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Advax augments B and T cell responses upon influenza vaccination via the respiratory tract and enables complete protection of mice against lethal influenza virus challenge

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ABSTRACT

Administration of influenza vaccines via the respiratory tract has potential benefits over conventional parenteral administration, inducing immunity directly at the site of influenza exposure as well as being needle free. In this study, we investigated the suitability of Advax™, a stable particulate polymorph of inulin, also referred to as delta inulin, as a mucosal adjuvant for whole inactivated influenza vaccine (WIV) administered either as a liquid or dry powder formulation. Spray freeze-drying produced Advax-adjuvanted WIV powder particles in a size range (1–5 μm) suitable for inhalation. The physical and biological characteristics of both WIV and Advax remained unaltered both by admixing WIV with Advax and by spray freeze drying. Upon intranasal or pulmonary immunization, both liquid and dry powder formulations containing Advax induced significantly higher systemic, mucosal and cellular immune responses than non-adjuvanted WIV formulations. Furthermore, pulmonary immunization with Advax-adjuvanted WIV led to robust memory B cell responses along with an increase of lung localization factors i.e. CXCR3, CD69, and CD103. A less pronounced but still positive effect of Advax was seen on memory T cell responses. In contrast to animals immunized with WIV alone, all animals pulmonary immunized with a single dose of Advax-adjuvanted WIV were fully protected with no visible clinical symptoms against a lethal dose of influenza virus. These data confirm that Advax is a potent mucosal adjuvant that boosts vaccine-induced humoral and cellular immune responses both in the lung and systemically with major positive effects on B-cell memory and complete protection against live virus. Hence, respiratory tract immunization, particularly via the lungs, with Advax-adjuvanted WIV formulation as a liquid or dry powder is a promising alternative to parenteral influenza vaccination.

1. Introduction

Influenza is a highly contagious disease affecting millions of people worldwide on annual basis [1,2]. Seasonal epidemics and sporadic pandemics of influenza are caused by the transmission of influenza virus via aerosols [3,4]. Since the respiratory tract is the portal of influenza virus entry, in-theory the best means of protection would be to use a vaccine to generate a local memory immune response able to neutralize the virus at the site of infection. However, the majority of the currently available influenza vaccines are administered via intramuscular or subcutaneous injection [5]. Injected vaccines generate strong systemic immunity but minimal mucosal immunity [6,7]. Moreover, injected vaccines can cause local reactions including pain, swelling and redness at the injection site, needle phobia, and transmission of infectious diseases due to needle stick injuries. An influenza vaccine formulation that could be administered via the respiratory tract would overcome these drawbacks of current injected formulations, is therefore needed.

Presently, live attenuated influenza vaccine (LAIV) is the only formulation approved for administration via the intranasal (i.n.) route, but

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due to the live nature of the virus, it is not approved for use in high risk groups. This problem could be avoided by the use of inactivated influenza vaccine formulations suitable for delivery via the respiratory tract. Already in 1969, Waldman et al. reported that pulmonary vaccine administration was as effective as the conventional i.m. administration for preventing influenza associated illness [8]. Pulmonary vaccines can be delivered as liquids or as dry powders [6,9,10]. In pre-clinical studies, pulmonary delivery of both liquid and dry powder influenza vaccine formulations has shown to induce mucosal as well as systemic immune responses [6,7,11,12]. However, the magnitude of immune responses evoked by these non-adjuvanted vaccines was low with low mucosal IgA titers and low numbers of memory cells; this might result in short lived protection against infection [7,12,13]. These issues might be solved by the use of a suitable adjuvant to boost the immune memory responses able to be elicited by respiratory tract administration of influenza vaccine.

Identification of an adjuvant suitable for administration via the respiratory tract is not as easy as for parenteral administration, with no adjuvant currently approved for intranasal or pulmonary use in clinic. The problems of developing a mucosal adjuvant for influenza vaccines are highlighted by the issue of rare cases of facial palsy in clinical trial subjects who were administered an intranasal inactivated influenza vaccine containing a latent toxin adjuvant, resulting in the vaccine being abandoned [14]. Of current approved alternatives, alum, is not effective in influenza vaccines [15], and in addition causes inflammasome activation, local cell necrosis with DNA release and forms insoluble aggregates rendering it unsuitable for pulmonary use [16,17]. The only other currently approved influenza vaccine adjuvants are based on squalene oil emulsions, which are restricted to subcutaneous or intramuscular use. Moreover, the administration of oil-based emulsions to the respiratory tract are most likely detrimental for the normal balance of the alveolar lining fluid; thus interfering with lung function. Newer experimental adjuvants such as toll-like receptor agonists work via activation of NFκB. However, NFκB is a key inducer of inflammatory responses, and therefore pulmonary administration of these agonists may induce unacceptable lung inflammation [18]. Hence, the number of candidate adjuvants likely to be suitable for respiratory tract use is very limited.

An adjuvant that has shown a good safety and tolerability record upon parenteral administration with inactivated and recombinant influenza vaccines in animal models and clinical trials is Advax [19–21]. Advax adjuvant is composed of the insoluble particulate polymorph of inulin, also referred to as delta inulin. The inulin that makes up Advax adjuvant is rapidly excreted from the body through renal excretion with complete clearance within approximately 3 weeks after parenteral administration [22]. Advax adjuvant comprises discoidal shape particles of 1–2 μm in diameter, formed by assembly of a series of lamellar crystalline sheets [23]. Adjuvantation of parenterally administered vaccines with Advax has shown to improve the immunogenicity and protective capacity of several vaccine candidates against hepatitis B, anthrax, severe acute respiratory syndrome (SARS) coronavirus, listeria and influenza [21,24–27]. The exact mechanism by which Advax boosts immune responses upon parenteral administration is still under investigation [23].

Till date, however, the use of Advax as an adjuvant for vaccines delivered via the respiratory tract, has been less well investigated. A single study by Murugappan et al. [28] showed that pulmonary co-administration of a liquid influenza vaccine formulation with Advax induced a more balanced Th1/Th2 profile with a modest increase of only nasal IgA titers [28]. No enhancement in other humoral and cellular immune responses was found at the used Advax dose of 200 μg [28]. Also, the potential of Advax to boost immune responses by the alternative more commonly used mucosal route such as intranasal or when incorporated in alternative physical form such as powders, was not investigated in that study.

In the present study, we investigated whether Advax adjuvant could augment immune responses to whole inactivated influenza vaccine (WIV) administered to the respiratory tract via intranasal (i.n.) or pulmonary routes as either a dry powder or liquid formulation. Further, we investigated the mechanisms whereby Advax enhanced the immune responses to influenza vaccine administered via the respiratory tract. Lastly, we explored whether a single pulmonary immunization with a low dose of WIV adjuvanted with Advax would provide protection against lethal viral challenge.

2. Materials and methods

2.1. Virus preparation

For the immunization study, Influenza A strain NIBRG 23, a reassortant virus from A/turkey/Turkey/1/2005 (H5N1) and A/PR/8/34 (H1N1) was grown in embryonated chicken eggs by allantoic inoculation of the seed virus and purified as described previously [12]. For the challenge experiments, a mouse-adapted Influenza A/PR/8/34 (H1N1) virus propagated in allantoic fluid of 10-day old embryonated hens eggs was used.

2.2. Vaccine preparation

Live virus was inactivated by an overnight treatment of 0.1% β-propiolactone (Acros Organics, Geel, Belgium) in citrate buffer (125 mM sodium citrate, 150 mM sodium chloride, pH 8.2) at 4 °C. Then, inactivated virus was dialyzed overnight against Hepes buffer (145 mM NaCl, 5 mM Hepes, pH 7.4, sterilized by autoclaving) to completely remove β-propiolactone. Protein content of the WIV preparation was determined by micro-Lowry assay and hemagglutinin (HA) was assumed to be 1/3rd of the total protein content of the inactivated virus [12].

2.3. Spray freeze drying

Spray-freeze drying (SFD) was performed by mixing WIV or WIV-Advax (Advax™ adjuvant, Vaxine Pty Ltd., Adelaide, Australia) (HA:Advax 1:100 (w/w)) with a water soluble form of inulin which was used as a lyoprotectant and bulking agent (4 kDa, Sensus, Roosendaal, The Netherlands). For WIV and WIV-Advax formulations, the HA:inulin weight ratio was 1:200 and 1:100, respectively, thus obtaining dispersions with composition HA:inulin 1:200 (w/w) and HA:Advax:inulin 1:100:100 (w/w/w). The HA:inulin weight ratios of 1:200 and 1:100 were based upon a dose of 5 μg HA with or without 500 μg of Advax in 1 mg of SFD powder. A two-fluid nozzle (diameter 0.5 mm) of a Buchi 190 Mini Spray Dryer (Buchi, Flawil, Switzerland) was used to pump the dispersions at a flow rate of 5 ml/min which was then sprayed in a vessel of liquid nitrogen using an atomizing airflow of 600 L_n/h. Drying was performed in Christ Epsilon 2–4 freeze dryer with a shelf temperature of −35 °C and at a pressure of 0.220 mbar; the shelf temperature was gradually increased to 4 °C over a time period of 32 h. For secondary drying, the temperature was further gradually increased to 20 °C and pressure was lowered to 0.05 mbar during the consecutive 12 h. The vaccine powder was collected in a climate box with relative humidity of 0% and was stored under airtight conditions.

