New strategies for simplifying influenza vaccination

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New strategies for simplifying influenza vaccination

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born on 1 August 1984
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Dedicated to mother nature

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Chapter 1

General Introduction

Introduction

Influenza is an infectious disease caused by influenza A viruses. Influenza occurs as seasonal epidemic and pandemic. Almost every human is susceptible to contract this virus. In seasonal outbreaks, the influenza virus causes illness ranging from mild fever to death. In future pandemics, there is a serious risk for high mortality rates because humans are naïve to new influenza virus strains especially the bird flu strains H5N1 and H7N9 [1]. Therefore, it is very important to control the spread of influenza virus during both epidemic and pandemic outbreaks.

The spread of influenza during outbreaks can be controlled either by the administration of antivirals (neuraminidase inhibitors) or by vaccination [2,3]. The neuraminidase inhibitors like oseltamivir and zanamivir are used to control the disease in seasonal and pandemic outbreaks. During the 2009 swine flu pandemic, oseltamivir was the drug of choice for the treatment and prophylaxis of swine flu (H1N1) [4]. However, it does not prevent one's ability to acquire the virus from an infected person. In principle, influenza vaccines are capable of inducing an immune response that can immediately neutralize the virus. Therefore, vaccination is considered to be more effective than the administration of antivirals. Influenza vaccines are available as inactivated vaccines i.e. subunit, split, virosome and whole inactivated virus (WIV) and live attenuated virus (e.g. Flumist®). WIV, subunit and split vaccines are widely used because virosomal and live attenuated virus vaccines are only approved in a few countries [5–7].

Although influenza vaccines are advantageous over antivirals there are several shortcomings related to the route of administration and the production and properties of the vaccine formulation. Except for the live attenuated virus vaccine, which is administered intranasally, all the other types of influenza vaccines are administered either through the intra muscular or subcutaneous route. These routes of vaccination require resources like syringes, sterile needles and the help of trained healthcare workers. These prerequisites further add up to the costs of vaccination. Moreover, due to needle phobia compliance can be compromised. Furthermore, the possibilities of needle stick injuries also add up to limitations of conventional vaccination routes. In terms of vaccine production capacities, both egg and cell culture based influenza vaccine production is limited. As a result during a pandemic, mass vaccination involving high and multiple vaccine doses may not be feasible at the present situation. Current influenza vaccines also have several shortcomings with respect to the immune response they elicit. Despite vaccination, people still can get sick and this could be overcome by inducing stronger immune response in the respiratory tract, i.e. the port of entry of virus. Finally, current influenza vaccines including the live attenuated virus vaccine are unstable and therefore require refrigerated storage and transport, the so-called cold chain. In particular for stockpiling pandemic and seasonal influenza vaccines, storage at ambient temperature would be highly advantageous, as it would significantly reduce the costs. Summarized, there is a need for an influenza vaccine that can be self-administered via a non-parenteral route,

induces a potent immune response at relatively low dose, and is stable. In this thesis, we explored two different strategies to develop a vaccine/vaccination routine that could possibly fulfill these requirements.

In the first strategy, we envisage to develop a dry and stable powder vaccine that can be administered through the pulmonary route. The main advantage of this route of administration is its ease of administration. Therefore, it requires a minimal involvement of trained health care workers [8]. A dry powder formulation is preferred over a liquid formulation because it has been shown before that when dried in the presence of stabilizing excipients, the antigenicity of the vaccine is not affected during drying and storage for extended period of times at ambient conditions. [9]. Moreover, with the application of proper drying techniques, powder particles can be produced that are suitable for inhalation [10–12]. Obviously, it is of utmost importance that during storage not only the antigenicity of the vaccine is maintained but also the physical powder properties. Changes in physical powder properties e.g. irreversible agglomeration, would severely compromise the suitability of the powder for pulmonary administration. As the physical characteristics of vaccine powders during storage have not been studied before, this issue will be addressed in this thesis. The feasibility of pulmonary administration of influenza vaccine has been studied before. In several studies it has been shown that pulmonary vaccination can induce not only potent systemic immune responses but can also elicit immune responses in the respiratory tract, the port of entry of virus [9,12]. However, it fails to induce a balanced Th1/Th2 immune response [9]. In addition, pulmonary vaccination induces poor IgA antibody responses in the nose. We hypothesize that the quality of immune response can be improved by the co-administration of adjuvants. Moreover, application of adjuvants may facilitate dose sparing. Therefore, in this thesis various adjuvants were incorporated in the dry powder formulations and evaluated in a mouse model.

In the second strategy, which might be in particular suitable during a pandemic outbreak, we envisage to develop a sublingual tablet containing a stabilized vaccine of a previous strain, which can be used as primer for a booster vaccine prepared from the current drifted strain. As described above, the influenza pandemic outbreaks create a big risk of vaccine shortage. It has been described that the immune response against newly drifted influenza strains can be primed with old vaccine strains [13]. The major advantage of this strategy is that the old vaccine can be produced in large quantities because time constraints lack. However, the priming doses used in that study were unstable liquid formulations, which will hamper stockpiling of these vaccines. Moreover, the liquid formulations were administered by injection, which has the shortcomings as described above. To overcome both the disadvantage of the antigen instability and the parenteral administration, we propose to develop a stable tablet formulation that can be administered sublingually. As described above, using stabilizing excipients and proper drying techniques, the vaccine can be brought in a dry and stable state. In this thesis, it is investigated whether or not this powder can be formulated into a tablet suitable for sublingual applications while the antigenicity

of the vaccine is maintained. We propose to apply a sublingual tablet because it is in particular suitable for mass vaccination campaigns during a pandemic. Furthermore, we have chosen for this route of administration because it has been shown in previous studies that the sublingual administration of influenza vaccines can successfully induce immune responses [14–16]. However, these studied were performed with unstable liquid influenza vaccines. Moreover, to our knowledge the sublingual prime and heterologous booster strategy has never been explored before.

Highlights of this thesis

The highlights of this thesis are,

- The storage stability for stockpiling influenza vaccines
- Improve the immune response by co-administering adjuvants with stabilized pulmonary influenza vaccines
- The prime-boost strategy for vaccination during pandemics with sublingual tablet and heterologous i.m. booster vaccine.

Outline of the thesis

In chapter 2, we reviewed the importance of particulate influenza vaccines. The current status of liposomes, virosomes, nanoparticles and virus like particles in influenza vaccination was discussed in a broader context. The advantages and limitations of these vaccine systems were described. This review also includes a discussion on regulatory challenges, compliance issues and perspectives of particulate influenza vaccines.

In chapter 3, the physical powder properties and the immunogenic stability of pulmonary influenza vaccine powders during storage was evaluated. The influenza vaccine was spray freeze dried in the presence of inulin, dextran and a mixture of dextran and trehalose. The dry powder formulations were stored at different temperatures for 3 months. The physical powder properties such as particle size and specific surface area before and after storage were evaluated. The immunogenic stability of the stored influenza vaccines was evaluated in mice.

In chapter 4, δ-inulin, a carbohydrate based adjuvant was evaluated for its adjuvant effect when co-administered with pulmonary influenza vaccines. The immune responses induced by adjuvanted and pulmonary influenza vaccines alone in mice were compared.

In chapter 5, monophosphoryl lipid A, a lipid based vaccine adjuvant was tested with pulmonary influenza vaccine powder in mice. The receptor binding capacity and immunostimulatory properties of the adjuvanted influenza vaccine was evaluated. The antibody levels and memory B cell levels induced by adjuvanted vaccine were compared to that of pulmonary vaccine alone. The advantages of

adding monophosphoryl lipid A to influenza vaccine are discussed in comparison to vaccine alone.

In chapter 6, a wide range of vaccine adjuvants were evaluated with pulmonary influenza vaccine powders in mice. The adjuvants tested were Pam3CSK4, monophosphoryl lipid A, CpG-ODN-1826 and GPI-0100. The immune responses induced by these adjuvanted influenza vaccines were compared. The choice of optimal adjuvant for pulmonary influenza vaccine is also discussed in this chapter.

In chapter 7, a prime-boost strategy of influenza vaccination was evaluated in an *in-vivo* study using mice. In this study, the immune responses were primed with a single dose of sublingual influenza vaccine. Later, the immune response was boosted with a heterologous i.m. influenza vaccine. Furthermore, the possibilities to formulate the influenza vaccine into stable sublingual tablet were explored.

In chapter 8, the results of the research described in this thesis are summarized and the perspectives are discussed.

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Chapter 2

Review on particulate influenza vaccines

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Introduction

Influenza is an airway infection caused by influenza viruses. Influenza viruses are classified into three types, Influenza A, B and C. Influenza A infections are more common compared to the other types. Influenza A virus mutates rapidly, hence humans are susceptible to frequent influenza virus attacks. In an influenza epidemic, the symptoms of infection range from cough, sore throat and fever, to a general feeling of illness. Antigenic shift in influenza may result in a pandemic strain that can be fatal. The burden of influenza epidemics is huge and both direct (health care) and indirect (a.o. economic losses due to reduced productivity) costs add to this burden. Direct health care costs alone are already estimated to be over 50 million Euro per million of inhabitants in Europe [1]. Both vaccines and antiviral drugs (neuraminidase inhibitors) can contain the spread of influenza virus. However, in terms of protection, vaccines provide protection for longer period compared to the current antiviral drugs.

Vaccination against the influenza virus is an effective strategy to control the spread of virus in pandemics. Currently, influenza vaccines are available as subunit, split, and live attenuated virus (LAV) and whole inactivated virus (WIV) vaccine. A subunit influenza vaccine comprise of surface antigen hemagglutinin and/or neuraminidase. Subunit vaccines are safe for vaccinating more fragile patient populations. Split influenza vaccines are prepared by splitting the viral membrane with diethyl ether and a detergent or a detergent alone to impair the replicating ability of the virus. Split vaccines usually include the essential viral proteins like hemagglutinin, neuraminidase along with other membrane proteins. Both the subunit and split influenza vaccines are considered to be safe. Nevertheless, they have their own limitations regarding stability and antigenicity. The WIV and LAV vaccines are more immunogenic compared to subunit and split vaccines. Previous studies have reported that high immunogenicity of WIV and LAV is due to the presence of genetic material. The RNAs are potent TLR 7 (toll-like receptor 7) agonists [2]. Due to their high immunogenicity WIV and LAV are not suitable for vaccination against influenza in high-risk patient populations. Usually, the high-risk group includes the elderly population (age above 65 years), children (age above 6 months), pregnant and lactating women and people with an impaired immune system.

High-risk patient populations are the main target for influenza vaccination programs during pandemic outbreaks. Since the WIV and LAV are not suitable for vaccination in this group, they are vaccinated either with subunit or split influenza vaccines. However, both subunit and split influenza vaccines are moderately immunogenic. Hence, it is very important to improve their immunogenicity. To improve the immunogenicity of these vaccines several options exist, they include (i) the administration of multiple vaccine doses, (ii) the use of an adjuvant or (iii) or formulation of the antigens in special delivery systems.

Influenza vaccines are supplemented with adjuvants either to improve their immunogenicity or to save doses during pandemics. The immunogenicity of vaccine can be improved with a variety of adjuvants **(Table 1)**. The licensed and widely used adjuvants for influenza vaccines include: alum, MF59, ASO3, and ASO4 [3]. Many other adjuvants have been described but they are mostly in the preclinical and clinical trial stages [4–6], and for most is still unclear whether they will ever meet the strict requirements necessary to gain regulatory approval.

Particulate vaccines are vaccines in which the key antigen is formulated together with one or more adjuvants in a special delivery system to form a particular structure. Most of the particulate-based vaccine (PBV) systems and the adjuvants used are still in the developmental stage, only a few products have made their way to market. Inflexal®, a virosomal influenza vaccine, is an example of this last category. A special feature of this product is that it is considered safe even in children [7].

Table 1. Overview of Influenza vaccine adjuvants classified in different categories

Category	Adjuvants	Refs
Alum		[8]
Nano-emulsion	ASO3, MF59	[9,10]
CpG oligonucleotide	CpG-A, CpG-B	[4]
Cytokines	IL-1, γ - IFN, IL-2, IL-4, IL-12	[11,12]
Fusion Proteins	Flagellin	[13]
Inulin	γ Inulin, δ Inulin	[14]
GEM		[15,16]
Lipids	Ceramide Carbomyl- Spermine, α-Galactosylceramide	[17,18]
Bacterial Toxin	CT, LT, mCTA-LTB	[19]
Saponin	GPI-0100	[20]
Other adjuvant	MPLA (TLR 4)	[21]

Particulate vaccines facilitate effective antigen presentation, but in a different manner compared to other adjuvants like alum, saponin or TLR ligands. The latter adjuvants will, in general, stimulate the immune response through mechanisms such as slow release of antigens (Alum), induction of proinflammatory cytokines (saponin), activation of TLR receptors induction of immunomodulatory cytokines, whereas, PBVs improve the immune response through antigen presentation.

Table 2. *Particulate influenza vaccine adjuvants and their route of delivery*

Formulation	Types	Route of administration	Refs
Liposomes	Cationic	i.m, i.n	[17,25–28]
	Ceramide carbamoyl-spermine		
	Lipid/DNA-complex		
	Mucoadhesive		
	Peptide coupled		
Virosomes	Heat labile toxin from *E.coli*	s.l., i.n.	[29,30]
	LpxL1		
Virus-like particles	Flagellin from *salmonella*	i.m, i.n	[31]
Proteosomes	Outer membrane proteins of *Neisseria meningitides*	i.n.	[32]
Nanoparticles	Chitosan	s.c., i.n., oral	[6,33]
	γ –glutamate / Chitosan		[34–36]
	poly-(ε-caprolactone) / Chitosan		
	Chitin		
Microparticles	Surf clam		
	Eudragit S and trehalose		
ISCOMS	Quil A glycoprotein, cholesterol, phospholipid	i.m., i.n.	[37,38]
Bliosomes	Bile salts	oral	[39]

The components used in PBV-based vaccine delivery systems target the antigen effectively to antigen presenting cells (APC). Additionally, PBVs are particulate in nature which may make their appearance similar to that of influenza virus particles [22–24]. At present the research on PBVs mainly focuses on systems like liposomes, nanoparticles and microparticles. Other PBV delivery systems include proteosomes and bilosomes, whereas nanoemulsion based systems like MF59 and ASO3 have been approved for human use. In figure 1 a schematic overview of the structure and appearance of different PBVs is presented.

Particulate vaccine delivery forms have their own specific advantages and limitations when used with influenza vaccines. An update on these vaccine delivery systems will
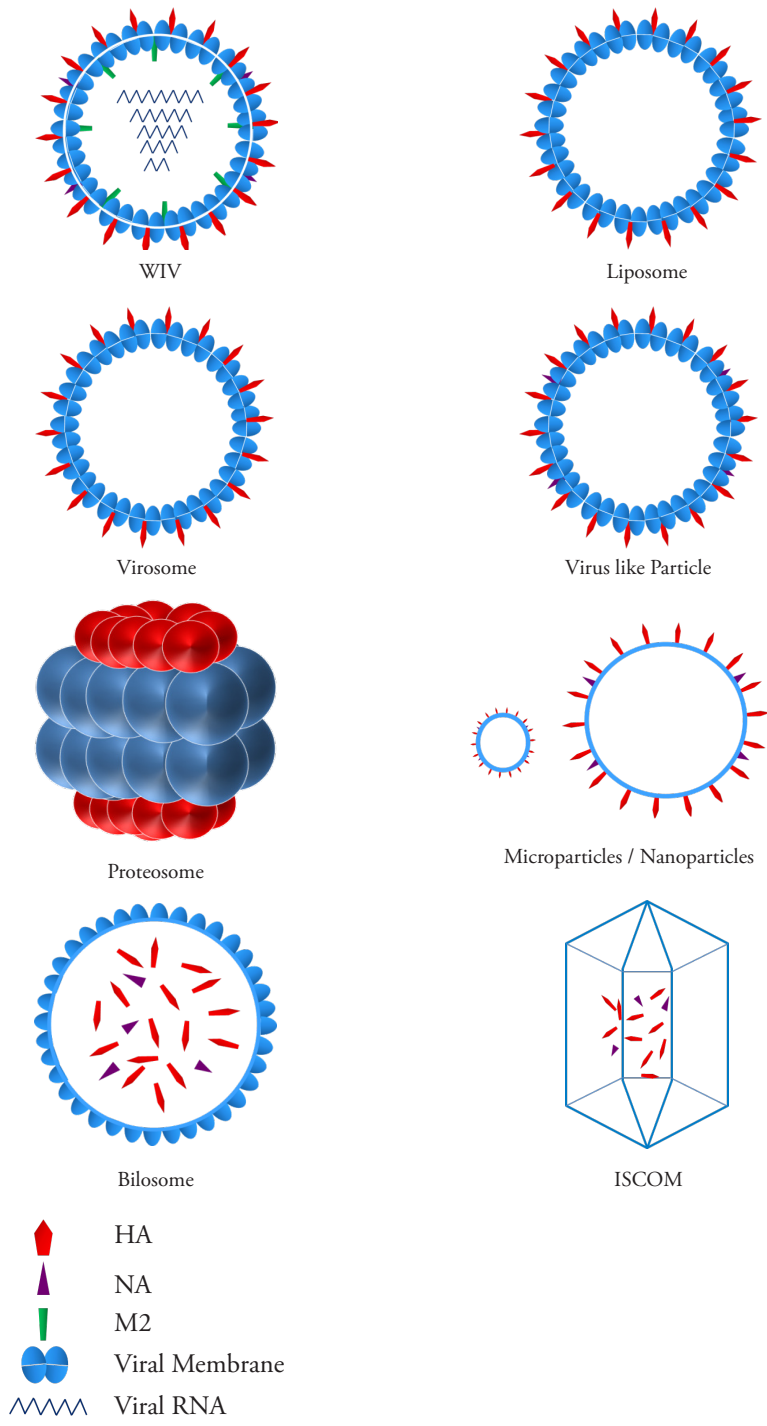
Figure 1. Schematic overview of the structure and appearance of different PBVs.

be presented in this review **(Table 2)**. In addition, the compliance and regulatory issues pertaining to different PBVs will be discussed briefly. Finally, the future perspectives of PBV also in relation to their intrinsic stability will be discussed.

1. Liposomes

Liposomes were first described in the early 1960's. Since their discovery, significant research efforts have been devoted to tap the potentials of liposomes. Liposomes can contain both hydrophilic and lipophilic drugs. The presence of hydrophilic inner core and lipophilic compartment in the bilayer made the liposome an attractive carrier for vaccine delivery. Nevertheless, the antigen entrapment in liposome is a challenging task. Only twenty year after their discovery, the liposomes were first investigated for possible adjuvant activity. The first study focused on the adjuvant activity of liposomes on tetanus toxoid vaccine [40]. Later, researchers investigated a wide range of antigens originating from bacteria, protozoa, and viruses. The immune responses induced by the liposomal antigen products were reported to be better than the responses induced by native antigens [41,42]. The main reason for effective antigen presentation of liposome is considered to be the lipid bilayer, which adsorbs the antigens with electrostatic charge. Moreover, the liposomal bilayers serve as a depot to supply antigens to APC at a slower rate [43].

Although the basic liposomal vaccine formulations were already shown to boost the immune response, research was progressed by investigating the effects of modified liposomes. The modifications included altering the size, entrapment efficacy and charge, as well as the incorporation of secondary adjuvants like cytokines, ceramide carbamoyl-spermine, lipid-DNA complex and peptides [44–47]. The size and the entrapment efficacy of liposomal influenza vaccines was shown to have some relation to the adjuvant activity [44]. It was reported that split influenza vaccine has the tendency to bind strongly to cationic liposomes formulated with cholesterol [48] this could potentially improve the antigen entrapment in the liposome which is still one of the major challenges in this type of products. The adjuvant effect in cationic liposomes mainly depends on the activation of extracellular signal regulated kinase and induction of CC chemokine by the cationic phospholipids [46]. It was shown that electrostatic forces of liposomal bilayer resulted in improved adsorption of antigens to bilayer and that improved adsorption resulted in good entrapment efficacy of antigen in liposome [49]. Liposomal flu vaccines supplemented with IL-2 proved to be safe in clinical trials and the incorporation of the cytokine in the liposomal formulation enhanced the immune response [47].

The parenteral administration of liposomal flu vaccines induced both humoral and cell mediated immune response. The liposomes form a vaccine-depot at the injection site which prolongs the antigen presentation and mediates a strong Th1 immune responses [50]. However, a liposomal formulation containing matrix 2 (Me2) proteins as antigen induce a Th2 immune response [123]. Hence, the type of immune response induced by liposomal vaccines depends on the type of antigen

in liposome vaccine formulation administered. Intra nasal (i.n.) administration of liposomal flu vaccines induced a mucosal immune response [51]. Influenza vaccines capable of inducing mucosal immune responses are considered superior to conventional influenza vaccines, as they induce the immune response at the port of entry of the virus (nose and airways). This boosts the research on mucosal liposomal influenza vaccines [52–54]. Muco-adhesive formulations of these liposomal flu vaccines improved the residence time of antigen for the uptake by APC in the mucosal sites [27].

In recent years, the inclusion of secondary adjuvants to liposomes has improved the transport and uptake of antigen from the mucosal surfaces. A synthetic cord factor from M. tuberculosis in liposomes was reported to improve the transport of antigens through the mucus membrane [53]. Transport of antigen in the mucosal layer can be adopted for vaccination against respiratory viruses like influenza. Liposomes with poly-cationic lipids like CCS can further boost the immune response of influenza antigens via the i.n. route and, has been demonstrated in mice and ferrets [55]. Furthermore, complexation of DNA with cationic liposomes improved the antibody responses by providing cross protection against drifted influenza strains [26,56].

2. Virosomes

The main difference between virosomes and liposomes is the presence of the surface antigens like HA or NA on the outside of the latter. Virosomes more or less resemble a virus particle without the genetic material of native influenza. The term virosome was proposed in 1975 to the modified liposomes with influenza surface proteins over the lipid bilayer [57]. In the virosomes the influenza surface protein HA is bound to the surface of the lipid bilayer, arranged in a mode similar to that of the HA in the influenza virus. This specific arrangement increases the fusion capacity of the virosomes with cell walls significantly [58,59].

In the early years of virosome research, they were developed as a carrier for biopharmaceuticals [60]. Virosomal vectors containing viral envelopes of stomatitis and sendai virus have been developed [61,62]. However, the virosome with influenza viral proteins is the most commonly used due to its preserved fusion activity [63]. Next to being an influenza vaccine type, influenza virosomes can also be used as a carrier or adjuvant for other antigens like hepatitis A. They can actively present the antigen to APC by binding to the sialic acid end receptor as they still maintain the binding capacity and fusion properties of the HA [64].

Vaccination studies in humans have reported that influenza virosomes are more immunogenic compared to influenza subunit vaccine, and they have been approved for human use, administered via i.m. injection [65,66]. The positive response after parenteral administration paved the way to investigate other routes of administration for virosomal vaccine formulations. Virosomal vaccines given intranasally to ferrets showed that virosomes elicited an immune response after mucosal vaccination [67]. Moreover, the virosomes induced a local immune response in addition to

the systemic immune response. Safety studies revealed that the phospholipids used in virosomes are safe and immuno-tolerable making them suitable for frequent influenza vaccination [68].

The addition of secondary adjuvants to virosomes can further improve the immune response (after mucosal administration). Preclinical studies of virosome with trivalent influenza vaccine and heat labile toxin from *E. coli* in different animals proved to be safe and efficient in inducing antibodies against the viruses [29]. The addition of lipopolysaccharide LpxL1 to influenza virosome shifted the immune response towards Th1 [30]. Sublingual administration of virosomal flu vaccines was shown to induce secretory IgA antibodies in the upper respiratory tract. Hence, virosomal influenza vaccines are considered effective formulations for controlling the virus in pandemics [69].

3. Virus-like particles

Virus-like particles (VLP) are developed to improve the immunogenicity of recombinant protein antigens that suffer from poor presentation to APC [70]. VLPs can actively present the antigen to the APC, which in turn increases the immune response against the native virus [71]. They resemble native virus particles, however they carry no genetic material in them. Hence, they cannot replicate, but they are immunogenic to the same extent as the native virus when loaded with antigens. VLPs can be produced by cloning the genome responsible for the expression of the antigenic material in the virus to the target vector plasmid [72]. Usually they are expressed in insect cell lines and in some cases even in plants [72–74]. The vaccine against the human papilloma virus and hepatitis B has been tested clinically and showed positive response in boosting the immune response [75].

Influenza VLPs comprising HA, NA and M1 proteins induced protective immune response against the virus in preclinical studies [76,77]. The i.n. immunization with influenza VLPs induced a broad spectrum of antibodies against heterologous influenza viruses [78]. In addition, cross-protection over different clades has been reported when animals were immunized with H5N1 VLPs [79]. Mucosal vaccination of VLPs can induce both systemic and mucosal immune responses [80,81]. One i.n. dose of influenza VLPs in mice was shown to induce an immune response comparable to that induced by two i.m. doses of VLP or adjuvanted VLPs, a response sufficient to protect against the virus [82]. This observation demonstrates once more that also the route of immunization affects the immune response. This was further confirmed by a study showing that the intradermal immunization of VLPs with micro-needles prompted an immune response superior to i.m. VLP immunization [83].

Influenza VLPs mixed with secondary adjuvants like Novosome and flagellin from salmonella further boosted the immune response [31,84]. The influenza VLPs with Me2 protein induced superior immune response with cross-protection against a variety of influenza virus strains [72]. Hence, this approach could open the avenues for research towards universal influenza vaccines. The Me2 protein (considered to

be the conserved epitope in influenza virus) with the VLP may improve the antigen presentation.

4. Proteosomes

Proteosomes are hydrophobic particles that include bacterial outer membrane proteins and trace amounts of purified lipopolysaccharide. The outer membrane proteins are isolated from *Neisseria meningitides* [85]. Two strategies have been adopted in the development of proteosome-based vaccines. In the first approach, the vaccine antigen is combined non-covalently with proteosomes by complexation. This approach may incur some antigen loss [86]. In the second approach, the vaccine antigen is simply mixed with proteosomes prior to vaccination [87]. The proteosome particles activate the APCs by both TLR2 and TLR4 activation [88]. Proteosome-based vaccines produced by both methods induce both mucosal and systemic immune response when administered via the mucosal route [86,87]. Furthermore, i.n. vaccination studies with influenza subunit vaccine with proteosomes showed induction of a mucosal immune response that protected mice from virus challenge [89,90]. The i.n. proteosome-based influenza vaccines induce a strong Th1 immune response [91]. After successful preclinical studies in animals the clinical studies were performed with healthy volunteers for the evaluation of safety and efficacy in man and the outcome this study showed that influenza proteosome vaccines are safe and effective in inducing immune responses [32,92]. In the last decade, research on proteosome-based influenza vaccines has been performed with the conserved epitope of the influenza virus. The i.n. vaccination of this proteosome–based vaccine with conserved epitopes resulted in a positive immune response [93]. Hence, influenza vaccines based on proteosomes can be used as a carrier/adjuvant to elicit a protective immune response against the influenza virus. Noticeably, the proteosome-based vaccines are effective when administered via i.n. route, making proteosomes interesting adjuvants for non-invasive influenza vaccination.

