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Short communication

Adjuvant effects of invariant NKT cell ligand potentiates the innate and adaptive immunity to an inactivated H1N1 swine influenza virus vaccine in pigs

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ABSTRACT

Pigs are considered as the source of some of the emerging human flu viruses. Inactivated swine influenza virus (SwIV) vaccine has been in use in the US swine herds, but it failed to control the flu outbreaks. The main reason has been attributed to lack of induction of strong local mucosal immunity in the respiratory tract. Invariant natural killer T (iNKT) cell is a unique T cell subset, and activation of iNKT cell using its ligand α-Galactosylceramide (α-GalCer) has been shown to potentiate the cross-protective immunity to inactivated influenza virus vaccine candidates in mice. Recently, we discovered iNKT cell in pig and demonstrated its activation using α-GalCer. In this study, we evaluated the efficacy of an inactivated H1N1 SwIV coadministered with α-GalCer intranasally against a homologous viral challenge. Our results demonstrated the potent adjuvant effects of α-GalCer in potentiating both innate and adaptive immune responses to SwIV Ags in the lungs of pigs, which resulted in reduction in the lung viral load by 3 logs compared to without adjuvant. Immunologically, in the lungs of pigs vaccinated with α-GalCer an increased virus specific IgA response, IFN-α secretion and NK cell-cytotoxicity was observed. In addition, iNKT cell-stimulation enhanced the secretion of Th1 cytokines (IFN-γ and IL-12) and reduced the production of immunosuppressive cytokines (IL-10 and TGF-β) in the lungs of pigs. In conclusion, we demonstrated for the first time iNKT cell adjuvant effects in pigs to SwIV Ags through augmenting the innate and adaptive immune responses in the respiratory tract.

1. Introduction

Pigs are constantly exposed to genetically and antigenically variant influenza A viruses in the commercial herds. Humans are periodically infected with zoonotic SwIV, which transmit among humans and occasionally causing global outbreaks such as the 2009 pandemic swine flu (Shinde et al., 2009). The swine influenza is a highly contagious disease characterized by weight loss, fever, cough, anorexia and nasal discharge in pigs. Economic impact of the SwIV on the global pig production and its interspecies transmission warrant the necessity of developing the cross-protective swine flu vaccines (Yassine et al., 2010).

Inactivated SwIV vaccines have been administered by intramuscular route, and they induce systemic antibody production and alleviate clinical disease, but they failed to control transmission of the infected virus in the swine herds (Rajao et al., 2014). Studies have demonstrated the advantages of a potent intranasal vaccine which generates both mucosal and systemic immunity (Holmgren and Czerkinsky, 2005). Intranasal vaccines against influenza, parainfluenza-3, and porcine reproductive and respiratory syndrome (PRRS) virus coadministered with a potent adjuvant elicits cross-protective immunity (Binjawadagi et al., 2014b; Guillonneau et al., 2009; Kamijuku et al., 2008; Karron et al., 1995; Renukaradhya et al., 2012). Thus, intranasal delivery of inactivated SwIV vaccine with a potent adjuvant may be an attractive strategy

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to induce protective immunity and reduce the flu virus transmission in swine herds.

Invariant natural killer T (iNKT) cell is a CD1d restricted lymphocyte subset, and it was shown to play an important role in antiviral immunity (Cerundolo et al., 2009). CD1d is a MHC class I-like molecule and it presents glycolipid antigen to iNKT cell. A well characterized CD1d ligand is α-Galactosylceramide (α-GalCer) (Kopecky-Bromberg et al., 2009). The α-GalCer activated iNKT cell rapidly secrets large amounts of Th1 and Th2 cytokines, and induces the expression of costimulatory molecules on dendritic cells and contributes to bystander activation of NK cells, T cells and B cells (Carnaud et al., 1999; Hermans et al., 2003; Vomhof-DeKrey et al., 2014). The α-GalCer has been shown to potentiate the cross-protective efficacy of influenza vaccine candidates in mice model (Guillonneau et al., 2009; Kamijuku et al., 2008; Ko et al., 2005; Kopecky-Bromberg et al., 2009). Until now such a novel adjuvant role of iNKT cell to influenza virus was not evaluated in a higher mammalian species which is a natural host for flu. Like humans, pig is a natural host for all three subtypes of influenza H1N1, H1N2, and H3N2 viruses. Pig has iNKT cell and CD1d transcripts, and recently iNKT cell in pig was discovered and its adjuvant effects to a model Ag was demonstrated using α-GalCer (Artiaga et al., 2014; Eguchi-Ogawa et al., 2007; Looringh van Beeck et al., 2009; Renukaradhya et al., 2010). Further, pigs genetically knocked out for CD1d were shown to be iNKT cell deficient (Yang et al., 2015). In this study, we examined adjuvant effects of α-GalCer to inactivated H1N1 SwIV Ags coadministered intranasally in pigs, and evaluated its efficacy against a homologous virus challenge.

