Short communication

Evaluation of CpG-ODN-adjuvanted polyanhydride-based intranasal influenza nanovaccine in pigs

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ABSTRACT

Influenza results in significant economic loss in the swine industry each year. A broadly protective swine influenza vaccine would have the dual benefit of protecting pigs from influenza A viruses (IAVs) and limiting their possible zoonotic transmission to humans. In this study, we developed polyanhydride nanoparticles-based swine influenza vaccine (KAg + CpG-nanovaccine) co-encapsulating inactivated/killed soluble antigen (KAg) and Toll-like receptor (TLR)-9 agonist (CpG-ODN). The immunogenicity and protective efficacy of KAg + CpG-nano-vaccine was compared with KAg vaccine containing five-times greater quantity of antigens following heterologous virus challenge. Prime-boost intranasally delivered KAg + CpG-nanovaccine induced significantly higher levels of cross-reactive antigen-specific IgA antibody responses in the nasal cavity, greater lymphoproliferative response in peripheral blood mononuclear cells (PBMCs), and higher IFN-γ secretion during antigen-induced recall responses of PBMCs and tracheobronchial lymph nodes cells compared to those immunized with KAg alone. Importantly, KAg + CpG-nanovaccine provided better protective efficacy through a significant reduction in influenza-induced fever, 16-fold reduction of nasal virus shedding and 80-fold reduction in lung virus titers compared to those immunized with soluble KAg. Our results indicated that CpG-ODN-adjuvanted polyanhydride nanovaccine can induce higher mucosal antibody and cellular immune responses in pigs; and provide better protection as compared with intranasally delivered soluble KAg.

1. Introduction

Influenza A virus (IAV) infection is a constant threat in the global pig industry and remains as one of the top three health challenges for U.S. pig farms (Vincent et al., 2017). Due to the possibility of concurrent infection with avian and human origin IAVs, pigs are considered as the 'mixing vessel' for the generation of novel IAV reassortants of zoonotic and pandemic potential (Hass et al., 2011). Effective vaccines can efficiently prevent influenza in pigs and its spillover to humans. Intramuscular monovalent or multivalent whole inactivated virus vaccines are predominantly used in the U.S. swine industry and over 50% of them are autogenous vaccines (Vincent et al., 2017). The intramuscular inactivated vaccines primarily induce systemic antibody responses and protect against homologous IAV strains but not against unrelated heterologous IAVs (Vincent et al., 2008, 2017). Whole inactivated vaccine formulations delivered through intramuscular routes also induce inefficient cell-mediated immunity and

mucosal antibody responses (Van Reeth and Ma, 2013; Vincent et al., 2017). Further, vaccine-associated enhanced respiratory disease (VAERD) and maternal antibody interference in piglets are also observed occasionally following intramuscular administration of inactivated vaccines (Kitikoon et al., 2006; Vincent et al., 2008). Thus, there is a need for novel vaccine delivery technologies that induce efficacious mucosal and cellular immune responses, override maternal antibodies, protect pigs against broad-spectrum of swine influenza virus (SIV), and do not result in VAERD.

