Original Article

DOI: 10.5582/bst.2013.v7.3.129

# A recombinant protein containing highly conserved hemagglutinin residues 81-122 of influenza H5N1 induces strong humoral and mucosal immune responses

**Ye Li[1,2], Lanying Du[2,*], Hongjie Qiu[3], Guangyu Zhao[3], Lili Wang[2], Yusen Zhou[3], Shibo Jiang[2,4], Jimin Gao[1,*]**

[1] *School of Medical Laboratory Science, Wenzhou Medical College, Wenzhou, Zhejiang, China;*
[2] *Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY, USA;*
[3] *State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China;*
[4] *Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai, China.*

**Summary** **Influenza has long been considered a serious global health threat. The highly pathogenic avian influenza A virus (IAV) H5N1, particularly the currently identified IAV/H7N9 in humans in China, illustrates that influenza is still a significant public health problem. Due to the high mortality of H5N1, development of safe and effective vaccines against divergent strains of H5N1 influenza virus, especially the one capable of inducing both strong systemic and local immune responses in the vaccinated targets, is a challenge of immediate importance. In the present study, we designed two recombinant proteins containing highly conserved hemagglutinin (HA) residues 81-122 of H5N1 fused with Fc of human IgG (HA-81-122-Fc) and/or foldon (Fd) trimeric motif (HA-81-122-Fdc), and identified their immunogenicity in vaccinated mice. We found that HA-81-122-Fc and HA-81-122-Fdc proteins formed high molecular weight dimer and oligomer, respectively, and induced potent IgG antibodies in vaccinated mouse sera and lung wash. Stronger IgG1 (Th2-associated) and IgG2 (Th1-associated) antibody responses could be raised in the sera of mice following last vaccination of HA-81-122-Fdc than those raised by HA-81-122-Fc vaccination. Importantly, HA-81-122-Fdc is able to elicit high titers of IgA antibodies in vaccinated mouse lung wash and sera through the parenteral immunization pathway. Our data demonstrated that the recombinant protein containing highly conserved HA residues 81-122 of H5N1 fused with Fd and Fc could induce strong local mucosal and systemic humoral immune responses in the vaccinated animals, revealing the possibility of developing an effective Fc-mediated mucosal influenza vaccine.**

***Keywords:*** Influenza, H5N1 vaccine, hemagglutinin, conserved sequences, mucosal responses

## 1. Introduction

The highly pathogenic avian influenza A virus (IAV) H5N1, particularly the currently identified IAV/H7N9 in humans in China (*1*), illustrates that influenza is still a significant public health problem. According to the WHO report, H7N9 maintains about 25% of death rate as of May 08, 2013 (*http://www.who.int/csr/don/2013_05_08/en/*), while H5N1 is more pathogenic, with the cumulative fatality rate approaching 60% (*http://www.who.int/influenza/human_animal_interface/EN_ GIP_20130426CumulativeNumberH5N1cases.pdf*). Accordingly, the H5N1 virus remains a greater concern of influenza pandemic, although the possibility of efficient human-to-human transmission of the virus has been rare (*2-4*). Therefore, development of antiviral agents and efficient vaccines against highly pathogenic avian influenza

*Address correspondence to:
Dr. Lanying Du, Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065, USA.
E-mail: Ldu@NYBloodCenter.org

Dr. Jimin Gao, School of Medical Laboratory Science, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China.
E-mail: jimingao64@163.com

(HPAI) H5N1 virus is an urgent task.

Recombinant subunit vaccines incorporating antigenic viral membrane glycoproteins, especially hemagglutinin (HA), are attractive vaccine candidates since they are able to induce virus-neutralizing antibodies (*5-7*). The HA subunit 1 (HA1) has been previously shown as an important antigen to induce neutralizing antibodies and protect against IAV challenge (*5,8*). The amino acids of HA1 tend to develop continuous mutation, but some conserved regions with limited changes are still found throughout the passage. Thus, development of novel vaccines based on these conserved sequences would be practicable against divergent virus strains. Using a broadly neutralizing antibody, we have identified a novel and highly conserved conformational epitope centered on residues 81-122 of HA1 of H5N1 virus (*9*), further providing important information to develop a universal vaccine based on the identified sequence. In this study, we fused the conserved HA sequences covering residues 81-122 of influenza H5N1 with Fc of human IgG and/or foldon (Fd) to express recombinant proteins, and detected their ability to induce systemic and local immune responses in vaccinated mice in order to develop an Fc-mediated, HA1 conserved sequence-based vaccine to prevent H5N1 influenza virus infection.

## 2. Materials and Methods

### 2.1. *Ethics statement*

The study of animals was approved by the Institutional Animal Care and Use Committee at the New York Blood Center (Approval #322.02). All animal studies were carried out in strict accordance with the recommendations of the American Veterinary Medical Association (AVMA) Guidelines and the approved protocols.

### 2.2. *Construction, expression and purification of recombinant proteins*

The construction, expression and purification of recombinant HA-81-122 proteins fused with Fc plus Fd (HA-81-122-Fdc) or without Fd (HA-81-122-Fc), were performed following our previously described protocols with some modifications (*5,10*). Briefly, the genes encoding HA1 residues 81-122 of A/Anhui/1/2005(H5N1) (AH/1) (GenBank:ABD28180) fused with Fd were amplified by polymerase chain reaction (PCR) using our previously constructed HA1-Fdc plasmid (*5*), and overlapping primers covering Fd as the template and inserted into Pfuse-hIgG1-Fc2 expression vector (hereinafter named Fc, InvivoGen, San Diego, CA, USA) to construct HA-81-122-Fdc recombinant. HA-81-122-Fc was constructed by directly digesting HA-81-122 PCR product and inserting into Fc vector. The sequence-confirmed recombinant plasmids were transfected into 293T cells (ATCC, Manassas, VA, USA) seeded 24 h prior to transfection, using the calcium phosphate method. Culture medium was replaced by fresh Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA) 10 h later, and supernatant was collected 72 h post-transfection. The recombinant HA-81-122-Fdc and HA-81-122-Fc proteins in the supernatant were purified by Protein A affinity chromatography (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. Constructed recombinants were shown in Figure 1A.

