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Comparison of chicken immune responses after inoculation with H5 avian influenza virus-like particles produced by insect cells or pupae

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Abstract

Introduction: Novel clade 2.3.4.4 H5 highly pathogenic avian influenza virus (HPAIV) outbreaks have occurred since early 2015 in Taiwan and impacted the island economically, like they have many countries. This research investigates the immunogenicity of two HPAIV-like particles to assess their promise as vaccine candidates. **Material and Methods:** The haemagglutinin (HA) gene derived from clade 2.3.4.4 H5 HPAIV and matrix protein 1 (M1) gene were cloned into the pFastBac Dual baculovirus vector. The resulting recombinant viruses were expressed in *Spodoptera frugiperda* moth (Sf)21 cells and silkworm pupae to generate Sf21 virus-like particles (VLP) and silkworm pupa VLP. Two-week-old specific pathogen–free chickens were immunised and their humoral and cellular immune responses were analysed. **Results:** The silkworm pupa VLP had higher haemagglutination competence. Both VLP types elicited haemagglutination inhibition antibodies, anti-HA antibodies, splenic interferon gamma (IFN-γ) and interleukin 4 (IL-4) mRNA expression, and $CD4^+/CD8^+$ ratio elevation. However, chickens receiving silkworm pupa VLP exhibited a significantly higher anti-HA antibody titre in ELISA after vaccination. Although Sf21 VLP recipients expressed more IFN-γ and IL-4, the increase in IFN-γ did not significantly raise the $CD4^+/CD8^+$ ratio and the increase in IL-4 did not promote anti-HA antibodies. **Conclusion:** Both VLP systems possess desirable immunogenicity *in vivo*. However, in respect of immunogenic efficacy and the production cost, pupa VLP may be the superior vaccine candidate against clade 2.3.4.4 H5 HPAIV infection.

Keywords: avian influenza virus, clade 2.3.4.4, silkworm pupa, vaccine, virus-like particle.

Introduction

Avian influenza is a highly contagious disease caused by the type A influenza virus. The low-pathogenic H6N1 virus was first isolated in Taiwan in 1972 (16) and subsequently isolated frequently from chickens. Sporadic infections were observed in chickens in Taiwan from 2003 to 2012 with the H5N2 strain of the virus (10), which has 3–4 basic amino acids at the haemagglutinin (HA) protein cleavage site. In early 2014, novel clade 2.3.4.4 H5 highly pathogenic avian influenza viruses (HPAIVs) outbreaks were reported in poultry and wild birds in China, Laos, Vietnam, Japan and Korea (11, 13, 28). Outbreaks caused by clade 2.3.4.4 H5 HPAIVs with six basic amino acids at the HA cleavage site have been found in Taiwan since January 2015 (12), and have led to deaths on a large-scale in land and waterfowl and massive economic losses. Therefore, it is imperative to develop an efficient vaccine against this epizootic.

Virus-like particles (VLPs) are multimeric nanostructures assembled from viral structural proteins that are devoid of any genetic material. They contain the functional viral proteins responsible for cell penetration by the virus, ensuring efficient cell entry (2). VLPs provide delivery systems that combine good safety profiles with strong immunogenicity, which could be a better alternative to traditionally attenuated or inactivated vaccines (2, 15). There are two major VLP production platforms, the *Spodoptera frugiperda* (Sf) insect cell line and silkworm (*Bombyx mori*) larvae/pupae expression

system (14). The insect cell system has been broadly applied in laboratories (4, 17, 23, 29), and the silkworm system has been employed for large-scale production in recent years (21, 22). The VLP produced by the insect cell system demonstrated its ability to induce humoral and cellular immune responses in vaccinated animals (4). Regarding the VLP generated by the silkworm system, high haemagglutination inhibition antibody titres in vaccinated animal sera were demonstrated (21, 22), while evidence of cell-mediated immunity was still absent. No vaccine efficacy comparisons between these two VLP production systems have been documented so far.

Finding a vaccine candidate superior in safety, productivity, and protectiveness is important for the poultry industry. In this study, we developed VLP vaccines against the currently prevailing clade 2.3.4.4 H5 HPAIV in Taiwan and compared the differences in chicken immunogenic efficacy in two VLP expression systems, Sf21 cells and silkworm pupae. The produced VLPs were verified using transmission electron microscopy and assayed for their haemagglutination activity. The VLPs' immunogenicity was validated, and the humoral and cellular immune responses in chickens immunised using the two VLP systems were compared. The results observed in this study could provide crucial information on VLP formulation options for production at scale, which would be useful for future vaccine development against avian influenza viruses.

