mitotic Bcl-2 phosphorylation is JNK-independent. The Journal of Biological Chemistry. 2004; 279: 11957-11966. DOI: 10.1074/jbc.M304935200

[37] Wang C, Li XK, Zhang CJ, Wu TC, Li YJ, Cheng XC. A eukaryotic expression plasmid carrying chicken interleukin-18 enhances the response to Newcastle disease virus vaccine. Clinical and Vaccine Immunology. 2015; 22: 56–64. DOI: 10.1128/CVI.00636-14

[38] Bhardwaj N. Processing and presentation of antigens by dendritic cells: implications for vaccines. Trends in Molecular Medicine. 2001; 7: 388–394. DOI: 10.1016/S1471-4914(01)02101-3

[39] Schijns VE. Immunological concepts of vaccine adjuvant activity. Current Opinion in Immunology. 2000; 12: 456–463. DOI: 10.1016/S0952-7915(00)00120-5

[40] Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunology and Cell Biology. 2004; 82: 488–496. DOI: 10.1111/j.0818-9641.2004.01272.x

[41] Cai MH, Zhu F, Wu HC, Shen PP. A new recombinant hybrid polypeptide and its immunologic adjuvant activity for inactivated infectious bursal disease vaccine. Biotechnology Letters. 2014; 36: 1431–1437. DOI: 10.1007/s10529-014-1499-0

[42] Charoenvit Y, Goel N, Whelan M, Rosenthal KS, Zimmerman DH. CEL-1000—a peptide with adjuvant activity for Th1 immune responses. Vaccine. 2004; 22: 2368–2373. DOI: 10.1016/j.vaccine.2003.11.062

[43] Gagnon L, DiMarco M, Kirby R, Zacharie B, Penney CL. D-LysAsnProTyr tetrapeptide: a novel B-cell stimulant and stabilized bursin mimetic. Vaccine. 2000; 18: 1886–1892. DOI: 10.1016/S0264-410X(99)00374-6

[44] Li WY, Lu HM, Guo Q, Hu WM, Zhang ZD. Effects of thymosin alpha1 on immune effector molecules of mouse. Sichuan da xue xue bao Yi xue ban = Journal of Sichuan University Medical science edition. 2014; 45: 400–404.

[45] Kreijtz JH, Bodewes R, van Amerongen G, Kuiken T, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. Vaccine. 2007; 25: 612–620. DOI: 10.1016/j.vaccine.2006.08.036

[46] Wen L, Chen SJ, Zhang W, Ma HW, Zhang SQ, Chen L. hsBAFF regulates proliferation and response in cultured CD4(+) T lymphocytes by upregulation of intracellular free Ca(2+) homeostasis. Cytokine. 2011; 53: 215–222. DOI: 10.1016/j.cyto.2010.11.006

[47] Kumar DA, Manikandan P, Sumitra M, Raju KV, Gayathri C, Arutselvan N, Puvanakrishnan R. A novel peptide derivative exhibits anti inflammatory and antioxidant activity in adjuvant induced arthritis in rats. Molecular and Cellular Biochemistry. 2002; 229: 9–17. DOI: 10.1023/A:1017980024029

[48] Lee CW, Song CS, Lee YJ, Mo IP, Garcia M, Suarez DL, Kim SJ. Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza viruses and assessment of the

pathogenic potential of isolate MS96. Avian diseases. 2000; 44: 527–535. DOI: 10.2307/1593091

[49] Soda K, Asakura S, Okamatsu M, Sakoda Y, Kida H. H9N2 influenza virus acquires intravenous pathogenicity on the introduction of a pair of di-basic amino acid residues at the cleavage site of the hemagglutinin and consecutive passages in chickens. Virology Journal. 2011; 8: 64. DOI: 10.1186/1743-422X-8-64

IntechOpen