### 2.3. *SDS-PAGE, N-PAGE, Cross-linker and Western blot*

Purified HA-81-122 proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as previously described (*5*) using our developed anti-HA HA-7 monoclonal antibody (mAb). Briefly, purified proteins were either non-boiled or boiled at 95ºC for 5 min and separated by 10% Tris-Glycine SDS-PAGE gels, which were then stained with Coomassie Blue or transferred to nitrocellulose membranes. After blocking overnight at 4ºC, the blots were incubated with HA-7 mAb (1:3,000) for 1 h at room temperature. After three washes, the blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000, Invitrogen) for 1 h at room temperature. Signals were visualized with ECL Western blot substrate reagents and Amersham Hyperfilm (GE Healthcare).

Native PAGE (N-PAGE) and cross-linker analyses were done as before (*10*). For N-PAGE, the proteins were first separated by 6% N-PAGE gels using N-PAGE sample buffer and running buffer (Invitrogen), followed by the same protocols as above. For protein cross-linker detection, 4.5 μg of purified proteins were respectively mixed with 20 μl of 0.1% glutaraldehyde (final concentration 4 mM) and left at room temperature in the dark for 2 h before SDS-PAGE and Coomassie Blue staining as described above.

### 2.4. *Vaccination protocol*

Groups of five female BALB/c mice at 6-8 weeks were respectively subcutaneously (*s.c.*) primed-vaccinated with 20 μg/mouse of HA-81-122-Fdc and HA-81-122-Fc proteins resuspended in phosphate buffered saline (PBS) in the presence of Montanide ISA 51 adjuvant (SEPPIC, Fairfield, NJ, USA) and boosted twice with 10 μg/mouse of immunogen containing adjuvant ISA 51 at 3-week intervals. Control mice were *s.c.* injected with the same volume of PBS/Montanide ISA 51.

### 2.5. *Sample collection*

Sera were collected before immunization and 10 days

post-each vaccination to detect HA-specific antibodies by a rapid, simple, and humane submandibular bleeding method (*11*) with some modifications. Briefly, mice were anesthetized and held from the scruff of the neck in the air to establish the most relaxed situation. A sterile 18G1 needle was then poked to the cheek of mice with enough force to create a small stick hole so that drops of blood would exude from the point of penetration. This bleeding method may consistently yield a reasonable blood volume, and it is much more humane. Collected sera were retained for enzyme-linked immunosorbent assay (ELISA) analysis. For the collection of lung wash, after sacrifice of mice, a midline incision was made over the neck aspect of the ventral. A small hole was cut in the trachea with small surgical scissors. Around 800 μL/mice of sterile PBS were slowly injected into the alveoli through tracheal lumen of mice and then drained from the alveoli by using a 1 mL syringe. This procedure was repeated 3 times.

### 2.6. *ELISA*

The IgG antibody responses and/or subtypes were evaluated by ELISA in the collected mouse sera and lung wash, as previously described (*5*) with some modifications. Briefly, 96-well ELISA plates were precoated, respectively, with recombinant HA-81-122-Fdc and HA-81-122-Fc fusion proteins at 4ºC overnight and blocked with 2% non-fat milk at 37ºC for 2 h. Serially diluted mouse sera and lung wash were added to the plates and incubated at 37ºC for 1 h, followed by four washes. Bound antibodies were incubated with HRP-conjugated goat anti-mouse IgG (1:2,000, Invitrogen), anti-mouse IgG1 (1:2,000, Bethyl Laboratories, Montgomery, TX, USA) or anti-mouse IgG2a (1:2,000, Invitrogen), respectively, for 1 h at 37ºC. The reaction was visualized by substrate 3,3',5,5'-Tetramethylbenzidine (TMB) (Invitrogen) and stopped by 1 N $H_2SO_4$. The absorbance at 450 nm (A450) was measured by ELISA plate reader (Tecan, San Jose, CA, USA).

Secretory anti-HA IgA antibody responses in lung wash were measured by ELISA using protocols similar to those described above, except for the addition of lung lavage fluid at 50 μl/well or sera (1:50, 50 μL/well) in duplicate wells of the plates. The HRP-conjugated goat anti-mouse IgA (Invitrogen) was added at a dilution of 1:2,000, followed by measuring the absorbance at A450.

### 2.7. *Statistical analysis*

Values were presented as mean with standard deviation (SD). Statistical significance among different groups was calculated by Student's *t*-test using *Stata* statistical software. *P* values less than 0.05 were considered significant.

## 3. Results

### 3.1. *Recombinant HA-81-122-Fc and HA-81-122-Fdc proteins formed high molecular weight dimeric and oligomeric structures*

The highly conserved sequences of HA-81-122 of A/Anhui/1/2005 (H5N1-HA) containing 44 amino acid residues were fused with or without Fd, followed by insertion in-frame to the Fc vector, generating recombinant HA-81-122-Fdc and HA-81-122-Fc, respectively, with IL2ss signal sequence of the Fc vector as the signal sequence (Figure 1B). The recombinant proteins were expressed in the culture supernatant of transfected 293T cells, followed by purification of the protein using protein A columns. The purified proteins were analyzed by SDS-PAGE, N-PAGE and Cross-linker, followed by Coomassie Blue staining, and the reactivity was determined by using an HA1-specific monoclonal antibody (HA-7 mAb) developed in our laboratory (*9*). As shown in Figure 1C, one clear band was observed in the corresponding samples of both non-boiled and boiled HA-81-122-Fc and HA-81-122-Fdc proteins analyzed by reducing SDS-PAGE, with the molecular weight of the non-boiled proteins (dimers) equaling almost 2-fold that of the boiled proteins (monomers), which contain influenza HA-81-122 with the Fc region (CH2 and CH3 domains) of the human IgG1 heavy chain and the hinge region and/or Fd. The above results demonstrated that highly purified proteins could be obtained from the transfected culture supernatant and that the expressed proteins fused with Fc and/or Fd formed conformational structures. These purified HA-81-122 proteins could be further recognized by the HA1-specific HA-7 mAb, as indicated by Western blot (Figure 1D), revealing their high specificity to the HA1 of H5N1.