5. Nanoparticles and micro-particles

Nanoparticles are used as a carrier of a variety of biopharmaceuticals including vaccines, toxins and other antigens. A wide range of materials is used to formulate these nanoparticles. The most commonly used materials are chitosan, chitin and combination of these materials with γ-glutamate or poly-(ε-caprolactone). Preclinical studies have been performed to ascertain the safety and efficacy of the vaccine nanoparticles [94]. The encouraging results of these nanoparticle based vaccine studies opened new avenues in vaccine research. Later, research on nanoparticle influenza vaccines administered through i.n. route showed that i.n. vaccination is effective. The immune response induced by these influenza nanoparticles are comparable to those induce by conventional vaccine [94]. Additionally, influenza nanoparticle vaccination via i.n. route induced mucosal immune response. Although effective immune responses after i.n. administration to humans of chitosan formulations have been reported, little progress was seen in this field over the past decade. The

inclusion of Poly (γ-glutamic acid) in chitosan nanoparticles with influenza subunit vaccine further improved the immune response, by providing protection against the influenza virus challenge [6,95]. It was also reported that the adjuvant activity of the chitosan / poly(γ-glutamic acid) nanoparticles is mediated through both humoral and cellular immune response [96]. Simple physical mixing of trimethyl chitosan nanoparticles with WIV also boosted immune response in mice with protection against the virus [97]). The chitosan nanoparticles with poly-(ε-caprolactone) also induced superior immune response with protection against influenza [33].

Similar to nanoparticles, microparticles can be formulated with chitosan and chitin. Chitin microparticles vaccination through the i.n. route induces immune responses that protect against a viral challenge [34,98]. The micronized particles of surf clam shell showed adjuvant activity with i.n. influenza vaccines [99]. The i.n. administration of influenza vaccines adjuvanted with surf clam microparticles reduced the lung virus titers after challenge [100]. Oral administration of microparticles formulated with Eudragit S and trehalose induce cross protective immune response against different influenza virus [36]. Since these microparticles induce good level of immune response compared to conventional influenza vaccines, chitosan based delivery systems may act as potential influenza vaccines in the future. Recent study on safety of the chitosan revealed that it is safe for use in humans for i.n. administration [101]. The preclinical studies on chitosan based nanoparticles have showed promising results. However, the safety and immunogenicity of nanoparticles based influenza vaccines have to be reaffirmed with successful clinical studies.

6. Bilosomes

Bilosomes are lipid vesicles formulated with bile salts. The bile salt vesicles protect the conformation of proteins/antigens even at low pH values like that in the stomach. Moreover, this kind of vaccine formulations were reported to effectively transport the antigen through the GI mucosa to gut-associated lymphoid tissue [102]. Studies on influenza subunit vaccine in bilosome revealed that it induces Th2 with no Th1 type of immune response. Additionally, it induces mucosal immune response [103]. Tailoring of immune response with influenza bilosome is achieved by altering the size of the bilosome [39].

7. ISCOMs

ISCOMs are composed of Quil A glycoprotein matrix, cholesterol and phospholipid. The hydrophobic nature of matrix facilitates the antigen attachment [104]. Administration of ISCOM adjuvanted vaccines via the i.m., i.n. and intraperitoneal route was studied for effectiveness [105–107]. The ISCOMs formulated with influenza glycoprotein showed superior immune responses compared to native influenza glycoproteins. Moreover, it also protected the animals against the lethal virus challenge [108]. However, the vaccine failed to protect the animals against the virus challenge with distant drifted influenza virus [109]. Indicating that, ISCOM

influenza vaccine may provide better protection against the closely related influenza strains but not against distant drifted variants.

Regulatory challenges

Currently adjuvants are not licensed separately; they are licensed only in combination with a specific vaccine. This is a logical approach since efficacy can never be proven in formulations without any antigen and because the adjuvant activity may vary between different vaccines. The regulatory challenges involved in licensing of these adjuvanted vaccines normally includes mode of action, non-clinical toxicity studies, clinical safety and post marketing surveillance [110]. Hence, it can take a while before a vaccine for human use will be approved. The EMEA and WHO guidelines stress that adjuvanted vaccines should be non-toxic, safe and effective. Moreover, the WHO also suggests that the new adjuvanted vaccines should prove their effectiveness over the standard adjuvant i.e. the alum adjuvanted vaccine [111,112] or over the non-adjuvanted formulations (when the current standard is non-adjuvanted).

Compliance issues

The particulate influenza vaccines have to be safe when compared to the conventional vaccines. The preclinical studies showed that several PBVs are safe and immunogenic. However, safety and immunogenicity studies in humans are required and their success will determine the future of PBVs. In addition to administration via conventional (parenteral) routes, PBVs are also developed to enhance the immune response after administration via non- invasive routes like sublingual, pulmonary, intranasal or oral administration.

Almost all the PBVs were shown to boost the immune response after i.n. administration in animal studies, however, none has be approved for human use so far. An important aspect of i.n. vaccination is neurotoxicity, because in recent past a virosomal influenza vaccine with CTL adjuvant induced Bell's palsy in humans. Hence, it is very important to study the toxicity of adjuvants used in influenza vaccine formulations before proceeding to the clinical studies.

Influenza vaccination via the sublingual mucosa has gained interest in recent years, because s.l. vaccination with adjuvanted influenza vaccine was shown to induce local immune responses along with serum level protection in preclinical studies. This route, however, requires high doses of antigen to elicit an adequate immune response as the tolerance level is high for microbial antigens in the oral mucosa. Hence, a potent adjuvant is required for s.l. vaccines. Lipid-based delivery systems like liposomes and virosomes are likely to provide the best chances for transporting the vaccines across the mucosal membrane [69], although the success of this approach in humans has not been proven so far.

The oral route of vaccination was considered ineffective in flu vaccination. However, research on oral vaccination has demonstrated that administration via this route can

also elicit immune responses once the antigen is protected against the low gastric pH and enzymatic degradation [113,114]. Although it is to be mentioned that so far this concept was proven effective in animals only. Novel oral delivery approaches like bilosomes can protect the antigen from denaturation or inactivation. Furthermore, they can transport the antigen effectively across the g.i. mucosa to elicit an immune response thereby improving the compliance level of vaccination.

Perspectives of particulate influenza vaccines

At present, only a few PBVs are commercially available and they are all liquid formulations. Liquid formulations have a major drawback regarding stability. The particulate structure mixed with or containing the antigen has to be stabilized since its efficacy is thought to depend on its structure. A complicating factor is that in several of the new formulation types the antigen degrades faster than in the native WIV or LAV type formulations, since the intrinsic stabilizing effects of the virus wall components are missing in the new formulations. One of the options described to prevent the degradation of antigens in the particulate vaccine formulations is to convert them into solids [115–117]. Thereby, the movement of the antigens is restricted at the molecular level [118]. This can be achieved by lyophilizing the antigen with suitable lyoprotectants. Lyoprotectants that have been described include trehalose, mannitol, inulin and dextran. Choosing the corrected stabilizer is an important parameter as different materials provide different degrees of stabilization. For novel vaccination formulations with an improved compliance level, the particulate vaccines should be formulated in a form that is suitable for their intended route of administration.

The liquid vaccines seem best suited for the i.n. vaccination as suitable delivery devices exist. However, devices for nasal powder administration have also been described [119,120]. Administration via the s.l. or oral route requires a suitable unit dosage form too. Formulating the stabilized vaccine into a tablet will further improve the compliance and reach of vaccination among the population during pandemics. Alternatively, the novel particulate vaccines can be formulated as powder vaccines for pulmonary delivery. Pulmonary vaccination of particulate influenza vaccines may improve immune response through active presentation of antigen to the APC in lung [121,122]. Mucosal vaccination with PBV formulations may increase the immune response. Especially, the induction of local IgA responses may be of interest since this may actively neutralize the virus at the port of entry.

Conclusion

The formulation of influenza antigens in novel particulate-based formulations may boost the immune response after vaccination and open new (non-invasive) routes of administration. Moreover, the immune response could be boosted by combining these new PBVs with other potent adjuvants. PBVs mainly boost the immune response through an improved (often active) presentation of the antigen to APCs. However, several particulate vaccine formulations may need stabilizing formulations

since they are intrinsically unstable. Processing them into solid formulations may be a suitable solution for this problem, whereas the solid material may also be more suitable for various alternative routes of administration like the pulmonary route. Additionally, administration via a non-invasive route may be more suitable for rapid self-administration during mass vaccination campaigns. In conclusion, the particulate influenza vaccine formulations may be the suitable candidates in the future for mass vaccination programs during pandemic outbreaks. However, major efforts in preclinical and clinical development are still necessary before the new type of PBVs can be introduced in the market.

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Chapter 3

Physical and immunogenic stability of spray freeze dried influenza vaccine powder for pulmonary delivery: comparison of inulin, dextran or a mixture of dextran and trehalose as protectants

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Abstract

One of the advantages of dry influenza vaccines over conventional liquid influenza vaccines is that they can be used for alternative routes of administration. Previous studies showed that spray freeze drying is an excellent technique to prepare vaccine containing powders for pulmonary delivery (Amorij,J-P.; et al, 2007; Audouy,S.A.; et al, 2011). The aim of this study was to investigate the physical and immunogenic stability of spray freeze-dried whole inactivated virus influenza vaccine prepared by using inulin, dextran and a mixture of dextran and trehalose as protectants. Physical and biochemical characteristics of the vaccine powder were maintained at temperatures up to 30 °C for three months. In addition, in vivo data indicate that also the immunogenic properties of the vaccine were maintained under these storage conditions. On the other hand, in vivo results also revealed that subtle changes in powder characteristics were induced during storage at 30 °C. However, laser diffraction measurements showed that problems associated with these subtle changes can be overcome by using dry powder inhalers with an efficient powder dispersing capacity.

1. Introduction

Influenza vaccination is the main strategy for the containment of the virus during influenza outbreaks. Unfortunately, every year many doses of influenza vaccines are discarded as the potency of the vaccine is lost due to the limited stability of the aqueous dispersion[1,2]. A major disadvantage of current influenza vaccines is that they remain stable within the narrow temperature range of 4 to 8 °C only, which limits their widespread use especially during pandemic outbreaks. In addition, liquid vaccines are mainly administered via conventional routes like intramuscular (i.m.) and subcutaneous (s.c.) injection. Drawbacks like pain and needle fear reducing compliance, the chance for needle stick injuries and the need for trained health care workers to administer the injection, make injection a far from ideal method for vaccination [3]. Finally, influenza vaccines administered by injection predominantly induce a systemic immune response, which only provides protection at the systemic level and is not considered to be very effective in protecting the most vulnerable population, e.g. young children and elderly adults [4-6]. Although intranasally administered (i.n.) live attenuated influenza vaccine provides both local and systemic immune responses [7,8], it shares other drawbacks with conventional vaccines such as the requirement of a cold chain. Furthermore, this vaccine is not suitable for immune-compromised persons and children.

An attractive alternative to the current dosage forms could be a dry powder for pulmonary administration. Already more than 40 years ago several studies were performed to demonstrate the potential of pulmonary immunization using liquid influenza vaccine formulations [9,10]. The outcomes of these studies indicate that pulmonary immunization can induce same level of systemic immune responses compared to immune responses induced by vaccine administered via injection in humans. In addition, more recent studies show that pulmonary immunization also induce a local immune response, which bestows additional protection against influenza virus infection at the site of virus entry and might even provide cross-protection against infection by heterologous viruses [11]. A further advantage of pulmonary immunization is the improved patient compliance. In addition, these vaccines do not require the involvement of trained health care personnel as they can be self-administered. This procedural simplicity would be an advantage in particular during a pandemic.

In the early studies in man liquid vaccine formulations were used and they were administered via nebulizers which are currently known for their poor and irreproducible dosing in the lung [12]. These problems may have been the reason for discontinuation of this research in the seventies of the previous century. However, modern technologies can overcome these problems. Moreover, scientific progress in drying technologies and dry powder inhalation systems has opened new possibilities for the development of stable dry powder inhalation systems for vaccines.

Pulmonary administration of influenza vaccines recently has regained interest because it became apparent that biopharmaceuticals such as vaccines can be brought

in a dry and stable state by incorporating them in a matrix of a sugar glass by freeze drying, spray drying or spray freeze drying (SFD) [13-16]. In previous studies we have shown that various types of influenza vaccines can be stabilized by freeze drying using the oligosaccharide inulin as stabilizer [17-19]. For pulmonary delivery we envisage that whole inactivated virus (WIV) influenza vaccine would be the best option since it is more immunogenic than other types of influenza vaccines [20-23]. For an effective deposition in the lungs, WIV has to be formulated into powder particles with an aerodynamic particle size ranging from 1-5 μm [12,24,25], which can be achieved by SFD [26]. Recently, Audouy et al [27] showed that WIV can be SFD in the presence of inulin without loss of its immunogenicity and that the powder is suitable for pulmonary administration. However, neither the physical nor the immunogenic storage stability of the SFD WIV powder was investigated.

The aim of the present study was to investigate these aspects of storage stability for a period of three months at various temperatures. The following parameters were evaluated: (i) physical powder characteristics, (ii) biochemical integrity of WIV, and (iii) in vivo antigenicity after pulmonary administration to mice. Additionally, we studied the possibilities to replace inulin by dextran or a mixture of dextran and trehalose (dex/trh) as a stabilizer. Dextran has excellent amorphous bulking properties when lyophilized [28] and low molecular weight dextran can also act as a lyoprotectant [29]. Also trehalose, a disaccharide, can preserve biopharmaceuticals during lyophilization [30-32]. However, due to its large specific surface area, SFD trehalose rapidly absorbs moisture when exposed to air and due to its relatively low glass transition temperature (Tg) of 121 °C, the powder easily becomes sticky (personal observations). These properties of SFD trehalose will impose problems while handling and dispersing a powder to an aerosol for inhalation. Therefore, to circumvent problems with stickiness of SFD trehalose we investigated a mixture of trehalose with dextran as the latter possesses a much higher Tg (220 °C). Hence, dextran and dex/trh [30] were investigated next to inulin in this study.

2. Material and Methods

2.1 Virus

Live influenza virus A/Hiroshima/52/2005 (A/Hir/H3N2) was kindly provided by Solvay Biologicals (Weesp, The Netherlands).

2.2 Vaccine preparation

WIV was produced by inactivating live A/Hir/H3N2 virus by overnight incubation with 0.1 % β-propiolactone (Acros Organics, Geel, Belgium) at room temperature in citrate buffer (125 mM $Na_3C_6H_5O_7$, 150 mM NaCl, pH 8.2) under continuous rotation. The inactivated virus was then dialyzed overnight at 4 °C against hepes buffered saline (HBS, 2 mM hepes, 125 mM NaCl, 0.9 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2$; pH 7.4). WIV protein content was determined by micro-Lowry

assay and its purity was analyzed by SDS-PAGE under reducing and non-reducing conditions followed by silver staining.

2.3 Spray freeze-drying

WIV was SFD together with inulin (4 kD; Sensus, Roosendal, The Netherlands) or dextran (6 kD; Sigma-Aldrich, Zwijndrecht, The Netherlands) or dex/trh (Cargill, Kerfeld, Germany) at a weight ratio of 1:1. A dispersion of WIV in HBS buffer containing 5 % w/v stabilizer was prepared at an HA:sugar weight ratio of 1:200. For placebo powder, a solution containing 5 % w/v stabilizer in HBS buffer without WIV was used. The dispersion/solution was pumped at a flow rate of 5 ml/min through a two-fluid nozzle (diameter 0.5 mm) of a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) and sprayed using an atomizing air flow of 600 ln/hour in liquid nitrogen. The liquid nitrogen was allowed to evaporate after which the frozen droplets were placed on the shelf (pre-cooled to a temperature of -55 °C) of a Christ Epsilon 2-4 freeze dryer. Drying was performed at a pressure of 0.220 mBar with a condenser temperature of -85 °C. The shelf temperature was gradually increased from -55 °C to 4 °C over 32 hours. Thereafter, the pressure was decreased to 0.055 mBar and the shelf temperature was gradually increased to 20 °C over 11 hours. The powder vaccine was then collected in a hood at a relative humidity of 10% or less and was stored at -20, 4, 30, and 40 °C in hermetically sealed injection vials for various periods of time.

2.4 Transmission electron microscopy

SFD vaccines were reconstituted with sterile water. Liquid and SFD formulations were dialyzed against ammonium acetate buffer (75 mM ammonium acetate, 2.5 mM Hepes, pH 7.4) overnight at 4 °C. Dialyzed samples were placed on a glow discharged 200 mesh copper grid covered with Formvar film. Samples were stained with 3 % ammonium molybdate, pH 7.2 and analyzed on Philips CM 12 transmission electron microscope (TEM).

2.5 Hemagglutination assay

WIV containing 0.1 µg/µl of hemagglutinin (HA) was diluted 1:10 (w/v), in PBS (154 mM NaCl, 12 mM Na_2HPO_4 0.9 mM KH_2PO_4, pH 7.4) and 50 µl was added in 96-well V-bottom plates containing 50 µl of PBS and serially diluted twofold in PBS. Subsequently, 50 µl of 0.1 % guinea pig red blood cells (RBC; Harlan, Zeist, The Netherlands) in PBS was added. Hemagglutination was determined two hours after incubation at room temperature. Hemagglutination titers were expressed as $\log_2$ of the highest dilution showing agglutination of RBC and recorded as one hemagglutination unit (HAU).

2.6 Physical characterization of the powders

Scanning electron microscopy (SEM) was performed with a JEOL JSM 6301-F microscope (JEOL Ltd., Tokyo, Japan). Samples were prepared by placing the powders on double-sided sticky carbon tape on a metal disk. Then the particles were coated with a layer of approximately 10 nm of gold using a Balzers 120B sputtering device (Balzer UNION, Liechtenstein). Images were captured at a magnification of 1000x.

The geometric particle size distribution of the SFD powders was measured using a HELOS compact model KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany). The powders were dispersed using a RODOS dispersing system at a pressure of 1 bar or using the dry powder insufflator (Penn-Century Inc., Wyndmoor, USA).

The specific surface area of the SFD powders was determined using a Tristar surface analyzer (Micrometrics Instrument Corp., USA). The samples were loaded on the surface area analyzer, and the surface area was determined using the multipoint BET method from the nitrogen adsorption isotherm at 77 K.

2.7 Immunization of mice

Animal experiment handling and work protocols were approved by the local animal welfare and use committee of the University of Groningen, The Netherlands. An in vivo study was carried out in 6-8 weeks old female BALB/c mice (Harlan, Zeist, The Netherlands).

Mice were immunized twice at an interval of three weeks with the different WIV formulations containing 5 µg HA. For pulmonary vaccination, mice were anesthetized by inhalation of isoflurane/O_2. Then the mice were intubated in vertical position with a modified Autograde catheter (Becton Dickinson, Breda, The Netherlands). SFD vaccine powder was delivered using a dry powder insufflator (Penn-Century Inc., Wyndmoor, USA). Approximately 1 mg of SFD vaccine powder was delivered to the lungs by giving three puffs with the insufflator. 50 µl of liquid aerosol vaccine was administered using an IA-1C Micro-sprayer aerosolizer for mice attached to the FMJ-250 high-pressure syringe (Penn-Century Inc., Wyndmoor, USA), and 50 µl of liquid aerosol of HBS was administered to the control group. Mice were placed in a recovery incubator at a temperature of 25 °C for two hours, and then placed back in the housing facility.

One week after the second dose, mice were sacrificed for evaluation of the immune response. After sacrifice, blood was withdrawn by heart puncture. Serum was stored at -20 °C until used. Nose washes and broncho-alveolar lavages (BAL) were obtained using 1 ml PBS, pH 7.4, containing complete protease inhibitor cocktail tablets (Roche, Almere, The Netherlands).

2.8 ELISA

Influenza specific IgG, IgG1, and IgG2a antibody amounts in serum and IgA antibody levels in nose wash, BAL and serum were measured using ELISA. The ELISA plates (Greiner bio-one, Alphen a/d Rijn, The Netherlands) were coated overnight at 37 °C with 500 ng/well of A/Hir/H3N2 WIV. Coated plates were washed once with coating buffer (17.8 mM Na_2CO_3, 22.5 mM $NaHCO_3$, pH 9.6) and blocked with 2.5 % milk powder in coating buffer. After washing once with coating buffer and twice with PBS containing 0.05 % Tween 20 (PBST, pH 7.2), serial dilutions of the serum samples were applied to plates. Plates were then incubated at 37 °C for 90 minutes and were subsequently washed with PBST. Then, 100 µl of horseradish peroxidase (HRP) conjugated antimouse IgG, anti-mouse IgG1, anti-mouse IgG2a or anti-mouse IgA (Southern Biotech, Birmingham, USA) diluted 1:5000 in PBST was added followed by incubation at 37 °C for 60 minutes for the detection of IgG, IgG1, IgG2a and IgA, respectively. After extensive washing, 100 µl phosphate-citrate buffer (0.2 M NaH_2PO_4, 0.1 M citric acid, pH 5, containing 0.04% o-phenylenediamine and 0.012 % H_2O_2) was added. The enzymatic reaction was allowed to proceed at room temperature for 30 minutes and stopped by adding 50 µl of 2 M H_2SO_4. The absorbance was measured at 492 nm using a Synergy HT reader (BioTek, Winooski, USA). Average IgG titers were determined as log_{10} of the reciprocal of the sample dilution corresponding to an absorbance of 0.2 at a wavelength of 492 nm. IgA titers are presented as average of maximum absorbance of 1:1 diluted nose and lung washes. IgG1 and IgG2a concentrations were determined using a calibration curve made by overnight coating 0.1 µg anti mouse IgG at 37 °C. Following extensive washing, increasing concentrations of 100 µl of IgG1 or IgG2a (Southern Biotech, Birmingham, USA) was added to the plates. Average influenza HA-specific IgG1 or IgG2a responses are presented as concentrations µg/ml.

2.9 Hemagglutination inhibition (HI) assay

HI titers were determined by HI assay as described before [33]. In brief, 75 µl of serum (pooled per experimental group) was inactivated by incubation at 57 °C for 30 minutes. In order to prevent the non-specific hemagglutination, 225 µl of 25 % kaolin was added and incubated for 20 minutes. The suspension was then centrifuged for 2 min at 1400 x g. 50 µl of the resulting supernatant was added in duplicate to 96-well V-bottom plates containing 50 µl of PBS and, diluted serially till the last well of the plate. Subsequently, 50 µl of PBS containing 4 HAU was added to each well and mixed with a multichannel pipette. The plates were then incubated at room temperature for 40 minutes. Finally, 50 µl of 1 % guinea pig RBC was added and hemagglutination was allowed to proceed at room temperature for 2 h. The highest serum dilution capable of preventing hemagglutination was recorded as the HI titer.

2.10 Statistical analysis

The antibody titers are mentioned as geometric mean ± standard error mean. The difference in antibody titers was analyzed by one tailed Mann Whitney U-test at a confidence interval of 95 % (P≤0. 05). The significance was denoted by an increase in the number of symbols: one symbol (P≤0.05); two symbols (P≤0.01).

3. Results

3.1 Physical characterization of powders

Several physical characteristics of the SFD vaccine and placebo powders were evaluated in this study i.e. particle size, powder morphology, and specific surface area.

SEM of SFD powders

Analysis of the morphology of freshly prepared SFD inulin, dextran and dex/trh by SEM revealed that all three sugars formed highly porous spherical particles with interconnected pores and a particle size ranging from 1 to 10 μm **(Figure1)**. No difference in powder morphology was observed for the different sugars. The high porosity and interconnectivity of the pores can be contributed to the removal of the ice crystals by sublimation, which were formed during freezing. Hence, mirror images of ice crystals are seen in the SEM images.
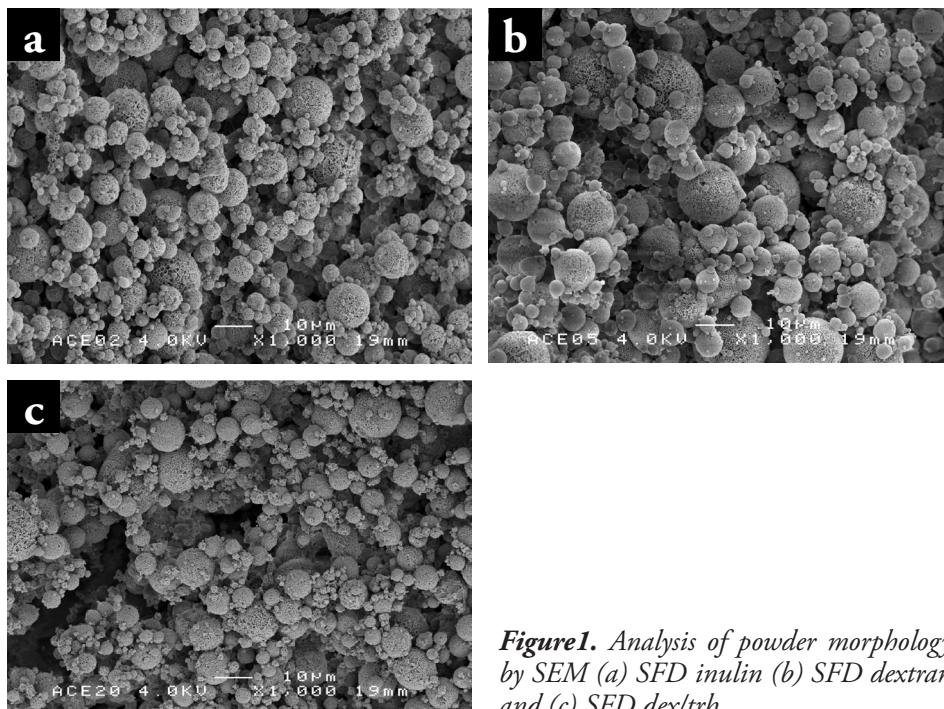

Figure1. *Analysis of powder morphology by SEM (a) SFD inulin (b) SFD dextran and (c) SFD dex/trh.*

Particle size of vaccine and placebo powders

To be deposited in the lungs after inhalation, a particle should have an aerodynamic particle size between 1 and 5 μm. Larger particles are likely to be deposited in the throat while smaller particles will be exhaled after inhalation [34]. Laser diffraction measurements revealed that SFD yielded powder particles with cumulative geometric size distribution of X10, X50 and X90 of 3.5, 8 and 15 μm, respectively, **(Table1)** which may seem too large. However, laser diffraction measurements yield the geometric particle size and not the aerodynamic particle size. The aerodynamic particle size can be calculated from the geometric particle size by the following equation [35]:

$$d_{ae} = d_e \sqrt{\frac{\rho_p}{\rho_o \chi}}$$

Where, dae is the aerodynamic diameter, de the geometric particle size, ρ_p the density of the particles (g/cm³), ρ_0 the unit density (1 g/cm3) and, χ the dynamic shape factor. The atomized liquid droplet leaving the nozzle was measured by the laser diffraction and had an average geometric particle size of approximately 8 μm that was similar to the particle diameter of SFD powders. Apparently, the particle size of liquid droplet did not shrink during freeze-drying. Therefore, the solid content of the solution before SFD can be used to calculate the density of the final SFD powder particles. Since the total solid concentration of the solution before SFD was 50 mg/ml, the density of the SFD powder particles will be: = 0.05 g/cm³. The dynamic shape factor of spherical particles is 1 and the SEM **(Figure 1)** confirmed the spherical shape of particles. Hence, it can be calculated that the aerodynamic diameter of X10, X50 and X90 of the SFD vaccine powder were around 0.8, 1.8 and 3.3 μm, respectively, indicating that except for a small fraction these powders were suitable for inhalation.

