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The optimized fusion protein HA1-2-FliCΔD2D3 promotes mixed Th1/Th2 immune responses to influenza H7N9 with low induction of systemic proinflammatory cytokines in mice

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ABSTRACT

H7N9 influenza virus has an unusually high fatality rate of approximately 40%, and a safe and effective vaccine against this subtype is urgently needed. Flagellin, a Toll-like receptor (TLR) 5 agonist, has been deemed as a potent adjuvant candidate. However, its high antigenicity and potential for causing inflammatory injury might restrict its clinical application. Previously, we demonstrated that a fusion protein, HA1-2-FliC, comprising the hemagglutinin globular head protein (HA1-2) of H7N9 influenza virus and the full-length *Salmonella typhimurium* flagellin protein (FliC), had high efficiency against H7N9 in mouse and chicken models. Here, we constructed an improved fusion protein, HA1-2-FliCΔD2D3, with HA1-2 fused to the FliCΔD2D3 (lacking the hypervariable-region domains D2 and D3 of FliC). HA1-2-FliCΔD2D3 exhibited efficient immunoreactivity and TLR5 agonist efficacy, and promoted innate immune-response activation in mouse macrophages, peripheral blood mononuclear cells, and splenocytes, based on cytokine- and chemokine-expression profiles. Mice immunized with HA1-2-FliCΔD2D3 showed significantly lower systemic inflammatory responses (compared with HA1-2-FliC) and highly reduced flagellin-specific antibody production, without affecting HA1-2-specific antibody production and cellular immune responses. Enhanced IFN-γ/IL-4 generation suggested that HA1-2-FliCΔD2D3 maintained balanced Th1/Th2 immune responses. Furthermore, virus challenge was performed in a chicken model. The results showed that chickens receiving FliCΔD2D3 adjuvant vaccine induced high levels of serum neutralizing antibodies, and exhibited a significant reduction of viral loads in throat and cloaca compared to chickens receiving only HA1-2. In conclusion, we constructed the H7N9 influenza subunit vaccine candidate HA1-2-FliCΔD2D3, with reduced immunogenicity against FliC and lower adverse events. The improved adjuvant FliCΔD2D3 can potentially help in developing safe and effective universal protein-based influenza vaccines for humans.

1. Introduction

The avian influenza A(H7N9) virus continues to cause human infections in China and is a major ongoing public health concern. Five epidemic waves of H7N9 infection have occurred since 2013, and in the current fifth epidemic wave, a highly pathogenic avian influenza (HPAI) H7N9 virus emerged (Yang et al., 2018). After introduction into the Pearl River Delta region, the origin LPAI H7N9 virus acquired four amino acid (KRTA) insertions in the hemagglutinin (HA) protein cleavage site and mutated into the HPAI H7N9 virus in late May 2016. Afterward, the HPAI H7N9 viruses further reassorted with LPAI H7N9 or H9N2 viruses locally and generated multiple different genotypes. As of 14 July 2017, the HPAI H7N9 viruses had spread from Guangdong to at least 12 other provinces (Yang et al., 2017; Zhu et al., 2017).

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Furthermore, they retain a series of genetic features contributing to the ability to infect humans (e.g. 186 V in the HA protein and 627 K in the PB2 protein) that raise concerns regarding their pandemic potential (Zhu et al., 2017).

Human infection caused by H7N9 virus causes severe respiratory illness and a fatality of ~40% (Wang et al., 2017a), making safe and effective anti-H7N9 vaccines imperative. Several new types of H7N9 influenza vaccines for pandemic preparedness are in development, including recombinant subunit vaccines, recombinant replicative H7N9 vaccines based on hemagglutinin (HA) (Song et al., 2017a; Wang et al., 2017b), live attenuated influenza vaccines (Kong et al., 2015), and cell culture-derived inactivated whole-virus vaccines (Wodal et al., 2015). Including adjuvants can improve influenza vaccines by enhancing immune responses.

Toll-like receptor (TLR) agonists stimulate "broadly specific" proinflammatory immune responses and enhance adaptive immune response to defined antigens (Maisonneuve et al., 2014). Several studies have described the adjuvant properties of flagellin (a TLR5 agonist) in combination with recombinant vaccines against pathogens like *Yersinia pestis* and West Nile virus (McDonald et al., 2007; Mizel et al., 2009). Flagellin offers plasticity when generating antigen–flagellin fusion proteins (Kitzmüller et al., 2018; Mohabati Mobarez et al., 2017). Several vaccine candidates employing this strategy have reached early-stage clinical studies (Frey et al., 2017; Treanor et al., 2010). Previously, we demonstrated that a fusion protein, HA1-2-FliC, with the globular-head domain of H7N9 HA (HA1-2) directly fused to the N-terminus of *Salmonella typhimurium* flagellin (FliC), was a highly potent systemic and mucosal vaccine candidate in mouse and chicken models (Song et al., 2017a).

However, the potent antigenicity of flagellin and severe systemic inflammation induced by high-dose flagellin indicates that immunity to flagellin might affect its potency and induce side effects, as an adjuvant (Nempont et al., 2008; Xiao et al., 2015). Thus, efforts must be made to reduce potential adverse effects induced by flagellin while maintaining its adjuvanticity. Developing improved flagellin adjuvants with unique properties is a major challenge in manipulating immune responses.

Flagellin is the major structural protein of bacterial flagella (Ramos et al., 2004). The FliC protein from *Salmonella typhimurium* is a 494-aa protein with two distinct domains. The highly conserved N-terminal and C-terminal (domains D0/D1; 170 and 90 aa, respectively) form an essential domain for TLR5-agonist activity. The middle hypervariable region (domains D2/D3; amino acids 170–400) is essential for flagellin antigenicity (Smith et al., 2003; Yoshioka et al., 1995).

Here, we constructed an improved H7N9-subunit vaccine candidate, HA1-2-FliCΔD2D3, by fusing HA1-2 to FliCΔD2D3 (lacking the FliC D2 and D3 domains). HA1-2-FliCΔD2D3 exhibited efficient immunoreactivity and TLR5-agonist efficacy, and promoted innate immune-response activation *in vitro* and *ex vivo*. Moreover, deletion of hypervariable region of flagellin significantly reduced its antigenicity and potential for inducing related systemic inflammatory responses, without altering adjuvant activity in mice. In addition, chickens receiving HA1-2-FliCΔD2D3 candidate vaccines significantly decreased viral loads in throat and cloaca following H7N9 influenza virus challenge.

2. Materials and methods

2.1. Ethics statement

Female, 6–8-week-old BALB/c mice were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. Two-week-old specific-pathogen free (SPF) White Leghorn chickens were purchased from poultry institute, Shandong academy of agricultural science. All mice and birds were housed in isolators with controlled temperature, light, and ventilation. Pathogen-free water and diet were supplied *ad libitum*. All animal studies were approved by the Committee on the Ethics of Animal Experiments of Yangzhou University (Approval ID: SYXK [Su] 2012-0029). All animals were randomly assigned to groups before immunization.

Table 1
PCR primers used for construction of HA1-2-fliCΔD2D3 in this study.