Since the reducing SDS-PAGE could not reflect the actual size of the expressed protein, we used a crosslinking assay and N-PAGE for further detection of the molecular weight of these proteins. Results from the crosslinking analysis showed that the crosslinked HA-81-122-Fc and HA-81-122-Fdc formed high molecular weight dimer or oligomer, respectively, while non-crosslinked proteins retained their monomeric status (Figure 1E). N-PAGE analysis identified oligomeric structures of these proteins, with HA-81-122-Fdc showing the highest molecular weight (Figure 1F). The above results indicate that the expressed HA1 fused with Fc and/or Fd is able to polymerize into high molecular weight dimer or oligomer, maintaining conformational structures.

### 3.2. *HA-81-122 protein fused with Fd and Fc induced strong humoral immune responses in both vaccinated mouse sera and lung wash*

In order to evaluate the ability of HA-81-122-Fdc and

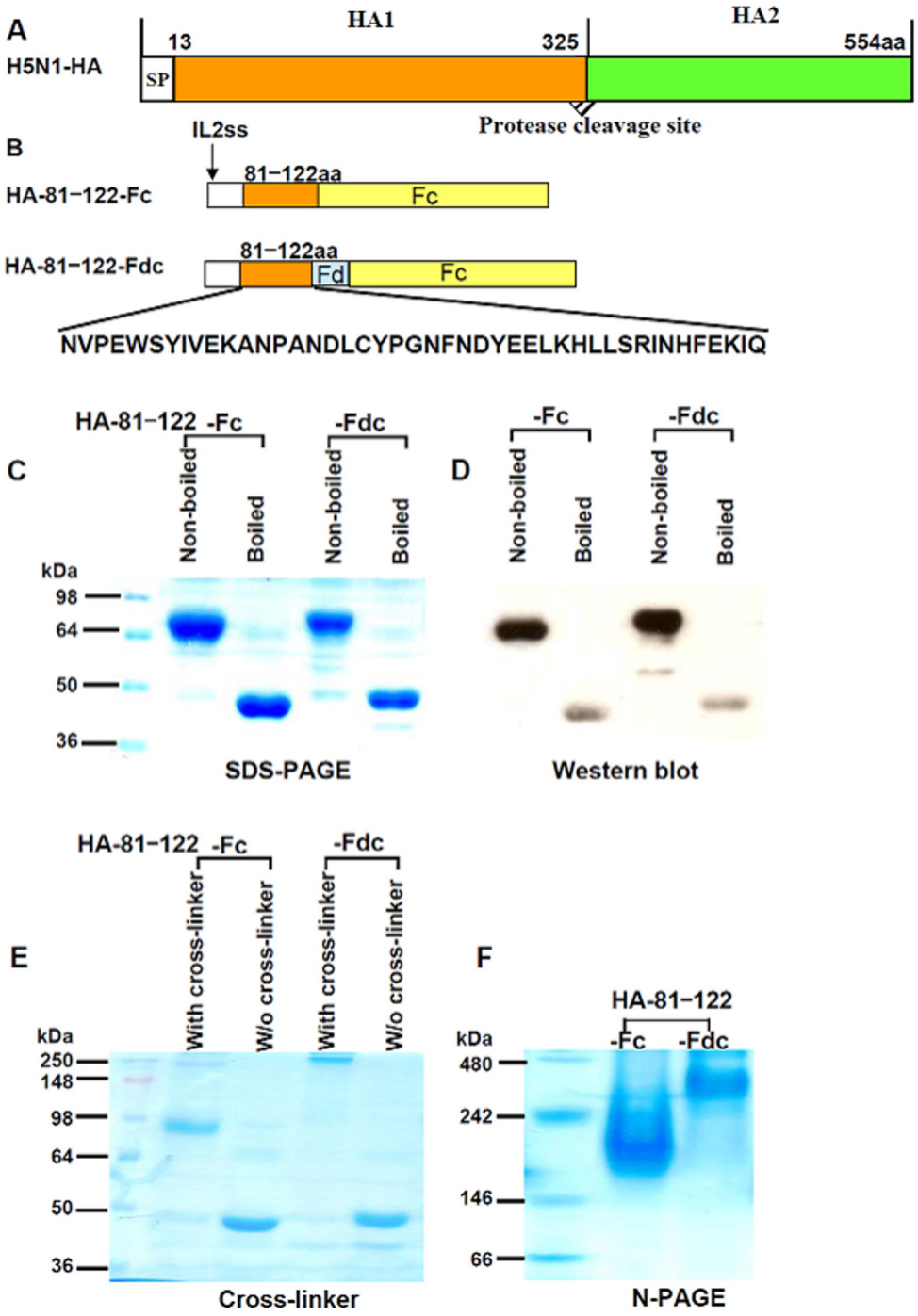


**Figure 1. Construction of recombinant protein fragments and analysis of the expression of HA-81-122-Fc and HA-81-122-Fdc proteins. (A)** Schematic outline of A/Anhui/1/2005(H5N1) HA protein (H5N1-HA). H5N1-HA is composed of signal peptide (SP) at the 5' terminus, HA1 subunit with protease cleavage site at its 3' terminus, and HA2 subunit with fusion peptide (FP) at its 5' terminus. **(B)** Construction of HA-81-122 recombinant fragments, respectively fused with Fc (HA-81-122-Fc) and Fd plus Fc (HA-81-122-Fdc). Signal peptide IL2ss was constructed at the 5' terminus of the recombinants to lead expressed proteins to the culture supernatant. Lower panel shows the amino acid residues of HA-81-122. The expression of the HA-81-122 proteins fused with Fc and/or Fd was detected by SDS-PAGE followed by Coomassie Blue staining **(C)**, Western blot using an HA-specific HA-7 mAb **(D)**, Cross-linker **(E)** and N-PAGE **(F)**. The proteins without cross-linker (w/o cross-linker) were used as the controls. The protein molecular weight marker (kDa) is indicated on the left.