2.4. Characterization of influenza vaccine formulations and Advax adjuvant

The size of WIV before and after addition of Advax was determined by Dynamic Light Scattering (DLS) (Malvern Zetasizer ZS90, Malvern, United Kingdom). Likewise, the size of Advax was also measured before and after addition of WIV. For sample preparation, WIV and Advax were either used alone or mixed in an HA:Advax ratio of 1:100 (w/w). Particle size analysis was done using the Zetasizer software.

Transmission electron microscopy (TEM) images were captured

using a Philips CM120 transmission electron microscope. SFD powder containing Advax was reconstituted in sterile filtered water. Liquid and reconstituted SFD Advax containing formulations were placed on a plain carbon grid and after rinsing with water samples were stained twice with 5 μl of 2 wt-% uranyl acetate. Images were taken with a Gatan type UltraScan 4000SP CCD Camera at a magnification of 17,000×.

The morphology of the SFD powders was analyzed by scanning electron microscopy (SEM) using a Jeol JSM 6301-F microscope. A double sided sticky carbon tape on a metal disc was used and powders were placed on it. Then, the particles were coated with 30 nm of gold using a Balzer's 120B sputtering device (Balzer, Union, Austria). Images were captured at a magnification of 500× and 5000×.

Primary particle size distribution of SFD powders, was determined by laser diffraction. Powders were dispersed at a pressure of 0.1 bar and RODOS (Sympatec, Clausthal-Zellerfeld Germany) was used as the disperser. A 100 nm (R3) lens was used. Fraunhofer theory was used to calculate the geometric particle size distribution.

The receptor binding activity of WIV after SFD was assessed by the hemagglutination assay as described previously [12]. Briefly, WIV was reconstituted in PBS and 50 μl was added to 96 V bottom plates containing 50 μl of PBS. Two-fold serial dilutions were prepared after which 50 μl of 1% guinea pig red blood cells suspension was added to each well. Plates were incubated for 2 h at room temperature and hemagglutination titers were read after 2 h. Hemagglutination titers are expressed as $\log_2$ of the highest dilution where RBC agglutination could be seen.

2.5. Immunization and samples collection

Animal experiments were approved by The Institutional Animal Care and Use Committee of the Université Catholique de Louvain, Brussels, Belgium (Permit number: 2012/UCL/MD/006), University of Groningen, Groningen, The Netherlands (Permit number: AVD1050020165 99) and Flinders University, Adelaide, Australia (Permit number: 838/12). In-vivo experiments were carried out on 6–8 weeks old female BALB/c mice (Elevage Janvier, Le Genets-St-Isle, France). Mice were randomly divided into eight groups consisting of 6 mice/group. In order to investigate whether co-administration of Advax with influenza vaccine would boost immune responses, a weakly immunogenic strain of influenza virus (NIBRG-23) was chosen. Mice were immunized with WIV, however, as is routine in the influenza vaccine field given that HA is the dominant protective antigen, the dose used for immunization is represented by its HA content (~1/3rd of the total protein content of the inactivated virus). Mice were vaccinated twice at 3 weeks interval with vaccine formulations containing 5 μg HA of NIBRG-23. For intramuscular (i.m.) vaccination, 50 μl of vaccine formulation containing 5 μg HA without adjuvant was divided over both hind legs. For intranasal (i.n.) immunization, 15 μl of vaccine formulation containing 5 μg HA with or without 500 μg Advax (HA:Advax 1:100) was slowly administered using a pipette in both nares (7.5 μl in each nare).

For pulmonary administration of liquid vaccines (Pul Liq), 25 μl of vaccine containing 5 μg HA with or without 500 μg Advax (HA:Advax 1:100) was administered in the trachea of mice via microsyringe; followed by insufflation of 200 μl of air to assure deep lung deposition [11]. For vaccine powder delivery (Pul Pow), 1 mg of powder containing 5 μg HA with or without 500 μg Advax (HA:Advax 1:100), was administered to lungs of each animal by applying three puffs of 200 μl air via a dry powder insufflator, as described previously [7]. Negative control animals were left untreated.

On the day of second immunization, blood was collected by retro-orbital puncture. One week after the second vaccine dose, mice were sacrificed and the obtained sera was stored at −20 °C until further analysis. Nose washes and bronchioalveolar lavages (BAL) were collected by flushing 1 ml PBS containing complete protease inhibitor cocktail tablets (Roche, Almere, Netherlands), through nasopharynx and lungs, respectively. Lavages were stored at −20 °C until further use. Spleens and lungs were collected in complete IMDM media containing 100 U/ml penicillin, 100 mg/ml streptomycin, 0.05 M 2-mercaptoethanol (Invitrogen, Breda, The Netherlands) and 5% fetal calf serum (Lonza, Basel, Switzerland). Spleens were processed to single cell suspensions and passed through cell strainers; followed by RBC lysis using hypotonic medium (0.83% NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). Bone marrows were treated in a similar way as spleens to process single cell suspension. Lungs were processed to single cell suspensions as described previously [29]. Splenocytes and bone marrow cells were used for individual mice and lung lymphocytes were pooled per experimental group. Lung lymphocytes were pooled for each experimental group due to lack of enough cells in individual animals for a number of the readouts investigated in the study.

2.6. ELISA

Sera, nose washes and BAL were used for the determination of influenza-specific antibody responses. IgG, IgG1, IgG2a and IgA antibodies were detected by overnight coating of ELISA plates (Grenier Bio-One, Alphen, The Netherlands) with 500 ng/well of WIV at 37 °C. *ELISA* was performed as described previously [7]. Absorbance was measured at 492 nm using a Synergy HT Reader (BioTek, Winooski, USA). For the determination of average IgG, IgG1 and IgG2a titers, $\log_{10}$ of the reciprocal of the sample dilution corresponding to an absorbance at 492 nm of 0.2 was used. Nose and lung IgA levels are presented as average of the absorbance at 492 nm for undiluted nose and lung washes.

2.7. Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assay was performed as described previously [30]. Briefly, sera were pooled from each experimental group and 4 hemagglutination units (4 HAU) of inactivated virus were added to two-fold diluted serum samples. Sera were pooled as the sample volume in individual animals was not enough for the assay. HI titers were recorded as the highest serum dilution capable of preventing hemagglutination of RBCs.

2.8. Microneutralization assay

Microneutralization assay (MN) was performed as described previously [29]. Briefly, 50$TCID_{50}$/well of NIBRG-23 virus were added to two-fold serial dilution of sera samples and incubated at 37 °C for 2 h. After 2 h, the virus-serum mixture was transferred to MDCK cells and incubated at 37 °C for 1 h. Thereafter, virus-serum mixture was discarded and culture supernatants were supplemented with medium containing 5 μg/ml of TPCK trypsin and were incubated for an additional 72 h. Subsequently, MN titers were calculated by recording hemagglutinating activity as the highest serum dilution capable of preventing hemagglutination.

2.9. ELISpot

2.9.1. B-cell ELISpot

B cell ELIspot was performed as previously described with some modifications [31]. MultiScreenHTS-HA filter plates (Millipore, Billerica, Massachusetts) were coated with 10 μg/ml of NIBRG-23 overnight at 4 °C. Cells were washed three times with PBS containing 0.01% Tween 20 and twice with PBS. Plates were then blocked with 1% BSA for 2 h at 37 °C. 1×10^6 lymphocytes from lungs or splenocytes in 100 μl IMDM complete medium with 10% FBS were added to wells and incubated for 4 h at 37 °C with 5% CO_2. Following incubation, cells were washed with PBS containing 0.01% Tween 20. Subsequently, alkaline phosphatase labeled anti-mouse IgA antibody (Sigma-Aldrich

Chemie B.V., Zwijndrecht, The Netherlands) or horse radish peroxidase labeled anti-mouse IgG antibody (SouthernBiotech, Birmingham, USA) was added to the wells and incubated for 37 °C for 1 h. Wells were washed thoroughly with PBS containing 0.01% Tween 20. The numbers of IgA and IgG antibody secreting cells (ASC) were identified using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) and 3-amino-9- ethylcarbazole (AEC) substrate (Roche, Almere, The Netherlands), respectively. Spots were counted by using ELISpot reader (A.EL.VIS ELISpot reader, Hannover, Germany).

2.9.2. T-cell ELISpot

The number of IFN-γ and IL-4 producing cells in spleens were determined using Ready SET-Go ELIspot kits (eBioscience, Vienna, Austria). Briefly, 5×10^5 splenocytes or lymphocytes from lung were added to MultiScreenHTS-HA filter plates pre-coated with anti-IFN-γ or anti-IL-4 antibodies. Then, plates were incubated overnight at 37 °C with 5% CO_2 in IMDM complete medium with or without 10 μg/ml WIV (NIBRG-23). For IFN-γ and IL-4 ELISpot, plates were stained as per manufacturer's protocols. Spots were counted by using an A. EL.VIS ELISpot reader.

