

Pandemic A/H1N1 2009 Influenza Virus-like Particles Elicited Higher and Broader Immune Responses than the Commercial *Panenza* Vaccine

Naru Zhang^{1,*}, Yongping Lin^{1,*}, Min Chen^{1,*}, Ho Chuen Leung¹, Chung Sing Chan¹, Kwok Man Poon¹, Jie Zhou¹, Chung Yan Cheung¹, Liwei Lu² and Bojian Zheng¹

1. Department of Microbiology, Li Ka Shing Faculty of Medicine, University of Hong, Hong Kong, China.

2. Department of Pathology, Li Ka Shing Faculty of Medicine, University of Hong, Hong Kong, China.

Received: October 14, 2013 / Accepted: December 03, 2013 / Published: February 28, 2014.

Abstract: Objectives: The aim was to construct 2009 pandemic A/H1N1 influenza VLPs (virus-like particles) and compare the immunogenicity and protection efficacy with the commercial *Panenza* vaccine in BALB/c mouse model. Methods: VLPs derived from influenza A/Hong Kong/01/2009 (H1N1) virus were constructed by Bac-to-Bac baculovirus expression system. VLPs were purified by sucrose density gradient ultracentrifugation and then characterized by Western blotting analysis and transmission electron microscopy. After single dose vaccination with 3 µg of VLPs and equal amount of *Panenza* vaccine, the immune responses and efficacy of protection induced by VLPs were compared with those elicited by the *Panenza* vaccine in 6-8 weeks old female BALB/c mice. Key findings: VLPs could induce higher antibody titer as determined by hemagglutinin inhibition and microneutralization assay. Furthermore, we demonstrated that VLPs induced better antibody response to neuraminidase. In addition, VLP vaccinated mice had stronger cell-mediated immune response. As a result, our VLPs conferred 100% protection while the *Panenza* vaccine only conferred 67% protection. Conclusion: From the results, we concluded that influenza VLPs are highly immunogenic and they are promising to be developed as an alternative strategy to vaccine production in order to control the spread of influenza viruses.

Key words: Influenza virus, virus-like particle, *Panenza* vaccine, BALB/c mice.

1. Introduction

Influenza A virus belongs to the Orthomyxoviridae family and its genome contains 8 negative-sense, single stranded RNA segments encoding 11 viral proteins. On the viral surface, there are two glycoproteins named HA (hemagglutinin) and NA (neuraminidase). The structural matrix protein (M1) is the most abundant which is found underneath the lipid membrane. In April, 2009 CDC of the United States of America announced the detection of a novel strain of influenza virus in humans [1]. This novel virus transmitted

rapidly among humans throughout the world, and on May 1st, Hong Kong declared a state of health emergency following the first confirmed case of swine influenza A H1N1 flu in the territory. On June 11th, the World Health Organization raised the worldwide pandemic alert level into phase 6 [2].

Vaccination is widely considered to be one of the most effective preventive strategies for the control of seasonal as well as pandemic influenza viruses. The current influenza vaccine is usually prepared from virus that is grown in embryonated chicken eggs. The virus is isolated from the allantoic fluids and then inactivated with formaldehyde or β-propiolactone. Alternatively, the purified virus is treated with detergent for “split” or “subunit” vaccine formulation

* Equal contribution.

Corresponding author: Bojian Zheng, Ph.D., professor, research fields: virology and immunology. E-mail: bzheng@hkucc.hku.hk.

[3]. VLPs (Influenza virus like particles) are being developed as a new generation of non-egg based cell culture-derived vaccine against influenza infection during a pandemic threat [4]. This new generation vaccine has several advantages over the traditional egg-based strategies, especially in its high yield and short production time [5]. Several laboratories have successfully constructed influenza VLPs [6-13], however, there has been no report comparing the immunogenicity between VLPs with the commercially available vaccine. To afford a better understanding of VLPs' immunogenic efficacy, we constructed VLPs derived from an influenza A (H1N1) 2009 virus and then made a comparison on induction of immune responses and protection efficacy with the commercial *Panenza* inactivated vaccine for the first time in mice.

2. Materials and Methods

2.1 Recombinant Plasmid Construction

Viral RNA was extracted from influenza A/Hong Kong/01/2009 (H1N1) (HK/01) virus isolate according to the manufacturer's instructions (RNeasy Mini

Handbook, QIAGEN) and cDNA was synthesized by influenza unit 12 primer and superscript II reverse transcriptase (Invitrogen). Segment PCR of HA and NA and full length of M1 PCR were then conducted using primers for the synthesis of the HA, NA, and M1 listed in Tables 1–3, respectively and cDNA fragments were then cloned into PCR2.1-TOPO vector (Invitrogen). The nucleotide sequences of the HA, NA, and M1 genes were identical to the online published sequences (GenBank GQ168606.1, GQ457486.1 and FJ966954).