Vaccination through the intranasal (IN) route presents antigens to the immune cells in a manner similar to natural infection. The IN route of vaccine delivery provides a larger surface area for antigen deposition and has less chance of enzymatic and chemical degradation compared to oral delivery. In addition, the nasal passage is highly vascularized and also contains a large number of microfold (M) cells which may enhance antigen uptake and elicit mucosal antibody and cellular immune responses compared to other routes (Davis, 2001). However, the
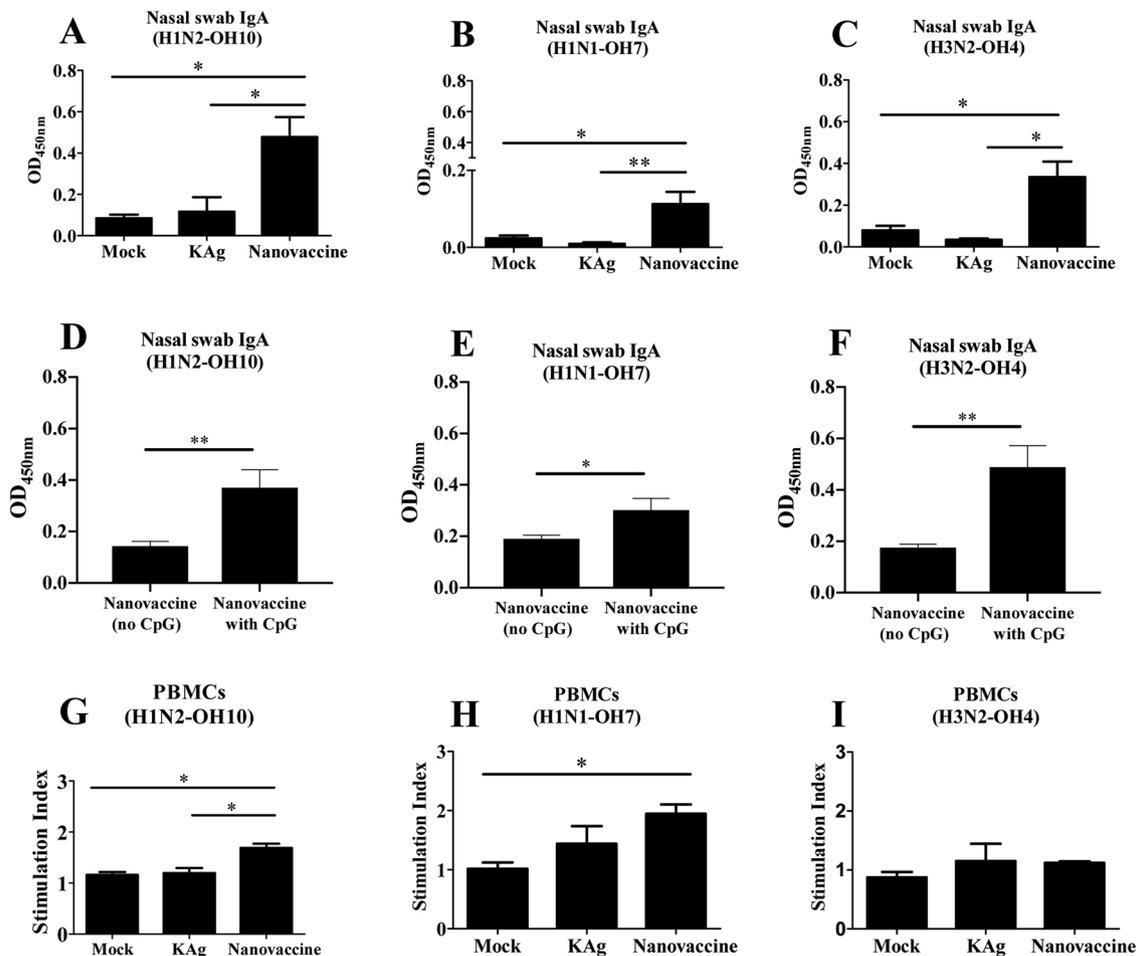
Fig. 1. KAg + CpG-nanovaccine enhanced cross-reactive IgA antibody response in nasal cavity and improved lymphocytes proliferative response in peripheral blood mononuclear cells (PBMCs) after prime-boost vaccination. Pigs were prime-boost vaccinated with respective formulation and at 35 days after first vaccination, IgA antibody responses were tested in nasal swabs against (A) vaccine virus H1N2-OH10; (B) heterologous/challenge virus H1N1-OH7; and (C) heterosubtypic virus H3N2-OH4. IgA antibody responses in nasal cavity were also compared side-by-side between pigs that received similarly formulated KAg-nanovaccine in our previous study with pigs received KAg + CpG-nanovaccine of this study against (D) vaccine virus H1N2-OH10; (E) heterologous/challenge virus H1N1-OH7; and (F) heterosubtypic virus H3N2-OH4. PBMCs isolated from pigs at 35 DPV were stimulated *in vitro* for 72 h with (D) vaccine virus H1N2-OH10; (E) heterologous/challenge virus H1N1-OH7; and (F) heterosubtypic virus H3N2-OH4 and analyzed for lymphocyte stimulation index values. Each bar represents mean ± SEM of 3 to 6 animals. Asterisk refers to significant difference between two indicated groups (*p < 0.05, **p < 0.01).

IN route has limitations such as rapid mucociliary clearance, inefficient uptake of soluble antigens and lack of appropriate mucosal adjuvants to augment the vaccine response (Partidos, 2000). Therefore, an efficient IN vaccine delivery platform is required that ensures antigen availability in the nasal cavity for longer duration, facilitates antigen uptake by antigen presenting cells, and stimulates innate immunity.

Particulate vaccines based on biodegradable and biocompatible polymeric nanoparticles, such as polyanhydrides, represent an attractive platform for IN vaccine delivery. Copolymer nanoparticles consisting of 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaocatane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) have previously been shown to enhance stability and sustained release of influenza virus antigens (Ross et al., 2014b). Polyanhydride nanoparticles are biocompatible, safe and have demonstrated enhanced antigen internalization by antigen presenting cells resulting in activation of dendritic cells (DCs) as observed through the expression of costimulatory markers and cytokines (Huntimer et al., 2013; Petersen et al., 2011; Ross et al., 2014a). Finally, polyanhydride nanoparticle-based vaccines (i.e., nanovaccines) induced both cellular and humoral immune responses when used to deliver virus-specific antigens (McGill et al., 2018; Zacharias et al., 2018). Recently, we showed that the polyanhydride-based nanovaccine encapsulating killed/inactivated influenza virus antigen (KAg) enhanced antigen-specific cell-mediated immunity in pigs after IN

administration resulting in reduced clinical signs of disease following challenge, but did not reduce lung virus titers of a heterologous challenge virus, associated with lack of induction of mucosal antibody responses (Dhakal et al., 2017a). In this study, we developed polyanhydride nanoparticles-based killed swine influenza virus vaccine co-encapsulating KAg and Toll-like receptor (TLR)-9 adjuvant (CpG-ODN) (KAg + CpG-nanovaccine) and evaluated its immunogenicity and protective efficacy compared with soluble KAg vaccine containing five-times greater quantity of antigen following heterologous virus challenge infection in pigs.

