Vaccine 36 (2018) 6103–6110

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The type of adjuvant in whole inactivated influenza a virus vaccines impacts vaccine-associated enhanced respiratory disease

Carine K. Souza [a,b], Daniela S. Rajão [a], Matthew R. Sandbulte [a], Sara Lopes [c], Nicola S. Lewis [c], Crystal L. Loving [a], Phillip C. Gauger [d], Amy L. Vincent [a,*]

[a] Virus and Prion Research Unit, USDA-ARS, 1920 Dayton Avenue, P.O. Box 70, Ames, IA 50010, USA
[b] Laboratório de Virologia, Universidade Federal do Rio Grande do Sul–UFRGS, Av. Bento Gonçalves, 9090, CEP: 91540-000 Porto Alegre, Rio Grande do Sul, Brazil
[c] Department of Zoology, University of Cambridge, Cambridge, UK
[d] Iowa State University – ISU, Ames, IA 50010, USA

ARTICLE INFO

Article history:
Received 15 June 2018
Received in revised form 23 August 2018
Accepted 29 August 2018
Available online 1 September 2018

Keywords:
Vaccine
VAERD
Influenza
Swine
Adjuvant

ABSTRACT

Influenza A virus (IAV) causes a disease burden in the swine industry in the US and is a challenge to prevent due to substantial genetic and antigenic diversity of IAV that circulate in pig populations. Whole inactivated virus (WIV) vaccines formulated with oil-in-water (OW) adjuvant are commonly used in swine. However, WIV-OW are associated with vaccine-associated enhanced respiratory disease (VAERD) when the hemagglutinin and neuraminidase of the vaccine strain are mismatched with the challenge virus. Here, we assessed if different types of adjuvant in WIV vaccine formulations impacted VAERD outcome. WIV vaccines with a swine δ1-H1N2 were formulated with different commercial adjuvants: OW1, OW2, nano-emulsion squalene-based (NE) and gel polymer (GP). Pigs were vaccinated twice by the intramuscular route, 3 weeks apart, then challenged with an H1N1pdm09 three weeks post-boost and necropsied at 5 days post infection. All WIV vaccines elicited antibodies detected using the hemagglutination inhibition (HI) assay against the homologous vaccine virus, but not against the heterologous challenge virus; in contrast, all vaccinated groups had cross-reactive IgG antibody and IFN-γ responses against H1N1pdm09, with a higher magnitude observed in OW groups. Both OW groups demonstrated robust homologous HI titers and cross-reactivity against heterologous H1 viruses in the same genetic lineage. However, both OW groups had severe immunopathology consistent with VAERD after challenge when compared to NE, GP, and non-vaccinated challenge controls. None of the WIV formulations protected pigs from heterologous virus replication in the lungs or nasal cavity. Thus, although the type of adjuvant in the WIV formulation played a significant role in the magnitude of immune response to homologous and antigenically similar H1, none tested here increased the breadth of protection against the antigenically-distinct challenge virus, and some impacted immunopathology after challenge.

1. Introduction

Influenza A viruses (IAV) of H1 and H3 subtypes are shared between humans and pigs, and swine-adapted IAV occasionally cause zoonotic infections of public health concern. Periodic spillovers of human seasonal IAV to swine significantly contributed to the genetic and antigenic diversity of IAV in U.S. swine populations [1–3]. After the emergence of the pandemic H1N1 (H1N1pdm09) in humans [4], repeated transmission from humans to swine occurred globally, highlighting the role of reverse zoonosis [5] and further increasing the diversity of IAV in swine [2].

Vaccine protection against this vast antigenic diversity of IAV [2,3,6] in the U.S. swine population is a challenge since strains are not systematically updated as undertaken through the human IAV vaccine strain selection system. Although new IAV vaccine platforms are licensed for use in swine [7,8] commercial whole-inactivated virus (WIV) vaccines formulated with oil in water (OW) adjuvant are the most commonly used vaccine to control IAV in swine [9]. WIV vaccines induce protective immune responses against homologous and antigenically-related IAV strains; however, cross-protection against heterologous IAV can be limited and cross-reactive immune responses have been associated with enhanced lung pathology [10]. A model of vaccine associated enhanced respiratory disease (VAERD) has been reported when pigs are vaccinated with a monovalent WIV followed by a heterologous challenge containing the same hemagglutinin (HA)

* Corresponding author.
E-mail addresses: carine.souza@ars.usda.gov (C.K. Souza), daniela.rajao@uga.edu (D.S. Rajão), nilewis@rvc.ac.uk (N.S. Lewis), crystal.loving@ars.usda.gov (C.L. Loving), pcgauger@iastate.edu (P.C. Gauger), amy.vincent@ars.usda.gov (A.L. Vincent).

https://doi.org/10.1016/j.vaccine.2018.08.072
0264-410X/Published by Elsevier Ltd.

subtype. This model has been experimentally reproduced with several IAV strain combinations and with pigs at different ages at vaccination and challenge [11–16]. The outcome is severe disease characterized by prolonged fever with severe bronchointerstitial pneumonia, necrotizing bronchiolitis, and peribronchiolar lymphocytic cuffing [11,12]. Although the mechanisms responsible for VAERD are not completely understood, non-neutralizing IgG antibodies were shown to be targeted to a conserved region of the HA2 stalk close to the fusion peptide domain of the heterologous HA, and led to enhanced virus infectivity by promoting virus membrane fusion activity [17]. In addition, pro-inflammatory and anti-viral cytokine dysregulation were associated with the severe lung pathology and may contribute to neutrophil infiltration into the lungs [13]. An HA subunit vaccine formulated with OW adjuvant followed by a mismatched challenge also resulted in VAERD [15]. However, immunity to the neuraminidase (NA) protein also played an important role in abrogating VAERD when the NA of the vaccine and challenge strains were matched [14]. In contrast, age of vaccination and timing of challenge did not alter the VAERD outcome in growing pigs [16].