The full length of HA gene was cloned as a SalI-NotI DNA fragment downstream of the AcMNPV polyhedrin promoter within pFastBac1 transfer vector (Invitrogen). The full length of NA and M1 genes were cloned as EcoRI-XhoI DNA fragments into the same enzyme digested pFastBac1 plasmid. The three resulting baculovirus transfer plasmids were designated pHA, pNA, and pM1, located downstream of the AcMNPV polyhedrin promoter and upstream of the SV40 polyadenylation signal. Then pNA was digested with SnaBI and HpaI, and the fragment was ligated into the HpaI site of pHA, which resulted the

Table 1 Primers used for HA gene amplification.

Primer name	Sequence (5'--3')
F-VLP-HA-F	ACGCGT <u>CGACAT</u> GGAAGGCAACTAGTAG
F-VLP-HA-MR	TCTAGAAGGTTTACAGAGTGTG
F-VLP-HA-MF	CACACTCTGTAAACCTTCTAGA
F-VLP-HA-R	ACGCGC <u>GCGCCGCT</u> TAAATACATATTCTACTG

The underlined nucleotides indicate recognition sites of SalI and NotI.

Table 2 Primers used for NA gene amplification.

Primer name	Sequence (5'--3')
F-VLP-NA-F	CCGGAAT <u>TTCAT</u> GGAATCCAAACCAAAGAT
F-VLP-NA-MR	GCTGATGTTTACATATGTCTG
F-VLP-NA-MF	CAGACATATGTAAACATCAGC
F-VLP-NA-R	CGCCG <u>CTCGAGT</u> TACTTGCAATGGTAAATGGCA

The underlined nucleotides indicate recognition sites of EcoRI and XhoI.

Table 3 Primers used for M1 gene amplification.

Primer name	Sequence (5'--3')
F-VLP-M1-F	CCGGAAT <u>TTCAT</u> GAGTCTTCTAACCGAGG
F-VLP-M1-R	CCGCCG <u>CTCGAGT</u> CACTTGAATCGCTGCATCTGCAC

The underlined nucleotides indicated recognition sites of EcoRI and XhoI.

pHANA recombinant DNA. Then the fragment of pM1 digested with SnaBI and AvrII was ligated into pHANA fragment digested with HpaI and AvrII, thus yielding the recombinant plasmid pHANAM1.

2.2 Generation of Recombinant Baculoviruses

The recombinant baculoviruses were generated by Bac-to-Bac baculovirus expression system (Invitrogen). Briefly, the plasmid pHANAM1 was transformed into *E. coli* DH10Bac competent cells (Invitrogen). The recombinant bacmid DNA was extracted and transfected into *Spodoptera frugiperda* (Sf9) insect cells for recombinant baculovirus packaging with Lipofectamine 2000 reagent (Invitrogen). After 3 days, the recombinant baculovirus in the supernatant was collected as P1 viral stock and further amplified into P2 and P3 viral stock. The virus titer of P3 viral stock was determined by plaque assay using Sf9 insect cells.

2.3 VLP Production and Purification

The Sf9 insect cells were infected with P3 recombinant baculoviruses (MOI = 3) at a cell density of 2×10^6 /mL in 200 mL suspension culture. The culture supernatant was harvested at 72 h post-infection and the VLPs were pelleted by ultracentrifugation (Rotor-SW32Ti) and resuspended in phosphate-buffered saline (PBS) solution (pH 7.2), loaded onto a 20%-60% (w/v) discontinuous sucrose in NTE buffer (100 mM NaCl, 10 mM Tris-Cl pH 7.4, 1 mM EDTA) and ultracentrifuged (Rotor-SW41Ti). Fractions were collected and analyzed by 10% polyacrylamide gel electrophoresis and Western blot using anti-HA, anti-NA and anti-M1 rabbit polyclonal antibodies (Immune technology). Bovine serum albumin was used as a standard to quantitate the amount of HA protein [14]. Quantitative densitometry of proteins stained with coomassie blue was performed using the Odyssey application software version 3.1 (Li-Cor Bioscience).

2.4 Transmission Electron Microscopy

The fractions containing influenza VLPs were collected, negatively stained and then examined by transmission electron microscope. For negative staining, VLPs were loaded onto plastic carbon-coated 400-mesh copper grids for 2 min, stained with 1% uranyl acetate (pH 6.5) for 2 min and then rinsed gently with a few drops of distilled water. The grids were wicked dry with filter paper and then observed by transmission electron microscope (Philips EM 208s).

