RESEARCH REPORT
SWINE HEALTH

Title: Development of modified live attenuated virus vaccine expressing porcine IL-18 – **NPB #13-031** revised

Investigator: Wenjun Ma

Institution: Kansas State University

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Industry Summary: This proposal evaluated a live attenuated virus vaccine expressing porcine cytokine IL-18 in pigs that is expected to provide enhanced protection against different swine influenza virus (SIV) strains. A killed vaccine with adjuvants and a modified live attenuated virus vaccine that does not express IL-18 were used as controls. The results showed that the modified live virus expressing IL-18 was able to provide broad protection against different SIV strains including different subtype viruses, similar to the control modified live vaccine. However, the inactivated vaccine induced enhanced respiratory disease in vaccinated pigs (evidenced by enhanced lung pneumonia). While no difference was observed in efficacy between two live vaccines, this was probably due to the fact that the vaccine expressing IL-18 is too attenuated to replicate efficiently in pigs. The specific adjuvants can enhance efficacy of the whole virus inactivated vaccine when the vaccine strain does not match with the challenge virus. This study proves the concept that SIV vaccines could be improved with specific adjuvants or additional cytokines. The proposed project has improved our knowledge of influenza vaccine development to better control emerging and endemic swine influenza in US swine herds.

Keywords: swine influenza virus, modified live attenuated vaccine, IL18, heterologous challenge, heterologous protection, efficacy

Scientific Abstract: Novel swine influenza vaccine development should focus on efficacy, route of delivery and improvement of heterosubtypic immunity. This proposal utilizes a new strategy to develop a modified live influenza vaccine expressing porcine IL-18 (TX/98NS1Δ126-swIL18) since IL-18 has been shown to be an important immuno-modulator that is able to enhance the development of antigen-specific immunity and vaccine

efficacy. The TX/98NS1Δ126-swIL18 has been shown to stably express bioactive porcine IL18, and was tested in pigs challenged with heterovariant H3N2 and heterosubtypic H1N1 SIVs using the modified live influenza vaccine TX/98NS1Δ126 and the whole virus inactivated vaccine as controls. The results showed the TX/98NS1Δ126-swIL18 was able to reduce lung lesions, decrease viral nasal shedding and provide broad protection against multiple SIV strains including heterovariant and heterosubtypic SIVs, similar to the control vaccine TX/98NS1Δ126. However, the inactivated vaccine induced enhanced respiratory disease (evidenced by higher lung lesions) despite also decreasing virus shedding and virus replication. No difference was observed in efficacy between two live vaccines is probably due to the fact that the TX/98NS1Δ126-swIL18 is too attenuated to replicate efficiently in pigs. The specific adjuvants enhance cell-medicated immunity of the whole virus inactivated vaccine that most likely contributes to decreasing virus shedding and clearance. This study proves the concept that heterosubtypic immunity can be improved with vaccines containing specific adjuvants or additional cytokines. The proposed project has improved our knowledge on influenza vaccine development and correlates of heterosubtypic immunity to better control swine influenza in swine herds.

Introduction: Although commercial vaccines are available and are widely used in US swine herds, swine influenza is still not efficiently controlled and has become an endemic disease, resulting in significant economic losses for the US swine industry. Current SIV vaccines are traditional inactivated trivalent virus vaccines containing SIV H3N2 and 2 different H1N1 subtype viruses. There are 3 major difficulties with the use of current SIV vaccines: 1) SIV is antigenically changing faster than novel traditional vaccines can be developed; 2) Inactivated SIV vaccines do not provide good cross-protection among different SIV isolates, especially against heterovariant and heterosubtypic viruses; and 3) Passively acquired immunity can interfere and block vaccine efficacy in piglets. In the past years, this has led to a significant decrease in the sale of licensed commercial inactivated vaccines and to a significant increase in the production and use of autogenous vaccines (presently about 50% of the U.S. market). A priority for novel SIV vaccine development is the improvement of heterovariant and heterosubtypic immunity.

