RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Development of an epitope-based vaccine against swine influenza A virus using a non-toxic enterotoxin as the carrier-adjuvant - **NPB#09-163**

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Industry Summary

Influenza A virus causes a highly contagious respiratory disease in a variety of avian and mammalian hosts, including humans and pigs. Pigs as an intermediate host facilitate the genetic reassortment between avian and human influenza viruses, which results in the emergence of new, human-proficient viruses. The primary means for controlling influenza virus epidemics is vaccination. However, the efficacy of vaccination towards influenza virus is limited by frequent modifications and antigenic variations of its glycoproteins. In addition, unlike the human vaccine, which is updated annually based on the prediction of circulating stains, funding is not currently available to the swine industry to update swine influenza vaccines annually. In order to develop a vaccine that can be broadly effective against the various strains of the virus, the objective of this study is to develop an epitope-based vaccine using a set of influenza A subtype consensus swine influenza virus (SIV) epitopes. To enhance the immunogenicity of the epitope-based vaccine, a detoxified bacterial heat-labile enterotoxin mutant (LT_{192}) was used to construct the epitope-toxin chimeric antigen. The recombinant SIV epitope-toxin antigen was expressed in *E. coli*. The potential application of this epitope-toxin chimera in SIV vaccine development was determined in a pig model. Pigs were immunized with epitope-toxin chimeric antigen, and challenged with H1N1 or H3N2 virus. In comparison to the non-vaccinated pigs, vaccinated pigs showed protection from H1N1 virus challenge, with significant reduction of H1N1 induced fever and pneumonic lesions. In addition, significant reduction of the viral load in nasal secretion was observed in vaccinated pigs that challenged with H1N1 virus. This study established a model system for future construction of peptide-based vaccines against swine pathogens. For detailed information, please contact Dr. Ying Fang at South Dakota State University (Phone: 605-688-6648; E-mail: ying.fang@sdstate.edu).

Keywords: swine influenza A virus, enterotoxin LT, adjuvant, epitope vaccine

Scientific Abstract

Influenza A virus causes a highly contagious respiratory disease in a variety of avian and mammalian hosts, including humans and pigs. Pigs as an intermediate host facilitate the genetic reassortment between avian and human influenza viruses, which results in the emergence of new, human-proficient viruses. The primary means for controlling influenza virus epidemics is vaccination. However, the efficacy of vaccination towards influenza virus is limited by frequent modifications and antigenic variations of its glycoproteins. In addition, unlike the human vaccine, which is updated annually based on the prediction of circulating stains, funding is not currently available to the swine industry to update swine influenza vaccines annually. In order to develop a vaccine that can be broadly effective against the various strains of the virus, the objective of this study is to develop an epitope-based vaccine using a set of influenza A subtype consensus swine influenza virus (SIV) epitopes. To enhance the immunogenicity of the epitope-based vaccine, a detoxified bacterial heat-labile enterotoxin mutant (LT_{192}) was used to construct the epitope-toxin chimeric antigen. The recombinant SIV epitope-toxin antigen was expressed in *E. coli*. The potential application of this epitope-toxin chimera in SIV vaccine development was determined in a pig model. Pigs were immunized with epitope-toxin chimeric antigen, and challenged with H1N1 or H3N2 virus. In comparison to the non-vaccinated pigs, vaccinated pigs showed protection from H1N1 virus challenge, with significant reduction of H1N1 induced fever and pneumonic lesions. In addition, significant reduction of the viral load in nasal secretion was observed in vaccinated pigs that challenged with H1N1 virus. This study established a model system for future construction of peptide-based vaccines against swine pathogens.

Objectives of Research Project

1) To construct an 'SIV epitope:LT192' fusion immunogen using a set of subtype consensus SIV epitopes and a strong adjuvant LT192;

2) To assess the efficacy of 'SIV epitope:LT192' in prevention of SIV infection using a heterologous virus challenge pig model.

Introduction

Swine have long been considered as a mixing vessel for generation of new influenza viruses that can infect humans. Pigs have receptors that can bind both avian and mammalian influenza viruses, which increases the chance for viruses to exchange genetic sequences and produce new reassortant viruses capable of causing novel disease and posing potential to be pandemics in the human population. Cases of swine influenza virus infects human have been recently reported in the US (California and Texas) and Mexico (http://www.who.int/csr/don/2009_04_24/en/). Therefore, control of influenza virus infection in swine is critical to reduce the cross-species adaptation and minimize the risk of animals being the source of the next influenza pandemic (Thacker and Janke, 2008). The best preparation for an influenza outbreak arising from a newly emerging virus requires the availability of a vaccine that can induce cross-strain immunity, and be manufactured in large amounts with low cost. Current swine influenza vaccines that are inactivated, whole-virus vaccines are produced in embryonated chicken eggs. Although in most interpandemic influenza seasons this method of production works well, this type of vaccine is strain dependent, which do not offer effective protection against heterologous strain infection, especially to the newly emerging strains (Fedson, 2003; Webby and Webster, 2003). The embryonated chicken egg production system poses problems in vaccine production for highly pathogenic strains, due to the inability to obtain high yields of viruses (Wood, 2001; Zambon, 1998). Unlike the human influenza vaccine that is produced annually based on the prediction of the circulating stains, funding is not available to update the swine influenza vaccine annually. Thus, broadly protective vaccine strategies are required to combat the emergence of new influenza viruses. One novel approach for vaccine development is the utilization of specific immunogenic epitopes (Olszewska and Steward, 2001; Sette and Fikes, 2003; Jiang et al., 2006). Both humoral and cellular arms of the immune system recognize and react with only specific regions of the pathogen - epitopes. Because they are more exquisitely targeted, epitope vaccines are less likely to induce undesired immune responses. Epitopes that are short peptides are relatively easy to manufacture, safe to deliver, and stable in storage. Administration of epitope vaccines greatly reduces the risk of transmission of infections and makes easy to immunize a large population of animals. Furthermore, a cocktail of epitopes can induce a variety of different immune responses simultaneously. This prompts the design of epitope-based vaccines that can be effective in a heterogeneous pathogen population.