2.5 BALB/c Mice Immunization and Influenza Virus Challenge

Female BALB/c mice at the age of 6-8 weeks were used in this study. All of the mice were housed in the animal facility in accordance with the animal care protocol. The animal studies have been approved by the CULATR (Committee on the Use of Live Animals in Teaching and Research) of the University of Hong Kong. Three groups each consisting of fifteen mice were vaccinated on day 0. Mice were anesthetized with Phenobarbital and then group 1 were inoculated intramuscularly (i.m.) with 3 µg (based on HA content) of the commercial *Panenza* vaccine. Group 2 were inoculated with 100 µL VLPs containing the same amount of HA protein. The negative control group 3 received equal volume of PBS. Serum samples were taken before inoculation as well as on day 21. On day 21, 3 mice in each group were sacrificed for spleen extraction and the remaining mice were challenged intranasally with 10LD₅₀ of mouse adapted influenza HK/01 virus [15]. Animals are monitored for daily activity after viral challenge. On day 5 post-viral challenge, 3 mice in each group were sacrificed for lung tissue extraction. Half of the lung was fixed in 10% formaldehyde for histological analysis and the other half was homogenized for virus titer titration in MDCK (Madin-Darby canine kidney) cells by plaque assay. The remaining 9 mice in each group were used for the calculation of the survival rate.

2.6 Histopathologic Examination

For microscopic evaluation of the pathologic changes in the lung tissues, 3 mice in each group were sacrificed and the lungs were immediately fixed in 10% paraformaldehyde. After fixation, they were embedded in paraffin. Sections were made at 4 to 6 μm thickness and mounted on slides. Histopathological changes caused by influenza virus infection were examined by H&E (haematoxylin and eosin) staining under light microscope.

2.7 ELISA for Detection of HA Specific IgG Antibodies

ELISA was conducted to measure the levels of HA-specific IgG antibodies in immunized mouse sera. Briefly, 96-well plates were coated with 100 ng recombinant HA protein per well overnight at 4 $^{\circ}\text{C}$. After washing with PBST (PBS containing 0.05% Tween 20), the plates were blocked with 200 μL blocking buffer overnight at 4 $^{\circ}\text{C}$. After washing, sera with serial dilutions were added in triplicates and incubated at 37 $^{\circ}\text{C}$ for 1 h. After washing, the plates were incubated at 37 $^{\circ}\text{C}$ for 30 min with a secondary goat anti-mouse antibody conjugated with horseradish peroxidase (Invitrogen) diluted in diluent buffer. After washing, the plates were incubated for 10 min with TMB substrate solution. The reaction was stopped by 1M H_2SO_4 and the absorbance was then measured at 450 nm using an ELISA reader (Tecan Group Ltd., Switzerland).

2.8 HI (Hemagglutinin Inhibition) Assay

The primary assay for determining the amount of influenza-specific antibody present in vaccinated mouse sera is the HI assay [16]. To inactivate non-specific inhibitors, one part of mice sera were treated with three parts of RDE II (receptor destroying enzyme II) and incubated at 37 $^{\circ}\text{C}$ for 18 h. Then the RDE II was inactivated at 56 $^{\circ}\text{C}$ water bath for 30 min and PBS was added, creating 1:10 starting dilution of sera. RDE II-treated sera were two-fold serially diluted in triplicates in 96-well plates (Greiner bio-one,

Germany). An equal volume of HK/01 virus, adjusted to 8 HAU/50 μL was added into each well. The plates were incubated at 37 $^{\circ}\text{C}$ for 1 h followed by the addition 50 μL per well of 0.5% turkey erythrocytes. The plates were then incubated at room temperature for 1 h. The HI titer was determined as the highest serum dilution able to completely inhibit hemagglutination [17].

2.9 Neutralization Assay

Mouse sera were tested on MDCK cells for neutralization assay according to the WHO manual. Briefly, sera were heat inactivated at 56 $^{\circ}\text{C}$ for 30 min and prepared at 1:10 starting dilution, followed by 2-fold dilutions. The serially diluted sera were incubated with HK/01 virus of 100TCID₅₀/60 μL at 37 $^{\circ}\text{C}$ for 1 h. The 96-well plates with MDCK cells were washed with PBS and then 100 μL of neutralized samples were transferred to the cell culture plates. Virus back titration was also included during the procedure. The plates were incubated at 37 $^{\circ}\text{C}$ for 3 days to record cytopathic effect.

2.10 Quantification of Released Viral RNA by Quantitative Real-Time PCR

MDCK cells were infected with HK/01 virus at high MOI of 3. After 2 h infection, the cells were washed 3 times and the cell culture plates were refilled with fresh medium containing different concentrations of sera. At 6 h post-infection, both the supernatant and cell pellets were collected and viral RNA from infectious viruses was quantified by real-time PCR with SYBR green PCR Master Mix (Stratagene). Primers of real-time PCR for HA gene were: HA-Forward: 5'-CAATAAGACCCAAAGTGAGGG-3'; HA-Reverse: 5'-AATCGTGGACTGGTGTATCTG-3'. β -actin primers which were used to detect viral RNA copies in the cell pellet were β -actin-Forward: 5'-GAGACCTTCAACACCCCGG-5' and β -actin Reverse: 5'-ATGTCGCGCACG ATTTCCC-3'.