In order to improve heterovariant (within same subtype) and heterosubtypic protection, a vaccine needs to be developed which has the ability to induce a balanced immune response including humoral, mucosal and cell-mediated immunity. The advantage of modified live virus vaccines (MLV) is its enhanced stimulation of cell-mediated immunity, most likely directed against the conserved nucleoprotein (NP), thus providing more heterovariant and heterosubtypic protection. Attenuated SIV expressing a truncated NS1 protein with 126 amino acids (TX/98NS1Δ126) with promising vaccine potential has been generated via modification of the viral NS1 gene of an H3N2 (A/Swine/Texas/4199-2/98, TX/98) and shown that it is highly attenuated in pigs. Pigs vaccinated with TX/98NS1Δ126 virus were completely protected against a challenge with the homologous

TX/98 virus and partially protected against a challenge with a heterosubtypic H1N1 virus. A series of experiments have demonstrated that the NS1-truncated MLV vaccine appears to be more efficacious when compared to the inactivated vaccine, indicating that it is a promising vaccine candidate against SIVs.

Cell mediated or Th1 immunity towards virus antigens is dependent on the coordinated expression of several cytokines, including interferon-γ (IFN-γ) to assist in the production of antigen-specific cytotoxic T cells. Interleukin-18 (IL18) is a potent pleiotropic cytokine that induces the production of IFN-γ, enhances natural killer cell cytotoxicity and stimulates Th1 cell differentiation. IL18 is required for optimal cytokine production by influenza virus-specific $CD8^+$ T cells. A previous study has shown that co-administration of plasmid-derived IL18 as an adjuvant produced stronger $CD4^+$ and $CD8^+$ T cells responses in immunized mice, thereby enabling DNA vaccines to achieve enhanced immune responses. In addition, IL-18 has been demonstrated to shorten the induction time for CTLs and enhance antigen-specific lympho-proliferative responses and IFN-γ release when it was applied as a vaccine adjuvant in various animal species including mice, cats, rhesus macaques, pigs and chickens. All of these results indicate that IL-18 is an important immuno-modulator that is able to enhance the development of antigen-specific immunity and vaccine efficacy.

Novel SIV vaccine development should focus on efficacy, route of delivery and improvement of heterosubtypic immunity. This proposal utilizes a new strategy to develop a modified live influenza vaccine expressing porcine IL-18 (TX/98NS1Δ126-swIL18) that has been shown to stably express bioactive porcine IL18. This new vaccine candidate is expected to have enhanced heterovariant and heterosubtypic immunity compared to the former vaccine TX/98NS1Δ126 without IL-18.

Objectives: The overall aim of this project is to evaluate the efficacy of the MLV TX/98NS1Δ126 expressing porcine IL18 (TX/98NS1Δ126-swIL18) against challenge with heterovariant and heterosubtypic SIVs in pigs using both inactivated and MLV TX/98NS1Δ126 as controls and to determine the cellular and humoral responses induced by the different vaccine candidates.

Specific objectives:

a) To investigate pathogenicity of the TX/98NS1Δ126-swIL18 in pigs.

b) To evaluate efficacy of the TX/98NS1Δ126-swIL18 against challenge with heterovariant and heterosubtypic SIVs in pigs using both inactivated and MLV TX/98NS1Δ126 as controls.

c) To determine the cellular immune response induced by different swine influenza vaccines.

Materials & Methods:

Viruses and vaccines: Both MLV TX/98NS1Δ126 and MLV TX/98NS1Δ126-swIL18 viruses were used in the following vaccine study. A heterovariant A/swine/Kansas/10-91088/2010 (KS10 H3N2) that is a recent isolate belonging to the H3N2 Cluster IV, and a heterosubtypic H1N1 virus A/swine/Kansas/77778/2007 (KS07

H1N1) that is highly virulent for pigs were used for the challenge study. The whole virus inactivated vaccine was prepared by inactivating A/swine/Texas/4199-2/98 (TX/98 H3N2) using ultraviolet irradiation and adding a commercial adjuvant (Emulsigen D, MVP Labs). Per dose of inactivated vaccine includes 64 HA units of inactivated virus and 20% of Emulsigen D (v/v).

