New strategies for simplifying influenza vaccination

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Comparison of adjuvants for a spray freeze-dried whole inactivated virus influenza vaccine for pulmonary administration

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Abstract
Influenza virus is transmitted by aerosols and accordingly the presence of IgA antibodies in the upper respiratory tract plays an important role in preventing initial infection and spread of the virus to the lower respiratory tract. Parentally administered influenza vaccines do not induce IgA antibody production and thus do not provide protection at the port of virus entry. Previously, we showed that non-adjuvanted inactivated influenza vaccines administered as a dry powder to the lungs successfully induce IgG antibody in serum but are poorly effective in eliciting nose IgA antibody responses. Here we investigated the suitability of a range of Toll-like receptor (TLR) ligands and a non-pattern recognition receptor (PRR)-binding compound, GPI-0100, to serve as pulmonary adjuvant in a whole inactivated virus (WIV) dry powder influenza vaccine formulation. For this purpose, adjuvants were spray freeze dried (SFD) along with WIV using inulin as cryoprotectant. In vitro analysis of the SFD vaccine formulations on a NFkB reporter cell line showed that the immune stimulating properties of the adjuvants were not affected by stress during spray freeze drying. Comparative in vivo immunization revealed that the TLR ligands palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4), Monophosphoryl lipid A (MPLA) and CpG oligodeoxynucleotides (CpG ODN) did not stimulate potent mucosal immune responses to a pulmonary delivered WIV vaccine in mice although all except Pam3CSK4 did increase systemic IgG responses. In contrast, vaccination with WIV adjuvanted with GPI-0100 induced robust mucosal antibody responses in nose and lungs and resulted in the strongest systemic immune responses of all vaccines studied. Moreover, in the GPI-0100 group but not in the other vaccination groups partial protection against lethal heterologous influenza virus challenge was obtained as confirmed by reduced lung virus titres. These data highlight that adjuvants can be SFD along with WIV without loss of adjuvant function. Moreover, they underline that a properly adjuvanted dry powder influenza vaccines delivered directly to the lungs is capable of inducing effective mucosal IgA and systemic IgG antibody responses which can contribute to heterologous protection.
1. Introduction

Influenza virus is well known for its ability to cause seasonal epidemics and occasional pandemics. The virus is transmitted by aerosols and binds to and infects epithelial cells in the respiratory tract\cite{1,2}. Pre-existing secretory IgA (S-IgA) antibodies in the nose play a vital role in preventing binding of influenza virus to its cellular receptor and further spreading of the virus in the respiratory tract\cite{3-5}. Moreover, IgA antibodies are known for their ability to provide cross protection against heterologous influenza virus\cite{4,6}. Together with IgG antibodies, IgA antibodies were shown to inhibit infectivity of influenza virus in the respiratory tract\cite{5,7}. To prevent influenza infection and further dissemination of the virus to the lower respiratory tract or to the environment, it is therefore important that a vaccine induces IgA antibody responses. However, currently available inactivated influenza vaccines that are administered by the intramuscular, subcutaneous or intradermal route do not induce mucosal immunity\cite{8,9}.

To induce mucosal IgA antibody production as well as systemic immunity, mucosal vaccine delivery is mandatory. In this context, the lung is a particularly attractive target. Upon interaction with respiratory viruses, airway epithelial cells produce cytokines and chemokines promoting recruitment of inflammatory cells and help directing the adaptive immune response according to stimuli received\cite{10}. Antigen presenting cells (APC) like dendritic cells (DC) that are present beneath the epithelial layer and alveolar macrophages (AM) that are present in the lumen constantly protect the large surface area of the lung\cite{11-13}. Upon activation by pathogens, these epithelial cells, DC and AM effectively bypass the steady state anti-inflammatory T helper 2 (Th2) responses in the lung and initiate innate and adaptive immunity against the invading pathogen\cite{13}.

We previously showed that pulmonary delivery of spray freeze dried (SFD) whole inactivated virus (WIV) influenza vaccine induced the production of systemic IgG antibody to the levels similar to those evoked by standard intramuscular (i.m.) immunization without causing inflammation in the lungs\cite{14-16}. Pulmonary vaccination also resulted in induction of IgA antibody in nose and lungs. However, the levels of IgA in the respiratory tract and the amount of systemic IgG2a antibodies, the protective antibody subtype in mice, were low\cite{14}. An approach to overcome this drawback is addition of a potent adjuvant to the pulmonary administered vaccine.

Toll-like receptor (TLR) ligands are pathogen associated molecular patterns or substances mimicking such patterns that are recognised by TLR present on the host cells. Binding of TLR ligands to TLR activates signals that are important for initiation of innate and adaptive immune responses\cite{17}. Our previous study showed that addition of a low amount (0.625 μg) of monophosphoryl lipid A (MPLA), a TLR 4 agonist, to pulmonary delivered WIV improved IgA levels in the lung, but had little effect on IgA titers in the nose\cite{18}. Therefore to find a more effective pulmonary adjuvant a head-to-head comparison of several candidate adjuvants is needed. TLR ligands like palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4,
recognised by TLR 1 and TLR 2), MPLA (TLR 4) and CpG oligodeoxynucleotide (CpG-ODN-1826, TLR 9) are attractive adjuvant candidates. They have been described to improve humoral responses against parenterally administered split or subunit influenza vaccines [19-22]. The semi-synthetic saponin adjuvant GPI-0100 is not recognised by known pattern recognition receptors (PRR). Yet, we earlier showed that pulmonary vaccination with GPI-0100-adjuvanted influenza subunit vaccine induced potent mucosal and systemic antibody responses as well as efficient protection against virus challenge[23].