The influenza hemagglutinin (HA), nucleocapsid (NP) and matrix protein 2 (M2) are common targets for vaccine development. The surface glycoprotein, HA is the major antigen that induces protective immune response to prevent infection. The HA protein contains epitope peptide sequences that are conserved within specific subtypes. The NP protein is highly conserved within the type, and NP was demonstrated to induce strong CD8+ T cell response, and the immune response is cross-reactive within the type of influenza. In previous studies, HA 91-108 (B cell epitope), HA 307-319 (Th epitope) and NP335-350 (CTL epitope) were tested in mouse models. The results showed that the construct containing the HA B-cell epitope alone led to

partial protection. However, the addition of the two T-cell epitopes augmented the protection in a significant manner. Mice were protected against sub-lethal and lethal infection challenge by different strains of influenza virus, including H3N2 and H1N1 (Shapira et al., 1985; Schulze-Gahmen et al., 1988; Ikeda and Katagiri, 1993; Levi and Arnon, 1996; Adar et al., 2009). These results indicate that HA91-108, HA307-319 and NP 335-350 peptides are good candidates for peptide-based vaccine development. The extracellular domain of M2 protein, M2e contains a 23-amino acid that is conserved in all influenza A strains (Face et al., 1999; Neirynck et al., 1999). The M2e is poorly immunogenic (Feng et al., 2006), however, when presented with an appropriate carrier, such as hepatitis B virus core particles, it induces a high titer of antibody response and provided good level of protection in mice challenged with a lethal influenza strain (Fiers et al., 2004). It is suggested that M2e could be used to develop as a "universal' influenza A vaccine (Fiers et al., 2004; Tamura et al., 2005). The ability of M2e to provide protection appears to be low, compared with that of HA protein. In this study, we linked the M2e with epitopes from HA and NP to form an 'epitope cocktail' antigen.

The main limitation of peptide-based vaccines is their low immunogenicity. However, this will be solved by conjugation to an appropriate adjuvant and expression in an efficient delivery system. Thus, the identification and use of an appropriate adjuvant/carrier system would be essential for the success of epitope-based vaccines. Two bacterial proteins, heat-labile (LT) enterotoxin produced by E. coli and cholera toxin (CT) of Vibrio cholerae, are the best mucosal adjuvants and have been successfully used to enhance mucosal immune responses in developing vaccines against various pathogens, including pathogens in respiratory diseases (McNeal et al., 2007; Smiley et al., 2007; Lemere et al., 2002; Cheng et al., 1999; Hathaway et al., 1995; Xu-Amano et al., 1994). However, native LT and CT are very potent toxins that may produce adventitious effects in vaccine development. LT192, an LT mutant, maintains full adjuvant function as native LT but with much reduced toxicity, becomes the ideal choice of adjuvant to enhance immune responses. LT192 adjuvant has been successfully used in our laboratory to increase antigenicity of the non-immunogenic STa and STb antigens in developing vaccines against porcine post-weaning diarrhea (PWD) disease (Francis and Zhang, 2007). In this study, we adapted this technology to fuse the swine influenza A virus epitopes with the LT192, and this fusion construct 'SIV epitope:LT192' was expressed in *E. coli* as the fusion antigen. The feasibility of using this fusion antigen as a potential vaccine was assessed in a pig challenge model.

Materials and Methods

The main purpose of this project is to construct a SIV epitope-toxin chimera, and to determine its potential application in SIV vaccine development. A total of six epitopes (Table 1) from the HA, NP and M2 proteins of SIV H1N1 and H3N2 were linked into multiple epitopes, and then genetically fused with the non-toxic LT192 gene. To assess the protective efficacy, 'SIV

epitope: LT192' immunized pigs were challenged with SIV H1N1 or H3N2. Protective immunity elicited by the 'SIV epitope: LT192' were evaluated based on the measurement of body temperature, observation of clinical signs, and determination of the level of viral shedding, serum antibody response and cellular immune response.

Table 1. SIV epitopes included in the vaccine construction.

Epitope	Sequence	Homology to swine influenza strains
1. HA 91-108 B-cell epitope	SSWSYIVETSNSDNGTCY	H1N1
2. HA 307-319 Th epitope	LPFQNIHPVTIGE	H1N1
3. HA 91-108 B-cell epitope	HCDGFQNEKWDLFVERSK	H3N2
4. HA307-319 Th epitope	DKPFQNVNKITYG	H3N3
5. NP 335-350 CTL epitope	SAAFEDLRVSSFIRGK	H1N1, H1N2, H3N2
6. M2 2-24 B- and CTL epitope	SLLTEVETPIRNGWGCKCNDSSD	H1N1, H1N2, H3N2

Swine influenza strains: The SIV A/Sw/IA/15/1930 (IA30, H1N1 subtype) and A/swine/Texas/4199-2/98 (TX/98, H3N2 subtype) were used for multi-epitope construction. The SIV A/Sw/IA/40766/92 (IA92, H1N1 subtype) and A/Sw/IA/41305/98 (IA98, H3N2 subtype) were used for animal challenge. A non-pathogenic *E. coli* strain 1836-2 that naturally expresses the K88ac fimbria, but no enterotoxin, was used as a parental strain for antigen expression.

'SIV epitope:LT192' chimera construction: Initially, each of the SIV epitopes was linked into a multi-epitope peptide. The corresponding region of each individual SIV epitope as shown in Table 1 was amplified by RT-PCR from SIV H1N1 (IA30) or H3N2 (TX/98) and cloned into a protein expression vector pET-28a(+). Each individual epitope region was sequentially linked into a single pET-28a (+) vector (Novagen) to generate the multi-epitope using a modified method described previously (Sun et al., 2004). After linking all the epitopes, the SIV multi-epitope was removed from the pET-28(a) vector by PCR and cloned into the pBR322 vector that contains the LT192 gene to generate 'SIV epitope:LT192' fusion construct (Figure 1). The pBR322 plasmid containing SIV multi-epitope:LT192 was transformed into *E. coli* strain 1836-2 to express the fusion antigen.

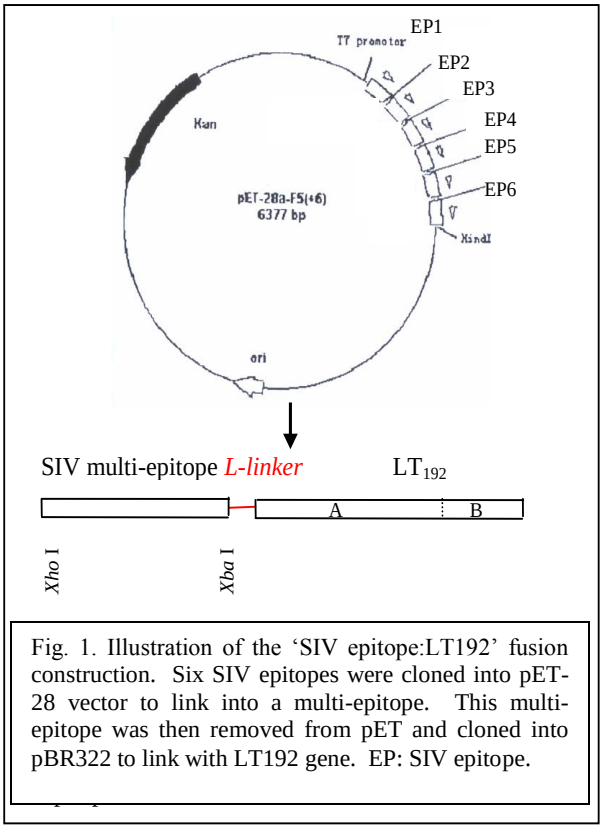


Fig. 1. Illustration of the 'SIV epitope:LT192' fusion construction. Six SIV epitopes were cloned into pET-28 vector to link into a multi-epitope. This multi-epitope was then removed from pET and cloned into pBR322 to link with LT192 gene. EP: SIV epitope.