2. Methodology

2.1. Antigen and TLR-9 ligand preparation

Preparation of SIV KAg of SW/OH/FAH10-1/10 (H1N2-OH10) was performed as described previously (Dhakal et al., 2017b). Briefly, the virus was propagated in Madin-Darby canine kidney epithelial (MDCK) cells and the virus culture fluid was clarified, ultracentrifuged with sucrose cushion and pellet was suspended in phosphate-buffered saline (PBS). The virus preparation was titrated and then inactivated by binary ethyleneimine (BEI) method (Dhakal et al., 2017b). The TLR-9 agonist (CpG ODN 2007), which was used earlier by us in pigs (Ouyang
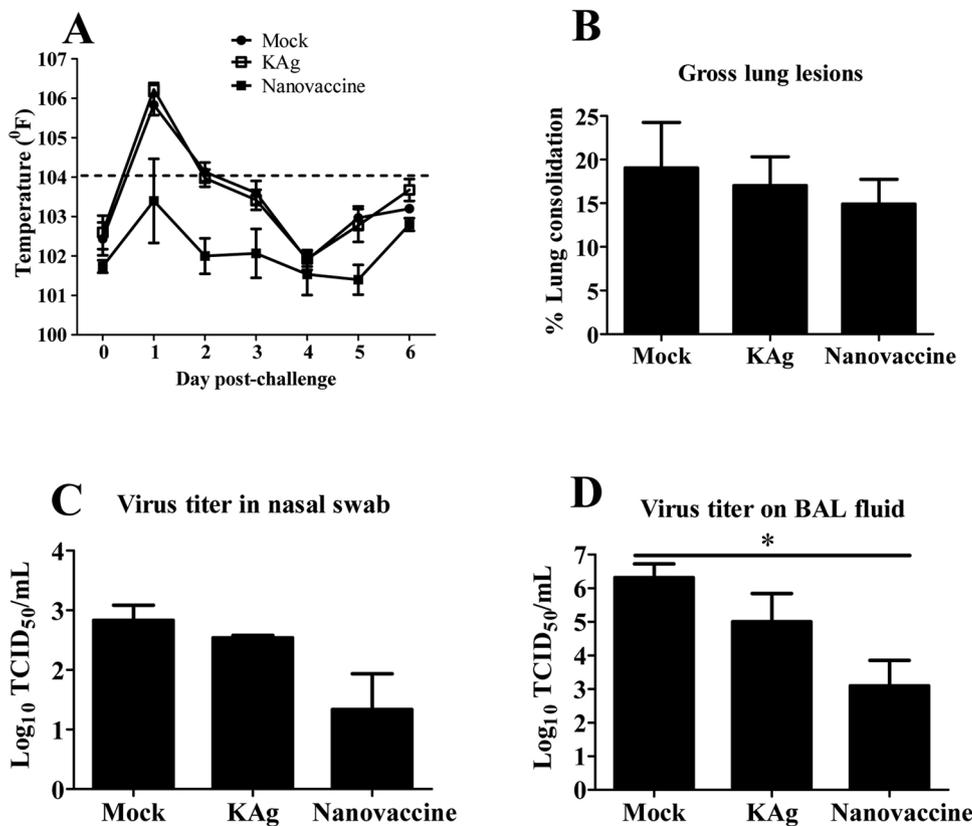
Fig. 2. KAg + CpG-nanovaccine administered pigs had reduced fever, lower lung lesions and reduced virus titers in respiratory tracts. (A) Rectal temperature was recorded from 0 to 6 days post-challenge (DPC); (B) pneumonic lesions in lungs were scored at 6 DPC; and infectious virus titers in (C) nasal swabs and (D) BAL fluid samples were determined at 6 DPC. Data are shown as mean ± SEM of 3 to 6 pigs. Asterisk refers to significant difference between two indicated groups (*$p < 0.05$).

et al., 2016) with the sequence T CG T CG TTGT CG TTTTGT CG TT in phosphorothioate backbone was custom prepared (Integrated DNA Technologies, IA).

2.2. Nanovaccine synthesis

CPTEG and CPH diacids were synthesized as previously described, and the subsequent 20:80 CPTEG:CPH copolymer was synthesized via melt polycondensation as described previously (Torres et al., 2006). The resulting polymer was characterized with ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR; DXR 500, Bruker, Billerica, MA) to assess molecular weight, composition, and purity. Nanovaccines were prepared by solid-oil-oil double emulsion. First, 20:80 CPTEG:CPH polymer was dissolved 20 mg/mL in methylene chloride. Lyophilized antigens and adjuvants were co-dissolved with the polymer as 1.75 wt. % KAg +1.75 wt. % CpG. The resulting solution was sonicated for 30 s to ensure the components were fully dissolved and evenly distributed. Next, nanoparticles were precipitated by pouring the solution into chilled pentane (−10 °C; 1:250 methylene chloride:pentane) and collected via vacuum filtration. Nanoparticle morphology and size were verified with scanning electron microscopy (FEI Quanta 250, FEI, Hillsboro, OR). Following synthesis, the protein encapsulation efficiency of the nanoparticles was determined. Briefly, 10 mg/mL nanoparticles were suspended in 40 mM sodium hydroxide. After the nanoparticles were degraded, the released protein in the supernatant was quantified using a microBCA assay (Thermo Scientific, Waltham, MA). The protein encapsulation efficiency for KAg + CpG-nanovaccine was 8.9 ± 2.7%.