To find an optimal adjuvant that effectively enhances systemic and mucosal immune responses to pulmonary administered dry powder vaccine, WIV was SFD along with minimal concentrations of above mentioned adjuvants using inulin as stabilizer[14]. The SFD vaccines were used for pulmonary immunization of mice. Our results show that all adjuvants tolerated the stresses encountered during spray freeze drying well. All adjuvants except Pam3CSK4 enhanced systemic influenza-specific antibody levels. Yet, only GPI-0100-adjuvanted vaccine effectively induced mucosal antibody titres and provided partial protection from heterologous virus challenge. To our knowledge, this is the first study comparing different adjuvants for their suitability to be used in SFD, pulmonary administered vaccine.

2. Material and methods:

2.1. Virus

A/PR/8 H1N1 was cultured in embryonated eggs by inoculation of the seed virus into the allantoic fluid. Allantoic fluid was harvested 4 days post seed virus inoculation and virus was purified as described previously[14].

2.2. Vaccine preparation.

WIV derived from A/California/07/2009 (H1N1) was kindly provided by Solvay Biologicals (Weesp, The Netherlands). WIV was dialysed overnight at 4 °C against HBS, pH 7.2, and protein content was determined by micro-Lowry assay. Purity of the WIV was confirmed by SDS PAGE followed by silver staining. The hemagglutinin (HA) content was assumed to be one third of the total protein for whole inactivated virus (based on the known protein composition of influenza virus and the molecular weight of the viral protein). Adjuvanted vaccine solutions were made by adding to WIV the minimum amount of adjuvant recommended for one dose in mice based on the literature. The amount of adjuvants used per vaccine dose, i.e. 2.5 µg HA of WIV, were 2.5 µg Pam3CSK4, 2.5 µg MPLA, 10 µg CpG ODN 1826 or 12.5 µg GPI-0100.
2.3. Spray freeze-drying
Spray freeze-drying of vaccine solutions was performed using 5 % (w/v) inulin from dahlia tubers, MW ~5000 (HA of WIV: inulin = 1:200) (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands) as described before[15,14].

2.4. Analysis of adjuvants after spray freeze drying
NFκB activation was detected using the NFκB reporter cell line RAW-Blue™ cell line (InvivoGen, Toulouse, France) following the manufacturer’s protocol. For stimulation, 1 x 10^5 RAW-Blue™ cells were added to flat bottom 96-well plates (Corning Incorporated, Corning, USA). Cells were stimulated with 5 µg/ml lipopolysaccharide (LPS) or adjuvants or liquid or dissolved powder vaccines containing 2.5 µg of HA of WIV obtained from A/California/07/2009 (H1N1) with or without adjuvants. The amount of adjuvant which was present in one vaccine dose (see above) was used for stimulating cells. Following stimulation, cells were incubated at 37 °C with 5% CO₂ for 16 hours. 40 µl of cell supernatant was collected in 96-well ELISA plates (Greiner Bio One, Alphen a/d Rijn, The Netherlands) to which 160 µl QUANTI-Blue™ (InvivoGen, Toulouse France) was added. The plates were incubated at 37 °C for 1 hour. Secreted embryonic alkaline phosphatase (SEAP) levels were determined by measuring absorbance at 620 nm. NFkB activation is expressed as the activity of the adjuvants or vaccine relative to that of LPS.

2.5. Physical characterization of powder vaccine
Scanning electron microscope (SEM) images of powder vaccines were taken using a JEOL JSM 6301-F microscope (JEOL Ltd., Tokyo, Japan) as described previously[15]. Images were taken at magnification of 1000x.

Geometric particle size distribution of powder vaccines was measured using a HELOS compact model KA laser diffraction apparatus (Sympatec GmbH, Germany) and the RODOS dispersing system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) as described previously[15]. The aerodynamic size distribution was calculated from the geometric particle size distribution using the equation from[24]

\[ d_{ae} = \frac{d_{e}}{\sqrt{\rho_p / \rho_o \chi}} \]

where \( d_{ae} \) = aerodynamic diameter, \( d_{e} \) = geometric diameter, \( \rho_p \) = density of powder particles (0.05 g/cm³), \( \rho_o \) = unit density (1g/cm³), \( \chi \) = dynamic shape factor which is 1 for spherical particles[25].

The specific surface area of powder vaccines was measured using a Tristar surface analyser (Micrometrics Instrument Corp., Norcross, USA) as described previously[15].
2.6. Immunization and challenge of mice

Animal experiments were approved by The Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG), The Netherlands. In vivo experiments were performed in female BALB/c mice (Harlan, Zeist, The Netherlands) which were 6-8 weeks old.

Mice (n=6) were vaccinated via the pulmonary route twice, with an interval of 3 weeks. Each dose of unadjuvanted or adjuvanted powder vaccine contained 2.5 µg HA of WIV derived from A/California/07/2009 (H1N1). Non-treated mice served as naive controls.

For pulmonary vaccination, mice were anaesthetised with isoflurane/O₂. Mice were brought to a vertical position and intubated with a modified Autograde catheter (Becton Dickinson, Breda, The Netherlands). Powder vaccine was delivered using a dry powder insufflator (DPI), (Penn-Century Inc., Wyndmoor, USA). Approximately 500 µg of powder vaccine containing 2.5µg HA was delivered to the lungs by applying a single puff of 200 µl. Before mice were brought back to the housing facility after anesthesia, mice were placed in a recovery incubator with a temperature of 25 °C for 2 hours.

The protective efficacy of the vaccines was determined by challenge of the immunized mice with live virus of a heterologous strain (A/PR/8/H1N1) one month after the booster vaccination. Briefly, mice were anaesthetised with isoflurane/O₂ and 40 µl of HBS containing 200 PFU A/PR8/H1N1virus were slowly administered via the nostrils. Mice were observed daily for weight loss and ruffled fur. Three days post challenge, mice were sacrificed under isoflurane/O₂ anesthesia. Blood, nose washes, lungs and spleens were collected and processed for further use. Nose washes were obtained using 1 ml PBS (pH 7.4), containing Complete protease inhibitor cocktail tablets (Roche, Almere, The Netherlands) as described previously[26]. Lungs were collected in 1 ml Iscove’s Modified Dulbecco’s Medium (IMDM) complete medium (Gibco, Life Technologies BV, Bleiswijk, The Netherlands) containing 5 % FBS (Lonza, Basel, Switzerland), 100 U/ml penicillin, 100 mg/ml streptomycin and 0.05 M 2-mercaptoethanol (Invitrogen, Breda, The Netherlands) after perfusion with 10 ml PBS containing 0.1 µg/ml heparine. Spleens were collected in 5 ml complete IMDM medium.

