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Research paper

Passive inhalation of dry powder influenza vaccine formulations completely protects chickens against H5N1 lethal viral challenge

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ABSTRACT

Bird to human transmission of high pathogenicity avian influenza virus (HPAIV) poses a significant risk of triggering a flu pandemic in the human population. Therefore, vaccination of susceptible poultry during an HPAIV outbreak might be the best remedy to prevent such transmissions. To this end, suitable formulations and an effective mass vaccination method that can be translated to field settings needs to be developed. Our previous study in chickens has shown that inhalation of a non-adjuvanted dry powder influenza vaccine formulation during normal breathing results in partial protection against lethal influenza challenge. The aim of the present study was to improve the effectiveness of pulmonary vaccination by increasing the vaccine dose deposited in the lungs and by the use of suitable adjuvants. Two adjuvants, namely, Bacterium-like Particles (BLP) and Advax, were spray freeze dried with influenza vaccine into dry powder formulations. Delivery of dry formulations directly at the syrinx revealed that BLP and Advax had the potential to boost either systemic or mucosal immune responses or both. Upon passive inhalation of dry influenza vaccine formulations in an optimized set-up, BLP and Advax/BLP adjuvanted formulations induced significantly higher systemic immune responses than the non-adjuvanted formulation. Remarkably, all vaccinated animals not only survived a lethal influenza challenge, but also did not show any shedding of challenge virus except for two out of six animals in the Advax group. Overall, our results indicate that passive inhalation is feasible, effective and suitable for mass vaccination of chickens if it can be adapted to field settings.

1. Introduction

Outbreaks of avian influenza in poultry are the major source of H5N1 infection in humans [1–3]. These outbreaks, caused by highly pathogenic strains of avian influenza virus, pose a significant risk of poultry-human transmission. Currently, the only remedy to control these outbreaks is to cull the poultry. For safety reasons animals are not only culled on infected farmyards but also on non-infected neighboring farmyards [4,5]. The slaughter of millions of poultry not only leads to severe economic losses but also raises ethical questions and incomprehension within the society.

To combat outbreaks of avian influenza virus, it could be useful to rapidly vaccinate the poultry in areas surrounding the outbreak, in a ring fencing strategy. However, this would require that very large numbers of poultry, potentially in the tens or hundreds of millions, should be able to be immunized within a very short time period. This would require a vaccine formulation and route of immunization that are suitable for such mass application. Liquid or powder influenza vaccine formulations that can be aerosolized and administered via the respiratory tract could be appropriate formulations for mass vaccination. Liquid formulations of inactivated influenza virus have been shown to be inadequate for aerosol vaccination by single administration [6]. Powder formulations, on the other hand, have shown an advantage over liquid formulations because of their long-term storage stability

Abbreviations: HPAIV, high pathogenicity avian influenza virus; BLP, bacterium-like particles; WIV, whole inactivated virus vaccine; SFD, spray freeze drying; HI, hemagglutination inhibition; EID_{50}, 50% embryo infectious dose; $TCID_{50}$, 50% tissue culture infective dose

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[7–9]. This long term stability facilitates stockpiling and thus provides ease of administration during mass vaccination [10]. In a previous study, we have shown that pulmonary immunization by dispersion of a dry powder influenza vaccine directly at the syrinx of chickens (active administration) was able to completely protect these animals against lethal viral challenge [11]. However, in realistic situations, due to extreme time pressures, active administration of chickens is unsuitable for mass vaccination. A more realistic approach would be to let chickens inhale aerosolized dry powder influenza vaccine formulation during breathing (passive administration). Indeed, passive inhalation of dry powder influenza vaccine formulation was feasible in chickens, but the animals were only partially protected indicated by a delay in time to death and reduced virus shedding [11]. Hence, the efficiency of influenza immunization by passive inhalation must be improved for pulmonary vaccination to become feasible. Possible approaches could be to increase the concentration of aerosolized vaccine, to expose the animals to aerosolized powder for longer periods of time and to include an adjuvant in the vaccine formulation.

An effective adjuvant should not only be cheap, readily available and potent, but also safe. An adjuvant that has been generally recognized as safe is BLP. BLP are produced by hot acid treatment (pH 1 for 30 min at 99 °C) of *Lactococcus lactis*, a non-pathogenic, food-grade Gram-positive bacterium [12,13]. BLP act as Toll-like receptor-2 ligand and have shown potent immune boosting properties when administered together with vaccines against influenza, *Yersinia pestis*, malaria, and pneumococcal disease [14–17]. Besides Toll-like receptor ligands, naturally derived polysaccharides, for example Advax™ adjuvant comprising an insoluble isoform of inulin, boosts vaccine responses through mechanism that are still not fully characterized [18]. In (pre) clinical studies Advax has been shown to enhance immune responses induced by a wide variety of vaccines including vaccines against Hepatitis B, SARS coronavirus, listeria and influenza [19–24]. Upon parenteral administration, Advax has been shown to have a good safety and tolerability record both in animal studies and clinical trials [25–27].

The aim of the current study was to investigate whether passive administration with dry non-adjuvanted or adjuvanted influenza formulations has the potential to completely protect chickens against lethal HPAIV challenge. For this, we initially tested whether (a) BLP or Advax could be co-formulated with influenza vaccine into dry powder formulations that are suitable for pulmonary immunization; (b) the adjuvants have the potential to boost systemic and mucosal immune responses to influenza; (c) passive administration with either non-adjuvanted or adjuvanted influenza formulations would protect chickens against a lethal HPAIV challenge.

2. Materials and methods

2.1. Virus

For immunization, a reassortant virus, NIBRG-23, prepared by reverse genetics from A/turkey/Turkey/1/2005 (H5N1) virus and A/PR/8/34 (H1N1) virus was used (NIBSC code: 08/156). The virus was cultured in embryonated chicken eggs by allantoic inoculation of the seed virus. The virus was purified and inactivated as described previously to obtain whole inactivated virus vaccine (WIV) [11,28].

For challenge, a highly pathogenic avian influenza virus strain A/turkey/Turkey/1/2005 (H5N1) (Clade 2.2.1), obtained from the Animal Health and Veterinary Laboratories Agency, Weybridge, UK was used.