2.10. Cytokine ELISA

To determine IFN-γ and IL-4 levels in the spleens of immunized mice, Ready SET-Go ELISA kits (R&D systems Biotechne, Minnesota, USA) were used according to manufacturer's protocols. Briefly, 5×10^5 splenocytes or lymphocytes from lung were added to round bottom plates and incubated overnight at 37 °C with 5% CO_2 in IMDM complete medium with or without 10 μg/ml WIV (NIBRG-23). Cell supernatant was collected and stored at −20 °C until used.

2.11. Flow cytometry

1×10^6 cells splenocytes or bone marrow cells from each mouse or lung lymphocytes pooled per experimental group were added to flow cytometry tubes (Corning Incorporated, New York, USA). Separate tubes were used for B and T cell analysis. Cells were washed three times with fluorescence-activated cell sorting (FACS) buffer containing 0.1% bovine serum albumin in PBS, pH 7.4, and centrifuged at 1200 rpm for 5 min at 4 °C. Pelleted cells were resuspended in 100 μl FACS buffer containing 1 μg Fc Block (BioLegend, San Diego, USA) for 30 min.

For B cell staining, anti-CD19 PerCP, anti-IgM PE/Dazzle™ 594, anti-IgD PE/Dazzle™ 594, anti-IgG PE/Cy7, anti-CD69 PE (all antibodies from BioLegend), anti-IgA FITC (eBioscience) were added and incubated for 30 min in dark at 4 °C. Cells were washed once and 100 μl FACS buffer containing 10 μl BD Horizon™ Brilliant Stain Buffer (BD Bioscience, Vianen, Netherlands) was added to cells. Immediately thereafter, a mixture of anti-CD38 BV510 (BD Bioscience), anti-CXCR3 BV421 (BioLegend) in 100 μl FACS buffer was added to cells and incubated for 30 min in dark at 4 °C. Cells were washed three times with 1 ml FACS buffer and analyzed using LSRFortessa™ (BD Bioscience).

For T cell staining, anti-CD3 PerCP, anti-CD4 Alexa Fluro 488, anti-CD8a PE/Dazzle™ 594, anti-CD44 PE/Cy7, (all antibodies from BioLegend) were added and incubated for 30 min in dark at 4 °C. Cells were washed once and 100 μl FACS buffer containing 10 μl BD Horizon™ Brilliant Stain Buffer was added to cells. Immediately thereafter, a mixture of anti-CXCR3 BV421 (BioLegend), anti-CD103 BV786 (BD Bioscience) in 100 μl FACS buffer was added to cells and incubated for 30 min in dark at 4 °C. Cells were washed three times with 1 ml FACS buffer and analyzed using LSRFortessa™.

Obtained data was analyzed using FlowJo flow cytometry analysis software version 10.2. Gating strategy for B and T cells is shown in Fig. S1. Representative FACS plots of B and T cells are shown in Figs. S2 and S4.

2.12. Challenge study

For the challenge study, female BALB/c mice 6–8 weeks of age (n = 3) were immunized once via pulmonary route with 0.1 μg of A/PR/8/34 WIV with or without 1 mg of Advax adjuvant. The rationale for the 0.1 μg WIV immunization dose for challenge study was that this was found to be the optimal vaccine dose to see differences between groups in clinical outcomes, whereas 5 μg HA was found to be the optimal dose to see differences between groups in immunogenicity measures such as HI and MN. The vaccine was administered under anaesthesia using an intratracheal intubation and a microsprayer. Two weeks after the immunization, animals were challenged with a live virus (A/PR/8/34). The 50% mouse lethal dose (LD_{50}) of the virus was estimated in adult BALB/c mice by the Reed-Muench method [32]. One LD_{50} corresponded to 1250 $TCID_{50}$ on MDCK cells (data not shown) and the virus challenge dose used was 10,000 $TCID_{50}$ (8xLD_{50}) administered intranasally in a volume of 30 μl which gave 100% lethality in control non-immunized mice. Daily weights and a sickness scoring system based on coat condition, posture and activity was used to assess the extent of clinical disease with mice evaluated daily. Ruffled fur (absent = 0; slightly present = 1; present = 2), hunched back (absent = 0; slightly present = 1; present = 2) and activity (normal = 0; reduced = 1; severely reduced = 2). The final score was the addition of each individual symptom score (e.g. an animal showing slightly ruffled fur (1), slightly hunched back (1) and reduced activity (1) was scored as 3. Mice were euthanized if they had developed a clinical score of 6.

3. Statistical analysis

Mann Whitney *U* Test was performed for statistical analysis of data. A two tailed test was performed to compare non-adjuvanted vs adjuvanted or i.m. vs adjuvanted WIV formulations. p values < 0.05 were considered to be significant. *, ** and *** represent p values less than or equal to 0.05, 0.01 and 0.001, respectively. A Cox-Mantel log rang test was used to compare the difference in survival between Advax-adjuvanted WIV group and WIV alone i.e. without adjuvant.

4. Results and discussion

4.1. Physical and biological characterization of Advax-adjuvanted formulations

For the use of Advax as a mucosal adjuvant for WIV, it is essential that it has no detrimental effects on the physical and biological properties of inactivated virus particles; and that SFD has no impact on the physical characteristics of Advax. DLS measurements revealed that mixing with Advax had a negligible effect on the size of WIV with liquid WIV without adjuvant having a size of ~185 nm and Advax-adjuvanted liquid WIV formulation having a size of ~186 nm (Fig. 1A). Likewise, the size of Advax particles remained unaltered for Advax only (1522 nm) and Advax-adjuvanted WIV formulation (1535 nm).

Furthermore, we evaluated whether SFD had an impact on the physical appearance of Advax particles. For this, Advax was SFD without WIV, but in the presence of water soluble inulin as the stabilizer. TEM analysis revealed that Advax particles had comparable morphology before and after SFD (Fig. 1B).

In order to investigate whether Advax had an effect on the physical characteristics of SFD powder formulation, the physical appearance of powder particles was analyzed by SEM. SEM images revealed intact spherically shaped particles with an interconnected porous structure for both SFD WIV without adjuvant (Fig. 1C) and SFD Advax-adjuvanted WIV formulations (Fig. 1D). Further, upon dispersion from RODOS, the average geometric particle size (X_{50}) of SFD Advax-adjuvanted WIV formulation was found to be comparable to SFD non-adjuvanted WIV formulation, i.e. 8.64 and 9.12 μm, respectively (Fig. 1E). An important criterion for particles to be suitable for inhalation is their aerodynamic

A

Sample	WIV (nm ± SEM)	Advax (nm ± SEM)
WIV alone	185.6 ± 0.2	
Advax alone		1522 ± 0.4
WIV-Advax	186.4 ± 0.3	1535 ± 0.2