Primers	Sequence (5′-3′)	Restricted site
HA1-2-F	ccggaattcaaagggaaaaggacagttgacc	*Eco*R I
HA1-2-R	cacctccgcttccacctccaccggcatcaacctgtactc	$(Gly_4Ser)_3$
N*fliC*-F	aggtggaagcggaggtggtggaagcatggcacaagtcatta	$(Gly_4Ser)_3$
N*fliC*-R	cacctccgcttccacctccacccagcgtatccagacccagg	$(Gly_4Ser)_3$
C*fliC*-F	aggtggaagcggaggtggtggaagcgctgctacaaccaccg	$(Gly_4Ser)_3$
C*fliC*-R	tgctctagattaacgcagtaaagagaggacg	*Xba* I

2.2. Viruses

The avian influenza H7N9 virus (A/chicken/Shandong/LY301/2017) used in this study was preserved by Jiangsu Key Laboratory of Zoonosis of Yangzhou University. The inactivated virus using 0.1% formalin was used as hemagglutination inhibition (HI) antigen, and the live virus was used in the microneutralization assay and viral challenge experiment.

2.3. Recombinant plasmid construction

Based on the sequence of recombinant HA1-2-*fliC* gene (Song et al., 2015), three primer pairs were designed to amplify the fragments of interest (Table 1). The HA1-2 gene fragment was PCR-amplified from pCold-HA1-2 (Song et al., 2015) with primers HA1-2-F and HA1-2-R. Similarly, the N-terminal fragment of *fliC* (D0-D1 fragment, N*fliC*) and the C-terminal fragment of *fliC* (D1-D0 fragment, C*fliC*) were amplified using forward (N*fliC*-F, C*fliC*-F) and reverse (N*fliC*-R, C*fliC*-R) primers from the plasmid pCold-HA1-2-*fliC* (Song et al., 2015). The hypervariable region (D2 and D3)-deleted clone *fliCΔD2D3* was generated by linking the N*fliC* and C*fliC* fragment by overlap-PCR using primers N*fliC*-F and C*fliC*-R. The HA1-2-*fliCΔD2D3* fusion gene was generated by joining the HA1-2 gene fragment with *fliCΔD2D3* using forward primer HA1-2-F and reverse primer C*fliC*-R (Fig. 1A). The PCR products of HA1-2-*fliCΔD2D3* were digested by *Eco*RI/*Xba*I and cloned into the pCold vector (Takara, Dalian, China), generating the construct pCold-HA1-2-*fliCΔD2D3*. The resulting construct was sequenced by GenScript (Nanjing, China).

2.4. Protein expression, purification, and characterization

The pCold-HA1-2-*fliCΔD2D3* construct was transformed into Chaperone Competent Cell pTf16/BL21 (Takara), and the pTf16 chaperone plasmid increased soluble protein expression. Then, the HA1-2-FliCΔD2D3 protein was purified using the His•Bind Purification Kit (Novagen, Billerica, MA, USA). The purified HA1-2-FliCΔD2D3 protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting with mouse polyclonal anti-FliC or anti-HA1-2 antibodies. Recombinant His-tagged HA1-2 and HA1-2-FliC proteins were prepared as described (Song et al., 2015). Endotoxin was removed from all proteins using the ProteoSpin™ Endotoxin Removal Kit Maxi for protein & peptides (Norgen Biotek Corp., Thorold, Canada). Residual endotoxin levels were measured using a Chromogenic Endpoint Tachypleus Amebocyte Lysate (CE TAL) Assay Kit (Chinese Horseshoe Crab Reagent Manufactory Co., Xiamen, China).

2.5. TLR5 activity assay

The TLR5-agonist activities of recombinant HA1-2, HA1-2-FliC, and HA1-2-FliCΔD2D3 were evaluated by measuring IL-8 secretion and nuclear factor kappa B (NF-κB) activation of HEK293-mTLR5 cells. For

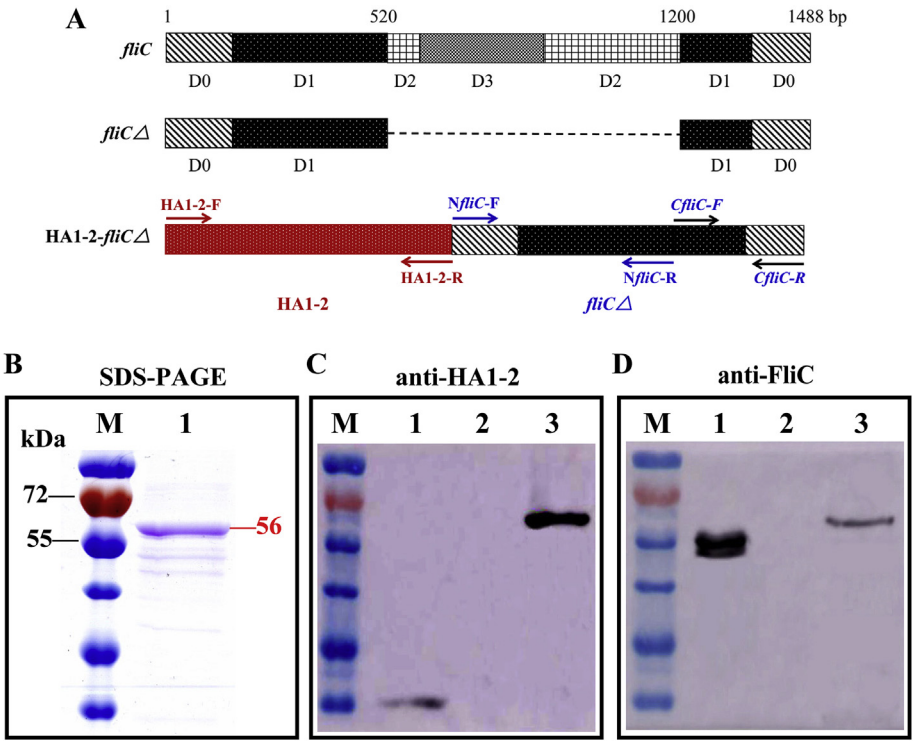

Fig. 1. Schematic diagram of the fusion protein HA1-2-FliCΔD2D3 and identification of the recombinant protein. (A) Diagram of the *fliC*, *fliCΔD2D3*, and HA1-2-*fliCΔD2D3* genes. The broken line indicates the deleted D2 and D3 regions of fliC. The HA1-2 gene was fused upstream of the *fliCΔD2D3* gene to form HA1-2-*fliCΔD2D3*, and the resulting fusion protein was connected by a flexible peptide linker $(Gly_4Ser)_3$. Arrows indicate the direction of primer amplification. (B) SDS-PAGE analysis of purified HA1-2-FliCΔD2D3. Lanes: M, protein marker; 1, HA1-2-FliCΔD2D3. (C) Western blotting with mouse polyclonal antibodies against HA1-2. Lanes: M, protein marker; 1, HA1-2; 2, FliC; 3 HA1-2-FliCΔD2D3. (D) Western blotting with mouse polyclonal antibodies against FliC. Lanes: M, protein marker; 1, FliC; 2, HA1-2; 3, HA1-2-FliCΔD2D3. The results are representative of at least three (B) or four (C, D) experiments.