2. Materials and methods

2.1. Pigs and virus

Influenza-specific antibodies are present in a majority of conventional pigs due to either vaccination or natural infection. Therefore, in this study we used naturally delivered and bovine colostrum fed conventional Large White-Duroc crossbred baby pigs. Pigs were raised and housed in a BSL2 facility at the FAHRP, OARDC, Wooster. Prior to beginning the study, pigs were confirmed negative for influenza antibody by ELISA using semipurified H1N1 virus Ags coated plates including the known positive and negative control sera. A zoonotic SwIV H1N1 (Sw/OH/24366/07, SwIV OH07) (Yassine et al., 2009) was propagated in Madin-Darby canine kidney (MDCK) cells, cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, penicillin-streptomycin and amphotericin B (Gibco). For the preparation of virus stock and titration, MDCK cells were cultured in DMEM supplemented with TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone) treated trypsin (1 μg/ml) at the time of virus infection and treating infected pig samples. The vaccine virus was inactivated by UV-treatment by using a UV-lamp (245 nm) placed 2 in. above the opened 6-well plate containing the virus in a biosafety cabinet, and the inactivation was confirmed in MDCK cells kept for 4 days.

2.2. Immunization and infection of pigs

Fifteen 4-week-old pigs from two litters were randomly allocated into five groups (n = 3 per group), and vaccinated once using mock (DMEM) (Group 1 and 2), UV-inactivated SwIV OH07 (1×106 $TCID_{50}$) (Group 3), or UV-inactivated SwIV OH07 (1×10^6 $TCID_{50}$) coadministered with α-GalCer (50 or 250 μg/pig) (Groups 4 and 5) in the final volume of 2 ml as intranasal drops. Adjuvant α-GalCer used in this study was synthesized and provided by Dr. JG Hague (Department of Chemistry, University of California Davis). Pigs with similar body weight received either 50 or 250 μg dose of α-GalCer. The dose of α-GalCer used in this study was 50% and 90% less than the dose which caused airway hyperreactivity in pigs (Renukaradhya et al., 2011) and monkeys (Matangkasombut et al., 2008) delivered by intratracheal route. Pig groups 2–5 were challenged three weeks later using the homologous SwIV OH07 (1×10^6 $TCID_{50}$ per pig) intranasally and euthanized at day post-challenge (DPC) 6.

2.3. Detection of virus replication in the lung tissue

Homogenate (wt/vol) (lysate) of a piece of cranial lobe of the lung was prepared in DMEM containing antibiotics, aliquoted and stored at −80 °C until used for virus detection (Khatri et al., 2010; Renukaradhya et al., 2010). Briefly, MDCK cells cultured in 96-well tissue culture plates were inoculated with serial 10-fold dilution of the lung homogenate in DMEM and supplemented with TPCK (1 μg/ml). The virus induced cytopathic effect was examined after 72 h of incubation of plates at 37 °C, and the titers were calculated by the standard Reed-Muench method.

2.4. Determination of cytokine concentrations in lung by ELISA

Lung homogenate samples were subjected to cytokine analysis by ELISA (Khatri et al., 2010; Renukaradhya et al., 2010) to detect the levels of innate (IFN-α), proinflammatory (IL- 6), Th1 (IFN-γ and IL-12), and immunosuppressive [IL-10 and transforming growth factor β (TGF-β)] cytokines. Briefly, ELISA plates were coated with predetermined concentration of cytokine coating antibodies, IFN-α (Thermo Scientific), IFN-γ (BD Biosciences), IL-6 and IL-12 (R&D Systems), IL-10 and TGF-β (Invitrogen) and incubated overnight at 4 °C. Plates were washed and blocked and the test samples were added along with respective cytokine standards and incubated at RT. Washed plates were incubated with respective cytokine detection antibody followed by streptavidin-Horse radish peroxidase (HRP) at RT. Finally, Ag-Ab reaction was developed using TMB substrate and the reaction was stopped after appropriate intensity of color developed using 1 M Phosphoric acid, and the OD was read at 450 nm. Concentration of the cytokines was calculated by fitting the OD values of the samples against a standard curve of respective cytokine standards and expressed as picogram/ml.

2.5. ELISA for antigen specific antibodies

Influenza antigen-specific IgA antibodies were analyzed by ELISA as described previously (Khatri et al., 2010) with a few modifications. Briefly, Nunc Maxisorp 96-well plates were coated with a pretitrated concentration (15 μg/ml, 50 μl/well) of UV-inactivated semipurifed SwIV OH07 antigens derived from sucrose gradient centrifugation and incubated overnight at 4 °C. Uninfected MDCK cells derived Ags prepared similarly was used in the assay as a control. Plates were blocked at RT with 2% bovine serum albumin (BSA) in TBST (0.1 M Tris, 0.17 M NaCl, 0.05% Tween 20) and washed using PBST (phosphate-buffered saline plus 0.05% Tween 20). Serum and lung lysate samples were added (50 μl/well) in duplicate and the plate was incubated at RT. Samples of previously defined positive and negative control sera were included in the assay. Plates were washed and incubated with HRP-labeled anti-porcine IgA secondary antibody (KPL). Finally, plates were treated with TMB substrate to develop specific Ag-Ab interaction and the OD was read at 450 nm using a microplate reader (Molecular Devices SpectraMax Plus 384). To eliminate the background activity, plate coated with uninfected MDCK cells derived Ags was used as the control, blocked and treated exactly as described above. The OD values obtained from the experimental plates were subtracted from that of the control plate to obtain corrected OD values.