2.7. ELISA

Nose washes, lung supernatants and serum samples were used for evaluation of humoral responses after vaccination. For detection of influenza-specific IgG, IgG1, IgG2a and IgA antibodies, ELISA plates (Greiner Bio One, Alphen, The Netherlands) were coated with 500 ng/well of WIV derived from A/California/07/2009 (H1N1) or A/PR/8 (H1N1) overnight at 37 °C. ELISA was performed as previously described[15]. Average IgG titres were determined as log 10 of the reciprocal of the sample dilution corresponding to an absorbance at 492 nm of 0.2. IgA levels are presented as average of maximum absorbance of 1:1 diluted nose and lung washes.
2.8 Hemagglutination inhibition

Hemagglutination inhibition (HI) assay was performed as described previously with minor changes[14] using pooled sera of mice from each experimental group. Four hemagglutination units (HAU) inactivated A/California/07/2009 (H1N1) virus was added to the diluted serum samples. HI titres are expressed as log₂ values of the highest dilution preventing hemagglutination.

2.9 FACS staining

Spleens were collected in complete IMDM medium three days after the second immunization and were processed to single cell suspension as described previously[15]. Briefly, spleens collected in IMDM were processed to single cell suspensions using GentleMACS C tubes along with a GentleMACS dissociator (Mitenyi Biotec B, Leiden, The Netherlands). RBCs were lysed using ACK buffer (0.83% NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH 7.2). Similarly, lungs were collected in complete IMDM medium and were processed as described previously[27]. After extensive washing 1 x 10⁶ cells were added to FACS tubes (Corning Incorporated, New York, USA). Cells were centrifuged at 1200 rpm for 5 min at 4 °C. Pelleted cells were resuspended and stained with Alexa Fluro 647 anti-GL7 (0.1 µg/100µl) and PE anti-B220 PE (0.2 µg/100 µl) in FACS buffer (1% Bovine serum albumin (BSA) in PBS, pH 7.4) at 4 °C for 60 min. Cells were then washed three times with FACS buffer and analysed on a MACSQuant flow cytometer (Miltenyi Biotec B, Leiden, The Netherlands). Data was analysed using Kaluza flow cytometry analysis software version 1.2 (Beckman Coulter, Woerden, The Netherlands).

2.10 IFNγ or IL4 ELIspot

The numbers of influenza specific IFNγ- and IL4-producing cells were determined using ELIspot. IFNγ was performed using a murine IFNγ ELIspot kit (Gen-Probe Diacloine SAS, Besancon Cedex, France) according to the manufacturer's protocol. IL4 ELIspot was performed using an in-house made protocol. After extensive washing, 5 x 10⁵ splenocytes or lymphocytes from lungs were added to MultiScreenHTS-HA filter plates (Millipore, Billerica, Massachusetts) coated with anti IFNγ or anti IL4 (BD Biosciences, Breda, The Netherlands) antibodies. Splenocytes were incubated overnight at 37 °C with 5 % CO₂ in IMDM complete medium with or without WIV obtained from A/California/07/2009 (H1N1) (10 µg/ml). Lymphocytes from lungs were incubated at 37 °C with 5 % CO₂ in IMDM completed medium without WIV. IFNγ- or IL4-producing cells were detected using alkaline phosphatase (AP)-labelled anti-mouse IFNγ or IL4 antibodies (eBioscience, Vienna, Austria). Plates were washed and IFNγ- or IL4-specific spots were detected using BCIP/NBT substrate (Roche, Almere, The Netherlands). Spots were allowed to develop and the reaction was stopped by washing plates with tap water.
2.11 Cytokine ELISA

5 x 10^5 splenocytes were added to round bottom 96-well plates (Corning incorporated, New York, USA) and were incubated for 72 hours at 37 °C with 5 % CO\textsubscript{2} in IMDM complete medium with or without WIV obtained from A/California/07/2009 (H1N1) (10 µg/ml). Cell supernatant was collected and stored at -20 °C until used. IFN\textsubscript{γ} and IL-4 levels in samples were determined using Ready-SET Go ELISA kit (Ebioscience, Vienna, Austria) according to manufacturer protocol.

2.12 Virus titration

Perfused lungs collected after challenge were homogenised in PBS (pH 7.4) and centrifuged at 1200 rpm for 10 minutes. Supernatants were collected, snap-frozen in liquid nitrogen and stored at -80 °C until use. Lung virus titers were determined by infecting MDCK cells grown in 96-well plates with serial dilutions of the lung homogenate supernatants as described previously [14]. Virus titers are presented as 10^log titre per gram of lung.

2.13 Statistical analysis

Mann Whitney U-test was used for data analysis. One-tailed tests were performed for comparison of data from groups immunized with non-adjuvanted vs adjuvanted vaccines. For other comparisons two-sided test was employed. P values < 0.05 were considered to represent statistically significant differences. *, **, *** signify p<0.05, p<0.01, p<0.001 respectively.

3. Results

3.1 Physical characterization of SFD powder vaccines

In order to evaluate whether addition of adjuvants to WIV prior to spray freeze drying affects the physical characteristics of the obtained powder particle size, surface area and morphology of powder vaccines were analysed. Firstly, the geometric particle size distribution of vaccine powders was studied by laser diffraction (Figure-1a). X10, X50 and X90 values of the geometric diameter were determined from cumulative undersize curves and the span [span = (X90 – X10)/X50] was calculated (Table 1). It was found that 90 % of the powder particles had a geometric size < 17 µm with a narrow size distribution (stated as span) between 1.4 and 2.1 µm. These results demonstrate that addition of the adjuvants did not substantially affect the geometric particle size of obtained powder particles.