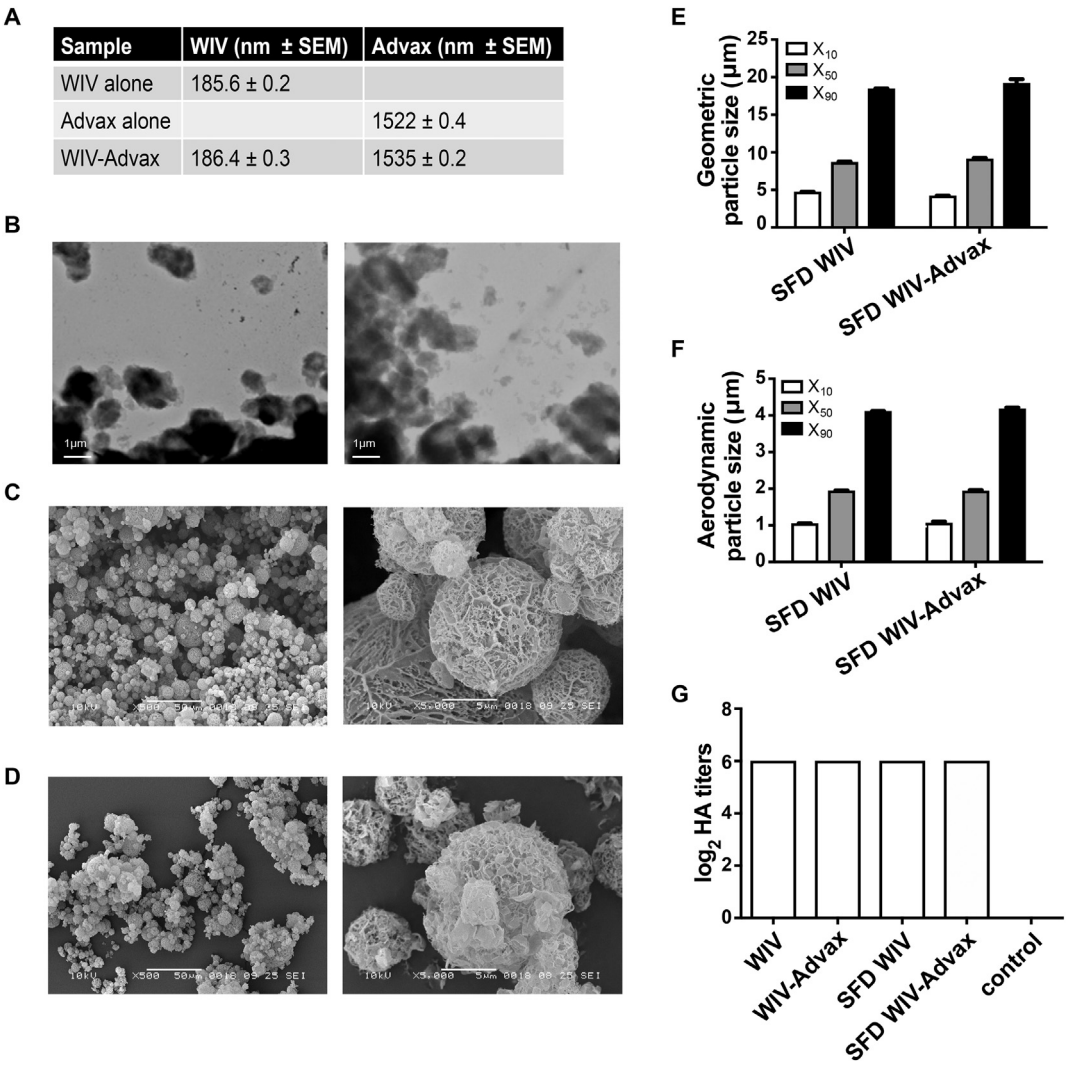

Fig. 1. Characterization of Advax-adjuvanted liquid and powder formulations. (A) DLS measurements representing z-average particle size of WIV, Advax and Advax-adjuvanted WIV formulations (n = 6). (B) TEM images of Advax before and after SFD. SEM images of (C) SFD WIV alone or (D) SFD Advax-adjuvanted WIV. Left and right side SEM pictures are captured at a magnification of 500× and 5000×, respectively. (E) Geometric particle size of SFD WIV or Advax-adjuvanted WIV after dispersion from RODOS (n = 6). (F) Aerodynamic particle size of SFD WIV or Advax-adjuvanted WIV (G) Hemagglutination titers of WIV and Advax-adjuvanted WIV before and after SFD (n = 3); no differences were found among the triplicates within a group. Data are presented as average ± standard error of the mean for Fig. 1A, E and F.

particle size, which ideally should be 1–5 μm [33,34]. Aerodynamic particle size was calculated according to the formula described by Bhide et al. [35]. Aerodynamic particle size of both WIV and Advax-adjuvanted WIV after SFD were found to be in the required size range, i.e. 1–5 μm, thus indicating the suitability of both these formulations for pulmonary immunization (Fig. 1F). Thus, upon SFD of WIV formulated either with or without Advax, powder particles with a similar size and morphology were formed making a fair comparison between the non-adjuvanted and Advax-adjuvanted SFD powders possible.

It is well known that the existence of HA in its native conformation is crucial for its receptor binding activity and the induction of immune responses [9]. Thus, in order to evaluate whether or not the receptor binding activity of HA was preserved after the addition of Advax and after SFD, hemagglutination assay was performed. All formulations showed similar hemagglutination titers indicating that admixing WIV with Advax and SFD did not have destabilizing effects on HA (Fig. 1G). Overall, the data showed that SFD can be used to produce an Advax-adjuvanted WIV dry powder formulation suitable for pulmonary administration.

4.2. Systemic immune responses

Previous pre-clinical and clinical studies have shown that co-administration of influenza vaccine with Advax via the conventional parenteral route substantially enhanced systemic immunity [19,27,36]. Thus, in order to investigate the potential of Advax as a mucosal adjuvant, systemic immune responses were determined either three weeks after the first (day 21) or one week after the second immunization (day 28) or at both these time points. Non-vaccinated animals were used as negative control. It had been found in previous studies that immunization with Advax alone had no detectable effect on immune parameters when compared to mice injected with saline (unpublished data). Moreover, only influenza specific immune responses were measured. Therefore, any parameters that might be induced by the use of Advax alone could not be quantified. Hence, Advax alone group was not included in this study.

We first evaluated the number of IgG or IgA ASC in splenocytes of mice vaccinated with non-adjuvanted and Advax-adjuvanted WIV formulations (Fig. 2A). We found that respiratory tract immunization with Advax-adjuvanted WIV formulations either as liquid or powder led to a

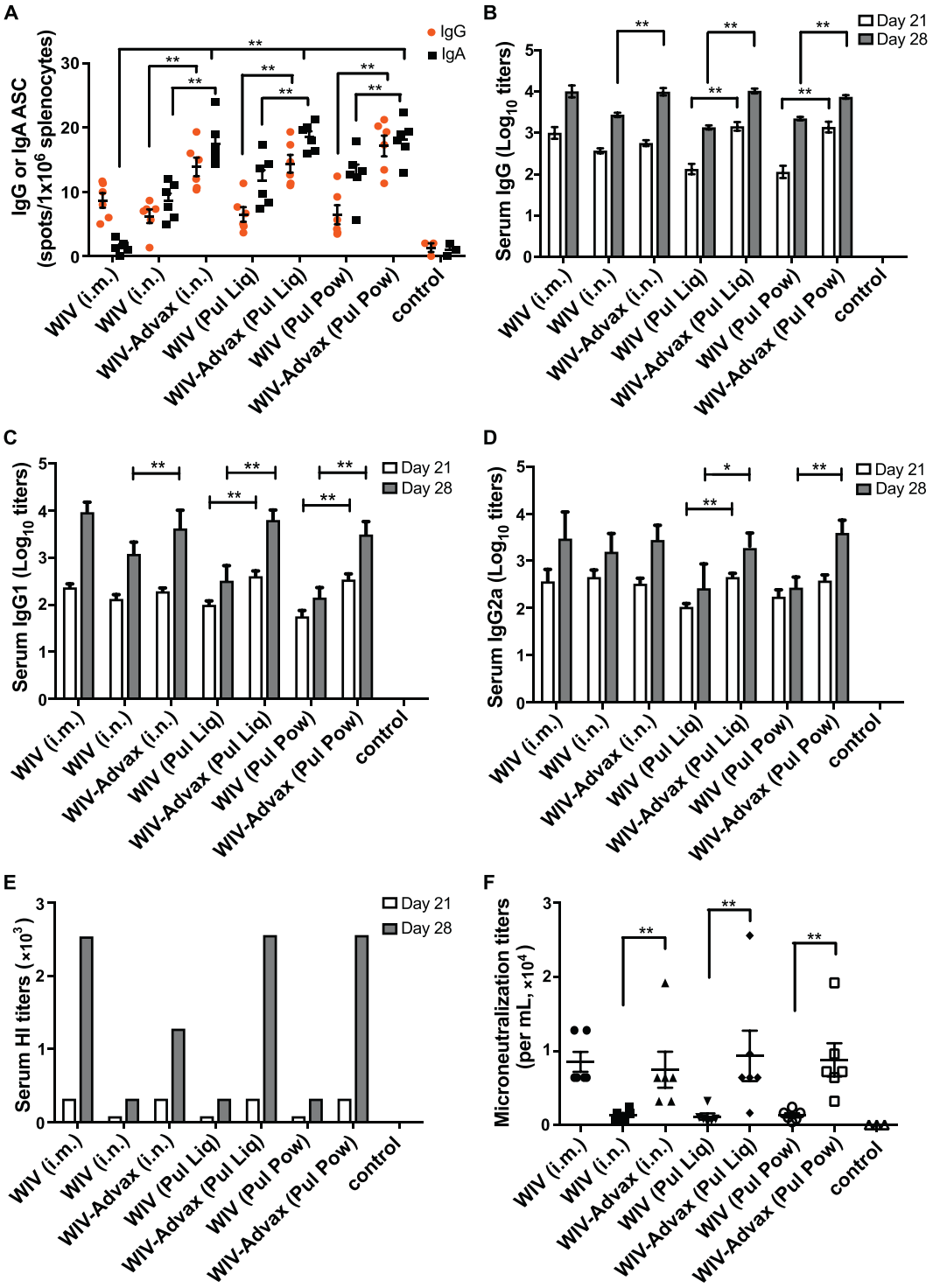

Fig. 2. Systemic immune responses after respiratory tract immunization. Mice were immunized twice on day 0 and day 21 with 5 μg HA of NIBRG-23 with or without 500 μg of Advax in liquid or powder form (i.n. or pulmonary). A week after the second vaccination, mice were sacrificed to determine (A) IgG or IgA antibody secreting splenocytes, (B) Serum IgG titers at day 21 (white bars) and at day 28 (grey bars), (C) Serum IgG1 titers, (D) Serum IgG2a titers, (E) HI titers for sera pooled per experimental group, (F) MN titers. Data are presented as average ± standard error of the mean unless stated otherwise (n = 6). Levels of significance are denoted as *p ≤ 0.05 and **p ≤ 0.01.

significantly higher number of IgG and IgA ASC than immunization with corresponding non-adjuvanted WIV formulations. As expected, delivery of WIV via the i.m. route led to the production of only few IgA ASC but a considerable number of IgG ASC in the spleen (Fig. 2A).