2.6. Collection of serum and bronchoalveolar lavage (BAL) fluid and isolation of the lung and tracheobronchial lymph nodes (TBLN) mononuclear cells

Blood sample was collected on the day of vaccination and at DPC 0, 3 and 6, and serum was aliquoted and stored at −20 °C until used in assays. BAL fluid was collected from euthanized pigs by perfusing the harvested lungs with 20–30 ml of ice cold PBS containing 0.03% (w/v) EDTA and 100 Units/ml of penicillin and 100 μg/ml of streptomycin sulphate, and the collected lavage fluid was centrifuged at 2095 xg for 15 min at 4 °C and the supernatant was aliquoted and stored at −80 °C until used in the assays. Lung mononuclear cells (LMNC) and TBLN MNC were isolated on the day of necropsy as described previously (Khatri et al., 2010). Briefly, samples of lungs and TBLN collected in DMEM were cut into small pieces and treated with collagenase and DNase and homogenized in stainless steel Cellectors. Homogenates were passed through the mesh, cells washed and the pellet was resuspended in RPMI containing 43% Percoll and centrifuged to remove the debris. RBCs in the cell pellet were lysed and MNCs washed and resuspended in enriched RPMI [RPMI-1640, 10% fetal bovine serum, gentamicin (100 μg/ml), ampicillin (20 μg/ml), 20 mM HEPES, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 nM of 2-ME], and live-dead cell count was performed by trypan blue dye exclusion method.

2.7. Influenza virus specific recall cytokine response in the lungs and estimation of virus specific IFN-γ secreting cells in the lungs and TBLN

We analyzed the recall/memory cytokine response in LMNC as described previously (Dwivedi et al., 2011) with a few modifications. Briefly, 5 million LMNC were restimulated with the inactivated semipurifed SwIV OH07 antigens (100 μg/ml) for 48 h at 37 °C in enriched RPMI plated in 24-well tissue culture plates. Supernatants were harvested after 48 h and performed ELISA to estimate the levels of cytokines IFN-γ, IL-6, IL-12, IL-10, TGF-β and IFN-α as described previously (Ouyang et al., 2014).

The frequency of IFN-γ-secreting cells in LMNC and TBLN MNC was determined by ELISPOT assay as described previously (Dwivedi et al., 2011; Khatri et al., 2010). Briefly, LMNC and TBLN MNC were plated (5×10^5 cells/well) in enriched RPMI in a 96-well MultiScreen plate (Millipore, Billerica, MA) precoated overnight with the mouse anti-pig IFN-γ mAb (BD PharMingen, CA) at 4 °C. Cells were restimulated with SwIV OH07 Ags (100 μg/ml) for 24 h at 37 °C in a CO_2 incubator. Washed plates were incubated with biotinylated anti-pig IFN-γ detection antibody followed by streptavidin-HRP conjugate, and the reaction was developed using an insoluble tetramethylbenzidine with H_2O_2 peroxidase substrate system (KPL Inc., MD). The frequency of influenza specific IFN-γ-secreting cells was counted using an AID® ELISpot Reader System. The background counts of the respective unstimulated cells were subtracted from their restimulated cells and the immune response was expressed as the number of IFN-γ-secreting cells per 0.5 million MNC.

2.8. NK cell cytotoxicity assay

A colorimetric NK cell-cytotoxcity assay was performed to determine the percent lysis of target cells as described previously (Renukaradhya et al., 2010). Briefly, LMNC were used as the source of NK cells (effectors) and K-562 human myeloblastoid leukemia cells as targets. Different ratios of effectors and targets were incubated for 24 h at 37 °C in a CO_2 incubator and the amount of released lactate dehydrogenase (LDH) into the supernatant was measured by the LDH substrate. The percent NK cell-specific lysis of target cells was calculated after subtracting the spontaneously released LDH due to nonspecific lysis of targets.