2.3. Animal experiment and sample collection

Influenza virus antibody free piglets were raised in the biosafety level (BSL)-2 facility at The Ohio State University and used in the vaccine trial as described previously (Dhakal et al., 2017b). Piglets of both sexes were randomly assigned into different groups: (1) Mock

group (n = 3) that received Dulbecco's Modified Eagle Medium; (2) KAg group (n = 4), each pig received 1×10^7 TCID$_{50}$ equivalent of inactivated triple reassortant H1N2-OH10 virus (Dhakal et al., 2017a); and (3) KAg + CpG-nanovaccine group (n = 6), each pig received 2×10^6 TCID$_{50}$ equivalent of H1N2-OH10 virus (5 times lower than in KAg group) and 70 μg of CpG-ODN encapsulated in 20 mg polyanhydride nanoparticles. Pigs received the first dose of vaccine at five weeks of age and a similar booster dose at eight weeks through IN route as mist. For challenge virus infection, a heterologous triple reassortant SW/OH/24366/2007 (H1N1-OH7) IAV (Dhakal et al., 2017b) grown in MDCK cells was inoculated two weeks after the booster immunization, and each pig received 6×10^6 TCID$_{50}$ in 2 mL with 1 mL through IN and 1 mL by intratracheal route. A heterosubtypic virus to the vaccine virus, A/Turkey/OH/313053/2004 (H3N2-OH4) IAV, was used in immunological assays (Dhakal et al., 2018). The H1N2-OH10 vaccine virus and H1N1-OH7 challenge virus are heterologous to each other with 77% HA gene identity, whereas H3N2-OH4 virus, originally isolated from turkeys, is heterosubtypic to other two SIV with HA gene identity 63% (Ali et al., 2012; Yassine et al., 2007, 2009). Pigs were euthanized at six days post-challenge (DPC). Serum and nasal swab samples were collected at 35 days post-vaccination (DPV) for antibody assays. Blood samples collected at 0 and 6 DPC were used for isolation of peripheral blood mononuclear cells (PBMCs). Rectal temperature was recorded daily from 0 to 6 DPC. At necropsy (6 DPC), lungs were scored for macroscopic pneumonic lesions and nasal swab and bronchoalveolar lavage (BAL) fluid were collected for virus titration. Tracheobronchial lymph nodes (TBLN) were collected at 6 DPC for isolation of mononuclear cells as described previously (Dhakal et al., 2018).

