# ORIGINAL ARTICLE

# Protection of guinea pigs by vaccination with a recombinant swinepox virus co-expressing HA1 genes of swine H1N1 and H3N2 influenza viruses

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Abstract Swine influenza (SI) is an acute respiratory infectious disease of swine caused by swine influenza virus (SIV). SIV is not only an important respiratory pathogen in pigs but also a potent threat to human health. Here, we report the construction of a recombinant swinepox virus (rSPV/H3-2A-H1) co-expressing hemagglutinin (HA1) of SIV subtypes H1N1 and H3N2. Immune responses and protection efficacy of the rSPV/H3-2A-H1 were evaluated in guinea pigs. Inoculation of rSPV/H3-2A-H1 yielded neutralizing antibodies against SIV H1N1 and H3N2. The IFN- $\gamma$  and IL-4 concentrations in the supernatant of lymphocytes stimulated with purified SIV HA1 antigen were significantly higher (P < 0.01) than those of the control groups. Complete protection of guinea pigs against SIV H1N1 or H3N2 challenge was observed. No SIV shedding was detected from guinea pigs vaccinated with rSPV/ H3-2A-H1 after challenge. Most importantly, the guinea pigs immunized with rSPV/H3-2A-H1 did not show gross

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and micrographic lung lesions. However, the control guinea pigs experienced distinct gross and micrographic lung lesions at 7 days post-challenge. Our data suggest that the recombinant swinepox virus encoding HA1 of SIV H1N1 and H3N2 might serve as a promising candidate vaccine for protection against SIV H1N1 and H3N2 infections.

# Introduction

Swine influenza (SI) is an acute respiratory infectious disease of swine caused by influenza A virus of the genus Influenzavirus A of the family Orthomyxoviridae. Swine influenza virus (SIV) H1N1 and H3N2 are the dominant subtypes causing SI diseases in China and other countries in the world [9]. Infection with SIV damages swine health and decreases swine production yields. More importantly, swine can be infected simultaneously by avian and mammalian influenza viruses because swine lung epithelial cells possess receptors that are recognized by both avian and mammalian influenza viruses [6]. Swine have been identified as an intermediate host in the process of genetic reassortment between influenza viruses from different hosts, which can lead to new pandemic strains of human influenza virus [21]. Therefore, it is clear that controlling swine influenza is important for preventing formation of new influenza virus strains.

The genome of SIV consists of eight segments of singlestranded, negative-sense RNA. The gene of RNA segment 4 encodes the large hemagglutinin (HA) glycoprotein, which is considered the main immune antigen, and it can induce protective humoral and cellular immune responses in animals [4]. HA is split into the glycoprotein subunits HA1 and HA2, with HA1 containing most of the antigenic epitopes of the HA glycoprotein [1]. Swinepox virus (SPV) is the only member of the genus *Suipoxvirus*, which is one of eight genera within the subfamily *Chordopoxvirinae* of the family *Poxviridae*. SPV is only known to infect porcine species [13]. Infection in nature is usually mild and occasionally causes localized skin lesions that heal naturally [19]. Poxviruses potentially induce both humoral and cellular immune response, and they have been shown to display foreign antigens to the immune system in various disease models [8, 18]. In addition, laboratory manipulation of poxvirus DNA for various applications has become almost standard practice, making it easy to develop recombinant vaccines.

To date, no commercial vaccines against SI are available in China even though SIV is common in swine populations in this country. There is an urgent need to develop effective strategies to control SI to prevent influenza virus replication in swine and thus decrease the possibility of creating potentially pandemic influenza viruses by reassortment. The aim of the present study was to construct a recombinant swinepox virus that simultaneously expresses HA1 genes of SIV H1N1 and H3N2, and to examine its immunogenicity and protective efficacy in guinea pigs.

# Materials and methods

#### Viruses and cells

Wild-type swinepox virus (wtSPV, Kasza strain, ATCC VR-363) and porcine kidney cells (PK-15, ATCC CCL-33) were purchased from the American Type Culture Collection. A crude viral stock was prepared, and the recombinant SPV and wtSPV titers were determined as described previously [8]. SIV H1N1 (A/swine/Shanghai/1/2005) and H3N2 (A/swine/ Guangxi/1/2004) strains in this study, provided by Dr. Xian Qi (Jiangsu Provincial Center for Disease Prevention and Control), were propagated only once in specific-pathogenfree embryonated eggs (Nanjing Veterinary Drug and Instrument Factory, Nanjing, China). Madin-Darby canine kidney cells (MDCK) were used for SIV isolation and titration assay. All experiments involving SIV H1N1 and H3N2 were conducted using biosafety level 3 procedures.