Material and Methods

Recombinant virus generation. Synthesis of the clade 2.3.4.4 H5 haemagglutinin (HA) gene and H6 matrix protein 1 (M1) gene was carried out by Protech Technology (Taipei, Taiwan). The HA gene was isolated from A/chicken/Taiwan/a2888/2015 (clade 2.3.4.4 H5N2), and the M1 gene from A/chicken/Taiwan/2838v/00 (H6N1). The synthesised genes were optimised based on insect codons. The amino acid sequence connecting the H1 and H2 subunits of the HA protein was modified from RERRRKRG to RDTRG to mitigate the virus' virulence, because the multiplicity of basic amino acids in this connecting sequence contributes to high pathogenicity. A 6 × His tag was added to the C-terminus of the H2 peptide. A 6 × His tag was also added to the C-terminus of the M1 protein.

For the recombinant bacmid DNA used in the Sf21 system, the resulting 1713-nt codon-optimised sequence encoding HA and 777-nt codon-optimised sequence encoding M1 were cloned into the pFastBac Dual baculovirus expression vector (Thermo Fisher Scientific, Waltham, MA, USA) and these were then transformed into *Escherichia coli* DH10Bac (Thermo Fisher Scientific) for generation of this bacmid DNA. The recombinant bacmid DNA was transfected into Sf21 insect cells. Recombinant Bac-H5M1 baculovirus was then generated after three passages and the virus titre was determined using a plaque assay in the Sf21 cells.

To generate recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV), the pFastBac Dual expression vector containing codon-optimised sequences encoding HA and M1 was first electroporated into *Escherichia coli* DH10Bac/BmNPV for production of recombinant BmNPV bacmid DNA. Next, the BmNPV bacmid DNA was transfected into silkworm Bm-N cells. Recombinant H5M1-BmNPV was generated after three passages, and the virus titre was determined using a plaque assay in the Bm-N cells.

VLP production and purification. Sf21 cells were infected with rBac-H5M1 baculovirus at 0.02 multiplicity of infection (MOI) at 28°C for Sf21VLP formation. Silkworm pupa VLPs were produced by injecting 5 μL of rH5M1-BmNPV (1×10^6 pfu/mL) into pupae on the third day of pupation and incubating them for 4 days at 25°C. Next, the infected pupae were ground and homogenised using a sonicator (Misonix, Farmingdale, NY, USA) in phosphate buffered saline (PBS) containing 0.01% formalin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μM phenylthiourea (Sigma-Aldrich) as antioxidants. Sonication was performed and the resultant homogenate was then centrifuged at 4,500 × g for 30 min. The supernatant was further centrifuged at 22,000 × g for 30 min. The final supernatant was filtered using a 0.45 μm filter and pelleted by centrifugation at 150,000 × g for 1 h at 4°C. The Sf21 VLP and silkworm pupa VLP pellets were resuspended in a buffer of 10 mM Tris base, 1 mM EDTA, and 100 mM NaCl. Following this, sucrose gradient centrifugation was performed to obtain purified VLPs. The total purified VLP protein amount was quantified using the Pierce Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The VLP HA protein concentration was quantified using an ELISA developed by RuenHuei Biopharmaceuticals Inc. (Taipei, Taiwan).

Western blot analysis. The protein expression of synthetic H5 and M1 genes was verified using Western blot. Both purified Sf21 VLPs and silkworm pupa VLPs were run on 12% polyacrylamide gels alongside a PageRuler Plus prestained protein ladder (Thermo Fisher Scientific). The separated proteins were then transferred onto Immobilon-P membranes (Sigma-Aldrich). The membranes were blocked and further incubated with mouse anti-His tag antibody (Sigma-Aldrich) and horseradish peroxidase (HRP)–conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The signal was visualised after development with 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich) at room temperature.

Transmission electron microscopy. Purified VLPs were absorbed onto a plasma-discharged copper grid for 3 min and fixed with 2% phosphotungstic acid (catalogue number P4006, Sigma-Aldrich) for 1 min. VLPs were then imaged using an EM-1400 transmission electron microscope (Jeol USA, Peabody, MA, USA).

Chicken immunisation. Two-week-old specific pathogen-free (SPF) chickens were obtained from JD-SPF

Biotech (Miaoli, Taiwan). Animals were randomly divided into three groups (n = 6 per group) receiving Sf21 VLPs, silkworm pupa VLPs or PBS. Briefly, VLPs of each group containing 20 μg HA protein were emulsified with complete Freund's adjuvant (Thermo Fisher Scientific) at a 1:1 ratio and used for the primary immunisation *via* a subcutaneous route on day 1. For the booster dose, the same amount of the antigen was mixed with incomplete Freund's adjuvant (Thermo Fisher Scientific) and injected on day 14. Chicken serum was collected at 7, 21, 38 and 46 days post immunisation (dpi), and all chickens were sacrificed thereafter using CO_2 inhalation.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests. The HA and HI tests were performed with standard WHO protocols (30). The HA activities of purified Sf21 VLPs and silkworm pupa VLPs were tested against 1% chicken red blood cells. The HA titres were recorded as the highest dilution exhibiting complete haemagglutination. For the HI tests, chicken sera were pre-treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) and incubated with 4 HA units of Sf21 VLPs, silkworm pupa VLPs, or A/Anhui/1/2005 (H5N1, clade 2.3.4). The chicken serum HI titre was recorded as the highest serum dilution exhibiting complete haemagglutination inhibition.