2.9. Phenotypic analysis of NK cells in the lungs

LMNC were immunostained using the pig lymphocyte specific cell surface markers and analyzed by flow cytometry as described previously (Binjawadagi et al., 2014a; Manickam et al., 2013). Briefly, LMNC were washed using fluorescence activated cell sorting (FACS) buffer (Hanks balanced salt solution [HBSS] containing 0.1% BSA and 0.02% sodium azide) and plated in U-bottom 96-well plates. Cells were blocked for the Fc receptors using FACS buffer containing 2% pig serum for 20 min and immunostained using monoclonal antibody (MAb) directly conjugated to fluorochrome, biotinylated or purified pig specific cell surface markers CD3ε (clone PPT3, Southern Biotech), CD4α (clone 74-12-4) and CD8α (clone 74-2-11) (BD Biosciences). Respective isotype controls were included in the assay. Cells were washed with FACS buffer and treated with streptavidin-conjugated fluorochrome or respective anti-species isotype-specific secondary antibody conjugated with fluorochrome. Subsequently, cells were

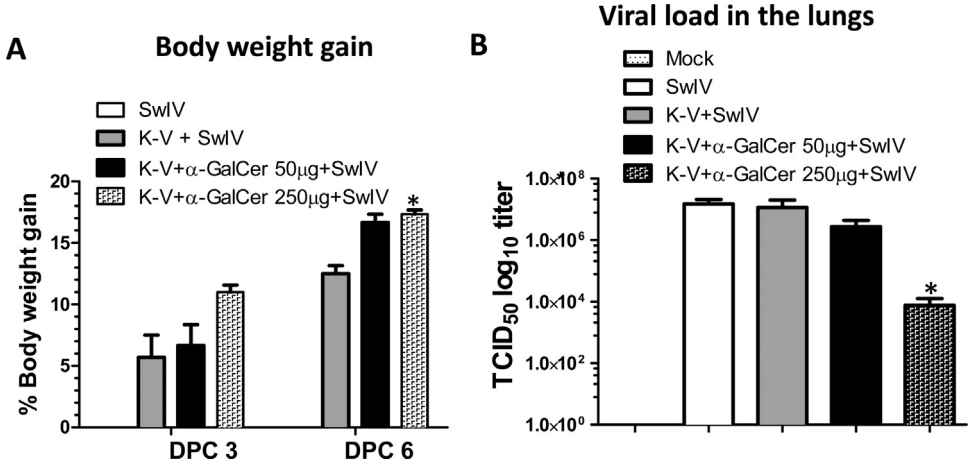

Fig. 1. Improved body weight gain and reduced viral load by iNKT cell adjuvanted inactivated SwIV vaccine in pigs. Pigs were vaccinated with inactivated SwIV H1N1 Ags coadministerd with or without iNKT cell adjuvant α−GalCer and challenged with a homologous virus and euthanized at DPC 6. (A) Percent body weight gain until DPC 6 considering the weight of pigs at DPC 0 as 0% (base line) was calculated. (B) Lung lysate samples were analyzed for the infective SwIV titer by cell culture method. Each bar represents an average viral load in the lungs or percent body weight gain of 3 pigs ± SEM. Asterisk denotes statistical significance between SwIV challenged pigs received virus Ags without adjuvant (K-V) with either low or high dose of the adjuvant.

fixed with 1% paraformaldehyde, and 100,000 events were acquired using FACS AriaII Flow cytometer (BD Biosciences) and analyzed using the FlowJo Software (Tree Star, Inc., OR).

2.10. Ethics statement

Pigs were maintained, inoculated and challenged as per the guidelines of Institutional Animal Care and Use Committee (Protocol Number: 2010A00000195). Physical condition of pigs were monitored daily twice and maintained under standard husbandry conditions with *ad libitum* water and balanced diet. All efforts were taken to minimize suffering of the pigs and euthanized as per the approved protocol.

2.11. Statistical analysis

All the data were expressed as the mean +/− SEM of three pigs. Statistical analyses were performed by one-way ANOVA followed by Tukey's *t*-test to establish differences between the pig groups. Statistical significance was assessed as $P < 0.05$.

3. Results

3.1. Clinical findings and viral load in the lungs of SwIV challenged pigs

Pigs were vaccinated with inactivated SwIV OH07 coadministerd with two different concentrations of adjuvant α-GalCer (50 and 250 μg), and challenged with the homologous virus. Clinically, unvaccinated virus-challenged pigs had occasional cough, anorexia and lethargia for first 3–4 days. Body weight gain in the vaccinated pigs was shown as percent of weight gain over unvaccinated-infected animals (indicated as 'SwIV') (Fig. 1A). Our data indicated that vaccine without adjuvant received pigs had 5 and 10% gain in the weight at DPC 3 and 6, respectively, compared to unvaccinated-infected animals (Fig. 1A). Pigs vaccinated with the higher dose of the adjuvant had a significant higher body weight gain (18%) compared to animals vaccinated without adjuvant at DPC 6 (K-V + SwIV) (Fig. 1A).

The lung viral load in both unvaccinated and vaccine without adjuvant received and virus-challenged pig groups were approximately 7 logs at DPC 6 (Fig. 1B). While in the lungs of inactivated SwIV coadministered with the higher dose of α-GalCer (250 μg) received pigs a significant reduction in the viral load by 3 logs was observed compared to viral Ags without adjuvant group (Fig. 1B); suggesting the potent adjuvant effects of iNKT cell to inactivated SwIV vaccine in pigs.