2.11 ELISpot Assay

Mouse spleens were extracted for ELISpot assay on day 21 before virus challenge. Diluted antibodies for IFN- γ , IL-2 and IL-4 were coated onto plates and incubated overnight at 4 °C. The plates were washed thoroughly with PBS and blocked by RPMI-1640 containing 10% FBS and 1% P/S, and incubated at room temperature for more than 2 h. During incubation, the splenocytes were isolated and determined as previously described [5]. Splenocytes in culture medium at 1×10^6 /well were stimulated with recombinant HA protein (Immune Technology) and HA, NA peptides (HAI-535: IYSTVASSL and NAI-73: CPISGWAI) (Gl Biochemistry Shanghai Ltd) in 5% CO₂; 37 °C humidified incubator for 36 h. Then the plates were washed thoroughly with 0.05% PBST, the diluted detection antibodies were added and the plates were incubated at room temperature for 2 h. After washing, diluted streptavidin peroxidase was added into each well and the plates were incubated at room temperature for 45 min. The plates were washed again and TMB substrate solution was added into each well. The spot development was monitored with the help of hand-held magnifier and the color development was terminated with ddH₂O. The plates were air dried and spots were counted by an ImmunoSpot ELISPOT reader (Cellular Technology Ltd., USA).

2.12 Statistical Analysis

Data are presented as the arithmetic mean values of two experiments plus SD (standard deviation). Statistical significance compared between VLP and *Panenza* vaccinated mouse samples was indicated by student's *t*-test.

3. Results and Discussion

3.1 VLP Generation and Characterization

The recombinant transfer bacmid DNA was successfully constructed containing the three genes of HA, NA and M1 and they were within their own

expression cassette, downstream of the AcMNPV polyhedrin promoter and upstream of the SV40 polyadenylation signal (Fig. 1a). Bovine serum albumin was used as a standard to quantitate the amount of HA protein in VLPs (Fig. 1b). In order to confirm that the influenza HA, NA, and M1 proteins were co-expressed, the VLPs fractions were analyzed by Western blotting using polyclonal antibodies to the three proteins. As shown in Fig. 1c, the three proteins were successfully co-expressed. Aliquot of concentrated VLPs were negative stained for transmission electron microscopy and Fig. 1d shows that the three proteins were self-assembled into particles with morphology similar to the real virus particles.

3.2 VLP Vaccination Provided Complete Protection against Lethal Challenge with HK/01 Virus

The BALB/c mice were i.n. inoculated with 10 LD₅₀ of influenza HK/01 virus. All the mice in the negative control group which was vaccinated with PBS did not survive beyond day 8 after virus challenge, whereas all VLP vaccinated mice survived. In contrast, *Panenza* provided incomplete protection against lethal viral challenge, in which only about 67% (8/12) mice survived (Fig. 2a). The result indicated that VLP vaccination could provide more potent protection against lethal challenge with HK/01 virus. Virus titer in lung tissues was detected by plaque assay and it was found that the virus titer in VLP vaccinated mouse lungs was significantly lower than that in *Panenza* vaccinated mouse lungs (Fig. 2b).

3.3 Absence of Histopathological Changes in Lung Tissues of Mice Vaccinated with VLPs

Histopathological changes of mouse lung sections collected at 5 days post-challenge were further examined by H&E staining (Fig. 3). Compared to the uninfected mice (Fig. 3d), lung tissues of PBS injected (unvaccinated) mice showed severe inflammation with intensive infiltration of lymphocytes and marked tissue