ELISA analysis. An equivalent amount of purified Sf21 VLPs, silkworm pupa VLPs, or A/Anhui/1/2005 (H5N1, clade 2.3.4) containing10 ng HA protein was coated onto a flat-bottomed 96-well microplate (Asahi Glass, Tokyo, Japan). After blocking with a 5% antibody blocker (LTK BioLaboratories, Taoyuan City, Taiwan), the tested chicken sera from the 46 dpi blood samples were serially diluted and incubated with the corresponding VLPs or A/Anhui/1/2005 for 1 h. Following a wash protocol, HRP conjugated goat anti-chicken IgY containing heavy and light chains (Jackson ImmunoResearch Laboratories) was dispensed into each well and incubated for another 1 h. After three additional washes, the wells were incubated with TMB substrate (Sigma-Aldrich). The colour was allowed to develop and the reaction was stopped by adding H_2SO_4 stop solution (Sigma-Aldrich). The optical density at 450 nm was read using an automated ELISA reader (Tecan, Männedorf, Switzerland).

VLP T cell response in chickens. To analyse antigen-specific T cell responses in chickens, chicken spleens were harvested at 46 dpi. Following splenocyte isolation and stimulation with the corresponding VLPs, the splenocytes were incubated with fluoroisothiocyanate mouse anti-chicken CD4 (Southern Biotech, Birmingham, AL, USA) and mouse anti-chicken CD8a-PE (Southern Biotech). Following three washes, the splenocytes were resuspended in 0.5 ml PBS containing 0.4% paraformaldehyde and filtered with a cell strainer (Corning, Corning, NY, USA). The samples were read using an FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) and analysed using Kaluza software (Beckman Coulter). The splenocyte total RNA was extracted and 1μg of purified RNA was reverse transcribed to cDNA using the SuperScript IV First-Strand Synthesis System and oligo-dT primers (Thermo Fisher Scientific). Subsequently the splenic T cell IL-4 and IFN-γ mRNA were quantified (27) using a qTOWER^{3}G real-time PCR (Analytik Jena, Jena, Germany). Finally, the expressed IFN-γ and IL-4 mRNA levels were calculated *via* Ct value conversion and recorded as relative percentages.

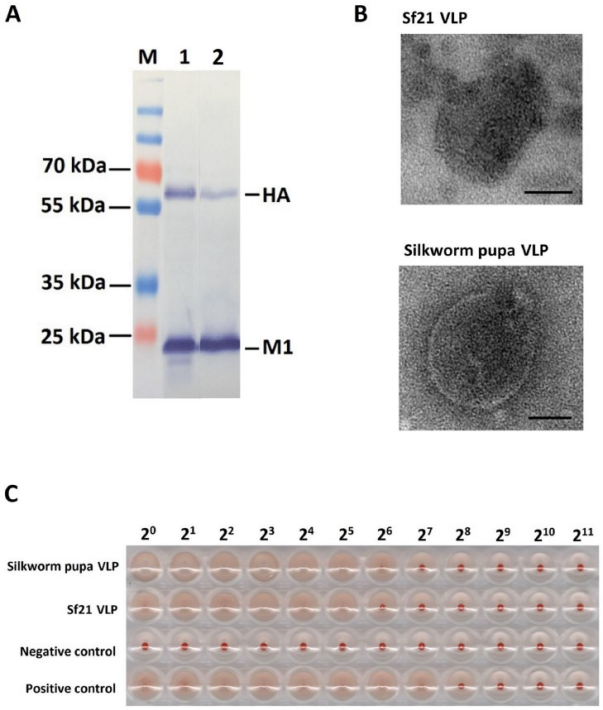

Fig. 1. Sf21 VLP and silkworm pupa VLP characterisation. A – Detection of HA protein and M1 protein in VLPs using Western blot; M – molecular weight markers; 1 – Sf21 VLP; 2 – silkworm pupa VLP. B – Detection of Sf21 VLP (up) and silkworm pupa VLP (down) under a transmission electron microscope; scale bar = 50 nm. C – Haemagglutination activity of the purified Sf21 VLP and silkworm pupa VLP