2.4. Antibody assay

IAV specific immunoglobulin (Ig) A antibodies in the nasal swab samples were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Dhakal et al., 2017a). Briefly, ELISA
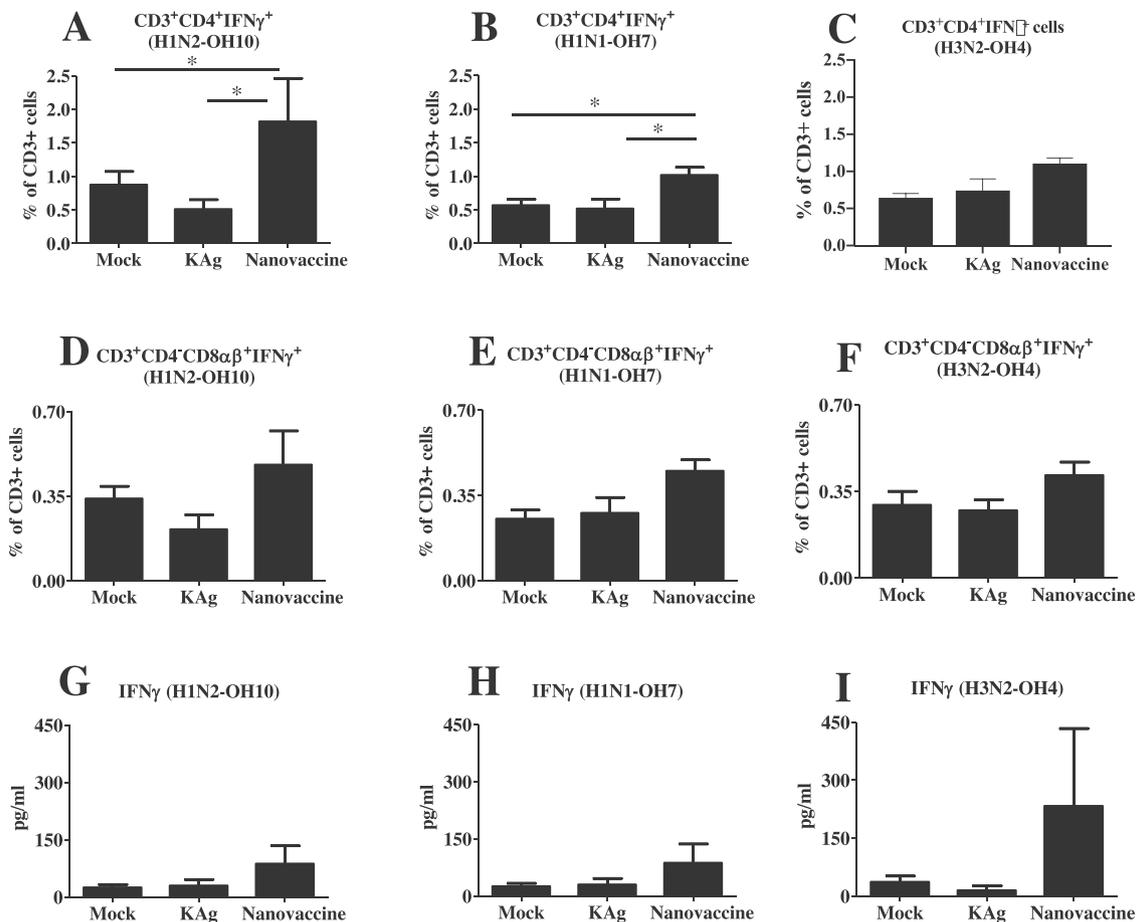
Fig. 3. KAg + CpG-nanovaccine administered pigs had greater recall IFN-γ response in PBMCs after heterologous virus challenge. Peripheral blood mononuclear cells (PBMCs) isolated from pigs at 6 DPC were stimulated with (A, D & G) vaccine virus H1N2-OH10; (B, E & H) heterologous/challenge virus H1N1-OH7; and (C, F & I) heterosubtypic virus H3N2-OH4. Cells were immunostained for analysis of the frequency of IFN-γ secreting T-helper cells (A-C) and cytotoxic T cells (D-F) by flow cytometry. Secreted IFN-γ from stimulated cells culture supernatant was measured by ELISA (G-I). Each bar represents mean ± SEM of 3 to 6 pigs. Asterisk refers to significant difference between two indicated groups (*p < 0.05).

plates were coated with pretitrated IAV antigen (5 μg/mL) overnight (4°C), blocked with 5% skim-milk powder for 2 h at room temperature (RT), and incubated with prediluted test samples (nasal swab 1:2 and serum 1:100 ratios) overnight at 4°C. Plates were washed and treated with goat anti-pig IgA antibody horse-radish peroxidase conjugated (Bethyl Laboratories Inc., TX, USA) for 2 h at RT and incubated with substrate for 15–20 min at RT. Color development was stopped using 1 M phosphoric acid and optical density (OD) values were recorded at 450 nm.

### 2.5. Antigen-specific cell proliferation assay

PBMCs were isolated at 35 DPV from vaccinated pigs, seeded at 1 million cells/well in triplicate wells and stimulated with medium control, H1N2-OH10, H1N1-OH7 and H3N2-OH4 IAVs at 0.1 multiplicity of infection (MOI) as described previously (Dhakal et al., 2017b). After 72 h of incubation at 37°C in a 5% CO_2 incubator, a mixture of a tetrazolium compound and an electron coupling reagent (MTS + PMS) (Promega, WI) was added. Plates were incubated for an additional 4 h and OD values were recorded at 490 nm. The lymphocyte stimulation index values were determined by dividing the average OD of virus stimulated PBMCs with OD of medium control PBMCs of respective pig (Dhakal et al., 2017b).

2.6. Virus titration

To determine the virus titer, nasal swabs and BAL fluid samples were 10-fold serially diluted in serum-free medium containing TPCK-trypsin (1 μg/mL) and transferred in quadruplets into 96-well plates containing MDCK cells at 80–90% confluency (Dhakal et al., 2017b, 2018). After 72 h of incubation at 37°C in a 5% CO_2 incubator, cells were fixed with 80% acetone and stained with IAV nucleoprotein-specific antibody (CalBioreagents, CA) followed by AlexaFluor 488 conjugated goat anti-mouse IgG (H + L) antibody (Life Technologies, OR). The virus specific immunofluorescence was recorded using the fluorescent microscope (IX51, Olympus, Tokyo, Japan) and virus titers were determined using the Reed and Muench method (Reed and Muench, 1938).