3.2. Mucosal antibody response in the lungs of vaccinated pigs

Since the vaccine was delivered to pigs as nasal drops, we analyzed the virus specific antibody response both in the airways and lungs. Our results identified a significantly increased SwIV specific IgA secretion both in the lungs and airways of pigs received SwIV Ags with the higher dose of α-GalCer (250 μg) compared to without adjuvant (Fig. 2A and C). Similarly, though statistically not significant, specific IgA secretion was high in tracheal wash of the

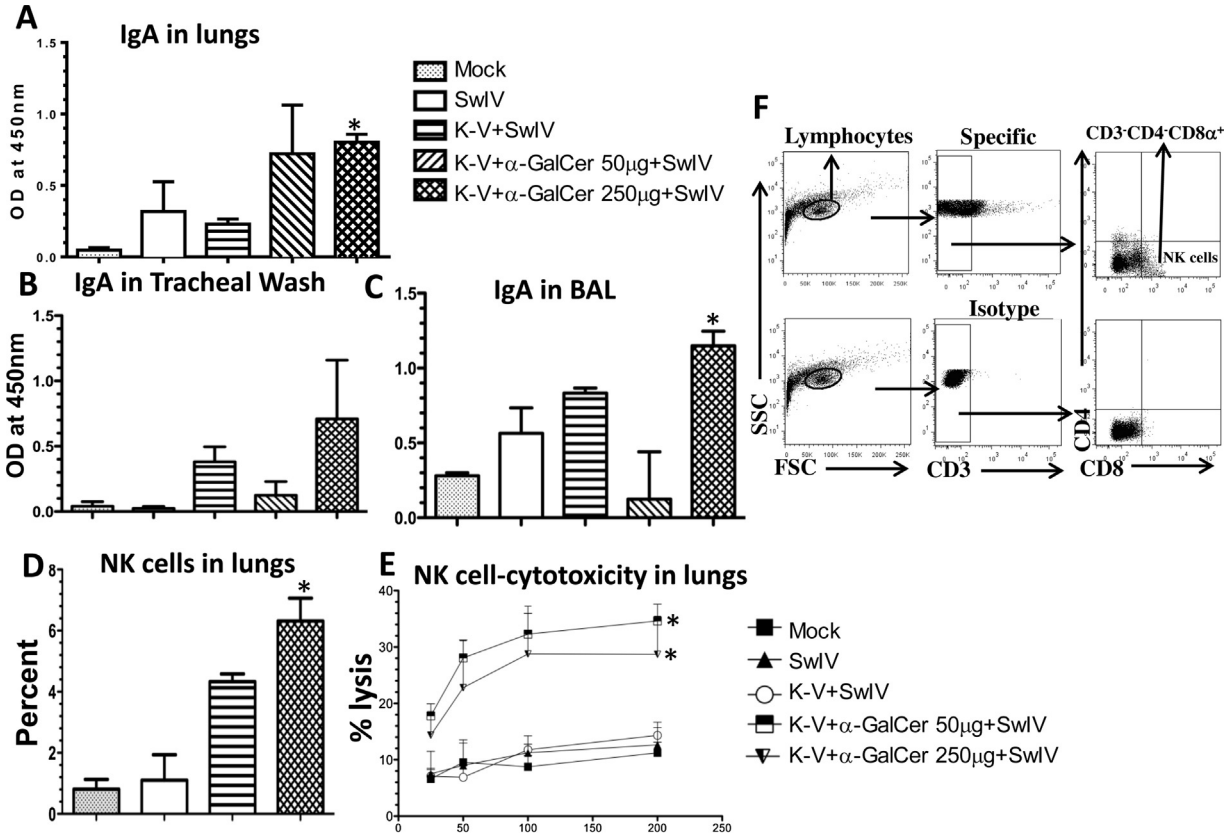

Fig. 2. Elevated levels of SwIV specific IgA antibody response and improved innate NK cell- cytotoxicity in the lungs of iNKT cell adjuvanted inactivated SwIV vaccinated pigs. Samples of lung lysate, tracheal wash and BAL fluid collected at DPC 6 from pigs vaccinated as indicated in figure legend 1 were analyzed for SwIV specific (A, B and C) IgA antibodies by ELISA. LMNC isolated at DPC 6 were (D) immunostained to analyze the frequency of NK cells ($CD3^-CD4^-CD8\alpha^+$) by flow cytometry; and (E) NK cell-cytotoxicity was determined using LMNC as source of NK cells (effectors) cocultured with target cells (K562) at different E:T ratios. (F) A representative flow cytometry plots depicting porcine NK cells. Cells were immunostained using different fluorochrome conjugated pig specific CD3ε, CD4α and CD8α lymphocyte markers and identified the frequency of $CD3^-CD4^-CD8\alpha^+$ NK cells. Each bar or data point represents an average OD value, percent of NK cells or percent NK cell-lysis from 3 pigs ± SEM. Asterisk denotes statistical significance between SwIV challenged pigs received virus Ags without adjuvant (K-V) with either low or high dose of the adjuvant.

high dose α-GalCer compared to lower dose and without adjuvant in inactivated SwIV received pig groups (Fig. 2B).