Results

Characterisation of Sf21 VLP and silkworm pupa VLP. Following obtainment of purified VLPs, the HA and M1 proteins were detected using Western blotting. Fig. 1A shows 63 kDa HA and 25 kDa M1 bands, indicating the successful expression of the two proteins in both Sf21 VLP and silkworm pupa VLP. Under transmission electron microscopy, the Sf21 VLP and silkworm pupa VLP were identified as predominantly spherical, pleomorphic enveloped particles approximately 150 nm in diameter and containing spikes protruding from the VLP envelope (Fig. 1B). The average total protein and HA protein content of Sf21 VLP were 1.81 mg/mL and 0.18 mg/mL, respectively. The equivalents for silkworm pupa VLP were 2.36 mg/mL and 0.11 mg/mL. Even though there was less HA protein content, the haemagglutination activity per 25 μL of silkworm pupa VLP was 2^6, which was higher than that of Sf21 VLP at 2^5 (Fig. 1C). This indicated that the silkworm pupa VLP had higher haemagglutination competence than Sf21 VLP in this study.

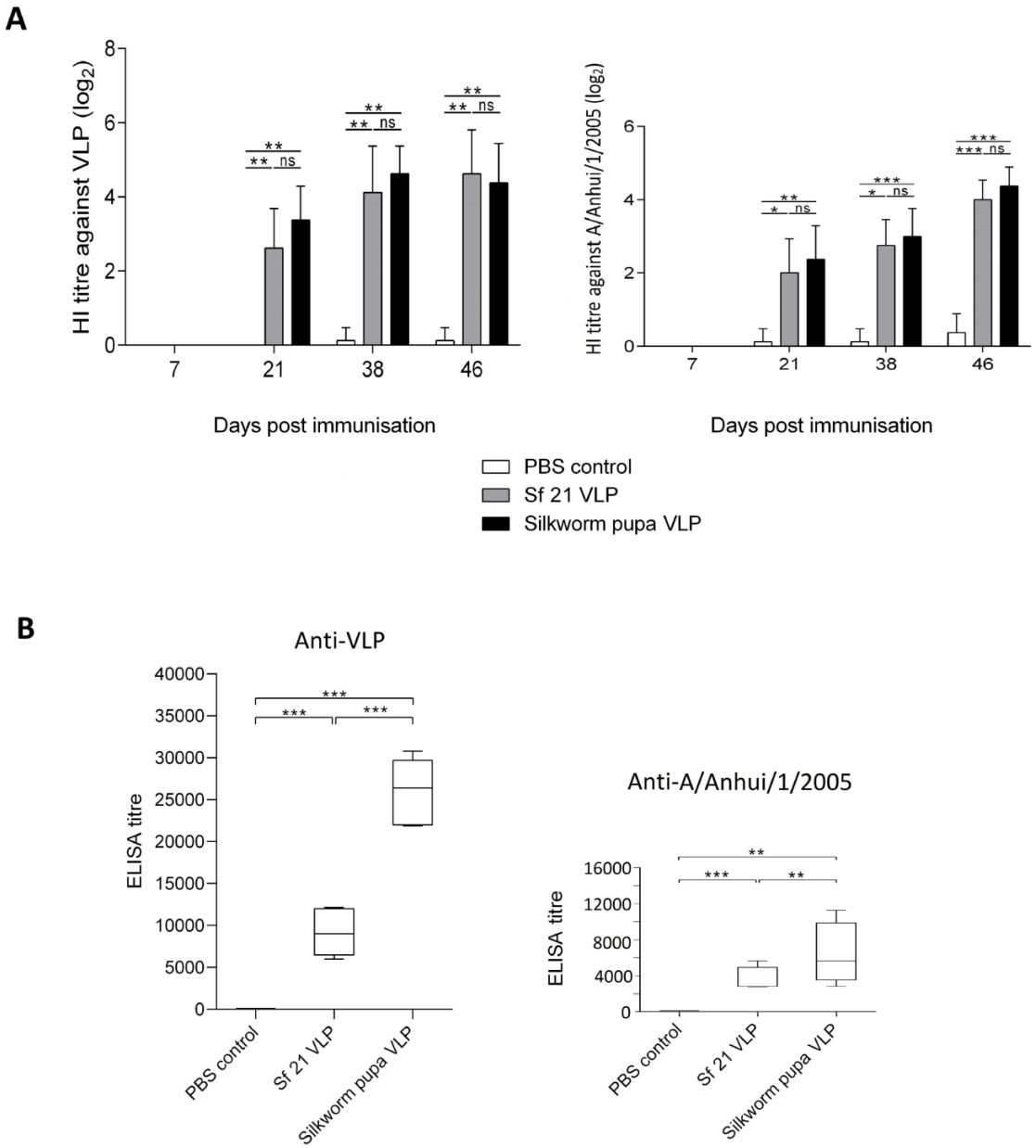

Fig. 2. Immunogenicity of Sf21 VLP and silkworm pupa VLP. A – Serum HI titres against the corresponding VLP (left) or A/Anhui/1/2005 (H5N1, clade 2.3.4) (right). Error bars are mean ± SEM. ns – not significant. B – ELISA IgY titres on day 46 post-immunisation using the corresponding VLP (left) or A/Anhui/1/2005 (H5N1, clade 2.3.4) (right) as antigens. Lines and boxes represent the upper extreme, 25th, 50th, and 75th percentiles, and the lower extreme. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$