Adjuvants are widely used in vaccine formulations to modulate antigen-specific immunity by magnifying or shaping T-helper immune responses [18,19]. The humoral response against IAV provides protection through specific neutralizing antibodies to the HA [20], and cell-mediated immunity (CMI) also play a role in augmenting the B-cell response and providing partial protection against heterologous or heterosubtypic IAV strains [21,22]. There is a need to develop IAV vaccines for pigs that confer cross-protection against heterologous infection without resulting in VAERD. Although VAERD was associated with the use of WIV-OW vaccination, it was unknown if the type of adjuvant in the WIV formulation would impact the immunopathology of VAERD. Here, we demonstrate that the type of adjuvant in the WIV vaccine formulation impacted the magnitude of immune response and subsequent degree of immunopathology after heterologous challenge highlighting the role of the adjuvant component on the spectrum of quality and quantity of immunity.

2. Material and methods

2.1. Viruses and vaccine formulations

The vaccine virus, A/sw/MN/02011/08 H1N2 (δ1-H1N2), was grown in Madin-Darby canine kidney (MDCK) cells with OptiMEM (Gibco, Thermo Scientific, USA) and was inactivated and prepared as previously described [23]. Four commercial adjuvants, OW1, OW2, nano-emulsion (NE) and gel polymer (GP), were used to formulate WIV vaccines according to the manufacturer's recommendations (Table 1).

2.2. Experimental design

At three weeks of age, pigs were obtained from a herd free from IAV and porcine reproductive and respiratory syndrome virus (PRRSV) and treated prophylactically with ceftiofur (Zoetis, Parsippany, NJ) according to label directions to reduce potential bacterial respiratory pathogens. Prior to vaccination, pigs were screened for antibody against IAV nucleoprotein (NP) by ELISA (MultiS ELISA, IDEXX, Westbrook, Maine, USA) to ensure the absence of preexisting antibodies against IAV. Pigs were housed in biosafety level 2 (BSL2) containment during the vaccination and challenge phases of the study and cared for in compliance with the Institutional Animal Care and Use Committee (IACUC) of the National Animal Disease Center (NADC).

Pigs were vaccinated with 2 mL of the assigned WIV, by the intramuscular route, as described in Table 1 and challenged with 10^5 50% tissue culture infectious dose ($TCID_{50}$) per mL of A/CA/04/2009 (H1N1pdm09), with 2 mL instilled in the trachea and 1 mL instilled in the nose [24]. Nasal swabs were collected at 0, 1, 3, and 5 days post infection (dpi). At 5 dpi, pigs were humanely euthanized with a lethal dose of pentobarbital (FatalPlus, Vortech, Dearborn, MI, USA). Rectal temperatures were recorded daily until necropsy. Broncho-alveolar fluid (BALF) samples were collected at necropsy, as previously described [14].

2.3. Macroscopic and microscopic lung lesions

Lungs were removed at necropsy and the percentage of the surface area affected with pneumonia was visually estimated as previously described [12]. Tissue samples from the trachea and the right middle or affected lung lobe were fixed in 10% buffered formalin for histopathologic examination. Slides were stained with hematoxylin and eosin (H&E) or immunohistochemistry (IHC) and evaluated by a veterinary pathologist blinded to the treatment groups [12].

2.4. Virus titration

Nasal swabs and BALF samples were used for virus titration on MDCK cells, as previously described [25]. MDCK-inoculated monolayers were evaluated for cytopathic effect (CPE) at 48 h post-infection and subsequently fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry (ICC) with anti-influenza A nucleoprotein monoclonal antibody [26]. A $TCID_{50}$ titer was calculated for each sample [27].

Table 1
Study design and adjuvant properties.

WIV group	N	Vaccination (4 wk)	Boost (7 wk)	Adjuvant	Major immunostimulatory component[§]	(v/v)	Route	Type of immune response
*OW1	10	δ1-H1N2	δ1-H1N2	Emulsigen-D ™ MVP	Oil in water (OW) emulsion	20%	IM	Humoral response (strong short-term immunity)
*OW2	10	δ1-H1N2	δ1-H1N2	Seppic MONTANIDE™ ISA 15A VG	Mineral OW based emulsion	15%	IM	Humoral response (strong short-term immunity)
*NE	10	δ1-H1N2	δ1-H1N2	Invivogen AddaVax™	Squalene-based nano-emulsion (similar to MF59)	50%	IM	Humoral and/or cellular responses. Cytokines and chemokines
*GP	10	δ1-H1N2	δ1-H1N2	Seppic MONTANIDE™ Gel 01	Sodium polyacrylate polymer in water	10%	IM	Humoral and/or cellular responses. Cytokines and chemokines
*NV/C	10	–		–	–	–	–	–
NV/NC	5	–		–	–	–	–	–

IM: intramuscular route.
§ Information from manufacturer's label. The proportion of adjuvant and the route of administration were performed following the manufacture recommendations.
* All WIV groups and NV/C were challenged with H1N1pdm09 strain at 10 weeks of age.

2.5. Hemagglutination inhibition and ELISA

Serum samples collected prior to vaccination, pre-challenge (42 days post vaccination-dpv or dpi 0) and dpi 5 were heat inactivated at 56 °C for 30 min and hemagglutination inhibition (HI) assays were conducted against the homologous vaccine virus (δ1-H1N2) and heterologous challenge virus (H1N1pdm09), using turkey red blood cells, as described [28]. HI titers were divided by 10 and $\log_2$ transformed, analyzed, and reported as the geometric mean titer (GMT) [27]. Pre-challenge sera were also evaluated by HI assays using genetically-related but heterologous H1 strains listed in Supplementary material 1 and 2.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.08.072.

Independent ELISAs were conducted using whole virus preparations to detect IgA and IgG antibody specific to δ1-H1N2 and H1N1pdm09 following a described protocol [29]. ELISA results were reported as mean OD for each duplicate sample and the mean of each group was compared.

2.6. Neuraminidase inhibition assay

Neuraminidase specific antibodies were measured in post boost (dpi 0) sera by neuraminidase inhibition (NI) assays. Antigens were viruses with homologous NA of δ1-H1N2 (homologous N2-98), a drifted N2-98 (A/swine/Texas/4199-2/1998) within the swine 1998-lineage, or a heterologous N2-02 (A/turkey/Ohio/313053/2004) from the swine 2002 N2 lineage, each paired with an HA gene from an unrelated strain (A/guinea fowl/HK/WF10/1999 H9N2) [30]. NI titer of each serum sample was defined as the highest dilution that resulted in 50% inhibition of NA activity [30].