Recombinant protein expression: To determine the expression of 'SIVepitope: LT192' fusion protein from the transformed *E. coli*, the bacterial construct was grown in Casamino acids and yeast extract broth with lincomycin (45 µg/ml) and ampicillin (100 µg/ml) at 37 ºC overnight. The overnight-grown culture was pelleted by centrifugation, and recombinant protein was prepared using bacterial protein extraction reagent (B-PER, Pierce, Rockford, IL) as we described previously (Fang et al., 2008; Brown et al., 2009). Western blot was used for detection the expression of SIV multi-epitope peptide and LT protein. The recombinant protein preparation was separated in a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into an immuno- blot using the method we described previously (Ferrin et al., 2004). Transferred membrane blots were probed with swine anti-sera to SIV or anti-LT rabbit antisera (Zhang et al., 2006).

Animals / challenge groups: Thirty 2- 3 weeks old pigs free of SIV, PRRSV and Mycoplasma hyopneumoniae were obtained, and randomly divided into five groups housed separately in animal isolation facilities at Iowa State University (ISU). The experimental design was presented in Table 2. After a 4 day acclimation period, group 3 and 5 pigs were immunized with SIVepitope: LT192' fusion protein, while other groups of pigs remain unimmunized. The immunized pigs receive a second immunization two weeks later with the same dose. After two more weeks, group 2-5 pigs challenged with SIV A/Sw/IA/40766/92 (IA92, H1N1 subtype) and A/Sw/IA/41305/98 (IA98, H3N2 subtype). Group 1 pigs were mock-challenged with cell culture medium as negative control. Challenge virus was administered intratracheally to pigs with a dose of 2 ml per pig at 1 x 10^7 50% TCID50/ml. Pigs were observed daily for clinical signs and body temperatures taken for 2 days prior to infection and five days after challenge. Blood samples were obtained from all pigs on 0, 7, 14, 21, 27, and 33 days post 1st immunization (dpi). Nasal swabs were collected the day prior to infection and 2, 3, 5 days post challenge. Pigs were euthanized at 5 days after challenge to evaluate lung lesions, and tissue samples including lung, tonsil, lymph node and ileum were harvested.

Table 2. Design of animal study (***dpi:** days post 1st immunization)

Group	Vaccine construct (0, 14 **dpi***)	Challenge virus (28 dpi)	Collection of blood samples (dpi)	Nasal swab collection (dpi)
1 (n = 6)	No	No	0, 7, 14, 21, 27, 33	27, 30, 31, 33
2 (n = 6)	No	H1N1	0, 7, 14, 21, 27, 33	27, 30, 31, 33
3 (n = 6)	1836-2/SIVepitope:LT_{192}	H1N1	0, 7, 14, 21, 27, 33	27, 30, 31, 33
4 (n = 6)	No	H3N2	0, 7, 14, 21, 27, 33	27, 30, 31, 33
5 (n = 6)	1836-2/SIVepitope:LT_{192}	H3N2	0, 7, 14, 21, 27, 33	27, 30, 31, 33

Quantification of the level of viral shedding: For determination of the level of virus shedding, nasal swab samples were examined using real-time PCR, which is routinely performed at the SD Animal Disease Diagnostic Laboratory of SDSU.

Determination of humoral immune response: To determine humoral antibody responses, all serum samples were evaluated using the HI assay. Both challenge viruses IA/92 and IA/98 were used as the antigen for the HI assays.

Determination of cellular immune response: To measure the cellular immune responses induced by the epitope-toxin chimera, PBMCs were isolated from the whole blood as described previously (Meier et al., 2003) and plated in a 24-well plate (4 x 106 cells/ml). PBMCs were stimulated with inactivated H1N1 or H3N2 viruses (100 HA units/100 ul). For controls, cells were stimulated with the mitogen Concanavalin A (positive control) or PBS only (negative control). Stimulated cultures were incubated at 37 ^{0}C, and culture supernatant was harvested after 24 hours. Serum samples and culture supernatant from PBMCs were evaluated for expression of selected swine cytokine proteins targeting immune markers for innate immunity: IL-1β, IL-8, IFN-α, TNF-α, IL-12; Th1 immunity: IFN-γ; Th2 immunity: IL-4; and regulatory T cell response: IL-10. Detailed FMIA procedure for cytokine protein detection was described in our previous publication (Lawson *et al*., 2010). Relative quantification of target cytokine expression was evaluated using mean fluorescent intensity (MFI) values, and each group's results were compared with the mean values for control pigs (or control cultures).

Data Analysis: Analysis of variance (ANOVA) was used to analyze the data. Significant differences between treatment groups were evaluated using the Tukey-Kramer Honestly Significant Difference multiple comparison test. All statistical analysis was performed using JMP 5.1 (SAS Institute, Cary, NC).

Results

Construction of an 'SIV epitope:LT_{192}' fusion immunogen using a set of subtype consensus SIV epitopes and a strong adjuvant LT_{192}.

Multiple epitopes (Table 1) from SIV H1, H3, NP, and M2e proteins were genetically linked and further fused to the LT_{192} gene to produce a fusion gene, designated as 'SIV epitope:LT92.' (Fig.1). This fusion gene was introduced into nonpathogenic porcine *E. coli* strain (1836-2). To facilitate the detection of fusion proteins, anti-LT polyclonal antibody and anti-HA3 91-108 monoclonal antibody (mAb) were made in our laboratory. The reactivity of anti-HA3 91-108 was confirmed in an immunofluorescent assay (Fig. 2). The expression of full-length LT192 – SIV multi-epitope and subunit LTB –SIV multi-epitope fusion proteins were detected in Western blot using these antibodies (Fig. 3).

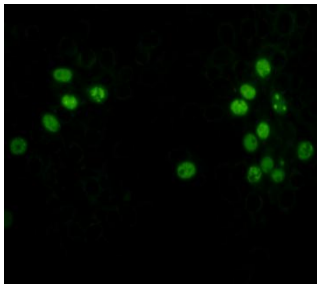


Fig. 2. Immunofluorescent assay detection of expression of HA proteins in SIV infected MDCK cells. MDCK cells were infected by swine influenza virus A/swine/Texas/4199-2/98, and 48 h after infection, cells were fixed and stained by FITC conjugated anti-HA3 monoclonal antibody.