For pulmonary delivery the aerodynamic diameter is more relevant than the geometric diameter since it determines the behaviour of particles in an air stream like during inhalation. It has been earlier demonstrated that particles having an aerodynamic diameter of 1-5 µm are suitable for pulmonary delivery[28][29]. Calculation of the
Figure 1. Physical characterization of SFD powder vaccines

WIV derived from A/California/07/2009 (H1N1) was mixed with different adjuvants and SFD using inulin as stabilizer. Evaluation of (a) geometric particle size [X10 (white bars), X50 (grey bars) and X90 (black bars)] and (b) aerodynamic particle size of SFD powder vaccines by laser diffraction using RODOS. (c) Analysis of the surface area of SFD vaccine formulations. Scanning electron microscope images of SFD vaccines at 1000X magnification representing (d) WIV, (e) WIV-Pam3CSK4, (f) WIV-MPLA, (g) WIV-CpG ODN 1826 and (h) WIV-GPI-0100.
aerodynamic size showed (Figure 1b) that the diameter of 90% of the particles was < 5 µm for all the vaccine powders. These powders thus have the dimensions required for pulmonal delivery. Analysis of the surface area of SFD vaccines revealed that all vaccine formulations had high specific surface areas > 60 m²/g proving that the obtained powder vaccines were highly porous as desired for pulmonary delivery (Figure 1c). Among the SFD vaccines, WIV-Pam3CSK4 had the lowest surface area (65 m²/g) while WIV-GPI-0100 had the highest (97 m²/g). The surface area of WIV-MPLA and WIV-CpG was 87 m²/g and 84 m²/g respectively.

The physical appearance of the powder particles was further investigated by scanning electron microscopy (SEM). SEM images revealed that all adjuvanted powder formulations consisted of spherical particles with interconnected pores (Figure 1 d-h). For each powder there was some minor variation in particle size distribution. Yet, the general appearance of the powders was very similar.

Overall the results of the physical characterization of the SFD powders indicate that addition of the adjuvants had negligible effects on the powder size and porosity.

### 3.2 Biological activity of vaccine components in vitro

**Table 1.** Analysis of powder vaccines by laser diffraction for geometric size distribution. Particle diameters are indicated in µm. X10, X50, X90 gives the average size of 10, 50, 90%, respectively, of the powder particles after spray freeze drying.

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Laser diffraction (µm)</th>
<th>Span</th>
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<tbody>
<tr>
<td></td>
<td>X10</td>
<td>X50</td>
</tr>
<tr>
<td>SFD WIV</td>
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<td>5.637</td>
</tr>
<tr>
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<td>6.263</td>
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<tr>
<td>SFD WIV MPLA</td>
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<td>SFD WIV CpG ODN 1826</td>
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<td>5.747</td>
</tr>
<tr>
<td>SFD WIV GPI-0100</td>
<td>1.893</td>
<td>5.497</td>
</tr>
</tbody>
</table>

**Figure 2.** Biological activity of vaccine components in vitro

Analysis of NFκB activation by SFD vaccines on RAW-BlueTM cells. Relative absorbance was calculated by dividing OD620 values of supernatants from cells incubated with the vaccines by the OD620 value of the supernatant from cells incubated with LPS. Relative absorbance for adjuvants (white bars), non SFD vaccines (grey bars) and SFD vaccines (black bars).
To study in how far spray freeze drying affects the biological activity of adjuvants and vaccines, different adjuvants, untreated or SFD together with WIV and then reconstituted, were analysed on RAW-BlueTM cells for their capacity to activate NFκB (Figure 2). Analysis of adjuvants alone demonstrated that all adjuvants except GPI-0100 were able to activate NFκB in RAW-BlueTM cells, with Pam3CSK4 and MPLA being somewhat more active in this respect than CpG. NFκB activation was further increased when cells were incubated with adjuvant/WIV formulations. WIV itself had little effect and when present in adjuvanted formulations had an additive but not a synergistic effect on NFκB activation. Spray freeze drying did not affect the capacity of the WIV-adjuvant combinations to induce NFκB activation in a negative way. Indeed, for Pam3CSK4-adjuvanted and CpG-adjuvanted WIV the SFD formulations were more potent in activation of RAWBlue cells than the non-SFD formulations. SFD inulin alone had no effect on RAWBlue cells (results not shown) proving the absence of LPS contamination from inulin. These results indicate that the stresses encountered by the adjuvants during spray freeze drying did not have an adverse effect on their immune stimulating capacities.

3.3 Systemic antibody responses after pulmonary vaccination

The immunological properties of the adjuvanted powder vaccines were evaluated in BALB/c mice that received two doses of 2.5 µg HA (approximately 8 µg total viral protein) by the pulmonary route. We compared the humoral immune response generated by mice vaccinated with SFD adjuvanted WIV with that generated by administration of plain SFD WIV. For this purpose blood was collected 21 days after the first immunization or 7 and 30 days following the second immunization (i.e. day 28 and 51 after the first immunization, respectively). Analysis of haemagglutination inhibition (HI) activity (Figure 3a) revealed that after a single immunization only mice immunized with WIV-GPI-0100 developed HI titres greater than 40. An HI titre > 40 is considered as protective in humans[30,31]. This titre was further boosted to >4000 after the second immunization with WIV-GPI and was the highest among all immunized groups. The other adjuvanted vaccine formulations, except WIV-Pam3CSK4+, also induced HI titres higher than unadjuvanted WIV but titres greater than 40 were reached only after the second immunization. These titres remained constant for a month after booster.