2.7. Flow cytometry assay and interferon gamma (IFN-γ) cytokine ELISA

PBMCs isolated at DPC 6 were stimulated with 0.1 MOI of H1N1, H1N2 and H3N2 IAVs. After 72 h of incubation (37°C, 5% CO_2 incubator), cells were harvested and labeled for porcine-specific T cell surface markers and intracellular IFN-γ as previously described (Dhakal et al., 2017b). Antibodies used in flow cytometry were anti-porcine CD3 (Southernbiotech, AL), CD4α (Southernbiotech, AL), CD8α (Southernbiotech, AL), CD8β (BD Biosciences, CA) and IFNγ (BD Biosciences, CA). TBLN-MNCs isolated at 6 DPC were also similary stimulated with 0.1 MOI of H1N1, H1N2 and H3N2 for 72 h. The supernatants collected

A IFNγ (H1N2-OH10)

B IFNγ (H1N1-OH7)

C IFNγ (H3N2-OH4)
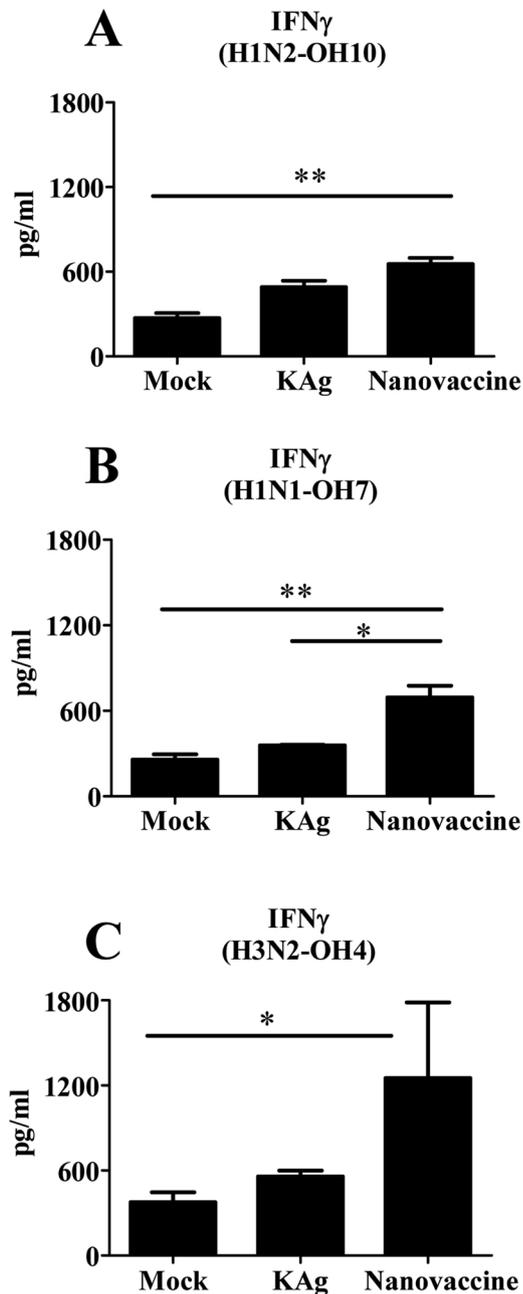
Fig. 4. KAg + CpG-nanovaccine group secreted higher IFN-γ cytokine after antigenic stimulation of tracheobronchial lymph nodes derived mononuclear cells (TBLN-MNCs). IFN-γ secretion by TBLN-MNCs at 6 DPC following 72 h stimulation with (A) vaccine virus H1N2-OH10; (B) challenge virus H1N1-OH7; or (C) heterosubtypic virus H3N2-OH4 was measured by ELISA. Each bar represents mean ± SEM of 3–6 pigs. Asterisks refer to significant difference between two indicated groups (*p < 0.05, **p < 0.01).

from stimulated PBMCs and TBLNs were used for analysis of secreted IFNγ by using cytokine ELISA as previously described (Dhakal et al., 2017b).

2.8. Statistical analysis

Data are presented as mean ± standard error of mean. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) and comparison between two vaccine groups was made by Mann Whitney test. A p-value less than 0.05 was considered as statistically significant.

3. Results

3.1. Nanovaccine immunization enhanced cross-reactive mucosal IgA antibody response in pigs

Mucosal IgA antibody responses were evaluated in nasal swabs collected after prime-boost vaccination at 35 DPV in pigs. The observed IgA response induced by the KAg + CpG-nanovaccine was significantly higher compared to unvaccinated and KAg-vaccinated pigs against vaccine virus H1N2-OH10 (Fig. 1A). In addition, pigs administered with the KAg + CpG-nanovaccine had significantly higher IgA titers compared to the other mock and KAg-vaccinated groups against both heterologous H1N1-OH7 (Fig. 1B) and heterosubtypic H3N2-OH4 (Fig. 1C) IAVs. The hemagglutination inhibition (HAI) antibody titers against H1N2-OH10 virus in KAg and KAg + CpG-nanovaccine groups were 18 ± 5 and 32 ± 7 (mean ± SEM), respectively; and IgG antibody response in serum after prime-boost vaccination against H1N2-OH10 virus in KAg and KAg + CpG-nanovaccine groups were 1.1 ± 0.3 and 1.3 ± 0.2 (mean ± SEM of OD_{450}nm), respectively, were not statistically different between KAg and KAg + CpG-nanovaccine groups.