Table 1. *Comparison of geometric diameter of spray freeze-dried vaccine and placebo powder as determined by laser diffraction using the RODOS disperser.*

Sample	Vaccine powder (μm±SD)			Placebo powder (μm±SD)		
	X_{10}	X_{50}	X_{90}	X_{10}	X_{50}	X_{90}
Inulin	3.56 ± 0.01	8.01 ± 0.03	14.84 ± 0.02	2.94 ± 0.12	7.48 ± 0.39	20.93 ± 0.11
Dextran	3.67 ± 0.02	8.30 ± 0.04	15.42 ± 0.01	2.29 ± 0.12	7.64 ± 0.05	17.95 ± 0.17
Dex/trh	3.25 ± 0.01	7.65 ± 0.31	14.78 ± 0.23	2.78 ± 0.83	6.96 ± 0.12	14.85 ± 0.33

Laser diffraction measurements revealed that immediately after production the geometric particle size distributions of the SFD inulin, dextran and dex/trh powders containing WIV and the corresponding placebo powders were similar **(Table 1)**. Since for physical characterization huge amounts of powder are required and because the sugars were in large excess in the powders (weight ratio HA/sugar = 1/200),
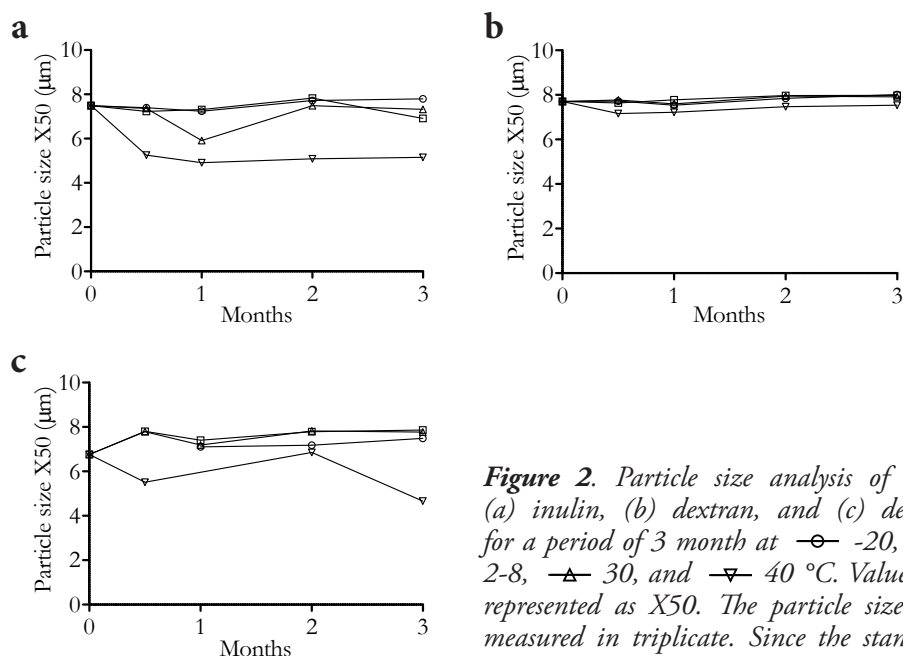
a

b

c

Figure 2. *Particle size analysis of SFD (a) inulin, (b) dextran, and (c) dex/trh for a period of 3 month at* ―⊖― *-20,* ―⊟― *2-8,* ―△― *30, and* ―▽― *40 °C. Values are represented as X50. The particle size was measured in triplicate. Since the standard deviations were small the error bars are not visible.*

placebo powders were used to evaluate the effects of storage on the shape and size characteristics.

To evaluate whether the particle size of the SFD powders changed during storage, the powders were analyzed by laser diffraction at various time intervals. The measurements **(Figure 2)** revealed that the particle size of SFD inulin, dextran and dex/trh stored at -20, 4 or 30 °C did not change. However, the particle size of SFD inulin stored at 40 °C was reduced 15 day after storage. The particle size of the SFD dextran was found to remain the same for 3 months at 40 °C. In case of the SFD dex/trh stored at 40 °C, particle size slightly reduced after 15 days of storage, and substantially after 3 months of storage.

Evaluation of dry powder insufflator performance

The powder dispersing capacity of the dry powder insufflator was evaluated with the SFD inulin, dextran and dex/trh powders using laser diffraction. It was found that the particle size distribution of powder leaving the dry powder insufflator was highly irreproducible between the puffs. Moreover, it was found that the dry powder insufflator was dispersing particles with a bimodal distribution, i.e. particles with a size of about 10 μm and some with a size of about 100 μm. The X10, X50 and X90 values of a typical measurement with SFD powders dispersed by the insufflator are shown in **Table 2**. Laser diffraction measurements using the RODOS disperser showed no bimodal particle size distribution but small particles only (average of

Table 2. *Results of a typical example of a laser diffraction measurement of SFD inulin, dextran and mixture of dextran and trehalose using the dry powder insufflator.*

Sample	Freshly prepared powder (μm)		
	X_{10}	X_{50}	X_{90}
Inulin	21.99	100.55	153.13
Dextran	19.81	92.04	149.55
Dex/trh	4.06	13.96	119.84

3

a

b

c
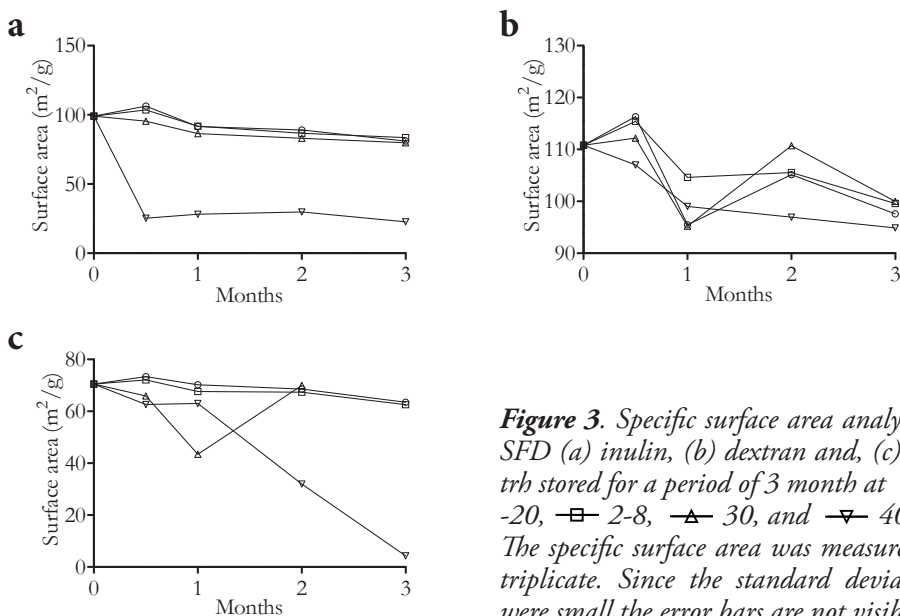
Figure 3. *Specific surface area analysis of SFD (a) inulin, (b) dextran and, (c) dex/trh stored for a period of 3 month at ─○─ -20, ─□─ 2-8, ─△─ 30, and ─▽─ 40 °C. The specific surface area was measured in triplicate. Since the standard deviations were small the error bars are not visible.*

around 8 μm; see **Table 1**). Dry powder insufflator has been reported efficient in dispersing spray dried powders [36]. However, our results show that the dry powder insufflator was not able to deagglomerate the highly fluffy SFD powders efficiently.

Specific surface area of placebo SFD powders

The specific surface area of placebo SFD powders before and after storage at different temperatures up to three months was evaluated by BET analysis **(Figure 3)**. This analysis indicated that, immediately after preparation, the produced powders, irrespective of the sugar used, had a high surface area, thus confirming the presence of highly porous particles as observed by SEM. Yet, SFD inulin and SFD dextran both had a higher specific surface area (100-120 m²/g) than dex/trh (around 75 m²/g). As mentioned in section 1.2., the density of the SFD particles were the same for all three sugars, which implies that the porosity was same in all cases. Since the specific surface area of SFD inulin and dextran was smaller than that of SFD dex/trh, it can be concluded that the pore sizes in the SFD inulin and dextran particles were smaller than those in the SFD dex/treh particles. This indicates that during the
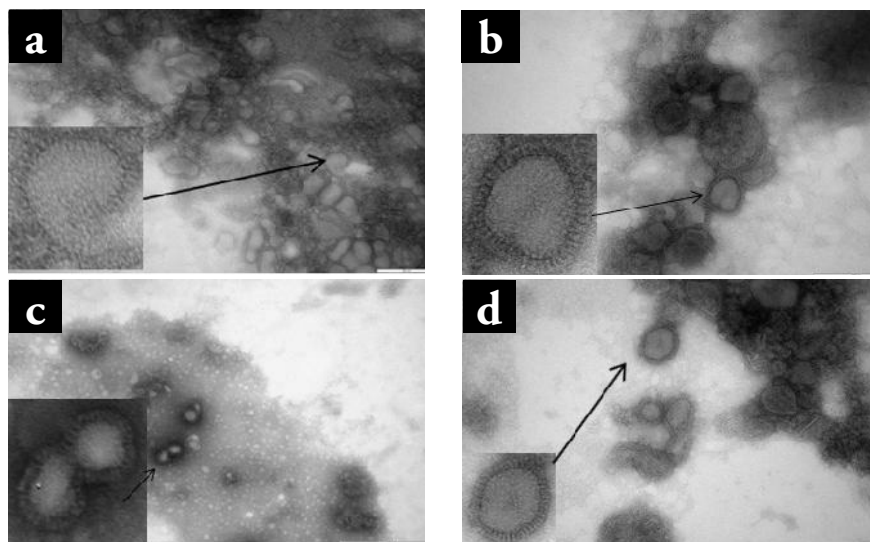
Figure 4. TEM images of (a) unprocessed WIV and WIV SFD in the presence of (b) inulin, (c) dextran and (d) dex/trhand then reconstituted.

freezing step of the SFD process, nucleation of ice crystals was slower in the inulin and dextran solution than in the dex/trh solution which might be related to the lower viscosity of the latter.

The specific surface area of all SFD powders remained unchanged during storage for 3 months at -20, 4 or 30 °C. However, at 40 °C the specific surface area of the SFD inulin was significantly reduced after 15 days of storage. Also the specific surface area of the SFD dex/trh was not stable during storage at 40 °C as it was reduced to 40 m^2/g after 2 months and to 5 m^2/g after 3 months. In contrast, the specific surface area of the SFD dextran sample was maintained during storage at 40 °C for 3 months.

In conclusion, all three SFD powders could be stored at temperatures up to 30 °C for at least 3 months without substantial change of their physical powder characteristics. However, higher storage temperatures led to changes in the physical properties of SFD inulin and dex/trh but the powder properties were retained in SFD dextran.

3.2 Characterization

Morphology of WIV

The immunogenicity of WIV relies on the presence of intact virus particles, which retain the structure of the live virus and harbor all virus components including the single stranded viral RNA which is an important trigger of innate immune reactions [37]. To evaluate whether the particulate nature of WIV was preserved during the drying process, the SFD powders were reconstituted and then analyzed by TEM. **(Figure 4)**.
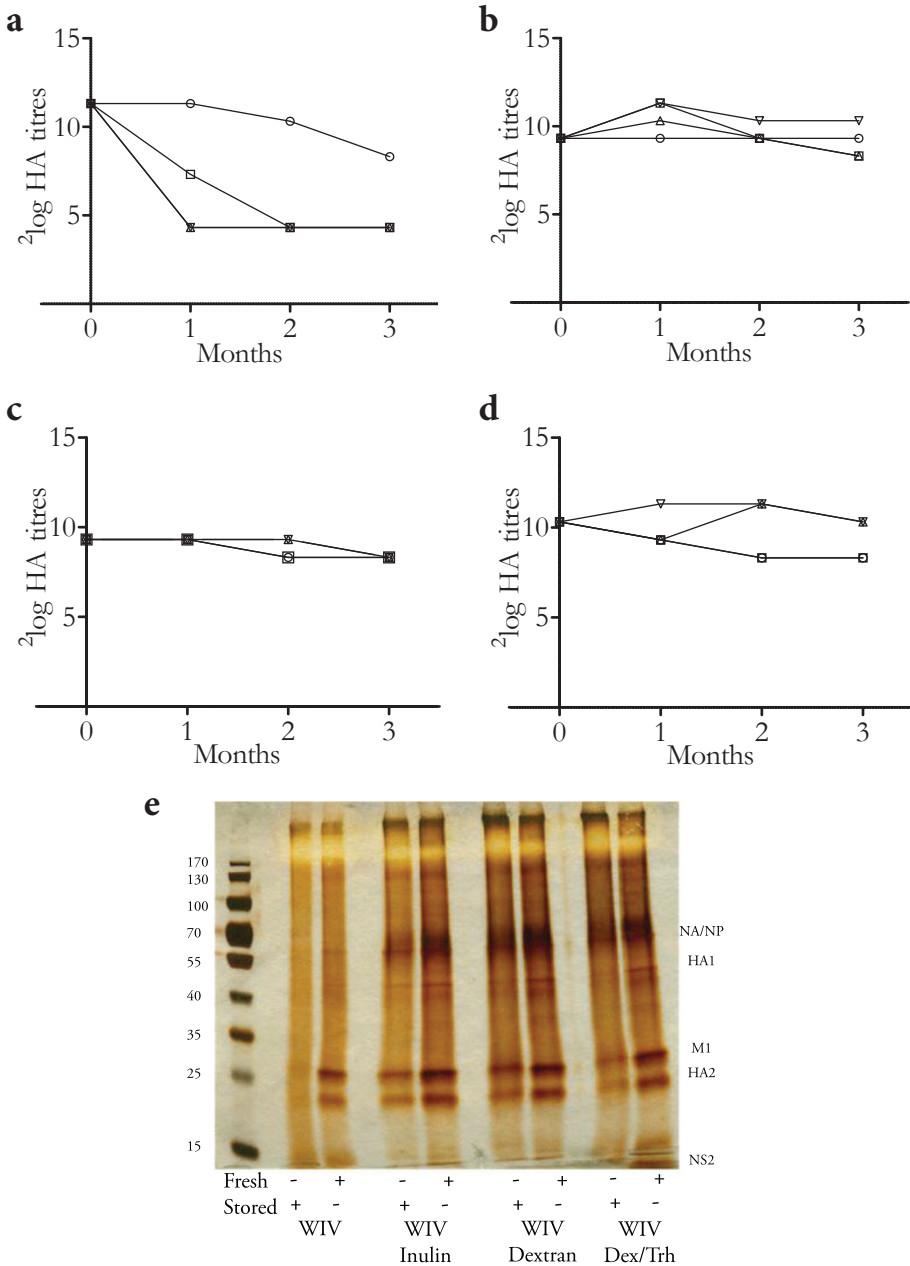
Figure 5. *Hemagglutination titers of WIV in (a) unprocessed liquid, (b) SFD inulin, (c) SFD dextran and (d) SFD dex/trh stored for 3 months at ─○─ -20, ─□─ 2-8, ─△─ 30, and ─▽─ 40 °C. The HA titers of SFD dex/trh stored at 30 °C are superimposed with HA titers of samples stored at 40 °C from 2 months. The HI titers were measured in triplicate. Since no differences in HA titers were found no error bars are shown. (e) SDS PAGE analysis of freshly prepared and SFD and reconstituted WIV stored for three months at 30 °C.*

TEM images of liquid WIV and WIV SFD in the presence of inulin, dextran or dex/trh and then reconstituted with water revealed that stress related to SFD did not affect the particulate nature of WIV. Presence of WIV particles with sizes of 100 nm to 150 nm and clearly visible spikes on the viral membrane indicates that inulin, dextran and dex/trh are suitable stabilizers during SFD of WIV.

Hemagglutination titers

The biological activity of HA after SFD with inulin, dextran or dex/trh and subsequent storage at -20, 4, 30 or 40 °C for up to three months was evaluated by measuring virus binding to a target cell membrane in a hemagglutination assay **(Figure 5)**.
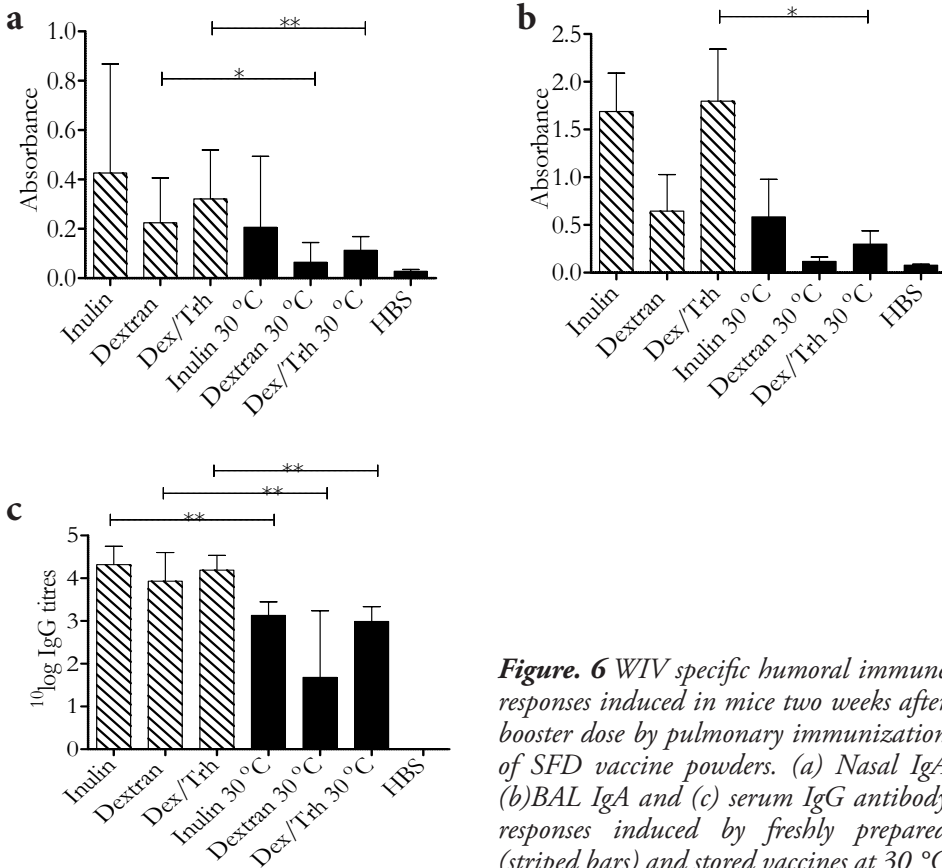

Figure. 6 WIV specific humoral immune responses induced in mice two weeks after booster dose by pulmonary immunization of SFD vaccine powders. (a) Nasal IgA (b)BAL IgA and (c) serum IgG antibody responses induced by freshly prepared (striped bars) and stored vaccines at 30 °C (black bars).

The activity of HA in unprocessed WIV **(Figure 5a)** was reduced by more than 100-fold ($\log_2 7$ when stored at 30 °C or 40 °C for 1 month. In addition, there was a gradual loss in hemagglutination titer for samples stored at -20 and 4 °C. The hemagglutination titers of WIV after SFD with inulin, dextran or dex/trh were found to be unchanged after storage for a period of 3 months at temperatures up to 40 °C **(Figure 5b-d)**.

To further determine the molecular stability of the proteins present in WIV, freshly prepared vaccines and vaccines stored for three months at 30 °C were analyzed by SDS-PAGE. The gel of freshly prepared WIV showed a band pattern consistent with the structural proteins present in WIV **(Figure 5e)**. Four bands associated with proteins of WIV were observed on the gel, viz., HA1: 47 kD, NA: 50 kD and HA2: 29 kD. No difference was found in the band pattern between freshly prepared liquid or reconstituted SFD formulations and SFD formulations stored at 30 °C. Yet, the band color intensity was observed to be lower for the SFD formulations stored at 30 °C. However, a larger difference was observed in the banding pattern between freshly prepared WIV and liquid WIV stored at 30 °C. Bands of HA1 and HA2 which could be easily identified in freshly prepared WIV were almost absent in WIV stored at 30 °C for 3 months.

Preservation of the hemagglutinating function of HA and the unchanged protein patterns revealed by SDS-PAGE demonstrate that the stresses of the SFD process and storage of the SFD powders at 30 °C for three months did not result in aggregation or cleavage of the WIV proteins.

3.3 Immune response after vaccination

Evaluation of humoral immune responses evoked by SFD and reconstituted vaccines

To evaluate the effect of the different sugars on preservation of the immunogenic properties of the vaccines mice were immunized twice via the pulmonary route with the different SFD vaccine powders which were either freshly prepared or stored at 30 °C for 3 months. Nose washes, BAL and serum samples were collected one week after the second dose and analyzed by ELISA **(Figure 6a-c)**. No significant differences were found in the immune responses evoked by freshly prepared SFD vaccine powders with the different sugars except for BAL IgA which was somewhat lower for the SFD vaccine powder based on dextran . Comparison of IgA antibody responses in nose and BAL washes between mice that received freshly prepared and stored SFD vaccine powders showed that in all cases there was a decreased IgA antibody production in the mice vaccinated with the stored formulations although the differences were not always significant **(Figure 6a-b)**. Similarly, evaluation of IgG antibody titers in serum revealed that SFD vaccine powders stored at 30 °C for three months induced significantly lower IgG antibody titers than freshly prepared SFD formulations vaccine powders **(Figure 6c)**, and the same trend was seen for the HI titers of the sera (data not shown).
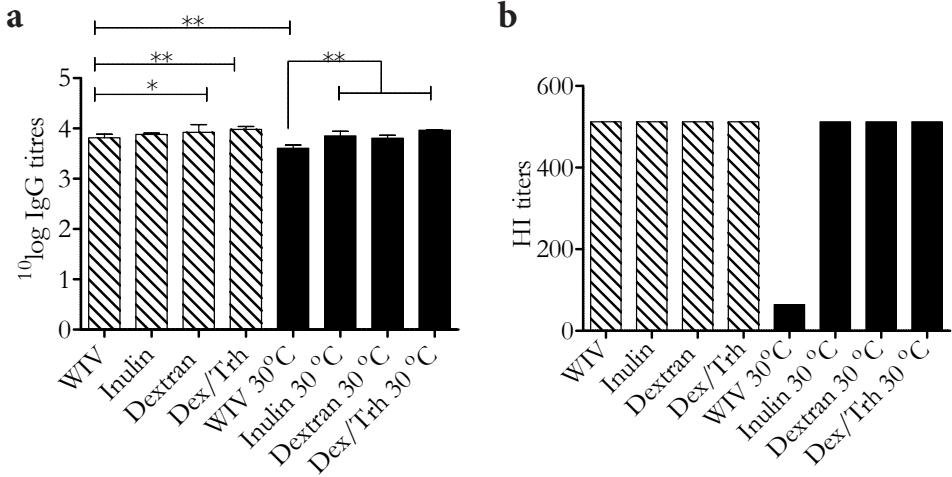
a

b

Figure 7. *WIV specific humoral immune responses evoked by reconstituted SFD vaccines. (a) Serum IgG antibody responses and (b) Serum HI titers induced by freshly prepared (striped bars) and stored vaccines at 30 °C (black bars) administered in liquid form in mice.*
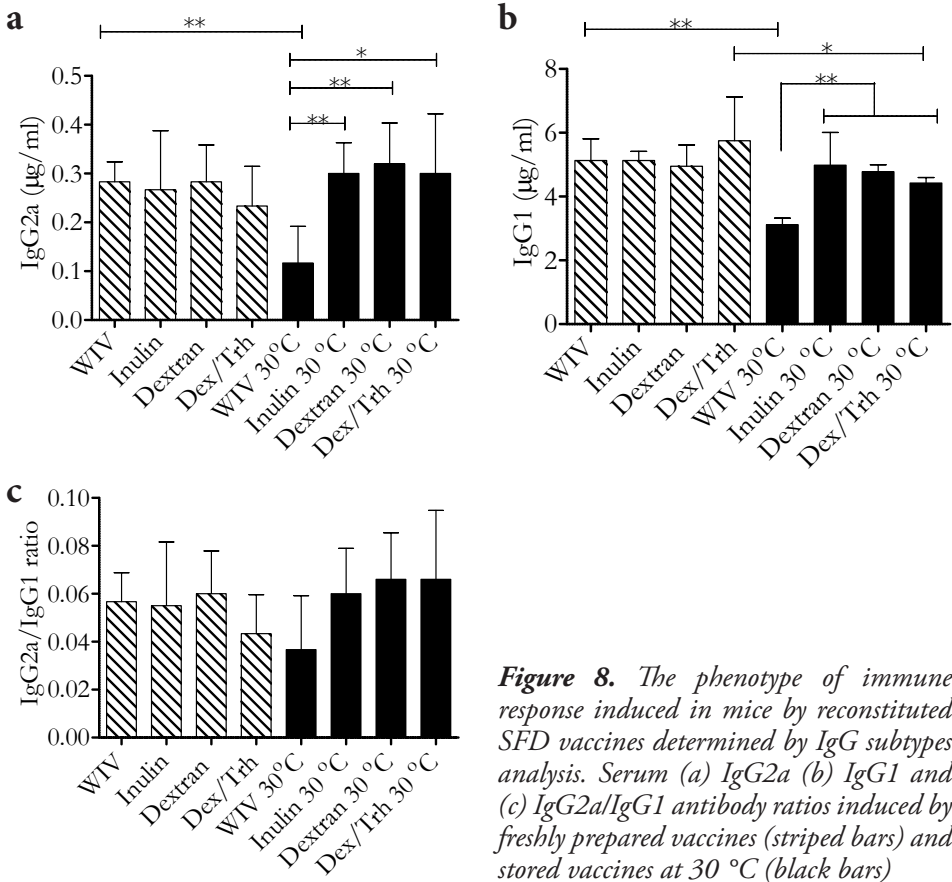
a

b

c

Figure 8. *The phenotype of immune response induced in mice by reconstituted SFD vaccines determined by IgG subtypes analysis. Serum (a) IgG2a (b) IgG1 and (c) IgG2a/IgG1 antibody ratios induced by freshly prepared vaccines (striped bars) and stored vaccines at 30 °C (black bars)*

The lower mucosal and systemic antibody induction in mice after pulmonary administration of SFD vaccine powders stored at 30 °C could be due to loss of antigenicity of the vaccine or due to changes in physical powder characteristics after storage leading to less effective vaccine administration or due to both. However, our in vitro studies showed that both the physical powder characteristics and the integrity of the vaccine did not change substantially during storage. To elucidate this inconsistency, freshly prepared as well as stored SFD vaccine powders were reconstituted with water and then pulmonary administered to mice with the micro-sprayer. The serum IgG antibody response as determined by ELISA **(Figure 7a)** showed that stored liquid WIV induced significantly lower levels of IgG antibody titers in mice than freshly prepared WIV or SFD vaccine powders reconstituted after storage. Furthermore, SFD vaccine powders, reconstituted after storage, induced a similar level of IgG antibody titers as SDF vaccine powders reconstituted immediately after preparation. HI titers were in line with the IgG antibody titers as the stored liquid WIV elicited significantly decreased HI titers compared to SFD powders reconstituted immediately after preparation or after storage or the freshly prepared liquid formulation **(Figure 7b)**.