We next evaluated serum anti-influenza IgG titers both after the first and second immunization. Both i.m. and respiratory tract delivery of WIV formulations, with or without Advax, induced serum IgG responses after the first immunization that were further increased after the booster dose (Fig. 2B). Furthermore, respiratory tract delivery of Advax-adjuvanted WIV formulations, either as liquid or powder, generated significantly higher IgG titers than the corresponding non-adjuvanted WIV formulations after the second immunization. The higher

serum IgG titers induced by Advax-adjuvanted WIV formulations were in line with a significant increase in splenic IgG ASC Serum IgG titers generated by respiratory tract administered Advax-adjuvanted WIV formulations were comparable to those generated by non-adjuvanted WIV formulation given via the i.m. route at both day 21 and day 28 (Fig. 2B). Coherent with IgG titers, IgG1 responses were significantly enhanced in mice immunized with Advax-adjuvanted WIV formulations via the respiratory tract versus mice immunized with non-adjuvanted WIV formulations (Fig. 2C). However, IgG2a responses were only significantly enhanced for Advax-adjuvanted WIV formulations, both liquid and powder, administered to the lungs but not by i.n. administration (Fig. 2D). Moreover, a balanced IgG2a:IgG1 ratio was observed, indicating that Advax- adjuvanted WIV induces a balanced Th1/Th2 type of immune response in agreement with our previous study where a balanced Th1/Th2 ratio was observed after pulmonary administration of a liquid, Advax-adjuvanted WIV formulation [28].

The functional potential of IgG antibodies in serum was assessed by the HI and MN assay. Both at day 21 and day 28, Advax-adjuvanted WIV formulations administered to the lungs induced substantially higher HI titers than non-adjuvanted WIV formulations (Fig. 2E). In line with the HI titers, approximately five-six fold higher MN titers were seen for Advax-adjuvanted WIV formulations administered to the respiratory tract than for corresponding non-adjuvanted WIV formulations (Fig. 2F). The higher HI and MN titers for Advax-adjuvanted WIV were consistent with the higher serum IgG titers, thus indicating the functional effectiveness of the vaccine-induced IgG antibodies in these groups.

Thus, Advax-adjuvanted WIV formulations administered to the respiratory tract induced comparable systemic immune responses as WIV administered via the i.m. route and considerably higher immune responses than non-adjuvanted respiratory tract administered WIV.

4.3. Mucosal immune responses

An important goal of influenza vaccination is the induction of antibodies in the respiratory tract, the portal of influenza virus entry [37,38]. The traditional parenteral route of influenza vaccine administration is inefficient in inducing mucosal immune responses. Similarly, pulmonary immunization with non-adjuvanted WIV generally induces only low levels of local or mucosal immunity [9,12]. In order to investigate the potential of Advax to boost local mucosal immunity, respiratory tract immunity was determined a week after the second immunization by assessment of nasal IgA and BAL anti-influenza IgA and IgG levels along with ASC in lungs. As expected, WIV administered via the i.m. route failed to induce substantial nasal or lung IgA titers (Fig. 3A, B). Compared to the i.m. route, higher nose IgA titers were found for Advax-adjuvanted WIV formulations administered to the respiratory tract, which, however, were only significantly higher for the liquid formulation administered to the nose and the powder formulation administered to the lungs (Fig. 3A). Yet, compared to non-adjuvanted WIV formulation, only the Advax-adjuvanted liquid formulation administered to the lungs elicited significantly higher nasal IgA titers. By contrast, a significant effect of Advax adjuvant was seen on BAL IgA in both the i.n. and pulmonary vaccine groups with approximately four-eight-fold higher lung IgA titers than mice immunized with corresponding non-adjuvanted WIV formulations (Fig. 3B). Hence, Advax either administered i.n. or into the lungs increased lung but not nasal IgA production. This might be due to the relatively smaller surface area of the nasal mucosa compared to that of the lower respiratory tract [39]. Since 1 ml of PBS was used for collecting both nasal and lung washes, the concentration of IgA in the lung washes would be expected to be much higher than in the nasal washes if the amount of IgA per specific surface area in the lung and nose would be the same. The fact that only pulmonary powder but not liquid delivery where adjuvanted or WIV alone induced increased nasal IgA, might suggest powder particles may have been exhaled by the mice back up from the bronchi into the nasal nares after the insufflation procedure, whereas the liquid vaccine may have been more likely to instantly adhere to the bronchial walls and thereby not remain suspended in air and able to escape into the nose.

Advax-adjuvanted WIV formulations administered to the respiratory tract significantly increased anti-influenza IgG titers in the lungs in accordance with the increased serum IgG titers seen in these animals when compared to corresponding non-adjuvanted WIV immunizations (Fig. 3C). Interestingly, lung IgG titers of mice immunized with WIV-Advax formulations administered to the respiratory tract were significantly higher than those immunized with non-adjuvanted WIV formulation via the i.m. route (Fig. 3C). The boost in lung IgA and IgG titers after Advax-adjuvanted respiratory tract immunization of WIV, is consistent with the increased number of IgA and IgG ASC found in the lungs of these mice (Fig. 3D).

Hence, the inclusion of Advax in WIV formulations resulted in significantly higher mucosal humoral immune responses than non-adjuvanted WIV formulation administered via the respiratory tract or via i.m. route.

4.4. Cellular immune responses

The phenotype of an immune response (skewed Th1 or Th2 or balanced Th1/Th2) is considered to be of importance for its protective potential [28,40,41]. A balanced Th1/Th2 response is preferable because it aids in both virus neutralization and clearance [41]. In order to investigate whether incorporation of Advax in a WIV formulation and delivery of the adjuvanted vaccine to the respiratory tract has an influence on the type and magnitude of cell-mediated immune responses induced, the frequency of influenza-specific IFN-γ and IL-4 secreting splenic T cells was determined. In addition, IFN-γ and IL-4 levels were measured in supernatants of splenocytes stimulated *in- vitro* with WIV. Compared to WIV alone, Advax-adjuvanted WIV formulation was associated with a significant increase in the number of IFN-γ secreting influenza-specific T cells (Fig. 4A). Likewise, increased production of IFN-γ was seen in Advax-adjuvanted WIV groups when compared to non-adjuvanted WIV, although, the differences were only significant for the pulmonary immunized groups (Fig. 4B). Moreover, Advax-adjuvanted WIV was associated with significantly higher frequencies of both IL-4 secreting T cells as well as significantly higher amounts of IL-4 in the culture supernatants as compared to WIV alone (Fig. 4C, D). By contrast, i.m. administered WIV induced a high number of IL-4 secreting T cells but low numbers of IFN-γ secreting T cells (Fig. 4A, C), which was matched by the IFN-γ and IL-4 levels measured in the culture supernatants (Fig. 4B, D).

Thus, immunization with Advax-adjuvanted WIV via the respiratory tract led to a balanced Th1/Th2 (IFN-γ/IL-4) response whereas i.m. immunization with WIV alone predominantly induced a Th2-type (predominant IL-4) immune response.

4.5. Mechanistic insights

4.5.1. Memory B cell responses and expression of lung localization factors

Advax-adjuvanted WIV, administered either as liquid or powder formulation, induced comparable humoral and cellular immune responses when administered via the pulmonary route. Hence, only liquid Advax-adjuvanted WIV was used as a representative formulation for further mechanistic investigations into the types of B and T cells responding to immunization.