IL-8 assay, the cells were seeded in 96-well plates (5×10^4/well) and maintained at 37 °C in 5% CO_2 overnight and subsequently stimulated with proteins at different concentrations (0.001, 0.01, 0.1, 1 or 5 μg/ml). The supernatants were collected after 5 h to detect IL-8 with an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, Franklin Lakes, NJ, USA). For the NF-κB activation assay, HEK293-mTLR5 cells were transfected with pGL4.32[luc2P/NF-κB-RE/Hygro] NF-κB reporter plasmid and pRL-TK renilla luciferase control reporter plasmid (Promega, Madison, WI, USA). The cells were plated in 96-well flat-bottom plates at a density of 5×10^4 cells/well. On the day of stimulation, cells were washed twice and then stimulated with proteins at different concentrations (0.001, 0.01, 0.1, 1 or 5 μg/ml) for 5 h. The firefly luciferase levels were measured and normalized to the activity of pRL-TK-derived renilla luciferase. Three independent replicates were used in the evaluation.

2.6. Macrophage activation in vitro

Mouse RAW264.7 cells were grown in dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA) at 37 °C in 5% CO_2. The cells were seeded overnight in 24-well plates (2×10^5 cells/well) and then stimulated with 1 μg/ml HA1-2, 3 μg/ml HA1-2-FliC, 2 μg/ml HA1-2-FliCΔD2D3 (containing 1 μg/ml HA1-2, respectively), or DMEM control. After 5 h, the cells were collected and stored at −70 °C before RNA extraction, to assess the mRNA levels of cytokines and chemokines. The results were from three independent replicates.

2.7. Preparation and stimulation of splenocytes and peripheral blood mononuclear cells (PBMCs) ex vivo

PBMCs and splenocytes were prepared from 6–8-week-old female BALB/c mice (Song et al., 2017b). Single lymphocyte suspensions were seeded in 24-well plates (1×10^6 cells/well, 0.5 ml) in complete RPMI 1640 containing 10% foetal bovine serum and 1% penicillin-streptomycin (Gibco). Cells were stimulated with 3 μg/ml HA1-2, 9 μg/ml HA1-2-FliC, 6 μg/ml HA1-2-FliCΔD2D3 (containing 3 μg/ml HA1-2, respectively), or RPMI 1640 control. After 4 h, the cells were washed in phosphate-buffered saline (PBS), lysed in RNeasy Lysis (RLT) buffer (Qiagen, Hilden, Germany), and stored at −70 °C until RNA extraction. The supernatants were collected at 12 h post-stimulation and cytokine levels were quantified using commercial ELISA kits against IL-6, IL-12p40, and TNF-α (BD Biosciences) and IL-23p19 (eBioscience, San Diego, CA, USA). Three independent replicates were used in the evaluation.

2.8. RNA extraction and real-time PCR (RT-PCR) quantification of cytokines and chemokines

Total mRNA from RAW264.7 cells, PBMCs, and splenic lymphocytes was obtained using an RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesised from mRNA using a PrimeScript RT Reagent Kit (Takara). The cytokine and chemokine mRNA levels were detected as described (Song et al., 2017b). The sequences of primers used for RT-PCR are shown in Table 2. Data were calculated using the $2^{-\Delta\Delta CT}$ approach, with normalisation to expression of the reference gene, β-actin.

2.9. Serum cytokine levels following immunization

Groups of 6–8-week-old female BALB/c mice ($n = 6$) were intraperitoneally (i.p.) immunized with PBS (control) or 10 μg HA1-2, 30 μg HA1-2-FliC, or 20 μg HA1-2-FliCΔD2D3 (containing 10 μg HA1-2, respectively), in a 100-μl volume. At 4 and 12 h post-administration, sera were analyzed for IL-6, IL-12p40, TNF-α, and IL-23p19 levels using commercial ELISA kits.

2.10. Mouse-vaccination and sample-collection schedules

Female BALB/c mice (6–8-week-old) were randomly divided into 4 groups ($n = 6$) and i.p.-immunized with HA1-2 (10 μg), HA1-2-FliC (30 μg), HA1-2-FliCΔD2D3 (20 μg) (containing 10 μg HA1-2, respectively), or PBS alone in a 100-μl volume. Immunization was performed twice, with priming on day 0 and boosting on day 14. Sera were collected on day 26 and analyzed for HA1-2- and flagellin-specific

Table 2
Sequences of primers used for quantitative real-time PCR.

Gene	Primer sequences (5′-3′)	Product size (bp)	Accession no.
IL-1β	F: gcccatcctctgtgactcat R: aggccacaggtattttgtcg	230	NM_008361.4
IL-6	F: agttgccttcttgggactga R: tccacgatttcccagagaac	159	NM_031168.2
TNF-α	F: agcccccagtctgtatcctt R: ctccctttgcagaactcagg	212	NM_013693.3
IFN-γ	F: actggcaaaaggatggtgac R: tgagctcattgaatgcttgg	237	NM_008337.4
IL-12	F: catcgatgagctgatgcagt R: cagatagcccatcaccctgt	163	NM_001159424.2
MCP-1	F: aggtccctgtcatgcttctg R: tctggacccattccttcttg	249	NM_011333.3
MIP-2	F: aagtttgccttgaccctgaa R: aggcacatcaggtacgatcc	180	NM_011333.3
KC	F: gctgggattcacctcaagaa R: aggtgccatcagagcagtct	208	NM_008176.3
IP-10	F: ggatggctgtcctagctctg R: ataacccttgggaagatgg	211	NM_021274.2
β-actin	F: agccatgtacgtagccatcc R: ctctcagctgtggtggtgaa	228	NM_007393.5

antibody responses by indirect ELISA. Two weeks after the second (booster) immunization, all immunized mice were euthanized and splenic lymphocytes were obtained from immunized mice to study cellular immune responses in BrdU, IFN-γ, and IL-4 enzyme-linked immunospot (ELISpot) assays.

2.11. Indirect ELISA

HA1-2- or flagellin-specific antibody serum titers were assessed using indirect ELISA (Song et al., 2015). Briefly, HA1-2 (0.25 μg/well) and FliC (0.5 μg/well) were coated in 96-well plates overnight in 50 mM carbonate buffer at 4 °C. After washing and blocking, two-fold-diluted samples were added for 2 h at 37 °C. Horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000) (Invitrogen, Carlsbad, CA, USA) was added and incubated for 1 h at 37 °C, with 3, 3′, 5, 5′-tetramethybenzidine as the substrate. The reaction was stopped by adding H_2SO_4 and the OD at 450 nm was determined on a microplate reader (BioTek, Winooski, VT, USA). The cutoff value was defined as the mean + 2 standard deviation of negative control. The antibody titers were defined as the reciprocal of the highest dilution of samples that had a reading above the cutoff value.

2.12. HI assay

Animals were bled 12 days after the second immunization. Serum samples were analyzed by HI assays. Briefly, serum samples collected from mice were treated with receptor destroying enzyme II overnight, heat inactivated (56 °C, 30 min), diluted in 96-well V-bottomed microtiter plates, and incubated with 4 HA units of inactivated avian influenza A (H7N9) virus for 30 min at room temperature. Then, 1% chicken erythrocytes were added, mixed briefly, and incubated for 30 min at room temperature. The highest dilution of serum that inhibited hemagglutination was considered the HI titer.