The subtypes of IgG antibodies (IgG2a and IgG1) induced after pulmonary administration of stored (three months at 30 °C) or freshly prepared, liquid WIV or reconstituted SFD vaccine powders were analyzed by ELISA. It was found that stored liquid WIV induced significantly lower amounts of IgG2a and IgG1 antibodies than freshly prepared or stored SFD vaccine powders or freshly prepared WIV **(Figure 8a-b)**. Furthermore, storage of the SFD vaccine powders did not affect the induction of IgG1 and IgG2a antibody titers. Analysis of the IgG2a/IgG1 ratio **(Figure 8c)** shows that pulmonary vaccination induced a IgG1-dominated antibody response which is in line with a previous study [27].

Overall, the HI titers, IgG titers and the IgG2a and IgG1subtype titers demonstrate that the antigenicity of WIV stored at 30 °C for three months was better preserved in SFD than in liquid vaccine formulation. Furthermore, the stabilizers used for SFD had no effect on the preservation of the immunogenicity of the vaccines.

4. Discussion and conclusion

In this study, we prepared a dry powder influenza WIV formulation by SFD using inulin, dextran, or dex/trh as stabilizing excipients. The aerodynamic diameter of the SFD powders was about 0.8, 1.8 and 3.3 μm for X10, X50 and X90 indicating that almost all of the powder particles were suitable for inhalation. Furthermore, we showed that for all three stabilizers the SFD vaccine powders could be prepared without loss of the particulate nature, biochemical integrity and receptor-binding property of the incorporated WIV as shown by TEM, SDS-PAGE and HA assay, respectively.

During storage of the SFD powders for three months at temperatures up to 30 °C, the physical powder characteristics, i.e. particle size distribution and specific surface area, remained the same. However, when stored at 40 °C, the size and specific surface area of SFD particles of inulin and dex/trh were reduced. These results indicate that the inulin and dex/trh particles shrunk during storage, yet without displaying macroscopic viscous flow. These observation can be related to the Tg of the powders. The Tg of dry dextran was found to be 220 °C, whereas the Tg of dry inulin and dry dex/trh was in both cases 154 °C. All these Tg values are far above storage temperatures implying that all three sugars are suitable as stabilizers. However, it is to be realized that residual moisture is a potent plasticizer for sugar glasses. Thus, residual moisture present in the samples (although not determined) will strongly reduce the Tg [38]. As all powders were prepared by an identical procedure it can be assumed that all contained equal amounts of residual moisture. For inulin and dex/trh this moisture might have been sufficient to reduce the real Tg values from 154 °C to close to 40 °C. In contrast, for dextran with a high initial Tg of 220 °C, the residual moisture was possibly insufficient to lower the Tg to 40 °C. The shrinkage of the inulin and dex/trh containing particles is in line with this reasoning. The shrinkage indicated that there was some translational molecular mobility but not to such an extent that macroscopic viscous flow occurred.

Furthermore, WIV incorporated in the SFD powders retained its particulate nature, biochemical integrity and receptor-binding properties during three months storage at temperatures up to 30 °C. As expected, the unprocessed liquid WIV formulation was less stable. Replacing inulin by dextran and dex/trh revealed that both these sugars are also excellent stabilizers for WIV during SFD and subsequent storage. Additionally, WIV incorporated in dextran was even stable at temperatures as high as 40 °C. Overall, WIV SFD in the presence of all three sugars yielded a stable product when stored at 30 °C. Hence, the SFD vaccine powders stored at 30 °C for three months were selected for the immunization of mice. The pulmonary administration of the SFD vaccine powders induced IgA in the nose and BAL washes, serum IgG and serum HI titers. However, to our surprise the stored SFD vaccine powders were found to elicit significantly lower antibody and HI titers than the freshly prepared SFD vaccine powders. In contrast, immunization experiments with reconstituted SFD vaccines demonstrated that the immunogenicity of WIV was not decreased during storage of the SFD powders. This indicates that although not detected by laser diffraction and BET measurements, the physical properties of the SFD vaccine powders must have changed during storage such that delivery to the lungs was hampered. Possibly, the results can be explained as follows: for the determination of the particle size distribution by laser diffraction, the RODOS disperser was used. It is well known that the RODOS dispenser is a very powerful disperser [39]. In contrast, the dry powder insufflator was found to exhibit very poor dispersing capacities for the SFD powders as not only primary particles left the insufflator but also large agglomerates. Therefore, it could be that during storage subtle changes to the physical properties of the SFD powder occurred that were not significant enough

to be measured by BET and laser diffraction using the RODOS, but caused the poor delivery of the SFD powders into the lungs of the mice by the dry powder insufflator. However, in a clinical situation, powder delivery can be improved by using inhalers like the Novolizer® or Twincer®, which disperse powders better than the dry powder insufflator used in this study [40-43].

In previous studies, we demonstrated that various influenza vaccines can be stabilized by incorporating them in inulin matrices which is in agreement with the present study [18,19,27,33]. Additionally, our results demonstrate that dextran and dex/trh can stabilize WIV equally well. Consequently, it is expected that vaccine powders prepared with either of these sugars can be safely stored at room temperature for extended period. The stabilized and powdered influenza vaccines can overcome the limitations imposed on liquid vaccines by cold chain requirements. Furthermore, the possibility of self-administration makes vaccination campaigns independent of the availability of trained health care personnel and may improve compliance with vaccination recommendations in high-risk populations.

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3

Chapter 4

Enhanced pulmonary immunization with aerosolized inactivated influenza vaccine containing delta inulin adjuvant

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Abstract

Vaccination is the primary intervention to contain influenza virus spread during seasonal and pandemic outbreaks. Pulmonary vaccination is gaining increasing attention for its ability to induce both local mucosal and systemic immune responses without the need for invasive injections. However, pulmonary administration of whole inactivated influenza virus (WIV) vaccine induces a Th2 dominant systemic immune response while a more balanced Th1/Th2 vaccine response may be preferred and only induces modest nasal immunity. This study evaluated immunity elicited by pulmonary versus intramuscular (i.m.) delivery of WIV, and tested whether the immune response could be improved by co-administration of delta (δ)-inulin, a novel carbohydrate-based particulate adjuvant. After pulmonary administration both unadjuvanted and δ-inulin adjuvanted WIV induced a potent systemic immune response, inducing higher serum anti-influenza IgG titers and nasal IgA titers than i.m. administration. Moreover, the addition of δ-inulin induced a more balanced Th1/Th2 response and induced higher nasal IgA titers versus pulmonary WIV alone. Pulmonary WIV alone or with δ-inulin induced hemagglutination inhibition (HI) titers > 40, titers which are considered protective against influenza virus. In conclusion, in this study we have shown that δ-inulin adjuvanted WIV induces a better immune response after pulmonary administration than vaccine alone.

1. Introduction

Influenza vaccines are important in controlling virus infection and spread during seasonal and pandemic outbreaks. Conventionally, inactivated influenza vaccines are administered by subcutaneous or intramuscular injection, with just live attenuated vaccines being administered by intranasal spray. However, non-invasive routes of inactivated influenza vaccine administration, including intranasal, sublingual, intradermal and pulmonary delivery have gained increasing attention [1–4]. The pulmonary route is an attractive route given its non-invasive nature, ease of administration and lack of need for trained health care workers. A further potential advantage of pulmonary immunization is the ability to induce local mucosal immune protection in the respiratory tract, e.g. via secretory IgA production [5]. However, there are also some short comings of pulmonary route of vaccination including a propensity to induce a dominant Th2 immune response [6,7]. A more balanced Th1/Th2 vaccine response is preferred as the combination of cellular with humoral immunity protects the host better against invading influenza viruses [8]. Furthermore, although pulmonary influenza vaccines elicit local immune responses in the respiratory tract, their ability to induce intranasal IgA antibody titers are only modest [6,7,9]. A strong mucosal response in the nose and lungs is desired to provide maximal protection at the port of entry for the influenza virus [10].

Addition of a suitable adjuvant to a pulmonary influenza vaccine could help improve vaccine immunogenicity and thereby its ability to protect against a viral infection. Carbohydrate-based adjuvants have gained recent attention with their ability to combine a high level of efficacy, tolerability and safety [11]. The lack of reactogenicity of carbohydrate-based adjuvants namely δ-inulin and δ-inulin, as demonstrated in both animal models and human vaccine trials [12], suggests their suitability for pulmonary use although to date they have predominantly been used by i.m. and s.c. routes [13]. δ-inulin, the most immunologically active inulin polymorph [14], was shown to have potent adjuvant activity when co-administered i.m. with seasonal or pandemic inactivated or recombinant influenza vaccines in the form of Advax™ adjuvant [12,15,16]. Therefore, we hypothesized that δ-inulin might similarly be able to enhance influenza vaccine immunogenicity when administered via the pulmonary route. This study therefore set out to compare mucosal and systemic WIV immunogenicity when administered by pulmonary versus i.m. routes with or without δ-inulin adjuvant.

2. Material and method

2.1 Vaccine

The WIV antigen (A/California/7/2009 (H1N1)) was produced and inactivated as previously reported [6]. After inactivation and dialysis, the protein content of the vaccine was determined by the micro Lowry assay [6]. The adjuvanted vaccine was prepared by mixing 200 μg of δ-inulin adjuvant (Advax™ adjuvant, 1 mg/ml,

Vaxine Pty Ltd, Adelaide, Australia) with WIV equivalent to 5 µg of HA (0.1 µg/µl) immediately prior to vaccination.

2.2 Immunization studies and sample analysis

The local animal welfare and use committee of the University of Groningen approved all animal experiment handling and work protocols. The study was carried out in 6-8 week old female BALB/c mice (Harlan, Zeist, The Netherlands). Mice were immunized twice at a two week interval (day 0 and 14) with WIV formulations containing 5 µg HA with or without 200 µg of δ-inulin adjuvant. For pulmonary immunization, mice were anesthetized by inhalation of isoflurane/O_2 then intubated in vertical position with a modified Autograde catheter (Becton Dickinson, Breda, The Netherlands). 50 µl of vaccine was delivered to the lungs of the mice using a IA-1C micro-sprayer attached to a FMJ-250 high-pressure syringe (Penn-Century Inc., Wyndmoor, USA). For i.m. immunization, 50 µl of vaccine was administered by dividing the dose equally between both hind limbs. Mice were then placed in a recovery incubator at a temperature of 25 °C for 2 hours, and then placed back in the housing facility.

Two weeks after the first dose, blood samples were withdrawn by facial vein technique for evaluation of the immune response. Two weeks after the second dose, the mice were sacrificed. After sacrifice, blood samples were withdrawn by heart puncture. Serum was stored at -20 °C until used. Nose wash was obtained using 1 ml PBS, pH 7.4, containing complete protease inhibitor cocktail tablets (Roche, Almere, The Netherlands). The collected samples were analyzed by ELISA to evaluate anti-influenza IgG, IgG1, IgG2a, and IgA antibody titers. Serum samples were also analyzed for anti-influenza HI titers, as previously described [7].

2.3 Statistical analysis

Immunoglobulin and HI titers are reported as geometric mean ± standard error mean. The differences in titers were analyzed by two-tailed Mann Whitney U-test at a confidence interval of 95 % (P≤0. 05). The significance level in figures is denoted by an increase in the number of "*" symbols: one symbol (P≤0.05); two symbols (P≤0.01) and three symbols (P≤0.001).

3. Results

3.1 Anti-influenza IgG responses

The immune responses evoked in mice by i.m. or pulmonary immunization with or without δ-inulin adjuvant were first evaluated by measuring total serum anti-influenza IgG titers 14 days after the first and second immunization. Both i.m. and pulmonary immunization with or without δ-inulin adjuvant induced potent IgG responses even after the first immunization with further increases in anti-influenza
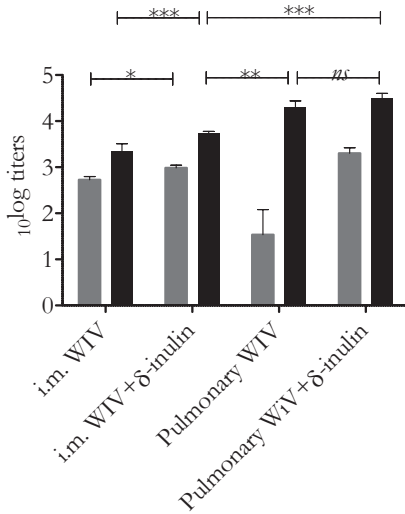
Figure 1. *WIV specific serum IgG antibody titers induced on day 14 (gray bars) and day 28 (black bars) after i.m. and pulmonary vaccination on day 0 and day 14.*
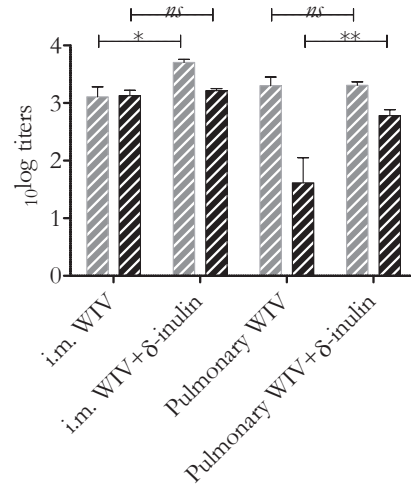
Figure 2. *WIV specific serum IgG subtypes, IgG1 (gray bars) and IgG2a (black bars) antibody titers induced on day 28 after i.m. and pulmonary vaccination on day 0 and day 14.*

IgG titers seen after the second immunization **(Figure 1)**. While after just a single immunization, anti-influenza IgG titers were significantly lower in the group receiving WIV alone by the pulmonary route when compared to WIV alone given by i.m injection, this reduced immunogenicity in the singe dose pulmonary group was more than compensated for by the addition of δ-inulin adjuvant, with the single dose WIV+ δ-inulin pulmonary group achieving higher IgG titers than the equivalent single dose i.m. groups.

Furthermore, while after the second immunization in the i.m groups the increases in anti-influenza IgG were relatively modest (approximately 1 log), the increases in IgG titers after the second immunization in the pulmonary groups were much greater (approximately 3 logs for the WIV alone group and 1.5 logs for the WIV+ δ-inulin adjuvant group). Hence after two immunizations pulmonary immunization with or without δ-inulin adjuvant induced significantly higher IgG antibody titers than two doses of i.m. vaccine, either with or without adjuvant. Two weeks after the second vaccination, the mice that received i.m. vaccine with δ-inulin adjuvant achieved a significantly higher anti-influenza IgG response than mice that received i.m. WIV vaccine alone (P=0.0002), whereas two weeks after the second vaccination, there were no longer significant differences in anti-influenza IgG titers in mice that received pulmonary WIV vaccine with δ-inulin adjuvant compared to those that received pulmonary WIV vaccine alone.

Phenotype of i.m versus pulmonary vaccine responses

Anti-influenza IgG subtypes were evaluated two weeks after the second vaccination **(Figure 2)**. Pulmonary immunization with WIV alone induced primarily an IgG1 response, consistent with a Th2 dominant immune response, consistent with the results of previous studies [6,7]. This compared to the i.m groups with or without adjuvant where the titers of IgG1 and IgG2a were approximately equal **(Figure 2)**, consistent with a more balanced Th1/Th2 response. The addition of δ-inulin adjuvant to the pulmonary immunization group significantly increased the IgG2a antibody titers when compared to WIV alone given pulmonary without compromising the IgG1 antibody titers, thereby resulting in a more balanced Th1/Th2 response. This was interesting as by contrast the addition of δ-inulin adjuvant to the i.m immunization group resulted in primarily in enhancement of the IgG1 rather than IgG2a response.

Hemagglutination inhibition titers after i.m. and pulmonary vaccination

Hemagglutination inhibition (HI) antibody titers reflect the subfraction of anti-influenza immunoglobulin that is able to block influenza virus attachment to mammalian sialic acid receptors and thereby more directly measures the amount of neutralizing antibodies induced by an influenza vaccine, with an HI titer of 40 considered to be protective [17]. After the second immunization, all groups whether
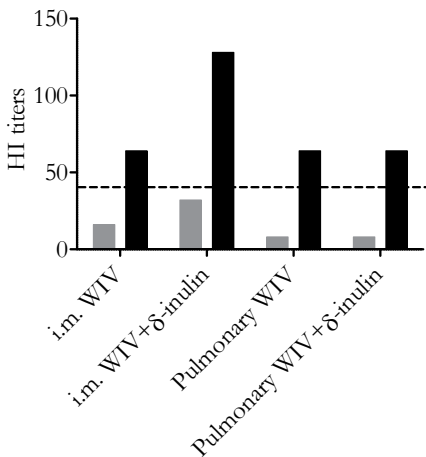

Figure 3. WIV specific serum HI levels induced on day 14 (gray bars) and day 28 (black bars) after i.m. and pulmonary vaccination on day 0 and day 14. The HI titers were measured in triplicate. Since no differences in HI titers were found no error bars are shown
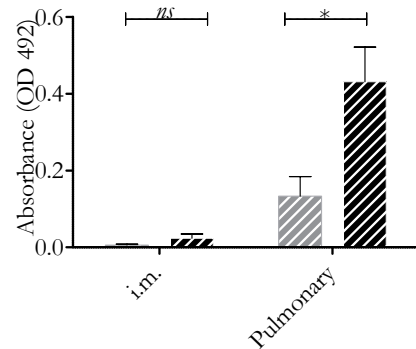
Figure 4. WIV specific nose IgA antibody levels induced by unadjuvanted (gray bars) and adjuvanted WIV (black bars) vaccine on day 28 after i.m. and pulmonary vaccination on day 0 and day 14.

i.m and pulmonary, achieved an HI titer > 40, consistent with protection (Figure 3). There were no significant difference between the i.m WIV alone group and both pulmonary vaccine groups, with the only group having significantly higher HI titers than the i.m WIV alone group being the δ-inulin adjuvanted i.m. vaccine group **(Figure 3)**.

Mucosal response after i.m. versus pulmonary immunization

The production of local secretory IgA at relevant mucosal respiratory surfaces was shown to be an important first line of defense against influenza virus attachment and infection. In general, mucosal IgA is not seen after intramuscular immunization. The mucosal immune response to i.m. or pulmonary immunization was therefore evaluated by measuring anti-influenza IgA levels in nasal washings of immunized mice **(Figure 4)**. WIV vaccine given pulmonary with or without adjuvant induced significantly higher anti-influenza IgA titers in nasal washings than obtained with the i.m. vaccine. Furthermore, δ-inulin adjuvant significantly enhanced the anti-influenza IgA titers in nasal washings compared to WIV vaccine alone given pulmonary (P=0.02) **(Figure 4)**.

4. Discussion and conclusions

To our knowledge, this is the first study to confirm that δ-inulin maintains its vaccine adjuvant activity when administered via the pulmonary route. Mice receiving δ-inulin adjuvanted influenza vaccine administered via the pulmonary route demonstrated a more balanced Th1/Th2 immune response whereas pulmonary administration of WIV alone demonstrated a marked Th2 bias in their antibody response. This Th1 enhancement of the antibody response to a pulmonary vaccine by δ-inulin is in contrast to the results seen when it was given i.m where it primarily enhanced IgG1 production consistent with a more Th2 action, and suggests that the immune pathways activated in the lung by δ-inulin may differ from the pathways it activates after i.m. injection. Another feature of δ-inulin when given pulmonary was its ability to enhance mucosal anti-influenza IgA titers. The most marked pulmonary effect of δ-inulin adjuvant on IgG titers was seen after a single immunization, when it enabled higher anti-influenza IgG titers to be achieved than obtained after even two i.m immunizations with WIV alone, or a single i.m immunization with WIV + δ-inulin. Previously, pulmonary or intranasal vaccines have generally required multiple booster vaccine doses to achieve satisfactory immunogenicity [6,18]. Based on these findings it may be possible to utilize δ-inulin adjuvant along with antigen dose optimization and delivery strategies to achieve single dose pulmonary vaccine protection, and this will be tested in future influenza challenge studies. While pulmonary delivery of WIV when compared to i.m. delivery induced a broader antibody isotype response than included IgA, and after two doses achieved HI titers considered protective, for as yet unexplained reasons there was a divergence such that whilst the anti-influenza IgG titers were substantially higher, the HI titers induced by pulmonary vaccination were

substantially lower than those induced by i.m. vaccination. Typically, WIV vaccines induce a Th1-biased immune response upon i.m. vaccination while Th2 dominant responses have usually been seen after pulmonary vaccination suggesting that the lung may have an innate Th2 bias [6,19]. The Th2 immune response helps to neutralize the virus whereas the Th1 immune response assists in clearing virally-infected cells from the host [8]. Hence, a balanced Th1/Th2 type of immune response should be optimal for influenza virus neutralization and clearance. Our results show that the δ-inulin adjuvanted pulmonary vaccine was capable of inducing a more balanced Th1/Th2 immune response than the WIV alone pulmonary vaccine. Furthermore, mucosal IgA is beneficial because it neutralizes the influenza virus at the port of entry and thus enhances protection [20,21]. Moreover, IgA antibodies are known for their broad spectrum protection, i.e. they are cross-protective against a range of drifted, heterologous influenza strains [22]. The results of this study show that significant anti-influenza IgA was only induced by pulmonary but not i.m. vaccination and was further improved by the δ-inulin adjuvant. Future studies will test the extent to which this enhanced mucosal IgA production might contribute to enhanced protection against influenza infection.

A wide variety of adjuvants including cholera toxins, squalene oil emulsions, saponins, CpG oligonucleotides, and ISCOMS have been tested for efficacy in animal models of intrapulmonary or intranasal immunization with variable results [23–28]. However, the single most important consideration for an adjuvant to be administered to the lung, nose or other mucosal surfaces is safety, given previous mucosal adjuvant misadventures most particularly the occurrence of facial nerve palsy in human trials of a Escherichia coli heat-labile toxin adjuvanted intranasal inactivated influenza vaccine [29], an event later shown to be directly attributable to the Escherichia coli heat-labile toxin adjuvant [30]. With these safety issues in mind, it is reassuring that δ-inulin has a strong safety and tolerability record when administered by the i.m. route in multiple preclinical and human clinical studies [12,15,16]. Of course, this i.m administration data does not guarantee against an unforeseen effect specific to lung administration and hence formal preclinical safety studies of δ-inulin's intrapulmonary use will be required to exclude such a possibility, prior to any commencement of any human studies. However, no differences in animal well-being, e.g. weight loss, were observed between the pulmonary groups with or without adjuvant and the i.m groups (data not shown). The absence of any obvious pulmonary or systemic adverse events in mice in this study receiving δ-inulin adjuvant is reassuring and supports the idea that inulin particles might be safely administered to the lung as part of an intrapulmonary vaccine formulation.

In conclusion, our results clearly indicate that δ-inulin has adjuvant activity when administered through the pulmonary route with an inactivated influenza vaccine, inducing changes to the balance of IgG isotypes resulting in a more balanced Th1/Th2 response and promoting the production of secretory IgA in the nose. The extent to which this contributes to protective immunity will need to be clarified in future influenza virus challenge studies. These studies will also look at the effect of

changes in the dose of either the influenza antigen, the δ-inulin adjuvant or both on optimization of the anti-influenza immune response, with the ultimate goal of testing whether protection against influenza with a single dose of intrapulmonary vaccine is in fact achievable.

Acknowledgements

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Chapter 5

Evaluation of monophosphoryl lipid A as adjuvant for pulmonary delivered influenza vaccine

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Abstract

Prophylaxis against influenza could be improved by the development of a stable, easy to deliver, potent mucosal vaccine. In this study, we spray-freeze-dried (SFD) whole inactivated virus influenza vaccine (WIV) alone or supplemented with monophosphoryl lipid A (MPLA) using inulin as a lyoprotectant. Physical characterization revealed that the SFD powder consisted of highly porous particles with a size distribution suitable for pulmonary administration. The receptor-binding properties of WIV and the immunostimulatory properties of MPLA were preserved after spray-freeze-drying as indicated by unchanged hemagglutination titers and a retained ability of the vaccine to activate NFκB after incubation with a reporter cell line, respectively. Pulmonary vaccination of mice with MPLA-adjuvanted liquid or powder WIV resulted in induction of higher mucosal and systemic antibody concentrations than vaccination with non-adjuvanted formulations. When exposed to influenza virus, mice immunized with MPLA-adjuvanted pulmonary vaccine showed similar protection in terms of reduction in lung virus titers and prevention of weight loss as mice immunized intramuscularly with subunit vaccine. Characterization of the antibody response revealed a balanced IgG2a-to-IgG1 profile along with induction of both memory IgA- and IgG-producing B cells in mice immunized with MPLA-adjuvanted vaccine. These studies suggest that the mucosal and systemic immune responses to pulmonary delivered influenza vaccines can be significantly enhanced by using MPLA as adjuvant. MPLA-adjuvanted SFD vaccine was particularly effective implying that delivery of adjuvanted vaccine powder to the lungs can be an attractive way of immunization against influenza.

1. Introduction

Influenza virus spreads via aerosols and is therefore easily transmitted from person to person. According to the World Health Organization (WHO), around 250,000 to 500,000 people yearly die because of influenza infections, and around 3 to 5 million people suffer from severe illness[1]. During a pandemic, this number can increase by several-fold, as demonstrated by the 1918 Spanish Flu with 40 to 50 million deaths[2,3]. Today, the spread of a virus would be much faster than in previous pandemics because of global travel and contacts, and therefore vaccine distribution and administration should be similarly rapid. Hence, in the context of pandemic preparedness there is a need for a vaccine which can be stockpiled for years[4,5], is easy to administer, and when administered, evokes a serum IgG antibody response along with a robust mucosal IgA antibody response which neutralizes the virus at the port of entry and provides some degree of cross-protection[6].

Inactivated influenza vaccines available today are administered by intramuscular (i.m.) injection. These formulations have several disadvantages for use as a pandemic vaccine. First, current vaccines are liquid and require a cold chain until vaccine delivery, making vaccine distribution difficult in remote areas. Second, for administration of needle-based vaccines trained medical personnel is required[6]. Third, vaccine injection is time consuming, especially when dealing with people suffering from needlephobia[7-9]. Finally, current inactivated influenza vaccine, being administered parenterally, induce serum IgG but no mucosal IgA. Pulmonary vaccination is an attractive method for vaccine delivery. This vaccination strategy targets the lungs which are highly vascularized organs and offer a large surface area with strategically located dendritic cells and macrophages for antigen capture and presentation [6,10]. Pulmonary immunization can be performed with aerosolized liquid vaccine or with vaccine formulated as dry powder. Dry powder influenza vaccines are particularly attractive since they offer higher stability than liquid vaccine at low as well as at elevated temperatures [11,12]. Pulmonary delivery strategies have been successfully employed for several vaccines. The development of this strategy is most advanced for the live attenuated measles vaccine which has been successfully tested in several field trials [13]. In the context of influenza vaccines, early studies by Waldmann et al and Haigh et al[14,15] indicated the feasibility of pulmonary immunization strategies in a clinical setting but pulmonary influenza vaccination has been studied most extensively in mice. Immunization with liquid or dry powder subunit or split influenza vaccine formulations resulted in immune responses comparable to or higher than those induced by i.m. immunization[11,16,17]. Moreover, pulmonary immunized mice showed the same level of protection against virus growth in the lungs as mice that were conventionally immunized by the i.m. route[18].