2.2. Spray freeze drying (SFD)

For active administration, 5.0, 1.0, and 0.2 μg HA of NIBRG-23 WIV was SFD either as such or admixed with adjuvants (BLP, Mucosis, Groningen, The Netherlands or Advax, Vaxine, Adelaide, Australia) in various ratios. For active administration, the dose of BLP and Advax in 1 mg of SFD powder was 150 μg and 500 μg respectively. WIV was mixed with BLP in weight ratios of HA:BLP of 1:30, 1:150 and 1:750. For WIV-Advax, the weight ratios of HA:Advax were 1:100, 1:500 and 1:2500. Likewise, for passive administration, 5 μg HA of NIBRG-23 was SFD either as such or mixed with 300 μg of BLP or 500 μg of Advax (this dose corresponds to the amount of HA and adjuvants in 1 mg of SFD powder). Both non-adjuvanted and adjuvanted formulations were SFD using inulin (4 kDa, Sensus, Roosendaal, The Netherlands) as the stabilizer. Inulin used for stabilization of influenza vaccine is amorphous and water soluble with potentially no adjuvant effect whereas Advax (delta inulin) is crystalline and insoluble in water with potent adjuvant activity. In brief, a 5% (w/v) solution of WIV and inulin with or without adjuvants was pumped at a flowrate of 5 ml/min through a two-fluid nozzle of a Büchi 190Mini SprayDryer (Büchi, Flawil, Switzerland). An atomizing airflow of 600 L/h was used to spray vaccine preparations in a vessel of liquid nitrogen. Then, the frozen vaccine preparations were placed in a Christ Epsilon 2–4 freeze dryer precooled to a shelf temperature of −35 °C and at a pressure of 0.220 mbar. The shelf temperature was slowly increased from −35 °C to 4 °C within the time period of 32 h. During the next 12 h, the temperature was further increased to 20 °C and pressure was lowered to 0.05 mbar. The dry vaccine powder was collected in a climate box with a relative humidity of < 1% and stored under airtight conditions.

2.3. Physical and biological characterization of influenza vaccine and adjuvants before and after SFD

2.3.1. Transmission electron microscopy

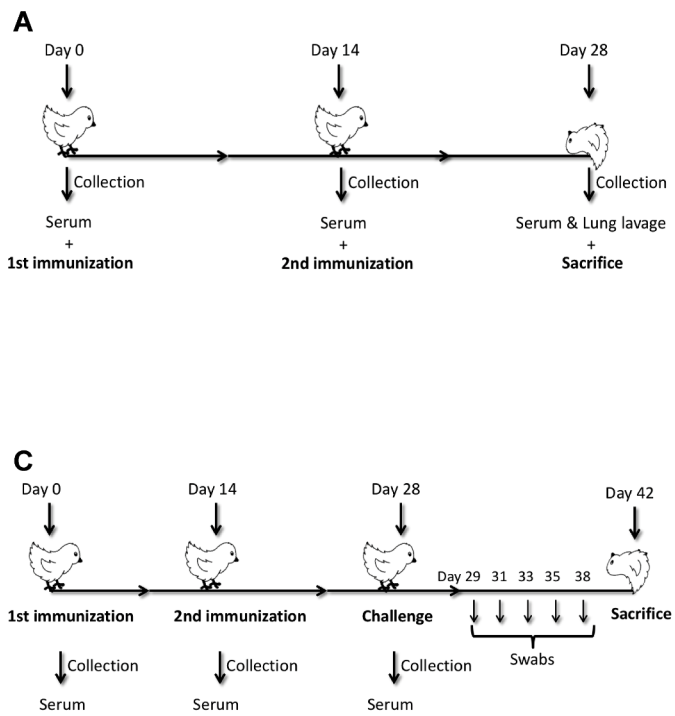
A Philips CM120 transmission electron microscope was used to make transmission electron microscopy images. SFD powders containing WIV (5 μg HA formulation) with or without adjuvants i.e. BLP (300 μg in 1 mg of SFD powder), Advax (500 μg in 1 mg of SFD powder) were reconstituted in sterile filtered water. Liquid and reconstituted SFD formulations were placed on a plain carbon grid, rinsed with water and then samples were stained twice with 5 μL of 2 wt-% uranyl acetate. A Gatan type UltraScan 4000SP CCD Camera at a magnification was used to take images.

2.3.2. Hemagglutination assay

The receptor binding activity of WIV after SFD with or without adjuvants was assessed by hemagglutination assay. SFD powders were reconstituted in phosphate buffer saline (PBS) to a concentration of 20 μg/ml of HA. Then, 50 μL of the preparation was added to 96-well V bottom plates containing 50 μL of PBS. The entire mixture was two-fold serially diluted, then 50 μL of 1.5% guinea pig red blood cell suspension was added to each well. The plate was allowed to stand undisturbed for two hours at room temperature. Hemagglutination titers read after two hours were expressed as $\log_2$ of the highest dilution where red blood cell agglutination could be seen.

2.3.3. Quanti-blue assay

The capacity of BLP to activate NF-κB via Toll-like receptor-2 was evaluated using RAW-Blue™ cells (InvivoGen, Toulouse, France). RAW-Blue™ cells have a number of pattern recognition receptors which when bound to agonists leads to activation of NF-κB and thus the production of secreted alkaline phosphatase. A schematic illustration is shown in Fig. S1. Cells were maintained in DMEM with high glucose (Gibco Life Technologies BV, Bleiswijk, The Netherlands), 10% FBS (Lonza, Basel, Switzerland), 100 μg/ml Normocin™ (InvivoGen, Toulouse, France), 2 mM L-glutamine and passaged when 70–80% confluency was reached. Approximately 5×10^4 cells were added to 96-well flat bottom plates and were stimulated with 1.7 μg untreated BLP or with liquid and reconstituted SFD powder formulations with (5 μg HA + 300 μg BLP formulation) or without 1.7 μg BLP. The incubation was maintained for 18 h at 37 °C with 5% CO_2. To measure alkaline phosphatase levels, 150 μL QUANTI-Blue™ (InvivoGen, Toulouse,



B

Group No.	Vaccine dose (HA in µg)	Adjuvant	Adjuvant dose (µg)	HA:adjuvant	HA:inulin
1	5	n/a	n/a	n/a	1:200
2	1	n/a	n/a	n/a	1:1000
3	0.2	n/a	n/a	n/a	1:5000
4	5	BLP	150	1:30	1:170
5	1	BLP	150	1:150	1:850
6	0.2	BLP	150	1:750	1:4250
7	5	Advax	500	1:100	1:100
8	1	Advax	500	1:500	1:500
9	0.2	Advax	500	1:2500	1:2500
10	0*	n/a	n/a	n/a	n/a

D

Group No.	Vaccine dose (HA in µg)	Adjuvant	Adjuvant dose (µg)	HA:adjuvant	HA:inulin
1	50	n/a	n/a	n/a	n/a
2	50	BLP	3000	1:60	1:140
3	50	Advax	5000	1:100	1:100
4	50	Advax and BLP#	Advax: 5000, BLP: 3000	1:100, 1:60	1:100, 1:140

Fig. 1. An overview of the immunization scheme and immunized groups. Active administration: immunization scheme (A), immunized groups (B). Passive administration: immunization scheme (C), immunized groups (D). * Represents group which received only inulin (placebo), # represents 1st immunization with Advax and 2nd immunization with BLP. In the passive inhalation set up, the vaccine and adjuvant dose states the theoretical/calculated dose.

France) was added to the cell supernatant and after 1 h, absorbance was measured at 630 nm using a spectrophotometer.