Elicitation of haemagglutination inhibition antibodies by Sf21 VLP and silkworm pupa VLP in immunised chickens. Fig. 2A displays that all chickens showed significantly increased serum HI titres from 21 dpi. The average HI titres of all experimental groups at 46 dpi reached approximately 2^4–2^5 regardless of whether VLPs or the complete virion were used. There were significant differences compared to the negative control chickens receiving PBS, which validates the ability of both Sf21 VLP and silkworm pupa VLP to elicit anti-HA immunisation responses in chickens. However, there were no significant differences between chickens receiving Sf21 VLP and those inoculated with silkworm pupa VLP.

IgY immunoglobulin titre induction by silkworm pupa VLP and Sf21 VLP. When the corresponding VLPs were employed as the ELISA antigen, the serum antibody of Sf21 VLP–immunised chickens was found to be at an elevated median titre of 9,000 while that of silkworm pupa VLP–immunised chickens was nearly 300% higher at a median titre of 26,400. There was a significant difference between the two VLP-immunised sera, and the P value was 1.24×10^{-5} (<0.001) (Fig. 2B left). Similarly, when A/Anhui/1/2005 (H5N1, clade 2.3.4) was used as the antigen, the median antibody titres of Sf21 VLP and silkworm pupa VLP–immunised sera were 2,820 and 5,640, respectively. There was again a significant difference between the two VLP-immunised sera, and the P value was 7.25×10^{-3} (<0.01) (Fig. 2B right).

The results showed that both VLPs elicited antigen-specific humoral responses. The silkworm pupa VLP was demonstrated to induce a higher IgY titre and may possess superior capability in humoral immune response development than Sf21 VLP.

T cell response elicitation in chickens: splenic IFN-γ and IL-4 mRNA expression and $CD4^+/CD8^+$ ratios. It was observed that immunisation with both VLPs increased the IFN-γ and IL-4 levels, indicating elicitation of both type 1 and type 2 helper T cell directed immune responses (5, 19). The Sf21 VLP induced significantly more splenic IFN-γ ($P = 6.1 \times 10^{-3}$, <0.01) and IL-4 ($P = 3.84 \times 10^{-2}$, <0.05) mRNA expression than silkworm pupa VLP (Figs 3A and 3B). Flow cytometric analysis on day 46 post immunisation showed both VLP immunisations to have increased $CD4^+/CD8^+$ ratios. However, no statistical significance in $CD4^+/CD8^+$ ratio was observed (P = 0.051) between the Sf21 VLP and the silkworm pupa VLP groups (Fig. 3C).

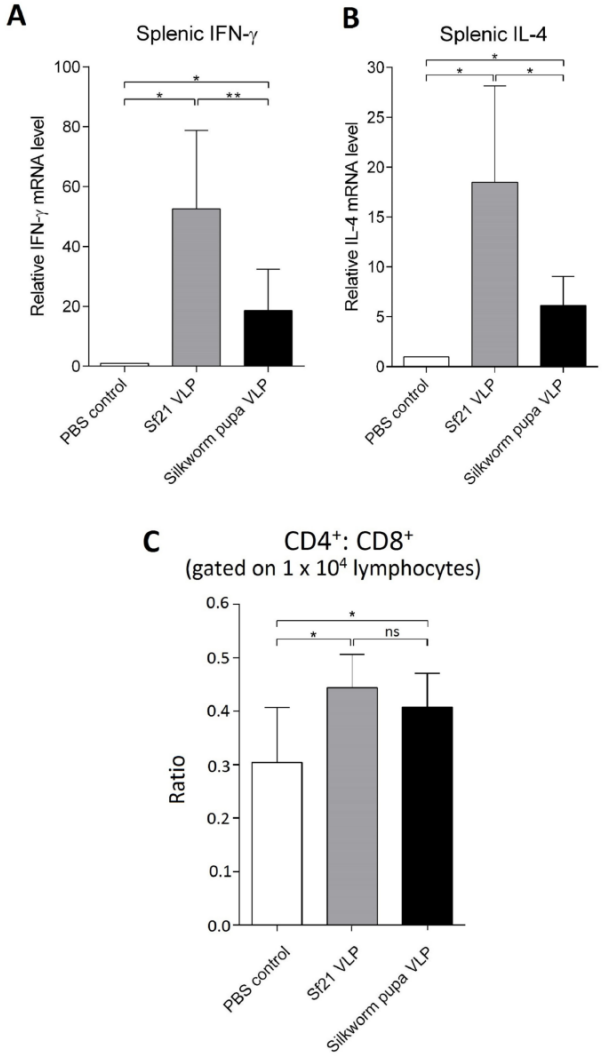

Fig. 3. Cell-mediated immune response induced by Sf21 VLP and silkworm pupa VLP in chickens. A – Splenic IFN-γ mRNA amplification in real-time PCR. B – IL-4 mRNA amplification in real-time PCR. C – Splenic $CD4^+/CD8^+$ ratios found using flow cytometry. Error bars are mean ± SEM. ns – not significant. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$