However, despite these promising results immune responses achieved by pulmonary immunization need further optimization. While pulmonary immunization with unadjuvanted influenza subunit or WIV vaccines did result in induction of IgA antibodies in the lungs, only some of the immunized mice mounted measurable IgA

responses in the nose and in those that did antibody titers were low[17,18]. Next to IgA also IgG participates in protection from influenza[19]. In mice the Th1 related IgG subtype IgG2a is particularly effective[20]. Yet, antibody responses observed in mice after pulmonary vaccination with whole inactivated virus (WIV) or subunit influenza vaccine were dominated by the Th2-related antibody subtype IgG1[21] [18]. Although the relevance of different IgG subtypes for protection in humans is so far unclear we expect a Th1 type response to be favorable for protection. One potential solution to the above mentioned problems might involve the addition of an adjuvant to the vaccine in order to steer the immune response to the desired Th1 phenotype and to enhance the stimulation of mucosal immune responses. Initiation of Th1 response leads to activation of pro-inflammatory cytokines which promotes IgG2a antibody production. Toll-like receptor (TLR) ligands are acknowledged for their ability to induce potent immune responses to antigen with which they are co-administered[22]. One such TLR ligand is monophosphoryl lipid A (MPLA), a low toxicity derivative of lipopolysaccharide (LPS) derived from the cell wall of gram-negative bacteria. As LPS, MPLA is a ligand of TLR 4 which is expressed on immune as well as on respiratory epithelial cells[23,24]. MPLA is recognized for its ability to skew responses towards Th1 thus promoting IgG2a antibody production in mice. In addition, MPLA has been shown to effectively stimulate IgA antibody responses against mucosally administered Hepatitis B surface antigen, tetanus toxoid, respiratory syncytium virus (RSV) vaccine and influenza subunit vaccine[25,26] . MPLA, together with alum is part of the adjuvant AS04, which is FDA approved and commercially used in the Hepatitis B vaccine Fendrix and in the Human Papilloma Virus (HPV) vaccine Cervarix[27,28]. Moreover, the combination of MPLA and a TLR7 ligand, as also present in WIV in the form of single stranded viral RNA, is particularly effective in inducing long-lasting immune responses[29].

In the present study, we investigated whether inclusion of MPLA as an adjuvant in liquid or dry powder influenza vaccine promotes mucosal and systemic (Th1-skewed) immune responses after pulmonary vaccination. To this end, WIV influenza vaccine and a mixture of WIV and MPLA were either used as such or spray-freeze-dried using inulin as a lyoprotectant. Our results indicate that MPLA is a suitable adjuvant for use in liquid as well dry vaccine formulations and enhances systemic and mucosal immune responses upon pulmonary delivery together with influenza vaccine.

2. Material and Methods

2.1. Virus preparation

A/Hiroshima/52/2005 (A/Hir/H3N2) influenza virus was kindly provided by Abbott (Weesp, The Netherlands). A/PR/8/34 H1N1 viruses were cultured in embryonated eggs by allantoic inoculation of the seed virus as described previously[18].

2.2. Vaccine preparation

For preparation of WIV, live A/Hir/H3N2 or A/PR/8/H1N1 viruses were inactivated by overnight incubation at room temperature under continuous rotation in 0.1 % β-propiolacton prepared in sterile citrate buffer (125 mM citrate, 150 mM NaCl, pH 8.2). The inactivated virus was then dialysed overnight at 4 °C against hepes buffered saline (HBS; 145 mM NaCl, 5 mM Hepes, pH 7.4). Subunit vaccine was produced by solubilizing WIV for 3 hours at 4 °C under continuous rotation at a final concentration of 800 μg/ml in sterile HBS containing 12 mg/ml Tween 80 (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and 6 mg/ml hexadecyltrimethylammonium bromide (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Virus solubilization was followed by centrifugation at 50000 RPM at 4 °C for 30 minutes in a TLA100.3 rotor. The supernatant was recovered and detergents were removed using absorbent Bio-Beads SM-2 (Bio-Rad, Veenendaal, The Netherlands). Vaccine protein content was determined by micro-Lowry assay and purity was analyzed by SDS PAGE followed by silver staining. The hemagglutinin (HA) content was assumed to be one third of the total protein weight of the whole inactivated virus (based on the known protein composition of influenza virus and the molecular weight of the viral proteins) and to be equal to the total amount of protein for subunit vaccine (based on the SDS analysis). WIV-MPLA was obtained by mixing WIV with MPLA (InvivoGen, Toulouse, France) at a weight ratio of 8:1 (HA of WIV: MPLA) prior to spray-freeze-drying or immunization.

2.3. Spray-freeze-drying

Spray-freeze-drying by mixing WIV or a solution containing WIV and MPLA (in a ratio of 0.125 μg MPLA/μg HA) with a 5 % (w/v) solution of inulin 4 kD (Sensus, Roosendaal, The Netherlands) in HBS, pH 7.2 to a final HA:inulin weight ratio of 1:200. The mixture was pumped at a flow rate of 5 ml/min through a two-fluid nozzle (diameter 0.5 mm) of a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland), and sprayed using an atomizing airflow of 500 l/hr in liquid nitrogen. The liquid nitrogen was evaporated in a Christ Epsilon 2-4 freeze dryer precooled at a shelf temperature of -55 °C. Drying was performed at a pressure of 0.220 mBar with a condenser temperature of -85 °C. The shelf temperature was steadily increased from -55 °C to 4 °C over a time period of 32 hours. The pressure was decreased to 0.055 mBar and the shelf temperature was gradually increased to 20 °C in a time period of 11 hours. The vaccine powder was collected in a cabinet with a relative humidity less than 10 % and stored at 4 °C under air tight conditions.

2.4. Analysis of influenza WIV

The size of WIV derived from A/PR/8/H1N1 before and after addition of MPLA was analyzed using a nanoparticle tracking system (LM-14, NanoSight, Wiltshire, United Kingdom). One dose of vaccine corresponding to 5 μg HA of WIV and

0.626 µg MPLA was diluted with 5 ml PBS (Gibco Life Technologies B.V., Bleiswijk, The Netherlands) to a final concentration 1 µg/ml and 0.125 µg/ml, respectively. Particle size analysis was done using Nanoparticle Tracking Analysis (NTA) software (NanoSight) and particle size distribution plots were obtained.

Transmission electron microscopy (TEM) was performed using a Philips CM 12 transmission electron microscope (Philips, Eindhoven, The Netherlands). SFD vaccines were rehydrated using sterile-filtered water. Liquid and reconstituted SFD formulations were dialysed against ammonium acetate buffer (75 mM ammonium acetate, 2.5 mM Hepes, pH 7.4) overnight at 4 °C. Dialyzed samples were added to glow-discharged 200 mesh copper grids covered with Formvar film. Samples were stained with 3% ammonium molybdate, pH 7.2 and analyzed.

The capacity of WIV derived from A/PR/8/H1N1 to bind to cells was assessed by a hemagglutination assay, performed as described previously[18]. WIV was dissolved in PBS to a final concentration of 0.1 µg HA/µl and 50 µl was added to 96-well V-bottom plates containing 50 µl of PBS. Two-fold dilutions were performed followed by addition of 50 µl of a 1% guinea pig red blood cell (RBC) suspension prepared in PBS. Hemagglutination was read 2 hours after incubation at room temperature. Hemagglutination titers were expressed as $\log_2$ of the highest dilution showing agglutination of RBC.

Activation of NFκB by the vaccines was detected using the RAW-BlueTM cell line (InvivoGen, Toulouse, France). RAW-Blue™ cells are reporter cells expressing a range of pattern recognition receptors and secreting embryonic alkaline phosphatase (SEAP) upon induction of NfκB following ligand binding to one of these receptors. The assay was performed according to the manufacturer's protocol. Briefly, RAW-BlueTM cells were maintained in DMEM with high glucose (Gibco Life Technologies BV, Bleiswijk, The Netherlands) containing 10 % Fetal Bovine Serum (FBS, Lonza, Basel, Switzerland), 100 µg/ml Normocin™ (InvivoGen, Toulouse, France), 2 mM L-glutamine. For stimulation, 1×10^5 RAW-Blue™ cells were added to flat bottom 96-well plates (Corning Incorporated, Corning, USA). Cells were stimulated with 1.25 µg MPLA (6.25 µg/ml) or with liquid vaccine or reconstituted vaccine powder containing 5 µg (25 µg/ml) of HA of A/PR/8 H1N1 WIV with or without 1.25 µg MPLA and were incubated at 37 °C with 5 % CO_2 for 16 hours. 40 µl of cell supernatant was collected in a 96-well ELISA plate (Greiner Bio One, Alphen a/d Rijn, The Netherlands) to which 160 µl/well QUANTI-Blue™ (InvivoGen, Toulouse France) was added. The plate was incubated at 37˚C for 1 hour. Secreted embryonic alkaline phosphatase (SEAP) levels from cells were determined by measuring absorbance at 620 nm.

2.5. Physical characterization of vaccine powders

Scanning electron microscopy (SEM) was performed with a JEOL JSM 6301-F microscope (JEOL Ltd., Tokyo, Japan). Samples were prepared by placing powders onto double-sided sticky carbon tape on a metal disk. Then particles were coated with 30 nm

of gold using a Balzer's 120B sputtering device (Balzer UNION, Liechtenstein, Austria). Images were taken at magnification of 1000x and 5000x.

Geometric particle sizes of SFD vaccine powders were measured using a HELOS compact model KA laser diffraction apparatus (Sympatec GmbH, Germany). The RODOS dispersing system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) was used to disperse vaccine powders at a pressure of 1 bar.

The specific surface area of vaccines powders was determined using a Tristar surface analyser (Micrometrics Instrument Corp., Norcross, USA). The specific surface area was determined using the multipoint BET method from the nitrogen adsorption isotherm at 77 K as described previously [11].

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 carried out in 8-10 weeks old female BALB/c mice obtained from Harlan (Zeist, The Netherlands).

Mice received two immunizations with an interval of 3 weeks using vaccine formulations containing 5 µg HA of A/Hir/H3N2 or A/PR/8/H1N1 WIV. Subunit vaccine was administered via the i.m. route. 50 µl of vaccine containing 5 µg of HA was divided over both hind legs.

For pulmonary vaccination, mice were anaesthetized by subcutaneous injection of dormitor (0.75 mg/kg body weight) and ketamine (50 mg/kg body weight). Then mice were brought to a vertical position and intubated with a modified Autograde catheter (Becton Dickinson, Breda, The Netherlands). 50 µl of liquid vaccine was administered using an IA-1C Microsprayer Aerosolizer for mice attached to a FMJ-250 High Pressure Syringe (Penn-Century Inc., Wyndmoor, USA). Vaccine powders were delivered using a dry powder insufflator (DPI), (Penn-Century Inc.). Approximately 1 mg of vaccine powder containing 5 µg HA was delivered to the lungs by applying 3 puffs of 200 µl. Antisedan (0.75 mg/kg body weight) was administered subcutaneously for awakening mice. Before they were brought back to the housing facility after awaking, mice were placed in a recovery incubator with a temperature of 25 °C for 2 hours.

One week after the second vaccine dose, mice (n=4) were sacrificed for evaluation of the immune response. After sacrifice, blood was obtained by heart puncture. Serum was collected and stored at -20 °C until use. Nose washes and bronchioalveolar lavages (BAL) were obtained using 1 ml PBS (pH 7.4), containing Complete protease inhibitor cocktail tablets (Roche, Almere, The Netherlands) as described previously[17].

The protective efficacy of the vaccines was determined by challenge of the immunized mice (n=6) with live virus (A/PR/8/H1N1) 4 weeks after the booster vaccination.

Briefly, mice were anaesthetized with isoflurane/O_2 and 40 μl of HBS containing 200 plaque forming units (PFU) A/PR8/H1N1 viruses were slowly administered via the nostrils. Mice were observed daily for weight loss followed by sacrifice on three days after challenge by heart puncture under the anesthesia with isoflurane/O2. Nose wash, lungs and spleens were collected and processed for further use.

2.7. ELISA

Nose washes, BAL and serum samples were used for ELISA and HA-specific IgG, IgG1, IgG2a and IgA antibodies were determined. ELISA plates (Greiner Bio One, Alphen, The Netherlands) were coated with 200 ng/well of A/Hir/H3N2 or A/PR/8 H1N1 subunit vaccine overnight at 37 ˚C. ELISA was performed as previously described[18] except that the substrate solution consisted of citrate phosphate buffer, pH 5, containing 0.04 % (w/v) o-phenylenediamine and 0.012 % (v/v) H_2O_2. The enzymatic reaction was allowed to proceed at room temperature for 30 minutes and stopped using 50 μl of 2 M H_2SO_4. Absorbance was measured at 492 nm using a Synergy HT reader (BioTek, Winooski, USA). Average IgG titers 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 the maximum absorbance of 1:1 diluted nose and lung washes. IgG1 and IgG2a concentrations were determined using calibration curves made by overnight coating of ELISA plates with 0.1 μg anti IgG (Southern Biotech, Birmingham, USA) at 37 °C. Following extensive washing increasing concentrations of 100 μl of IgG1 or IgG2a (Southern Biotech, Birmingham, USA) were added to the plates. Average influenza HA-specific IgG1 or IgG2a responses are presented as concentration (μg/ml).

2.8. Hemagglutination inhibition

Hemagglutination inhibition (HI) assay was performed as described previously with minor changes[18]. The changes were as follows: pooled sera from vaccinated mice were used for determining HI titers, and 4 Hemagglutination units (HAU) live virus was added to the diluted serum samples. HI titers are expressed as the highest serum dilution preventing hemagglutination.

2.9. Virus titration

Lungs collected after challenge were homogenized in PBS (pH 7.4) and centrifuged at 1200 rpm for 10 minutes, supernatants were 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[18]. $\log_{10}$ virus titers were calculated as per milliliter of lung homogenate supernatant.

2.10. Determination of influenza specific antibody secreting cells

The number of influenza-specific antibody secreting cells (ASC) was determined using the protocol as described previously [30]. Spleens collected in Iscove's Modified Dulbecco's Medium (IMDM) complete medium (Gibco, Life Technologies B.V., 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) were processed to single cell suspensions using cell strainers (BD Biosciences, Breda, The Netherlands) followed by RBC lysis using hypotonic medium (0.83 % NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). After extensive washing, 1 x 10^6 splenocytes, pooled per experimental group, were added to 6-well plates (Corning incorporated, New York, USA) followed by incubation at 37 °C with 5 % CO_2 for 6 days in complete IMDM medium with or without subunit vaccine (5 μg/ml) in the presence of imiquimode (5 μg/ml, InvivoGen, Toulouse, France). After incubation, cells were recovered and washed with complete IMDM medium followed by addition of 5 x 10^5 cells to MultiScreenHTS-HA filter plates (Millipore, Billerica, Massachusetts) coated with subunit vaccine 0.5 μg /well. Memory IgA or IgG ASC were detected using alkaline phosphatase labeled anti-mouse IgA antibody (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) or horse radish peroxidase labeled anti-mouse IgG antibody (Southern Biotech, Birmingham, USA). Plates were washed and memory influenza-specific IgA ASC spots were stained using 3-amino-9-ethylcarbazole substrate (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Plates were washed with PBS again and memory influenza-specific IgG ASC spots were stained using 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium substrate (Roche, Almere, The Netherlands). Spots were allowed to develop and the reaction was stopped by washing plates with tap water. Spots were counted manually using a microscope (Wild Heerbrugg, Gais, Switzerland).

2.11. Cytokine ELISA

To determine IFNγ levels in the lungs of the challenged mice, lung supernatants were obtained as described above. Lung supernatants were stored at -80 °C until use. IFNγ levels in lung supernatants were determined using Ready-SET-Go ELISA kits (Ebioscience, Vienna, Austria) according to the manufacturer protocols.

2.12. 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 tests were employed. P values less than 0.05 were considered to represent statistically significant differences. *, **, *** represent p<0.05, p<0.01, p<0.001 respectively.
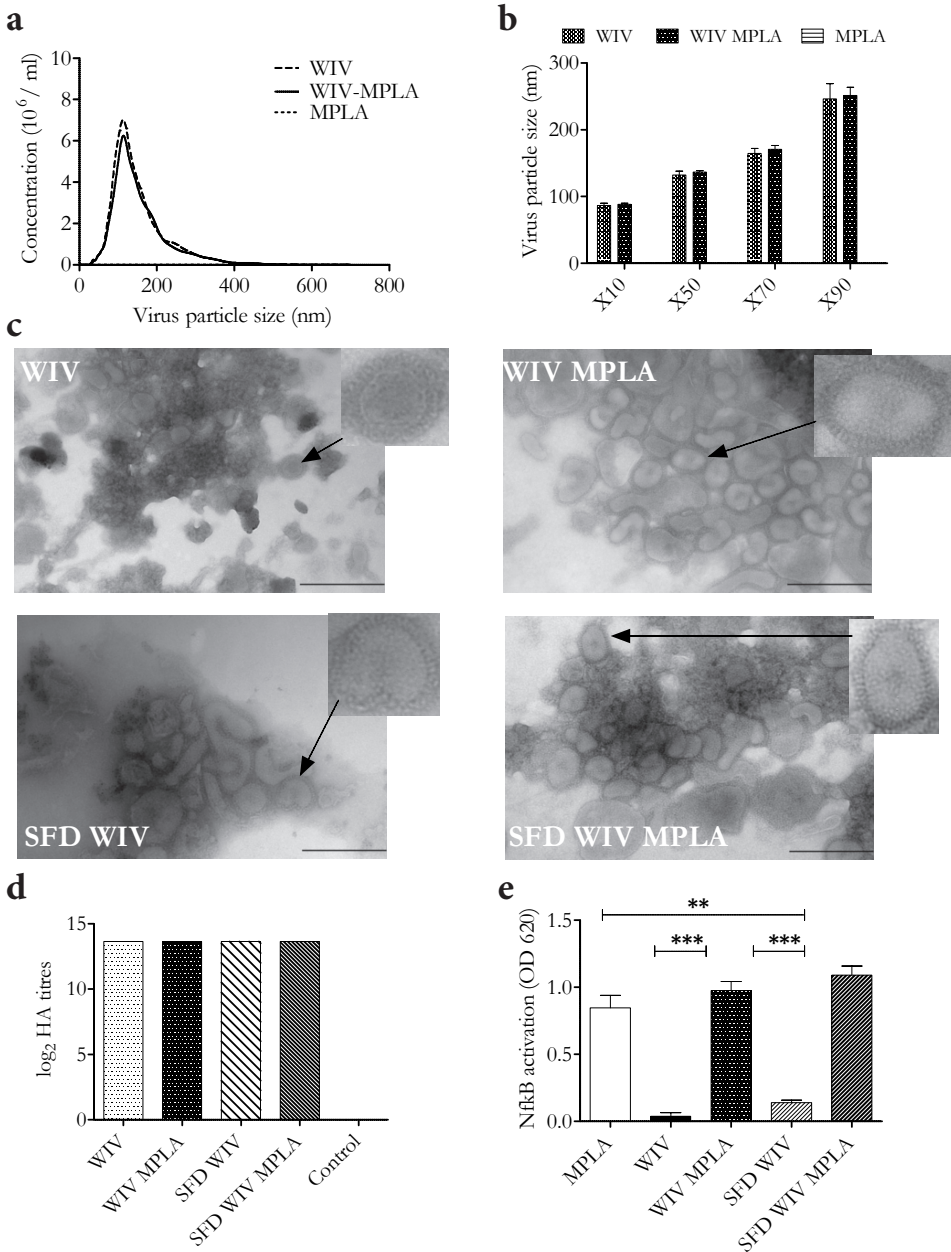
*Figure 1. Characterization of virus particles in liquid and SFD vaccine. (a) Number size distribution of WIV, WIV in combination with MPLA, and MPLA only (c) Transmission electron microscope images of WIV and WIV MPLA before and after spray-freeze-drying (bar represents 200 nm). (d) Hemagglutination activity of WIV and WIV MPLA before and after spray-freeze-drying (e). NfκB activation by MPLA, WIV, and WIV-MPLA before and after spray-freeze-drying. Levels of significance are presented as *p<0.5, **p<0.01, ***p<0.001.*

3. Results

3.1. Analysis of effects of MPLA on the structure and biological properties of whole inactivated virus influenza vaccine

In order to further improve immune responses elicited by pulmonary administered WIV influenza vaccine we decided to evaluate MPLA as an adjuvant. MPLA is an amphiphilic molecule which potentially could interact with the membrane of the WIV particles. To determine the effect of MPLA on morphology and size of WIV, the particle size of WIV derived from A/Hir/H3N2 was evaluated before and after addition of MPLA using NanoSight, a microscope-based nanoparticle tracking system, to characterize and measure nanoparticles in liquid dispersion. The size distribution of plain and adjuvanted virus particles showed that liquid WIV and liquid WIV-MPLA consisted of a relatively homogenous population of virus particles as indicated by a single narrow peak at 115 nm and 114 nm respectively **(Figure 1a)**. No particles were observed when MPLA was analyzed alone. Cumulative undersize curves showed virus particles had a size range between 80 to 300 nm and that the distribution of sizes was very similar for non-adjuvanted and adjuvanted WIV. This result suggests that addition of MPLA did not affect virus particle size.

In order to further assess whether addition of MPLA to WIV and stress of spray-freeze-drying induced structural changes in the virus particles, WIV derived from A/Hir/H3N2 was SFD with or without addition of MPLA using inulin as a lyo-protectant. The morphological integrity of the virus particles in non-treated and reconstituted SFD WIV and SFD WIV-MPLA was evaluated by TEM **(Figure 1b)**. The morphology of liquid WIV-MPLA was similar to that of liquid WIV. Moreover, the morphology of WIV and WIV-MPLA reconstituted from SFD formulations was similar to that of the non-spray freeze-dried counterparts. The virus structure was intact with clearly visible spikes protruding from the virus membrane. These results suggest that addition of MPLA to WIV did not have effects on the structure of the virus particles in WIV vaccine.

To evaluate whether addition of MPLA or spray-freeze-drying or both affected the biological activity of HA, in particular its capacity to bind to its cellular receptor, a hemagglutination assay was performed using the reconstituted vaccines prepared from A/PR/8/H1N1 and guinea pig RBCs. Evaluation of liquid WIV and liquid WIV-MPLA suggests that addition of MPLA to WIV did not affect the hemagglutinating properties of the virus particles **(Figure 1c)**. Moreover, after spray-freeze-drying, the haemagglutination activity of the reconstituted vaccine formulations was similar to that of the liquid vaccine formulations, indicating that the capacity of WIV to bind to RBC was fully preserved after spray-freeze-drying.

We next evaluated in how far spray-freeze-drying had any effect on the biological activity of MPLA. For this purpose, adjuvanted liquid and reconstituted powder vaccine formulations were tested for their capacity to activate NFκB using RAW-
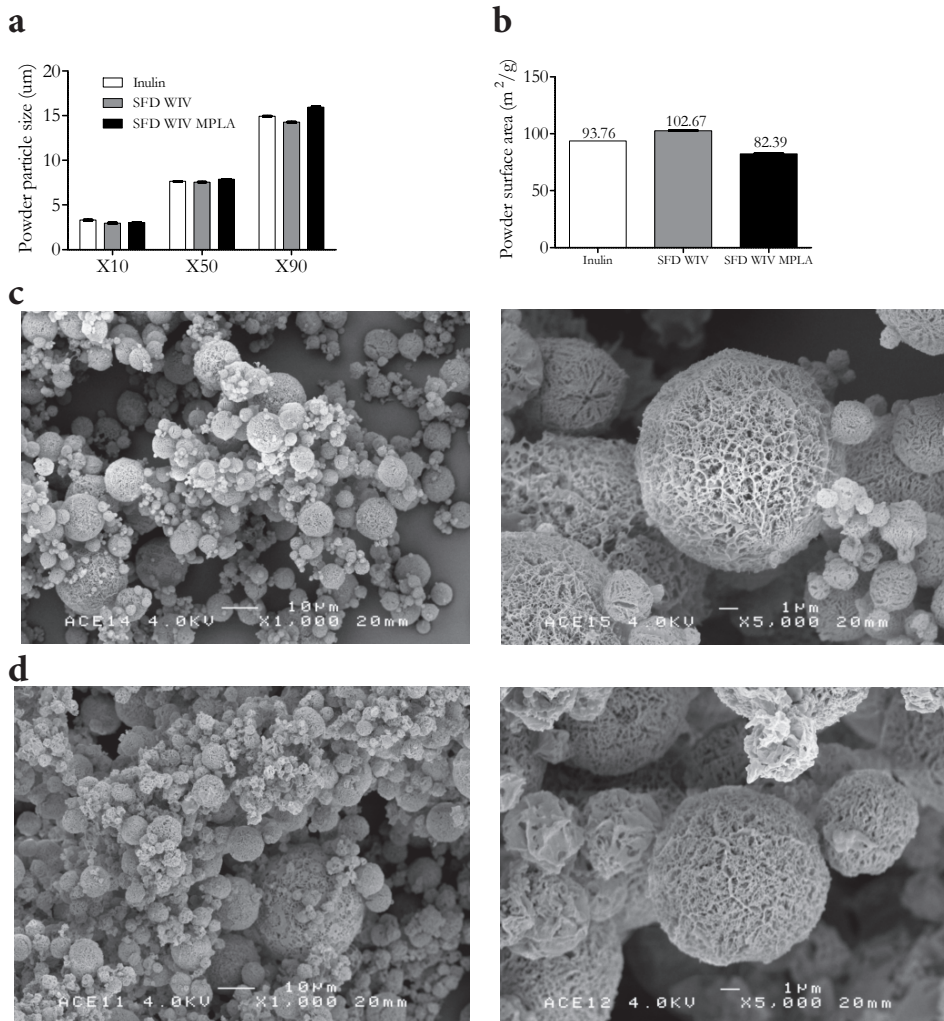
Figure 2. *Physical characterization of spray freeze dried vaccine powder.*

(a) X10, X50 and X90 undersize values of the cumulative geometric volume size distributions of SFD particles containing inulin alone or in combination with WIV or WIV-MPLA. (b) Specific surface area of SFD particles containing inulin alone or in combination with WIV or WIV-MPLA. Scanning electron microscope images at 1000X and 5000X of (c) SFD WIV and (d) SFD WIV-MPLA.

BlueTM cells **(Figure 1d)**. Unexpectedly, WIV derived from A/PR/8/H1N1 by itself was poorly capable of activating NFκB. MPLA-adjuvanted A/PR/8/H1N1 WIV activated NFκB to a significantly higher extent than WIV alone. The capacity of MPLA to activate NFκB was fully retained after spray-freeze-drying, suggesting that stress during the drying procedure did not affect MPLA's efficacy to bind and activate TLR4. MPLA-adjuvanted WIV tended to have a higher capacity to activate RAW-BlueTM cells than MPLA alone; this trend was significant for SFD WIV-MPLA.

The above results demonstrate that addition of MPLA to WIV did neither change the physical nor the biological characteristics of WIV. Moreover, spray-freeze-drying had no detrimental effect on the physical and biological properties of WIV or MPLA.