2.3.4. Scanning electron microscopy

SFD-WIV preparations were imaged using a JEOL JSM 6301-F microscope (JEOL. Ltd., Tokyo, Japan). Powders were placed on double-sided sticky carbon tape on a metal disc and the particles were coated with approximately 10 nm of gold using a Balzer's 120B sputtering device (Balzer UNION, Liechtenstein). A magnification of 500× and 5000× was used to capture the images.

2.3.5. Particle size analysis

Geometrical particle size distribution of the powders were measured using a HELOS compact model KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany). To disperse the powders, the highly efficient RODOS dispersing system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) was used at a pressure of 1 bar. Aerodynamic particle size distribution was calculated using the equation described by Bhide et al. [29].

2.4. Vaccination-challenge

2.4.1. Active administration and sample collection

Sixty 3-week old specific-pathogen free chickens (White Leghorn) were randomly divided into 10 groups of 6 animals. Animals were immunized twice i.e. on day 0 and day 14. SFD-WIV formulations (either non-adjuvanted or adjuvanted with BLP or Advax) were administered to the animals using a DP-4-C Dry Powder Insufflator (Penn-Century Inc., Philadelphia, USA). A custom length delivery tube designed to deliver the powder directly at the syrinx was used. Three puffs of 1 ml air were used to aerosolize 1 mg of powder filled in the insufflator. The animals were sacrificed on day 28.

Blood samples were taken on day 0 (before the 1st immunization), day 14 (two weeks after the 1st immunization) and day 28 (two weeks after the 2nd immunization). On the day of sacrifice (day 28), lung lavages were collected by flushing lungs with 20 ml of PBS as described by Holt et al. [30]. The obtained sera and lung lavages were stored at −20 °C until further use.

An overview of the immunization scheme and groups is shown in Fig. 1A and B.

2.4.2. Passive administration and challenge

For passive administration of non-adjuvanted and adjuvanted WIV formulations, 24 3-week old specific-pathogen free chickens were randomly divided into 4 groups of 6 chickens. One animal died of unknown causes before the start of the experiment. Each group was placed in a customized box with a volume of 0.035 m^3. A 5 ml Eppendorf tube filled with 100 mg of SFD powder containing 0.5 mg HA of NIBRG-23 WIV with or without adjuvants was punctured at the bottom with a 29G hypodermic needle. The lid of the Eppendorf tube was fitted to a pressurized air container while the bottom conical part of the tube was inserted airtight in a hole drilled in the customized box for the dispersion of powders. The SFD powders were aerosolized in the box by using several pulses of pressurized air, resulting in a theoretical concentration of 14 mg HA/m^3 (HA dose for dispersion: 0.5 mg, inhalation box volume: 0.035 m^3). For the first vaccination, the animals were exposed to the aerosolized vaccine for 20 min and after every 2 min short pulses of medicinal oxygen (20 s; flow rate 0.5 L/min) were supplied through another hole in the box. For the second vaccination (2 weeks later i.e. on day 14) the exposure time was 12 min with short pulses of oxygen (13 s; flow rate 0.5 L/min) every minute. Based on the respiration rate for chickens of 44 L/h/kg body weight [31,32] these exposure times would result in a maximum theoretical dose of 50 µg HA per animal per application.

The EID_{50} (50% embryo-infectious dose) titre of the challenge strain stock of A/turkey/Turkey/1/2005 (8.1 $\log_{10}$ EID_{50}/ml) was used to calculate the dilution for the challenge virus. The 50% chicken lethal dose of A/turkey/Turkey/1/2005 virus is 2.5 $\log_{10}$ EID_{50} [33]. On day

28, animals were challenged with a lethal dose of 5.0 $\log_{10}$ EID_{50} of this virus by the combined intranasal/intratracheal route (liquid suspension; 0.1 ml each). For back titration, we determined $TCID_{50}$ (50% tissue culture infectious dose). The titre (10log $TCID_{50}$/ml) after back-titration of the challenge virus that we received from the stables after the challenge inoculation was 4.2 whereas the titre of the original stock was 6.8. Thus, a titre of 8.1 EID_{50}/ml corresponds to 6.8 $TCID_{50}$/ml. The titre of 4.2 $TCID_{50}$/ml for the back-titration should therefore correspond to at least 5.0 EID_{50}/ml. Since the 50% chicken lethal dose of the challenge virus is 2.5 $\log_{10}$ EID_{50}, a dose of 4.3 10logEID_{50} (0.2 ml of 5.0 EID_{50}/ml) is more than sufficient (> 60× CLD_{50}) to kill non-vaccinated animals. In previous studies with the same virus batch and dilution, we found that this dose killed all non-vaccinated control group animals within 2–3 days after the challenge [11,34].

In this study, after challenge, animals were observed for clinical signs and on 1, 3, 5, 7 and 10 days post challenge choanal and cloacal swabs were collected for virus quantification. On day 42, the animals were sacrificed. An overview of the immunization-challenge scheme along with groups is shown in Fig. 1C and D. Passive inhalation of dry formulations could be seen in video file (supplementary data).

2.4.3. Virus quantification by RT-PCR

Virus titers in choanal and cloacal swabs were determined as per the method described by van der Goot et al. [35]. Challenge virus was ten-fold serially diluted, followed by RNA extraction, and a standard curve consisting of the RNA from these serial dilutions was prepared. As per the standard curve, PCR data was converted into equivalent virus titers (eq$TCID_{50}$/ml).

2.4.4. ELISA

Serum samples and lung washes were used for the determination of IgY and IgA antibody titers. For the determination of IgY titers, ELISA was performed as previously described [36], except that the secondary antibody consisted of goat anti chicken IgY-HRP (Southern Biotech, Birmingham, USA). IgY titers were determined as $\log_{10}$ of the reciprocal of the sample dilution corresponding to an absorbance of 0.2 at the wavelength of 492 nm. IgA titers were determined in lung washes using the commercially available chicken IgA ELISA kit as per manufacturer's instructions (Abcam, Cambridge, UK).

2.4.5. Hemagglutination inhibition (HI) assay

HI assay was performed as described previously [11]. Briefly, 8 hemagglutination units of inactivated virus A/turkey/Turkey/1/05 (H5N1) were added to two-fold diluted sera samples. HI titers were recorded as highest serum dilution capable of preventing hemagglutination. HI titers are presented on $\log_2$ scale.

2.4.6. Micro-neutralization assay

Micro-neutralization titers were determined as previously described [37]. Briefly, two-fold serial dilutions of serum samples were prepared and 50 $TCID_{50}$ of NIBRG-23 virus was added to each well of 96 well plate. After 2 h of incubation at 37 °C, the mixture of serum and virus was transferred to MDCK cells (ATCC, Germany) cultured in 96-well flat bottom plate. After 1 h of incubation at 37 °C, the supernatant was discarded and replaced with Episerf-medium (100 U/ml penicillin, 100 mg/ml streptomycin, 1 M HEPES and 7.55% sodium bicarbonate, all Life Technologies™ BV, Bleiswijk, The Netherlands) supplemented with 5 μg/ml of TPCK trypsine (Sigma-Adrich, The Netherlands). After 72 h of incubation, the supernatant was transferred to 96 V bottom plate. Micro-neutralization titers were calculated by finding the highest serum dilution capable of preventing hemagglutination. Micro-neutralization titers are presented on $\log_{10}$ scale.