Discussion

The clade 2.3.4.4 viruses have caused severe outbreaks in Taiwan since early 2015 (12), resulting in a huge negative impact on the poultry industry. Thorough slaughtering is the principal policy of the government, but the development of a safe and effective vaccine offers hope of a solution which preserves bird stock.

A VLP, which consists of one or more structural proteins but no viral genome, resembles the authentic virion but cannot replicate in cells (14). It is devoid of virus reassortment possibility and, therefore, a safe vaccine candidate. Insect cells and silkworm larvae or pupae have been utilised for VLP production. The former production technique uses *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) as the vector and has been applied extensively. The latter employs *Bombyx mori* nucleopolyhedrovirus (BmNPV) as the vector (14, 18). Choosing the better platform is a crucial issue for vaccine efficacy and best cost effectiveness.

The *Bombyx mori* silkworm larva or pupa is one of the optimal systems for mass production of recombinant eukaryotic proteins. The BmNPV expression system is attractive because it is not only minimally biohazardous but also highly efficient and costs approximately 90% less than insect cell-based expression (20). Silkworm pupae have therefore been exploited for large-scale production of non-conventional influenza VLP vaccine in recent years (21, 22). However, its immunisation efficacy compared to that of the insect cell system remains to be established. In the present study, VLPs were generated against clade 2.3.4.4 H5 HPAIVs from both Sf21 cells and silkworm pupae, and their immune response discrepancies in chickens were validated for the first time.

From the HI and ELISA results illustrating the humoral immune response, the clade 2.3.4.4 VLP–immunised chickens could be cross-protected against clade 2.3.4 virus (Figs 2A and 2B). ELISA titre values were significantly higher for the chicken sera vaccinated by silkworm pupa VLP than for the sera of those vaccinated by Sf21 VLP (Fig. 2B). This indicated that the silkworm pupa VLP might induce more anti-HA antibody generation than the Sf21 VLP, either against its homologous VLP or the heterologous clade 2.3.4 virus.

Examining cellular immune responses, we observed that Sf21 VLP could elevate splenic IFN-γ and IL-4 expression more than silkworm pupa VLP could, the difference in the intensification being statistically significant. This implies that Sf21 VLP induced higher activity of T helper cells (with the surface marker $CD4^+$) (5). Type 1 T helper cells secrete IFN-γ and TNF-β, which allow these cells to be particularly effective in protecting against intracellular infections by viruses and bacteria that grow in macrophages and in eliminating cancerous cells (8). Interferon gamma can, in turn, cause more $CD4^+$ differentiation through positive feedback loops (26). Nevertheless, the $CD4^+/CD8^+$ ratio did not

show significant differences between the Sf21 VLP and silkworm pupa VLP groups. Type 2 T helper cells secrete IL-4 and activate B cells, which can upregulate antibody generation (5). However, Sf21 VLP did not provoke more antibody production to the extent that the sera of chicken administered this inoculum than the sera of silkworm pupa VLP–inoculated birds. This implied that Sf21 VLP could not prompt a stronger immune response than silkworm pupa VLP, although it induced more IFN-γ and IL-4 expression.

The insect cell line can assemble N-glycan precursors in order to produce high mannose or paucimannosidic end products. However, the cells failed to elongate the trimmed N-glycan to produce complex products containing terminal galactose and sialic acid residues (9). Enzyme assays revealed that the insect cell lines had little or none of the galactosyltransferase and sialyltransferase activities involved in N-glycan elongation; however, these enzymes would exist in silkworms (3, 7, 25). To a certain extent, this may account for the differences in immune response induced by Sf21VLP and silkworm pupa VLP.

Because VLP immunogenicity is primarily contributed by the HA protein (1, 6, 24), we constructed the VLP vaccines using the HA gene of clade 2.3.4.4 H5N2 (A/chicken/Taiwan/a2888/2015) for protection against clade 2.3.4.4 H5 HPAIV infection. The M1 component of VLPs having also demonstrated immunogenicity in a previous study (4), and the H6N1 virus being common in Taiwan, we employed the M1 gene of A/chicken/Taiwan/2838v/00 isolate of H6N1 virus for VLP production. However, the immunity-conferring capacity of the VLP-M1 fraction against avian influenza viruses still needs to be verified in future studies.