Antigen-activated B cells undergo isotype class switching and change the production of antibody subtype from IgM and IgD to IgG, IgA or IgE [42]. In order to characterize the phenotype of class switched B cells, we determined the fraction of memory B cells among the total number of class switched B cells after i.n. or pulmonary delivery of WIV alone or with Advax adjuvant. A previous study has shown that memory B cells, particularly in lungs, play a key role in protection against

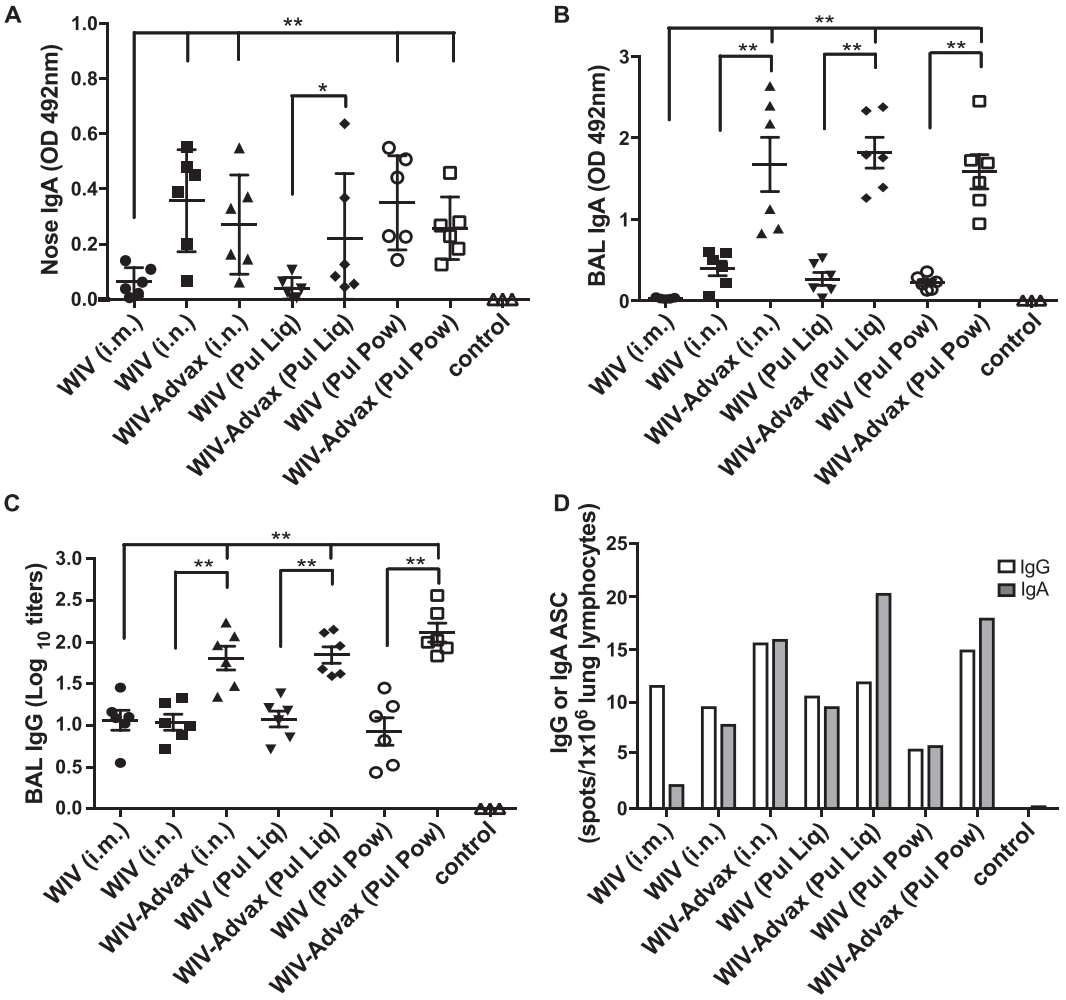

Fig. 3. Mucosal immune responses after respiratory tract immunization. Mice were immunized twice on day 0 and day 21 with 5 μg HA of NIBRG-23 with or without 500 μg of Advax in liquid or powder form (i.n. or pulmonary). A week after the second vaccination, mice were sacrificed to determine (A) Nose IgA, (B) BAL IgA, (C) BAL IgG, (D) IgG or IgA antibody secreting lung lymphocytes pooled per experimental group. Data are presented as average ± standard error of the mean (n = 6) unless stated otherwise. Levels of significance are denoted as *p ≤ 0.05 and **p ≤ 0.01.

influenza re-infection [43]. These memory B cells can be identified by the expression of CD38 [43–45]. Hence, cells isolated from lungs, spleen and bone marrow were stained for both IgM/IgD (to identify IgM/IgD$^-$ class switched cells) and the memory B cell marker, CD38 (Fig. 5A–B). Advax-adjuvanted WIV administered via the pulmonary route led to an 8-fold increase in the frequency of memory B cells in the lungs, 4-fold in spleen and about 10-fold in bone marrow in comparison to administration with WIV alone (Fig. 5A–B). Further analysis of these cells revealed that in lungs and spleen the percentage of memory B cells was particularly high among IgG producing cells (Fig. 5C, D) while in bone marrow it was high among IgA producing B cells (Fig. 5D). By contrast, much lower numbers of CD38$^+$ B cells were seen in the i.n. immunized groups although still a 2–3 fold increase in lung memory B cells among IgG or IgA producing cells was observed in the Advax-adjuvanted WIV group when compared to the WIV alone group (Fig. 5A, C). Our data suggests that respiratory tract immunization, in particular, pulmonary immunization with Advax-adjuvanted WIV induces a large number of both class-switched IgG$^+$ and IgA$^+$ memory B cells with the IgG$^+$ memory B cells primarily trafficking to the lungs and spleen and the IgA$^+$ memory B cells instead trafficking to the bone marrow.

Previous studies have shown that CXCR3 and CD69 promote lung homing of B cells and effector T cells after infection with influenza virus [43,46,47]. Pulmonary immunization with Advax-adjuvanted WIV increased the percentage of class-switched B cells expressing the lung localization marker, CXCR3 (Fig. 5E), by about 4-fold, with a slight increase in the percentage expressing CD69 (Fig. 5F). Interestingly, i.n. immunization with Advax-adjuvanted WIV induced a 2-fold increase in CD69$^+$ B cells but no increase in CXCR3 expressing B cells (Fig. 5E, F). Likewise, pulmonary but *not* i.n. immunization of Advax-adjuvanted WIV enhanced the number of class-switched B cells expressing CXCR3 (Fig. S3).

Overall, respiratory tract delivery, in particular pulmonary delivery, of Advax-adjuvanted WIV increased the frequency of class-switched memory B cells and enhanced the expression of localization factors i.e. CXCR3 and CD69 on these class-switched B cells.

4.5.2. Memory T cell responses and expression of lung localization factors

Memory CD4$^+$ T cells are assumed to be the key players in promoting the production of long-lived ASC and memory B cells, thus facilitating rapid production of antibodies in cases of antigen recall [48,49]. Effector/memory T cells are identified by the expression of CD44 and absence of CD62L and are thus denoted as CD44$^+$CD62L$^-$. Pulmonary immunization with Advax-adjuvanted WIV led to a ~ 3-fold increase in lung effector/memory CD4$^+$ T cells in comparison to administration of WIV alone (Fig. 6A). Previous studies have shown that even in the absence of B cells and CD8$^+$ effector/memory T cells, CD4$^+$ effector/memory T cells can provide at least partial protection against influenza infection with recruitment of CD4$^+$ T effector/memory cells