2.4.7. Statistics

Mann-Whitney one tailed test was used to compare whether the differences between non-adjuvanted and adjuvanted influenza vaccine formulations were significant. Levels of significance are denoted as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ respectively.

3. Results

3.1. Physical and biological characterization of influenza vaccine and adjuvants after SFD

In order to investigate the potential of BLP and Advax as mucosal adjuvants in chickens, it was essential to investigate whether the physical and biological properties of WIV and adjuvants remained unaltered during SFD.

To evaluate whether admixing adjuvants with WIV and SFD had an influence on the physical appearance of WIV, BLP or Advax particles, transmission electron microscopy analysis was performed for these samples before and after SFD. The powder samples were reconstituted prior to use. Transmission electron microscopy pictures showed that WIV, BLP and Advax particles had comparable morphological appearance before and after SFD (Fig. 2A–C). Thus, neither the stress encountered during SFD nor admixing adjuvants with WIV had any adverse effect on their physical appearance.

The biological activity of WIV needs to be preserved both after the addition of adjuvants and after SFD. In order to investigate whether the receptor binding activity of HA was preserved, hemagglutination assay was performed. No differences in hemagglutination titers could be detected either by the addition of adjuvants or by SFD, thus indicating no detrimental effects of adjuvants or SFD on the biological activity of HA (Fig. 2D).

Furthermore, the effect of SFD on the biological activity of BLP was evaluated. For this purpose, reconstituted BLP-adjuvanted SFD WIV formulations were compared to unprocessed dispersion of liquid WIV and BLP for their capacity to activate NFκB using RAW-BLUE™ cells. NIBRG-23 derived WIV alone was found to be a poor activator of NFκB. Reconstituted BLP-adjuvanted WIV formulations activated NFκB to a similar extent as native liquid WIV-BLP dispersion. Thus, stress encountered during SFD had no effect on the biological activity of BLP to activate Toll-like receptor 2 (Fig. 2E).

3.2. Physical characterization of powders

To assess whether the incorporation of BLP or Advax in the SFD formulation had an effect on the physical characteristics of powder particles, scanning electron microscopy was used to analyze physical appearance of SFD formulations. Spherical shaped intact particles with an interconnected porous structure could be seen for both non-adjuvanted and adjuvanted WIV formulations. No major differences could be observed in the morphology of SFD particles by varying the dose of HA and by the addition of adjuvants. Representative pictures of non-adjuvanted, BLP and Advax adjuvanted WIV formulation at an HA dose of 0.2 μg per mg of powder are shown in Fig. 3A–C. Further, upon determination of geometric particle size, average particle size (X_{50}) was found to be between 8 and 12 μm both for non-adjuvanted and adjuvanted WIV formulations (Fig. 3D). However, for inhalation, an aerodynamic particle size of 1–5 μm is considered to be most suitable [29]. The average aerodynamic particle size of non-adjuvanted as well as adjuvanted WIV formulations was calculated according to the formula described by Bhide et al. [29]. The average aerodynamic particle size was found to be ≤3.7 μm and the majority of the particles (X_{90}) had size ≤5 μm (Fig. 3E). Particles with a size range between 1 and 3.7 μm are considered to be suitable for deposition in the entire respiratory tract of chickens [38–40].

Overall, our results indicated that SFD can produce BLP and Advax-adjuvanted WIV particles with physical and biological characteristics that make them suitable for pulmonary vaccination.