Regarding viral challenge experiments in chickens using clade 2.3.4.4 field viruses, strict oversight measures have been implemented by the government. However, regulatory compliance is burdensome because of the biosecurity demands imposed, and few facilities are available. This was the major limitation of the present study, and opportunities for further cooperation with national certified institutes will need to be sought. More direct evidence may provide a ray of hope for the clinical application of the VLP vaccine.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC Approval No: NTU105-EL-00142).

References

1. Chang G.R.-L., Lai S.-Y., Chang P.C., Wang M.-Y.: Production of immunogenic one-component avian H7-subtype influenza virus-like particles. Process Biochem 2011, 46, 1292–1298, doi: 10.1016/j.procbio.2011.02.019.
2. Chroboczek J., Szurgot I., Szolajska E.: Virus-like particles as vaccine. Acta Biochim Pol 2014, 61, 531–539.
3. Dojima T., Nishina T., Kato T., Uno T., Yagi H., Kato K., Park E.Y.: Comparison of the *N*-linked glycosylation of human beta1,3-*N*-acetylglucosaminyltransferase 2 expressed in insect cells and silkworm larvae. J Biotechnol 2009, 143, 27–33, doi: 10.1016/j.jbiotec.2009.06.013.
4. Hu C.-M.J., Chien C.-Y., Liu M.-T., Fang Z.-S., Chang S.-Y., Juang R.-H., Chang S.-C., Chen H.-W.: Multi-antigen avian influenza a (H7N9) virus-like particles: particulate characterizations and immunogenicity evaluation in murine and avian models. BMC Biotechnol 2017, 17, 2, doi: 10.1186/s12896-016-0321-6.
5. Kaiko G.E., Horvat J.C., Beagley K.W., Hansbro P.M.: Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology 2008, 123, 326–338, doi: 10.1111/j.1365-2567.2007.02719.x.
6. Kang S.M., Pushko P., Bright R.A., Smith G., Compans R.W.: Influenza virus-like particles as pandemic vaccines. Curr Top Microbiol Immunol 2009, 333, 269–289, doi: 10.1007/978-3-540-92165-3_14.
7. Kato T., Kajikawa M., Maenaka K., Park E.Y.: Silkworm expression system as a platform technology in life science. Appl Microbiol Biotechnol 2010, 85, 459–470, doi: 10.1007/s00253-009-2267-2.
8. Kidd P.: Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003, 8, 223–246.
9. Kost T.A., Condreay J.P., Jarvis D.L.: Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 2005, 23, 567–575, doi: 10.1038/nbt1095.
10. Lee C.C., Zhu H., Huang P.Y., Peng L., Chang Y.C., Yip C.H., Li Y.T., Cheung C.L., Compans R.W., Yang C., Smith D.K., Lam T.T.Y., King C.C., Guan Y.: Emergence and evolution of avian H5N2 influenza viruses in chickens in Taiwan. J Virol 2014, 88, 5677–5686, doi: 10.1128/JVI.00139-14.
11. Lee D.-H., Bertran K., Kwon J.-H., Swayne D.E.: Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. J Vet Sci 2017, 18, 269–280, doi: 10.4142/jvs.2017.18.S1.269.
12. Lee M.S., Chen L.H., Chen Y.P., Liu Y.P., Li W.C., Lin Y.L., Lee F.: Highly pathogenic avian influenza viruses H5N2, H5N3, and H5N8 in Taiwan in 2015. Vet Microbiol 2016, 187, 50–57, doi: 10.1016/j.vetmic.2016.03.012.
13. Lee Y.J., Kang H.M., Lee E.K., Song B.M., Jeong J., Kwon Y.K., Kim H.R., Lee K.J., Hong M.S., Jang I., Choi K.S., Kim J.Y., Lee H.J., Kang M.S., Jeong O.M., Baek J.H., Joo Y.S., Park Y.H., Lee H.S.: Novel reassortant influenza A(H5N8) viruses, South Korea, 2014. Emerg Infect Dis 2014, 20, 1086–1089, doi: 10.3201/eid2006.140233.
14. Liu F., Wu X., Li L., Liu Z., Wang Z.: Use of baculovirus expression system for generation of virus-like particles: successes and challenges. Protein Expr Purif 2013, 90, 104–116, doi: 10.1016/j.pep.2013.05.009.
15. López-Macías C., Ferat-Osorio E., Tenorio-Calvo A., Isibasi A., Talavera J., Arteaga-Ruiz O., Arriaga-Pizano L., Hickman S.P., Allende M., Lenhard K., Pincus S., Connolly K., Raghunandan R., Smith G., Glenn G.: Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine in a blinded, randomized, placebo-controlled trial of adults in Mexico. Vaccine 2011, 29, 7826–7834, doi: 10.1016/j.vaccine.2011.07.099.
16. Lu Y.S., Sugimura T., Shieh H.K., Lee Y.L., Jong M.H.: Isolation and identification of an influenza A virus in ducks in Taiwan. Jour Chinese Soc Vet Sci 1985, 11, 23–24.