2.7. Interferon-γ ELISpot assay

Peripheral blood mononuclear cells (PBMC) were collected at 28 days post vaccination (dpv; or 1 week post-boost) and at 42 dpv (or pre-challenge) and stimulated with homologous (δ1-H1N2) and heterologous (H1N1pdm09) strains, positive control (Pokeweed mitogen) or media (sham) to evaluate the number of interferon-gamma secreting cells (IFN-γ SCs) in an ELISpot assay (porcine IFN-γ ELISpot assay, R&D systems, Minneapolis, MN), using a previously described protocol [31]. Plates were scanned and spots enumerated using CTL-ImmunoSpot® S5 UV Analyzer and ImmunoSpot 5 Software. The reported values were calculated from the average number of spots counted for duplicate wells receiving virus, subtracted by the number of spots in the mock stimulated wells.

2.8. Antigenic cartography and antibody landscaping

HI data (Supplementary material 1) were used to create 2 dimensional (2D) antigenic maps to quantify the inter-relationships among delta (1B.2)-lineage strains (Supplementary material 2) using antigenic cartography [6,32]. Antibody landscapes were generated as previously described using RStudio 1.0.44 [33]. The HI titers were plotted in the 3rd dimension as a smooth landscape over the 2D antigenic maps (Supplementary material 3). Since the minimum dilution used in the HI assays was 1:10, undetectable titers were given a set value of ≤10. The distance between the viruses in the x- and y-axis represent antigenic distance while the height of the landscape (z-coordinate) represent antibody titers (geometric mean value) to the respective virus strains. The antibody landscapes visualize the breadth of antibody response of each WIV-adjuvant group against H1 strains. The colors used for strains in the antibody landscaping were based on genetic lineage, as previously described [34].

2.9. Statistical analysis

Lung lesions scores, $\log_{10}$ transformed virus titers, $\log_2$ HI titers, mean OD ELISA IgA and IgG antibody levels and number of IFN-γ SC were analyzed using analysis of variance (ANOVA) with a p value ≤ 0.05 considered significant (Graph Pad Prism Version 5.00, San Diego, CA). Response variables shown to have significant differences among groups were subjected to pair-wise comparison using the Tukey-Kramer test.

3. Results

3.1. OW vaccinated pigs with heterologous challenge resulted in VAERD

All pigs were negative for IAV NP-specific antibodies prior to vaccination. BALF samples were negative for PCV2, *M. hyopneumoniae* and PRRSV and aerobic bacterial pathogens were not detected. Upon heterologous infection, OW-vaccinates demonstrated clinical signs such as coughing and increased respiratory effort, consistent with previous findings in other VAERD studies. In contrast, NE and GP groups demonstrated only mild signs of respiratory disease after challenge. OW1 pigs demonstrated febrile responses, as defined as from 2 to 5 dpi and were significantly higher than NV/NC controls, OW2 pigs were febrile at 2 and 3 dpi, and the NE pigs were febrile at 3 dpi (data not shown).

OW1 (22.1%) and OW2 (21.7%) had significantly higher group mean percentages of macroscopic lung lesions when compared to NE (11.3%) and GP (9.3%) which were more similar to NV/C controls (6.1%) (Fig. 1A). OW1 (14.4) and OW2 (14.6) presented significantly higher microscopic lung lesions scores compared to GP (7.8) and NV/C control (5.1) (Fig. 1B). OW1 (5.6) had a mean microscopic trachea lesions score that was significantly higher compared to other adjuvant and control groups (Fig. 1C). IAV antigen detection was confirmed in the lungs and trachea in all vaccinated groups by IHC, but mean scores were not statistically different between groups.

3.2. Virus shedding and replication in the lungs

All groups were negative for IAV in nasal swabs pre-challenge. NE and GP groups shed virus at low titers at 1 dpi (data not shown). At 3 and 5 dpi, all WIV groups shed H1N1pdm09 at titers similar to NV/C (Fig. 1D and E). In addition, all vaccinated groups demonstrated high virus titers in the lungs (Fig. 1F).

3.3. Peripheral and local antibody responses

All WIV groups demonstrated HI titers against the homologous δ1-H1N2 vaccine strain at 0 (Fig. 2A) and 5 dpi (Fig. 2B), with highest pre-challenge titers in the OW1 and OW2 groups. However, none of the vaccinated groups elicited detectable HI titers to the heterologous challenge strain (H1N1pdm09, data not shown).

OW1 and OW2 showed significantly higher anti-δ1-H1N2 and cross-reactive anti-H1N1pdm09 IgG levels compared to other vaccinated groups and controls at 0 and 5 dpi (Fig. 2C–F).

All vaccinated groups showed similar levels of anti-δ1-H1N2 IgA in BALF. However, no cross-reactive anti-H1N1pdm09 IgA responses were observed (Fig. 2G and H). In contrast, all vaccinated groups demonstrated significantly higher levels of anti-δ1-H1N2 and cross-reactive anti-H1N1pdm09 IgG responses in BALF