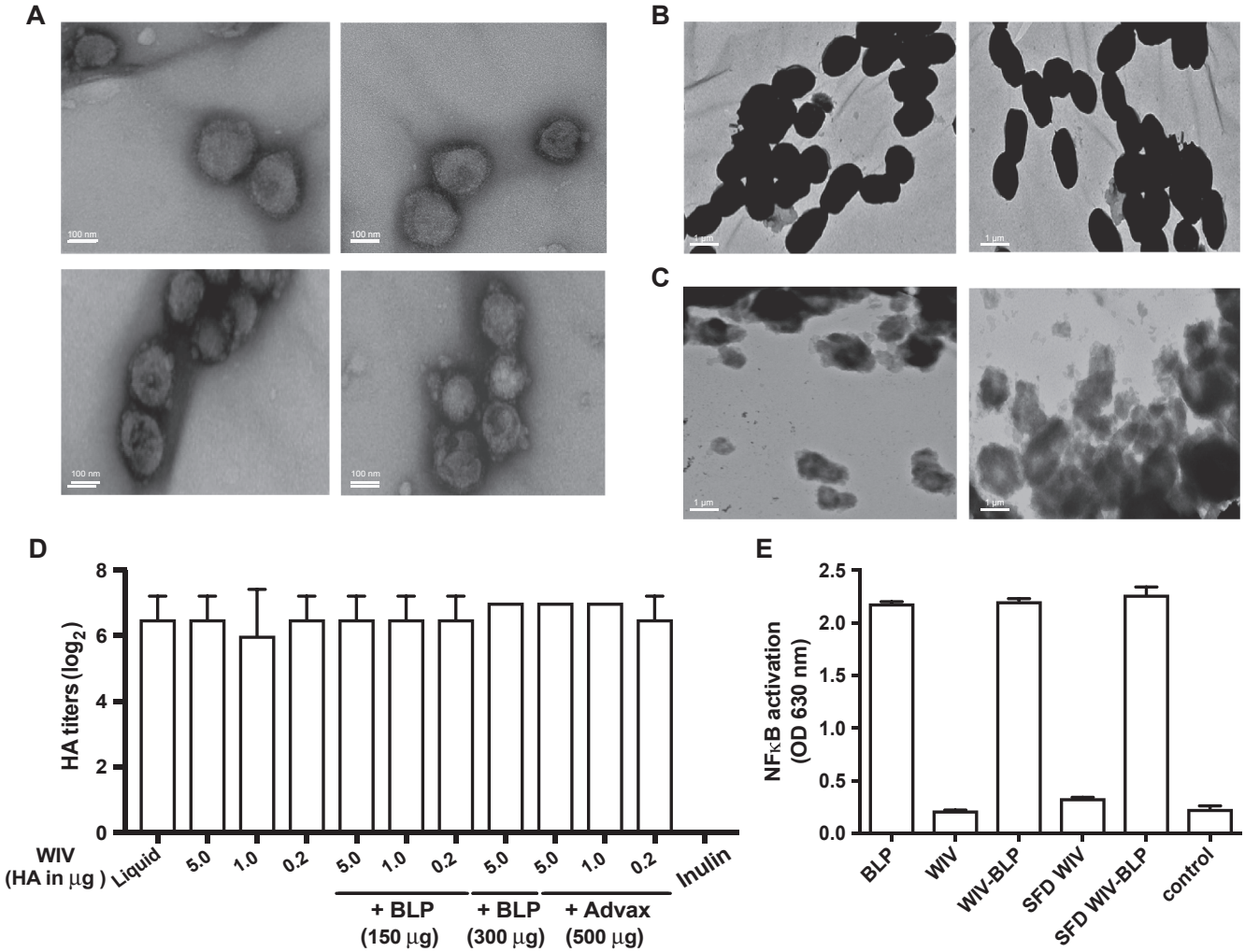


Fig. 2. Physical and biological characterization of influenza vaccine and adjuvants. (A–C) Transmission electron microscopy pictures of (A) WIV (left-upper panel), WIV-BLP showing WIV (right-upper panel), SFD WIV (left-lower panel), SFD WIV-BLP showing WIV (right-lower panel), (B) WIV-BLP showing BLP (left), SFD WIV-BLP showing BLP (right), (C) WIV-Advax showing Advax (left), SFD WIV-Advax showing Advax (right); (D) Hemagglutination titers of non-adjuvanted and adjuvanted WIV formulations; (E) NFκB activity of non-adjuvanted and BLP adjuvanted WIV formulations before and after SFD. Data of hemagglutination assay titers and NFκB are presented as average ± standard error of the mean (n = 6).

3.3. Immune responses induced by active administration

For active administration, the tip of the insufflator was placed directly at the syrinx of chickens and three puffs of 1 ml air were used to disperse the powders. We next evaluated the systemic and mucosal immune responses induced in chickens after active administration. Serum IgY and HI titers were measured both at day 14 and day 28 whereas serum MN, lung IgY and IgA titers were measured only at day 28. On day 14, non-adjuvanted as well as adjuvanted WIV formulations had induced considerable serum IgY titers. Advax-adjuvanted WIV formulations induced significantly higher serum IgY titers than the corresponding non-adjuvanted WIV formulations (Fig. 4A). However, no major differences were found among non-adjuvanted and BLP-adjuvanted WIV formulations, except between corresponding 0.2 µg HA formulations (Fig. 4A). Although, this difference was statistically significant, it was not major. However, on day 28, significantly higher titers were induced by BLP-adjuvanted WIV formulations than by the non-adjuvanted WIV formulation at an HA dose of 0.2 and 5 µg; the difference between non-adjuvanted and BLP-adjuvanted formulations was not significant at HA dose of 1 µg (Fig. 4B). For Advax-adjuvanted WIV formulation, significantly higher titers than corresponding non-adjuvanted WIV formulation were only induced by the 5 µg HA formulation (Fig. 4B).

Systemic immune responses were further assessed by determining serum HI titers. It was found that after the first vaccination i.e. at day 14, none of the vaccinated chickens had detectable HI titers (Fig. 4C). However, after the second vaccination, BLP-adjuvanted WIV formulations, at a dose of 5 and 0.2 µg HA, induced a trend towards higher HI titers (mean HI titers of 5.2 $\log_2$ and 2.7 $\log_2$ respectively) than corresponding non-adjuvanted WIV formulations (mean HI titers of 4.2 $\log_2$ and 0 respectively) (Fig. 4C). Though at an HA dose of 5 µg, the difference was not statistically significant, but at an HA dose of 0.2 µg, significantly higher titers could be seen for BLP-adjuvanted WIV formulation than corresponding non-adjuvanted formulation. Also, compared to non-adjuvanted WIV formulation (0), a small but non-significant increase in HI titers was seen for the Advax-adjuvanted WIV formulation at the lowest HA dose of 0.2 µg (0.58 $\log_2$) (Fig. 4C). At day 28, micro-neutralization titers were found to be in line with HI titers. In comparison to non-adjuvanted WIV formulation, Advax and BLP-adjuvanted WIV formulations at a dose of 0.2 µg HA induced an increase in the micro-neutralization titers of about five and eight fold, respectively (Fig. 4D). No major differences were seen at 5 µg and 0.2 µg HA dose.

Mucosal immune responses were assessed by determining IgA and IgY titers in the lung lavages obtained two weeks after the second immunization. Lung IgY titers were found to be consistent with serum IgY