2.13. Cell-proliferation ELISA based on 5-bromo-2′-deoxyuridine (BrdU)

Splenic lymphocytes were obtained from each mouse after the second vaccination (described above), and cell-proliferation assays were performed with splenic lymphocyte pre-treated with 10 μg/ml of HA1-2 using the cell-proliferation ELISA Kit Based on BrdU (Roche Diagnostics, Tokyo, Japan). Briefly, 100 μl/well of splenic lymphocytes (2×10^5 cells/well) were pre-treated with or without 10 μg/ml of HA1-2 in triplicate in 96-well plates and incubated at 37 °C, in a 5% CO_2 incubator for 48 h. Then, the BrdU labeling reagent was added at 10 μl/well. At 12 h, the cells were harvested by centrifugation and the plate was dried at 60 °C for 1 h. BrdU-labeled DNA in the cells was fixed and denatured by incubation with FixDenat solution for 30 min at room temperature. After washing, the BrdU-labeled DNA was stained with peroxidase-conjugated anti-BrdU antibody for 90 min at room temperature. The plate was washed again, and TMB substrate solution was added. The reaction was stopped by adding 1 M H_2SO_4 solution. Absorbance was measured at 450 nm using a microplate reader (BioTek).

2.14. IFN-γ and IL-4 ELISpot assays

Splenic lymphocytes were obtained from mice after the second vaccination, and single-splenocyte suspensions were prepared to quantify IFN-γ- or IL-4-producing cells using the ELISpot set (BD Biosciences). All ELISpot assays were performed as described previously (Song et al., 2016). Briefly, 96-well ELISpot microplates were coated with a capture antibody in sterile PBS, incubated overnight at 4 °C, and blocked (2 h at room temperature) with complete Roswell Park Memorial Institute (RPMI) medium. Splenic lymphocytes were counted and plated in ELISPot plates at 2×10^5 cells/well (in triplicate) in complete RPMI medium. Cells were mock stimulated with RPMI or stimulated with HA1-2 (10 μg/ml) for 24 h incubation at 37 °C and 5% CO_2. After the incubation period, ELISpot plates were processed following the manufacturer's specifications using a biotinylated antibody as a detection antibody, streptavidin-AKP (BD Biosciences) and a BCIP/NBT Liquid Substrate System (Sigma-Aldrich, St. Louis, MO, USA) for assay development. All assay plates were scanned and analyzed using an automated ELISpot reader system (Bioreader 5000-Vβ, BioSys, Karben, Germany).

2.15. Chicken vaccination and viral challenge

A total of 40 SPF chickens at two-week-old were randomized into 4 groups ($n = 10$). Prior to challenge, all birds were immunized intramuscularly with a final volume of 200 μl/dose/chicken candidate vaccines (10 μg HA1-2, 30 μg HA1-2-FliC, 20 μg HA1-2-FliCΔD2D3) or PBS on days 0, 14, and 28. Blood samples were collected 12 days after the third vaccination for determination of serum neutralizing antibodies. At two weeks after the third immunization (on day 42), chickens were inoculated intranasally with 10^6 50% egg infectious dose (EID_{50}) of H7N9 influenza virus in a 200 μl volume. The throat and cloaca swabs were collected from chickens at 9 day post inoculation, and resuspended in 1 ml PBS for viral RNA extraction followed by real-time PCR (RT-PCR) for quantitative analysis of virus.

2.16. Microneutralization assay

At 12 days after the third immunization, blood samples of chickens were collected and analyzed for neutralizing antibody titers in Madin-Darby canine kidney (MDCK) cells. The microneutralization assay was modified from a previous study (Ruan et al., 2018). Briefly, all serum samples were heat inactivated for 30 min at 56 °C, then two-fold serially diluted in a 50 μl opti-minimum essential media containing 1 μg/ml L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (Sigma). Subsequently, an equal volume of 100 $TCID_{50}$ of H7N9 viruses was added to each dilution and incubated at 37 °C for 2 h. 100 μl of serum and virus mixture was inoculated into MDCK cells on 96-well plates. The cell supernatant was harvested after 72 h and further verified by hemagglutination assay, and titers were calculated by the method of Reed and Muench.

2.17. Quantitative analysis of viral loads

Viral RNA was extracted from the swab samples of chicken using

TIANamp Virus RNA Kit (TianGen, Beijing, China). cDNA was synthesised from mRNA using a Primescript RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. For viral quantification, the primers forward: 5′-GGAGTTCTAATTATCAACAATC-3'; reverse: 5′- TCCCATAGATTTTCCTCTC-3'; and the TaqMan probe: 5′-(FAM) CCAGGAGCGAGACCACAAGTTA (TAMRA)-3′ were designed to amplify and detect a 185-base pair segment of HA gene of H7N9 influenza virus. The reaction was run on an ABI 7500 with the following steps: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Data analyses were performed using the 7500 software supplied with the instrument. The copy number of the HA gene was calculated on the basis of a standard curve using an HA-containing plasmid pCold-HA1-2 (Song et al., 2015) of known concentration as a standard. The copy numbers of the HA gene were quantified by the following equation: copies/μl = ((6.02 × 10^{23} copies/mol) × concentration of plasmid g/μl)/(number of base pairs of the plasmid × 2 × 324.5).

2.18. Statistical analysis

Data are expressed as mean ± SEM. Differences between two groups were analyzed by the one-way or two-way ANONA using Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance is indicated by *P < 0.05, **P < 0.01, ***P < 0.001, or no significant difference (n.s) (P > 0.05).

3. Results

3.1. Protein purification and identification of immunoreactivity

The recombinant protein, HA1-2-FliCΔD2D3 was efficiently expressed in *E. coli* BL21. The soluble HA1-2-FliCΔD2D3 was purified by His-tag affinity chromatography with an expected molecular weight of 56 kDa (Fig. 1B). Immunoreactivity was confirmed by western blotting using mouse anti-FliC or anti-HA1-2 polyclonal antibodies (Fig. 1C and D).

3.2. TLR5-agonist activity of the HA1-2-FliCΔD2D3 protein

HEK293-mTLR5 cells were used to test for TLR5-agonist activity. Cell supernatants were collected after 5 h stimulation to detect IL-8 secretion. The results showed that HA1-2-FliCΔD2D3 induced significantly higher levels of IL-8 than that in the HA1-2 group at indicated concentrations. In addition, HA1-2-FliCΔD2D3 induced IL-8 in a dose-dependent manner, which was similar to that elicited by HA1-2-FliC (Fig. 2A). This finding was supported by the results of an NF-κB activation assay. Both HA1-2-FliC and HA1-2-FliCΔD2D3 induced significantly higher NF-κB activation than HA1-2 control group (Fig. 2B).

3.3. Macrophage activation in vitro

Macrophage activation induced by vaccine candidates was evaluated by measuring inflammatory cytokine (IL-1β, IL-6, TNF-α) and chemokine (CXCL chemokines MIP-2, IP-10, and CCL chemokine MCP-1) expression in RAW264.7 cells treated with different proteins. Cells exposed to HA1-2-FliCΔD2D3 showed significantly increased expression of IL-1β (38.9-fold, P < 0.001), IL-6 (58.7-fold, P < 0.05), MCP-1 (4.0-fold, P < 0.05), MIP-2 (75.9-fold, P < 0.001), and IP-10 (24.0-fold, P < 0.001), compared to those levels in the HA1-2-treated group (Fig. 3). HA1-2-FliCΔD2D3 induced comparable cytokine and chemokine responses, although some (IL-1β, MIP-2, and IP-10) were less than that induced by HA1-2-FliC.