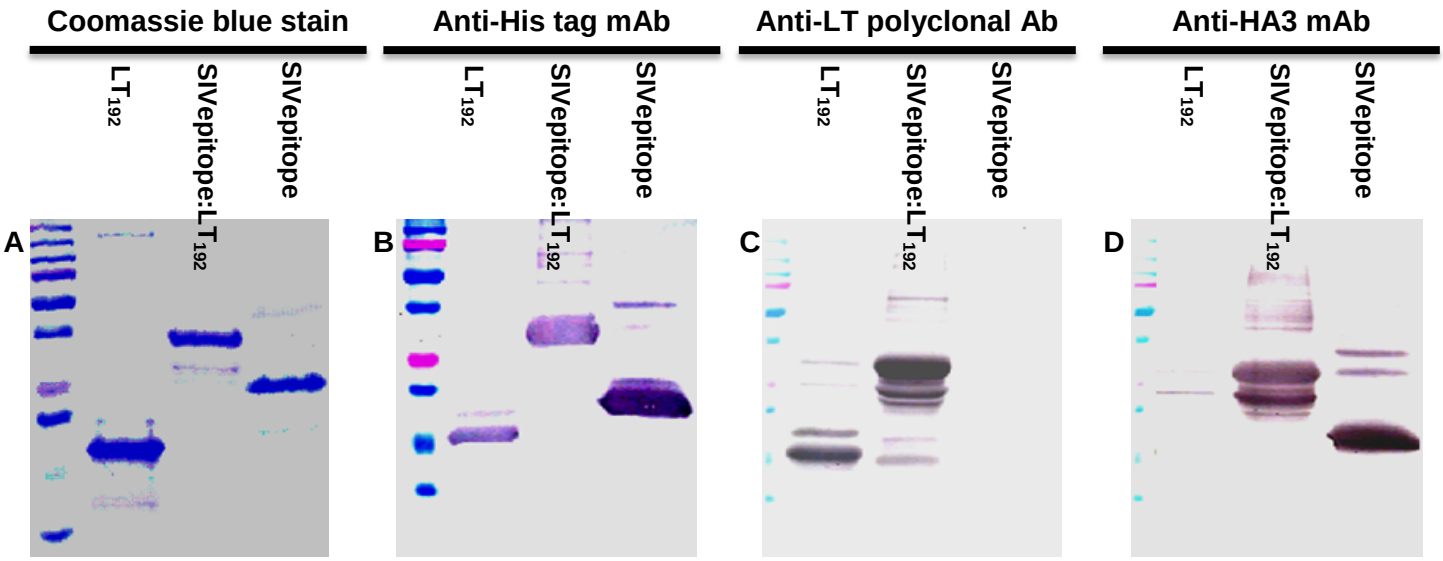


Fig. 3. SDS-PAGE and Western blot analysis of recombinant SIV epitope:LT_{192} fusion protein expression. Pooled fractions from Ni-agarose affinity chromatography purified recombinant proteins were separated on 15% polyacrylamide gel and transferred on nitrocellulose membranes. Membrane was probed by anti-LT rabbit sera (A) or anti-HA3 91-108 monoclonal antibody (B).

Assessment of the potential application of 'SIVepitope:LT_{192}' in SIV vaccines development.

Clinical and lung lesion evaluation: Clinical symptoms were monitored after the challenge. Respiratory signs were mild in all vaccinated pigs. In the nonvaccinated group 2, pigs challenged with H1N1 virus (IA92) developed significant respiratory disease compared to the negative control group (group 1). Respiratory symptoms include coughing, febrile and elevated rectal temperature (Figure 4). However, no significant clinical signs were observed in pigs challenged with H3N2 virus (IA98).

At necropsy, gross lung lesions were evaluated. As shown in Table 3 and Figure 5, the percentage of lung lesions was significantly decreased in group 3 pigs (vaccinated/challenge with H1N1 virus) compared to the group 2 pigs (non-vaccinated/challenge with H1N1 virus). However, no significant lung lesions were observed in group 4 and 5 pigs.

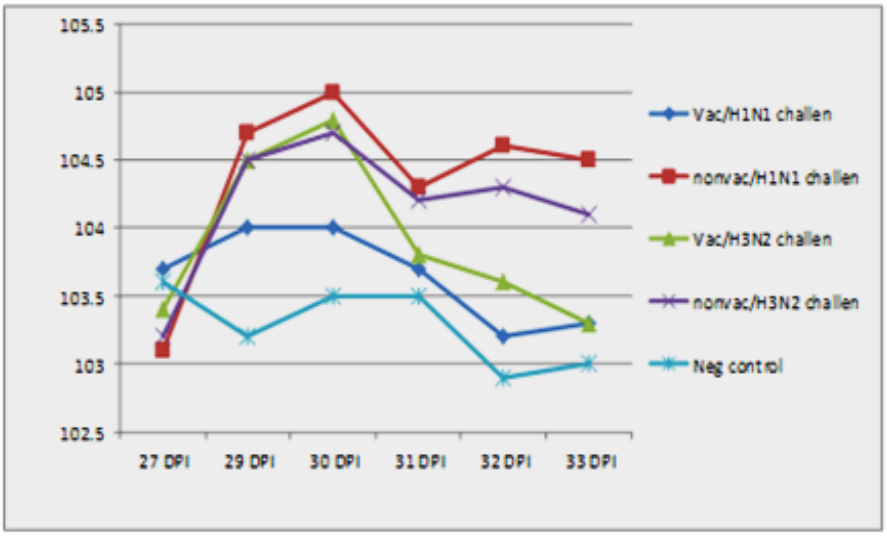


Figure4. Comparison of body temperatures among different treatment groups. Rectal temperature was taken one day prior to challenge, and each day after challenge. Each data point is presented as a mean value of rectal temperatures from six pigs.

Table 3. Lung lesion scores of vaccinated / nonvaccinated pigs challenge with H1N1 or H3N1 influenza A viruses