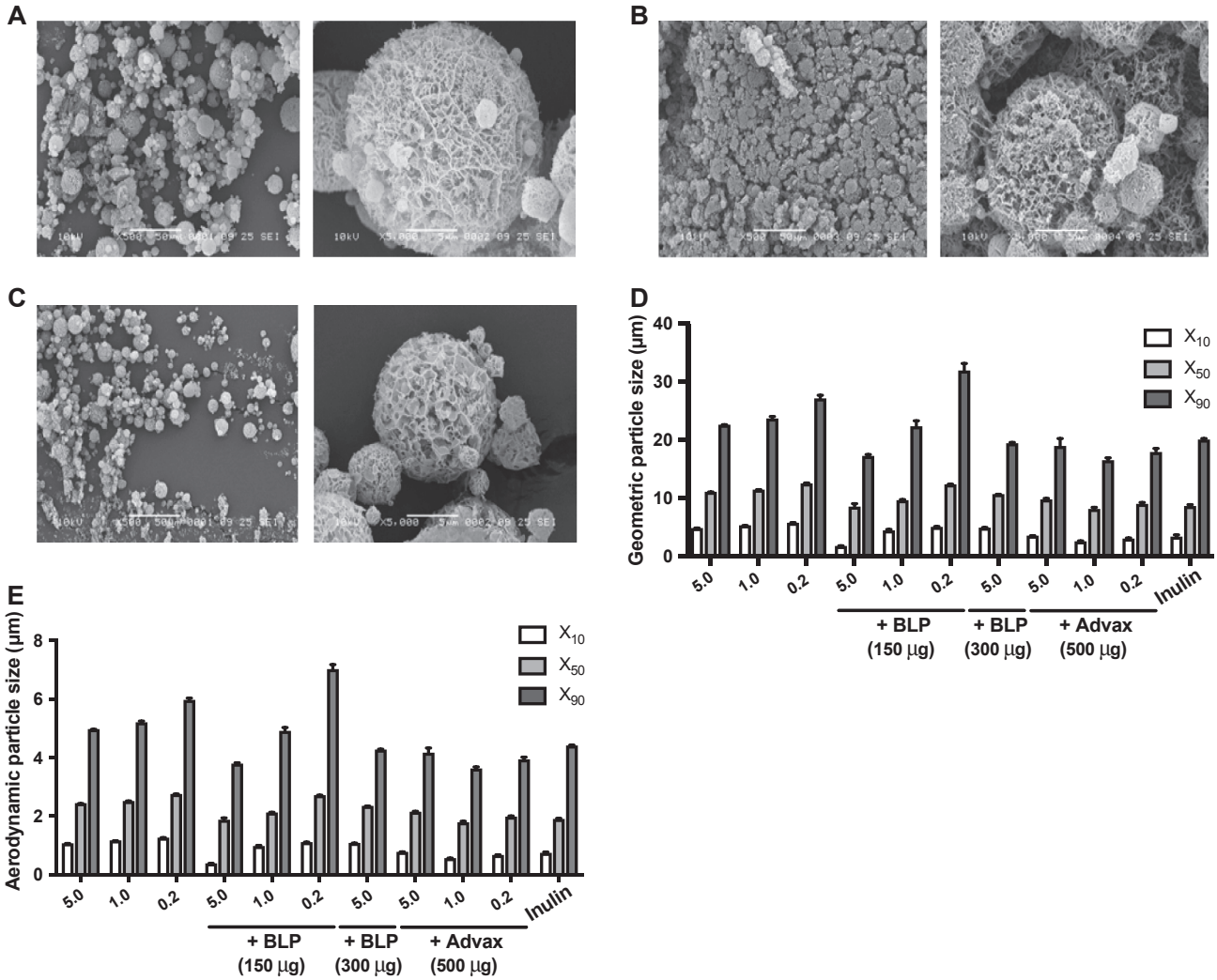


Fig. 3. Physical characterization of SFD vaccine powders. Scanning electron microscope pictures of non-adjuvanted and adjuvanted WIV formulations (0.2 µg HA dose) at a magnification of 500× (left) and 5000× (right). (A) WIV, (B) WIV-BLP, (C) WIV-Advax. (D) X_{10}, X_{50} and X_{90} undersize values of the geometric size distribution of non-adjuvanted and adjuvanted WIV formulations, (E) Aerodynamic particle size. Data of geometric and aerodynamic particle size are presented as average ± standard error of the mean (n = 4).

titers after the second immunization. BLP-adjuvanted WIV formulations induced significantly higher lung IgY titers than respective non-adjuvanted WIV formulations at a dose of 0.2 and 5 µg HA (Fig. 4E). In addition, Advax-adjuvanted WIV formulations induced higher lung IgY titers than non-adjuvanted WIV formulation at an HA dose of 0.2 µg, though the difference was not significant (Fig. 4E). Though non-adjuvanted WIV formulations were found to induce considerable lung IgA titers, the inclusion of BLP and Advax further boosted the immune responses (Fig. 4F). It was found that at a dose of 5 µg HA, BLP-adjuvanted WIV formulation boosted lung IgA titers (3.6 $\log_{10}$) by approximately four fold in comparison to corresponding non-adjuvanted WIV formulation (3.2 $\log_{10}$) (Fig. 4F). For Advax-adjuvanted WIV formulations, at a dose of 0.2 and 1 µg HA, lung IgA titers were augmented by three to five fold as compared to corresponding non-adjuvanted WIV formulations (Fig. 4F).

3.4. Immune responses induced by passive administration

Passive administration might be a suitable method for mass vaccination of chickens. For passive administration, chickens placed in a custom made box were allowed to inhale aerosolized vaccine powders during breathing using two applications 2 weeks apart. We next evaluated the systemic immune responses induced in chickens after passive administration of these aerosolized powders. Serum IgY titers were determined at day 14 and day 28 whereas micro-neutralization titers were determined at day 28. Adjuvanting with BLP or Advax resulted in significantly higher serum IgY titers at day 14; an increase of about three to five fold could be seen by the co-administration of BLP or Advax with WIV (Fig. 5A). However, after the second vaccination, only BLP and Advax/BLP adjuvanted WIV formulations had significantly higher serum IgY titers (4.8–5.1 $\log_{10}$) than non-adjuvanted WIV formulation (4.5 $\log_{10}$) (Fig. 5B). The augmentation in serum IgY titers was about six-fold for BLP-adjuvanted WIV and three fold for Advax/BLP-adjuvanted WIV (Fig. 5B).

Trends in HI titers were in agreement with serum IgY titers both after the first and second immunization. After the first immunization, all three formulations i.e. BLP, Advax and Advax/BLP adjuvanted WIV formulations showed significantly higher HI titers (mean HI titers between 3.0 and 3.5 $\log_2$) than non-adjuvanted WIV formulation (mean HI titer 1.9 $\log_2$) (Fig. 5C). Notably, after the second immunization, BLP adjuvanted and Advax/BLP-adjuvanted WIV formulations induced significantly higher HI titers (mean HI titers between 7.8 and 8.4 $\log_2$) than non-adjuvanted WIV or Advax adjuvanted WIV (mean HI titers of 5.9 $\log_2$). In addition, micro-neutralization titers determined after the