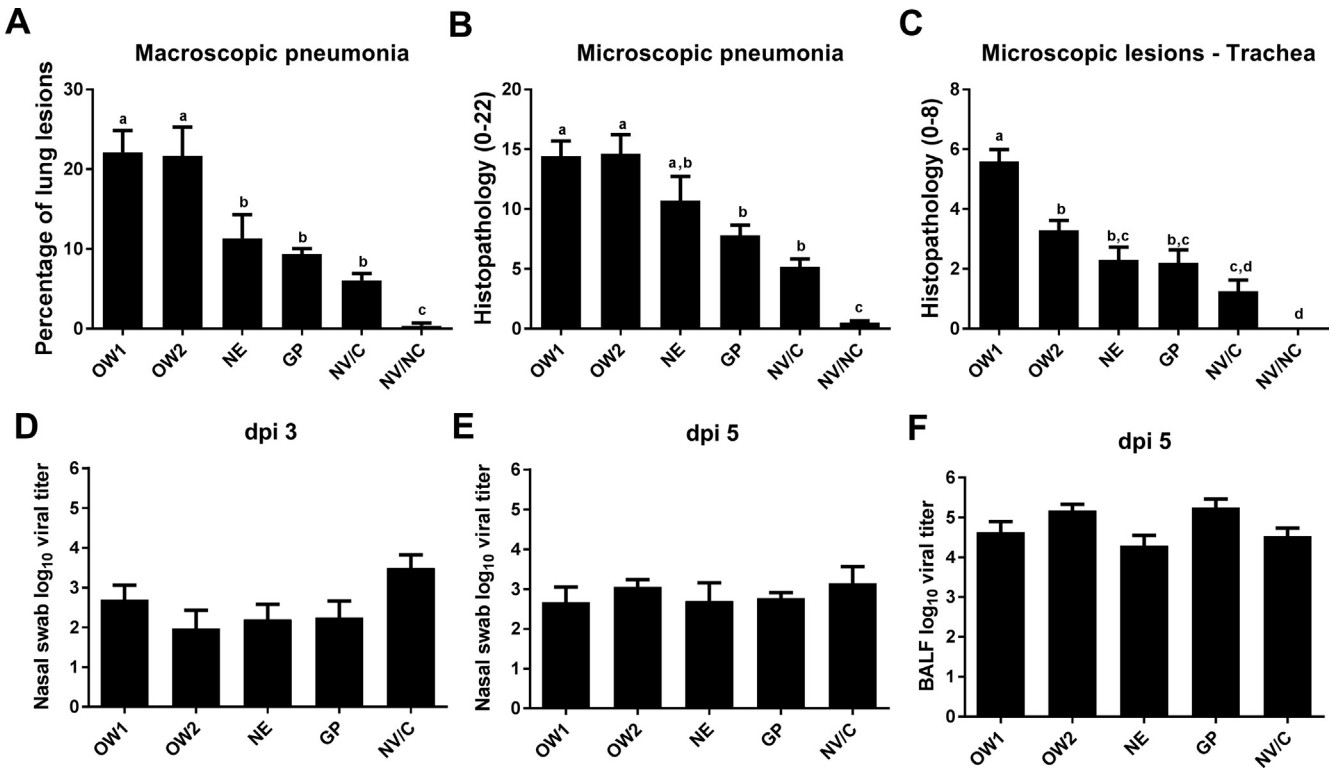

Fig. 1. Percentage of lung lesions of WIV-1δ-H1N2 vaccinated groups followed by a heterologous challenge (H1N1pdm09) and virus titers in nasal swab and lungs. OW1 and OW2 vaccinated pigs presented significantly higher percentage of lung lesions consistent with VAERD when compared with other adjuvants and controls (A). Group mean microscopic scores are significantly higher in OW groups in lungs (B) and trachea (C) compared to NV/C controls. Virus titers (Log_{10} $TCID_{50}$) of vaccinated/challenge pigs compared with controls (NV/C) in nasal swabs at 3 (D) and 5 (E) dpi and virus titers in the lungs (F). Treatment group means with statistically significant differences ($P \leq 0.05$) are identified by different lowercase letters.

compared to controls, with the OW groups showing the highest levels (Fig. 2I and J).

3.4. OW adjuvants elicited a broader antibody response against HA and NA

All vaccinated groups presented NI antibodies against the homologous antigen of the N2-98 genetic lineage, and both OW groups had significant cross-reactivity against the drifted N2-98 antigen compared to other adjuvants (Fig. 3A and B). However, no cross-reactivity was found against the heterologous N2-02 lineage antigen, regardless of the adjuvant type (Fig. 3C).

The breadth and magnitude of response between both OW adjuvants showed a similar range among genetically-related H1 δ-lineage strains in the antibody landscapes (Fig. 4A). In contrast, NE and GP landscapes demonstrated a more restricted span among H1 δ-lineage strains compared to the OW1 landscape (Fig. 4B and C). In accordance with the HI results (Supplementary material 1), the maximum height for each landscape corresponded to the antibody titer to the vaccine strain (δ1-H1N2), with the OW adjuvants stimulating higher homologous titers. None of the landscapes spanned over H1N1pdm09.

3.5. IFN-γ recall responses to δ1-H1N2 and H1N1pdm09 following vaccination

All WIV groups (OW1, OW2, NE and GP) showed significantly higher group mean numbers of IFN-γ secreting cells compared to NV controls at 28 dpv (61, 132, 80 and 26, respectively) and at pre-challenge (42 dpv) (33, 80, 36 and 35, respectively) against vaccine antigen δ1-H1N2 (Fig. 5A) and challenge antigen H1N1pdm09-specific at 28 dpv (51, 146, 39 and 29, respectively) and at 42 dpv (36, 88, 39 and 29, respectively) when compared to controls (Fig. 5B).

4. Discussion

Adjuvants can modulate antibody and cell-mediated adaptive immune responses, allow for the use of smaller doses of antigen, or may reduce the number of administrations required to achieve protection [35]. OW emulsions are broadly used in veterinary vaccines to improve immunogenicity and elicit potent immune responses [36], so two commercial sources of OW were tested in our study. The other two adjuvants selected were a squalene-based nano-emulsion adjuvant (NE) similar to MF59® formulation that is licensed for adjuvanted influenza vaccines for humans [37,38], and a stable dispersion of high molecular weight sodium polyacrylic gel polymer particles in water adjuvant (GP) reported to induce a balanced Th1/Th2 immune response [39,40].

WIV-OW vaccines have been associated with enhanced respiratory disease in pigs. VAERD has been reproduced in pigs using multiple combinations of IAV vaccines followed by a mismatched challenge strain [10,13–16,41], but only in the context of OW adjuvanted vaccines. Here, we demonstrated that the type of adjuvant did indeed impact the development of VAERD in this model. Both OW vaccinated groups demonstrated severe lung pathology consistent with previous VAERD studies [11,12,16], in contrast to other adjuvants (NE and GP) that showed mild lung lesions, similar to the non-vaccinated/infected controls. However, none of the WIV formulations protected pigs from virus infection with the mismatched H1N1pdm09.