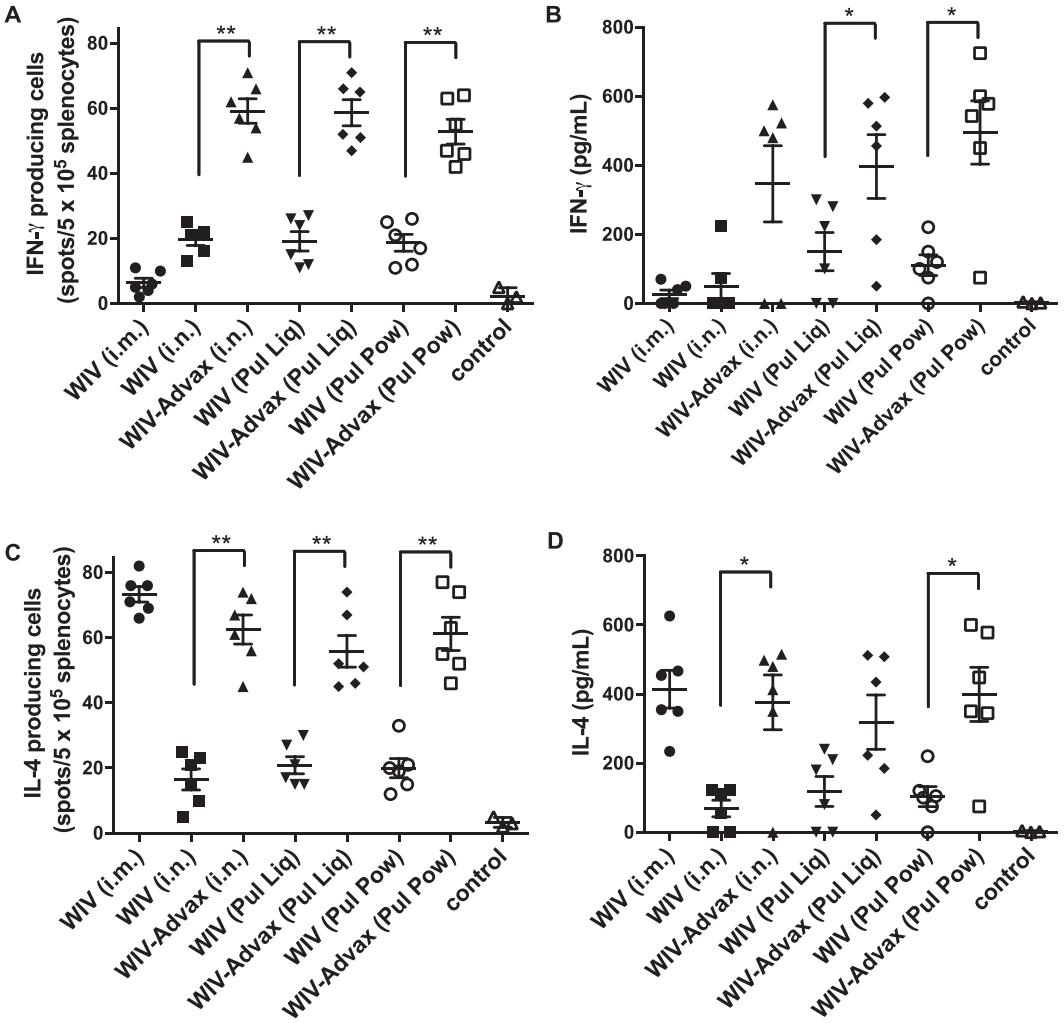

Fig. 4. Cellular immune responses after respiratory tract immunization. Mice were immunized twice on day 0 and day 21 with 5 μg HA of NIBRG-23 with or without 500 μg of Advax in liquid or powder form (i.n. or pulmonary). One week after the second vaccination, mice were sacrificed to determine (A) Frequency of IFN-γ secreting splenocytes and (B) IFN-γ levels. (C) IL-4 secreting splenocytes and (D) IL-4 levels. Data are presented as average ± standard error of the mean (n = 6). Levels of significance are denoted as *p ≤ 0.05 and **p ≤ 0.01.

to the lungs [50,51]. This recruitment is facilitated by the expression of lung localization factors on effector/memory T cells [46].

Tissue resident memory T cells (TRM) are a subset of memory T cells that express CD103 and lack the property of recirculation, so they remain restricted within tissues thereby making them readily available to protect against local infection [52,53]. Besides CD103, the expression of CXCR3 on effector/memory is known to promote their migration and localization to infected lungs [46,47,54]. We therefore characterized $CD4^+$ effector/memory T cells for the expression of the lung localization factor CXCR3 and the tissue resident T cell marker CD103. I.n. immunization with Advax-adjuvanted WIV formulation showed a minor increase in the percentage of $CXCR3^+$ cells as compared to the corresponding non-adjuvanted WIV formulation. By contrast, pulmonary administration of Advax-adjuvanted WIV enhanced the percentage of $CD4^+$ effector/memory expressing CXCR3 by 3-fold (Fig. 6B). Consistent with previous studies we also found that the augmented expression of CXCR3 on effector/memory T cells led to an increase in the migration of these cells to the lungs (Fig. 6A). Staining of the TRM marker, CD103, revealed that adjuvantation with Advax led to an approximately 2-fold increase in $CD4^+$ TRMs in the lungs for pulmonary as well as for i.n. administered vaccine (Fig. 6C). Thus, immunization of mice with Advax-adjuvanted WIV, in particular via the pulmonary route, increased effector/memory T cells with augmentation in the expression of CXCR3 and CD103 cells in the lungs. This is consistent with previous studies, which showed that mucosal administration of an antigen is necessary for the generation of local T cell responses [53,55,56].

Conclusively, co-administration of WIV with Advax resulted in an enhanced number of effector/memory $CD4^+$ T cells with a moderate increase in the expression of lung localization factors and TRM cell markers.

4.6. Challenge study

Respiratory tract immunization (i.n. and pulmonary) with Advax-adjuvanted WIV has shown in the experiments above to boost humoral and cellular immunity both systemically and locally in the lung. However, in the mechanistic studies, mainly, pulmonary immunization with Advax-adjuvanted WIV was found to boost memory responses and the expression of lung localization factors. Hence, the pulmonary route was chosen for a challenge study. In the challenge study, we explored whether the enhanced immunity translated into enhanced protection if mice were exposed to a lethal dose of influenza virus. To maximize the stringency of the model, mice received just a single dose of pulmonary WIV (A/PR/8/34) with or without Advax. An Advax alone (without antigen) control group was not included as in previous studies we

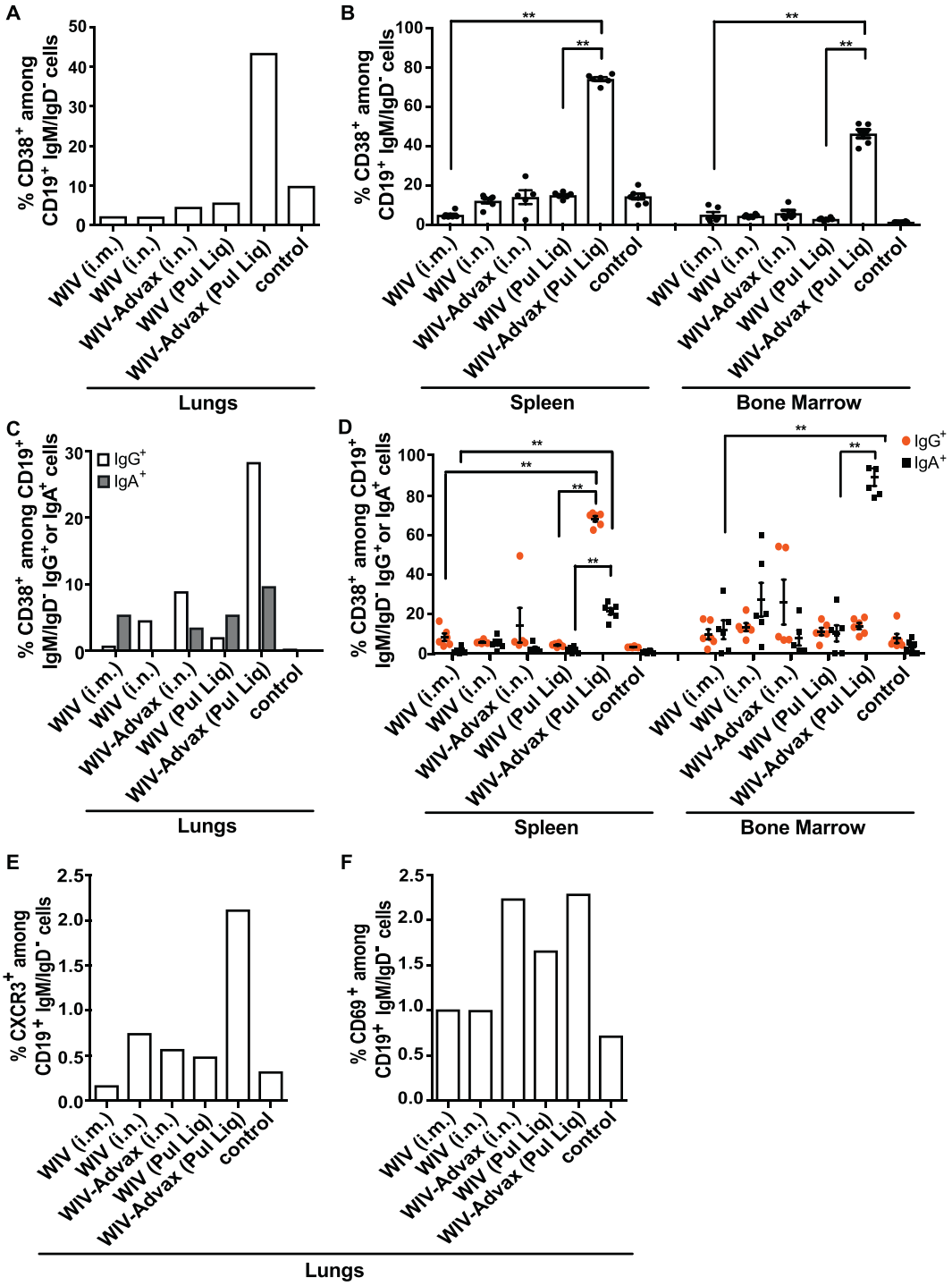

Fig. 5. Effects of respiratory tract immunization on memory B cells and expression of lung localization factors. Percentage of memory B cells among total class switched B cells (% $CD38^+$ among $CD19^+$ IgM/IgD^-) in lungs (A), spleen and bone marrow (B). Percentage of memory B cells among class switched IgG^+ or IgA^+ cells (% $CD38^+$ among $CD19^+$ IgM/IgD^- IgG^+ or IgA^+) in lungs (C), spleen and bone marrow (D). Percentage of lung cells expressing CXCR3 (E) or CD69 (F) among total class switched B cells (% $CXCR3^+$ or % $CD69^+$ among $CD19^+IgM/IgD^-$) The frequencies of cells are shown for pooled lung lymphocytes from each experimental group while data for spleen and BM data are presented individually as average ± standard error of the mean. Levels of significance are denoted as *$p \leq 0.05$ and **$p \leq 0.01$.