Animal studies

Pathogenicity of the MLV TX/98NS1Δ126-swIL18 in pigs

Experimental design (#1): The MLV TX/98NS1Δ126 has been shown to be highly attenuated in pigs and is a good vaccine candidate for swine influenza. To determine the pathogenicity of MLV TX/98NS1Δ126-swIL18 in pigs, nine 3-4 week-old SIV- and porcine reproductive and respiratory syndrome virus- seronegative pigs were allocated into 3 groups (3 pigs in infected groups and 3 pigs in the control group); pigs from each group were intratracheally inoculated with 10^6 $TCID_{50}$ of the MLV TX/98NS1Δ126-swIL18 or TX/98NS1Δ126 or with sera-free MEM as described previously. Clinical signs and rectal temperature were monitored daily for each pig during the course of the experiment. All infected pigs were necropsied at 5 days post infection (dpi). Nasal swabs were collected from each pig on 0, 3 and 5 dpi. During necropsy the lungs were removed *in toto* from pigs. The percentage of gross lesions on each lung lobe was scored by a single experienced veterinarian.

Efficacy of the MLV TX/98NS1Δ126-swIL18 against heterovariant and heterosubtypic SIVs in pigs

Experimental design (#2): Forty-eight 3-4 week-old SIV- and porcine reproductive and respiratory syndrome virus- seronegative pigs were allocated into 4 groups (12 pigs in each vaccine group and 12 pigs in the control group). Twelve pigs in groups 1 and 2 were intranasally inoculated with 10^6 $TCID_{50}$ of the MLV TX/98NS1Δ126 or TX/98NS1Δ126-swIL18 by slowly dripping 1 ml of inocula into the nasal cavity of each pig as indicated in Table 1 at day 21 before vaccination. Each pig in group 3 was intramuscularly injected with 1 ml of a whole inactivated vaccine (TX98 inactivated virus + adjuvant, HA titer 64) and each pig of the control group was intranasally inoculated with 1 ml of sera-free MEM. At 2 weeks post-vaccination, each pig in group 3 was boosted with 1 ml of the whole inactivated vaccine. One week after boost, 6 pigs in each vaccinated group and 6 pigs from the control group were moved to another individual room, then were intratracheally challenged with 10^6 $TCID_{50}$ of the heterovariant KS10 H3N2 or heterosubtypic KS07 H1N1 virus.

Table 1: Pig study design

	Pig numbers	**Vaccine**	**Boost**	**Challenge**
Group 1	12	MLV TX/98NS1Δ126	No	KS10 H3N2 (6 pigs) KS07 H1N1 (6 pigs)
Group 2	12	TX/98NS1Δ126-swIL18	No	KS10 H3N2 (6 pigs) KS07 H1N1 (6 pigs)
Group 3	12	Inactivated TX/98 H3N2	Yes	KS10 H3N2 (6 pigs) KS07 H1N1 (6 pigs)
Group 4	12	Control	No	KS10 H3N2 (6 pigs) KS07 H1N1 (6 pigs)

Clinical signs were monitored daily during the course of the experiment for each pig. Rectal temperatures for each pig were tested from 2 days before challenge to the end of experiment. Three challenged pigs from each room were necropsied on 3 and 5 day post infection (dpi). Nasal swabs were collected from each pig on 0, 3 and 5 dpi. Blood samples were collected from all pigs on day 0, 7, 14 and 21 post vaccination and on the day of necropsy. During necropsy the lungs were removed *in toto* from pigs. The percentage of gross lesions on each lung lobe was scored by a single experienced veterinarian. Bronchioaveolar fluid (BALF) was obtained by rinsing each lung with 50 mL MEM. The right cardiac lung lobe was collected and fixed in 10% buffered formalin, then stained with hematoxylin and eosion for histopathologic examination.