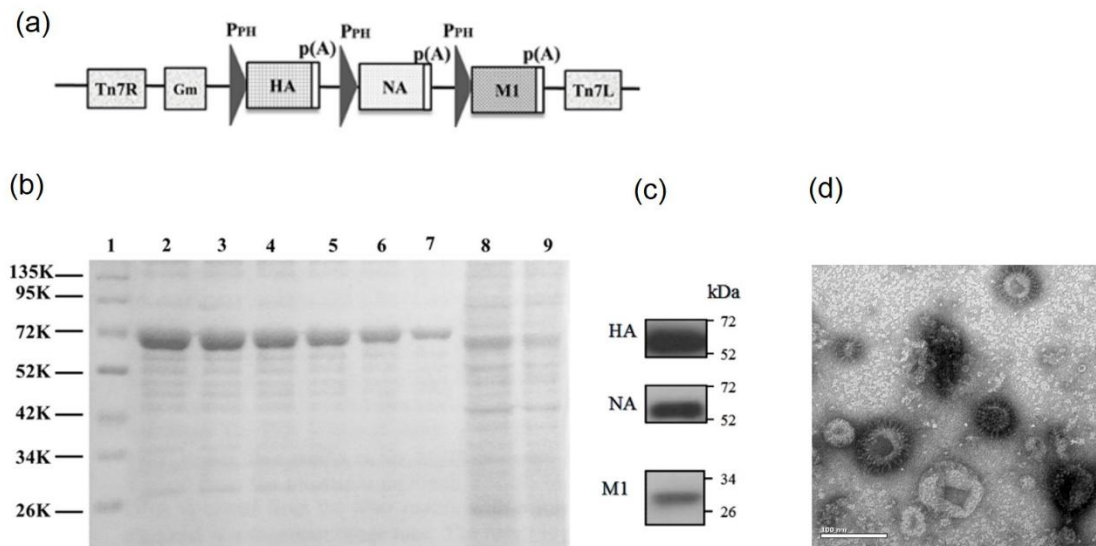


Fig. 1 (a) VLP expression cassette. Indicated are the polyhedrin promoter (P_{PH}), polyadenylation signal (p(A)), Tn7 regions, gentamicin resistance gene (Gm), and HA, hemagglutinin; NA, neuraminidase; M1, matrix 1 genes; (b) SDS-PAGE of purified VLPs stained with gel code reagent. Lane 1, Amersham full-range rainbow molecular weight marker; lanes 2-7, BSA 6 μg, 5 μg, 4 μg, 3 μg, 2 μg, 1 μg; lanes 8-9, 20 μL, 10 μL VLPs; (c) Western blot analysis of VLPs for HA, NA and M1 proteins; (d) Transmission electron micrograph of negatively stained VLPs (x 39,000).

damage (Fig. 3c). The lung sections of the mice vaccinated with *Panenza* exhibited some level of inflammatory responses, which were evidenced by lymphocyte infiltration in some area of the lung tissues, but no obvious tissue damage was observed (Fig. 3a). In contrast, no histopathological changes were found in lung tissues of the mice vaccinated with VLPs (Fig. 3b).

3.4 VLP Vaccination Induced Higher Levels of Protective Antibodies

To understand the underlined mechanism as to why VLP vaccination could provide more potent protection against the lethal challenge of the virus, serial assays were performed. As shown in Fig. 4a, the titer of HA-specific IgG antibody was much higher in VLP vaccinated group than that in *Panenza* vaccinated group. HI assay is considered to be the gold standard to evaluate the immunogenic efficiency in influenza vaccine studies, with an HI titer over 40 is considered as a representative marker for protection. While from Fig. 4b, the HI titer in *Panenza* vaccinated group was only around 20 which was not high enough to provide

potent protection. It is possible that VLPs are more immunogenic over the clinical *Panenza* vaccine because VLPs contain multiple copies of HA antigens presented in an organized array, thus allowing better activation of the immune responses. Neutralization antibody plays an important role in protecting against foreign antigens. Normally, neutralizing antibody titer reaching 20-40 may provide partial protection against lethal viral challenge, while it may provide complete protection against lethal viral infection when neutralizing antibody titer is over 40. From Fig. 4c, the neutralization titer in *Panenza* vaccinated group was only 20 which was not high enough to confer complete protection. However the neutralization titer in VLP vaccinated group reached 80 which was high enough to provide potent protection in mice.

3.5 VLP Vaccination Induced Higher Levels of Antibodies which Inhibited Virus Release from Infected MDCK Cells

It has been reported that NA vaccination can induce antibodies which may inhibit influenza virus release from the infected cells [18, 19]. The presence of NA