HA-81-122-Fc proteins with conformational structures in inducing specific immune responses, we vaccinated mice using these proteins and detected IgG antibody responses by ELISA in the collected mouse sera and lung wash. As shown in Figure 2A, recombinant HA proteins, particularly HA-81-122-Fdc, induced a high level of serum IgG antibody response specific to HA-81-122 fusion proteins, with the antibody titer quickly reaching a high level after the first vaccination. The increase of the boost vaccination did not significantly improve the antibody titer, suggesting that one immunization dose could elicit sufficient antibody

response. The IgG antibody response raised by HA-81-122-Fdc was relatively higher than that raised by HA-81-122-Fc. An average end-point antibody titer of 1:5.2 × $10^7$ was detected in the mouse sera collected at 10 days post-last boost (Figure 2B). In addition, HA-81-122-Fdc elicited a significantly higher level of HA-specific IgG than HA-81-122-Fc in the vaccinated mouse lungs (Figure 2C). In contrast, no IgG antibody response was detectable in the sera and lungs of control mice injected with PBS plus adjuvant (Figure 2).

To further evaluate whether immunization with HA-81-122-Fdc and HA-81-122-Fc fusion proteins could activate and differentiate native T lymphocytes into either CD4+ T helper 1 (Th1) or T helper 2 (Th2) cells and identify which T helper cell subsets were more functional, IgG1 (Th2) and IgG2a (Th1) subtypes induced by HA-81-122-Fdc and HA-81-122-Fc proteins were detected in the mouse sera collected at 10 days post-last vaccination. The results showed that the expressed HA proteins could elicit HA-specific antibodies in the vaccinated mouse sera, respectively belonging to the IgG1 (Th2-associated, Figure 3A) and IgG2a (Th1-associated, Figure 3B) subclasses, reaching an end-point antibody titer of 1:5.2 × $10^7$ and 2.1 × $10^8$, respectively. In particular, higher molecular weight HA-81-122 protein fused with Fd plus Fc (HA-81-122-Fdc) induced a higher level of IgG1 antibodies than lower molecular weight HA-81-122-Fc protein without Fd fusion. However, no HA-specific IgG1 or IgG2a antibody response was detected in the sera of PBS control mice (Figure 3).

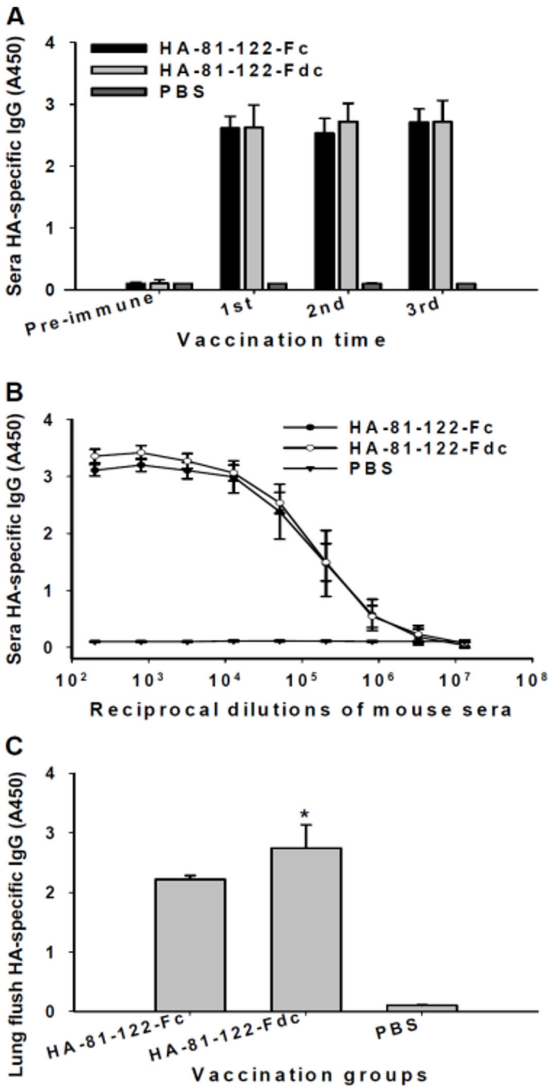


**Figure 2. Detection of antigen-specific IgG antibody responses by ELISA in HA-81-122 fusion protein-vaccinated mouse sera and lung wash.** PBS was used as the negative control. **(A)** Reactivity of antigen-specific IgG antibody response with HA-81-122-Fc or HA-81-122-Fdc protein in the vaccinated mouse sera. The ELISA plates were respectively coated with HA-81-122-Fc or HA-81-122-Fdc, and IgG was detected using sera (1:3,200) from mice before immunization (pre-immune) and 10 days after each boost. The data are presented as mean A450 ± S.D. of five mice per group. **(B)** Ability of IgG to bind the recombinant proteins was detected using mouse sera from 10 days post-last vaccination. The data are presented as mean A450 ± S.D. of five mice per group at various dilution points. **(C)** Reactivity of IgG with HA-81-122-Fc or HA-81-122-Fdc protein in the vaccinated mouse lung wash from 10 days post-last vaccination. The data are presented as mean A450 ± S.D. of five mice per group. * indicates significant difference ($p < 0.05$) between HA-81-122-Fdc and other groups.