Although, all vaccinated groups demonstrated IgA against the homologous vaccine strain (δ1-H1N2), no cross-reactive IgA mucosal responses to H1N1pdm09 were detected in the lungs, as

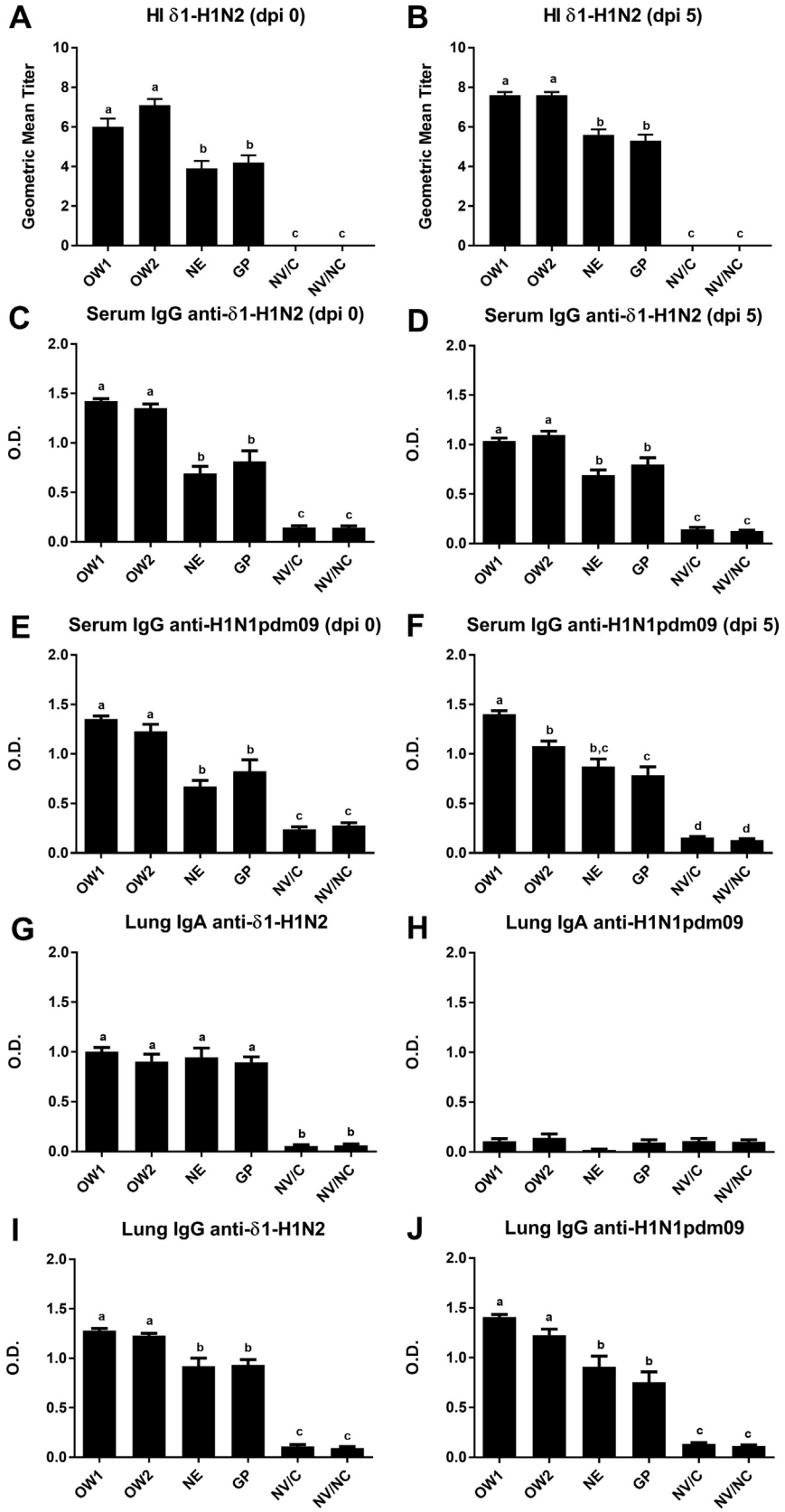

Fig. 2. Peripheral and local immune responses against the vaccine (δ1-H1N2) and challenge (H1N1pdm09) strains. Hemagglutination Inhibition (HI) geometric mean titer (Log_2) against the vaccine strain (δ1-H1N2) at 0 dpi (A) and 5 dpi (B). ELISA optical density (O.D.) of IgG anti-δ1-H1N2 at 0 dpi (C) and 5 dpi (D). Cross-reactive anti-H1N1pdm09 at dpi 0 (E) and dpi 5 (F) were significantly higher in both OW than other groups. Lung IgA responses against the vaccine (δ1-H1N2) (G) and challenge strains (H1N1pdm09) (H). Lung IgG anti-δ1-H1N2 (I) and cross-reactive anti-H1N1pdm09 (J) were significantly higher in both OW than other groups. Treatment group means with statistically significant differences ($P \leq 0.05$) are identified by different lowercase letters.

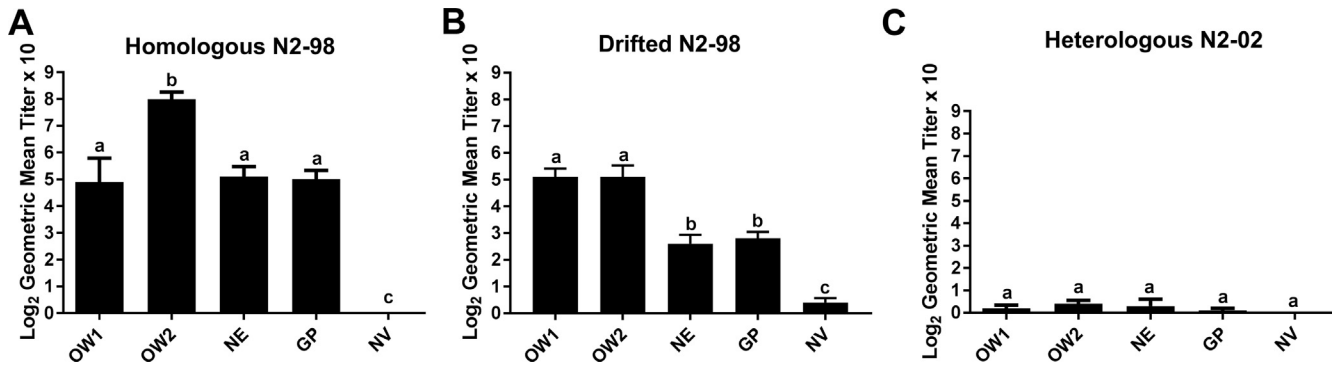

Fig. 3. Serum NI antibody responses to WIV containing δ1-H1N2 strain formulated with different adjuvants (OW1, OW2, NE and GP). All vaccinated groups demonstrated NI antibody response against homologous N2 (δ1-H1N2) (A). (B) NI antibody response against a drifted N2 within the same 1998-lineage of δ1-H1N2 was significantly higher in both OW groups. (C) None of the groups presented NI antibody response against heterologous N2, representing the 2002-lineage. Treatment group means with statistically significant differences ($P \leq 0.05$) are identified by different lowercase letters.