Viral titers of BALF and nasal swabs were determined in MDCK cells. Viral load in BALF was determined in a 96-well plate. Briefly, 10-fold serial dilutions of each sample were made in serum-free MEM supplemented with TPCK-trypsin and antibiotics. Each dilution (100 µl) was plated on PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for cytopathic effects after 24 to 72 h. At 72 h, plates were fixed with 4% phosphate-buffered formaldehyde and immunocytochemically stained with a monoclonal antibody to influenza A nucleoprotein. The $TCID_{50}$/ml was calculated for each sample by the method of Reed and Muench. Virus was isolated from nasal swab samples stored at -80°C by thawing and vortexing each sample for 15 sec, centrifuging it for 10 min at 640 x g, and passing the supernatant through 0.45-µm filters to reduce bacterial contamination. An aliquot of 100 µl was plated on confluent, PBS-washed MDCK cells in 48-well plates. After incubation for 1 h at 37°C, 500-µl serum-free MEM supplemented with 1 µg/ml TPCK trypsin and antibiotics was added. All wells were evaluated for cytopathic effects after 48–72 h. Subsequently, plates were fixed with 4% phosphate-buffered formaldehyde and stained as described above.

Cell mediated immunity assays

To measure CMI responses induced by different vaccines, IFNγ enzyme-linked immunospot (IFNγ-ELISpot) was performed. We cannot perform cell proliferation assay and phenotyping of T cell subsets (CD4+ and CD8+) due to the limited budget. Swine peripheral blood mononuclear cells (PBMC) were isolated from blood samples collected on day 14 and 21 post-vaccination and day 5 post infection. Nunc maxisorpt 96 well plates were coated with 10 µg/ml mouse anti-pig IFN γ (Thermo# MP70) in 0.1 M carbonate buffer (pH 9.6) for overnight at 4ºC. Plates were washed three times with PBS and subsequently incubated for two hours at 37°C with 100 µl per well of blocking buffer (PBS containing 4% dried skimmed milk). Plates were then washed three times with sterile PBS, and then incubated for 2 hours at 37°C with 50 µl per well of RPMI supplemented with 5% sera in 5% CO_2. Each well was seeded 5 x 10^5 of PBMC that mixed with the stimulus (10^6 $TCID_{50}$ of inactivated wild type TX98, KS07 or KS10 virus) or ConA as a positive control, then incubated for 3 days at

37°C in a 5% CO2, before harvesting supernatants to assess IFN γ production by ELISA or harvesting cells to quantify IFN γ –producing cells by ELISPOT. After 3 days incubation of PBMC cells with stimuli (inactivated wild type TX98, KS07 or KS10 virus), cells were washed off the plates using PBS containing 0.05% Tween-20, then 50 µl of biotinylated mouse anti-pig IFN γ (0.5 µg/ml, Thermo MP701B) per well was added to the plates for 1 hour at 37°C. Plates were washed three times with PBS containing 0.05% Tween-20, and then 50 µl of 0.31 ug/ml Streptavidin-HRP per well was added to the plates for 1 hour at 37°C. Plates were washed three times with PBS containing 0.05% Tween-20, and then 50 µl of TMB membrane peroxidase substrate per well was added until color change was observed. Plates were then rinsed with tap water and allowed to dry overnight at room temperature before counting the spots in a microscope. Results were expressed as IFN γ-producing cell number per 10^6 stimulated PBMC.