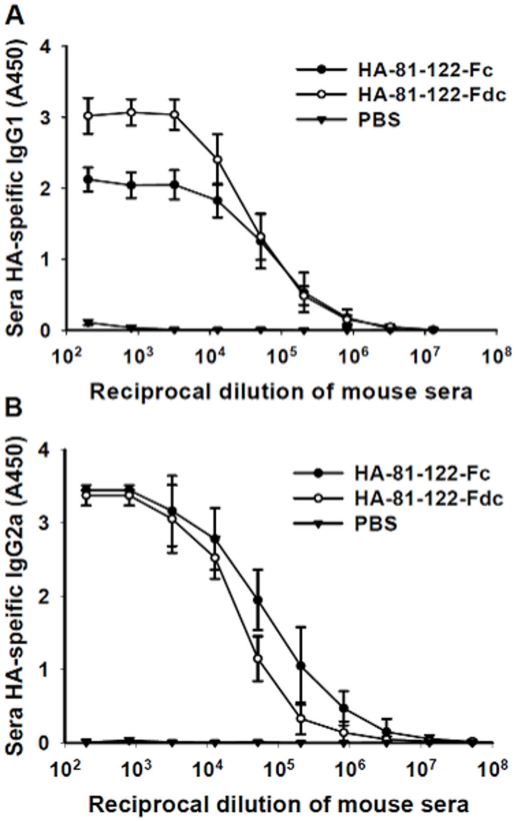


**Figure 3. Measurement of IgG1 and IgG2a antibody titers by ELISA in HA-81-122 fusion protein-vaccinated mouse sera. PBS was used as the negative control.** Ability of IgG1 **(A)** and IgG2a **(B)** antibodies to bind HA-81-122 proteins was detected using sera from 10 days post-last vaccination. The data are presented as mean A450 ± S.D. of five mice per group at various dilution points.

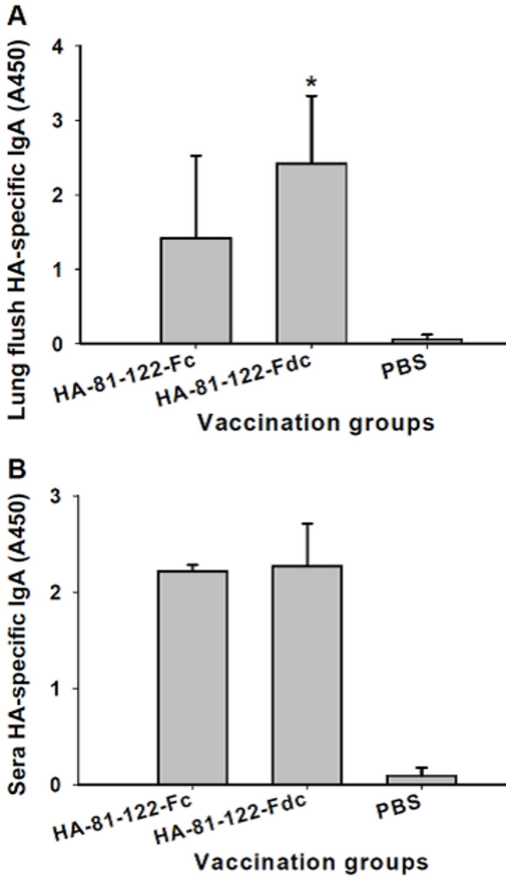


**Figure 4. Detection of IgA antibody response by ELISA in HA-81-122 fusion protein-vaccinated mouse sera and lung wash.** PBS was used as the negative control. Ability of IgA to bind HA-81-122 proteins was detected using lung wash **(A)** and sera **(B)** from 10 days post-last vaccination. The data are presented as mean A450 ± S.D. of five mice per group. * indicates significant difference ($p < 0.05$) between HA-81–122-Fdc and other groups.

The above data demonstrate that expressed HA-81-122 proteins, particularly HA-81-122-Fdc, can elicit strong humoral antibody responses specific to the HA protein of H5N1 virus, implying the high immunogenicity of HA-81-122-Fdc containing oligomeric structure in stimulating elevated humoral systemic immune responses in the vaccinated mice.

### 3.3. *HA-81-122 protein fused with Fd and Fc induced strong mucosal immune responses in both vaccinated mouse lung wash and sera*

To evaluate the regional mucosal immunity potentially induced by HA-81-122 proteins, vaccinated mice were collected for lung wash 10 days post-last vaccine for the detection of mucosal IgA by ELISA. As a comparison, IgA was also evaluated in the vaccinated mouse sera. As shown in Figure 4A, the recombinant HA-81-122-Fdc protein induced a significantly higher titer of mucosal IgA responses specific to the HA protein of H5N1 in the lung wash of the vaccinated mice than HA-81-122-Fc. In addition, a higher level of IgA antibody response could also be detected in the sera of mice vaccinated with HA-81-122-Fdc than with HA-81-122-Fc (Figure 4B). By comparison, no specific IgA was found in the lung wash or sera of the PBS control mice (Figure 4). The above data suggested that strong local immune responses could be specifically induced by HA-81-122-Fdc protein through the parenteral *s.c.* vaccination pathway.