3.3. NK cell response in vaccinated animals

NK cell is an important innate immune cell involved in viral clearance, and activated iNKT cell substantially augments NK cell function through increasing its cytotoxic ability and IFN-γ secretion (Carnaud et al., 1999). In vaccinated pig lungs, we analyzed both NK cell frequency and its cytotoxicity. In both the doses of adjuvant received vaccinated pig groups, a 2–3 fold increase in NK cell-cytotoxicity was detected compared to unvaccinated and vaccine without adjuvant recipient pig groups (Fig. 2E). The increase in NK cell-cytotoxicity was appeared to be mediated by iNKT cell adjuvant effect through augmenting the frequency of NK cells ($CD3^{-}CD4^{-}CD8\alpha^{+}$) (Fig. 2D). A representative plot showing the gating of NK cells is shown (Fig. 2F).

3.4. iNKT cell adjuvant boosted the SwIV H1N1 specific adaptive immune response in pigs

In situ secreted cytokines in the lungs of pigs. Activated iNKT cell has been shown to rapidly secrete high levels of cytokines and induces the expression of costimulatory molecules on dendritic cells, and thus augment both innate and adaptive immune responses (Carnaud et al., 1999; Hermans et al., 2003). Therefore, we measured the secretion of different cytokines in the lungs of vaccinated pigs. Our data showed a significantly elevated levels of the Th1 cytokine IFN-γ in the lungs (lung lysate) of pig group received SwIV Ags with the higher dose of adjuvant compared to without adjuvant group (Fig. 3A). In contrast, the levels of proinflammatory cytokine IL-6 and immunosupppressive cytokines, IL-10 and TGF-β, were at a significantly lower levels in that pig group compared to animals vaccinated without adjuvant (Fig. 3B, D and E). Like the observed enhanced innate NK cell