Results:

1. Pathogenicity of the MLV TX/98NS1Δ126-swIL18 in pigs. No obvious clinical symptoms were observed from pigs infected with either MLV TX/98NS1Δ126 or TX/98NS1Δ126-swIL18 virus, similar to the control animals. Only one pig infected with the TX/98NS1Δ126 showed fever (≥ 40ºC) for 2 days while no fever was observed from other pigs including those infected with the TX/98NS1Δ126-swIL18 virus. Minimal lung lesions were found in 2 out of 3 pigs infected with the TX/98NS1Δ126 virus, and only 1 out of 3 pigs infected with the TX/98NS1Δ126-swIL18 virus displayed very minimal lesions. No virus was detected from nasal swab samples collected from pigs infected with the TX/98NS1Δ126-swIL18 virus at 3 and 5 dpi, whereas virus with an average of 1,000 and 1,00 $TCID_{50}$/ml was detected from nasal swabs collected from all pigs infected with the TX/98NS1Δ126 virus at 3 and 5 dpi respectively. These results indicate that the TX/98NS1Δ126-swIL18 is much more attenuated than the TX/98NS1Δ126 virus.

2. Efficacy of the MLV TX/98NS1Δ126-swIL18 against a heterovariant SIV in pigs.

No obvious respiratory symptoms were observed in experimental pigs. In the heterovariant H3N2 KS10 virus challenge groups, all pigs (100%) from mock-vaccinated and inactivated vaccine immunization groups displayed fever, lasting for 2-5 days, whereas 4 out of 6 (66.7%) pigs immunized with either TX/98NS1Δ126 or TX/98NS1Δ126-swIL18 showed fever, lasting 2-3 days. When compared to the mock-vaccinated challenge group, the inactivated vaccine induced enhanced higher lung lesions, which were much higher than those found in the two live vaccine groups after challenge (Figure 1).

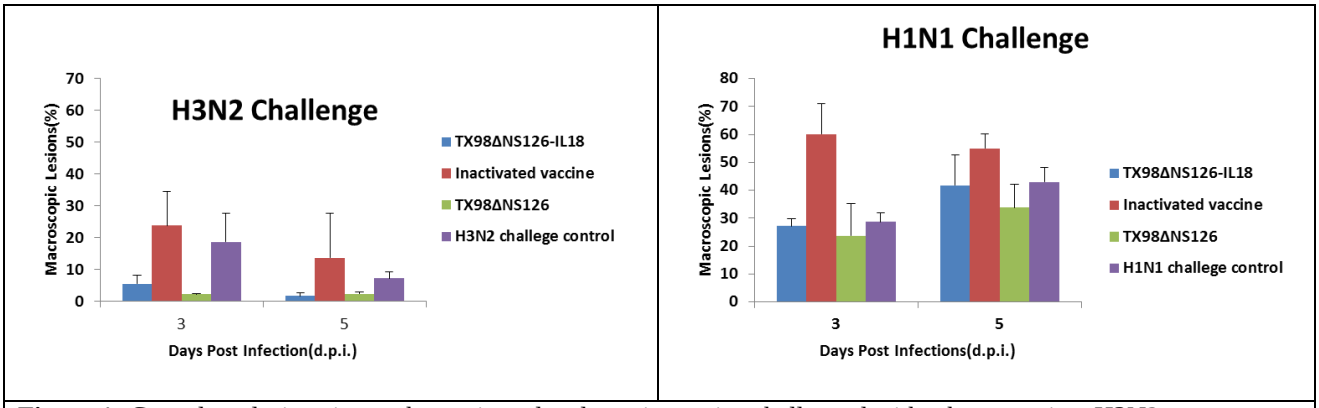

Figure 1: Gross lung lesions in mock-vaccinated and vaccinate pigs challenged with a heterovariant H3N2 or heterosubtypic H1N1 virus.

When compared to mock-vaccinated group, 3 vaccines showed different extents of protection against the heterovariant H3N2 challenge by inhibiting and blocking virus replication in pigs' lungs. Notably, no virus was detected in lungs of vaccinated pigs including inactivated vaccine, TX/98NS1Δ126 and TX/98NS1Δ126-swIL18 immunized groups at 5 dpi. However, significantly lower virus titer was detected in lungs of pigs immunized with TX/98NS1Δ126 virus when compared other 3 groups (Figure 2). Interestingly, when compared to mock-vaccinated group, 3 vaccines showed blocking nasal shedding after the heterovariant H3N2 challenge. On day 3 post challenge, lower titers from less pigs were detected from nasal swabs collected from pigs immunized with either of vaccines; no virus was detected in nasal swabs collected from pigs immunized with either of vaccines at day 5 post challenge (Figure 2). All results indicate that all 3 viruses are able to protect pigs against the heterovariant H3N2 challenge by inhibiting virus replication in lungs and virus nasal shedding.