Pig #	Room A	Room B	Group	Vaccine	Challenge	Lung Score	LN	Comments
19	136	110	H1N1	None	H1N1	23	normal	
20	136	110	H1N1	None	H1N1	14	normal	
21	136	110	H1N1	None	H1N1	19	normal	
22	136	110	H1N1	None	H1N1	7	normal	
23	136	110	H1N1	None	H1N1	24	normal	
24	136	110	H1N1	None	H1N1	24	normal	
43	136	110	H1N1	None	H1N1	35	normal	artificial lesions from captive bolt
25	136	136	H3N2	None	H3N2	0	normal	
26	136	136	H3N2	None	H3N2	0	normal	
27	136	136	H3N2	None	H3N2	0	normal	failed to collapse
28	136	136	H3N2	None	H3N2	0	normal	
29	136	136	H3N2	None	H3N2	0	normal	euthanasia lesions
30	136	136	H3N2	None	H3N2	0	normal	
44	136	136	H3N2	None	H3N2	0	normal	
13	136	106	Negative Control	None	None	0	normal	
14	136	106	Negative Control	None	None	0	normal	artificial lesions from captive bolt
15	136	106	Negative Control	None	None	0	normal	
16	136	106	Negative Control	None	None	0	normal	
17	136	106	Negative Control	None	None	0	normal	
18	136	106	Negative Control	None	None	0	normal	
7	134	108	VAC-H1N1	1836-2/SIVepitope:LT_{192}	H1N1	6	normal	
8	134	108	VAC-H1N1	1836-2/SIVepitope:LT_{192}	H1N1	9	normal	
9	134	108	VAC-H1N1	1836-2/SIVepitope:LT_{192}	H1N1	12	normal	
10	134	108	VAC-H1N1	1836-2/SIVepitope:LT_{192}	H1N1	3	normal	
11	134	108	VAC-H1N1	1836-2/SIVepitope:LT_{192}	H1N1	26	normal	
12	134	108	VAC-H1N1	1836-2/SIVepitope:LT_{192}	H1N1	9	normal	
1	134	134	VAC-H3N2	1836-2/SIVepitope:LT_{192}	H3N2	0	normal	failed to collapse
2	134	134	VAC-H3N2	1836-2/SIVepitope:LT_{192}	H3N2	0	normal	
3	134	134	VAC-H3N2	1836-2/SIVepitope:LT_{192}	H3N2	0	normal	failed to collapse
4	134	134	VAC-H3N2	1836-2/SIVepitope:LT_{192}	H3N2	0	normal	
5	134	134	VAC-H3N2	1836-2/SIVepitope:LT_{192}	H3N2	0	normal	
6	134	134	VAC-H3N2	1836-2/SIVepitope:LT_{192}	H3N2	0	normal	artificial lesions from captive bolt

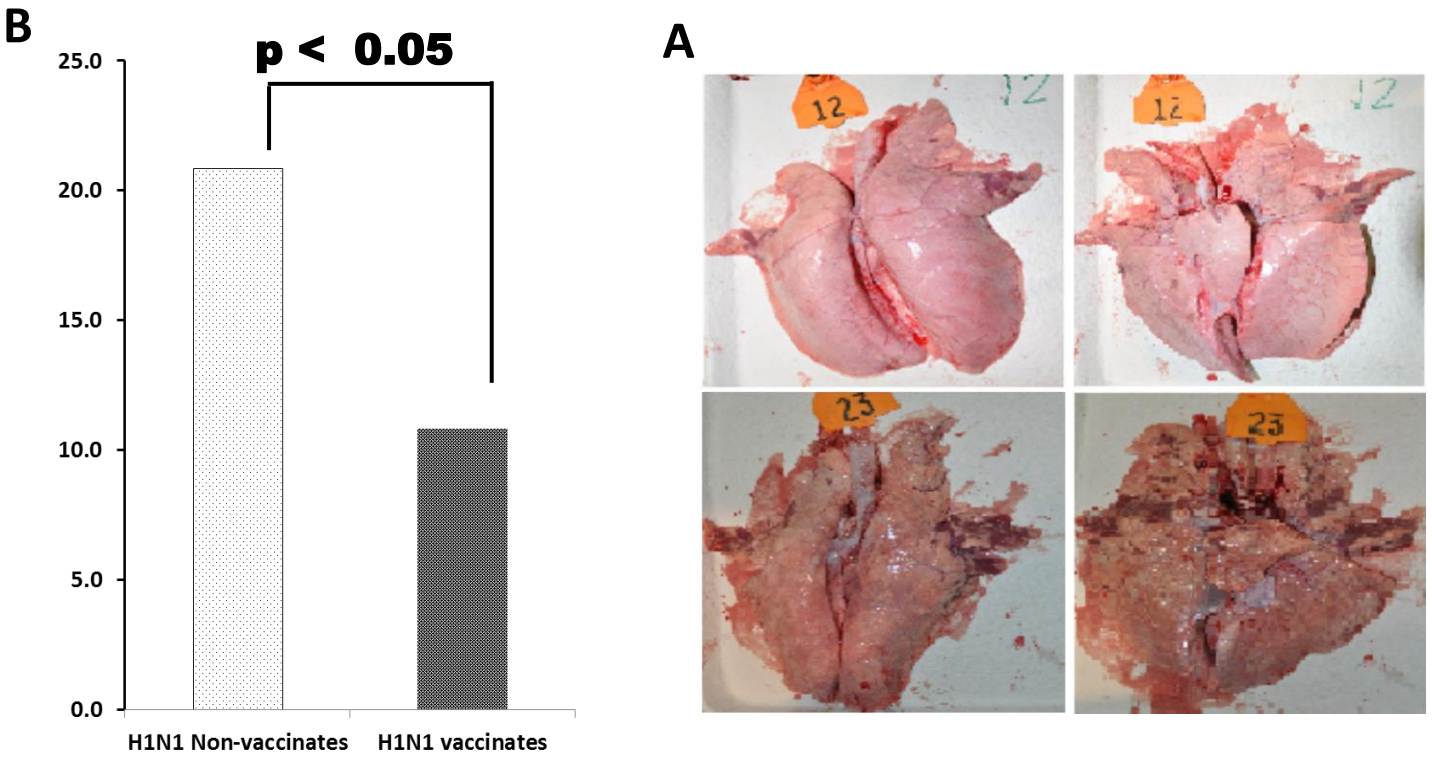
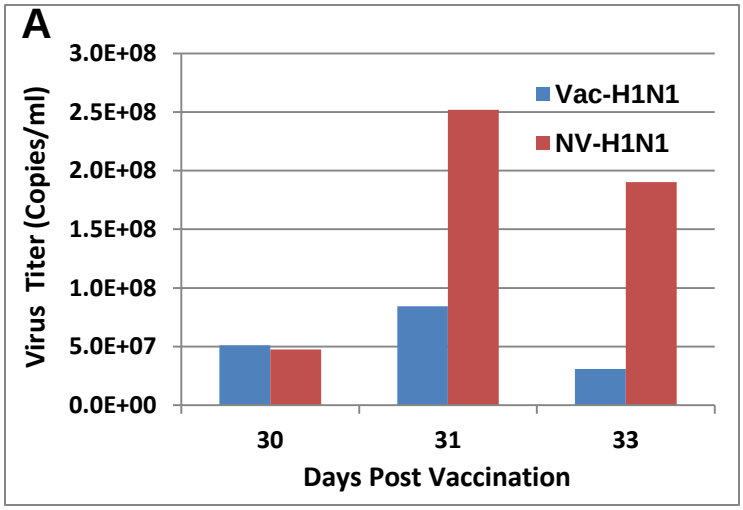


Figure 5. Comparison of the gross pathology and viral load in lungs from non-vaccinated and vaccinated pigs that challenged with H1N1 virus. **A**). Gross lung lesions from representative non-vaccinated/vaccinated pigs, which were subsequentially challenged with IA92 (H1N1) virus. Lung lesion score is assigned based on the evaluation of percent pneumonia in each lobe and the then added up for entire lung; **B**). Comparison of viral load in lungs from non-vaccinated and vaccinated group of pigs. Data points were presented as mean CT value of real-time PCR (n = 6). Note the statistical difference between the non-vaccinate and vaccinated groups of pigs.

Viral load in sera, nasal secretions and tissues: No viral RNA was detected by real-time PCR in sera, nasal swab and tissue sample from negative control pigs (group 1) at any time point during the study. No viral RNA was detected in serum samples at 5 DPC. In nasal secretions, group 3 pigs (vaccinated/challenged with H1N1 virus) shed significant lower amount of virus in comparison to that of group 2 pigs (non-vaccinated/challenge with H1N1 virus) at 30, 31, 33 days post vaccination (DPV) (Figure 6). In comparison to group 4 pigs, lower level of viral RNA was detected in nasal secretion from group 5 pigs on 30 dpi, although the levels were not significantly different between groups. In lung, tonsil and lymph node samples, lower level of virus was detected in group 3 pigs comparing to that of group 2 pigs (Figure 7). Minimal level of viral RNA was detected in tissue samples from H3N2 virus challenged group 4 and 5 pigs, and there was no significant difference on viral load between these two groups.



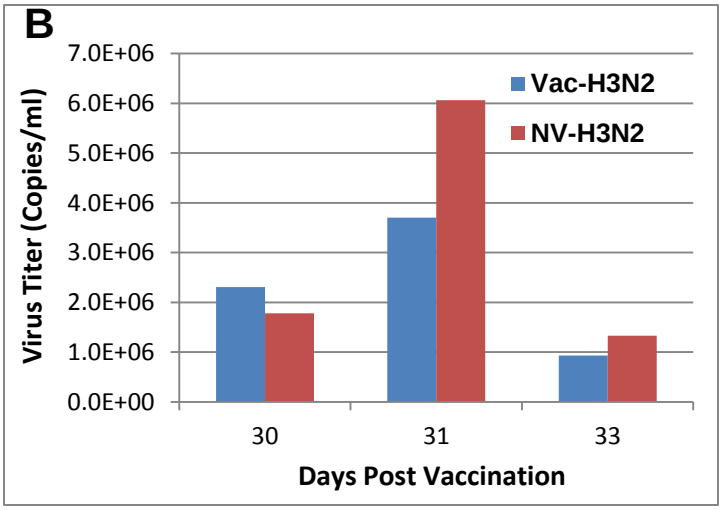
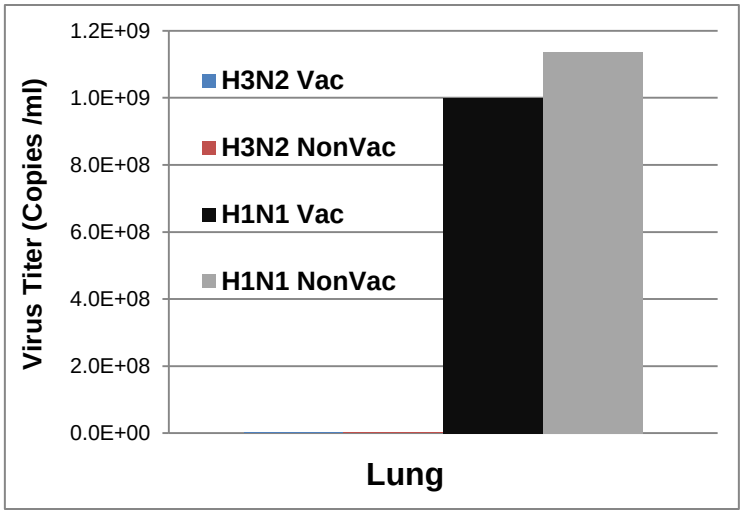
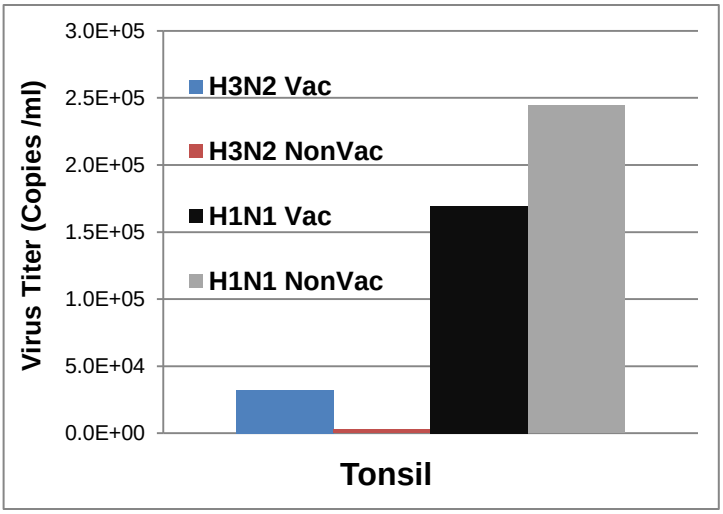


Figure 6. Comparison of viral load in nasal secretion from vaccinated or non-vaccinated pigs that challenge with H1N1 virus (**A**) or H3N2 virus (**B**). Viral load was quantified by real-time RT-PCR, and the result was interpreted as RNA copy numbers per ml.





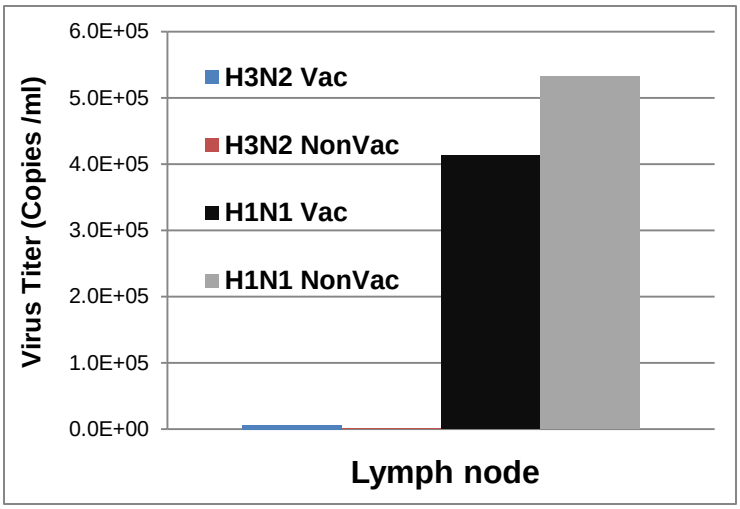


Figure 7. Comparison of viral load in lung, tonsil or lymph nodes from vaccinated or non-vaccinated pigs that challenge with H1N1 virus or H3N2 virus. Viral load was quantified by real-time RT-PCR, and the result was interpreted as RNA copy numbers per ml.

Antibody and cellular immune response: Serum samples from selected pigs in the individual groups were initially screened by the HI assay. The results frequently showed high titers in the Day 1 sera, with little or no increase in the Day 27 and Day 33 samples. In fact, some of the titers for the Day 27 and Day 33 samples were even lower than in the Day 1 sample for that pig. This was even seen in the negative control samples. Based on this result, we speculated that innate serum inhibitors may interfere with the HI results, and potentially overshadowing any antibody response that had developed. Serum samples were further tested using the microneutralization assay, as sera analyzed using this assay can demonstrate less inhibition from innate inhibitors. Figure 8 presents the result generated from microneutralizing assay. The data showed a few positive samples (1:10, 1:20, or 1:40 titer), with most of the titers being <10 (indicated on this graph with a value of 5). The titers of the positive samples are very low, and they are not consistent within a single group.

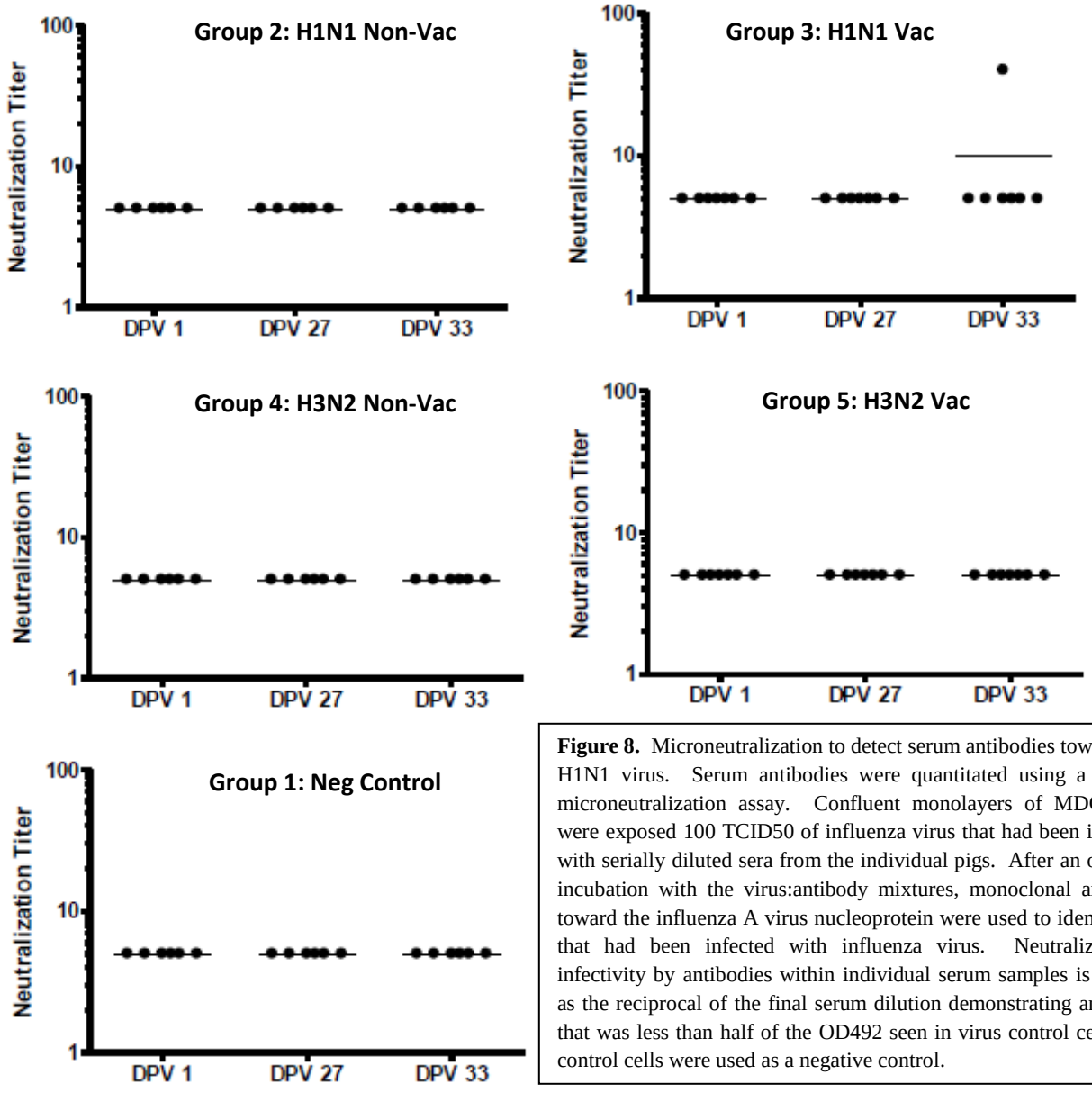


Figure 8. Microneutralization to detect serum antibodies toward IA92 H1N1 virus. Serum antibodies were quantitated using a standard microneutralization assay. Confluent monolayers of MDCK cells were exposed 100 TCID50 of influenza virus that had been incubated with serially diluted sera from the individual pigs. After an overnight incubation with the virus:antibody mixtures, monoclonal antibodies toward the influenza A virus nucleoprotein were used to identify cells that had been infected with influenza virus. Neutralization of infectivity by antibodies within individual serum samples is reported as the reciprocal of the final serum dilution demonstrating an OD492 that was less than half of the OD492 seen in virus control cells. Cell control cells were used as a negative control.

We further compared the temporal expression of a panels of selected innate and cellular immune proteins in PBMCs from vaccinate and non-vaccinated pigs. Serum samples and culture supernatant from PBMCs were evaluated using fluorescent immunomicrosphere assay (FMIA) to determine the expression of selected swine cytokine proteins, which includes immune markers for innate immunity: IL-1β, IL-8, IFN-α, TNF-α, IL-12; Th1 immunity: IFN-γ; Th2 immunity: IL-4; and regulatory T cell response: IL-10. As shown in Figure 9, certain level of IL-1β, IL-4, IL-8, IL-12 and IFN-γ expression was detected. However, due to large variation between individual pigs, there is no statistic difference among different treatment groups. No production of IL-10, IFN-a, and TNF-a were detected by FMIA.

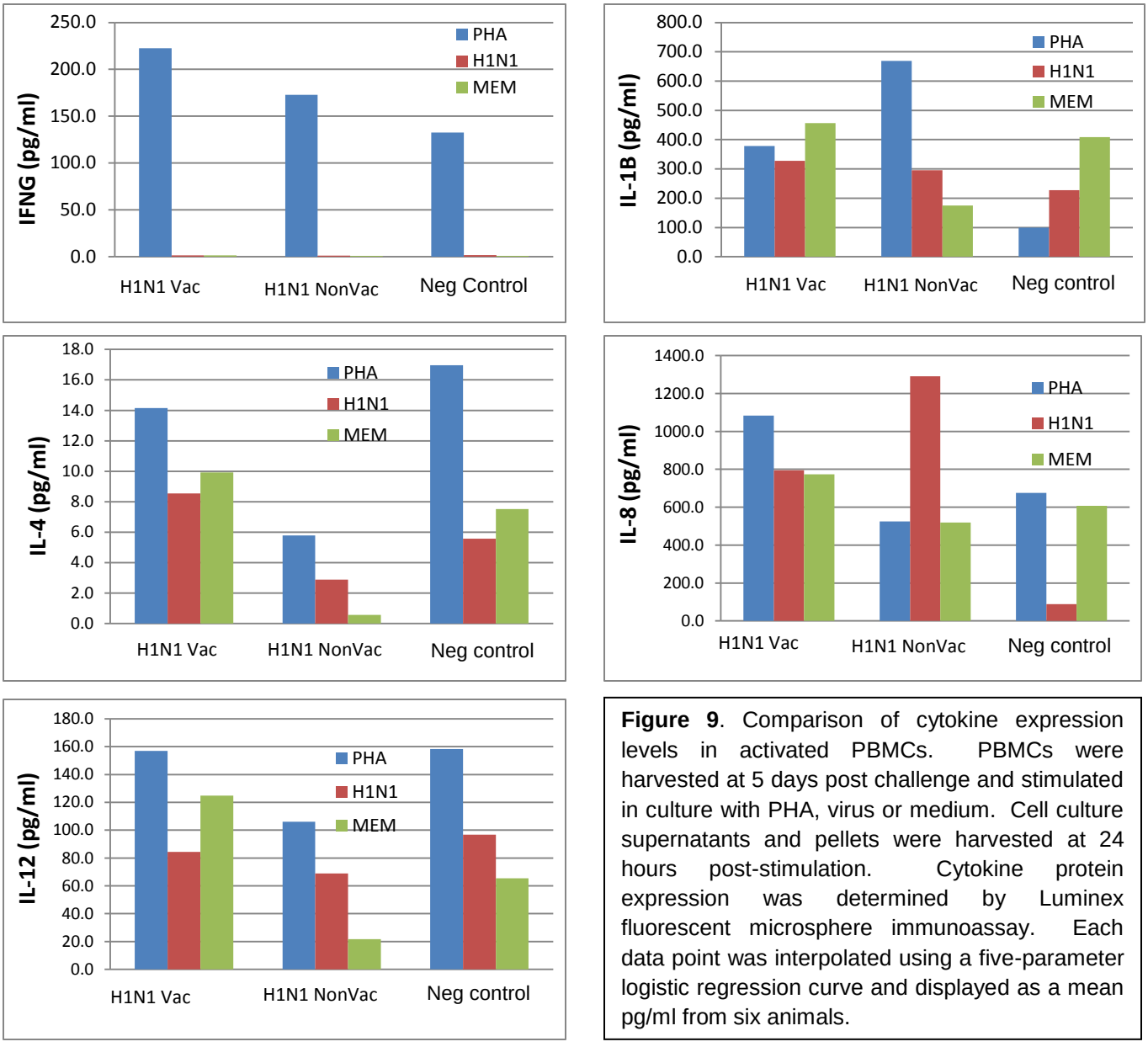


Figure 9. Comparison of cytokine expression levels in activated PBMCs. PBMCs were harvested at 5 days post challenge and stimulated in culture with PHA, virus or medium. Cell culture supernatants and pellets were harvested at 24 hours post-stimulation. Cytokine protein expression was determined by Luminex fluorescent microsphere immunoassay. Each data point was interpolated using a five-parameter logistic regression curve and displayed as a mean pg/ml from six animals.

Discussion

Swine influenza is one of the most important diseases for the swine industry, and causes substantial economic loss to swine producers worldwide. Development of new safe and effective vaccines can provide a means to control or prevent this disease, thus reducing cost for swine producers and increasing pork production. Most importantly, pigs as an intermediate host facilitate the genetic reassortment between avian and human influenza viruses,

Since pigs function as an intermediate host to facilitate the genetic reassortment between avian and human influenza viruses, human-proficient virus can be generated from pigs. An effective vaccine will block the intermediate transmission route to reduce the risk of transmission of influenza to the human population. As conventional vaccines fail to provide broad protection, efforts to develop new safe and effective vaccines against influenza virus should be strongly encouraged. In this study, we developed an epitope-based vaccine using a set of influenza A subtype consensus swine influenza virus (SIV) epitopes. To enhance the immunogenicity of the epitope-based vaccine, a detoxified bacterial heat-labile enterotoxin mutant (LT_{192}) was used to construct the epitope-toxin chimeric antigen. The recombinant SIV epitope-toxin antigen was expressed in *E. coli*. The potential application of this epitope-toxin chimera in SIV vaccine development was determined in a pig model. Pigs were immunized with epitope-toxin chimeric antigen, and challenged with H1N1 or H3N2 virus. In comparison to the non-vaccinated pigs, vaccinated pigs showed protection from H1N1 virus challenge, with significant reduction of H1N1 induced fever and pneumonic lesions. In addition, significant reduction of the viral load in nasal secretion was observed in vaccinated pigs that challenged with H1N1 virus.

We did not observe the significant effect of epitope-toxin chimera vaccine on prevention of H3N2 infection. This could be caused by the nature characteristics of H3N2 challenge virus. The IA98 (H3N2) virus is a laboratory attenuated strain, which may not be able to cause lung lesions. The real-time PCR result from lung tissue samples further support this notion. The result showed reduced level of viral load in vaccinated/H3N2 challenged pigs in comparison to the non-vaccinated/H3N2 challenged pigs, although the level of difference is not statistically significant.

The animal study is based on limited numbers of pigs (n = 6). The large variation among individual pigs could be a main reason not shown significant difference between different treatment groups in cytokine protein expression analysis. Future studies with larger number of animals are needed to fully evaluate the vaccine efficacy of this epitope-toxin chimera. On the other hand, we did not detected significant level of neutralizing antibody in serum samples, which suggested that the B cell epitope (HA 91-108) does not have the ability to stimulate a strong neutralizing response toward the entire HA molecule, and likely does not interfere with binding of the virus to chicken red blood cells in the HI assay. These results would suggest that the use of an ELISA for analysis of these sera may better detect antibodies toward this epitope.

We are performing the ELISA test using the concentrated whole virus or purified recombinant protein (HA 91-108) as the coating antigen.

In conclusion, we developed an epitope-toxin based SIV candidate vaccine. This study established basis system for peptide-based vaccine development against influenza. In the future, the epitope can be easily modified or new epitopes can be included in the construct based on the field epidemic strains. This new vaccine approach also provides a tool for developing vaccines against other pathogens.