#### Construction of recombinant swinepox virus plasmid

The swinepox virus vector pUSZ11 had been constructed previously [8]. The promoter in pUSZ11 is from the vaccinia virus P11 sequence. SIV H3N2 HA1 (named H3, 984 bp, nucleotides 78 to 1061 from GenBank ID FJ157986), foot-and-mouth disease virus 2A (plus the N-terminal proline of 2B; 51 bp, nucleotides 3865 to 3915 from GenBank ID HQ412603), and SIV H1N1 HA1 (named H1, 981 bp, nucleotides 84 to 1064 from GenBank ID EU502884) genes were chemically synthesized together (a start codon ATG was added before H3, and stop codons TAATAATAA were present at the end of H1) by Invitrogen Biotechnology Co. Ltd. (Shanghai, China). This synthetic DNA, named the "H3-2A-H1 gene" (2037 bp), was cloned first in pUC19 and then cloned into the BamHI site of pUSZ11. The correct insertion of the H3-2A-H1 gene in pUSZ11 was confirmed by DNA sequencing with specific primers (PF, 5'-gttataggtaccagtagaatttcattttg-3'; PR, 5'ctatttgggggatccttattatctagattg-3'), which we designated as pUSZ11/H3-2A-H1 (Fig. 1). The H3-2A-H1 gene was inserted between genes 020 and 021 in wtSPV.

Generation and screening of the recombinant swinepox virus

A pre-confluent monolayer of PK-15 cells grown in a 60-mm-diameter plate was infected with wtSPV (MOI 0.02) for 2 h, and subsequently transfected with 8 µg of the pUSZ11/H3-2A-H1 plasmid using Lipofectamine 2000 (Invitrogen, Shanghai, China). After 4 days of incubation, cells were collected and lysed. An appropriate dilution of the lysate was used to infect PK-15 cells. After adsorption of the virus, 3 ml of medium containing 1 % low-melting-point agarose (TaKaRa, Shanghai, China) was added to the cells, and the incubation continued for 5 days until foci became visible. Blue foci were visualized after 1 to 2 days by addition of a second overlay medium containing 200 µg/ml X-gal (Tiangen, Nanjing, China). Plugs of agarose surrounding the stained foci were resuspended in 0.3 ml of medium with 2 % FBS, and recombinant viruses were released by freezing and thawing. Isolation of blue foci was repeated for 5 to 6 rounds until all foci in a given well were stained blue. The recombinant SPV bearing HA1 of SIV H1N1 and H3N2 was designated as rSPV/H3-2A-H1. The growth kinetics of the recombinant SPV/H3-2A-H1 was determined and compared with wtSPV.

Identification of expressed SIV proteins by indirect immunofluorescence assay and western blot

An indirect immunofluorescence assay (IFA) was performed to detect SIV H1N1 and H3N2 HA1 gene expression from rSPV/H3-2A-H1. IFA was performed as described previously [8]. rSPV/H3-2A-H1- and wtSPV-infected PK-15 cells were incubated with SI H1N1- or H3N2- positive convalescent serum (1:1000 dilution), and cells were stained with staphylococcal protein A-FITC (SPA-FITC, Boshide, 1:10000 dilution). After a final wash, all wells were examined by fluorescence microscopy (Zeisss, Germany).

Protein expression by rSPV/H3-2A-H1 was further assessed by western blot (WB) analysis. The western blot was carried out as described previously [8] using



Fig. 1 Schematic diagram of the construction of SIV H3N2 and H1N1 HA1 recombinant SPV transfer vector pUSZ11/H3-2A-H1 using the 2A linker. H3, HA1 gene of SIV H3N2; 2A, 2A gene of

foot-and-mouth disease virus; H1, HA1 gene of SIV H1N1; LF, left flanking region; RF, right flanking region; AP<sup>r</sup>, anti-penicillin gene; lacZ, the *E. coli* lacZ gene

H1N1- and H3N2 SI-positive convalescent sera. The serum was diluted 1:1000 and used as the primary antibody, and then membranes were soaked in staphylococcal protein A-HRP (1:3000 dilution; Boshide, Wuhan, China). The membranes were then developed with 3, 3'-diaminobenzidine substrate (Tiangen, Nanjing, China) until optimum color development was observed.