3.4. Splenocyte and PBMC activation ex vivo

PBMCs and splenocytes were isolated from non-immunized mice and treated with recombinant proteins. Compared with the HA1-2 group, the mRNA-expression levels of IL-1β (1.5-fold, P < 0.05; 2.8-fold, P < 0.05), IL-6 (3.6-fold, P < 0.001; 10.9-fold, P < 0.001), and TNF-α (1.7-fold, P < 0.05; 3.4-fold, P < 0.05) were significantly higher in HA1-2-FliCΔD2D3-treated splenocytes and PBMCs, whereas IFN-γ mRNA expression was significantly higher (14.0-fold, P < 0.001) only in splenocytes (Fig. 4A, C). Regarding chemokines, significantly higher mRNA-expression levels of IP-10 (3.1-fold, P < 0.001) were detected in splenocytes treated with HA1-2-FliCΔD2D3 than in the HA1-2 group. KC (also designated as CXCL1, 6.2-fold, P < 0.001) and MIP-2 (2.7-fold, P < 0.001) expression were significantly higher only in PBMCs (Fig. 4B, D). Similar trends of increasing cytokine and chemokine expression were observed in the HA1-2-FliC-treated group, and no significant difference was observed, except for IP-10 in splenocytes and MIP-2 in PBMCs (Fig. 4A–D).

We tested cytokine secretion in culture supernatants after 12 h stimulations. IL-6, TNF-α, and IL-12p40 secretion levels by splenocytes and PBMCs showed that the immunocyte-activating efficacy of HA1-2-FliCΔD2D3 was significantly higher (2–4 fold) than that of HA1-2 (Fig. 4E and F). Similar results were found with the HA1-2-FliCΔD2D3 and HA1-2-FliC groups.

3.5. Systemic inflammatory responses in mice

To evaluate flagellin-induced inflammatory responses in mice i.p.-injected with vaccine candidates, proinflammatory cytokines IL-6, TNF-α, IL-12p40, and IL-23p19 in serum were measured by ELISA at 4 and 12 h post-immunization. All serum-cytokine levels were significantly upregulated at both times after HA1-2-FliC administration compared with the HA1-2 group, and the responses were higher at 4 h (Fig. 5). HA1-2-FliCΔD2D3 significantly lowered proinflammatory effects with all four cytokines in serum at 4 and 12 h versus HA1-2-FliC treatment,

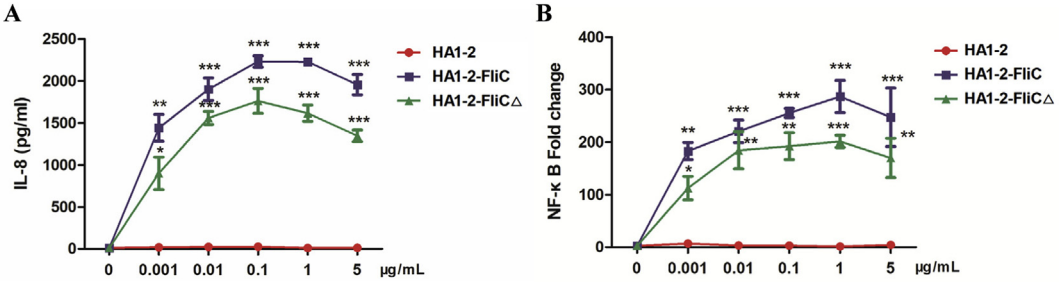

Fig. 2. TLR5 activity of the fusion protein. (A) Dose-response curve of IL-8 production. HEK293-mTLR5 cells were treated with indicated concentrations of HA1-2, HA1-2-FliC or HA1-2-FliCΔD2D3 for 5 h, and the supernatants were collected for IL-8 detection by ELISA. (B) NF-κB activity. HEK293-mTLR5 cells were transfected with pGL4.32[luc2P/NF-κB-RE/Hygro] NF-κB reporter plasmid and pRL-TK renilla luciferase control reporter plasmid. Twenty-four hours post-transfection, different concentrations (0.001–5 μg/ml) of purified proteins were added to the transfected cells and NF-κB luciferase activity was measured after stimulation for 5 h. Data are presented as mean ± SEM of three independent experiments.

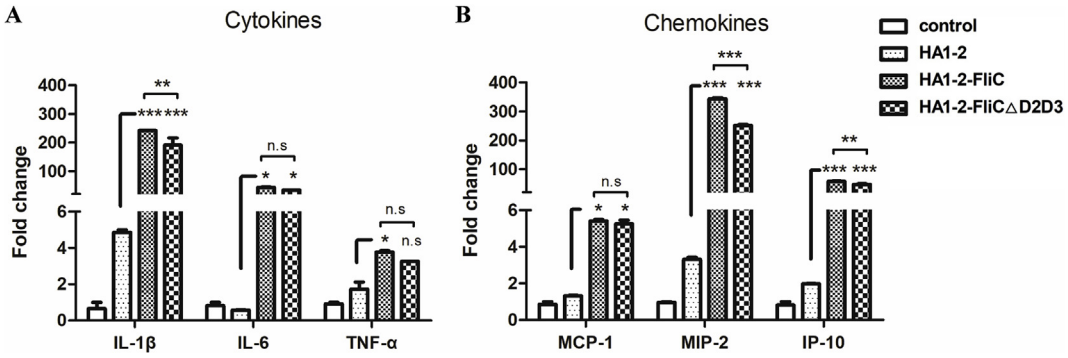

Fig. 3. Macrophage activation *in vitro*. RAW264.7 cells (4×10^5 cells/ml) were treated with recombinant HA1-2, HA1-2-FliC, or HA1-2-FliCΔD2D3 at the indicated concentrations for 5 h. The mRNA levels of cytokines (A) and chemokines (B) were measured via RT-PCR. Data are presented as mean ± SEM. The results are representative of three experiments.