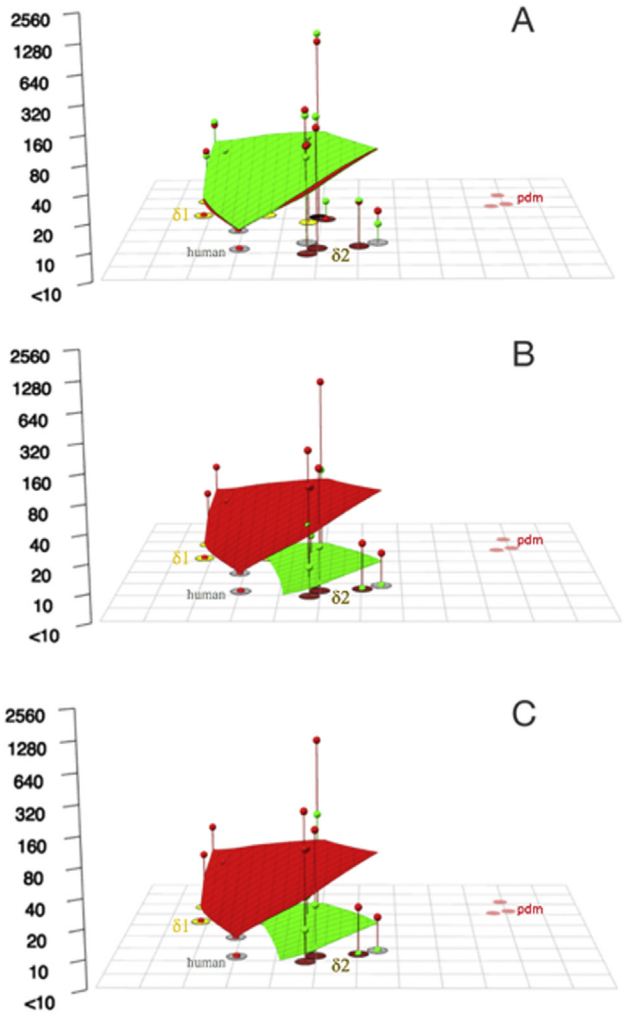

Fig. 4. Antibody landscaping representing the serological data from the vaccination of swine with WIV formulated with OW1 adjuvant (red) compared with the other adjuvants (green) (A) OW1 vs OW2. (B) OW1 vs GP. (C) OW1 vs NE. Swine δ1-H1N2 strains are colored in yellow, swine δ2-H1N2 in brown, human strains in grey, pandemic H1N1 2009 strain in red and vaccine strain (A/H1N2/Swine/Minnesota/02011/2008) in black. OW1 was chosen as a control adjuvant to compare with other landscapes. The landscape for OW1 was colored red, while OW2, NE and GP landscapes were colored green. Z axis represents the geometric mean of the HI titers at each antigenic point for each swine population. The x and y axis represent the coordinates of the virus strains derived from the 2D antigenic map.

previously observed [13]. All WIV groups induced anti-H1N1pdm09 cross-reactive IgG responses in serum and lungs, however OW groups demonstrated greater magnitude of cross-reactive IgG antibody response, in association with the observed immunopathology. Non-neutralizing, cross-reactive antibodies were previously shown *in vitro* to target a conserved epitope in the HA stalk and were associated with increased infectivity and virus fusion [17,42]. Cross-reactive, non-neutralizing antibodies can mediate activation of antibody-dependent cellular cytotoxicity (ADCC) and the complement cascade [14], that can employ a protective role on virus clearance [43,44]. Such immune responses can also lead to excessive production of pro-inflammatory cytokines and chemokines increasing neutrophil infiltration in IAV infected pigs resulting in immunopathology, as previously observed in VAERD affected-pigs [13,45]. MF-59® (similar to NE) is widely used in influenza vaccines formulations for humans and was shown to induce a broad antibody response in ferrets [46]. However, both OW groups demonstrated higher homologous titers and a broader range of HI antibody responses to genetically related H1 δ-lineage strains compared to NE and GP in the antibody landscapes. This broader HI antibody response against heterologous viruses in OW vaccinated pigs suggests an impact on B cell repertoire diversity that may also include broader recognition of individual HA protein domains or epitopes, including the HA stalk [17]. Based on findings with the HA, we assessed the range of antibody cross-reactivity against the N2 component of the δ1-H1N2 vaccine strain using NI assays. Similar to the HI results, both OW groups induced robust homologous NI titers, as well as cross-reactive titers to a drifted N2 within the same N2-98 phylogenetic lineage that were significantly higher than NE or GP, but not extended to the heterologous N2-02 lineage.

CMI response in IAV infection has been reported to play a role in virus clearance and reduction in severity of disease [43]. Previous findings showed that vaccination with WIV-OW elicited homologous and heterologous specific-IAV responses at 42 dpv without preventing lung pathology [31], so we assessed the number of peripheral IFN-γ secreting cells as a measurement of T-cell response. All types of adjuvants in this study activated CMI responses when stimulated by homologous δ1-H1N2 or heterologous H1N1pdm09, but the CMI responses were not associated with protection against heterologous challenge (H1N1pdm09), as measured by lung lesions or virus titers in the nose and lungs. Furthermore, OW groups displayed severe immunopathology in the lungs, despite the substantial antibody and CMI responses. Future studies are needed to determine if differential activation of $CD4^{\pm}$, $CD8^{\pm}$, or