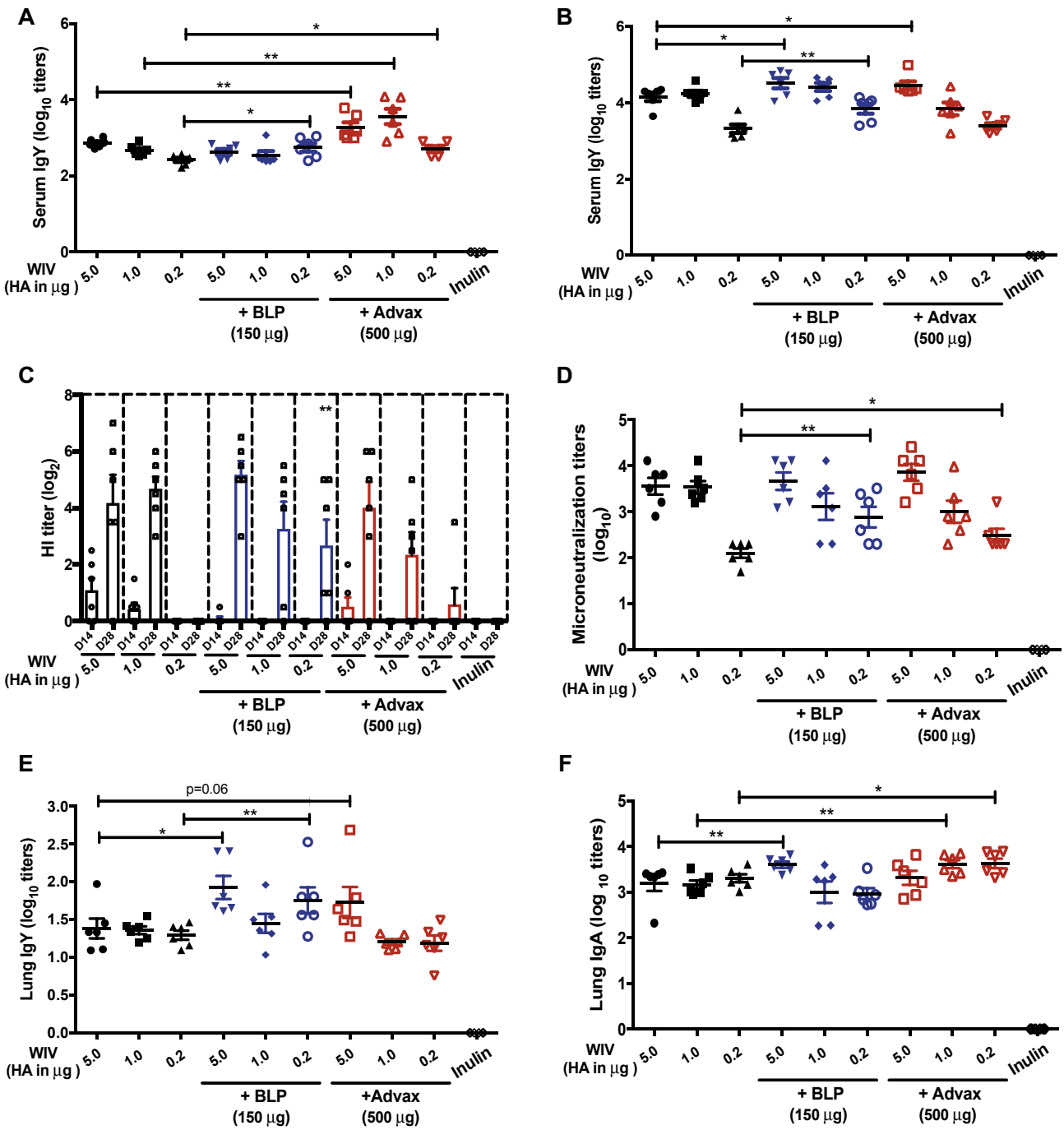


Fig. 4. Immune responses induced by active administration of chickens with various influenza vaccine formulations. Chickens were immunized twice with non-adjuvanted or adjuvanted WIV formulations delivered directly at the syrinx. Two weeks after the second immunization, chickens were sacrificed and immune responses were evaluated. Serum IgY titers on day 14 (A) and day 28 (B); (C) HI titers at day 14 and day 28; (D) Micro-neutralization titers at day 28; (E) Lung IgY titers; (F) Lung IgA titers. Data are presented as average ± standard error of the mean (n = 6). Levels of significance are presented as $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$; * in HI titers represent significance compared to corresponding non-adjuvanted WIV formulation.

second immunization were also in line with serum IgY and HI titers. BLP and Advax/BLP adjuvanted WIV formulations induced six-fold higher micro-neutralization titers (3.6 $\log_{10}$) than non-adjuvanted WIV formulation (2.9 $\log_{10}$) (Fig. 5D).

3.5. Shedding of challenge virus: passive administration

To assess whether passive administration of non-adjuvanted or adjuvanted influenza vaccine formulations, has the potential to reduce/diminish the shedding of challenge virus after lethal challenge, virus shedding was determined in choanal and cloacal swabs. In our previous study, we found out that non-vaccinated animals shed virus until they succumbed to infection (3 days post challenge) [11]. In this study, a similar dose of A/turkey/Turkey/1/2005 virus was used to challenge chickens by the combined intranasal/intratracheal route. No virus was found either in choanal or cloacal swabs of animals immunized with non-adjuvanted or BLP adjuvanted or Advax/BLP adjuvanted WIV formulations (Table 1). Only two animals immunized with Advax-adjuvanted WIV formulation showed virus in their choanal swabs (Table 1). One of the animals had cleared virus by day 3 post challenge

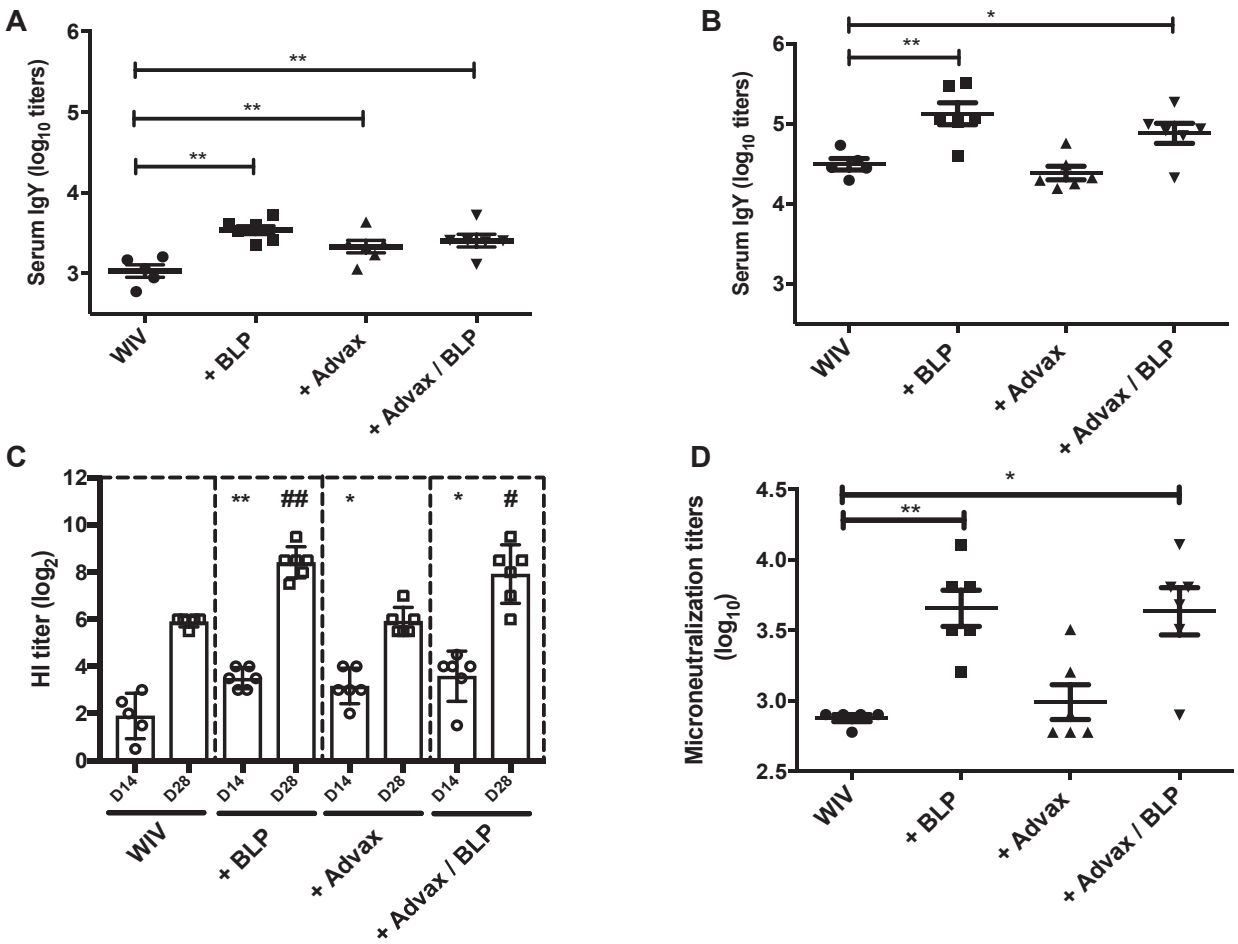


Fig. 5. Immune responses induced by passive administration of chickens with various influenza vaccine formulations. Chickens were immunized twice, by aerosolizing non-adjuvanted or adjuvanted WIV formulations in a custom made box. Two weeks after the second immunization, sera were collected and immune responses were evaluated. Serum IgY titers at day 14 (A) and day 28 (B), (C) HI titers at day 14 and day 28; (D) Micro-neutralization titers at day 28. Data are presented as average ± standard error of the mean (n = 6). Levels of significance are presented as $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$. * or $^{\#}$p...and ** or $^{\#\#}p < 0.01$. Significance is compared to non-adjuvanted WIV formulation.

and the other animal had virus cleared by day 7. None of these animals had virus shedding in their cloacal swabs except for one animal which had a very low level of virus shedding on 3 days post challenge.