Determination of IgG antibody titres in serum (Figure 3b) showed that mice immunized with WIV-MPLA, WIV-CpG or WIV-GPI-0100 produced significantly higher influenza specific IgG titres after the first immunization than mice that received WIV alone. Following the second immunization, the IgG titres were further increased 10-fold and remained at the same level for at least one month. Among the adjuvanted formulations studied WIV-Pam3CSK4 induced the lowest IgG titres and no difference in IgG titres was found as compared to non-adjuvanted WIV. Mice immunised with CpG or GPI-0100-adjuvanted vaccines had significantly higher titres on day 21, 28 and 51 than mice that received non-adjuvanted WIV. However,
Figure 3. Systemic antibody responses after pulmonary vaccination

Mice (n=6) were vaccinated via the pulmonary route on day 0 and 21 with powder vaccines containing 2.5µg HA of WIV derived from A/California/07/2009 (H1N1) alone or with adjuvants. Influenza virus specific humoral responses were evaluated in serum. (a) HI titres from pooled sera (b) IgG titres (c) IgG1 titres and (d) IgG2a titres and (e) IgG2a to IgG1 ratios on day 21 (white bar), day 28 (grey bars) and day 51 (black bars) following immunizations. HI titres after vaccination are expressed as reciprocal of the highest dilution of sera resulting in complete hemagglutination inhibition. IgG antibody levels are expressed as 10log titres. IgG1 and IgG2a levels are shown as µg per ml. Data shown are means ± s.m.e. Levels of significance are presented as *p<0.05, **p<0.01.
mice immunized with GPI-0100 adjuvanted vaccine displayed the highest IgG titres. Quantification of influenza specific IgG subclasses (Figure 3c-d) demonstrated that mice receiving adjuvanted formulations, except the one containing Pam3CSK4, developed significantly higher IgG1 levels than mice immunized with unadjuvanted vaccine. By far the highest IgG1 titres were measured in mice immunized with WIV-GPI-0100. Differences with titres in mice immunized with plain WIV were significant for d21, d28 and d51. Analysis of serum for IgG2a antibody revealed that after the first vaccination WIV-GPI-0100 induced higher IgG2a levels than any of the other vaccine formulations. However, after the second immunization mice vaccinated with WIV CpG displayed similar levels of IgG2a as WIV-GPI-0100-immunised mice. Other vaccine groups also showed increased IgG2a amount after the second immunization, however, the levels were lower than those observed for WIV-CpG or WIV-GPI-0100 immunized mice. Calculation of the IgG2a/IgG1 (Figure 3e) ratio showed that at least on day 51 all powder formulations induced IgG2a as the predominant antibody subtype (IgG2a/IgG1 ratio in the range of 1.1626 to 8.326) except for WIV GPI-0100 (IgG2a/IgG1 ratio = 0.281).

3.4 Mucosal antibody responses after pulmonary vaccination

Earlier we described that pulmonary immunization with non-adjuvanted WIV induced lung IgA but rather little IgA in the nose[14]. To find out whether the use of one of the adjuvants improved the mucosal immune response, nose washes and lung supernatants of the vaccinated and challenged mice were evaluated for antibody responses. Nose IgA responses in mice immunized with non-adjuvanted SFD WIV were higher than in our previous experiment, possibly due to some changes in vaccination technique. None of the adjuvanted vaccines induced significantly higher nose IgA titers than non-adjuvanted WIV (Figure 4a); yet, there was a trend towards higher nose IgA titers in mice immunized with WIV-CpG or WIV-GPI-0100. In contrast, mice vaccinated with WIV-Pam3CSK4 or WIV-MPLA had (significantly) lower levels of nose IgA than mice vaccinated with non-adjuvanted WIV.

Evaluation of lung supernatants for IgA antibody (Figure 4b) showed that mice immunised with WIV-GPI-0100 developed approximately 250-fold higher IgA titres than mice administered with unadjuvanted WIV. A trend towards higher IgA induction in the lungs was also observed in mice vaccinated with WIV-CpG, but was not statistically significant. Conversely, mice immunised with WIV-Pam3CSK4 showed lower lung IgA levels, while WIV-MPLA-immunised mice developed similar levels of IgA compared to mice that received non-adjuvanted WIV. Determination of IgG titres in the lungs supernatants demonstrated that all adjuvants except Pam3CSK4 significantly stimulated lung IgG responses. The IgG titres were highest in mice vaccinated with WIV-GPI-0100.
3.5 Germinal centre formation after pulmonary vaccination

Formation of germinal centres in the spleens was analysed 3 days post influenza virus challenge in immunized mice. For this purpose, splenocytes were analysed by flow cytometry for the presence of B cells expressing the germinal center marker GL7[32,33]. About 50% of splenocytes were B220-positive and no difference in the percentage of B cells was found between vaccinated and control mice (Figure 5a). However, all pulmonary vaccinated mice except those administered with WIV-CpG had significantly higher numbers of GL7+ B cells in the spleens than in the spleens of the control mice (Figure 5b). There was no difference in GL7+ B cell numbers between the mice that received unadjuvanted or adjuvanted vaccine.

To investigate to which extent formation of iBALT takes place upon pulmonary administration of adjuvanted vaccine, lymphocytes were isolated from the lungs of
immunized mice, pooled per experimental group and analysed for GL7+ B cells by flow cytometry (Figure 5c). In control mice (non-immunized, 3 days after challenge) about 1% of the lung lymphocytes were positive for B220 and GL7. In mice immunized with WIV-GPI-0100 the amount of GL7+B220+ lung lymphocytes was 2.2 times higher indicating an effect of the immunization on local B cell populations. The other adjuvants had little effect on the percentage of the size of the germinal centre B cell population.