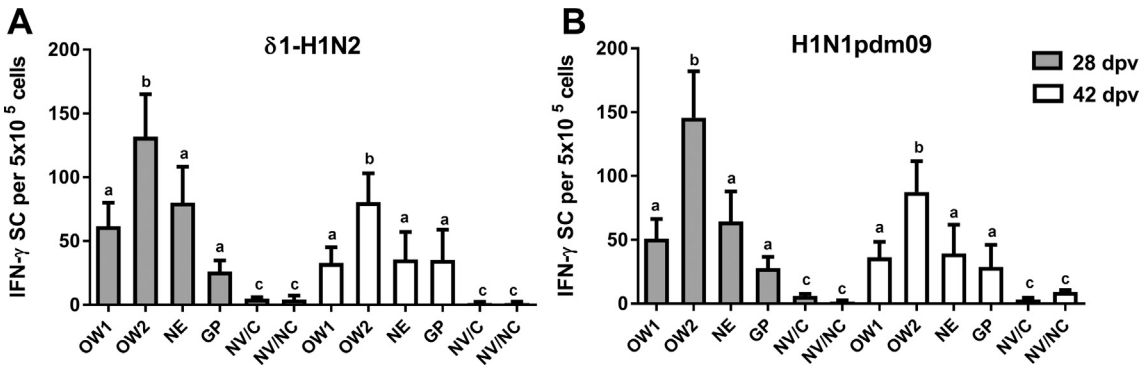

Fig. 5. Spot count (SC) number of antigen-specific IFN-γ secreting cells at 28 days post vaccination (dpv) and 42 dpv (pre-challenge) when stimulated with δ1-H1N2 (A) and H1N1pdm09 strain (B). Treatment group means with statistically significant differences ($P \leq 0.05$) are identified by different lowercase letters.

other T-cell subsets contributes to the immunopathologic mechanisms of VAERD.

In conclusion, the type of adjuvant in the WIV formulation when challenged with a mismatched strain impacted the VAERD outcome, showing that OW groups had more severe lung pathology compared to other adjuvants. However, OW were also associated with a greater magnitude of HI and NI antibody and CMI responses and cross-reactivity with heterologous strains within phylogenetic lineages of HA and NA, indicating a fine line of distinction between an efficacious adjuvant for WIV and the risk of immunopathology. Although this study focused on the impact of adjuvants in the context of VAERD, future vaccine studies should further evaluate the NE and other adjuvants in influenza vaccines, but in the full spectrum of breadth of protection from drifted strains to VAERD, in order to fully understand the extent and limitations of these vaccine components.

Acknowledgements

The authors thank Michelle Harland, Gwen Nordholm and Zahra Olson for technical assistance with lab assays, Jason Huegel, Tyler Standley and Jason Crabtree for assistance with animal care, Daniel Perez for reverse engineered NI antigens, and Dr. Susan Brockmeier for microbial screening of BALF. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Funding

CKS was a Capes-Brazil fellowship recipient of a sandwich PhD scholarship. Funding was provided by USDA-ARS. USDA is an equal opportunity provider and employer.

Author contributions

ALV, CKS and CLL contributed to conception and design of the study. CKS, DSR, CLL and ALV conducted the animal study. CKS, DSR, MRS, CLL, PCG, SL and NSL were involved in laboratory assays or data acquisition. All authors were involved in the interpretation of data and statistical analysis, drafting or revising the article, and approval of the final version of the manuscript.

Conflict of interest statement

The authors have no potential conflicts of interest to disclose.

References

[1] Rajao DS, Walia RR, Campbell B, Gauger PC, Janas-Martindale A, Killian ML, et al. Pandemic H1N1 in the United States resulted in influenza a viruses with diverse genetic constellations with variable virulence in pigs. J Virol 2009;2017:91.
[2] Lewis NS, Russell CA, Langat P, Anderson TK, Berger K, Bielejec F, et al. The global antigenic diversity of swine influenza A viruses. eLife 2016;5:e12217.
[3] Anderson TK, Macken CA, Lewis NS, Scheuermann RH, Van Reeth K, Brown IH, et al. A phylogeny-based global nomenclature system and automated annotation tool for H1 hemagglutinin genes from swine influenza a viruses. mSphere 2016:1.
[4] Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 2009;325:197–201.
[5] Nelson MI, Vincent AL. Reverse zoonosis of influenza to swine: new perspectives on the human-animal interface. Trends Microbiol 2015;23:142–53.
[6] Lewis NS, Anderson TK, Kitikoon P, Skepner E, Burke DF, Vincent AL. Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. J Virol 2014;88:4752–63.
[7] Vander Veen RL, Mogler MA, Russell BJ, Loynachan AT, Harris DL, Kamrud KI. Haemagglutinin and nucleoprotein replicon particle vaccination of swine protects against the pandemic H1N1 2009 virus. Vet Rec 2013;173:344.
[8] Genzow M, Goodell C, Kaiser TJ, Johnson W, Eichmeyer M. Live attenuated influenza virus vaccine reduces virus shedding of newborn piglets in the presence of maternal antibody. Influenza Other Respir Viruses 2018;12:353–9.
[9] Van Reeth KMW. Swine influenza virus vaccines: to change or not to change-that's the question. Curr Top Microbiol Immunol 2013;173–200.
[10] Vincent AL, Ciacci-Zanella JR, Lorusso A, Gauger PC, Zanella EL, Kehrli Jr ME, et al. Efficacy of inactivated swine influenza virus vaccines against the 2009 A/H1N1 influenza virus in pigs. Vaccine 2010;28:2782–7.
[11] Gauger PC, Vincent AL, Loving CL, Lager KM, Janke BH, Kehrli Jr ME, et al. Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. Vaccine 2011;29:2712–9.
[12] Gauger PC, Vincent AL, Loving CL, Henningson JN, Lager KM, Janke BH, et al. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. Vet Pathol 2012;49:900–12.
[13] Gauger PC, Loving CL, Lager KM, Janke BH, Kehrli Jr ME, Roth JA, et al. Vaccine-associated enhanced respiratory disease does not interfere with the adaptive immune response following challenge with pandemic A/H1N1 2009. Viral Immunol 2013;26:314–21.
[14] Rajao DS, Chen H, Perez DR, Sandbulte MR, Gauger PC, Loving CL, et al. Vaccine-associated enhanced respiratory disease is influenced by haemagglutinin and neuraminidase in whole inactivated influenza virus vaccines. J Gen Virol 2016;97:1489–99.
[15] Rajao DS, Loving CL, Gauger PC, Kitikoon P, Vincent AL. Influenza A virus hemagglutinin protein subunit vaccine elicits vaccine-associated enhanced respiratory disease in pigs. Vaccine 2014;32:5170–6.
[16] Souza CK, Rajao DS, Loving CL, Gauger PC, Perez DR, Vincent AL. Age at vaccination and timing of infection do not alter vaccine-associated enhanced respiratory disease in influenza a virus-infected pigs. Clin Vaccine Immunol: CVI 2016;23:470–82.
[17] Khurana S, Loving CL, Manischewitz J, King LR, Gauger PC, Henningson J, et al. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. Sci Transl Med 2013;5:200ra114.
[18] Egli A, Santer D, Barakat K, Zand M, Levin A, Vollmer M, et al. Vaccine adjuvants–understanding molecular mechanisms to improve vaccines. Swiss Medical Weekly 2014;144. w13940.