# Vaccination and virus challenge

Thirty female Hartly guinea pigs weighing 200-300 g each (provided by the Animal Center of Nanjing Medical University, Nanjing, China) were randomly divided into six groups (five guinea pigs per group). Each group was housed separately in an individual specific-pathogen-free isolation room.

Guinea pigs in groups 1 and 2 were inoculated in all four legs with  $0.4 \times 10^{7.0}$  TCID<sub>50</sub> of rSPV/H3-2A-H1 in 0.4 ml EMEM. In groups 3 and 4, each guinea pig was inoculated with  $0.4 \times 10^{7.0}$  TCID<sub>50</sub> wtSPV in 0.4 ml EMEM. In groups 5 and 6, each guinea pig was inoculated with 0.4 ml of EMEM. All inoculations were administered intramuscularly twice at 1 and 21 days post-inoculation (dpi). At 28 dpi, peripheral blood mononuclear cells (PBMCs) of five guinea pigs of each group were isolated to evaluate H1N1 and H3N2 SIV-specific T lymphocyte proliferation responses as well as H1N1 and H3N2 SIV- stimulated production of IFN- $\gamma$  and IL-4. At 35 dpi, serum samples (n = 5) were obtained for detection of antibodies against SIV H1N1 and H3N2.

At 35 dpi, the guinea pigs in groups 1, 3 and 5 were challenged by nasal inoculation with  $0.4 \times 10^{5.0}$  TCID<sub>50</sub> SIV H1N1, and those in groups 2, 4 and 6 were challenged with  $0.4 \times 10^{5.0}$  TCID<sub>50</sub> SIV H3N2. To reduce the possibility of secondary bacterial infections, oxytetracycline (25 mg/kg) was given IM at the time of challenge and once again at 2 days post-challenge (dpc). Clinical signs including hyponoia, inappetence, body temperatures, coughing, labored breathing, nasal discharge were recorded by observing the guinea pigs for 7 days. Each clinical symptom was given a numerical score of 0 to 3 points according to its severity. Finally, clinical sign scores were calculated as the sum of all clinical symptom scores of each guinea pig divided by 7. Nasal swabs from each

guinea pig were collected daily from 0 through 7 dpc to detect virus shedding by titration on MDCK cells. At 7 dpc, all of the guinea pigs were humanely euthanized. Lungs were examined for gross and microscopic lesions. The degree of consolidation on the surfaces of each lung was measured with a ruler. Lung pathological scores were calculated as the percent consolidation of each lung. Two lung tissue samples from each guinea pig were collected to test for the presence of virus.

All experimental protocols involving guinea pigs were approved by the Laboratory Animal Monitoring Committee of Jiangsu Province.

#### Assay for neutralizing antibodies

All serum samples from guinea pigs were heat inactivated (56 °C, 30 min) and used for neutralizing assay on MDCK cells. Serial twofold dilutions of serum were incubated with a virus dose of 100 TCID<sub>50</sub> of H1N1 or H3N2 SIV for 1 h at 37 °C and then transferred to preformed monolayers of MDCK cells in quadruplicate in 96-well tissue culture plates. Then, the plates were incubated and observed daily for up to 4 days for the appearance of a cytopathic effect (CPE). SIV H1N1- and H3N2-positive and negative sera were used as positive and negative controls, respectively. The titers of neutralizing antibodies (NA) were expressed as the highest serum dilution at which no CPE was observed.

#### Cytokine assay

PBMCs (5 × 10<sup>6</sup>/ml, 100 µl/well) from heparinized blood of guinea pigs at 28 dpi were stimulated with purified H1N1 or H3N2 SIV HA1 antigen at a final concentration of 10 µg/ml. After 66 h, the supernatant fluids were collected to examine the levels of the Th1-type cytokine IFN- $\gamma$  and the Th2-type cytokine IL-4 using commercially available cytokine ELISA kits (Groundwork Biotechnology Diagnosticate Ltd.) according to the manufacturer's instructions.