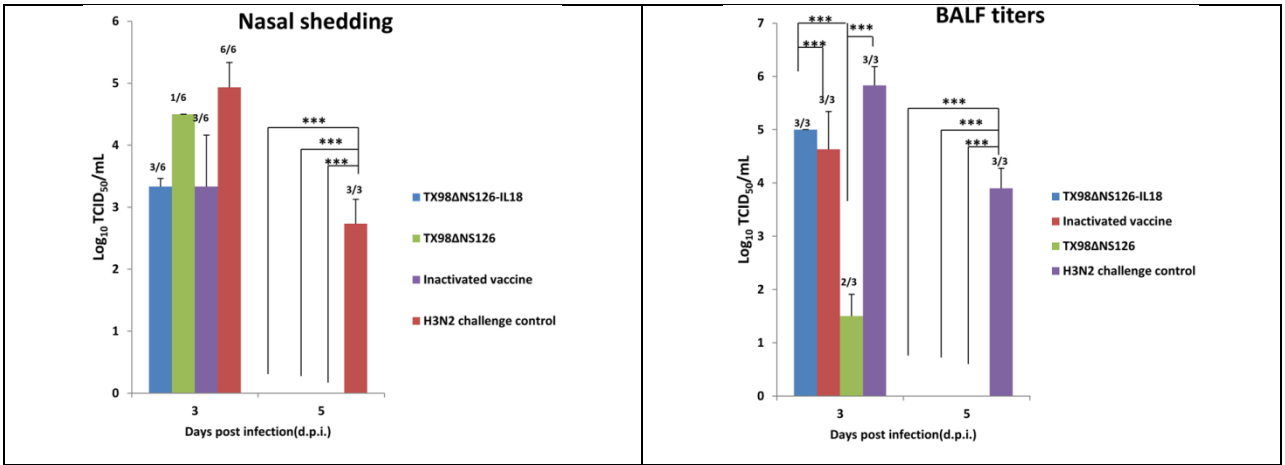

Figure 2: Virus titers in nasal swab and BALF samples collected from control and vaccinated pigs challenged with a heterovariant KS10 H3N2 virus

3. Efficacy of the MLV TX/98NS1Δ126-swIL18 against a heterosubtypic SIV in pigs.

No obvious respiratory symptoms were observed in experimental pigs. In the heterosubtypic H1N1 KS07 virus challenge groups, all pigs (100%) from mock-vaccinated and 3 vaccinated groups displayed fever,

lasting for 3-5 days. When compared to the mock-vaccinated challenge group, the inactivated vaccine induced higher lung lesions, which were much higher than those found in the two live vaccine groups after challenge (Figure 1).

There was no difference in virus replication in pigs' lungs between mock-vaccinated group and 3 vaccine groups on day 3 post challenge with the heterosubtypic H1N1 KS07 virus (the titer was $\geq 10^6$ $TCID_{50}$/ml). However, on day 5 post challenge, significantly lower virus titers were detected in lungs of pigs from 3 vaccine groups (Figure 3). There was no difference in virus titers among 3 vaccinated groups, but virus was only detected from one out of 3 pigs from both live vaccine groups in contrast to all 3 pigs from the inactivated vaccine group. All pigs including mock-vaccinated and 3 vaccinated groups shed virus efficiently via the nasal cavity on day 3 post challenge, while there was no significant difference in virus lung titers among 3 vaccine groups. On day 5 post challenge, no virus was detected in nasal swabs collected from inactivated vaccine immunized pigs; only 2 out of 3 pigs vaccinated with the TX/98NS1Δ126 shed virus via nasal cavity with lower titers compared to the mock-vaccinated pigs. All 3 pigs vaccinated with the TX/98NS1Δ126-swIL18 nasally shed virus, but the titer was lower than that of mock-vaccinated pigs (Figure 3).