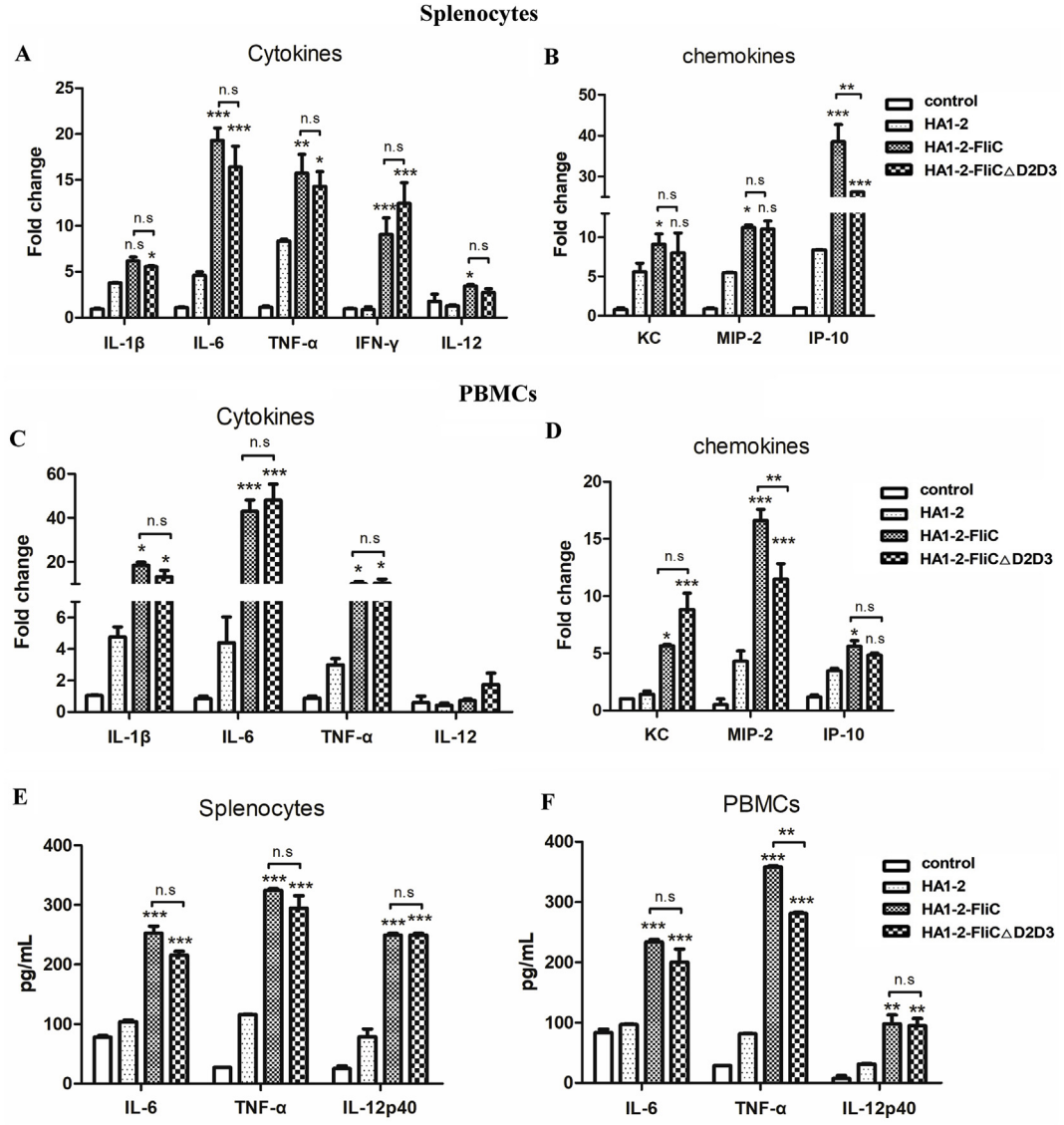

Fig. 4. Cytokine and chemokine levels in PBMCs and splenic lymphocytes following stimulation with fusion proteins. Splenic lymphocytes and PBMCs isolated from naïve BALB/c mice were treated with the indicated recombinant proteins for 4 or 12 h, and the cells and supernatants were analyzed. The mRNA levels of cytokines and chemokines in splenocytes (A and B) and PBMCs (C and D) were measured by RT-PCR, and IL-6, TNF-α, and IL-12p40 secretion into the culture supernatant from splenocytes (E) and PBMCs (F) was tested by ELISA. Data are presented as mean ± SEM. The results are representative of three experiments.

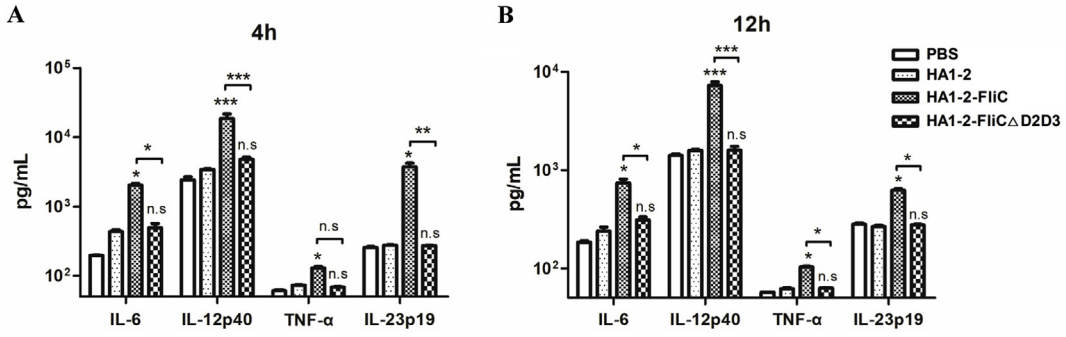

Fig. 5. Cytokine levels in serum. BALB/c mice were i.p. administered the indicated amount of HA1-2, HA1-2-FliC, and HA1-2-FliCΔD2D3 in 100 μl of PBS or PBS alone. Serum was collected to determine IL-6, IL-12p40, TNF-α, and IL-23p19 levels after 4 h (A) and 12 h (B) by ELISA. Data are presented as mean ± SEM.

being reduced by approximately 2–5-fold, or even > 10-fold (IL-23 at 4 h) (Fig. 5).

3.6. Antibody response in serum

BALB/c mice were immunized with different vaccine candidates on days 0 and 14, and HA1-2-FliCΔD2D3 immunogenicity was evaluated by detecting antigen-specific antibodies in serum 12 days after the second vaccination. HA1-2-FliC elicited a strong flagellin-specific IgG response (25,600 on average) in serum. Because the main antigenicity region of FliC was deleted, HA1-2-FliCΔD2D3 showed significantly lower flagellin-specific serum IgG titers (2533 on average) ($P < 0.01$) compared with HA1-2-FliC (Fig. 6B).

Notably, HA1-2-FliCΔD2D3 induced a slightly lower (non-significant) average HA1-2-specific IgG titer (61,440) compared with HA1-2-FliC (115,200). Both titers were significantly higher (~246-fold, $P < 0.05$; 460-fold, $P < 0.01$, respectively) than that induced by HA1-2 (250 on average) (Fig. 6A). Similar to the above results, HI titers in the adjuvant groups were significantly higher ($P < 0.01$) than HA1-2 antigen group (Fig. 6C).

3.7. Cellular immune responses

At 14 days after boosting, mice splenocytes were prepared, and cellular immune responses were assessed by monitoring lymphocyte proliferation with the BrdU assay. After the second vaccination, the average stimulation index (SI) of lymphocytes in the HA1-2-FliC and HA1-2-FliCΔD2D3 groups (2.9, $P < 0.001$; 3.1, $P < 0.001$, respectively) were significantly higher than in HA1-2 group (1.6), and no significant difference occurred between the two adjuvanted groups (Fig. 7A).

ELISpot assays were performed to further evaluate the ability of vaccine candidates to induce cellular immune responses and to estimate the immune types. Splenic lymphocytes were prepared 2 weeks after boosting and stimulated with HA1-2. The average numbers of IFN-γ- (63 and 87, respectively, $P < 0.05$) and IL-4- (72 and 61, respectively, $P < 0.05$) producing cells were significantly higher in the HA1-2-FliC- and HA1-2-FliCΔD2D3-vaccine groups than in the HA1-2 group (20 and 18, respectively), and no significant difference occurred between the HA1-2-FliC and HA1-2-FliCΔD2D3 groups (Fig. 7B). Notably, nearly equal numbers of IFN-γ- and IL-4-producing cells were observed in both adjuvanted groups, indicating that both Th1- and Th2-associated immune responses were induced by HA1-2-FliCΔD2D3 vaccination.