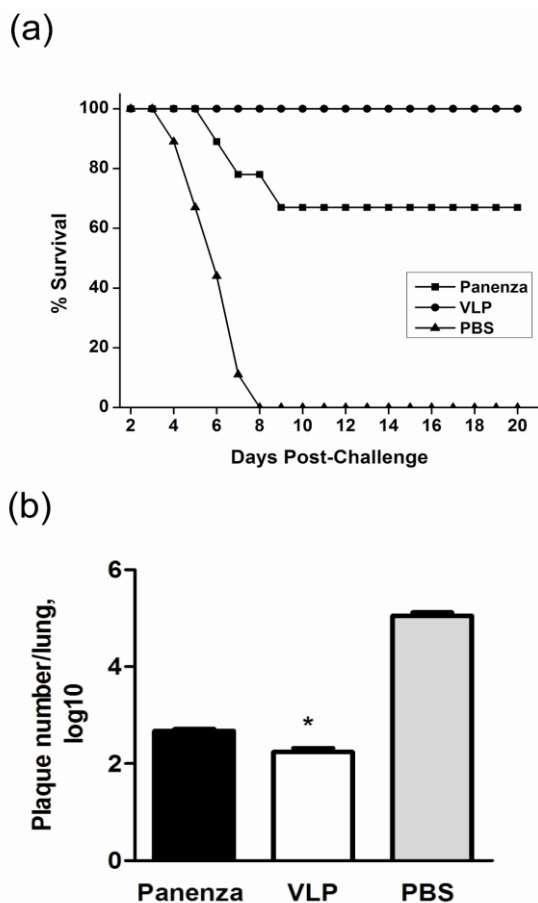


Fig. 2 Protection efficacy in BALB/c mice. On day 22 after vaccination, mice were challenged with 10LD₅₀ of influenza HK/01 virus. 5 days later, three mice in each group were sacrificed and the lungs were extracted for virus titer determination. The remaining 9 mice in each group were monitored for 20 days for survival rate calculation. (a) Mouse survival rate. Virus titer in lungs was determined by (b) plaque assay 5 days after lethal virus challenge. Statistical significance compared among VLP and *Panenza* vaccinated mice was indicated by student's *t*-test.

antibodies were verified by testing whether the sera from the vaccinated mice could inhibit virus release from infected cells. The serum samples were added to the cell cultures after the cells have been infected with high MOI (MOI = 3) of virus. The culture supernatant and cell pellets were collected at 6 h post-infection and the viral RNA copies were determined by real-time PCR. Viral RNA copies in cell pellets of all cultures showed similar levels (Figure not shown). Viral RNA copies in the culture supernatant in the presence of 20 folds diluted serum samples from mice vaccinated with

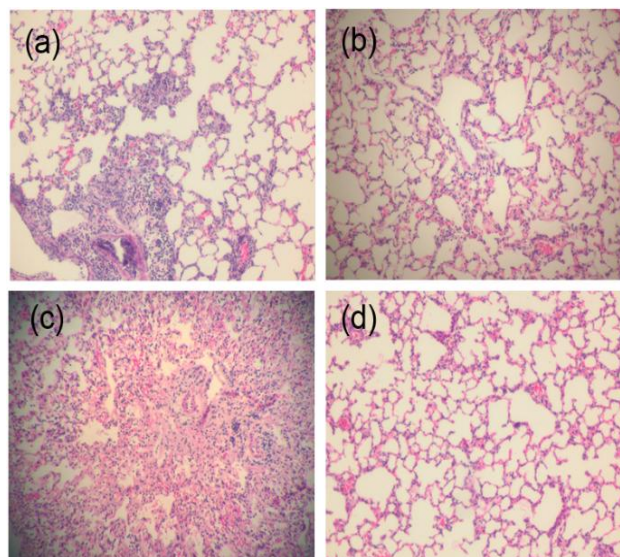


Fig. 3 Detection of histopathological changes in lung tissues after lethal challenge of HK/01 virus. On day 5 post-viral challenge, 3 mice in each group were sacrificed for lung tissue extraction. Lung tissues were collected from mice vaccinated with (a) *Panenza* vaccine; (b) VLP and (c) PBS control at 5 days post-virus challenge; (d) Lung section from uninfected normal mouse was included as control. Histopathological changes were detected by H&E staining.

VLP and *Panenza* vaccine were about 10 folds and 2 folds lower, respectively, than in the presence of sera from the mice vaccinated with the PBS control (Fig. 5). The results suggested that mice vaccinated with VLPs elicited higher levels of neutralizing NA antibodies than in those vaccinated with the *Panenza* vaccine.

3.6 VLP Vaccination Evoked Strong Cell-Mediated Immunity in Mice

The other important protective factors against viral infections that potentially induced by vaccination are cell-mediated immune responses [19, 20]. Thus, we further evaluated viral specific T help cell and CTL responses in the vaccinated mouse spleens (Fig. 6). The results showed that VLP vaccination indeed induced significantly higher levels of HA specific IL-2 and IFN- γ secreting Th1 cells than that of *Panenza* vaccination. Importantly, VLP vaccination elicited significantly higher levels of HA and NA specific CTL responses. T cell responses, particularly CTL responses, are very important in limiting viral infection and