SIV isolation and titration from nasal swabs and lungs

The presence of virus in the nasal swabs and lungs was determined by the appearance of CPE in MDCK cell cultures, and SIV titrations of positive nasal swabs were carried out as described previously [22]. The titer was calculated using the Reed-Muench method.

# Statistical analysis

Data were analyzed using ANOVA (single-factor analysis of variance), and P-values lower than 0.05 were considered statistically significant.

# Results

Characterization and growth kinetics of the recombinant swinepox virus

The correct insertion of the H3-2A-H1 gene in rSPV/H3-2A-H1 was confirmed by DNA sequencing as described in Materials and methods, with the specific primers PF and PR. The results of IFA showed that rSPV/H3-2A-H1infected PK15 cells could be stained with SIV-specific antibodies and SPA-FITC, but wtSPV-infected cells could not be stained (Fig. 2A). Meanwhile, the results of WB showed that the band of about 36 kDa is consistent with the predicted size of H1, and the band of about 38 kDa is consistent with the predicted size of H3-2A (Fig. 2B). Obviously, the polyprotein had not been completely separated by the 2A sequence, because a band about 75 kDa of the polyprotein H3-2A-H1 could also be seen in the WB (Fig. 2B). Thus, a recombinant SPV/H3-2A-H1 simultaneously expressing the HA1 proteins of SIV H1N1 and H3N2 in vitro had been identified.

Recombinant swinepox viruses were blue-focus-purified five times and titrated on PK-15 cells, and the  $TCID_{50}$  of

rSPV/H3-2A-H1 was determined to be  $10^{1.1}$ ,  $10^{3.0}$  and  $10^{7.0}$  TCID<sub>50</sub>/ml, and the TCID<sub>50</sub> of wtSPV to be  $10^{1.2}$ ,  $10^{3.2}$  and  $10^{7.1}$  TCID<sub>50</sub>/ml, at 2, 4 and 6 days after infection. Moreover, there was no visible difference between the plaque sizes of rSPV/H3-2A-H1 and wtSPV according to our observation.

rSPV/H3-2A-H1 induces production of neutralizing antibodies in guinea pigs

To evaluate whether rSPV/H3-2A-H1 could induce the production of neutralizing antibodies against SIV H1N1 and H3N2 in guinea pigs, serum samples were tested by microneutralization assay. The H1N1 or H3N2 SIV-specific neutralizing antibody titers at 35 dpi are shown in Table 1. The guinea pigs that were vaccinated with rSPV/H3-2A-H1 had a mean neutralizing antibody titer of 1:16 against SIV H1N1 and H3N2 at 35 dpi.

# rSPV/H3-2A-H1 induces Th1-type and Th2-type cytokine responses in guinea pigs

The concentrations of IL-4 and IFN- $\gamma$  in the supernatants of PBMCs from guinea pigs of groups 1, 3 and 5, stimulated with the purified SIV H1N1 HA1, were detected by ELISA. As shown in Fig. 3A, at 28 dpi, the IL-4 concentrations were 107.01 pg/ml, 110.14 pg/ml and 222.18 pg/ml, and the IFN- $\gamma$  concentrations were 107.44 pg/ml, 113.40 pg/ml and 250.88 pg/ml in the EMEM-, wtSPV- and rSPV/H3-2A-H1-inoculated group, respectively. Meanwhile, the concentrations of IL-4 and IFN- $\gamma$  in the supernatants of PBMCs from guinea pigs of groups 2, 4, and 6, stimulated with the purified SIV H3N2



**Fig. 2** Characterization of the recombinant swinepox virus. **A**: Identification of rSPV/H3-2A-H1 expression by IFA. **a**, **c**: rSPV/H3-2A-H1-infected PK-15 cells. **b**, **d**: wtSPV-infected PK-15 cells. **a** and **b**: the primary antibody was an SIV-H1N1-positive serum; **c**, **d**: the primary antibody was an SIV-H3N2-positive serum. The secondary

antibody was a staphylococcal protein A-FITC conjugate. **B**: Identification of rSPV/H3-2A-H1 expression by western blot analysis. **a**: Lane 1, wtSPV; lane 2, rSPV/H3-2A-H1. **b**: Lane 1, rSPV/H3-2A-H1; lane 2, wtSPV. In **a**, the primary antibody was an SIV-H3N2-positive serum. In **b**, the primary antibody was an SIV-H1N1-positive serum