3.6. Cellular responses after pulmonary vaccination

Cellular immune responses were assessed three days after virus challenge in immunised mice. Splenocytes obtained from the immunized and challenged mice were stimulated with influenza virus for 16 hours for performing ELISpot and for 72 hours for determination of the levels of secreted cytokines. Analysis of the number of IL4-producing cells (Figure 6a) demonstrated that the H1N1 specific T cell response was robust in mice immunized with WIV-GPI-0100. H1N1-specific IL4-forming cells were also observed in splenocyte cultures from mice immunised with WIV.
alone or WIV together with one of the other adjuvants. However, the numbers were significantly lower than those from mice immunised with WIV-GPI-0100. In line with the increased numbers of IL4-producing T cells, the supernatants of splenocyte cultures from mice immunised with WIV-GPI-100 also showed significantly increased levels of IL5 compared to those from mice that were immunized with any of the other formulations (supplementary Fig. 1a). IL-5 secretion was also high in the splenocyte cultures from the mice immunised with WIV alone although these mice did not possess large numbers of IL4 producing splenocytes. The other vaccines did not induce much production of IL5. Evaluation of splenocyte cultures from mice vaccinated with adjuvanted WIV did not show significant differences

**Figure 6. Cellular responses after pulmonary vaccination**

Effects of adjuvants on the number of influenza-specific T cells were evaluated three days after heterologous challenge. Splenocytes isolated were stimulated overnight with or without subunit vaccine derived from A/California/07/2009 (H1N1). (a) IL4- and (b) IFN\(\gamma\)-producing influenza-specific cells were calculated by subtracting spots formed by non-stimulated cells from spots formed by stimulated cells. Similarly, effects of adjuvants on cytokine-producing cells in lungs were evaluated three days after heterologous challenge. (c) Lymphocytes from the perfused lungs were pooled and analysed for IL4- (white bars) and IFN\(\gamma\)- (black bars) producing cells. The results are expressed as spot forming cells per 5 \times 10^5 cells. Levels of significance are presented as *p<0.5, **p<0.01.
in the number of H1N1-specific IFNγ spot-forming cells compared to those from mice that received non-adjuvanted formulations (Figure 6b). However, splenocytes cultures from vaccinated mice had significantly higher numbers of IFNγ producing cells than those from control mice. A similar trend was observed when supernatants of splenocyte cultures were analysed for secreted IFNγ (supplementary Fig. 1b). Irrespective of the immunising agent, stimulated splenocytes from all mice produced more of the Th1-related cytokine IFNγ than of the Th2-related cytokines IL4 or IL5 (compare supplementary Fig. 1a and b). The dominance of IFNγ was least pronounced for the mice immunized with WIV-GPI-0100.

Cellular immune responses in immunized and challenged mice were also analysed in the lungs. Higher numbers of IL4-producing cells were observed in the lungs of vaccinated and challenged mice than in the lungs of control mice which had only been challenged. Among the vaccinated mice the highest number of IL4 producing cells was seen in the lungs of mice that had received WIV GPI-0100. Not much difference was found between IL4-forming cells from the mice that received plain vaccine or vaccine supplemented with MPLA or CpG.

Analysis of lung lymphocyte cultures showed that influenza-specific IFNγ-producing T cells were present in the lungs of all mice, with the highest frequencies in WIV, WIV-MPLA and WIV-CpG-immunized mice and the lowest frequency in WIV-GPI-0100-immunized mice. Overall the lung ELIspot data suggest that all vaccine
formulations, irrespective of their composition, induced IL4- and IFNγ-producing cells in the lungs. The variation in the number of cytokine-producing T cells induced by the various formulations was higher for IL4- than for IFNγ-producing T cells.

3.7 Cross protection after pulmonary vaccination

In our earlier studies we proved that pulmonary vaccination is capable of providing protection against homologous virus challenge[14]. Now we investigated in how far pulmonary immunized mice were protected against challenge with a heterologous virus strain and whether adjuvants contributed to this protection. To this end, mice were immunized with (adjuvanted) SFD vaccines derived from A/California/07/2009 (H1N1pdm) and were challenged with a lethal dose of A/PR/8/34 (H1N1) one month after the second vaccination.

Analysis of serum samples for IgG titres post virus challenge revealed that pulmonary delivery of either of the SFD A/Cal vaccines induced IgG antibody cross-reactive with A/PR/8/34 (Fig. 7a). Adjuvantation of the vaccine with GPI-0100 but not with any of the other adjuvants significantly increased the titre of the cross-reactive IgG. Despite the presence of cross-reactive IgG antibodies in all experimental groups, only mice vaccinated with WIV-GPI-0100 had a detectable A/PR/8/34 (H1N1)-specific HI titer which was however low viz. 8.

Titration of lung supernatants for presence of virus showed that none of the vaccinated mice was able to completely clear the virus (Figure 7b). Yet, in mice vaccinated with WIV-GPI-0100 the lung virus titre was reduced by more than 1 log (>90%) as compared to the virus titre in mice vaccinated with unadjuvanted WIV and this difference was statistically significant. In contrast, mice immunised with any of the other adjuvanted vaccines did not show reduced virus load in the lungs in comparison with the control group.

4. Discussion

The aim of this study was to investigate the suitability of different adjuvants to enhance immune responses to pulmonary delivered whole inactivated virus (WIV) dry powder influenza vaccine. For this, WIV supplemented with different TLR ligands or the non-PRR binding, saponin-derived semi synthetic compound GPI-0100 was SFD using inulin as stabilizer. Physical characterization of SFD vaccines showed that all adjuvanted vaccines were ideally suitable for pulmonary delivery. NFκB activation in RAW-BlueTM cells confirmed that spray freeze-drying did not affect the immune stimulating properties of the adjuvants. A comparative pulmonary immunization study in mice highlighted that all adjuvants, except Pam3CSK4, enhanced the induction of systemic IgG. However, only GPI-0100 was capable of stimulating mucosal IgA responses. The enhanced humoral response in WIV-GPI-0100-immunised mice was associated with increased numbers of influenza-specific IFNγ- or IL4-secreting T cells. Mice immunised with GPI-0100-adjuvanted WIV
were partially protected against heterologous virus challenge while mice immunised with any of the other adjuvanted vaccines were not.