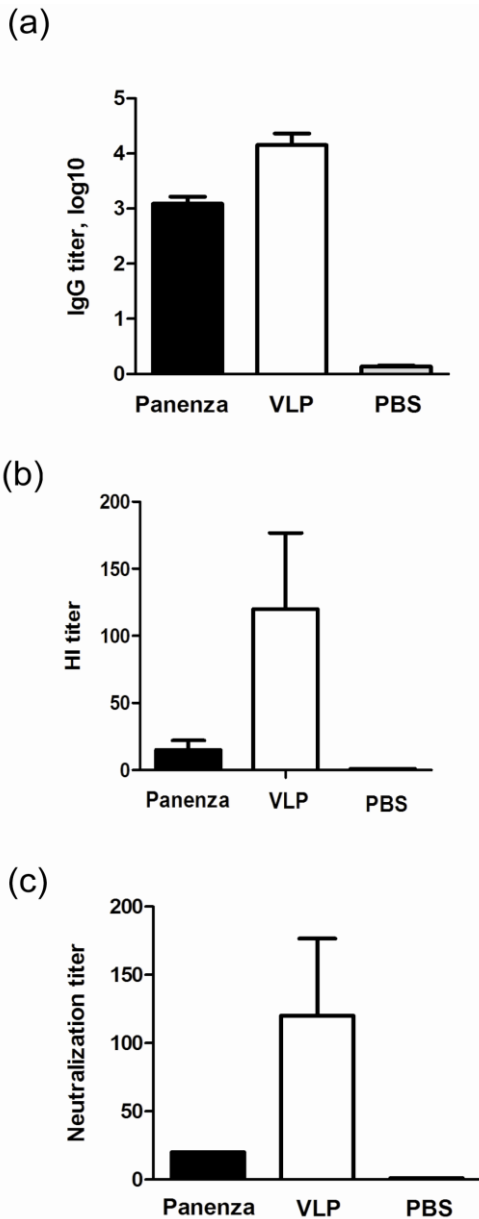


Fig. 4 Antibody responses. On day 21, mouse sera in all three groups vaccinated with *Panenza*, VLP and PBS were collected. (a) IgG titer; OD (Optical densities) were read at 450 nm; (b) HI titer and (c) Neutralization antibody titer, were determined. Results are expressed as the arithmetic means \pm SD ($n = 3$). Statistical significance compared between samples from VLP and *Panenza* vaccinated mice was indicated by student's *t*-test.

response to the virus clearance [21]. Thus, the results indicated that high levels of T cell responses evoked by VLP vaccination should play a key role, at least in part, in the potent protection against lethal viral infection.

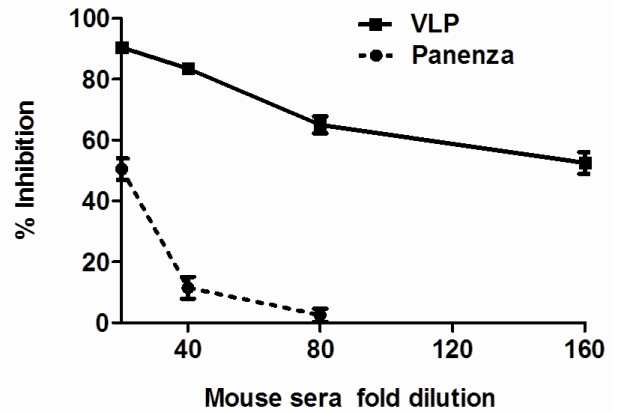


Fig. 5 Detection of inhibitory effect of vaccinated mouse sera to the virus release. MDCK cells were infected with HK/01 virus at MOI of 3. Mouse sera were added to the culture supernatants at 2 h post-infection, the supernatants were collected at 6 h post-infection and the viral RNA copies were measured by real-time PCR. The results are expressed as relative viral RNA copies \pm SD.

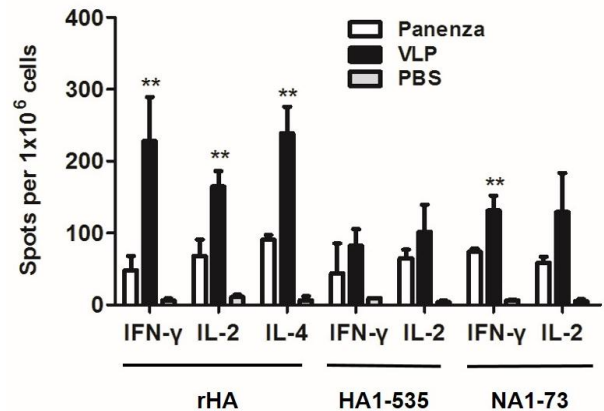


Fig. 6 Detection of specific T helper cell and CTL responses in vaccinated mice. Splenocytes were collected 21 days post-vaccination. Splenocytes (1×10^6) were stimulated with rHA (for T help cells), HA1-535 and NA1-73 (both for CTLs). Influenza HA specific IFN- γ , IL-2 and IL-4 screening T helper cells, HA1-535 and NA1-73 specific IFN- γ and IL-2 producing CTLs were detected by ELISpot. The results are presented as the mean values of two experiments \pm SD. Statistical significance (** $P < 0.05$) compared among VLP and *Panenza* vaccinated mice was indicated by student's *t*-test.

4. Conclusions

In this study, the immune responses and efficacy of protection induced by VLPs were compared with those elicited by the commercial *Panenza* vaccine in female BALB/c mice for the first time. Our results indicate

that VLPs are highly immunogenic and they can induce higher and broader immune responses than the clinically used *Panenza* vaccine, which reinforce that influenza vaccine using VLPs is quite a promising alternative in the future.

Acknowledgments

We would like to thank Professor KY Yuen (Department of Microbiology, HKU) who kindly offered us the clinically used *Panenza* vaccine. We would also thank Mr. WS Lee (Electron Microscope Unit, HKU) for his technical assistance.