In our previous study, we had shown that similarly formulated KAg-nanovaccine encapsulating the H1N2-OH10 antigens improved the cell-mediated immune response but not the mucosal IgA antibody response in pigs (Dhakal et al., 2017a). To understand whether the addition of CpG-ODN in KAg-nanovaccine enhanced IgA antibody response in pigs, we compared the nasal swab samples stored from previous study (KAg-nanovaccine) with the current study (KAg + CpG-nanovaccine). Despite the fact that, H1N2-OH10 KAg encapsulated in KAg + CpG-nanovaccine formulation was 5-times lower than in previous KAg-nanovaccine, it induced significantly greater IgA antibody response in nasal swab samples against homologous H1N2-OH10 virus (Fig. 1D), heterologous H1N1-OH7 (Fig. 1E) and heterosubtypic H3N2-OH4 (Fig. 1F) viruses compared to previous KAg-nanovaccine. This data suggest that inclusion of CpG-ODN in KAg-nanovaccine improved the cross-reactive mucosal antibody response in pigs.

3.2. Nanovaccine immunization induced greater antigen-specific cell-proliferation against vaccine virus in PBMCs

After prime-boost vaccination, at 35 DPV, PBMCs were isolated and restimulated *in vitro* with different IAVs and cell proliferation indices were compared. The proliferation of PBMCs was significantly higher in KAg + CpG-nanovaccine group compared to mock and KAg-vaccinated groups when re-stimulated with the vaccine virus, H1N2-OH10 (Fig. 1G). The stimulation index in cells from animals immunized with the nanovaccine was significantly higher compared with that of cells from animals that received the mock-vaccination when stimulated with the challenge virus, H1N1-OH7 (Fig. 1H), but no such differences were observed when stimulated with heterosubtypic H3N2-OH4 virus (Fig. 1I). We used the same live IAV H1N1 used to challenge pigs in our ex vivo lymphocyte stimulation assay, which is heterologous to the vaccine IAV H1N2 with 77% HA gene identify. It is likely that the observed lymphocytes responses are specific to conserved viral proteins such as nucleoprotein, matrix proteins, polymerase etc, in addition to HA and neuraminidase proteins.

3.3. Pigs immunized with nanovaccine had reduced fever and lowered virus titers in respiratory tract after heterologous virus challenge

Prime-boost vaccinated pigs were challenged with a virulent heterologous H1N1-OH7 virus at 35 DPV and rectal temperatures were recorded daily post-infection. Most of the pigs in the unvaccinated and KAg treatment groups demonstrated fever (≥104°F) until three DPC, while nanovaccine-administered animals were free from fever (Fig. 2A). The temperature profile in nanovaccine-administered pigs was significantly lower and closer to normal body temperature range

compared to all other treatment groups (Fig. 2A). The average lung consolidation in mock, KAg and nanovaccine groups were 19%, 17% and 15%, respectively, indicating that nanovaccine group had non-significant reduction of pneumonic lesions (Fig. 2B). The average virus titer in nasal swabs collected from nanovaccine-administered pigs was 32 and 16 times lower, respectively, when compared to mock and KAg-vaccinated animals, but data were not statistically significant (Fig. 2C). Likewise, virus titers in BAL fluid from animals that were immunized with the nanovaccine were significantly lower (> 1500 fold) than that of mock-vaccinated and virus challenged pigs, and 80 times lower than that of KAg vaccine-adminsitered pigs (Fig. 2D).

3.4. Recall IFN-γ response in PBMCs and TBLN-MNCs after virus challenge was higher in pigs immunized with nanovaccine

PBMCs isolated at 6 DPC were stimulated with different IAVs and analyzed for IFN-γ^+ lymphocyte subsets by flow cytometry. Our results identified a significantly higher frequency of IFN-γ^+ T-helper cells in the nanovaccine group compared to both mock and KAg-vaccine groups when stimulated with the vaccine virus H1N2-OH10 (Fig. 3A). Similarly, significantly higher frequencies of IFN-γ^+ T helper cells were observed in nanovaccine group when TBLN-MNCs were stimulated with the challenge virus H1N1-OH7 (Fig. 3B). While the average frequency of IFN-γ^+ T helper cells responding to the heterosubtypic antigen from the H3N2-OH4 virus and cytotoxic (CD8$\alpha\beta^+$) T cells against all three IAVs were higher in the KAg + CpG-nanovaccine group compared with mock and KAg-vaccinated pigs, the differences were not statistically significant between the groups (Fig. 3C–F). We also analyzed IFN-γ secretion into the culture supernatant of PBMCs isolated at six DPC following in vitro stimulation with IAV antigens and observed higher amounts of IFN-γ in the KAg + CpG-nanovaccine group compared to other treatment groups (Fig. 3G–I).

Similarly, to assess the mucosal immune response, TBLN-MNCs were isolated from pigs at six DPC and stimulated *in vitro* with different IAV antigens. The harvested culture supernatant was analyzed for secreted IFN-γ and the results indicated that MNCs isolated from pigs that received nanovaccine secreted significantly more IFN-γ regardless of the IAV antigens used relative to the IFN-γ secreted from MNCs recovered from control unvaccinated pigs (Fig. 4A–C). In addition, after stimulation with H1N1-OH7 virus, the nanovaccine group had significantly higher IFN-γ secretion compared to KAg vaccine group (Fig. 4B).