3.2. Physical characterization of the vaccine powder

We next evaluated whether addition of MPLA to WIV derived from A/Hir/H3N2 affects the characteristics of the SFD vaccine powder particles. The particle size distribution was measured by laser diffraction. The X10, X50 and X90 values were determined from cumulative undersize curves. The X50 values for SFD inulin, SFD WIV and SFD WIV-MPLA were found to be 7.64 μm, 7.56 μm and 7.87 μm respectively with span [span = (X90 – X10)/X50] of 1.52, 1.49 and 1.64 **(Figure 2a-2b)**. These results reveal that addition of MPLA to WIV did not noticeably change the particle size of the SFD powder. Laser diffraction measurements yield the geometric particle size, while for pulmonary administration the aerodynamic particle size is more relevant. The aerodynamic particle size **(Table 1)** was calculated from the geometric particle size using an approach described earlier[17,31]. From this calculation it can be concluded that powder particles obtained after spray-freeze-drying had aerodynamic diameters within the limits for pulmonary vaccination (1-5 μm)[32,33].

Table 1. *Aerodynamic particle size of SFD powder vaccines*

Sample	X10 (μm ± SD)	X50 (μm ± SD)	X90 (μm ± SD)
Inulin	0.768 ± 0.007	1.701 ± 0.009	3.166 ± 0.007
SFD WIV	0.651 ± 0.002	1.663 ± 0.004	3.135 ± 0.002
SFD WIV MPLA	0.669 ± 0.009	1.731 ± 0.009	3.509 ± 0.011

Porosity is important for dispersion of the powder from the DPI and for the solubility of the powder particles. As a measure of porosity, the specific surface areas of the SFD inulin, SFD WIV and SFD WIV-MPLA powders were determined. The specific surface areas were found to be 93.76 m²/g, 102.67 m²/g and 82.38 m²/g for the three formulations, respectively. These readings indicate that addition of MPLA to WIV had some effect on the specific surface area of the SFD powder; yet, the effects on
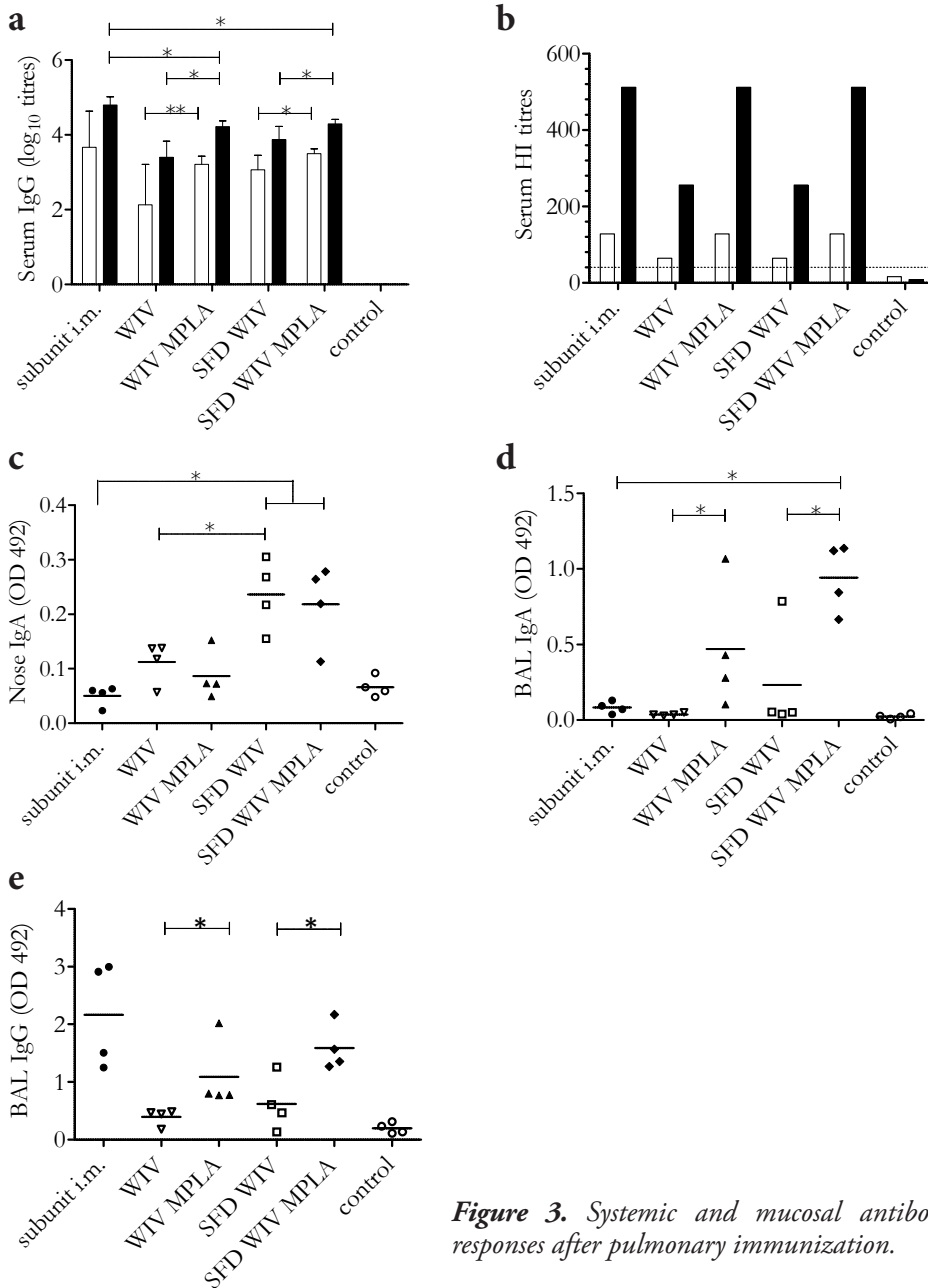
Figure 3. *Systemic and mucosal antibody responses after pulmonary immunization.*

Mice were immunized on day 0 and day 21 with 5 μg subunit vaccine (i.m.) or 5 μg HA of whole inactivated A/Hir/H3N2 with or without MPLA in liquid or powder form (pulmonary). Control mice received HBS (total respiratory track). Systemic responses were evaluated from serum three weeks after the first (white bars) and one week after the second immunization (black bars). (a) IgG titers of individual mice. (b) HI titers in sera pooled per experimental group. Mucosal antibody responses were evaluated one week after the second immunization

the overall particle architecture were limited. These findings were further confirmed by analysis of the vaccine powders using SEM **(Figure 2c,d)**. SEM analysis revealed highly porous structures with interconnected pores with little difference in particle architecture between SFD WIV and SFD WIV-MPLA. The geometric particle size ranged between 1 μm and 10 μm in both SFD WIV and SFD WIV-MPLA. The size of the particles, as seen in the SEM images, correlated well with the particle sizes as determined by laser diffraction. In line with the high porosity, all powders dissolved very easily within seconds when brought in contact with water.

Overall, the physical characterization of the vaccine powders highlighted that the addition of low amounts of MPLA to WIV did not affect the size and structure of the powder particles obtained by spray-freeze-drying in the presence of inulin.

3.3. Immune responses induced by pulmonary immunization of mice with plain or MPLA-adjuvanted WIV vaccines

To evaluate the adjuvant activity of MPLA upon pulmonary vaccination, BALB/c mice were immunized twice with non-adjuvanted or MPLA-adjuvanted WIV vaccine (derived from A/Hir/H3N2 virus) in liquid or SFD powder form. Evaluation of serum samples for IgG antibodies after the first and second immunization showed that all immunized mice developed detectable serum IgG titers after a single dose **(Figure 3a)**. These titers were further enhanced after the booster. Mice vaccinated with MPLA-adjuvanted formulations showed significantly increased IgG antibody titers after a single or after the booster dose compared to mice receiving non-adjuvanted vaccines. However, mice vaccinated with liquid WIV-MPLA and SFD WIV-MPLA showed lower levels of IgG antibody than mice which received a standard i.m. immunization with subunit vaccine. Analysis of HI titers **(Figure-3b)** revealed that all formulations induced HI titers greater than 40 (regarded as protective in humans[34,35]) after the first immunization dose. These titers were further enhanced following the booster immunization. HI titers detected in the liquid WIV-MPLA and the SFD WIV-MPLA group on day 21 and 28 were two-fold higher than those in the corresponding groups vaccinated with non-adjuvanted vaccines. Moreover, HI titers in mice receiving liquid WIV-MPLA and SFD WIV-MPLA were comparable to those in mice vaccinated i.m. with subunit vaccine.

Mucosal immune responses were analyzed on day 28 and one week after the second immunization. Evaluation of nose washes **(Figure 3c)** showed induction of nose IgA antibodies in mice immunized with powder formulations whereas little IgA antibody induction was observed in nose washes of mice vaccinated with liquid formulations. In contrast to the effect of MPLA on systemic antibody titers, no adjuvant effect of MPLA was observed on nasal IgA titers. However, in BAL **(Figure 3d-3e)**, both liquid WIV-MPLA and SFD WIV-MPLA vaccines induced significantly higher levels of IgA and IgG antibody than non-adjuvanted liquid WIV and SFD WIV vaccine. Levels of BAL IgA were similar for mice immunized with WIV-MPLA or SFD WIV-MPLA and were significantly higher than those found in mice vaccinated

i.m. with subunit vaccine. As expected, i.m. administered subunit vaccine failed to induce IgA antibody responses in nose and BAL while IgG was clearly detectable in BAL.

Overall, the data suggest that supplementation of pulmonary vaccines with MPLA adjuvant results in increased serum IgG antibody and HI titers along with enhanced IgA and IgG antibody levels in BAL. However, MPLA adjuvantation of the pulmonary administered vaccines did not improve nose IgA titers. The immune responses evoked by SFD vaccines tended to be higher than those evoked by the liquid versions but this trend was significant for nose IgA only.

3.4. Protection after influenza virus challenge

To compare the protective efficacy of MPLA-adjuvanted and non-adjuvanted, liquid or powder WIV or subunit vaccine derived from A/PR8/H1N1, mice were vaccinated twice on day 0 and 21 with 5 μg HA of WIV followed by a challenge with 200 PFU virus 5 weeks after the booster. The control group received plain HBS through the pulmonary route. Three days post infection, none of the mice in the
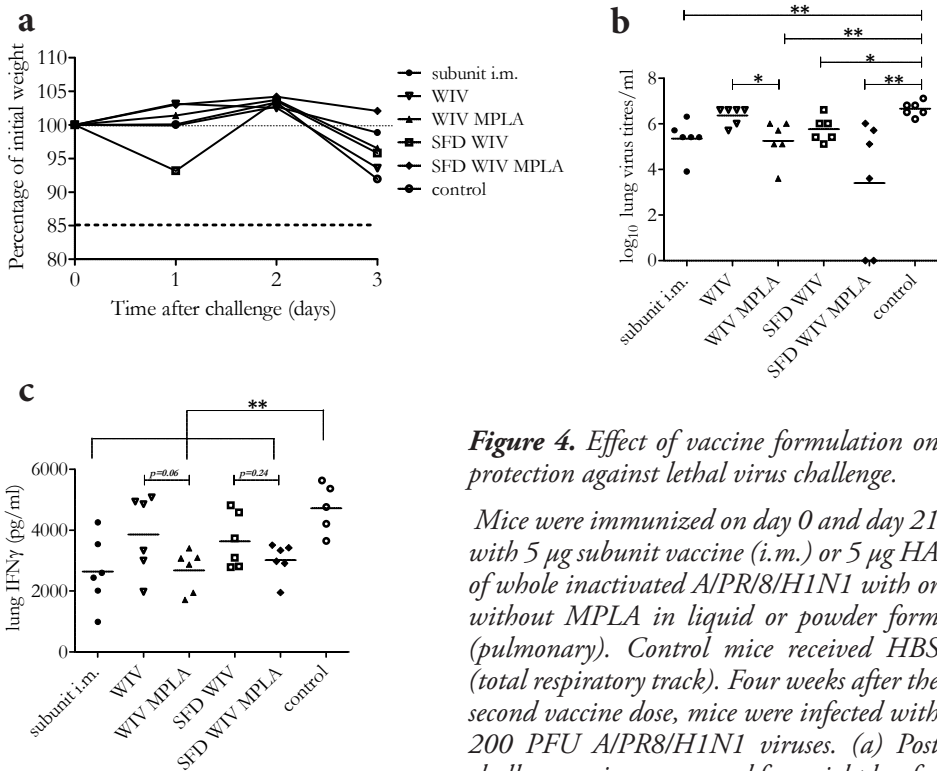
Figure 4. *Effect of vaccine formulation on protection against lethal virus challenge.*

*Mice were immunized on day 0 and day 21 with 5 μg subunit vaccine (i.m.) or 5 μg HA of whole inactivated A/PR/8/H1N1 with or without MPLA in liquid or powder form (pulmonary). Control mice received HBS (total respiratory track). Four weeks after the second vaccine dose, mice were infected with 200 PFU A/PR/8/H1N1 viruses. (a) Post challenge, mice were scored for weight loss for 3 days until sacrifice (85% weight loss, dotted line, was used as humane end point). (b) Three days upon challenge, lungs from mice were harvested and lung supernatants were evaluated for virus titers. Virus titers are expressed at log10 per ml of lung supernatant. Levels of significance are presented as *p<0.05, **p<0.01.*

immunized and control groups showed more than 15 % body weight loss which was set as humane end point in this experiment **(Figure 4a)**. However, control mice showed significantly more weight loss at day 3 than mice which received MPLA-adjuvanted liquid (p=0.0411) or powder (p=0.0087) vaccine formulations. Mice immunized with non-adjuvanted SFD WIV showed more weight loss on day 3 than mice immunized with SFD WIV-MPLA, though the difference was not significant (p=0.0660).

We next determined lung virus titers to assess the ability of infected mice to clear influenza virus from the lungs **(Figure 4b)**. On day 3 after challenge, all immunized groups showed lower lung virus titers than the non-immunized control group and this difference was significant for all groups except the group that received non-adjuvanted WIV. Mice vaccinated with the adjuvanted formulations showed reduced virus titers compared to mice that received non-adjuvanted formulations. MPLA-adjuvanted pulmonary vaccines and i.m. administered subunit vaccine resulted in the best protection. In the SFD WIV-MPLA group, the virus levels were below detection level for two mice. These results indicate that the adjuvanted powder vaccine elicits a more potent protection against influenza infection than the adjuvanted liquid vaccine.

In non-immune mice with a primary influenza infection, production of IFNγ by NK cells is an early response of the innate immune system to the infection and an indication of active virus replication[36-38]. We, therefore, determined IFNγ levels 3 days post challenge in lung supernatants as a measure for active viral replication **(Figure 4c)**. ELISA revealed substantial amounts of IFNγ in the lung supernatants of the control group and of mice vaccinated with non-adjuvanted vaccines. Lower levels of IFNγ were found in mice which received MPLA-adjuvanted formulations or i.m. subunit vaccine. In line with our expectation the observed IFNγ levels were proportional to the measured lung virus titers. These results imply that strong (local) immune responses effectively control virus replication in the lungs.

To evaluate systemic immune responses after virus infection, IgG antibody in serum **(Figure 5a)** and serum HI **(Figure 5b)** titers were determined in the virus-challenged mice. In line with the previous observations **(Figure 3)**, IgG antibody and HI titers were higher in mice immunized with MPLA-adjuvanted A/PR/8 WIV formulations than in mice immunized with non-adjuvanted formulations. For the MPLA-adjuvanted vaccines, IgG and HI titers were similar to those induced by i.m. administered subunit vaccine. Analysis of nose washes revealed that nose IgA titers **(Figure 5c)** after immunization with A/PR/8 WIV were generally low except for the SFD WIV-MPLA group. In the lungs, increased IgA **(Figure 5d)** and IgG **(Figure-5e)** antibody titers were observed in mice immunized with MPLA-adjuvanted formulations, compared to mice immunized with non-adjuvanted vaccine. Lung IgG antibody responses were in line with serum IgG responses. IgG1 and IgG2a responses were increased in mice immunized with MPLA-adjuvanted vaccines as compared to mice immunized with non-adjuvanted vaccine **(Figure 5f)**. However,
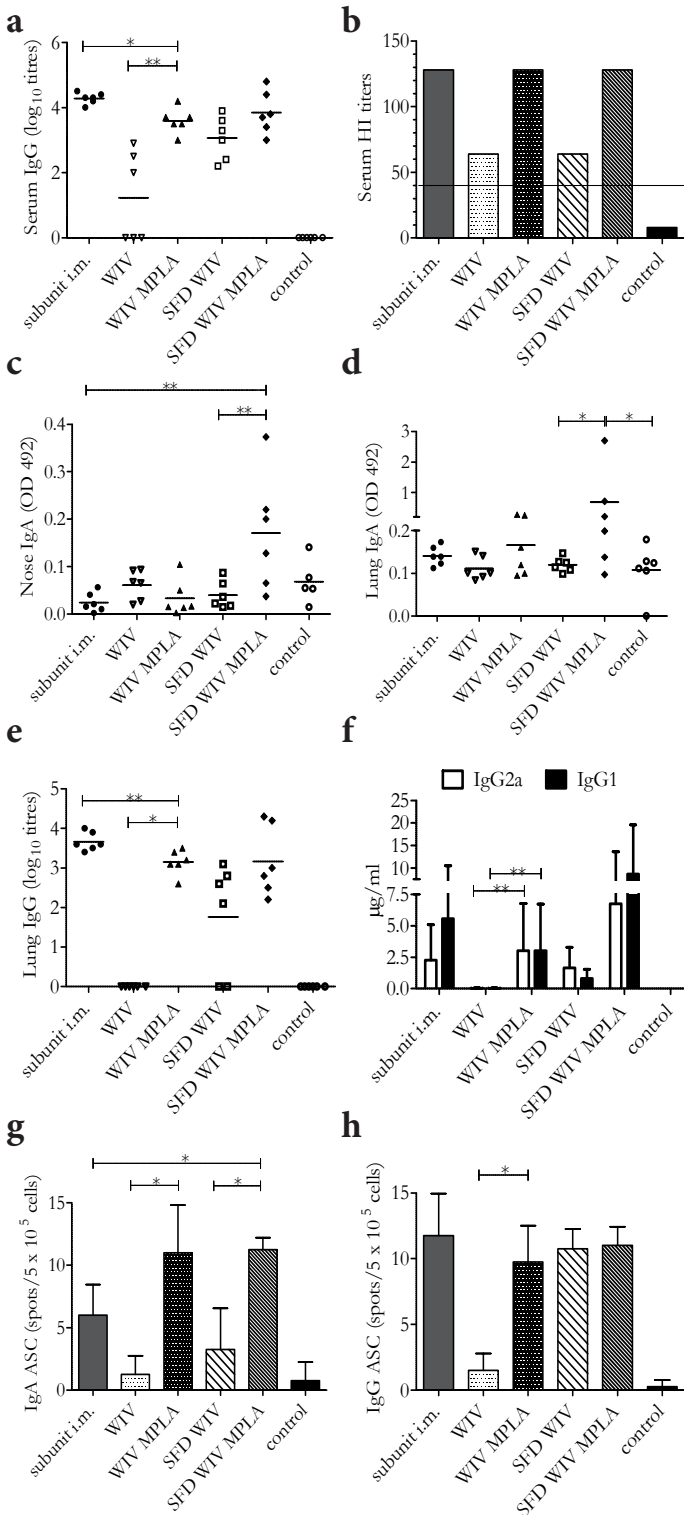
Figure 5. Effect of vaccine formulation on recall of immune response. Blood, nose wash, lungs and spleens from the mice described in the legend of Figure 4 were collected after vaccination and challenge and analyzed for (a) serum IgG (b) serum HI titers (c) nose IgA (d) lung IgA (e) lung IgG (f) serum IgG2a and IgG1 response. Serum and lung IgG antibody titers are expressed as log10 titers. Nose and lung IgA responses are represented as absorbance at OD492. HI titers after vaccination and challenge are expressed as reciprocal of the highest dilution of sera resulting in complete hemagglutination inhibition. Splenocytes were pooled per experimental group and IgA (g) and IgG (h) ASCs specific for A/PR/8 HA were enumerated by ELISPOT assay. Bars represent the number of ASCs per 5x105 cells +/- SD of quadruplicate measurements. Levels of significance are presented as *p<0.5, **p<0.01.

the differences reached statistical significance only for the groups immunized with liquid vaccine.

We next evaluated HA-specific memory B cells from immunized and challenged mice **(Figure 5g)**. Spleens of mice vaccinated via the pulmonary route with MPLA-adjuvanted vaccines contained significantly higher numbers of influenza-specific memory IgA-secreting B cells than spleens of mice vaccinated with non-adjuvanted formulations. Splenocytes from mice which received liquid WIV-MPLA showed approximately eight fold more memory IgA B cells compared to mice immunized with non-adjuvanted liquid WIV. Similarly, IgA-secreting memory B cells were approximately 3 times more frequent in splenocytes of mice immunized with SFD WIV-MPLA than in those of mice immunized with non-adjuvanted SFD WIV. Two-fold more memory IgA B cells were seen in spleens of in mice that received MPLA-adjuvanted pulmonary vaccines than in spleens of mice immunized i.m. with subunit vaccine. The number of memory IgG-secreting B cells **(Figure 5h)** was similar for all groups except for the group which received non-adjuvanted WIV via the pulmonary route. In this group the frequency of HA-specific memory IgG B cells was about 6 times lower than in the other groups.

Taken together, these results indicate that MPLA-adjuvanted vaccine formulations elicit more robust immune responses and protection, therefore causing less recruitment of IFNγ-producing NK cells to the lungs, than non-adjuvanted liquid WIV or SFD WIV. Across all parameters studied, SFD WIV-MPLA was found to be the best vaccine for its ability to induce more potent mucosal and systemic responses along with better protection than other vaccines.

4. Discussion

In the current study, we evaluated whether inclusion of MPLA as adjuvant in a liquid or powder influenza vaccine for pulmonary administration has advantageous effects on the immune response. WIV was chosen as basic vaccine formulation, since it is known to be superior to split or subunit vaccine formulations in inducing immune responses[39]. Analysis of liquid WIV and liquid WIV-MPLA by nanoparticle tracking and TEM highlighted that MPLA did not have any adverse effect on the size and structure of the inactivated virus particles in WIV. Dry powder vaccines were produced by spray-freeze-drying liquid WIV and liquid WIV-MPLA using inulin as stabilizer. Analysis of SFD WIV or SFD WIV-MPLA revealed that the stress encountered during spray-freeze-drying did not affect the biological activity of either MPLA or HA. Characterization of the vaccine powders showed that particle size, specific surface area and morphology of SFD WIV and SFD WIV-MPLA were comparable and both vaccine powders were found to be suitable for pulmonary immunization. In vivo studies demonstrated that pulmonary administration with MPLA-adjuvanted formulations, in particular the SFD formulation, resulted in superior antibody responses compared to non-adjuvanted formulations. Moreover, in mice, vaccinated with MPLA-adjuvanted formulations, decreased weight loss

and lower lung virus titers were observed after challenge with live influenza virus. Pulmonary immunization with MPLA-adjuvanted vaccines was at least as effective as i.m. immunization in reducing lung virus titers.

MPLA is an amphiphilic molecule and as such may have detrimental effects on the structure of the inactivated virus particles, like WIV. The structural integrity of the virus particles is important as only intact virus particles will provide protection for the ssRNA present in WIV; viral ssRNA has been shown to be recognized by TLR7 and to act as an intrinsic adjuvant in WIV[40]. Evaluation of the size and structure of the WIV virus particles by the nanoparticle tracking system and TEM did not reveal any effect of MPLA on these properties. Thus, addition of MPLA did not interfere with the structural integrity of the virus particles. HA-mediated binding of the WIV virus particles to cellular receptors is important for vaccine internalization by antigen-presenting cells. Moreover, a proper natural conformation of the HA would also be important to induce antibodies that recognize conformational epitopes and thus are able to interact with the native HA on infectious virus. We observed that the hemagglutination properties of WIV with and without MPLA were similar indicating that MPLA had no effect on the biological activity of HA. Thus, neither the structural integrity nor the cell-binding properties of the WIV virus particles were affected by addition of MPLA.

MPLA is known to retain its adjuvant activity but to be less toxic when integrated in a membranous environment[41]. Furthermore, LPS, the molecule from which MPLA is derived, was described to be more active when integrated into a virosomal membrane than when being present freely in solution[42]. Whether or not MPLA added to WIV in our experiments did integrate in the membrane of the virus particles remains elusive. MPLA easily inserts into lipid membranes of liposomes and virosomes when added during constitution of these structures[21,41]. If not inserted MPLA will form micelles when present in sufficiently high concentration (> 5 µM, [34]). We did not find evidence for either incorporation or the presence of micelles in our studies. The amount of MPLA used per vaccine dose (0.35 nmol) equals about 7% of the viral lipids in the vaccine. This amount is insufficient to increase the size of virus particles to an extent detectable by the nanoparticle tracking system or TEM. Indeed, we did not observe an increase in mean diameter of the WIV particles. We did not detect MPLA micelles either which might be due to the high dilution used for analysis by the nanoparticle tracking system by which the MPLA concentration was lower than its critical micelle concentration. The fate of MPLA added to WIV thus remains to be determined.

Earlier we reported that spray-freeze-drying is an effective technique to produce subunit or WIV vaccine powders suitable for pulmonary delivery[6,12,18]. Particle size distribution and specific surface area are key parameters for pulmonary delivery of the vaccine powder. The aerodynamic particle size determines the distribution of the powder vaccine in the respiratory tract while surface area, indicative of porosity, determines dispersion of powder particles from the DPI. Moreover, porous particles

dissolve instantaneously when they come in contact with aqueous solutions making them ideal for pulmonary vaccine delivery. These parameters may get affected by the presence of amphiphilic molecules like MPLA in vaccine formulations during spray-freeze-drying. Our data shows that the characteristics of powder particles generated from WIV in absence or presence of MPLA are similar. Possibly, the detrimental effects of MPLA are limited because we used a very high inulin to MPLA weight ratio (1600:1). Alternatively, interaction of the MPLA acyl chains with inulin may be prevented due to insertion of MPLA into the lipid bilayer of WIV.

After evaluating SFD vaccine powders for their physical characteristics, immune responses induced by pulmonary administered MPLA-adjuvanted or non-adjuvanted WIV were determined. MPLA-adjuvanted vaccine formulations induced significantly higher IgG and HI titers in serum than non-adjuvanted formulations, indicating a beneficial effect of including MPLA in pulmonary vaccines. These results are in agreement with previous observations demonstrating enhancement of immune responses to Hepatitis B surface antigen, tetanus toxoid, influenza subunit vaccine or RSV-derived virosomes administered to the total respiratory tract when MPLA was used as adjuvant[25,26,43].