## 4. Discussion

The HA of H5N1, the main surface protein of the virus, serves as an important target for inducing neutralization antibodies and/or protective immunity against HAPI H5N1 virus (*12,13*), and it makes a greater contribution toward the induction of neutralizing antibodies than other viral proteins, such as neuraminidase (NA), nucleoprotein (NP) and membrane protein (M2) (*12,14*). HA-based vaccines have been shown to elicit higher titers of neutralizing antibodies to prevent influenza virus infection in tested animals (*15-18*), as well as human clinical trials (*19-22*). The HA1 antigenic domain of HA has been demonstrated to induce an immune response equal to that of the full-size protein (*23*). Our previous studies have also indicated that recombinant vaccines containing the full-length HA1 fragment of H5N1 are able to induce strong immune responses in vaccinated mice, protecting against tested strains of H5N1 virus challenge (*5*), suggesting that the HA1 subunit of H5N1 virus is a major target for inducing protective immunity. Nevertheless, the frequent mutation of the HA1 protein makes it especially important to develop universal influenza vaccines based on the highly conserved epitopes of HA1 that potentially induce broad-spectrum protection against divergent strains of virus infections (*24*). It has been reported that the immunogenicity caused by direct expression of the major vaccine antigen HA protein is often low, requiring large doses of vaccines to generate a level of seroconversion consistent with protection (*25,26*). Because of the importance of structure-based antigen, oligomeric HA is more efficient than monomeric HA (*27*). However, oligomerization is usually ensured through the addition of an extraneous sequence of unknown risk for human immunization. Important to the present study, Fc fragment of human IgG is considered an important fusion tag for coexpression with several viral proteins in order to facilitate their purification and subsequent immunogenicity (*25,28*). Fc not only promotes correct folding of the fusion proteins following expression, but may also help to enhance binding of the antigen to antigen-presenting cells (APCs) and cell lines expressing Fc receptors (FcR) (*29,30*). As another antigen modification motif, the 27 amino acid-containing Fd derived from native T4 phage fibritin has been typically incorporated at the C-terminus of

collagen-like protein molecules to facilitate stabilization of protein trimers or oligomers (*31-34*), indicating that C-terminal Fd is essential for correct trimerization and folding of the protein.

Using a broadly neutralizing antibody, HA-7, we have previously identified a neutralizing epitope containing highly conserved sequences of residues 81-122 of H5N1 HA1, with residues $81^{N}$, $82^{V}$, $82A^{P}$, $83^{E}$, $117^{H}$, $118^{F}$, $119^{E}$, $120^{K}$, $121^{I}$, and $122^{Q}$ as the core epitope. We showed that residues 81 to 83 exist as a loop, while residues 117 to 122 adopt a β-strand conformation, manifesting the conformational nature of the epitope (*9*). In the present study, we aimed to develop a novel H5N1 vaccine by fusing these conserved sequences of HA1 with Fc immunoenhancer and/or Fd trimeric motif to form native conformational structures and then detect the ability of this vaccine to induce humoral and mucosal immunity in vaccinated mice. As expected, the expressed HA-81-122-Fdc and HA-81-122-Fc proteins formed high molecular weight dimer or oligomer, respectively, being able to maintain conformational structures, and indeed induced strong H5N1 HA-specific IgG antibody responses in the vaccinated mouse sera following one dose of immunization. HA-specific IgG, IgG1 (Th2) or IgG2a (Th1) antibody responses were detected in the vaccinated mouse lung wash and sera collected at 10 days post-last immunization. These results suggest that conformational HA-81-122 proteins, particularly oligomeric HA-81-122-Fdc, could induce strong humoral immune responses against virus infection.

In addition to humoral immune responses, mucosal immunity characterized by secretory IgA antibody could also play an essential role in the protection against virus infection, especially those caused by influenza A virus, a mucosal pathogen infecting humans through the respiratory tract (*35*). Thus, an influenza vaccine potentially inducing ample mucosal immunity in the vaccinated hosts would be considered an important factor in developing effective influenza vaccines. It is reported that the induction of IgA can broaden the vaccine-induced immune response and introduce local cross-protective immune responses to reduce viral load, ensuring the best possible preparedness for the next influenza pandemic (*36*). IgA has also been shown to be very effective in inhibiting initial pathogen colonization without causing tissue damage (*37,38*). However, it is generally believed that local protective antibody responses at the respiratory tract are not easily induced by parenteral vaccine pathways (*39*), such as *s.c.*, intramuscular (*i.m.*) or intradermal (*i.d.*) administration.

In this study, we found that parenteral *s.c.* immunization of mice with HA-81-122-Fdc and HA-81-122-Fc proteins could, indeed, induce high level of IgA antibodies in both vaccinated mouse lung wash and sera, suggesting that HA-81-122 recombinant proteins fused with Fc and/or Fd could elicit sufficient mucosal immunity, even when introduced parenterally, unlike some conventional inactivated vaccines which usually induce only limited local immunity *via* parenteral administration (*40,41*). This may partially result from the ability of FcR to transport FcR-targeted HA antigen to FcR-bearing APC cells within mucosal surfaces, enabling FcR-mediated transport of antigen across the mucosal barrier (*42*). The function of Fd in promoting the formation of oligomeric HA-81-122-Fdc protein could also play a role in increasing immunogenicity (*10*).

Antibody responses to virus HA provide essential immunity against IAV infection (*26*); thus, the antiviral effect of HA-81-122-based vaccines could be mediated by systemic and local antibodies to the HA antigen. Therefore, the induction of HA-81-122-specific antibody responses was necessary for HA-81-122-based vaccines in the prevention of H5N1 virus infection. Compared with the full-length HA1 protein, HA1 proteins containing residues 81-122 induced relatively lower level of HA-specific IgG antibodies in the vaccinated mouse sera (*5*). Although no data are currently available to compare the mucosal immunity induced by the full-length HA1 and the HA-81-122 protein, results from the present study indicated that HA-81-122, particularly HA-81-122-Fdc fused with Fd and Fc, was able to elicit high mucosal IgA antibody response which was detected in the vaccinated mouse lungs and sera (Figure 4). No side-effects were observed in the mice vaccinated with HA-81-122 proteins fused with Fc and/or Fd. Our study further confirms the ability of Fd trimeric motif and Fc immunoenhancer in the promotion of recombinant proteins forming correct conformational structures, thus enhancing immunogenicity (*10*). Further studies would be needed to test the efficacy of this vaccine in other vaccination pathways, as well as its cross-protective immunity against multiple strains of influenza virus infection.