4. Discussion

In this study, we encapsulated killed soluble viral antigen (KAg) of influenza virus H1N2-OH10 in CPTEG:CPH-based polyanhydride nanoparticles along with CpG (i.e. KAg + CpG-nanovaccine) and used this formulation for intranasal immunizations in pigs. The immunogenicity and protective efficacy of KAg + CpG-nanovaccine was compared with intranasally delivered soluble KAg vaccine group following a heterologous (H1N1-OH7) virus challenge. We also used five-times lower quantity of antigen in KAg + CpG-nanovaccine compared to control soluble KAg vaccine in pigs considering the inclusion of CpG-ODN-adjuvant in nanovaccine formulation, as well as the dose-sparing ability of CPTEG:CPH-based polyanhydride nanoparticles as shown with filarial worm treatment (Binnebose et al., 2015). Our data suggested that KAg + CpG-nanovaccine enhanced the induction of cross-reactive mucosal IgA antibody responses in the nasal cavity, induced better cell-mediated immune responses, relieved pigs from influenza virus-induced fever and reduced virus titers in the nasal cavity and BAL fluid by > 16 and 80-fold, respectively, compared with KAg alone.

Local mucosal IgA antibodies function as the first line of defense at the mucosal sites (Mestecky et al., 2005). In this experiment, KAg + CpG-nanovaccine induced a better IgA antibody response in the nasal cavity of pigs compared with KAg alone. More importantly, the

antibody response was greater not only against the homologous (vaccine) virus but also against heterologous and heterosubtypic viruses indicating the cross-reactive potential of the secreted antibodies. Polyanhydride-based nanovaccines have been shown to induce germinal center formation and result in long-lived antibody responses in mice (Vela Ramirez et al., 2016). Similarly, in calves vaccinated with the CPTEG:CPH polyanhydride-based nanovaccine encapsulating the recombinant post-fusion F and G proteins of bovine respiratory syncytial virus (BRSV), the secretion of BRSV-specific IgA antibodies in the nasal cavity was increased which corresponded reduction in nasal virus shedding (McGill et al., 2018). However, in pigs, we previously did not observe induction of better IgA antibody response with CPTEG:CPH-based KAg-nanovaccine compared with KAg alone (Dhakal et al., 2017a). We hypothesize that the inclusion of CpG-ODN might have contributed synergistically with polyanhydride nanoparticles in the induction of better mucosal IgA responses in KAg + CpG-nanovaccine in thais experiment, as CPG-ODN is known to induce better antigen-specific IgA antibody responses in mucosal surfaces (tested on nasal secretions, saliva and feces) after intranasal immunization with porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs (Zhang et al., 2007).

Polyanhydride nanovaccines have also been shown to induce excellent cell-mediated immune responses in mice, calves, and pigs (Dhakal et al., 2017a; McGill et al., 2018; Zacharias et al., 2018). Consistent with previous results, augmented lymphocyte proliferation of PBMCs in response to vaccine virus (H1N2-OH10) stimulation at DPV 35 (Fig. 1D) and greater frequencies of IFN-γ positive T-helper cells in PBMCs in respone to vaccine (H1N2-OH10) and challenge (H1N1-OH7) viruses at 6 DPC (Fig. 3A–B) were observed in KAg + CpG-nanovaccine administered pigs compared with the responses in KAg-nanovaccinated pigs (Dhakal et al., 2017a). Additionally, in this experiment we observed that IFN-γ secretion from TBLN-MNCs was in general greater in KAg + CpG-nanovaccine administered pigs with statistical significance observed after restimulation with challenge virus (H1N1-OH7). Inclusion of CpG-ODN in nanovaccine formulation may have also contributed in the induction of cell-mediated immune response in KAg + CpG-nanovaccine group as it is known as a potent T helper cell 1 (Th1) stimulant (Bode et al., 2011). CpG-ODN signals through TLR-9 and supports the maturation, differentiation and proliferation of multiple immune cells and induces production of cytokines that favor cell-mediated immunity (Bode et al., 2011). In this regard, CpG-ODN adjuvant has been shown to potentiate lymphocyte proliferation and IFN-γ production by porcine PBMCs following subcutaneous immunization with inactivated porcine reproductive and respiratory syndrome virus antigen (Linghua et al., 2007).

Both antibody and cell-mediated immune responses play an important role in the prevention of influenza virus infection and transmission. The superior mucosal antibody response and cell-mediated immunity induced by KAG + CpG-nanovaccine, that contained five-times lesser antigen than in soluble KAg vaccine group, protected pigs from fever after heterologous virus challenge and lowered infectious virus titers in the nasal passage (16 to 32 fold reduction compared to controls) and in lower respiratory tract (80 to > 1500 fold reduction compared to the controls). Further improvements are needed in the current nanovaccine formulation to achive even better virus clearance from the respiratory tract and relieve pigs from virus-induced pneumonia. Our study shows that intranasal polyanhydride-based IAV nanovaccine formulation in combination with CpG-ODN adjuvant has the potential to induce cross-reactive mucosal antibody and cellular immune responses in pigs.

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