4. Discussion

In this study, we demonstrated that BLP and Advax can be co-formulated with influenza vaccine into a dry formulation that is suitable for pulmonary immunization of chickens. Upon active administration of chickens, the adjuvants BLP and Advax were shown to augment systemic and mucosal immune responses. Remarkably, in an optimized set-up, passive administration of chickens either with non-adjuvanted or BLP or Advax adjuvanted WIV formulations induced robust immune responses that were sufficiently high to protect chickens against lethal viral challenge. Furthermore, BLP and Advax/BLP adjuvantation significantly raised the level of antigen-specific serum antibodies. In real life situations, where passive inhalation would be performed in large rooms, a portion of the aerosolized vaccine formulation would also end up in food and water. However, previous studies have shown that HA of the influenza vaccine undergoes conformational changes at acidic pH and is thus susceptible to proteolytic cleavage in the acidic environment [41,42]. Consequently, little-no neutralizing antibodies are induced by acidic pH treated influenza vaccine [41,42]. Therefore, it is speculated that the acidic environment in the stomach of chickens would not really add up to the lung induced mucosal immunity.

The crystalline nature of Advax and peptidoglycans on the surface of BLPs might have detrimental effects on influenza vaccine during the production of adjuvanted dry powder formulations. However, the inclusion of BLP or Advax together with WIV in a formulation led to the formation of dry powder particles of comparable physical and biological characteristics as those of non-adjuvanted WIV formulation. Moreover, the physical properties of adjuvants i.e. their size and shape were found to be unaltered after SFD. Also, the biological activity of BLP was found to be well preserved during SFD.

Previous studies have shown that administration of BLP or Advax adjuvanted influenza vaccine formulations via the respiratory tract results in substantial augmentation of systemic and mucosal immune responses in mice [16,43–46]. In this study we showed that after active administration, systemic immune responses (serum IgY titers) after one immunization were predominantly enhanced by Advax whereas after two immunizations they (serum IgY, HI titers, micro-neutralization titers) were mainly enhanced by BLP. After two immunizations, both non-adjuvanted and adjuvanted WIV formulations, at an HA dose of 5 μg, induced average HI titers above 3 $\log_2$ which was previously found to be required for protection against lethal dose of influenza virus [11]. With respect to mucosal immune responses, lung IgY titers were mainly enhanced by BLP whereas lung IgA titers were augmented by both BLP and Advax. Though both BLP and Advax were found to enhance either systemic or mucosal immune responses or both, the observed discrepancies might be due to the dose of BLP (150 μg) and Advax (500 μg) used in this study. Although doses of 150 μg of BLP and 500 μg of Advax were found to be sufficiently high to boost both

Table 1
Virus shedding after challenge.

Virus shedding (eq. TCID50/ml)												
Group No.	Vaccine	Animal No.	Choana swabs					Cloaca swabs				
			Day 1	Day 3	Day 5	Day 7	Day 10	Day 1	Day 3	Day 5	Day 7	Day 10
1	WIV	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	+ BLP	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	+ Advax	1	2.0	1.5	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
		3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		6	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	+ Advax/BLP	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

systemic and mucosal immune responses in mice, however, these doses might be too low for chickens with a 10x higher body weight. This might have resulted in the adjuvant dose to be a limiting factor in the active administration study.

The active administration study revealed a slight but significantly higher effect of both BLP and Advax adjuvants, in comparison to non-adjuvanted WIV formulations. Hence, for the passive administration study, the relative dose of BLP was increased to double, i.e 300 μg/mg of SFD powder while keeping the dose of Advax similar. Since Advax particles consisted of 50 wt% of the SFD powder formulation, enhancing the dose of Advax further would most likely have compromised the integrity of the powder particles. Moreover, a pattern of Advax being effective after one and BLP after two immunizations could also be determined in the active administration study. Therefore, one of the groups of passively administered animals inhaled Advax-adjuvanted WIV for the first immunization and BLP-adjuvanted WIV for the second immunization.

In our previous study, passive inhalation led to in-efficient delivery of influenza vaccine powders to the lungs of chickens and thus provided only partial protection [11]. Hence, for this study, we aimed for complete protection against lethal influenza viral challenge. This was achieved by enhancing vaccine powder delivery to the lungs: by increasing the vaccine concentration (by reducing the size of inhalation box), by increasing the exposure time, and by the use of adjuvants. Compared to our previous study, optimization of the vaccine concentration and exposure time resulted in an increment of ~7 fold in the theoretical vaccine dose (this study 2 × 50 μg HA/animal; previous study 3 × 5 μg HA/animal) [11]. Using this optimized set-up, passive inhalation of both non-adjuvanted and adjuvanted WIV formulations not only protected chickens against clinical signs after HPAIV challenge, but also almost completely prevented challenge virus shedding. These results are a significant improvement over our previous study in which chickens showed partial protection and challenge virus shedding even after three immunizations. Though the immunological mechanism that governed complete protection in chickens by passive administration of non-adjuvanted and adjuvanted WIV formulations still needs to be investigated, the shedding data indicate that mucosal immune responses were high enough to prevent production of substantial amounts of virus. In addition to mucosal immune responses, an important class of immune responses are those elicited systemically. At both time points, BLP and Advax/BLP adjuvanted WIV formulations, were found to induce higher systemic immune responses than non-adjuvanted or Advax-adjuvanted WIV preparation. In addition, we found out that, either of the two adjuvants i.e. Advax or BLP would be appropriate for first immunization, however, for second immunization, BLP was vital. Although these adjuvanted WIV formulations elicited significantly higher immune responses than non-adjuvanted WIV formulation, the fact that even the non-adjuvanted WIV formulations provided complete protection, suggests that the use of an adjuvant was not critical for protection via passive inhalation, but it might add to dose-sparing of influenza vaccines for future passive inhalation studies.

5. Conclusion

In conclusion, our results show that, vaccination by passive inhalation of dry influenza vaccine powders is suitable to induce protective immunity in chickens against highly pathogenic avian influenza virus. It not only has the potential to completely protect chickens from morbidity and mortality, and to prevent the virus from spreading, but also seems to be a feasible option for mass vaccination of chickens. The challenge now remains to translate passive inhalation vaccination from the box to real-world field settings.

Conflict of interest

NP is affiliated with Vaxine Pty Ltd, which has commercial interests in Advax adjuvant. The other authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2018.10.008.

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