A major aim of pulmonary vaccination is the induction of IgA responses at the site of virus entry. As expected, subunit delivered i.m., used as a golden standard in our experiments, was a rather poor inducer of lung IgA and failed to induce IgA in the nose. Pulmonary immunization with MPLA-adjuvanted vaccine as compared to non-adjuvanted formulations resulted in significantly increased IgA antibody levels in lung but not in nose. The low amounts of IgA in the upper respiratory tract may be explained by the fact that the antigen was directly delivered to the lungs, thus bypassing the nasal mucosa and its associated lymphoid tissue. Indeed, MPLA-adjuvanted RSV virosomes delivered to the total respiratory tract via the nose were found to effectively induce IgA in the lung as well as in the nose[26,44]. Another explanation could be that the amount of MPLA used in the vaccine was insufficient to induce nasal IgA upon pulmonary immunization. Based on in vitro experiments using the NFκB reporter cell line RAWBlue™, we chose a dose of 0.625μg MPLA per vaccine dose of 5 μg HA. This amount was obviously sufficient to stimulate production of IgA in the lungs, the site of vaccine administration, but not in the nose. In the studies on RSV virosomes, a much higher dose of MPLA was employed, i.e. 5 μg versus 0.625 μg used in the present study[26,44]. Thus, MPLA adjuvantation of pulmonary administered vaccines is capable of stimulating IgA production in the lungs but higher amounts of adjuvant may be necessary to also induce nasal IgA.

Analysis of lung virus titers and lung IFNγ levels upon influenza virus challenge revealed that MPLA-adjuvanted vaccines induced better protection in mice than non-adjuvanted formulations. For challenge, 200 PFU virus in 40 μl of buffer were inoculated in mice through the nasal route. In this model, the whole respiratory tract is challenged but most of the virus particles are delivered to the lungs because of the relative large volume used[45]. In mice immunized with the MPLA-adjuvanted

vaccines higher levels of IgA and IgG antibodies were produced in the lungs and might have played a vital role in neutralization of influenza virus and therefore reduction of virus titers. In addition, serum IgG antibody transuding into the lungs might have further helped in virus clearance[46]. Furthermore, MPLA-adjuvanted vaccines induced higher amounts of IgG2a than non-adjuvanted vaccines. In mice it has been shown that IgG2a antibody provides better protection than IgG1 against influenza virus upon challenge[20]. Thus, induction of higher levels of antibody and of the superior antibody subtype IgG2a probably contributed to the improved protection of mice immunized with MPLA-adjuvanted vaccine as compared to mice that received non-adjuvanted formulations.

In conclusion, our data indicate that MPLA is an effective adjuvant for pulmonary vaccines. When delivered together with WIV influenza vaccine MPLA improves local and systemic immune responses as well as the protective capacity of the vaccine. A stable adjuvanted vaccine can be formulated by spray-freeze-drying in the presence of inulin. In fact, among the vaccine formulations studied SFD WIV-MPLA was the most potent, inducing the highest serum and mucosal antibody responses and providing the best protection against virus challenge. To our knowledge, this is the first study to demonstrate that MPLA can be SFD along with an antigen formulation, in this case WIV, with full retention of its adjuvant activity. MPLA adjuvantation of pulmonary administered WIV certainly had a positive effect on humoral response induction and the effect can probably be further improved by using higher amounts of the adjuvant, especially with respect to nasal IgA titers. Pulmonary immunization with SFD WIV-MPLA was equally effective as i.m. immunization with subunit vaccine, used as golden standard in our study, in inducing humoral immune response and was better in inducing mucosal antibodies and protection. Pulmonary administration of adjuvanted influenza vaccines is therefore a promising approach for induction of protective immune responses by user-friendly inhalation.

Acknowledgements

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Chapter 6

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[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[3-5]. Moreover, IgA antibodies are known for their ability to provide cross protection against heterologous influenza virus[4,6]. Together with IgG antibodies, IgA antibodies were shown to inhibit infectivity of influenza virus in the respiratory tract[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[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[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[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[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[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[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[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[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

NFkB activation was detected using the NFκB reporter cell line RAW-Blue™ cell line (InvivoGen, Toulouse, France) following the manufacturer's protocol. For stimulation, 1×10^5 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% CO_2 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-BlueTM (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]

$$d_{ae} = d_e \sqrt{\frac{\rho_p}{\rho_o \chi}}$$

where d_{ae}= aerodynamic diameter, d_e= geometric diameter, ϱ_p= density of powder particles (0.05 g/cm^3), ϱ_0 = unit density (1g/cm^3), χ = 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/O_2. 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/O_2 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/O_2 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 Heamagglutination inhibition

Heamagglutination inhibition (HI) assay was performed as described previously with minor changes[14] using pooled sera of mice from each experimental group. Four heamagglutination units (HAU) inactivated A/California/07/2009 (H1N1) virus was added to the diluted serum samples. HI titres are expressed as $\log_2$ values of the highest dilution preventing heamagglutination.

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% NH_4Cl, 1 mM $KHCO_3$, 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 10^6 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 Diaclone 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 10^5 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 % CO_2 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 % CO_2 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

5×10^5 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_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. IFNγ 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
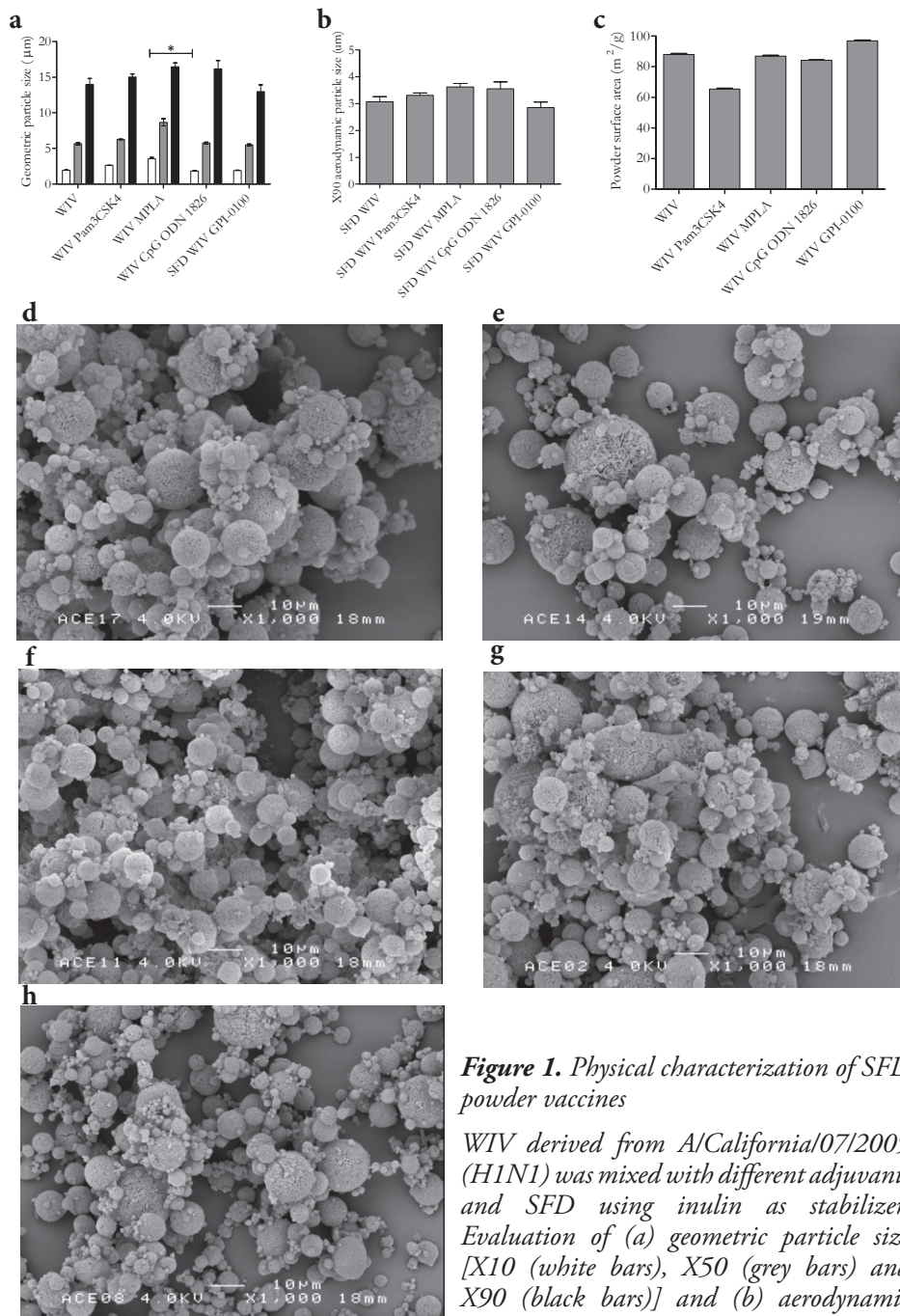
Figure 1. *Physical characterization of SFD powder vaccines*

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 m^2/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 m^2/g) while WIV-GPI-0100 had the highest (97 m^2/g). The surface area of WIV-MPLA and WIV-CpG was 87 m^2/g and 84 m^2/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.*

Vaccines	Laser diffraction (μm)			Span
	X10	X50	X90	
SFD WIV	1.93	5.637	13.953	2.133
SFD WIV Pam$_3$CSK$_4$	2.617	6.263	15.04	1.984
SFD WIV MPLA	3.537	8.66	16.46	1.492
SFD WIV CpG ODN 1826	1.82	5.747	16.16	2.495
SFD WIV GPI-0100	1.893	5.497	12.98	2.017

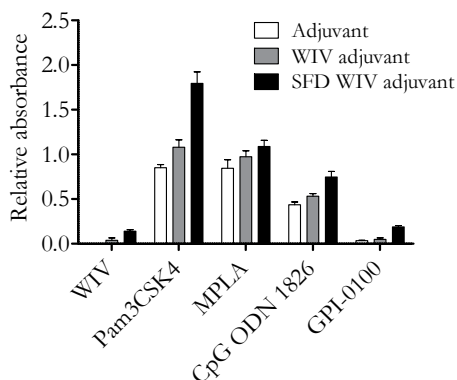**Figure 2.** *Biological activity of vaccine components in vitro*

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 NFkB in RAW-Blue™ cells, with Pam3CSK4 and MPLA being somewhat more active in this respect than CpG. NFkB 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 heamagglutination 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,
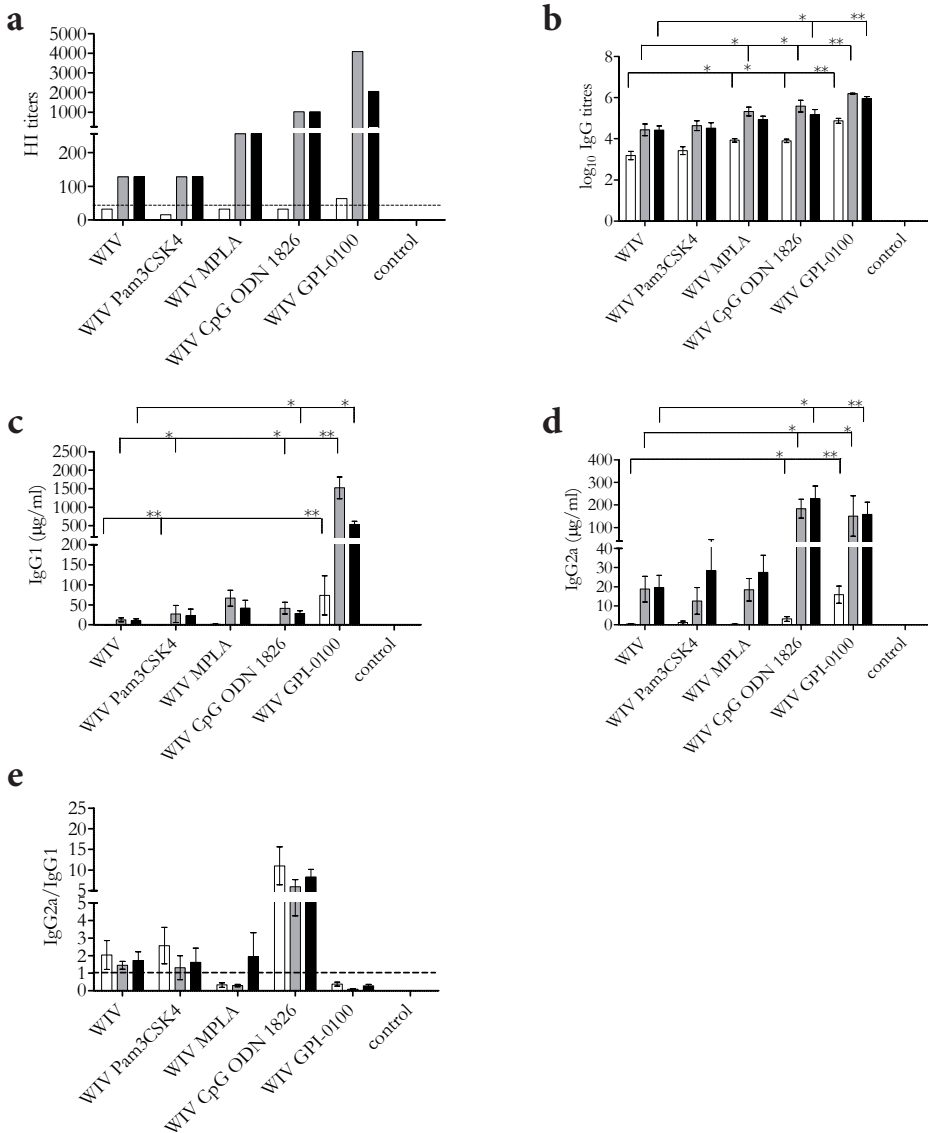
Figure 3. *Systemic antibody responses after pulmonary vaccination*

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 heamagglutination 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 IgG2as 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.
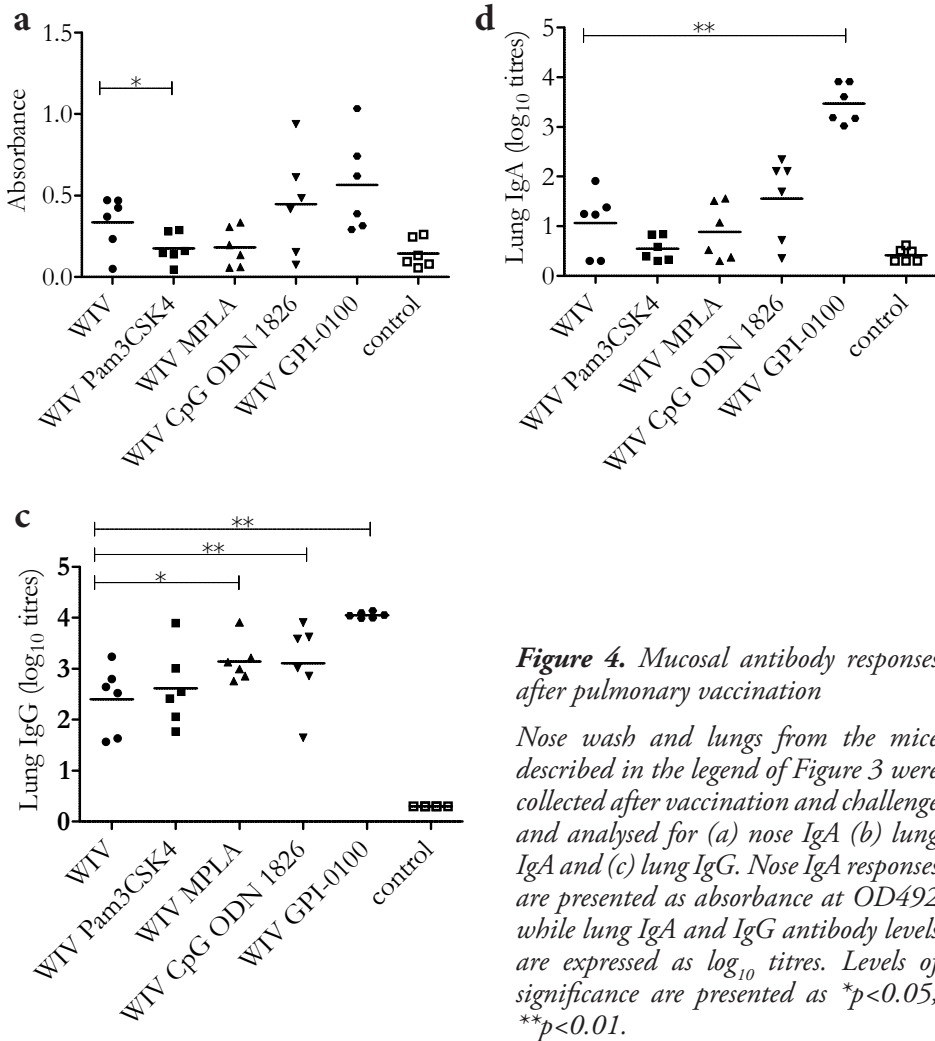
Figure 4. Mucosal antibody responses after pulmonary vaccination

Nose wash and lungs from the mice described in the legend of Figure 3 were collected after vaccination and challenge and analysed for (a) nose IgA (b) lung IgA and (c) lung IgG. Nose IgA responses are presented as absorbance at OD492 while lung IgA and IgG antibody levels are expressed as $\log_{10}$ titres. Levels of significance are presented as *p<0.05, **p<0.01.

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
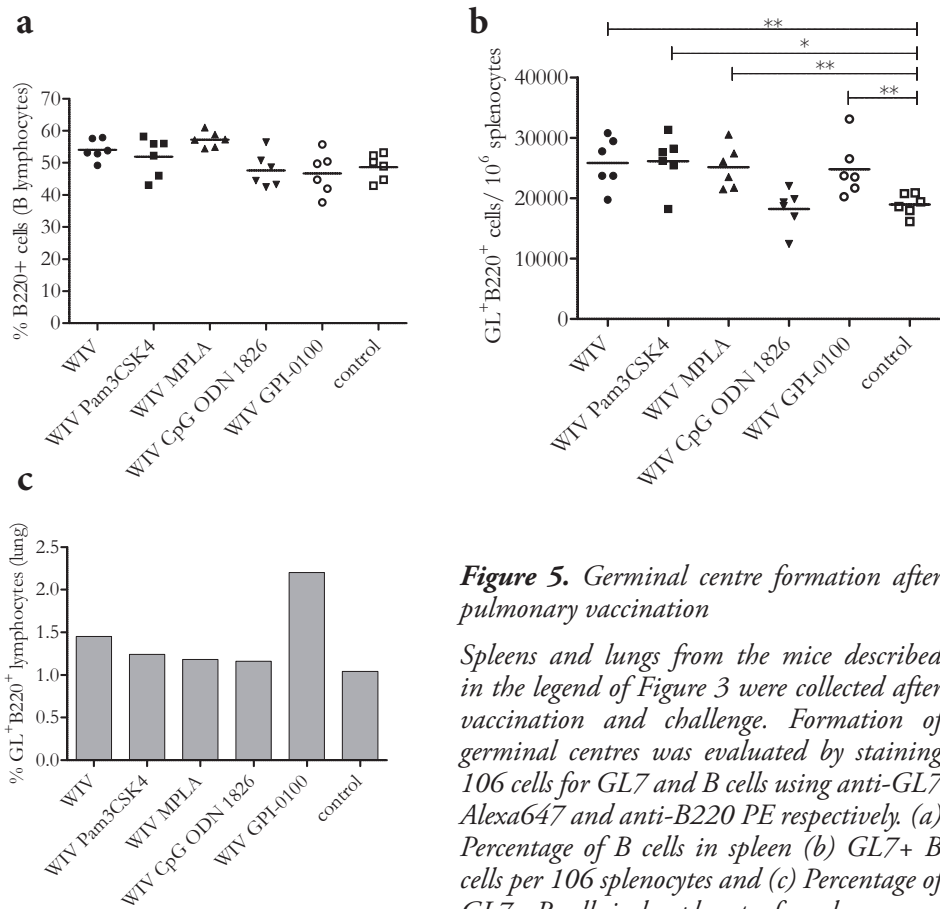
a

b

6

c

Figure 5. *Germinal centre formation after pulmonary vaccination*

Spleens and lungs from the mice described in the legend of Figure 3 were collected after vaccination and challenge. Formation of germinal centres was evaluated by staining 106 cells for GL7 and B cells using anti-GL7 Alexa647 and anti-B220 PE respectively. (a) Percentage of B cells in spleen (b) GL7+ B cells per 106 splenocytes and (c) Percentage of GL7+ B cells in lymphocytes from lungs.

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
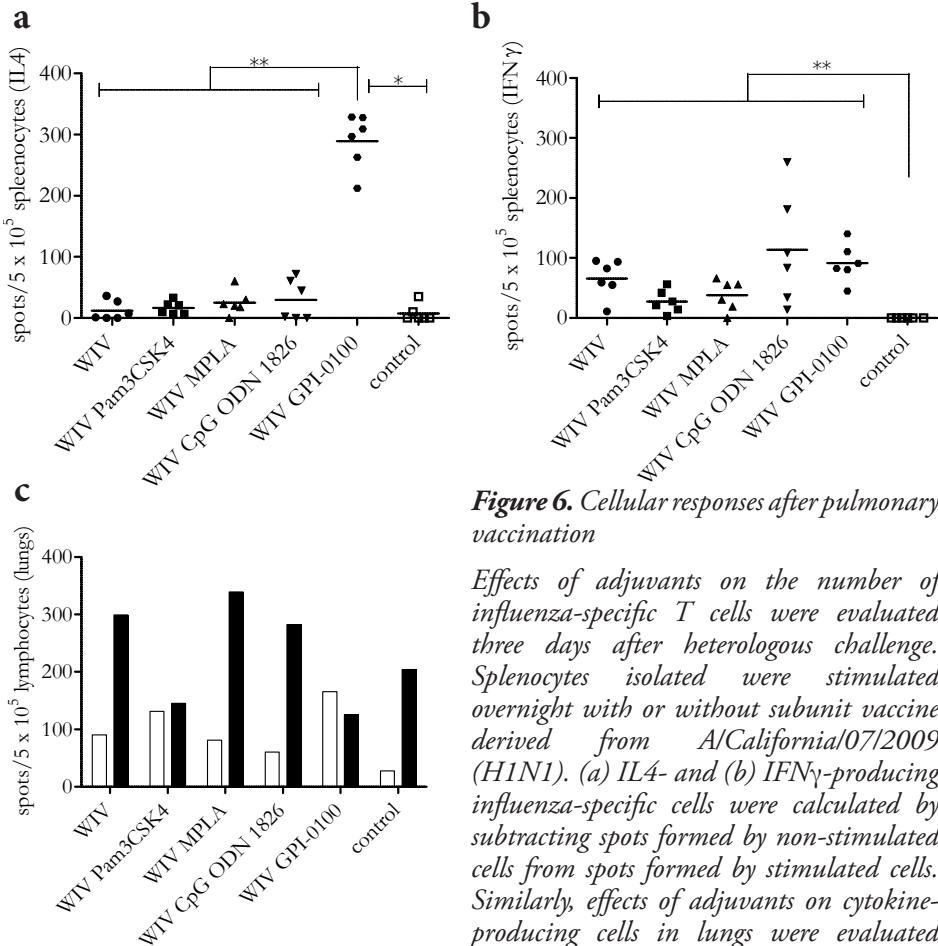
Figure 6. *Cellular responses after pulmonary vaccination*

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) IFNγ-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 IFNγ- (black bars) producing cells. The results are expressed as spot forming cells per 5 x 105 cells. Levels of significance are presented as *p<0.5, **p<0.01.

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
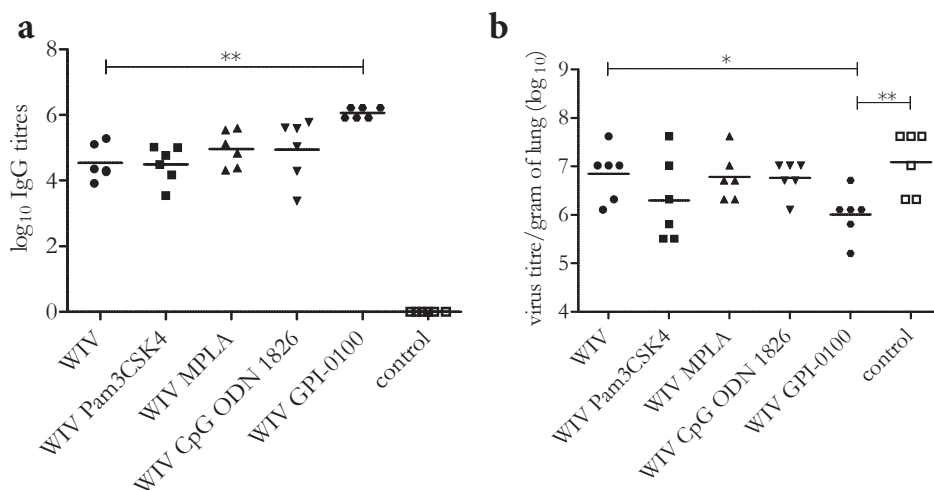
Figure 7. *Cross protection after pulmonary vaccination*

One month after the second vaccination, mice (n=6) were given a heterologous challenge with 200 PFU A/PR/8 (H1N1). Three days post challenge, mice were sacrificed. Serum samples were analysed for A/PR/8 (H1N1) specific (a) IgG. Lung supernatants were evaluated for (b) virus titres. IgG antibody levels are expressed as 10log titres per ml. HI titres after vaccination and challenge are expressed as reciprocal of the highest dilution of sera resulting in complete heamagglutination inhibition. Virus titres are expressed as 10log titres per gram lung. 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-Blue™ 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 mm^2 upper respiratory tract v/s 480 mm^2 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.