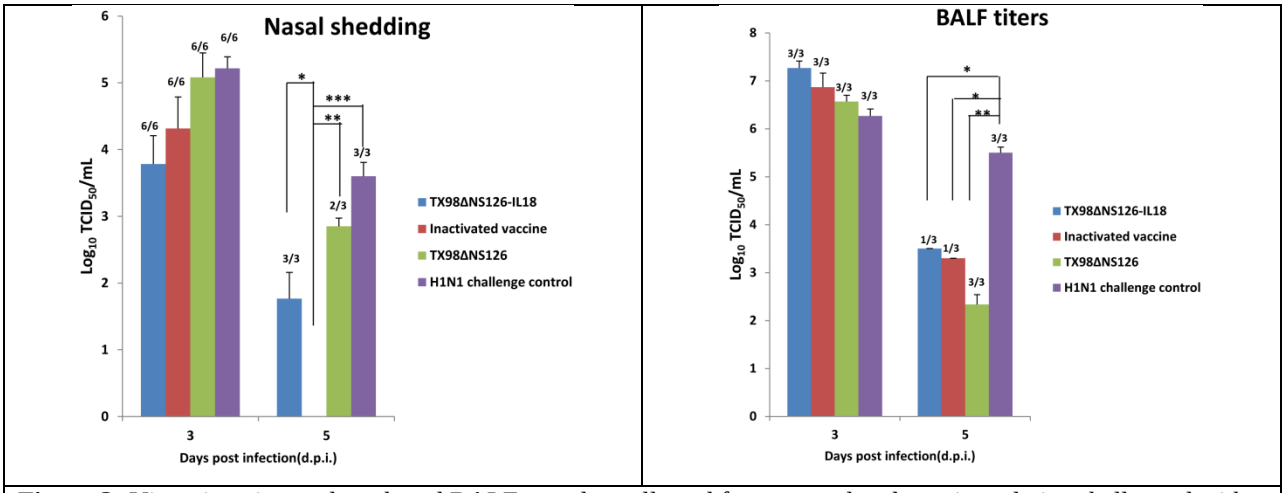

Figure 3: Virus titers in nasal swab and BALF samples collected from control and vaccinated pigs challenged with a heterovariant KS07 H1N1 virus

4. IFN γ response after vaccination and challenge

The swine IFNγ response after vaccination and challenge was measured by enzyme-linked immunospot (IFNγ-ELISpot). After immunization with the inactivated vaccine or modified live vaccines on days 14 and 21, a proportion of PBMC cells produced IFNγ stimulated by homologous TX98, heterovariant KS10, or heterosubtyic KS07 antigens when compared to the mock-vaccinated control group (Figure 4). The increased (double) percentage of PBMC cells collected from pigs immunized with the inactivated vaccine produced IFNγ when compared to TX/98NS1Δ126 and TX/98NS1Δ126-swIL18 vaccine groups. On day 3 post challenge, only

minimal percentage of PBMC cells stimulated with inactivated homologous TX98 H3N2 antigens were detected to produce IFNγ in the TX/98NS1Δ126-swIL18, inactivated vaccine and H3N2 challenge control groups. On day 5 post challenge, a higher percentage of PBMC cells stimulated by homologous TX98 or heterovariant KS10 antigens produced IFNγ, similar to control ConA in the vaccine groups including inactivated and 2 live vaccines (Figure 4). In the heterosubtyic KS07 antigen stimulated groups, a higher percentage of PBMC cells in the 3 vaccine groups produced IFNγ than the 2 challenge control groups (H3N2 and H1N1).