Table I Neutralizing
antibodies and protection result
of vaccination after challenge
in guinea pigs

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Table 1         Neutralizing           antibodies and protection results         of vaccination after challenge	Group <sup>a</sup>	Animal no.	NA (H1N1) at 35 dpi <sup>b</sup>	NA (H3N2) at 35 dpi	Clinical sign score <sup>c</sup>	Gross lesion score <sup>d</sup>	SIV in lungs at 7 dpc <sup>e</sup>
<ul> <li><sup>a</sup> Guinea pigs in groups 1 and 2 were inoculated with rSPV/H3- 2A-H1, those in groups 3 and 4 were inoculated with wtSPV, and those in group 5 and 6 were inoculated with EMEM. At 35 dpi, the animals in groups 1, 3 and 5 were challenged with SIV H1N1, and those in groups 2, 4 and 6 were challenged with SIV H3N2</li> <li><sup>b</sup> The NA titers are expressed as the reciprocal of the value of the highest serum dilution at which no CPE was observed</li> </ul>	1	1.1	16	16	0	0	_
		1.2	32	16	0	0	_
		1.3	32	32	0	0	_
		1.4	8	8	0	0	_
		1.5	16	32	0	0	_
	2	2.1	16	16	0	0	_
		2.2	16	16	0	0	_
		2.3	16	8	0	0	_
		2.4	8	16	0	0	_
		2.5	32	32	0	0	_
	3	3.1	<4	<4	4.2	64	+
		3.2	<4	<4	3.7	51	+
		3.3	<4	<4	5.4	75	+
		3.4	<4	<4	2.6	54	+
		3.5	<4	<4	3.1	61	+
	4	4.1	<4	<4	3.3	62	+
		4.2	<4	<4	4.6	58	+
		4.3	<4	<4	3.0	75	+
		4.4	<4	<4	3.4	69	+
<ul> <li><sup>c</sup> Each clinical sign was given a numerical score of 0 to 3 points according to its severity.</li> <li>Clinical sign scores were calculated as the sum of all clinical sign scores of each guinea pig divided by 7</li> <li><sup>d</sup> The gross lung lesion score was calculated as the percent consolidation of each lung</li> </ul>		4.5	<4	<4	4.2	51	+
	5	5.1	<4	<4	4.1	66	+
		5.2	<4	<4	3.8	69	+
		5.3	<4	<4	5.3	50	+
		5.4	<4	<4	2.7	61	+
		5.5	<4	<4	5.6	75	+
	6	6.1	<4	<4	3.0	71	+
		6.2	<4	<4	5.3	60	+
<sup>e</sup> Positive results were confirmed if one or two samples from one guinea pig were SIV positive		6.3	<4	<4	4.2	50	+
		6.4	<4	<4	3.9	68	+
		6.5	<4	<4	3.1	55	+

HA1, were also detected. As shown in Fig. 3B, at 28 dpi, the IL-4 concentrations were 99.01 pg/ml, 114.14 pg/ml and 220.40 pg/ml, and the IFN- $\gamma$  concentrations were 107.04 pg/ml, 119.39 pg/ml and 261.23 pg/ml in the EMEM-, wtSPV- and rSPV/H3-2A-H1-inoculated group, respectively. The concentrations of SIV H1N1- and H3N2-specific IL-4 and IFN- $\gamma$  in the rSPV/H3-2A-H1 groups were both significantly higher (P < 0.01) than those in the control groups. Moreover, the H1N1 and H3N2 SIV-specific T lymphocyte proliferation responses from the group of guinea pigs inoculated with rSPV/ H3-2A-H1 were significantly higher (P < 0.05) than those of either the wtSPV- or EMEM-treated guinea pigs at 28 dpi (data not shown). These results suggest that rSPV/ H3-2A-H1 potentiated the Th1-type and Th2-type cytokine responses.