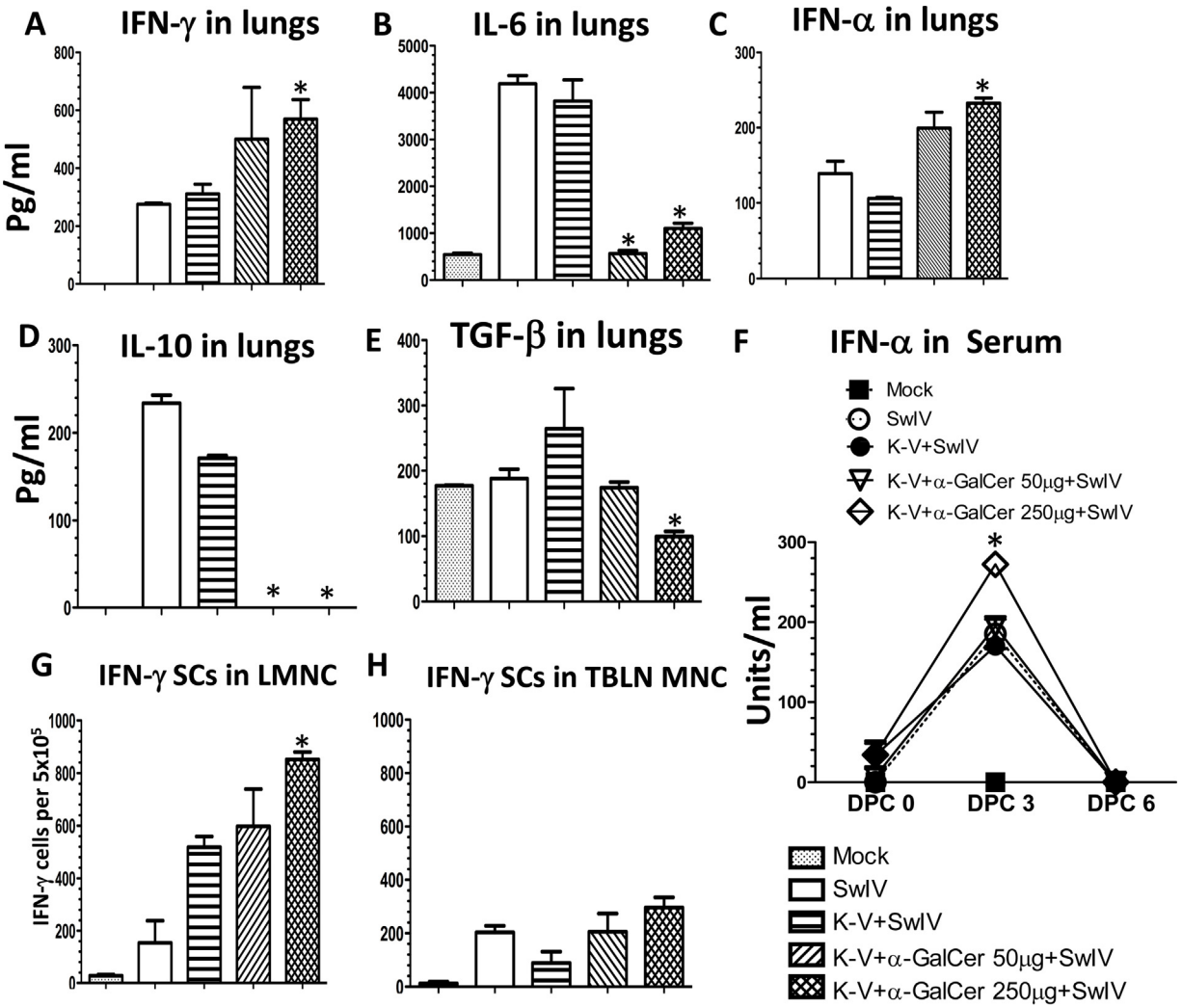

Fig. 3. Enhanced anti-viral and dampened immunosuppressive cytokines production in the lungs of iNKT cell adjuvanted inactivated SwIV vaccinated pigs. Lung lysate samples prepared at DPC 6 and serum samples collected at indicated three DPCs from pigs vaccinated as indicated in figure legend 1 were analyzed for cytokines: (A) IFN-γ, (B) IL-6, (C and F) IFN-α, (D) IL-10, and (E) TGF-β by ELISA. Lung (G) and TBLN (H) MNCs were restimulated *in vitro* with semipurified SwIV Ags and the frequency of IFN-γ secreting cells were determined by ELISPOT assay. Each bar or data point represents an average cytokine or number of IFN-γ secreting cells per 0.5 million MNC in 3 pigs ± SEM. Asterisk denotes statistical significance between SwIV challenged pigs received virus Ags without adjuvant (K-V) with either low or high dose of the adjuvant.

response, innate IFN-α production was also significantly increased both in the lungs and serum of pigs vaccinated using 250 μg dose of the iNKT cell adjuvant (Fig. 3C and F). Further, the frequency of SwIV specific IFN-γ secreting cells in the LMNC (but not TBLN) were significantly higher in pigs vaccinated with 250 μg of α-GalCer compared to animals vaccinated without adjuvant by ELISPOT assay (Fig. 3 G and H).

Recall secreted cytokines in the lungs of pigs. To identify the recall cytokine response in the lungs of pigs, LMNC were restimulated *in vitro* using SwIV Ags and the secreted cytokines were analyzed. Like in the lung lysate, a significantly increased production of IFN-γ by LMNC was observed in pigs vaccinated with the high dose α-GalCer (Fig. 4A). In addition, the levels of another Th1 cytokine IL-12 and innate IFN-α production were secreted at a significantly higher levels in iNKT cell adjuvanted SwIV Ags received pig group compared to Ags without adjuvant group (Fig. 4C and D). Also the levels of secreted IL-6, IL-10 and TGF-β cytokines were substantially lower in pigs vaccinated with adjuvanted SwIV Ags compared to without adjuvant (Fig. 4B, E and F).

4. Discussion

Immunity to influenza is dependent on both antibody and cell-mediated immune responses (Doherty et al., 2006; Stambas et al., 2008). To induce protective immunity against flu a collective and collaborative cross-talk between innate and adaptive immune components is critical. The iNKT cell adjuvant α-GalCer was shown to potentiate influenza virus specific adaptive immune response in mice vaccinated subcutaneously with both live and inactivated influenza vaccine candidates (Guillonneau et al., 2009). Intranasal immunization of mice with the hemagglutinin protein of H1N1, H3N2 and H5N1 strains of influenza virus with α-GalCer induces cross-protective immunity (Kamijuku et al., 2008). Intranasal delivery of inactivated influenza H1N1 with α-GalCer in C57BL/6 and BALB/c mice induces enhanced specific IgA and cytotoxic T cell response, resulting in 100% survival against a lethal viral challenge (Ko et al., 2005). But until now such studies are not performed in higher mammals.

We discovered iNKT cell in pigs and demonstrated its activation by treatment of PBMC, LMNC and splenocytes with α-GalCer *in vitro,* and *in vivo* in intratracheally delivered pigs (Renukaradhya et al., 2011). In this study, we observed that 50% less dose of α-GalCer (250 μg/pig) delivered as nasal drops potentiated mucosal response to coadministerd inactivated SwIV Ags. While the higher dose of α-GalCer (500 μg/pig) inoculated by intratracheal route induces airway-hyperreactivity (Renukaradhya et al., 2011).

Earlier in a study, pigs inoculated with H1N1 SwIV OH07 through intratracheal route caused severe lung inflammation and extensive replication of the virus in the lungs (Khatri et al., 2010). But in this study, our goal was to demonstrate the efficacy of inactivated SwIV Ags coadministered with α-GalCer in inducing mucosal immunity both in the upper and lower respiratory tract. Therefore, we administered the vaccine formulation (α-GalCer and inactivated SwIV Ags) and the challenge virus as nasal drops. Though the challenge virus did not cause severe clinical disease or lung lesions in unvaccinated pigs, but still high loads of infectious replicating virus was detected in the lungs.