observed no effect of administering Advax alone on influenza disease, with recipients of Advax alone having the same clinical scores and dying at the same rate as saline-injected controls, whether the Advax was given i.m. [27] or via the pulmonary route (unpublished data). Since a highly immunogenic influenza virus strain was used, a low dose of 0.1 μg WIV with or without 1 mg of Advax was found to be optimal to avoid complete protection that might be induced by antigen alone (without adjuvant) with a high dose. After lethal viral challenge, we found that, mice that received non-adjuvanted WIV were not protected against influenza infection, as evidenced by a rapid weight loss and a clinical sickness score of 6 within 8–9 days after challenge (Fig. 7A–C). By contrast, mice that received a single pulmonary dose of WIV formulated with Advax adjuvant were fully protected with no weight loss and no clinical disease symptoms after challenge (Fig. 7A–C). A Cox-

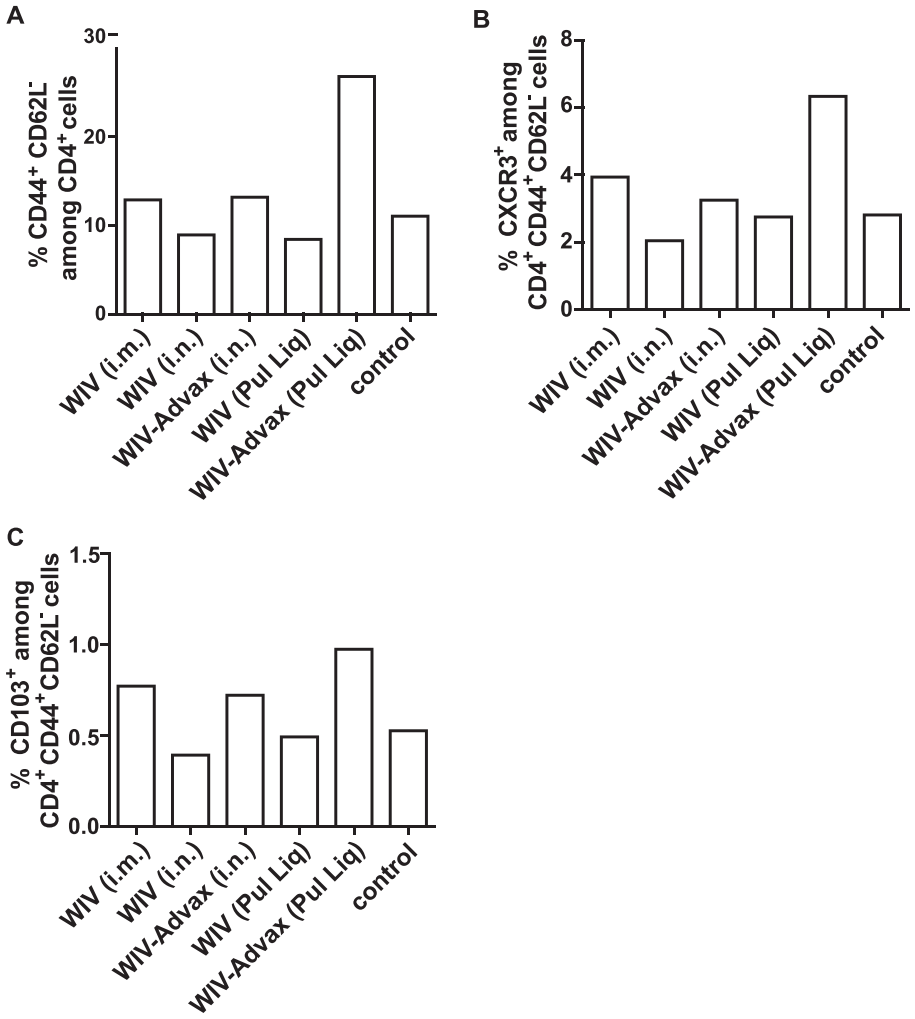

Fig. 6. Effects of respiratory tract immunization on memory B cells and expression of lung localization factors. (A) Percentage of effector/memory $CD4^+$ T cells in lungs (% $CD44^+$ $CD62L^-$ among $CD4^+$ cells), (B) Percentage of migratory $CD4^+$ T cells in lungs (% $CXCR3^+$ among $CD4^+$ $CD44^+$ $CD62L^-$ cells), (C) Percentage of tissue-resident memory $CD4^+$ T cells in lungs (% $CD103^+$ among $CD4^+$ $CD44^+$ $CD62L^-$ cells). The frequencies of cells are shown for pooled lung lymphocytes from each experimental group.

Mantel log rank test revealed that the difference in survival between non-adjuvanted WIV and Advax-adjuvanted WIV group was significant ($p = 0.029$). Similar outcome was obtained upon repetition of the experiments twice.

5. Conclusions

In the current study, we demonstrate that administration of Advax-adjuvanted WIV to the respiratory tract, either as liquid or dry powder, has the potential to boost influenza induced systemic, mucosal and cellular immune responses. To our knowledge, this is the first study to show that an effective Advax-adjuvanted dry powder influenza vaccine formulation with full retention of biological activity of the WIV antigen and the Advax adjuvant can be prepared by SFD. Though both liquid and dry powder influenza vaccine formulations can be used for pulmonary administration, a dry powder formulation is preferable due to its long-term stability at ambient temperatures, which facilitates stockpiling [10,57,58]. In cases of an influenza pandemic, a stockpiled dry powder formulation would be readily available and easy to administer in mass vaccination campaigns. For Advax-adjuvanted influenza formulations, the i.n. and pulmonary route were found to be equally effective in boosting humoral and cellular immunity, however, pulmonary route was found to be superior for the augmentation of memory responses as well as lung localization factors. Moreover, pulmonary immunization with Advax-adjuvanted WIV was found to be equally effective as an i.m. immunization with WIV in terms of induction of systemic and cellular immunity and was superior in terms of mucosal immunity. In addition, a single pulmonary administration with Advax-adjuvanted WIV at a low dose of 0.1 μg WIV not only protected the animals from weight loss and observable clinical symptoms but also led to their complete survival which is in contrast to the animals immunized with WIV alone. Moreover, no adverse effects (weight loss, sickness) were seen in animals that received pulmonary immunization with Advax adjuvant. Hence, inhalation of Advax-adjuvanted influenza vaccine as either a liquid or a dry powder formulation may be a promising alternative to conventional parenteral influenza vaccines.

Study limitations include the fact that the impact of the vaccine on direct measures of virus replication in the lung were not assessed, nor were studies of lung histology performed. These more detailed aspects of the mechanism of protection and of pulmonary adjuvanted vaccine safety will need to be studied in the future. However, we expect that the enhanced protection against clinical disease with WIV plus Advax, would be reflected in lower lung virus titers post-challenge. In future studies, it would also be interesting to investigate how the increased tissue resident memory B and T cell responses elicited after pulmonary immunization with Advax-adjuvanted WIV might contribute to long-term protection against influenza, as only short term protection was assessed in this study.

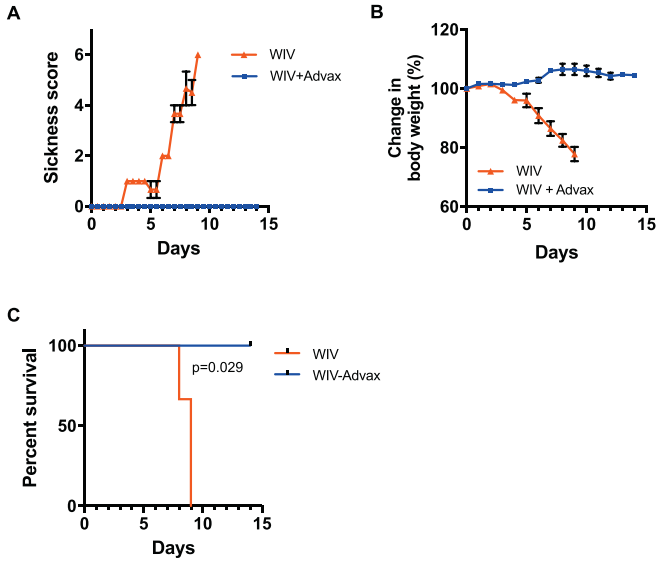

Fig. 7. Effect of Advax-adjuvanted WIV formulations on protection against live virus challenge. Mice were immunized once with 0.1 μg of WIV with or without 1 mg of Advax via the pulmonary route. Two weeks after the immunization, mice were challenged with a lethal dose ($8xLD_{50}$) of live virus and were followed for 14 days for clinical symptoms. (A) Clinical sickness score, (B) Percentage change in body weight, (C) Kaplan-Meier survival curve. A Cox-Mantel log rank test was used to calculate the difference in survival between Advax-adjuvanted WIV group and WIV alone group. Data shown is a representative example of one of three replicate experiments performed.

Conclusively, we have demonstrated that Advax is a highly effective mucosal adjuvant which can be formulated with influenza vaccine into dry powders and enables complete protection against lethal influenza virus challenge with just a single low dose of antigen. This approach may thereby provide a convenient needle free approach for influenza vaccination.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.09.006.

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Conflict of interest

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

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