Protective efficacy of rSPV/H3-2A-H1 against challenge in guinea pigs

#### Clinical signs

To determine the protective efficacy of rSPV/H3-2A-H1 against challenge with SIV H1N1 or H3N2, at 35 dpi, the animals in groups 1, 3 and 5 were challenged with SIV H1N1 (A/swine/Shanghai/1/2005), and those in groups 2, 4 and 6 were challenged with SIV H3N2 (A/swine/Guangxi/ 1/2004). Daily clinical evaluation was carried out for 7 days. Clinical sign scores are summarized in Table 1. During the 7-day observation period, labored breathing and increased production of mucus in the nasal passages were observed in the wtSPV and medium control groups, and especially, nasal discharge was significantly increased



**Fig. 3** Concentration of IL-4 and IFN- $\gamma$  in the supernatants of stimulated guinea pig PBMCs. The PBMCs isolated from guinea pigs (n = 5) at 28 dpi were stimulated with purified SIV H1N1 or H3N2 HA1 antigen. After 66 h, the supernatants were collected to examine the levels of IFN- $\gamma$  and IL-4. Data are shown as mean  $\pm$  SEM. **A**: the PBMCs were stimulated with SIV H1N1 HA1 antigen; **B**: the PBMCs were stimulated with SIV H3N2 HA1 antigen

2 days after infection. The guinea pigs immunized with rSPV/H3-2A-H1 did not show clinical signs (Table 1).

#### SIV isolation from nasal swabs and lungs

Viral growth in the nasal passages was assessed by collecting nasal swabs from all guinea pigs at 0, 1, 2, 3, 4, 5, 6 and 7 dpc. The results indicate that no SIV was shed from guinea pigs immunized with rSPV/H3-2A-H1 whether they were challenged with SIV H1N1 or H3N2. However, virus was shed from all test guinea pigs inoculated with wtSPV or EMEM after they were challenged with SIV H1N1 or H3N2 (Fig. 4A, B). Viral growth peaked at 2 and 3 dpc in



Fig. 4 Nasal shedding patterns after SIV H1N1 or H3N2 challenge. Data (n = 5) are shown as mean virus titer  $\pm$  SEM. All test guinea pigs were negative for SIV on the day of challenge. A: Nasal shedding patterns after SIV H1N1 challenge. B: Nasal shedding patterns after SIV H3N2 challenge

the nasal passages of animals challenged with SIV H1N1 or H3N2. Furthermore, virus was not cleared from the nasal passages up to 7 dpc. A decline in virus titer in nasal swabs from the control groups began at 4 dpc (Fig. 4). Most importantly, no SIV was isolated from the lungs in the rSPV/H3-2A-H1 group, but SIV was isolated from all lungs in the control groups at 7 dpc (Table 1).

#### Gross and microscopic lung lesions

The guinea pigs inoculated with wtSPV or EMEM (groups 3, 4, 5 and 6) experienced serious gross lung lesions and the area of surface consolidation of each guinea pig lung reached 50 % or more after they were challenged with SIV H1N1 or H3N2. However, guinea pigs inoculated with rSPV/H3-2A-H1 (groups 1 and 2) showed no gross pathological changes of the lung (Table 1). Consistent with the gross lung pathology observed above, guinea pigs inoculated with wtSPV and EMEM had prominent

histopathological changes of the lung, characterized by serious interstitial pneumonia, thickening of the interstitium of the alveolar walls by hyperplasia of interstitial substance, hyperemia, hemorrhage, fibrosis, and massive infiltration of lymphocytes and macrophages (Fig. 5C, D, E and F). In contrast, there was a significant reduction in the presence and severity of microscopic lesions in guinea pigs inoculated with rSPV/H3-2A-H1. Examination of lung sections of the guinea pigs in groups 1 and 2 revealed minimal interstitial pneumonia and slight alveolar wall thickening (Fig. 5A and B). All of these results indicate that rSPV/H3-2A-H1 inoculation provided complete protection against SIV H1N1 or H3N2 challenge in guinea pigs.

#### Discussion

In the light of the continuing threat of influenza virus, the availability of a safe and effective vaccine is crucial. In the present study, we have engineered a swinepox virus that co-expresses the HA1 proteins from SIV subtypes H1N1 and H3N2. Such a recombinant virus simultaneously expresses the HA1 antigens from H1N1 and H3N2 efficiently, induces neutralizing antibodies against SIV H1N1 and H3N2, potentiates strong Th1-type and Th2-type cytokine responses in guinea pigs, and demonstrates excellent protection efficacy in guinea pigs against challenge with the virulent homologous SIV H1N1 or H3N2. The protective role of antibodies against SIV has been