Our study aimed at delivering the WIV-adjuvant vaccines to the lungs as dry powder. For effective pulmonary delivery particle size, surface area and porosity of the powder vaccines are important parameters[24]. The particle properties of SFD vaccines are determined by the compounds present in the dried material, the sugar (disaccharide or polysaccharide) used for stabilization and the conditions employed for spray freeze-drying[14]. We therefore evaluated whether addition of any of the adjuvants to the vaccine/inulin mixture affected the powder properties. Our data indicates that addition of adjuvants to WIV had no adverse effects on the particle size of SFD powder vaccine. Also, all adjuvanted vaccines consisted of spherical and porous particles as desired for pulmonary immunization.

After analysing the SFD vaccines for their physical characteristics and the vaccine compounds for their biological activity, the systemic immune response induced by the adjuvanted pulmonary vaccines was evaluated. Our results show that most adjuvants stimulated the induction of HI titres to levels greater than those induced in mice vaccinated with unadjuvanted WIV. Yet, Pam3CSK4 performed poorly as an adjuvant. In a previous study, Pam3CSK4 administered i.m. along with influenza subunit vaccine did stimulate IgG antibody responses with IgG2a as the dominant antibody subtype[20]. Moreover, when administered to the total respiratory tract together with RSV virosomes, Pam3CSK4 elicited potent cellular and humoral responses[19]. The reason for a suboptimal humoral response in our study may be the combination of Pam3CSK4 and WIV. WIV contains ssRNA that is recognised by TLR7[34]. It has been shown that a combination of TLR2 and TLR7 ligands is not able to induce IL12 or IFNγ in human peripheral blood mononuclear cells[35]. IL12 is an important cytokine for induction of IgG as well as mucosal IgA antibody[36]. Furthermore, in the previous studies 5 or 10 µg Pam3CSK4 were used, these amounts exceeded earlier literature data. The lower amount of Pam3CSK4 in our study (2.5 µg) might have resulted in insufficient stimulation of APCs.

Another TLR ligand that was shown to be a safe and potent mucosal adjuvant is MPLA[37][38]. In our study, pulmonary delivery of MPLA together with WIV in powder form evoked effective serum and lung IgG responses but antibody subtype analysis revealed that the MPLA adjuvanted vaccine induced more IgG1 than IgG2a. Similar results were observed when 0.625 µg MPLA along with 5 µg of HA of WIV was delivered via the pulmonary route. Moreover these results are in line with immune responses induced when Mycobacterium tuberculosis antigen 85A together with MPLA was delivered to the total respiratory tract (TRT)[39]. However, these results contradict observations where TRT delivery of MPLA along with antigen stimulated Th1 responses and thus resulted in high IgG2a antibody titers[38]. The first reason for these contradictory results could be strict pulmonary delivery of vaccine as opposed to TRT delivery. In TRT delivery, APCs in nasal cavity, trachea as well as conducting airways gets activated because the vaccine flows from nasal cavity
to the lungs; while in strict pulmonary delivery only DCs (CD103+ CD11c+CD11b-langerin+) in the conducting airways along with alveolar macrophages(F4/80+ CD11c+CD11b+) might get activated[40,41]. Alveolar macrophages are thought to be immune suppressive and to have an anti-inflammatory role[42,43]. A second reason could be the use of a rather low amount of WIV and MPLA in the vaccine formulations used in our study. Antibody responses were shown to be dependent on the stimulation of innate immunity[44], and insufficient stimulation due to a low amount of MPLA might be the reason for low IgA induction in nose and lungs. Similar to MPLA, CpG enhanced IgG antibody responses in lungs and serum upon pulmonary immunisation. Moreover, pulmonary delivery of CpG-adjuvanted WIV induced four times more IgG2a than IgG1. These results are in line with previous observations where CpG along with bovine RSV successfully evoked production of IgG antibodies upon pulmonary delivery[45]. In this study it was also shown that CpG-adjuvanted pulmonary vaccine elicits highly skewed Th1 responses resulting in predominant induction of IgG2a antibodies in serum. This phenomenon was not only true for inactivated viruses but also for subunit antigen. For example, delivery of CpG together with Mycobacterium tuberculosis antigen 85A resulted in Th1-dominant cellular and humoral responses[39]. However, pulmonary delivery of CpG stimulated moderate IgA responses in nose and lungs. One way to increase IgA responses could be to increase the amount of CpG in the vaccine; a second way could be conjugation of CpG with a nanoparticle[46]. In contrast to the other adjuvanted vaccines, GPI-0100-adjuvanted vaccine induced robust HI and IgG titres upon pulmonary vaccination at all studied time points. These results are in line with previous observations where pulmonary delivery of ISCOMATRIX (IMX), another saponin-based adjuvant, with influenza split vaccine in sheep promoted robust systemic IgG responses[47]. However, analyses of serum antibody subtype and enumeration of cytokine-producing cells showed that GPI-0100 was unable to overcome the predominant Th2 microenvironment in the lungs: GPI-0100 stimulated a 25-fold increase in IgG1 as opposed to a 10-fold increase in IgG2a compared to unadjuvanted WIV. These results are in agreement with previous findings where GPI-0100-adjuvanted influenza subunit vaccine resulted in skewed Th2 responses after pulmonary immunisation[23]. Though GPI-0100 induced a Th2-dominant response, it enhanced both lung IgG and IgA titers by approximately 20 and 1600 fold respectively. Moreover, usage of GPI-0100 showed a trend towards increase of nose IgA demonstrating its potential to boost mucosal immunity. Earlier studies on mucosal immunity indicated that the microenvironment in the respiratory tract favours the development of a Th2-dominated immune response which is the result of anti-inflammatory cytokines such as IL4, IL5 and IL13[48-50]. These cytokines stimulate IgG1 antibody production and suppress production of the Th1-associated IgG2a antibody subtype[51,52]. This phenomenon has been observed in our previous studies where pulmonary immunisation with WIV resulted predominantly in production of IgG1 antibody although upon i.m. immunization
WIV induces a Th1-dominated immune response[34]. In the current study, use of adjuvants, except GPI-0100, resulted in a dominant Th1 response, the hallmarks of which are a high IgG2a/IgG1 ratio and high numbers of IFNγ producing cells. The vaccine formulation containing CpG induced robust IgG2a antibody production and relatively little production of IgG1 and the Th2-related cytokines IL4 and IL5. The amount of IgG2a antibody produced in mice immunized with GPI-0100-adjuvanted vaccine was equivalent to the amount produced in WIV-CpG-immunized mice. Yet, the amount of IgG1 in these mice was very high leading to an ‘unfavourable’ IgG2a/IgG1 ratio of 0.281. Previous studies show that both the IgG1 and the IgG2a antibody subtype play a role in controlling influenza spread in the host. IgG1 controls influenza by virus neutralisation while IgG2a performs the task by helping in clearance of influenza virus from the host by activation of the complement system and by stimulating uptake of opsonized virus by Fc receptor-bearing cells[53]. A study of Huber et al demonstrates that IgG2a alone can protect against influenza infection as effectively as a IgG1/IgG2a mix, while IgG1 is much less effective in protection[53,54]. Thus IgG2a is the more important isotype for protection and the IgG2a/IgG1 ratio is not that important as long as sufficient IgG2a is available. Therefore, though GPI-0100 adjuvanted vaccine gave rise to a dominant IgG1 response, it effectively evoked high IgG2a antibody levels resulting in control of influenza virus infection as seen from this and from previous studies[23,55].