[19] Di Pasquale A, Preiss S, Tavares Da Silva F, Garcon N. Vaccine adjuvants: from 1920 to 2015 and beyond. Vaccines 2015(3):320–43.
[20] Nauta JJ, Beyer WE, Osterhaus AD. On the relationship between mean antibody level, seroprotection and clinical protection from influenza. Biol: J Int Assoc Biol Standardization 2009;37:216–21.
[21] van de Sandt CE, Kreijtz JH, de Mutsert G, Geelhoed-Mieras MM, Hillaire ML, Vogelzang-van Trierum SE, et al. Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus. J Virol 2014;88:1684–93.
[22] Heinen PP, de Boer-Luijtze EA, Bianchi AT. Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. J Gen Virol 2001;82:2697–707.
[23] Vincent AL, Ma W, Lager KM, Richt JA, Janke BH, Sandbulte MR, et al. Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease. J Virol 2012;86:10597–605.
[24] Vincent AL, Lager KM, Faaberg KS, Harland M, Zanella EL, Ciacci-Zanella JR, et al. Experimental inoculation of pigs with pandemic H1N1 2009 virus and HI cross-reactivity with contemporary swine influenza virus antisera. Influenza Other Respir Viruses 2010;4:53–60.
[25] Vincent AL, Lager KM, Janke BH, Gramer MR, Richt JA. Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine. Vet Microbiol 2008;126:310–23.
[26] Kitikoon P, Nilubol D, Erickson BJ, Janke BH, Hoover TC, Sornsen SA, et al. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. Vet Immunol Immunopathol 2006;112:117–28.
[27] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 1938;27:493–7.
[28] Kitikoon P, Gauger PC, Vincent AL. Hemagglutinin inhibition assay with swine sera. Methods Mol Biol 2014;1161:295–301.
[29] Gauger PC, Loving CL, Vincent AL. Enzyme-linked immunosorbent assay for detection of serum or mucosal isotype-specific IgG and IgA whole-virus antibody to influenza A virus in swine. Methods Mol Biol 2014;1161:303–12.
[30] Sandbulte MR, Gauger PC, Kitikoon P, Chen H, Perez DR, Roth JA, et al. Neuraminidase inhibiting antibody responses in pigs differ between influenza A virus N2 lineages and by vaccine type. Vaccine 2016;34:3773–9.
[31] Olson ZF, Sandbulte MR, Souza CK, Perez DR, Vincent AL, Loving CL. Factors affecting induction of peripheral IFN-gamma recall response to influenza A virus vaccination in pigs. Vet Immunol Immunopathol 2017;185:57–65.
[32] Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, et al. Mapping the antigenic and genetic evolution of influenza virus. Science 2004;305:371–6.
[33] Fonville JM, Wilks SH, James SL, Fox A, Ventresca M, Aban M, et al. Antibody landscapes after influenza virus infection or vaccination. Science 2014;346:996–1000.
[34] Lorusso A, Vincent AL, Harland ML, Alt D, Bayles DO, Swenson SL, et al. Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. J Gen Virol 2011;92:919–30.
[35] Boyle J, Eastman D, Millar C, Camuglia S, Cox J, Pearse M, et al. The utility of ISCOMATRIX adjuvant for dose reduction of antigen for vaccines requiring antibody responses. Vaccine 2007;25:2541–4.
[36] Aucouturier J, Dupuis L, Ganne V. Adjuvants designed for veterinary and human vaccines. Vaccine 2001;19:2666–72.
[37] Khurana S, Chearwae W, Castellino F, Manischewitz J, King LR, Honorkiewicz A, et al. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. Sci Transl Med 2010;2:15ra5.
[38] Khurana S, Verma N, Yewdell JW, Hilbert AK, Castellino F, Lattanzi M, et al. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. Sci Transl Med 2011;3:85ra48.
[39] Vialle R, LD, Deville S, Bertrand F, Gaucheron J, Aucouturier J. Microgel particulate adjuvant: characterisation and mechanisms of action. Procedia Vaccinol 2010;2:12–6.
[40] Parker R, SD, Dupuis L, Bertrand F, Aucouturier J. Adjuvant formulation for veterinary vaccines: Montanide™ Gel safety profile. Procedia Vaccinol 2009;1:140–7.
[41] Rajao DS, Sandbulte MR, Gauger PC, Kitikoon P, Platt R, Roth JA, et al. Heterologous challenge in the presence of maternally-derived antibodies results in vaccine-associated enhanced respiratory disease in weaned piglets. Virology 2016;491:79–88.
[42] Gauger PC, Loving CL, Khurana S, Lorusso A, Perez DR, Kehrli Jr ME, et al. Live attenuated influenza A virus vaccine protects against A(H1N1)pdm09 heterologous challenge without vaccine associated enhanced respiratory disease. Virology 2014;471–473:93–104.
[43] Jegaskanda S, Vandenberg K, Laurie KL, Loh L, Kramski M, Winnall WR, et al. Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity in intravenous immunoglobulin as a potential therapeutic against emerging influenza viruses. J Infect Dis 2014;210:1811–22.
[44] O'Brien KB, Morrison TE, Dundore DY, Heise MT, Schultz-Cherry S. A protective role for complement C3 protein during pandemic 2009 H1N1 and H5N1 influenza A virus infection. PLoS One 2011;6. e17377.
[45] Van Reeth K, Nauwynck H, Pensaert M. Bronchoalveolar interferon-alpha, tumor necrosis factor-alpha, interleukin-1, and inflammation during acute influenza in pigs: a possible model for humans? J Infect Dis 1998;177:1076–9.
[46] Wang J, Hilchey SP, DeDiego M, Perry S, Hyrien O, Nogales A, et al. Broad cross-reactive IgG responses elicited by adjuvanted vaccination with recombinant influenza hemagglutinin (rHA) in ferrets and mice. PLoS One 2018;13. e0193680.