To summarize, our data showed that highly conserved HA residues 81-122 of influenza H5N1 fused with Fd and Fc of human IgG induced strong local mucosal and humoral systemic immune responses in the vaccinated animals. This study provides a sound scientific platform for the development of an effective and safe mucosal H5N1 vaccine based on the highly conserved HA residues 81-122 of influenza H5N1 to prevent future influenza outbreaks caused by avian influenza virus.

**Acknowledgements**

This work was supported by the National Institutes of Health (R03AI088449 to LD), the National 973 Basic Research Program of China (2011CB504706 to YZ), and Chinese Ministry of Science & Technology, Hong Kong, Macau, Taiwan Collaborative Program (201200007673 to SJ).

## References


1. Gao R, Cao B, Hu Y, *et al*. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013; 68:1888-1897.
2. Pappaioanou M. Highly pathogenic H5N1 avian influenza virus: Cause of the next pandemic? Comp Immunol Microbiol Infect Dis. 2009; 32:287-300.
3. Hunter P. H5N1 infects the biosecurity debate. Governments and life scientists are waking up to the problem of dual-use research. EMBO Rep. 2012; 13:604-607.
4. Peiris JS, de Jong MD, Guan Y. Avian influenza virus (H5N1): A threat to human health. Clin Microbiol Rev. 2007; 20:243-267.
5. Du L, Leung VH, Zhang X, *et al*. A recombinant vaccine of H5N1 HA1 fused with foldon and human IgG Fc induced complete cross-clade protection against divergent H5N1 viruses. PLoS One. 2011; 6:e16555.
6. Moon HJ, Lee JS, Talactac MR, Chowdhury MY, Kim JH, Park ME, Choi YK, Sung MH, Kim CJ. Mucosal immunization with recombinant influenza hemagglutinin protein and poly gamma-glutamate/chitosan nanoparticles induces protection against highly pathogenic influenza A virus. Vet Microbiol. 2012; 160:277-289.
7. Wang TT, Tan GS, Hai R, Pica N, Ngai L, Ekiert DC, Wilson IA, Garcia-Sastre A, Moran TM, Palese P. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc Natl Acad Sci U S A. 2010; 107:18979-18984.
8. Verma S, Dimitrova M, Munjal A, Fontana J, Crevar CJ, Carter DM, Ross TM, Khurana S, Golding H. Oligomeric recombinant H5 HA1 vaccine produced in bacteria protects ferrets from homologous and heterologous wild-type H5N1 influenza challenge and controls viral loads better than subunit H5N1 vaccine by eliciting high-affinity antibodies. J Virol. 2012; 86:12283-12293.
9. Du L, Jin L, Zhao G, Sun S, Li J, Yu H, Li Y, Zheng BJ, Liddington RC, Zhou Y, Jiang S. Identification and structural characterization of a broadly neutralizing antibody targeting a novel conserved epitope on the influenza virus H5N1 hemagglutinin. J Virol. 2013; 87:2215-2225.
10. Du L, Zhao G, Sun S, Zhang X, Zhou X, Guo Y, Li Y, Zhou Y, Jiang S. A critical HA1 neutralizing domain of H5N1 influenza in an optimal conformation induces strong cross-protection. PLoS One. 2013; 8:e53568.
11. Golde WT, Gollobin P, Rodriguez LL. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. Lab Anim (NY). 2005; 34:39-43.
12. Nayak B, Kumar S, DiNapoli JM, Paldurai A, Perez DR, Collins PL, Samal SK. Contributions of the avian influenza virus HA, NA, and M2 surface proteins to the induction of neutralizing antibodies and protective immunity. J Virol. 2010; 84:2408-2420.
13. Wei CJ, Xu L, Kong WP, Shi W, Canis K, Stevens J, Yang ZY, Dell A, Haslam SM, Wilson IA, Nabel GJ. Comparative efficacy of neutralizing antibodies elicited by recombinant hemagglutinin proteins from avian H5N1 influenza virus. J Virol. 2008; 82:6200-6208.
14. Patel A, Tran K, Gray M, Li Y, Ao Z, Yao X, Kobasa D, Kobinger GP. Evaluation of conserved and variable influenza antigens for immunization against different isolates of H5N1 viruses. Vaccine. 2009; 27:3083-3089.
15. Kreijtz JH, Suezer Y, de MG, van den Brand JM, van AG, Schnierle BS, Kuiken T, Fouchier RA, Lower J, Osterhaus AD, Sutter G, Rimmelzwaan GF. Preclinical evaluation of a modified vaccinia virus Ankara (MVA)-based vaccine against influenza A/H5N1 viruses. Vaccine. 2009; 27:6296-6299.
16. Prabakaran M, Madhan S, Prabhu N, Qiang J, Kwang J. Gastrointestinal delivery of baculovirus displaying influenza virus hemagglutinin protects mice against heterologous H5N1 infection. J Virol. 2010; 84:3201-3209.
17. Shoji Y, Bi H, Musiychuk K, *et al*. Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. Vaccine. 2009; 27:1087-1092.
18. Schwartz JA, Buonocore L, Suguitan A, Jr., Hunter M, Marx PA, Subbarao K, Rose JK. Vesicular stomatitis virus-based H5N1 avian influenza vaccines induce potent cross-clade neutralizing antibodies in rhesus macaques. J Virol. 2011; 85:4602-4605.
19. Schwarz TF, Horacek T, Knuf M, Damman HG, Roman F, Drame M, Gillard P, Jilg W. Single dose vaccination with AS03-adjuvanted H5N1 vaccines in a randomized trial induces strong and broad immune responsiveness to booster vaccination in adults. Vaccine. 2009; 27:6284-6290.
20. Leroux-Roels I, Roman F, Forgus S, Maes C, De BF, Drame M, Gillard P, van der Most R, Van MM, Hanon E, Leroux-Roels G. Priming with AS03 A-adjuvanted H5N1 influenza vaccine improves the kinetics, magnitude and durability of the immune response after a heterologous booster vaccination: An open non-randomised extension of a double-blind randomised primary study. Vaccine. 2010; 28:849-857.
21. Lakey DL, Treanor JJ, Betts RF, Smith GE, Thompson J, Sannella E, Reed G, Wilkinson BE, Wright PF. Recombinant baculovirus influenza A hemagglutinin vaccines are well tolerated and immunogenic in healthy adults. J Infect Dis. 1996; 174:838-841.
22. Treanor JJ, Schiff GM, Couch RB, Cate TR, Brady RC, Hay CM, Wolff M, She D, Cox MM. Dose-related safety and immunogenicity of a trivalent baculovirus-expressed influenza-virus hemagglutinin vaccine in elderly adults. J Infect Dis. 2006; 193:1223-1228.
23. Tonegawa K, Nobusawa E, Nakajima K, Kato T, Kutsuna T, Kuroda K, Shibata T, Harada Y, Nakamura A, Itoh M. Analysis of epitope recognition of antibodies induced by DNA immunization against hemagglutinin protein of influenza A virus. Vaccine. 2003; 21:3118-3125.
24. Du L, Zhou Y, Jiang S. Research and development of universal influenza vaccines. Microbes Infect. 2010; 12:280-286.
25. Loureiro S, Ren J, Phapugrangkul P, Colaco CA, Bailey CR, Shelton H, Molesti E, Temperton NJ, Barclay WS, Jones IM. Adjuvant-free immunization with hemagglutinin-Fc fusion proteins as an approach to influenza vaccines. J Virol. 2011; 85:3010-3014.
26. Garcia JM, Pepin S, Lagarde N, Ma ES, Vogel FR, Chan KH, Chiu SS, Peiris JS. Heterosubtype neutralizing responses to influenza A (H5N1) viruses are mediated by antibodies to virus haemagglutinin. PLoS One. 2009; 4:e7918.
27. Weldon WC, Wang BZ, Martin MP, Koutsonanos DG, Skountzou I, Compans RW. Enhanced immunogenicity of stabilized trimeric soluble influenza hemagglutinin.