Fig. 5 Histopathological examination of guinea pig lungs in rSPV/ H3-2A-H1 (**A** and **B**), wtSPV (**C** and **D**) and EMEM (**E** and **F**) inoculation groups at 7 dpc. **A**, **C** and **E**: Guinea pigs were

challenged with SIV H1N1. **B**, **D** and **F**: Guinea pigs were challenged with SIV H3N2. Hematoxylin and eosin staining (HE). Magnification,  $200 \times$ 

demonstrated in various studies [7, 10, 20]. Previous studies on animals have shown that influenza virus HA can provide protection within a subtype via antibody-mediated immunity, and protective immunity is established even at low or undetectable levels of serum antibody to HA [16, 22]. This also indicates that cell-mediated immunity promotes virus clearance and is an important factor in recovery from SIV infections [3, 11, 12, 20]. Our current study demonstrates that the guinea pigs vaccinated with rSPV/ H3-2A-H1 are well protected from challenge although the mean neutralizing antibody titers were only 1:16. This result confirms the role of rSPV/H3-2A-H1-induced cellular immunity in protection. IFN- $\gamma$ , produced by CD4+ T helper cell type 1 (Th1), CD8+ cytotoxic T cells and NK cells, is a major immune-modulator for antiviral immunity [14], and we have shown that the guinea pigs immunized with rSPV/H3-2A-H1 produce not only significantly high levels of IL-4 but also high levels of IFN- $\gamma$ . All of these data indicate that rSPV/H3-2A-H1 can potentiate both Th1-type- and Th2-type-mediated immunity. In line with our data, several other studies demonstrate that poxvirusvector-based vaccines are capable of inducing potent humoral and T-cell-mediated immune responses [5].

In previous studies, we have separately constructed the two recombinant swinepox viruses expressing HA1 against swine H1N1 and H3N2 influenza virus and evaluated their immune responses and protective efficacy in mice and pig models [23, 24]. Our results showed that the two recombinant swinepox viruses not only induced immune responses in mice and pigs but also demonstrated excellent protection efficacy in pigs against challenge with virulent homologous SIV H1N1 or H3N2. In the present study, in order to examine the large capacity of SPV to package recombinant DNA and its ability to express more than one foreign gene at the same time, we constructed a swinepox virus co-expressing the HA1 proteins from SIV subtypes H1N1 and H3N2. No doubt, our experimental results are promising and inspiring.

The 2A sequence of FMDV, which encode a selfcleavage protease, has been used to enable the expression of two proteins from one cistron [2, 15]. In our study, two HA1 genes encoding proteins were linked via a 2A sequence to form a single ORF. The 2A oligopeptide (plus the N-terminal proline of 2B) is able to self-cleave at the site corresponding to the 2A/2B junction (-NFDLLKLAG DVESNPG P-)[17]. The 2A sequence remains as a C-terminal extension of the upstream product (H3), and the last proline forms the N-terminus of the downstream protein (H1). The expected sizes of the two proteins are both about 36 kDa, corresponding to the molecular weight of HA1 (signal peptide deleted) of SIV subtypes H3N2 and H1N1, but H3-2A is about 38 kDa. Obviously, the polyprotein had not been completely separated [17], because a band about 75 kDa, corresponding to the polyprotein H3-2A-H1, was seen in the WB (Fig. 2B). Fortunately, the results of the animal experiments indicate that rSPV/H3-2A-H1 can induce immune responses and provide excellent protection against SIV H1N1 or H3N2 infection in guinea pigs. Although the results of this study are very promising with regard to the immunogenicity and protective efficacy of rSPV/H3-2A-H1 in guinea pigs, we will need to investigate its immunogenicity, safety and effectiveness in protecting pigs against SIV H1N1 and H3N2 infections in future work.

In summary, is the first report of the use of SPV as a delivery vector for co-expression of HA1 proteins of SIV H1N1 and H3N2. Our data indicate that rSPV/H3-2A-H1 can induce HA1-specific neutralizing antibodies as well as HA1-specific IFN- $\gamma$  in guinea pigs. Administration of rSPV/H3-2A-H1 provides complete protection against virulent homologous SIV H1N1 or H3N2 challenge in guinea pigs. Thus, the recombinant swinepox virus encoding HA1 of SIV H1N1 and H3N2 might serve as a promising candidate vaccine for protection against SIV H1N1 and H3N2 infections.

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