3.8. Serum neutralizing antibodies of chickens

The neutralizing antibodies induced by vaccine candidates in the chicken model were determined by microneutralization assay. At 12 days after the third immunization, similar high levels of serum neutralizing antibody titers were observed in both HA1-2-FliC (102 on average) and HA1-2-FliCΔD2D3 (128 on average) immunization groups. Furthermore, the neutralizing antibody titers in both flagellin adjuvanted groups were significantly higher than that in the non-adjuvanted HA1-2 group (Fig. 8A).

3.9. Virus loads in throat and cloaca of H7N9 virus challenged chickens

In order to investigate whether FliCΔD2D3 could improve the protective efficacy of HA1-2, we detected the viral loads in throat and cloaca of H7N9 virus challenged chickens. The result showed that the

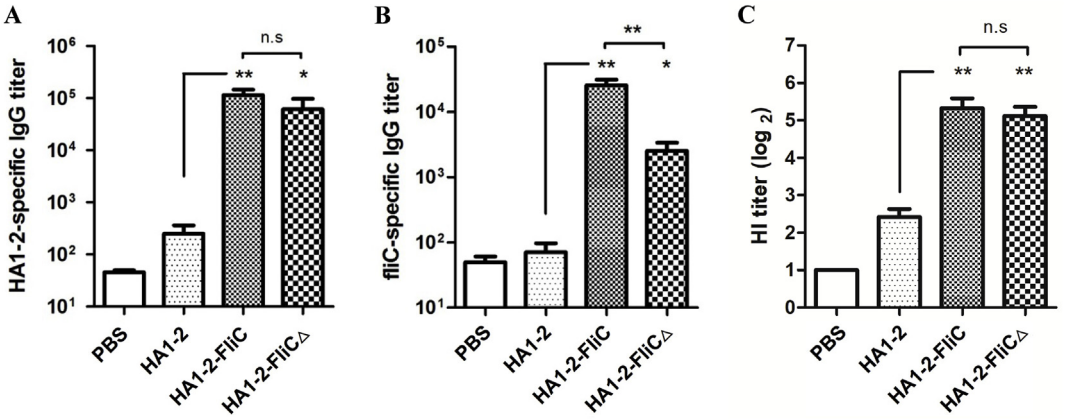

Fig. 6. Antigen-specific antibody immune responses. BALB/c mice ($n = 6$) were i.p.-vaccinated with two doses of HA1-2, HA1-2-FliC, HA1-2-FliCΔD2D3, or PBS on days 0 and 14. All animals were bled 12 days after the second immunization. Serum antibody titers were measured by indirect ELISA and HI assay. (A) HA1-2-specific IgG titers. (B) Flagellin-specific IgG titers. (C) HI titers. All data are presented as mean ± SEM.

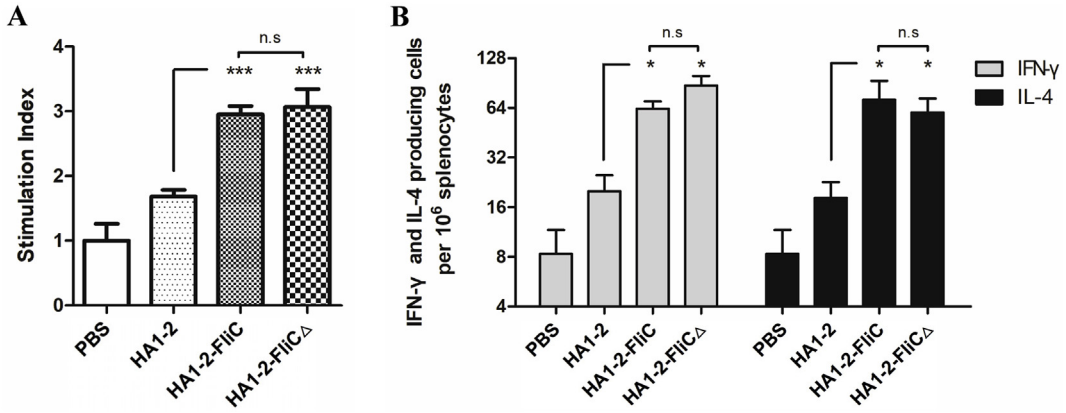

Fig. 7. Cellular immune responses. BALB/c mice ($n = 6$) were i.p.-vaccinated with two doses of HA1-2, HA1-2-FliC, HA1-2-FliCΔD2D3, or PBS on days 0 and 14. Splenocytes were prepared two weeks after the second immunization. All cells were stimulated with HA1-2 (10 μg/ml) *in vitro*. (A) Stimulation index (SI) of splenocytes. The SI of cells was calculated based on cell proliferation, as determined with the BrdU assay, using the following equation: SI = ($OD_{450} - OD_{690}$ of antigen-treated cells)/($OD_{450} - OD_{690}$ of untreated cells). (B) Analysis of IFN-γ and IL-4 production by performing ELISpot assays. The number of IFN-γ- or IL-4-producing cells stimulated with HA1-2 (10 μg/ml) was detected by ELISpot in triplicate wells. All data are presented as mean ± SEM.

average virus loads in throat and cloaca of chickens vaccinated with HA1-2-FliCΔD2D3 were significantly reduced (306, $P < 0.01$, and 684, $P < 0.05$, respectively) compared to chickens vaccinated with HA1-2 (1442 and 1460, respectively). Similarly, the average levels of virus were also significantly decreased in HA1-2-FliC vaccinated chickens (670, $P < 0.05$, and 609, $P < 0.05$, respectively) (Fig. 8B and C).

4. Discussion

Vaccination is the primary measure to control the spread of influenza virus in humans (Liu et al., 2012); therefore, enhancing the safety and reliability of vaccine adjuvants is critical. Previous results showed that approximately 10%, 30%, and 90% of antigenicity was generated by domains D0/D1, D3, and D2/D3, respectively (Yang et al., 2013).

Deletion of the D3 region partially reduced flagellin immunogenicity (Liu et al., 2010). Here, flagellin immunogenicity was evaluated by detecting flagellin-specific antibodies in sera at 12 days after boosting. HA1-2-FliCΔD2D3 showed ~90% less immunogenicity than HA1-2-FliC (Fig. 6B). Previous data suggested that wild-type flagellin, when used as an adjuvant, could induce pre-existing flagellin-specific antibodies and potentially attenuate the booster effect of the adjuvant (Nempont et al., 2008). Therefore, the > 10-fold decrease in flagellin-specific antibody responses induced by HA1-2-FliCΔD2D3 may be beneficial.

When developing a vaccine for human use, flagellin immunogenicity and potential flagellin-induced inflammatory responses should be restricted. Previous studies have shown that flagellin triggers a prototypical systemic inflammatory response (Bargieri et al., 2011; Taylor et al., 2011), which was strongly attenuated with a flagellin mutant lacking the hypervariable region (FliCΔ174-400) compared with wild-type flagellin (Nempont et al., 2008). To gain insight into the systemic inflammatory effect of HA1-2-FliCΔD2D3, cytokine responses were measured in mouse sera. HA1-2-FliCΔD2D3 induced significantly lower production of IL-6, IL-12p40, TNF-α, IL-23p19 compared with those in the HA1-2-FliC group (Fig. 5), in agreement with data showing that KFD-p24 3D (domains D2 and D3 of flagellin KF replaced with HIV p24) induced significantly lower levels of proinflammatory cytokines and chemokines in plasma, compared with KF-p24 (p24 fused to full-length KF) (Yang et al., 2013). The underlying mechanism for this reduction may be related to that the hypervariable domain was proactively involved in the flagellin's stimulation of cytokine production (Liu et al., 2010).