The aim of pulmonary vaccination is to raise IgA antibody titers at the mucosal surfaces so that immediate neutralization of influenza virus is obtained at the port of entry. In our study pulmonary immunisation with any of the vaccines stimulated rather moderate levels of IgA in the nose but high levels in the lungs. The reason could be that the vaccine was directly delivered to the lungs and the nasal mucosa with its antigen-presenting cells was bypassed. Upon antigen re-encounter, memory T and B cells from the draining lymph nodes were shown to preferentially home back to the earlier site of stimulation and then to the adjacent mucosa[56-58]. Thus, antigen-specific lymphocytes stimulated in the lungs would preferentially home back to the lungs and only to a limited extent to the nose. Another factor could be tissue-resident memory T and B cells which stay at the place of activation. For primary influenza infection it has been shown that, rather than homing to adjacent organs or the bone marrow, influenza-specific memory B and T cells remain in the lungs where they can provide immediate protection upon influenza re-encounter and do not home to adjacent organs[59]. Moreover, the surface area of the nasal mucosa is much smaller than that of the lower respiratory tract (20 mm² upper respiratory tract v/s 480 mm² lower respiratory tract) which probably contributes to moderate IgA levels in the nose.

In addition to antibody responses, we evaluated the ability of the adjuvanted vaccines to provide cross protection upon heterologous challenge. Upon doing ELISA, we found that pulmonary immunisation with A/California (H1N1) alone or with any of the adjuvants resulted in production of antibodies which cross-reacted with A/PR/8 (H1N1). However, a measurable HI titre (HI=8) against A/PR/8 (H1N1) was
only observed in mice immunised with GPI-0100 adjuvanted WIV. These results are in agreement with lung virus titers where only GPI-0100 immunised mice had significantly reduced virus in the lungs. However, these results contradict previous observations where i.m. administration of fusion-active, non-adjuvanted WIV was shown to provide protection against infection with a heterologous influenza virus strain[27]. In those experiments, protection could be attributed to CTLs rather than to antibodies [60]. The same paper shows that TRT immunisation does not elicit robust CTL responses. Instead, moderate cross-protective efficacy of TRT immunisation with WIV could be attributed to mucosal IgA[60]. Therefore, in our study the reason for reduced virus titres in the lungs of mice immunised with GPI-0100 could be the presence of IgA in lungs and serum (supplementary Fig. 2). The conditions of the virus challenge experiment were severe. Non-immunized mice challenged with 200 PFU of the used challenge virus are known to reach the humane endpoint (15% weight loss) by day 4 (unpublished observations). This severe challenge might have prevented better control of virus growth by vaccine-induced immune responses and thus lead to an under-estimation of the potency of pulmonary vaccination.

Taken together, our data show that all studied adjuvants tolerated the stresses associated with spray freeze drying and the use of all adjuvants was compatible with the production of powder particles as desired for pulmonary administration. Yet, the adjuvants differed in their capacity to enhance systemic and particularly mucosal immune responses, with GPI-0100 being clearly more effective than the other adjuvants in this respect. To our knowledge this is the first study which evaluates in a head-to-head comparison the capacity of different adjuvants to improve immune responses to pulmonary administered vaccine in vivo and does so by using dry powder vaccine formulations. The results are very encouraging and warrant further studies to determine the minimal amount of adjuvant and vaccine required for protection. In conclusion, pulmonary delivery of stable adjuvanted powder vaccine is a feasible approach for the production of potent mucosal vaccines suitable for preventing spread of seasonal and pandemic influenza infections.

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Supplementary Figure 1. Level of cytokine production in stimulated splenocytes.

Splenocytes were cultured overnight with or without subunit vaccine derived from A/California/07/2009 (H1N1). Cell supernatants from stimulated and non-stimulated splenocytes were analysed for (a) IL5 and (b) IFNγ. Results are presented as the difference in cytokine levels between stimulated and unstimulated splenocytes. Levels of significance are presented as *p<0.5, **p<0.01.

Supplementary Figure 2. Serum IgA responses.

Mice (n=6) were vaccinated via the pulmonary route on day 0 and 21 with powder vaccines containing 2.5 µg HA of WIV derived from A/California/07/2009 (H1N1) alone or with adjuvants. Influenza virus specific IgA response was evaluated from serum on (a) day 21 and (b) day 28.
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