References

- [1] B. Manicassamy, R.A. Medina, R. Hai, T. Tsibane, S. Stertz, E. Nistal-Villan, et al., Protection of mice against lethal challenge with 2009 H1N1 influenza A virus by 1918-like and classical swine H1N1 based vaccines, *PLoS Pathog.* 6 (1) (2010) e1000745.
- [2] WHO, Influenza A (H1N1): Special Insights, <http://www.who.int/en>, 2009.
- [3] T. Horimoto, Y. Kawaoka, Designing vaccines for pandemic influenza, *Curr. Top Microbiol. Immunol.* 333 (2009) 165-176.
- [4] S.M. Kang, J.M. Song, F.S. Quan, R.W. Compans, Influenza vaccines based on virus-like particles, *Virus Res.* 143 (2) (2009) 140-146.
- [5] T.M. Ross, K. Mahmood, C.J. Crevar, K. Schneider-Ohrum, P.M. Heaton, R.A. Bright, A trivalent virus-like particle vaccine elicits protective immune responses against seasonal influenza strains in mice and ferrets, *PLoS One* 4 (6) (2009) e6032.
- [6] D. Matassov, A. Cupo, J.M. Galarza, A novel intranasal virus-like particle (VLP) vaccine designed to protect against the pandemic 1918 influenza A virus (H1N1), *Viral Immunol* 20 (3) (2007) 441-452.
- [7] A. Prel, G. Le Gall-Recule, V. Jestin, Achievement of avian influenza virus-like particles that could be used as a subunit vaccine against low-pathogenic avian influenza strains in ducks, *Avian Pathol.* 37 (5) (2008) 513-520.
- [8] R.A. Bright, D.M. Carter, C.J. Crevar, F.R. Toapanta, J.D. Steckbeck, K.S. Cole, et al., Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle, *PLoS One* 3 (1) (2008) e1501.
- [9] K. Mahmood, R.A. Bright, N. Mytle, D.M. Carter, C.J. Crevar, J.E. Achenbach, et al., H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses, *Vaccine* 26 (42) (2008) 5393-5399.
- [10] P. Pushko, T.M. Tumpey, F. Bu, J. Knell, R. Robinson, G. Smith, Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice, *Vaccine* 23 (50) (2005) 5751-5759.
- [11] S.M. Kang, D.G. Yoo, A.S. Lipatov, J.M. Song, C.T. Davis, F.S. Quan, et al., Induction of long-term protective immune responses by influenza H5N1 virus-like particles, *PLoS One* 4 (3) (2009) e4667.
- [12] M.A. D'Aoust, P.O. Lavoie, M.M. Couture, S. Trepanier, J.M. Guay, M. Dargis, et al., Influenza virus-like particles produced by transient expression in *Nicotiana benthamiana* induce a protective immune response against a lethal viral challenge in mice, *Plant Biotechnol. J.* 6 (9) (2008) 930-940.
- [13] Z. Wen, L. Ye, Y. Gao, L. Pan, K. Dong, Z. Bu, et al., Immunization by influenza virus-like particles protects aged mice against lethal influenza virus challenge, *Antiviral Res.* 84 (3) (2009) 215-224.
- [14] J.M. Galarza, T. Latham, A. Cupo, Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge, *Viral Immunol.* 18 (1) (2005) 244-251.
- [15] A.L. Balish, J.M. Katz, A.I. Klimov, Influenza: Propagation, Quantification, and Storage, *Current Protocols in Microbiology.* (2013) 15G.1.1–15G.1.24.
- [16] R.J. Russell, L.F. Haire, D.J. Stevens, P.J. Collins, Y.P. Lin, G.M. Blackburn, et al., The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design, *Nature* 443 (7107) (2006) 45-49.
- [17] S. Kodihalli, H. Goto, D.L. Kobasa, S. Krauss, Y. Kawaoka, R.G. Webster, DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice, *Journal of Virology* 73 (3) (1999) 2094-2098.
- [18] J.H. Kim, J. Jacob, DNA vaccines against influenza viruses, *Vaccines for Pandemic Influenza* (2009) 197-210.
- [19] A. Allison, Cell-mediated immune responses to virus infection and virus-induced tumors, *British Medical Bulletin* 23 (1) (1967) 60-65.
- [20] G. Cambridge, J. Mackenzie, D. Keast, Cell-mediated immune response to influenza virus infections in mice, *Infection and immunity* 13 (1) (1976) 36-43.
- [21] G. Deliyannis, D.C. Jackson, N.J. Ede, W. Zeng, I. Hourdakakis, E. Sakabetis, et al., Induction of long-term memory CD8+ T cells for recall of viral clearing responses against influenza virus, *Journal of virology* 76 (9) (2002) 4212-4221.