Flagellin adjuvanticity is mainly caused by a conserved TLR5-activating motif (Jiao et al., 2010; Olive, 2012). Therefore, to balance TLR5-associated adjuvanticity and potential inflammatory responses, an ideal vaccine candidate should show moderate TLR5-activating

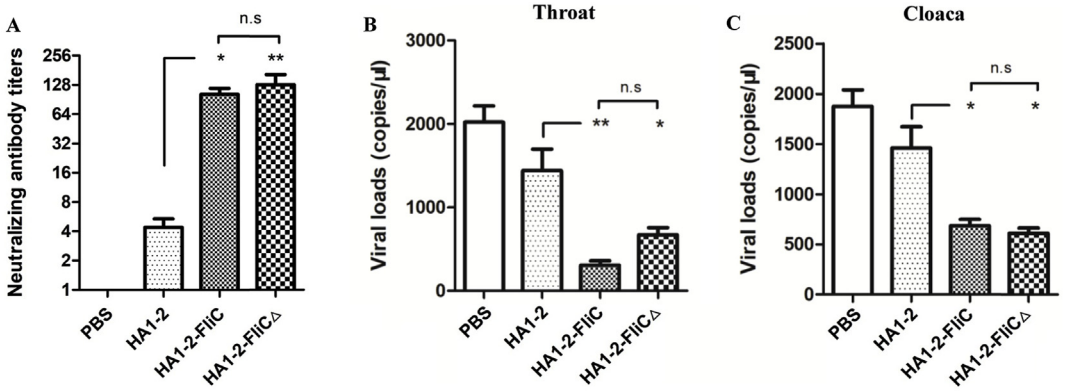

Fig. 8. Neutralizing antibodies in serum and viral loads in throat and cloaca. SPF chickens ($n = 10$) were immunized intramuscularly with three dose of candidate vaccines. At 12 days after the third immunization, blood samples of chickens were collected, and neutralizing antibody titers against H7N9 virus (A) were measured in MDCK cells. At two weeks after the third immunization, all birds were inoculated intranasally with 10^6 EID_{50} of H7N9 influenza virus after the third immunizations. The throat and cloaca swabs were collected from chickens at 9 day post inoculation, and resuspended in 1 ml PBS for viral RNA extraction followed by RT-PCR for quantitative analysis of virus. The viral loads in throat (B) and cloaca (C) were calculated according to the standard curve. All data are presented as mean ± SEM.

efficiency. We evaluated the TLR5-activating activity of HA1-2-FliCΔD2D3 using HEK293-mTLR5 cells. HA1-2-FliCΔD2D3 induced significantly higher levels of IL-8 and NF-κB activation than HA1-2 (Fig. 2), suggesting HA1-2-FliCΔD2D3 retained TLR5 recognition activity. These results were consistent with the known TLR5-stimulatory activity of *Listeria monocytogenes* flagellin, which almost completely lacks a variable region (Hayashi et al., 2001). Importantly, the HA1-2-FliCΔD2D3-dependent reduction of TLR5 activity may curtail inflammatory responses.

Flagellin activates various innate immune cells that secrete certain cytokines and chemokines, which triggers an adaptive immune response (Rolli et al., 2010). We evaluated the priming effects of HA1-2-FliCΔD2D3 on immune responses in PBMCs and splenocytes obtained from mice, and RAW264.7 macrophages by examining cytokine and chemokine gene transcription. IL-6, a marker of synthetic proinflammatory cytokines (Murthy et al., 2004), helps maintain T cell effector functions (Hunter and Jones, 2015). TNF-α is a proinflammatory and immune-regulatory cytokine that enhances leukocyte migration and promotes the transcription of several inflammatory genes (Fausel and Afzali, 2015). The mRNA levels of these cytokines and chemokines were upregulated in PBMCs and splenocytes treated with HA1-2-FliCΔD2D3, indicating that the absence of the hypervariable did not affect innate immune response activation of flagellin.

To evaluate HA1-2-specific acquired immune responses induced by HA1-2-FliCΔD2D3, serum and splenocytes from vaccinated mice were collected after boosting. Significantly higher HA1-2-specific IgG and HI titers were induced by adjuvanted groups than by HA1-2 (Fig. 6A, C), consistent with data obtained after the hypervariable D2 and D3 domains of *E. coli*-derived flagellin (KF) were replaced with HIV p24 (Yang et al., 2013). Flagellin could also promote serum IgG and HI antibody responses when used in combination with the whole inactivated virus or virus-like particle vaccines (Liu et al., 2016; Skountzou et al., 2010). HA1-2-FliCΔD2D3 induced Th1-biased IFN-γ and Th2-biased IL-4 production (Fig. 7B). Similarly, rSF-COE-3D, generated by domain D3 of recombined *Salmonella* flagellin (rSF) was replaced with the porcine epidemic diarrhea virus (PEDV) antigen "collagenase equivalent" (COE), induced mixed Th1/Th2 immune responses (Li et al., 2018). In a chicken model, the results showed that chickens vaccinated with flagellin-based HA1-2 vaccines exhibited significantly reduced viral loads in throat and cloaca compared to that vaccinated with HA1-2 vaccine, indicating that high levels of neutralizing antibody titers induced by flagellin-based HA1-2 vaccines could effectively neutralize the H7N9 viruses, and the presence of full length or truncated flagellin are capable of increasing virus clearance in throat and cloaca of chickens. Similar results were observed in cloaca of chickens challenged by low pathogenic H9N2 influenza virus (Singh et al., 2016). Collectively, these results suggested that deleting the hypervariable region did not affect the immune type induced by flagellin fused with H7N9 influenza antigen HA1-2. Therefore, FliCΔD2D3 might be used in combination with other licenced Th2-biased adjuvants, such as AS04, a mixed adjuvant of aluminium salt and a TLR4 agonist (Frenck et al., 2011).

In conclusion, we developed an improved recombinant subunit vaccine candidate, HA1-2-FliCΔD2D3, that was efficiently expressed in soluble form with retained immunoreactivity and TLR5-agonist efficacy. HA1-2-FliCΔD2D3 promoted innate immune-response activation *in vitro* and *ex vivo*, and induced much lower systemic inflammatory responses in mice compared with HA1-2-FliC. After intraperitoneal immunization, HA1-2-FliCΔD2D3 showed significantly lower flagellin-specific antibody responses, but comparable HA1-2-specific antibody responses. Moreover, HA1-2-FliCΔD2D3 still maintained mixed Th1/Th2 immune responses. In a chicken model, challenge-protection study showed that chickens receiving FliC and FliCΔD2D3 adjuvant vaccines exhibited a significant reduction of viral loads in throat and cloaca, indicating that it can be feasibly used to create successful H7N9 influenza vaccines.

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Authors' contributions

XJ, ZP, and LS designed the research; LS, DX, XK, YJ, KW and YZ performed the experiments; LS, DX and XZ analyzed the data; LS, XJ, ZP, DX and XZ participated in writing the paper. All authors reviewed the manuscript.

Disclosures

The authors declare that there are no conflicts of interest.

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