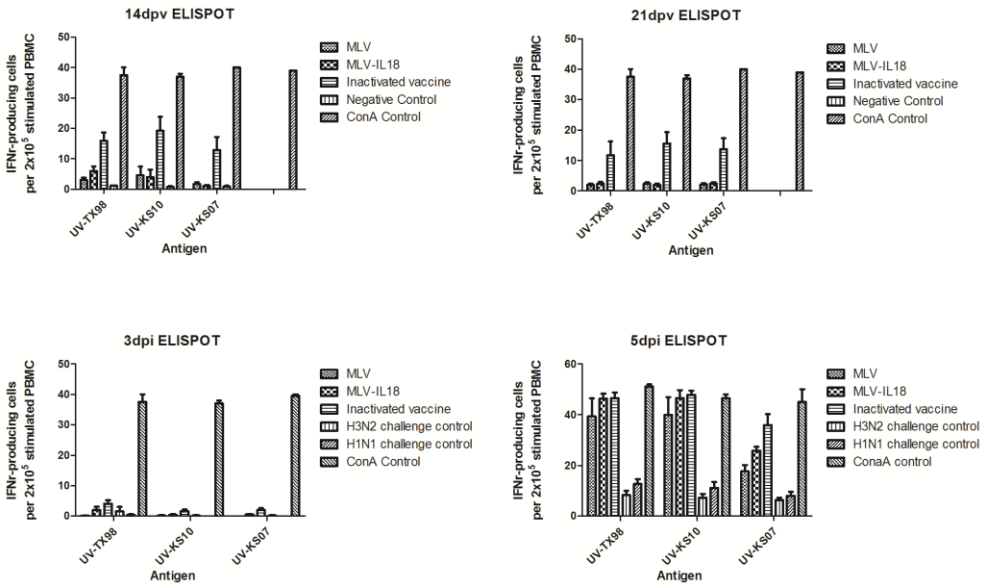

Figure 4: IFNγ response after vaccination and challenge.

Discussion: In this proposal, we evaluated a modified live attenuated virus vaccine expressing porcine IL-18 (TX/98NS1Δ126-swIL18) in pigs using the modified live influenza vaccine TX/98NS1Δ126 and the whole virus inactivated vaccine as controls. We have shown this vaccine candidate is able to stably express bioactive porcine IL18 which is expected to enhance heterologous immunity against challenge with heterovariant and heterosubtypic viruses. This is because IL-18 has been shown to be an important immuno-modulator that is able to enhance the development of antigen-specific immunity and vaccine efficacy. The vaccine study showed that the TX/98NS1Δ126-swIL18 was able to reduce lung lesions, decrease viral nasal shedding and provide broad protection against heterovariant and heterosubtypic SIVs when compared to mock-vaccinated and inactivated vaccine groups. However, no difference was observed when compared to the TX/98NS1Δ126 group. This is probably due to the fact that the TX/98NS1Δ126-swIL18 is too attenuated to replicate efficiently in pigs. Furthermore, there are no differences between the 2 vaccines in inducing the IFNγ. This data suggests that IL18 function might was diluted by attenuation of the TX/98NS1Δ126.

In contrast, vaccine associated enhanced respiratory disease (VAERD) as reported previously was observed in the inactivated vaccine group as the virus strains did not match the challenge strains including heterovariant H3N2 and heterosubtypic H1N1 viruses. However, a higher proportion of PBMC cells isolated from pigs immunized with the inactivated vaccine produced the IFNγ before challenge than those from pigs immunized with live vaccines, indicating that the adjuvants most likely play a critical role to induce the cell-medicated immunity, resulting in the decreasing virus nasal shedding and virus replication and clearance in lungs, similar to both live vaccines. This study proves the concept that heterosubtypic immunity can be improved with vaccines containing specific adjuvants or additional cytokines. The proposed project has improved our knowledge on influenza vaccine development and correlates of heterosubtypic immunity to better control swine influenza in swine herds.