17. Metz S.W., Gardner J., Geertsema C., Le T.T., Goh L., Vlak J.M., Suhrbier A., Pijlman G.P.: Effective chikungunya virus-like particle vaccine produced in insect cells. PLoS Negl Trop Dis 2013, 7, e2124, doi: 10.1371/journal.pntd.0002124.
18. Miele S.A., Garavaglia M.J., Belaich M.N., Ghiringhelli P.D.: Baculovirus: molecular insights on their diversity and conservation. Int J Evol Biol 2011, 2011, 379424, doi: 10.4061/2011/379424.
19. Mosmann T.R., Cherwinski H., Bond M.W., Giedlin M.A., Coffman R.L.: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986, 136, 2348–2357.
20. Motohashi T., Shimojima T., Fukagawa T., Maenaka K., Park E.Y.: Efficient large-scale protein production of larvae and pupae of silkworm by Bombyx mori nuclear polyhedrosis virus bacmid system. Biochem Biophys Res Commun 2005, 326, 564–569, doi: 10.1016/j.bbrc.2004.11.060.
21. Nerome K., Matsuda S., Maegawa K., Sugita S., Kuroda K., Kawasaki K., Nerome R.: Quantitative analysis of the yield of avian H7 influenza virus haemagglutinin protein produced in silkworm pupae with the use of the codon-optimized DNA: A possible oral vaccine. Vaccine 2017, 35, 738–746, doi: 10.1016/j.vaccine.2016.12.058.
22. Nerome K., Sugita S., Kuroda K., Hirose T., Matsuda S., Majima K., Kawasaki K., Shibata T., Poetri O.N., Soejoedono R.D., Mayasari N.L., Agungpriyono S., Nerome R.: The large-scale production of an artificial influenza virus-like particle vaccine in silkworm pupae. Vaccine 2015, 33, 117–125, doi: 10.1016/j.vaccine. 2014.11.009.
23. Pushko P., Kort T., Nathan M., Pearce M.B., Smith G., Tumpey T.M.: Recombinant H1N1 virus-like particle vaccine elicits protective immunity in ferrets against the 2009 pandemic H1N1 influenza virus. Vaccine 2010, 28, 4771–4776, doi: 10.1016/j.vaccine. 2010.04.093.
24. Pushko P., Pujanauski L.M., Sun X., Pearce M.B., Hidajat R., Kort T., Schwartzman L.M., Tretyakova I., Chunqing L., Taubenberger J.K., Tumpey T.M.: Recombinant H7 hemagglutinin forms subviral particles that protect mice and ferrets from challenge with H7N9 influenza virus. Vaccine 2015, 33, 4975–4982, doi: 10.1016/j.vaccine.2015.07.026.
25. Sasaki K., Kajikawa M., Kuroki K., Motohashi T., Shimojima T., Park E.Y., Kondo S., Yagi H., Kato K., Maenaka K.: Silkworm expression and sugar profiling of human immune cell surface receptor, KIR2DL1. Biochem Biophys Res Commun 2009, 387, 575–580, doi: 10.1016/j.bbrc.2009.07.065.
26. Schroder K., Hertzog P.J., Ravasi T., Hume D.A.: Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 2004, 75, 163–189, doi: 10.1189/jlb.0603252.
27. St Paul M., Barjesteh N., Paolucci S., Pei Y., Sharif S.: Toll-like receptor ligands induce the expression of interferon-gamma and interleukin-17 in chicken CD4+ T cells. BMC Res Notes 2012, 5, 616, doi: 10.1186/1756-0500-5-616.
28. Swayne D.E., Suarez D.L., Spackman E., Jadhao S., Dauphin G., Kim-Torchetti M., McGrane J., Weaver J., Daniels P., Wong F., Selleck P., Wiyono A., Indriani R., Yupiana Y., Sawitri Siregar E., Prajitno T., Smith D., Fouchier R.: Antibody titer has positive predictive value for vaccine protection against challenge with natural antigenic-drift variants of H5N1 high-pathogenicity avian influenza viruses from Indonesia. J Virol 2015, 89, 3746–3762, doi: 10.1128/jvi.00025-15.
29. Tao P., Luo M., Zhu D., Qu S., Yang Z., Gao M., Guo D., Pan Z.: Virus-like particle vaccine comprised of the HA, NA, and M1 proteins of an avian isolated H5N1 influenza virus induces protective immunity against homologous and heterologous strains in mice. Viral Immunol 2009, 22, 273–281, doi: 10.1089/vim.2009.0017.
30. World Health Organization: Manual for the laboratory diagnosis and virological surveillance of influenza. WHO Press, Geneva, 2011. http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf.