RESEARCH REPORT
SWINE HEALTH

Title: Development of novel mucosal vaccines against swine influenza in pigs - **NPB # 11-073**

Investigators: Mahesh Khatri (contact) and Renukaradhya J. Gourapura

Institution: The Ohio State University

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Industry Summary

Swine influenza is a highly contagious acute respiratory viral disease of swine. The causative agent swine influenza virus (SIV) has been widely prevalent in the US swineherds. Control of influenza outbreaks among herds and prevention of possible transmission of zoonotic SIV to humans is possible through protection of swineherds using potent vaccines. Current available vaccines are not highly efficacious due to the co-circulation of genetically variant viruses in the swine populations. Additionally, swine respiratory tract epithelial cells express receptor which facilitates simultaneous infection of both avian and mammalian influenza viruses; thus, pigs could be the potential source for the generation of novel new reassortant strains of influenza viruses. Therefore, in this study we made an attempt to develop an inactivated effective vaccine against SIV, aimed to reduce the economic losses to pig producers. We have demonstrated that by activating pig invariant natural killer T (iNKT) cells in the respiratory tract using an iNKT cell specific adjuvant, α-Galctosylceramide (α-GalCer), coadministered with a UV-inactivated SIV-H1N1 vaccine significantly reduce the burden of homologous virus infection. However, when pigs were vaccinated with a bivalent (SIV-H1N1 and H3N2) inactivated influenza virus vaccine coadministered with two iNKT cell adjuvants together twice intranasally, there was an increased shedding of pandemic 2009 H1N1 virus in the nasal discharge and its load in the lungs; immunologically, associated with dampened anti-viral immunity. Next, we tested poly I:C as mucosal adjuvant with bivalent inactivated vaccine. Maternal antibody positive pigs immunized twice with Poly I:C adjuvanted inactivated vaccine provided protection against antigenic variant H1N1 and heterologous H1N2 virus challenge.

Scientific Abstract

Swine influenza virus (SIV) causes an acute respiratory disease in pigs of all ages, and pigs are infectable by both avian and mammalian influenza viruses. Activation of innate immune cells including invariant natural killer T (iNKT) cells induces heightened heterologous protection against influenza viruses in rodent models. We discovered CD1d-restricted iNKT cell in pigs. Our goal was to determine the efficacy of UV-inactivated bivalent SIV vaccine, comprising of triple reassortant zoonotic H1N1 (Sw/OH/24366/07) and H3N2 (Sw/CO/99) viruses; coadministered intranasally with iNKT cell specific adjuvants, α-Galctosylceramide (α-GalCer) and phosphatidylinositolmannosides-2 (PIM2) or Poly I:C as mucosal adjuvants to provide cross-protective immunity against influenza in pigs. In monovalent vaccine inoculated homologous H1N1 virus

challenged pigs, reduced viral load in the lungs associated with increased IFN-γ^+ lymphocytes in the lungs and tracheobronchial lymph nodes by ELISPOT, and an increase in IFN-γ^+CD8$^+$ T cells and IFN-γ^+ $\gamma\delta$ cells was observed. Further, increased specific IgA and hemagglutination inhibition (HI) antibody responses in the BAL, and enhanced lung NK cytotoxicity were detected. However, in bivalent vaccine inoculated, heterologous pandemic 2009 H1N1 virus challenged pigs, enhanced nasal viral shedding and increased lung viral load was detected. Immunologically, reduced frequency of total lymphocytes, CD8$^+$ T cells, and frequency of total IFN-γ^+ T cells was detected in the lungs. On the other hand, Poly I:C adjuvanted vaccine was able to initiate cross-protective immunity and provided protection against antigenic variant H1N1 and heterologous H1N2 virus challenge in commercial pigs. In conclusion, Poly I:C may be a promising candidate for the development of novel cross-protective mucosal vaccine against influenza in pigs.

Keywords: Swine influenza virus; intranasal delivery; killed SIV vaccine; adjuvants; heterologous challenge

Introduction

Swine influenza is a highly contagious acute respiratory viral disease of swine. The causative agent, swine influenza virus (SIV), is a member of the influenza virus A genus. Clinical disease in SIV infected pigs include sudden onset of cough, respiratory distress, weight loss, fever, and nasal discharge. Control of influenza virus spread among pig herds and prevention of possible transmission to humans from SIV of zoonotic potential is possible only by effective vaccination, especially during pandemic influenza virus outbreaks. Currently available and routinely used SIV vaccines in the US swine herds are adjuvanted, inactivated, whole-virus vaccines, administered by intramuscular route. These vaccines stimulate high titers of IgG in serum which protect against clinical disease. Antibodies generated against the hemagglutinin (HA) protein of influenza virus appear to be protective, but protection against respiratory infection is incomplete. As a result, influenza viruses potentially circulate in swine herds with possible reassortment between field strains; resulting in generation of new reassortant viruses of pandemic potential. Therefore, there is an urgent and important public health need to develop effective vaccines against SIV. Moreover, the vaccine candidates must be continually updated to match the antigenicity of circulating viruses, because of the differences in antigenicity between the vaccine and the circulating viruses. In addition, it is difficult to predict which strain of the influenza virus will be responsible for pandemic outbreaks. In such a scenario, the ideal approach is to prepare a vaccine that confers strong cross-protective immunity against different subtypes and the antigenic drift variants.

Effective immunity against influenza is dependent on both antibody and cell-mediated immune components. Therefore, a collective and collaborative cross-talk among various immune cell types is critical. Invariant Natural Killer T (iNKT) cells have been reported to play an important role in generation of cross-protective immunity in rodent models against antigenically variant influenza viruses (1-7). iNKT cells are CD1d-restricted T lymphocyte subsets and are activated by a unique glycolipid called, α-GalCer (8, 9). Another agent derived from mycobacterial cell wall lipid called "phosphatidyl-*myo*-inositol mannosides" (PIM) (10) is a potent mucosal adjuvant (11-13), which also activates iNKT cells by bystander mechanism.

Synthetic dsRNA poly(I:C), a Toll-like receptor 3 (TLR3) agonist, has mucosal adjuvant activity. The poly(I:C) co-administered IN with an influenza HA vaccine, increased both the

mucosal and systemic immune responses, resulting in complete protection against challenge by homologous avirulent (H1N1) and highly pathogenic heterologous (H5N1) influenza viruses in mice (14, 15).Therefore, in this study to potentiate induction of influenza specific cross-protective immunity of UV-inactivated bivalent SIV vaccine (H1N1 and H3N2), we analyzed the adjuvant effects of two potent adjuvants, α-GalCer and PIM_2. *Our hypothesis was that stimulation of mucosal innate immune system in the respiratory tract of pigs with potent adjuvants will enhance the mucosal immunity to SIV vaccine, which would provide better cross-protective immunity.*

Objectives:

1. To evaluate the potential of killed SIV vaccine administered IN with adjuvant/s to generate anti-SIV specific mucosal immunity in pigs.
2. To examine the protective efficacy of killed SIV vaccine administered IN with adjuvant/s against heterologous and heterologous with different NA (termed as heterosubtypic in the proposal) influenza viral challenge in pigs.

Materials and Methods:

***Animal groups and inoculations (influenza antibody free pigs)*:**
The studies were conducted using conventional 4-week-old cesarean delivered-colostrum deprived healthy pigs, because a majority of commercial pigs possess circulating influenza-specific antibodies. Piglets were confirmed free from influenza specific antibodies prior to our study. Initially, to determine the appropriate dose of the adjuvant/s, α-GalCer and PIM2, we inoculated randomly assigned pigs at 4 - 5 weeks of age (N = 18, n = 3 pigs per group) with indicated dose of adjuvants coadministered with the UV-inactivated SIV-H1N1 ($5x10^6$ $TCID_{50}$ per pig) (A/swine/Ohio/24366/07) vaccine, once intranasally; and challenged on post-vaccination day 21 with the homologous SIV-H1N1-07 ($5x10^6$ $TCID_{50}$ per pig), intranasally (Table 1). Pigs were monitored for clinical disease and body weight gain. Blood and lung samples were analyzed for viral load and immune responses were determined at both the mucosal tissues in the respiratory tract and blood.

Table 1. Experimental design to study mucosal immune responses in pigs inoculated with the below vaccine adjuvants combination and challenged with a homologous H1N1 SIV. Total=18 pigs and n=3 pigs/group. All the inoculations by intranasal route
1. Mock
2. Mock + challenge
3. UV-monovalent (H1N1) vaccine + challenge
4. UV-monovalent (H1N1) vaccine with 50 µg α-GalCer + challenge
5. UV-monovalent (H1N1) vaccine with 250 µg α-GalCer + challenge
6. UV-monovalent (H1N1) vaccine with 250 µg PIM2 + challenge

In another *in vivo* experiment (Table 2), piglets derived from pregnant sows as described above were randomly assigned into one of the seven groups (N = 28, n = 4 pigs per group). To determine the extent of heterologous protection to influenza viruses, pigs were randomly assigned at 4-5 weeks of age (N = 28, n = 4 pigs per group) in to seven groups and inoculated with adjuvants, α-GalCer (250 µg/pig) and PIM2 (250 µg/pig), coadministered intranasally with UV-inactivated bivalent SIV-H1N1 and H3N2 (A/swine/Ohio/24366/07and Swine/CO99/H3N2) (each $5x10^6$ $TCID_{50}$ per pig) vaccine, twice at 2 weeks interval. Both vaccinated and control pigs were challenged at post-vaccination day 28 with the heterologous viruses, pandemic 2009 H1N1 (A/California/04/2009) or Sw/TX/1499-2/98, $5x10^6$ per pig, intranasally (Table 2). Pigs were monitored for clinical disease and body weight gain.

Table 2. Experimental design to study mucosal immune responses in pigs inoculated with a inactivated bivalent vaccine with adjuvants and challenged with heterologous SIVs. Total=28 pigs and n=4 pigs/group. All inoculations - intranasal route.
1. Mock
2. Mock + heterologous pandemic 2009 H1N1 challenge
3. Mock + heterologous H3N2 (TX/98) challenge
4. UV-bivalent (H1N1&H3N2) vaccine + heterologous pandemic 2009 H1N1 challenge
5. UV-bivalent (H1N1&H3N2) vaccine + heterologous H3N2 (TX/98) challenge
6. UV-bivalent (H1N1&H3N2) vaccine with α-GalCer & PIM2 + pandemic 2009 H1N1 challenge
7. UV-bivalent (H1N1&H3N2) vaccine with α-GalCer & PIM2 + H3N2 (TX/98) challenge

Heterologous challenge studies in maternal antibody positive pigs (Table 3): This trial was conducted in collaboration with Dr. David Francis, South Dakota State University. Three week old pigs were vaccinated with poly I:C (300 µg/pig) adjuvanted bivalent vaccine (PAV) or commercial vaccine (CV) twice at 2 weeks interval. Three weeks after the 2nd immunization, non-vaccinated (NV) and vaccinated pigs were challenged with heterologous viruses; Swine/MN/2073/2008 (H1N1) or Swine/NC/0036-2/2010(H1N2), $5x10^6$ per pig, intranasally (Table 3).

Table 3. Experimental design to evaluate protective efficacy of adjuvanted inactivated SIV vaccine in maternal antibody positive pigs. Total=28 pigs; n=4 pigs/group; Inoculations-intranasal
Three week old pigs immunized twice at 2 weeks interval and challenged 3 weeks after the 2nd immunization
1. Mock
2. Non-vaccinated challenged with H1N1 (Swine/MN/2073/2008)
3. Non-vaccinated challenged with H1N2 (Swine/NC/0036-2/2010)
4. Commercial vaccine challenged with H1N1
5. Commercial vaccine challenged with H1N2
6. UV-bivalent (H1N1&H3N2) vaccine with Poly I:C challenged with H1N1
7. UV-bivalent (H1N1&H3N2) vaccine with Poly I:C challenged with H1N2

The vaccine and challenges viruses were propagated in MK1-OSU cells (swine lung epithelial cells). Vaccine viruses were inactivated by UV-treatment using 254 nm UV rays for 1 hr, and the inactivation was confirmed in cell culture using both MK1-OSU and MDCK cells.

Pigs were examined daily for flu symptoms, and body weight recorded at day 0, 3 and 6 post-challenge. Blood, nasal discharge, and lung samples were analyzed for viral load and immune responses as described below.

Experimental procedures

1. ***Analysis of lung pathology:*** The gross pathology in lung and other organs were recorded during necropsy and scored as described previously (16).

2. ***Preparation of lung homogenate and isolation of lung mononuclear cells (lung MNCs)***

The lung homogenate was prepared as described previously (17). Briefly, one gram from right cranial lobe of the lungs was collected in 5 ml of DMEM and minced into tiny pieces and homogenized using Stomacher 400 laboratory blender (Seward, Long Island, NY) for 10 min, and the clarified supernatant was collected and preserved at -70°C. Lung MNCs (LMNC) from individual pigs were isolated as per the procedure described previously (18) .

3. ***Phenotypic analysis of immune cells:*** To determine the phenotype of immune cells and their activation status in the lungs, lung MNCs were immunostained with antibodies specific to pig cell surface markers: NK ($CD56^+$); γδ T cells ($CD8\alpha^+TcRN4^+$); T-cells [$CD3^+CD4^+CD8^-$ (T-helper cells); $CD3^+CD4^-CD8^+$ (cytotoxic T lymphocytes); $CD3^+CD4^+CD8^+$ (T-helper/memory phenotype)], and then subjected to flow cytometric analyses using our BD FACS AriaII (BD Biosciences) flow cytometer. We acquired 50,000 events for each combination of staining and the results were analyzed using FlowJo software (Tree Star, Inc. OR, USA).

   To determine IFN-γ^+ total lymphocytes and their subsets in the pig lungs, lung MNCs were restimulated with killed respective challenged influenza virus antigens (20 μg/ml) for 48 hrs, and the cells were immunostained first using surface specific markers for pig CD4, CD8, and $TcRN4^+$, and subsequently stained for IFN-γ^+ by intracellular staining using standard procedures. Stained cells were analyzed as described above.

4. **Recall cytokine response by immune cells:** To determine the functional response of *in vivo* primed immune cells in vaccine trial pigs, lung MNCs were subjected to *in vitro* restimulation with killed respective challenged influenza virus antigens (20 μg/ml) for 48 hrs and the culture supernatants harvested were analyzed for different swine cytokines. Cells cultured without any restimulation were included as control

5. ***Analysis of cytokine response:*** Lung homogenates prepared on the day of necropsy and *in vitro* restimulated primed immune cells culture supernatants were analyzed for innate (IFNα), proinflammatory (IL-6), Th1 (IFN-γ and IL-12), T-regulatory (1L-10 and TGFβ) and IL-17 cytokines by ELISA as described previously (19).

6. ***SIV specific neutralizing antibody response:*** Serum collected was tested for anti-SIV specific neutralizing antibody titers by standard immunofluorescence neutralization assay (18, 19).

7. ***Quantification of viral load*****:** For determination of viral load, the nasal wash and lung homogenate samples were analyzed for infective influenza virus titers by standard tissue culture method using MK1 and MDCK cells (19).

8. ***Pig NK cytotoxic assay:*** The innate NK cell-mediated cytotoxicity in the lungs of vaccine trial pigs was performed by a non-radioactive colorimetric assay standardized for pig NK cells by us (17, 20). Briefly, lung MNCs were used as source of NK cells (effectors) against K-562 (human myeloblastoid cell line) or Yac-1 (mouse T lymphoma cell line) (target cells) maintained in RPMI supplemented with FBS. Effectors were plated at 10-fold dilutions with fixed number of targets to get different E:T ratios. The seeded plate was incubated for 24 hr at 37^0C in a CO_2 incubator, and then supernatant was harvested and measured the amount of lactate dehydrogenase (LDH) present in the supernatant, which is directly proportional to NK specific lysis of target cells. The percent NK specific killing will be calculated using the formula: $[(OD_{E+T} - OD_E - OD_{T\text{-}spon})/OD_{T\text{-}total}\text{-}OD_{T\text{-}AM})]$ X 100. Where: E = effectors; T = targets; AM = assay medium; T-total = targets with 0.5% NP40; T-AM = targets with assay medium; T-spon = T-AM minus OD of AM control wells.

9. *Statistics:* Statistical analysis was performed to compare virus shedding, virus titers, frequency of immune cells, and cytokines production among the vaccine trial pig groups using non-parametric tests (Kruskal-Wallis test).

Results:

Results of Objective 1: Pigs vaccinated with monovalent inactivated SIV vaccine with a iNKT cell adjuvants and challenged with a homologous H1N1 virus had a significantly reduced viral load in the lungs (Fig. 1a). Clinically, adjuvanted vaccinated pigs did not suffer from flu symptoms (fever and anorexia), as a result the body weight of those pigs did not reduce compared to control challenged pigs (Fig. 1b). Immunologic response of lung MNCs identified that both the adjuvants (α-GalCer and PIM2) upregulated the secretion of an important innate immune cytokine IFN-α (Fig. 2a). NKT cells are known to activate NK cells and in pigs vaccinated with adjuvant α-GalCer (but not PIM2) a significant dose dependent increase in the NK cell-mediated cytotoxicity was detected, compared to control challenged pigs (Fig. 2b).

Further, immune correlates of protection were analyzed in the lungs of pigs. In a recall response, pig lung MNCs of SIV vaccine plus α-GalCer coadministered pigs had significantly increased frequency of IFN-γ secreting cells (Fig. 3a). The restimulated lung immune cells secreted significantly increased amounts of Th1 cytokines, IFNγ and IL-12 (Fig. 3b and c). In contrast, decreased production of both the immunosuppressive cytokines, IL-10 and TGF-β, in SIV vaccine plus α-GalCer or PIM2 coadministered pigs was detected (Fig. 3e and f). In addition, in SIV vaccine plus α-GalCer vaccinated pigs, along with reduced lung pathology significantly reduced secretion of a proinflammatory cytokine, IL-6, was detected (Fig. 3d).

As an indicator of *in situ* lung response in vaccinated SIV challenged pigs, we prepared lung homogenates and measured the amount of different cytokines by ELISA. Similar to our results on recall cytokine response, a significantly increased amounts of IFNα and IL-17, and reduced amounts of IL-6, IL-10 and TGF-β in SIV vaccine plus α-GalCer vaccinated pigs was detected (Fig. 4 a to f). While in SIV vaccine plus PIM2 vaccinated pigs a similar increased but reduced trend in cytokines was detected, and the differences in a few cytokines levels was not statistically significant. In addition, increased IFN-γ secreting total lymphocytes, $CD4^+$, $CD8^+$, $CD4^+CD8^+$ T lymphocyte subsets, and γδ cells were detected in the lungs of pigs immunized with killed SwIV vaccine with α-GalCer, compared to control virus challenged pigs (Fig. 5 a and b).

Results of Objective 2: Heterologous challenge studies with α-GalCer adjuvanted bivalent vaccine: Pigs challenged with either of the heterologous SIV-H1N1 and H3N2 viruses [pandemic 2009 H1N1 (A/California/04/2009) or Sw/TX/1499-2/98) irrespective of vaccinated or not vaccinated did not show any clinical disease. Also there was no difference in the net body weight gain. During necropsy we did not appreciate lung lesions typical of influenza virus infection in either virus challenged pigs. However, enhanced nasal viral shedding and lung viral load was detected in pandemic 2009 H1N1 challenged pigs (Fig 6. The viral titers in both the vaccine alone and vaccine plus adjuvant group were high and comparable (Fig 6). We did not detect any virus in the lungs of mock pigs challenged with pandemic 2009 H1N1 (Fig. 6). In H3N2 virus challenged pigs, we did not detect any virus in the nasal swabs, lung homogenate and blood.

Immunologically, reduced frequency of $CD4^+$ T cells, $CD8^+$ T cells, and NK cells were detected in H3N2 virus challenged pigs, irrespective of vaccinated or not vaccinated (Fig 7). But, in pandemic 2009 H1N1 virus challenged pigs, the frequency of NK cells and $CD4^+$ T cells in vaccinated pigs were significantly higher than mock challenged animals; and their levels were comparable in both vaccine with or without adjuvant received pigs (Fig. 7). In contrast, the frequency of $CD8^+$ T cells was significantly reduced in both the vaccinated, pandemic 2009 H1N1 virus challenged compared to mock challenged pigs (Fig. 7). The frequency of IFN-γ^+

total T cells, NK cells, $CD4^+$ T cells, and γδ T cells were neither significantly reduced nor increased in vaccinated H3N2 virus challenged pigs (Fig. 8). But, in pandemic 2009 H1N1 virus challenged vaccinated pigs, IFN-γ^+ total T cells, $CD4^+$ T cells, and γδ T cells were significantly reduced compared to mock challenged pigs (Fig. 8).

Heterologous challenge studies with Poly I:C adjuvanted bivalent vaccine: Commercial pigs that possessed maternal influenza virus antibodies, when immunized with PAV vaccine developed cross-reactive antibodies against the challenge viruses. Also immunized pigs when challenged with either H1N1 or H1N2 viruses showed significantly reduced lung lesions as compared to commercial vaccine-inoculated or non-vaccinated challenge pigs (Fig. 9) and nasal virus shedding of challenge viruses was also significantly less (Fig. 10).

Discussion

Swine influenza is a highly contagious, acute respiratory viral infectious disease of swine. Control of spread of SIV among pig herds and prevention of transmission of SIV of zoonotic potential to humans is important, and it is possible only by development and use of effective vaccines. Therefore, there is an urgent and important public health importance to develop effective vaccines against SIV. It is well established that stimulating the mucosal immune system results in production of both mucosal (local) and systemic immunity, which results in better block in entry of infectious agents into the body. It is known that SIV suppresses the host's innate immune responses; therefore, use of suitable adjuvants to potentiate the efficacy of SIV vaccines is crucial. In this proposal, we tested potent mucosal adjuvants α-GalCer and Poly I:C. Invariant Natural Killer T (iNKT) cells have been reported to play an important role in generation of cross-protective immunity against influenza virus vaccines in mouse models. Also poly I:C and its synthetic analogues have been shown to activate innate immune response and provide cross-protective immunity against homologous and heterologous including H5N1 influenza virus. Therefore, our goal was to coadminister the killed SIV vaccine with potent adjuvant/s to pigs, intranasally, to elicit better cross-protective immunity against heterologous SIV challenge. Immune correlates of protection included evaluation of clinical disease, analyses of viral load, immune cells, and secretion of cytokines in the lungs.

Our initial results suggested that activation of pig iNKT cells in the respiratory tract by intranasal delivery of iNKT cell adjuvant potentiated the influenza virus specific innate and adaptive immune responses to the homologous challenged virus. Associated with greater than two-log reduction in the lung viral load, significantly increased secretion of innate and Th1 cytokines, and reduced immunosuppressive cytokines production. Thus, our results using NKT cell adjuvanted UV-killed monovalent H1N1 SIV vaccine augment the protective immune response to genetically identical or very closely related SIV infection in pigs. In contrast, in α-GalCer adjuvanted inactivated bivalent vaccine inoculated, heterologous pandemic 2009 H1N1 virus challenged pigs, enhanced nasal viral shedding and lung viral load was detected. Immunologically, although increased frequency of NK cells and $CD4^+$ T cells were detected in both with and without dual adjuvant vaccinated, pandemic 2009 H1N1 virus challenged pigs; the frequency of IFN-γ^+ secreting total lymphocytes, $CD4^+$ T cells, and γδ T cells were significantly reduced in the lungs. Thus, inactivated SIV bivalent vaccine in pigs enhanced the pandemic 2009 H1N1 virus replication and dampened the anti-viral immune response; and the NKT cell adjuvant did not rescue the suppressive response induced by the inactivated SIV vaccine.

Poly(I:C) is a potent mucosal adjuvant that induce type I interferons (IFNs), and have the potential to bridge the gap between innate and adaptive immunity (21). Maternal antibody positive pigs vaccinated with poly I:C adjuvanted bivalent vaccine provided protection against heterologous H1N1 and H1N2 viruses. Experimental stuies in pigs have shown that poly I:c act as effective adjuvant and poly I:C adjuvanted foot and mouth disease (FMD) and PRRS vaccine provided protection against multiple antigenic FMD virus and PRRS virus (22, 23)

Summary: α-GalCer adjuvanted inactivated vaccine failed to provide adequate protection against heterologous virus challenge. However, ploy I:C adjuvanted inactivated vaccine was effective in providing protection against antigenic variant and heterologous virus challenge as indicated by reduced gross lung lesions and reduced nasal challenge virus shedding. Thus our data suggest that Poly I:C in promising mucosal adjuvant for the development of novel cross-protective vaccine against influenza virus.

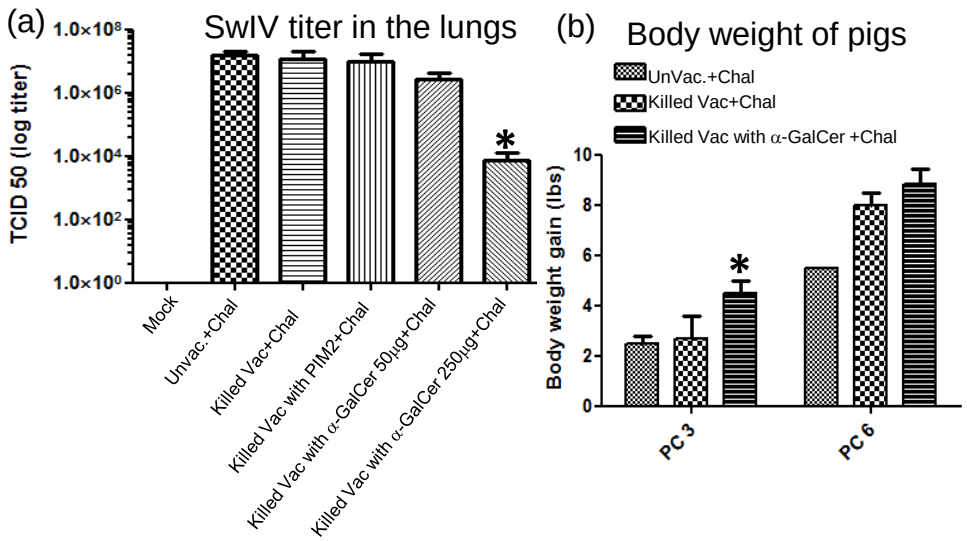

Fig.1. iNKT cell-mediated adjuvanticity to UV-killed SwIV H1N1 vaccine significantly reduced the viral load in the lungs and disease burden. Pigs were vaccinated as indicated and challenged on day post-vaccination 21 with a homologous SwIV. (a) SwIV titer in the lungs was determined, and (b) the body weight of the pigs was recorded on post-challenge (PC) days 3 and 6. Each bar is the mean value from 3 pigs ± SEM. Asterisk indicates statistically significant difference.

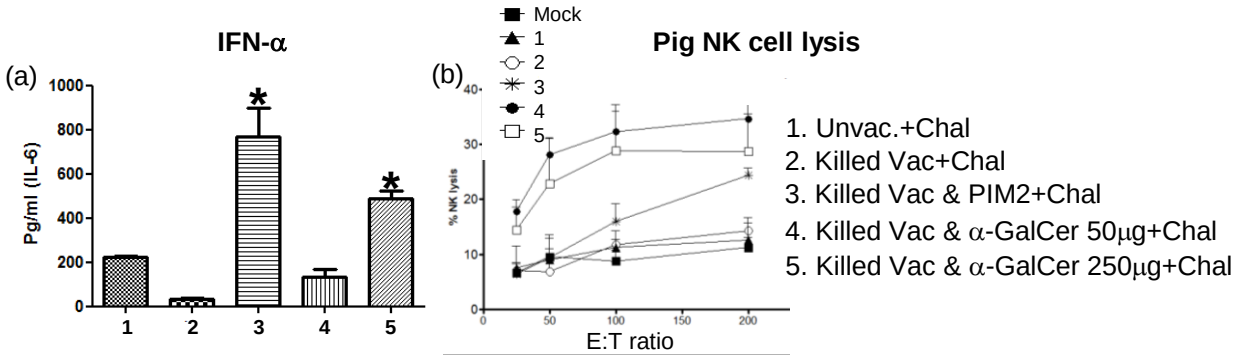

Fig. 2. iNKT cell-mediated adjuvanticity to UV-killed SwIV vaccine in pigs has significantly increased the production of innate cytokine and rescued the lung NK cell killing function. Pigs were vaccinated as indicated and challenged on day post-vaccination 21 with a homologous SwIV. (a) Lung lymphocytes were restimulated in vitro using killed SwIV antigens and the harvested supernatants were analyzed for cytokine IFN-α by ELISA. (b) Lung lymphocytes were cultured with NK target cells (K562) at indicated effector to target (E:T) ratio and the NK specific lysis was calculated by a standard lactate dehydrogenase assay. Each bar or data point is the mean value from 3 pigs ± SEM. Asterisk indicates statistically significant difference compared to both the control pig groups data. .

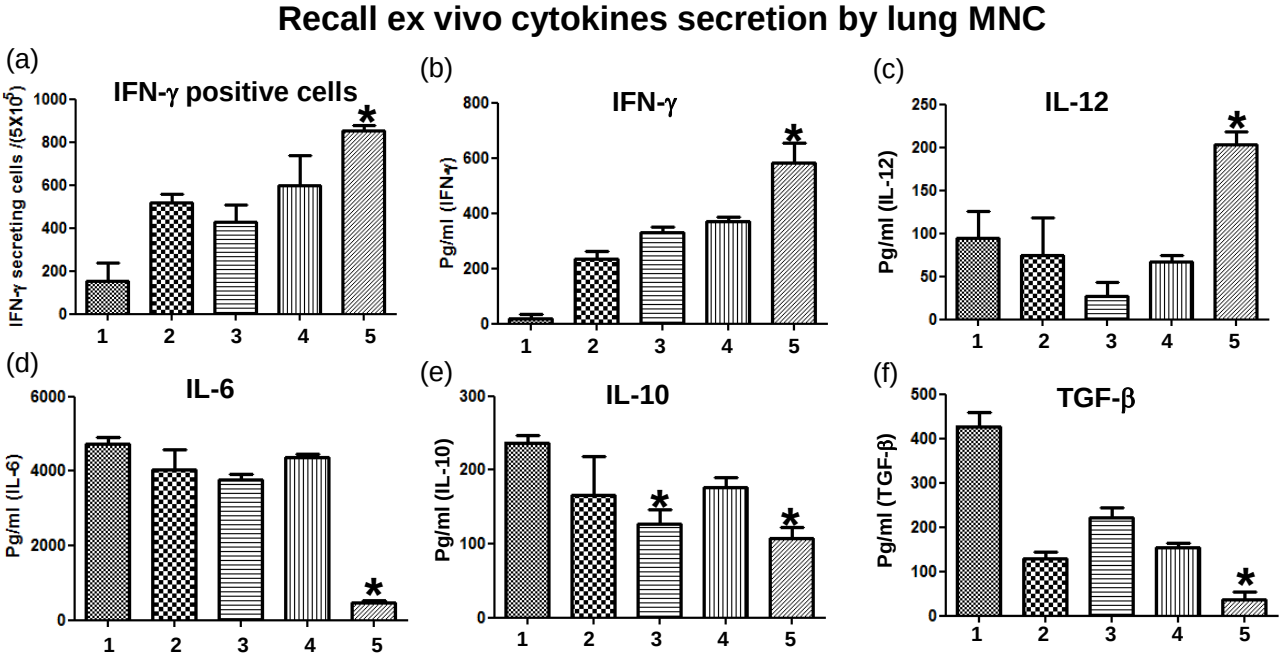

Fig. 3. iNKT cell-mediated adjuvanticity to UV-killed SwIV vaccine in pigs significantly increased the Th1 and suppressed the immunosuppressive cytokines. Pigs were vaccinated as indicated and on day 21 post-vaccination challenged with a homologous SwIV. Lung lymphocytes were restimulated in vitro using killed SwIV antigens: (a) frequency of IFN-γ secreting cells were analyzed by ELISPOT assay; and the harvested supernatants were analyzed for cytokines: (b) IFNγ; (c) IL-12; (d) IL-6: (e) IL-10; and (f) TGF-β by ELISA. Each bar is the mean value from 3 pigs ± SEM. Asterisk indicates statistically significant difference compared to both the control pig groups data. .

1. Unvac.+Chal
2. Killed Vac+Chal
3. Killed Vac & PIM2+Chal
4. Killed Vac & α-GalCer 50μg+Chal
5. Killed Vac & α-GalCer 250μg+Chal

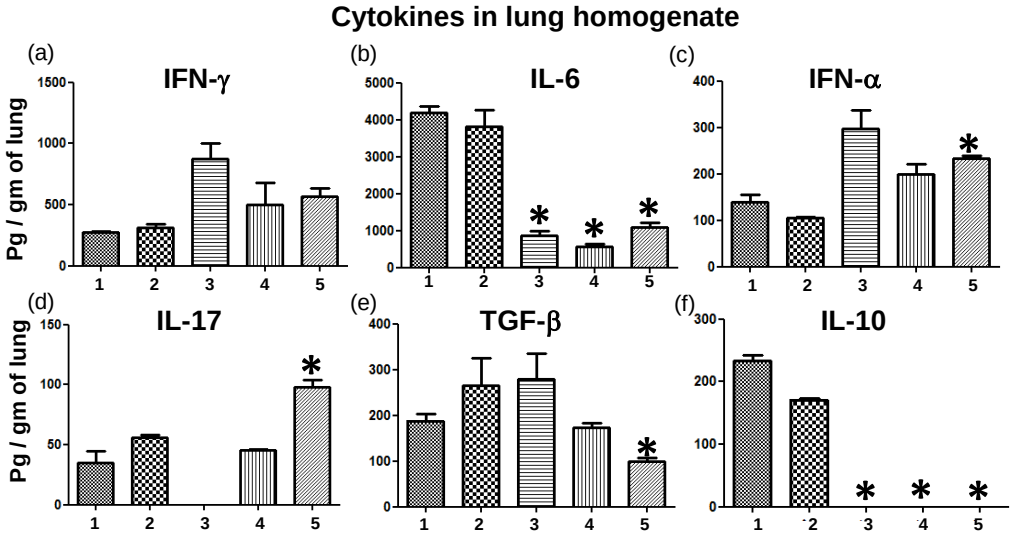

Fig. 4. iNKT cell-mediated adjuvanticity to UV-killed SwIV vaccine in pigs significantly increased the secretion of innate and Th1 cytokines and suppressed the immunosuppressive cytokines. Pigs were vaccinated as indicated and on day 21 post-vaccination challenged with a homologous SwIV. Lung homogenates were analyzed for cytokines: (a) IFNγ; (b) IL-6; (c) IFN-α; (d) IL-17; (e) TGF-β; (f) IL-10 by ELISA. Each bar is the mean value from 3 pigs ± SEM. Asterisk indicates statistically significant difference compared to both the control pig groups data. .

1. Unvac.+Chal
2. Killed Vac+Chal
3. Killed Vac & PIM2+Chal
4. Killed Vac & α-GalCer 50μg+Chal
5. Killed Vac & α-GalCer 250μg+Chal

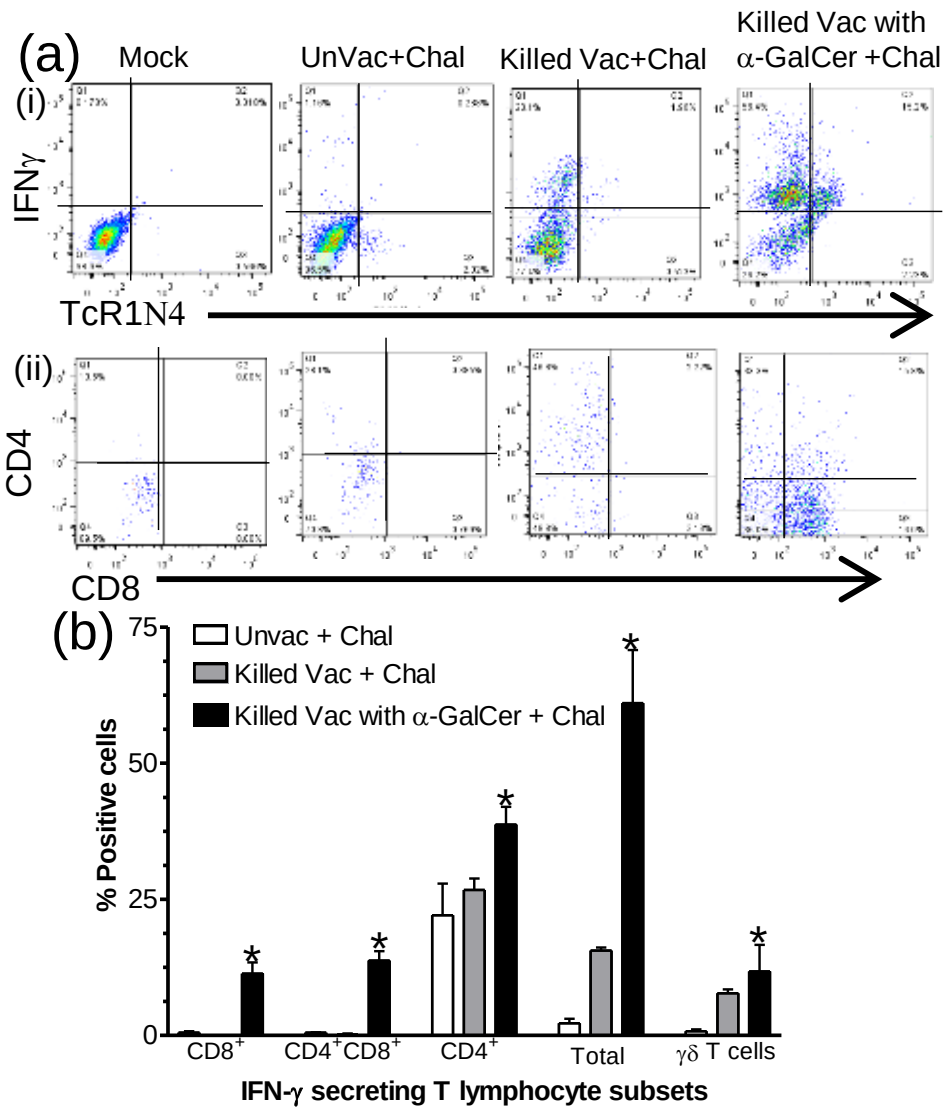

Fig. 5. iNKT cell-mediated adjuvanticity to UV-killed SwIV vaccine significantly enhanced the frequency of IFN-γ positive lymphocytes in the lungs of pigs. Pigs were vaccinated as indicated and on day 21 post-vaccination challenged with a homologous SwIV. (a,i) Lung lymphocytes were gated to show IFN-γ secreting γδ T cells, (a,ii) IFN-γ positive lung lymphocytes were gated to show $CD4^+$ and $CD8^+$ subsets, and (b) Percent IFNγ positive lung lymphocyte subsets are shown. Each bar is the mean value from 3 pigs ± SEM. Asterisk indicates statistically significant difference compared to other two control groups. .

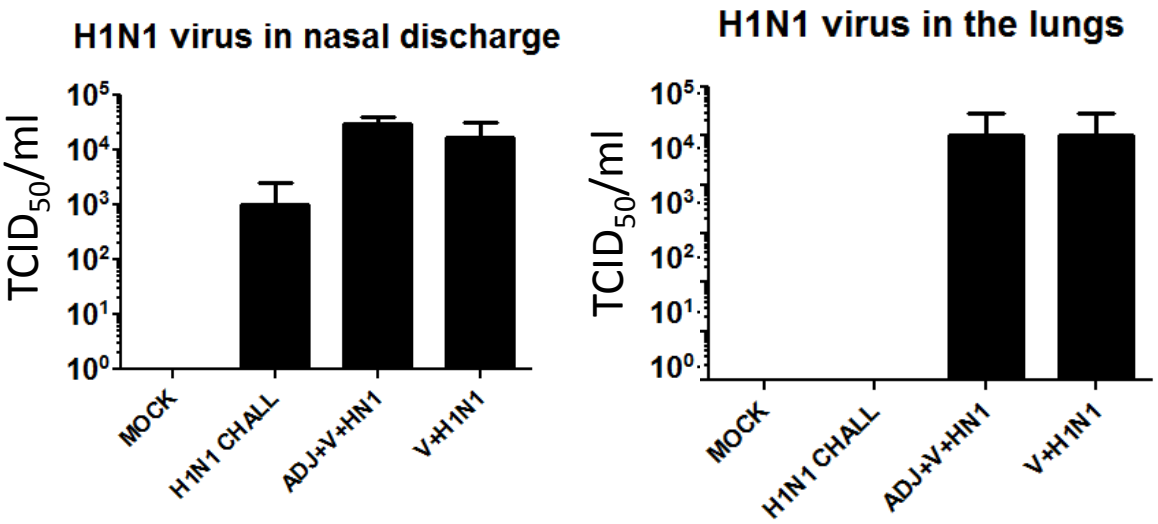

Fig. 6. iNKT cell-mediated adjuvanticity to UV-killed bivalent SwIV vaccine did not reduce the pandemic 2009 H1N1 virus load in the respiratory tract. Pigs were vaccinated with inactivated bivalent (H1N1 & H3N2) vaccine with adjuvants (α-GalCer+PIM2), twice intranasally at 2-weeks interval, and challenged with a heterologous pandemic 2009 H1N1 influenza virus. The nasal discharge and lung homogenate samples collected at post-challenge day six were analyzed for the challenged virus titer by indirect immunofluorescence assay. Each bar is the average titer from 4 pigs ± SEM.

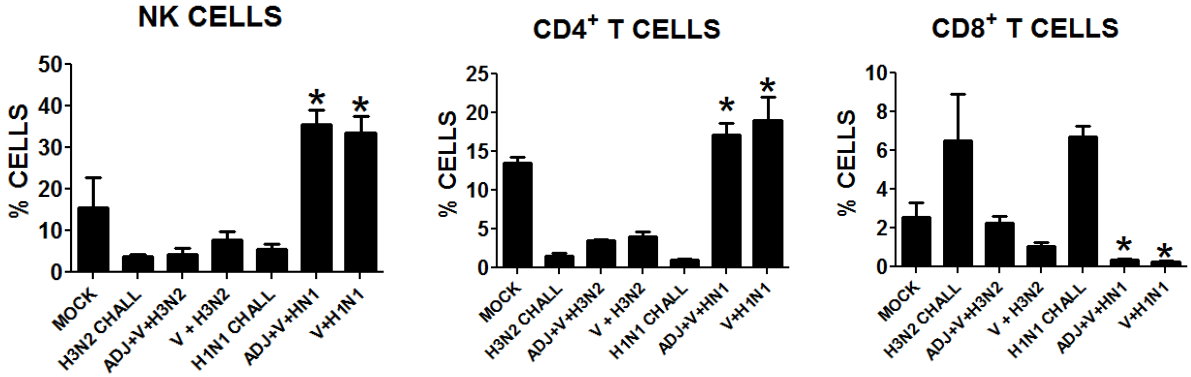

Fig. 7. iNKT cell-mediated adjuvanticity to UV-killed bivalent SwIV vaccine did not boost the frequency of important T cell subsets and NK cells in pandemic 2009 H1N1 virus challenged pigs lungs. Pigs were vaccinated with inactivated bivalent (H1N1 & H3N2) vaccine (V) with adjuvants (α-GalCer+PIM2) (ADJ), twice intranasally at 2-weeks interval, and challenged with a heterologous H1N1 or H3N2 influenza virus (pandemic 2009 H1N1 virus or H3N2 A/Swine/TX/4199-2/98 virus). Lung MNCs isolated at post-challenge day six were immunostained to analyze the frequency of NK cells, T-helper ($CD4^+$ T cells), and $CD8^+$ T cells by flow cytometry. Each bar is the average titer from 4 pigs ± SEM. Asterisk indicates statistically significant difference between respective virus challenged and vaccinated pigs.

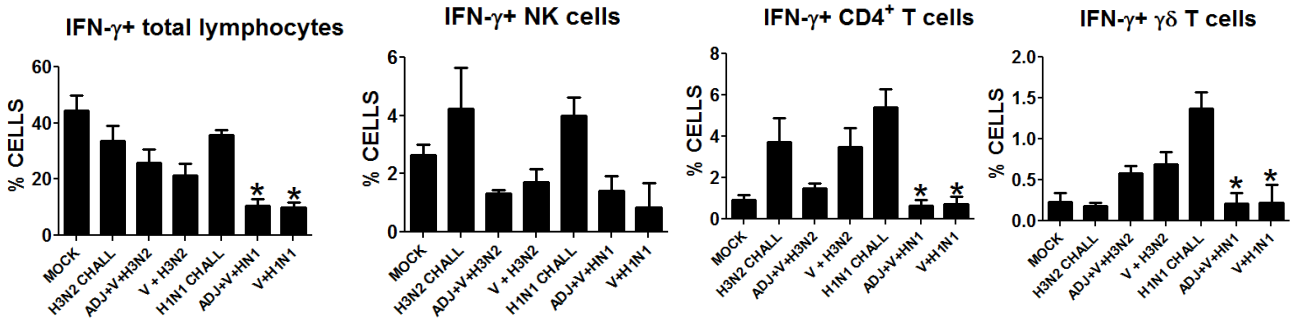

Fig. 8. iNKT cell-mediated adjuvanticity to UV-killed bivalent SwIV vaccine did not increase the frequency of IFN-γ secreting lymphocytes in the lungs of heterologous viruses challenged pigs. Pigs were vaccinated with inactivated bivalent (H1N1 & H3N2) vaccine (V) with adjuvants (α-GalCer+PIM2) (ADJ), twice intranasally at 2-weeks interval, and challenged with a heterologous H1N1 or H3N2 influenza virus (pandemic 2009 H1N1 virus or H3N2 A/Swine/TX/4199-2/98 virus). Lung MNCs isolated at post-challenge day six were cultured in the presence of killed pandemic 2009 H1N1 influenza virus antigens for 48 hrs, and then the cells were immunostained to analyze the frequency of IFN-γ secreting total lymphocytes, NK cells, $CD4^+$ T cells and γδ T cells by flow cytometry Each bar is the average titer from 4 pigs ± SEM. Asterisk indicates statistically significant difference between respective virus challenged and vaccinated pigs.

Gross lung lesions

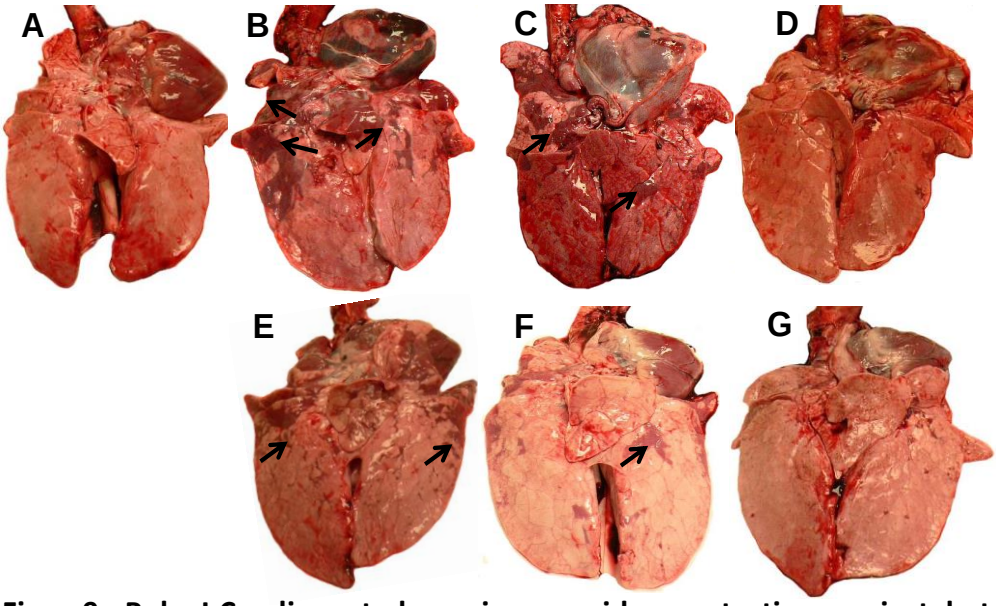

Fig. 9: Poly I:C adjuvanted vaccine provides protection against heterologous virus challenge. Three-week-old pigs were immunized intranasally with poly I:C adjuvanted bilvalent vaccine or commercial vaccine twice at 2 weeks intervals. Three weeks after the second immunization, groups of vaccinated pigs were challenged with swine H1N1 or H1N2 viruses. Six days after challenge, gross lung lesions were examined. Extensive areas of consolidation were observed in non-vaccinated H1N1 (B) or HiN2 (E) challenged pigs. Lung lesions were also observed in commercial vaccinated and H1N1 (C) and H1N2 (F) challenged pigs. Minimal lung lesions were observed in poly I:C adjuvanted bivalent immunized and H1N1 (D) and H1N2 (G) challenged pigs. No lung lesions were observed in mock-inoculated pigs (A).

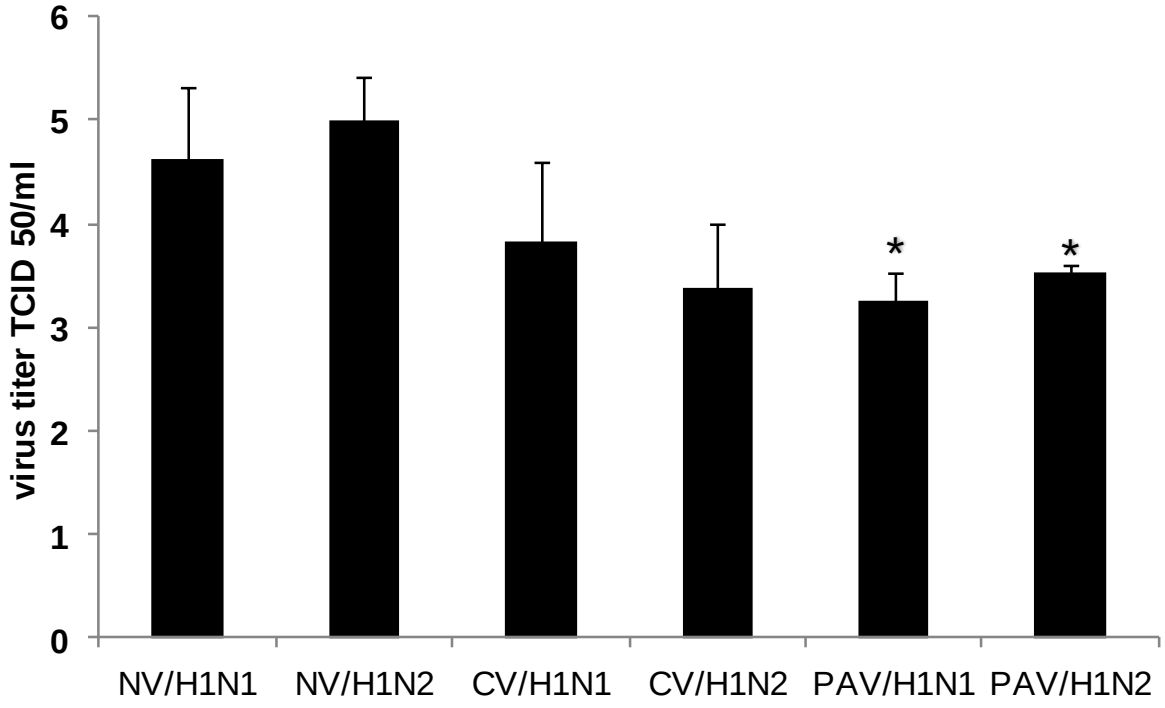

Fig. 10. Poly I:C adjuvanted bivalent vaccine reduces challenge virus shedding. Pigs were immunized intranasally with poly I:C adjuvanted inactivated bivalent (H1N1&H3N2) or commercial vaccine twice at 2 weeks interval. At 3 weeks after the second immunization, pigs were challenged with heterologous swine H1N1 or H1N2 influenza virus. Virus titer in nasal swabs collected at post-challenge day 3 were analyzed for the challenge virus by titration in MDCK cells. Each bar is the average from 4 pigs ± SD. Asterisk indicates significant differences compared to non-vaccinated virus challenged groups.

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