PLoS One. 2010; 5:e12466.
28. He Y, Lu H, Siddiqui P, Zhou Y, Jiang S. Receptor-binding domain of severe acute respiratory syndrome coronavirus spike protein contains multiple conformation-dependent epitopes that induce highly potent neutralizing antibodies. J Immunol. 2005; 174:4908-4915.
29. Chen H, Xu X, Jones IM. Immunogenicity of the outer domain of a HIV-1 clade C gp120. Retrovirology. 2007; 4:33.
30. Martyn JC, Cardin AJ, Wines BD, Cendron A, Li S, Mackenzie J, Powell M, Gowans EJ. Surface display of IgG Fc on baculovirus vectors enhances binding to antigen-presenting cells and cell lines expressing Fc receptors. Arch Virol. 2009; 154:1129-1138.
31. Du C, Wang M, Liu J, Pan M, Cai Y, Yao J. Improvement of thermostability of recombinant collagen-like protein by incorporating a foldon sequence. Appl Microbiol Biotechnol. 2008; 79:195-202.
32. Pakkanen O, Hamalainen ER, Kivirikko KI, Myllyharju J. Assembly of stable human type I and III collagen molecules from hydroxylated recombinant chains in the yeast Pichia pastoris. Effect of an engineered C-terminal oligomerization domain foldon. J Biol Chem. 2003; 278:32478-32483.
33. Letarov AV, Londer YY, Boudko SP, Mesyanzhinov VV. The carboxy-terminal domain initiates trimerization of bacteriophage T4 fibritin. Biochemistry (Mosc). 1999; 64:817-823.
34. Boudko SP, Londer YY, Letarov AV, Sernova NV, Engel J, Mesyanzhinov VV. Domain organization, folding and stability of bacteriophage T4 fibritin, a segmented coiled-coil protein. Eur J Biochem. 2002; 269:833-841.
35. Price GE, Soboleski MR, Lo CY, Misplon JA, Quirion MR, Houser KV, Pearce MB, Pappas C, Tumpey TM, Epstein SL. Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses. PLoS One. 2010; 5:e13162.
36. Cerutti A, Cols M, Gentile M, Cassis L, Barra CM, He B, Puga I, Chen K. Regulation of mucosal IgA responses: Lessons from primary immunodeficiencies. Ann N Y Acad Sci. 2011; 1238:132-144.
37. Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. Vaccine. 2007; 25:5467-5484.
38. Brandtzaeg P. Role of secretory antibodies in the defence against infections. Int J Med Microbiol. 2003; 293:3-15.
39. de Haan A, Haijema BJ, Voorn P, Meijerhof T, van Roosmalen ML, Leenhouts K. Bacterium-like particles supplemented with inactivated influenza antigen induce cross-protective influenza-specific antibody responses through intranasal administration. Vaccine. 2012; 30:4884-4891.
40. Ichinohe T, Ainai A, Tashiro M, Sata T, Hasegawa H. PolyI: PolyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants. Vaccine. 2009; 27:6276-6279.
41. Kreijtz JH, Osterhaus AD, Rimmelzwaan GF. Vaccination strategies and vaccine formulations for epidemic and pandemic influenza control. Hum Vaccin. 2009; 5:126-135.
42. Gosselin EJ, Bitsaktsis C, Li Y, Iglesias BV. Fc receptor-targeted mucosal vaccination as a novel strategy for the generation of enhanced immunity against mucosal and non-mucosal pathogens. Arch Immunol Ther Exp (Warsz). 2009; 57:311-323.

*(Received May 1, 2013; Revised May 16, 2013; Accepted May 30, 2013)*