We found dose-dependent response of α-GalCer to SwIV Ags in vaccinated pigs, with a significantly higher adjuvant effects with 250 μg dose compared to 50 μg dose delivered once intranasally. SwIV vaccines licensed in the US contain inactivated H1N1, H1N2, H3N2, and 2009 pandemic viruses (7). But unlike in human flu vaccine, the SwIV vaccine strains are not updated annually due to practical difficulties; and thus, it is important to enhance the level and breadth of immunity of swine flu vaccine using a potent adjuvant.

The iNKT cell adjuvant effects in pigs to intramuscular delivered α-GalCer with hen-egg lysozyme showed increased frequency of iNKT cells in circulation with enhanced Th1 and Th2 cytokines response (Artiaga et al., 2014). In intranasally vaccinated pigs, we found mucosal adjuvant effects of α-GalCer to inactivated SwIV Ags, indicated by enhanced innate and adaptive cytokine responses in the lungs. However, since we did not include a pig group inoculated with α-GalCer alone followed by a virus challenge, the observed adjuvant effects of α-GalCer to SwIV Ags in challenged pigs needs further investigation. But it is unlikely to be mediated

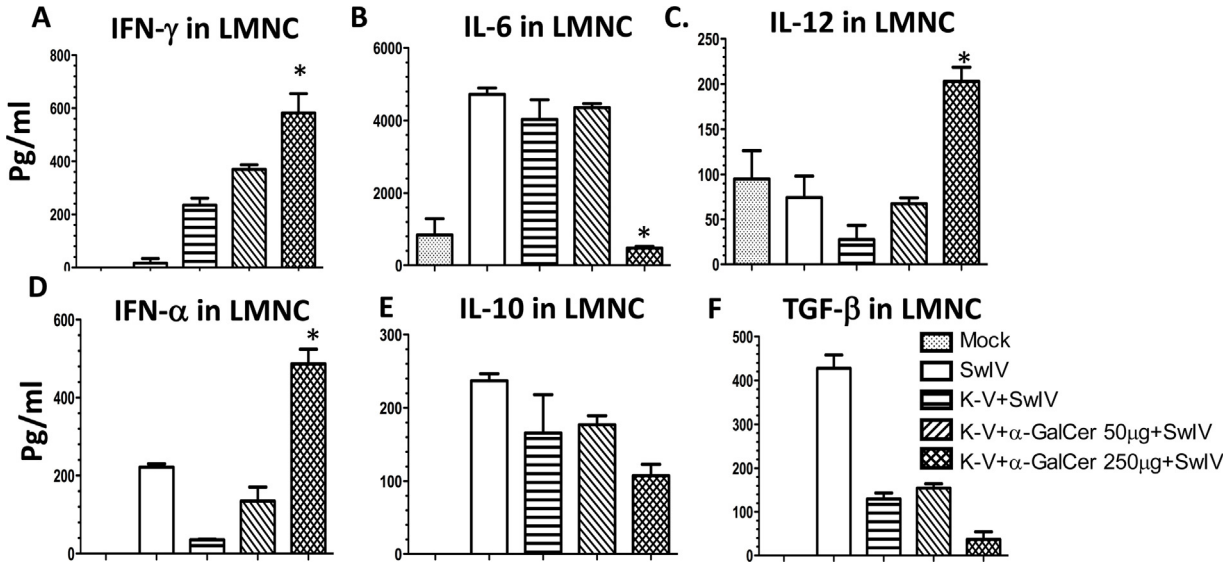

Fig. 4. Enhanced secretion of innate and Th1 and reduced immunosuppressive cytokines by cultured restimulated LMNC of iNKT cell adjuvanted inactivated SwIV vaccinated pigs. LMNC of pigs vaccinated as indicated in figure legend 1 were stimulated *in vitro* with semipurified inactivated SwIV antigens, and the culture supernatant harvested were subjected to analysis of cytokines: (A) IFN-γ, (B) IL-6, (C) IL-12, (D) IFN-α, (E) IL-10, and (F) TGF-β by ELISA. Each bar represents an average cytokine amount of 3 pigs ± SEM. Asterisk denotes statistical significance between SwIV challenged pigs received virus Ags without adjuvant (K-V) with either low or high dose of the adjuvant.

by the adjuvant alone, since we challenged pigs three weeks after the vaccination and by then innate effects of α-GalCer could have disappeared. Moreover, we performed iNKT cell analysis in LMNC on the day of necropsy (4 weeks after vaccination), and did not find any difference in their frequency among the pig groups.

Further, enhanced induction of Th1 cytokines in α-GalCer adjuvanted inactivated SwIV vaccine suppressed the immunosupppressive cytokines response in the lungs of pigs. Like in mice, iNKT cell adjuvant activity in pigs to inactivated SwIV Ags has helped in reducing the lung viral load. However, like in mice, to understand the adjuvant effects of α-GalCer in inducing heterologous and heterosubtypic immunity, we need to perform viral challenge studies in vaccinated pigs using genetically variant viruses. In conclusion, for the first time we demonstrated pig iNKT cell adjuvant effects in potentiating the innate and adaptive immune responses to intranasally vaccinated inactivated SwIV H1N1.

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