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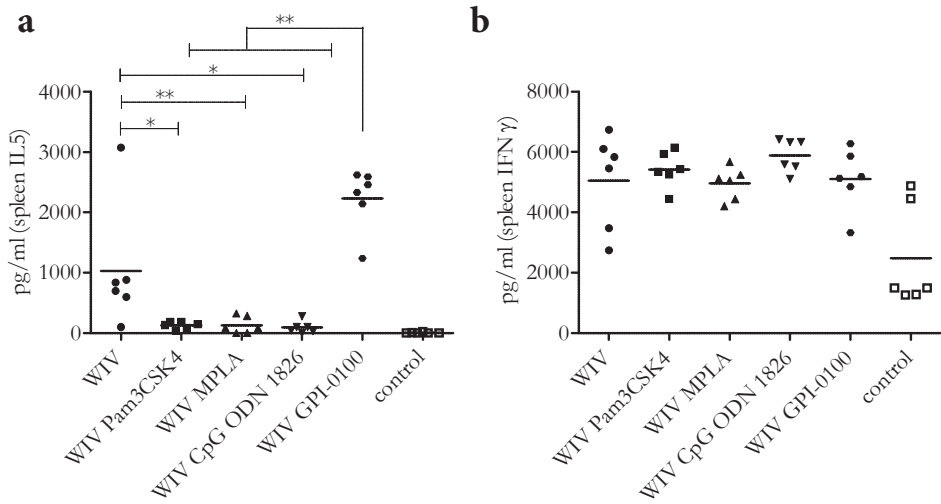
Supplementary Figure 1. *Level of cytokine production in stimulated splenocytes.*

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.
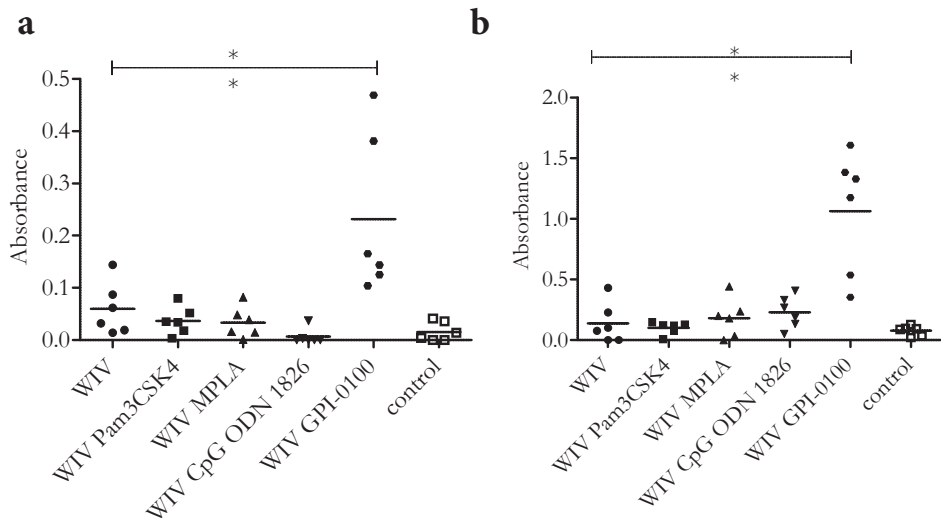
Supplementary Figure 2. *Serum IgA responses.*

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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Chapter 7

Simplifying influenza vaccination during pandemics: sublingual priming
and intramuscular boosting of immune responses with heterologous
whole inactivated influenza vaccine

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Abstract

The best approach to control the spread of influenza virus during a pandemic is vaccination. Yet, an appropriate vaccine is not available early in the pandemic since vaccine production is time consuming. For influenza strains with a high pandemic potential like H5N1, stockpiling of vaccines has been considered but is hampered by rapid antigenic drift of the virus. It has, however, been shown that immunization with a given H5N1 strain can prime the immune system for a later booster with a drifted variant. Here we investigated whether whole inactivated virus (WIV) vaccine can be processed to tablets suitable for sublingual (s.l.) use and whether s.l. vaccine administration can prime the immune system for a later intramuscular (i.m.) boost with a heterologous vaccine. In vitro results demonstrate that freeze-drying and tableting of WIV did not affect the integrity of the viral proteins or the hemagglutinating properties of the viral particles. Immunization experiments revealed that s.l. priming with WIV (prepared from the H5N1 vaccine strain NIBRG-14) four weeks prior to i.m. booster immunization with the same virus strongly enhanced hemagglutination inhibition (HI) titers against NIBRG-14 and the drifted variant NIBRG-23. Moreover, s.l. (and i.m.) immunization with NIBRG-14 also primed for a subsequent heterologous i.m. booster immunization with NIBRG-23 vaccine. In addition to HI serum antibodies, s.l. priming enhanced lung and nose IgA responses while i.m. priming enhanced lung IgA but not nose IgA levels. Our results identify s.l. vaccination as a user-friendly method to prime for influenza-specific immune responses towards homologous and drifted variants.

1. Introduction

Influenza is an infectious disease responsible for morbidity and mortality during yearly epidemics and occasional pandemic outbreaks. Vaccination is an efficient tool in containing the virus by stimulating the immune system and will be of eminent importance in case of a pandemic. However, the immediate availability of influenza vaccine during a pandemic is hindered by the laborious vaccine production process [1]. For this reason it is desirable to stockpile influenza vaccines for future pandemics, at least for influenza strains like H5N1 which have a high pandemic potential [2,3]. Yet, limited shelf life of current vaccine formulations and ongoing antigenic drift of the H5N1 virus hamper the stockpiling process [4–6]. Therefore, new strategies have to be developed to improve pre-pandemic preparedness.

H5N1 virus circulates mainly in aquatic birds and in poultry in South-east Asia and occasionally infects humans who are in close contact with the infected birds. Human to human transmission is rare and very few cases have been reported until today [7,8]. Yet, recent studies have shown that a very limited number of mutations may allow for efficient human to human transmission in the future [9–11]. Hence, H5N1 poses a constant threat of causing a new pandemic

Much effort has been put in the development of H5N1 vaccines. It appeared that unlike other influenza A vaccines, the H5N1 subtype requires two vaccine doses for effective protection [12,13]. The need for two doses of vaccine creates an obstacle to pre-pandemic preparedness and may contribute to a high risk of vaccine shortage during pandemic outbreaks. Furthermore, H5N1 viruses display distinct antigenic drift; currently 9 clades and several sub-clades are distinguished [14,15]. In some cases, influenza vaccines from one clade may provide cross protection against another clade(s). Yet, in other cases cross-reactivity is very limited [16]. Interestingly, a recent clinical study has demonstrated that immunization with one H5N1 virus strain, i.e. A/Vietnam/1203/2004 can prime for boosting with another strain, i.e. A/Indonesia/05/2005 and that the resulting antibodies can neutralize both the virus strains [17]. The heterologous boosting of immune responses with vaccines from drifted H5N1 virus has paved the way to pandemic preparedness. Indeed, several countries including the USA have currently stockpiled pre-pandemic H5N1 vaccines.

The instability of liquid influenza vaccine is an important problem in stockpiling of H5N1 vaccines [18]. Influenza vaccines can be stabilized by incorporating them into a glassy matrix of a sugar, e.g. inulin, dextran or trehalose, for example by means of freeze-drying techniques [19–22]. Dried vaccines can be reconstituted prior to intramuscular (i.m.) injection. Yet, ideally they would be used in dry form, e.g. as powder or formulated in a unit-dosage form like tablets. Tablets are easy to distribute and administer during challenging situations like the outbreak of an avian influenza pandemic.

Unfortunately, delivery of influenza vaccines to the gastro-intestinal tract as such induces suboptimal immune responses [23]. Recently, s.l. delivery of influenza vaccine has raised interest. Preclinical studies in mice have shown promising results: s.l. administered influenza vaccine is capable of inducing hemagglutination-inhibiting antibodies in serum as well as a local immune response in the upper respiratory tract [24–29]. Secreted IgA in the mucosa provides a local immune protection. IgA antibodies in the nose have an added advantage because they can neutralize the virus at the port of entry [21,30].

In this study, we tested the possibility of formulating a whole inactivated virus (WIV) H5N1 vaccine into a stable s.l. tablet and the suitability of such a tablet to prime for an immune response which can be boosted by i.m. immunization with a conventional vaccine formulation. We used inactivated A/Vietnam/1203/2004 vaccine virus (NIBRG-14) belonging to clade 1 for production of the s.l. tablets and NIBRG-14 or A/turkey/Turkey/1/2005 vaccine virus (NIBRG-23), a clade 2.1 virus, for the subsequent i.m. boost.

2. Materials and methods

2.1 Materials

The NIBRG-14 (a reassortant strain of A/PR/8/34 (H1N1) and A/Vietnam/1194/2004 (H5N1)) and NIBRG-23 (a reassortant strain of A/PR/8/34 (H1N1) and A/turkey/Turkey/1/2005 (H5N1)) vaccines strains were obtained from the National Institute for Biological Standards and Controls (NIBSC), Potters Bay, UK and were propagated on embryonated chicken eggs. Inulin 4 kD was procured from Sensus (Roosendaal, The Netherlands). The tablet excipients i.e. Avicel PH 102 (microcrystalline cellulose) and Ac-Di-Sol (cross-linked sodium carboxymethylcellulose) were purchased from FMC, Biopolymers (Philadelphia, USA), and mannitol from Bufa (Uitgeest, The Netherlands).

2.2 Vaccine

Whole inactivated influenza virus (WIV) was derived from NIBRG-14 and NIBRG-23 viruses by inactivating them with β-propiolactone as described previously [21]. Thereafter, WIV was purified by dialyzing overnight against HBS (2 mM Hepes, 125 mM NaCl, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4). The protein concentration of the inactivated vaccine solution was determined by micro-Lowry assay [31]. The HA content of the vaccine was considered to be one third of the total protein content.

2.3 Freeze-drying

The NIBRG-14 vaccine was freeze-dried as described previously [19]. In brief, the vaccine was mixed with an aqueous inulin solution at a HA: inulin weight ratio

of 1:500 and a final inulin concentration of 5 % w/v. Subsequently, 10 ml glass vials were charged with 2 ml of the vaccine dispersion. The samples were frozen by immersing the vials in liquid nitrogen for 10 minutes. After freezing, they were placed on the shelf of a freeze dryer (Christ Epsilon 2-4 freeze dryer; Salm and Kipp, Breukelen, The Netherlands). The shelf and condenser temperature were set at -35 °C and -85 °C, respectively. The freeze-drying process was initiated by reducing the pressure to 0.180 mBar and the shelf temperature was gradually increased to 4 °C over 32 hours. Thereafter, the pressure was further reduced to 0.05 mBar while the temperature was gradually increased to 20 °C over 11 hours. Freeze-drying was continued under these conditions for another 24 h. After freeze-drying, glass vials were closed in a nitrogen atmosphere with a controlled relative humidity of less than 10 % and stored at room temperature until further use.

2.4 Formulation and evaluation of s.l. vaccine tablets

S.l. tablets weighing 30 mg were formulated by mixing freeze-dried NIBRG-14 vaccine (25 % w/w) with Avicel PH 102 (55 % w/w), mannitol (10 % w/w) and Ac-Di-Sol (10 % w/w). The powder mixture was compressed using a single (6 mm x 2 mm capsule shaped die) tablet press with a compaction force of 10 kN, which was reached within 5s. The crushing strength of the tablet in radial direction was evaluated using a tablet tester (MODEL 6D (SG), Pharmatron, Switzerland). The tablet disintegration test recommended by pharmacopoeia could not be used, because the size of the tablets was too small for the meshes of the basket. Hence, we adopted a simple method as described by Rawas-Qalaji et al [32]. Briefly, the tablet was immersed into a test tube filled with 2 ml of water and the time required for breakdown of the tablet into smaller fragments was recorded by visual inspection.

2.5 SDS-PAGE

The biochemical integrity of proteins in freeze-dried NIBRG-14 vaccine was analyzed by SDS-PAGE under non-reducing conditions and compared with unprocessed NIBRG-14 vaccine. The freeze-dried samples and the tablets were reconstituted in water. The reconstituted and unprocessed samples and a pre-stained protein ladder (Page Ruler 10-170K, Thermo Scientific, USA) were incubated at 37 °C for 10 minutes. Thereafter, the samples were mixed with sample buffer (Novagen® 4X SDS Sample Buffer, Millipore Corporation, USA). Each sample was then loaded on a pre-cast gel (12 % polyacrylamide Mini-PROTEAN TGX Pre-cast Gels, Bio-Rad, USA) and resolved at 100 V for 1.5 hrs. Subsequently, the polyacrylamide gel was subjected to silver staining as reported earlier [33]. The gel was dried and scanned using an HP scanner.

2.6 Hemagglutination assay

The hemagglutination capacity of the unprocessed NIBRG-14 and NIBRG-23 vaccines, reconstituted freeze-dried vaccines and solubilized tablets was determined as described earlier [34]. In brief, a dispersion of the vaccine containing 5 µg of hemagglutinin (HA) in 50 µl phosphate buffer saline (PBS) was prepared and added to the first well of a V-bottom micro-titer plate (Corning Constar, USA). Subsequently, the solution was serially diluted twofold in PBS (pH 7.4). Subsequently, 50 µl of 1 % guinea pig red blood cells, (RBC) suspension was added to the wells and hemagglutination was allowed to proceed for 2 h at room temperature. The highest dilution of vaccine capable of agglutinating the RBC was recorded as 1 hemagglutination unit (HAU). The measurements were performed in triplicate.

2.7 Immunization studies

Animal experiments were evaluated and approved by the Committee for Animal Experimentation (DEC) of the University of Groningen, The Netherlands. Female Balb/c mice (6-8 weeks old) were purchased from Harlan (Zeist, The Netherlands). All procedures in mice were performed under Isofluran/ O_2 (inhalation) anesthesia.

The mice were immunized on day 0 and day 56 according to the immunization schedule depicted in **Table 1**. To ensure proper s.l. vaccination the dry vaccine powder was reconstituted in 10 µl PBS and pipetted carefully under the tongue of anesthetized mice. The freeze-dried vaccine powder was used for reconstitution because it was found that the reconstitution of the formulated tablet required more than 10 µl of water while the sublingual cavity of a mouse can only accommodate maximally 10 µl of liquid. After s.l. immunization the mice were placed on a flat surface for 30 minutes under anesthesia to ensure effective immunization. Mice were sacrificed on day 84.

Table I. *Immunization schedule*

Group	Prime (day 0)	Boost (day 56)	n
1	s.l. (NIBRG -14)	i.m. (NIBRG -23)	8
2	i.m. (NIBRG -14)	i.m.(NIBRG -23)	8
3	s.l. (NIBRG -14)	i.m. (NIBRG -14)	8
4	i.m. (NIBRG -14)	i.m. (NIBRG -14)	8
5	s.l. (PBS)	i.m. (NIBRG -23)	8

Blood samples were taken twice, i.e. on day 28 by orbital vein puncture and on day 84 via cardiac puncture. The samples were centrifuged and the serum was collected. Serum samples were stored at -20 °C until further analysis. Nasal wash and bronchoalveolar lavage (BAL) were performed as described earlier [34].

2.8 Hemagglutination inhibition assay

The antigen neutralizing capacity of the collected sera was evaluated by HI assay and was performed according to the procedure used by Audouy et al [21]. In short, serum was inactivated by incubating it with kaolin suspension at 56 °C for 20 minutes. Subsequently, after centrifugation at 1200 rpm the samples were transferred to the first well of a V-bottom 96 well plate in duplicate and serially diluted twofold in PBS (pH 7.4). Then, 50 µl of either NIBRG-14 or NIBRG-23 vaccine containing 4 HAU was added to the wells. After 40 minutes of incubation at room temperature, 50 µl of 1 % guinea pig erythrocyte in PBS was added to wells. After 2 h of incubation at room temperature, the highest serum dilution capable of preventing hemagglutination of RBCs was scored as HI titer. By convention titers below the detection limit (<8) were assigned a titer of 4 for calculation purposes. An HI titer of ≥ 40 is considered to be effective in reducing the chance of influenza infection by 50 % [35–37]. HI titers are presented in $\log_2$ scale; a titer of 40 equals a $_2\log$ titer of 5.3.

2.9 ELISA

ELISA was performed as described previously [34]. Briefly, ELISA plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated overnight with WIV (NIBRG-14 or NIBRG-23) containing 500 ng of total protein (WIV) and then blocked with a 2.5 % aqueous solution of milk powder in PBS at 37 °C. The plates were washed and then charged with pre-diluted samples (serum, nasal or lung lavages), which were serially diluted till the last well of the plate (12 times) and the plates were incubated for 1.5 h at 37 °C. Then, 100 µl of horseradish peroxidase (HRP) conjugated antimouse IgG, anti-mouse IgG1, anti-mouse IgG2a or anti-mouse IgA (Southern Biotech, Birmingham, USA) diluted 1:5000 in PBST was added followed by incubation at 37 °C for 60 minutes for the detection of IgG, IgG1, IgG2a and IgA, respectively. Thereafter, the antibodies were detected using 1,2-phenylen-diamine-dihyrochloride (Sigma Aldrich, USA) as substrate in the phosphate-citrate buffer. The absorbance at 492 nm was measured with a micro-plate reader (Synergy HT, BioTek, USA). IgG antibody titers are given as the reciprocal of the sample dilution calculated to correspond to A492 = 0.2 after background correction and IgA levels are presented as average of the maximum absorbance of 1:1 diluted nose and lung washes. The measurements were performed in duplicate.

2.10 Statistical analysis

The titers are given as the geometric mean ± standard error of the mean (SEM), unless stated otherwise. The differences in titers between groups were analyzed by Mann-Whitney U test at 95 % confidence interval (P<0.05). Significance is denoted by one symbol (P≤0.05) or two symbols (P≤0.01).

3. Results

3.1 Formulation of WIV as s.l. tablet and evaluation of tablet properties

In order to assess whether formulation of WIV influenza vaccine as tablet for s.l. administration is feasible, the inactivated NIBRG-14 was freeze-dried using inulin as stabilizer, mixed with other tablet ingredients and compressed. During this procedure, the vaccine encounters stress related to freeze-drying and tableting. Therefore, to investigate the stability of the vaccine in s.l. tablets the unprocessed vaccine, the freeze dried vaccine and the tablets were subjected to different tests such as SDS-PAGE and HA activity assay. Furthermore, the crushing strength and the disintegration time of the tablet were determined.

Stability during drying and tableting

First, the proteins in WIV (NIBRG-14) were investigated to establish that they remained intact during freeze-drying and tablet formulation. WIV proteins i.e. HA, neuraminidase (NA), matrix protein (M1), and nuclear export protein (NS2) of processed WIV were compared with those of unprocessed WIV by SDS-PAGE analysis under non-reducing conditions. Knowing their molecular weights, the WIV proteins could be identified by comparison with the standard protein mixture. The band patterns of HA, NA, M1 and NS2 did not change after processing of WIV by freeze-drying and subsequent compression into tablets **(Figure 1a, compare lanes 2, 3 and 4)**. The faintness of bands in lane 3 may be due to adsorbent properties
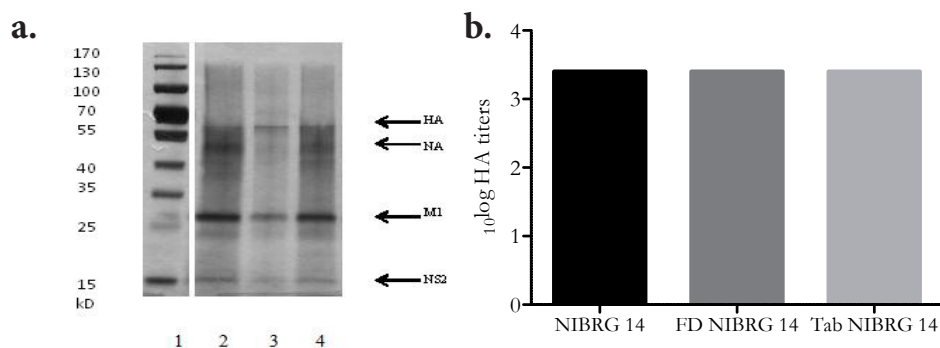

Figure 1. *Stability of NIBRG-14 WIV vaccine. (a) SDS-PAGE of unprocessed and processed WIV proteins under non-reducing conditions. Lane 1: molecular weight standard, lane 2: unprocessed WIV, lane 3: WIV freeze dried and subsequently processed into a formulated tablet, lane 4: freeze dried WIV. (b) HA titers of unprocessed WIV (black bar), freeze-dried WIV (dark gray bar) and freeze-dried WIV which was processed into a formulated tablet (light gray bar). The HA assay was performed in triplicate. As no differences in HA titers were found there are no error bars.*

of Avicel PH 102 and Ac-Di-Sol (which are insoluble) included in the tablet formulation. This result confirms the biochemical integrity of WIV proteins after freeze-drying and formulation.

Next, the structural stability of inactivated NIBRG-14 was investigated in vitro by determination of the hemagglutination activity. As shown in **Figure 1b**, the NIBRG-14 vaccine did not lose any hemagglutinating capacity during freeze-drying and processing of the freeze-dried powder into a tablet.

Tablet crushing strength and disintegration time

The tablets should have good mechanical stability in order to withstand the stress related to packaging and transport. The crushing strength of the formulated tablets was found to be 41.80 ± 0.92 N (n=10), which is high for these small tablets. In addition, the s.l. tablet should disintegrate rapidly to efficiently deliver the enclosed antigen and to prevent swallowing of the vaccine released from the tablet [38]. The disintegration time of the formulated s.l tablet was found to be 2 ± 1 sec (n = 10).

It can be concluded that inulin sugar glass technology can be successfully applied to prevent deterioration of WIV during stressful process conditions like freeze-drying and compression. The formulated tablets fulfilled requirements regarding strength and disintegration for s.l. tablets. Hence, a stable influenza vaccine in the form of sublingual tablet can be made using inulin glass technology.

3.2. Effect of s.l. priming on the immune response to i.m. immunization

In order to evaluate whether s.l. immunization can prime for a subsequent i.m. boost, mice were immunized s.l. with NIBRG-14 (20 μg HA) on day 0 followed by an i.m. booster immunization with NIBRG-14 or the drift variant NIBRG-23 on day 56 (5 μg HA). Control groups received prime and boost via the i.m. route (5 μg HA each) or received PBS on day 0 followed by i.m. immunization with NIBRG-23 (5 μg HA) on day 56. Serum antibodies towards NIBRG-14 and NIBRG-23 were evaluated on day 28 (after prime only) and on day 84 (28 days after the final immunization), respectively. Mucosal antibody responses in lung and nose were determined on day 84 after termination of the animals.

Hemagglutination inhibition titers

On day 28 after a single immunization with NIBRG-14 WIV serum HI titers against the homologous virus were below the detection limit ($\log_2 3$), irrespective of whether the immunization was given via the s.l. or the i.m. route **(Figure 2a and 2b, left parts, day 28)**. Similarly, no HI titer against the heterologous drift variant NIBRG-23 was observed 28 days after s.l. or i.m. immunization with NIBRG-14 **(Figure 2a and 2b, right parts, day 28)**. However, HI titers towards NIBRG14 were readily observed
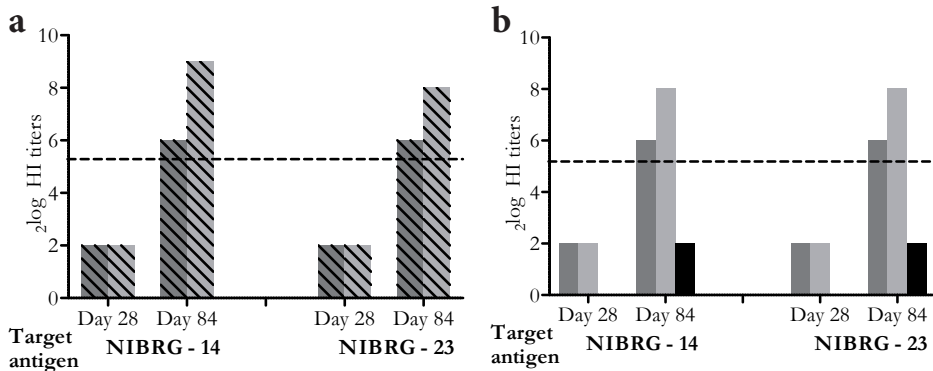
a

b

Figure 2. NIBRG-14 and NIBRG-23 specific HI titers of pooled sera after one or two immunizations with H5N1 WIV. (a) Mice were primed on day 0 with NIBRG-14 WIV given via the s.l. (dark gray bars) or the i.m. (light gray bars) route and boosted with NIBRG-14 WIV via the i.m. route on day 56. HI titers were measured in blood collected on day 28 and 84 against NIBRG-14 (left part) and NIBRG-23 (right part). (b) Mice were primed on day 0 by s.l. (dark gray bars) or i.m. (light gray bars) immunization with NIBRG-14 WIV and boosted i.m. with NIBRG-23 WIV on day 56. Control mice received PBS on day 0 and NIBRG-23 WIV via the i.m. route on day 56 (black bars). HI titers were measured at day 28 and day 84.

upon i.m. boosting of the s.l. or i.m. primed mice and exceeded the titer of 40 ($\log_2$ 5.3), regarded as protective in humans **(Figure 2a left part, day 84)**. Priming via the s.l. route (dark gray bar) was somewhat less effective than priming via the i.m. route (light grey bar) in this respect. The NIBRG-14 prime/boost immunization regimens resulted in comparable levels of HI antibodies directed against NIBRG-14 **(Figure 2a, left part, day 84)** and NIBRG-23 **(Figure 2a, right part, day 84)**.

Interestingly, s.l. (or i.m.) immunization with NIBRG-14 primed not only for a homologous i.m. boost with NIBRG-14 but also for a heterologous i.m. boost with NIBRG-23 vaccine **(Figure 2b, day 84)**. Priming with NIBRG14 followed by boosting with NIBRG23 resulted in $\log_2$ HI titers of 6 (s.l. priming) and 8 (i.m. priming) against NIBRG14 and NIBRG23. In contrast, i.m. immunization with NIBRG23 alone was not capable of inducing HI titers above the detection limit **(Figure 2b, black bars)**.

Serum IgG antibody titers and IgG subclasses

In contrast to HI antibodies, influenza-specific IgG (reacting with NIBRG-14 as well as NIBRG-23) could already be detected on day 28 after priming of the immune response with NIBRG-14 vaccine administered by the s.l. route **(Figure 3a and 3b, day 28, dark gray bars)**. These results clearly indicate that s.l. vaccination with WIV influenza vaccine induced a systemic immune response. Not surprisingly, the response elicited by s.l. priming was of somewhat lower magnitude than the response induced by i.m. priming **(Figure 3a and 3b, day 28, light gray bars)**. An i.m. boost with either NIBRG-14 **(Figure 3a)** or NIBRG-23 **(Figure 3b)** further
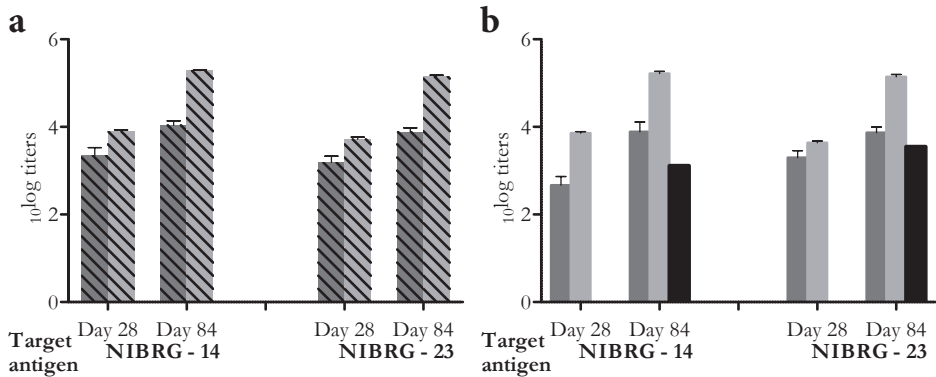
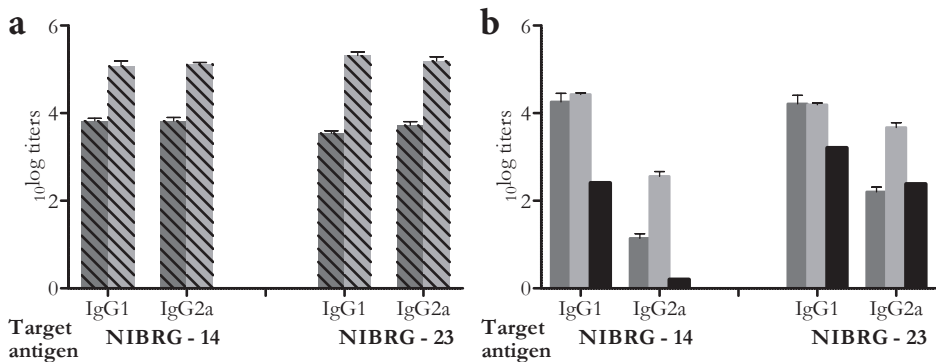**Figure 3.** *NIBRG-14 and NIBRG-23 specific IgG titers of sera after one or two immunizations with H5N1 WIV. (a) Mice were primed on day 0 with NIBRG-14 WIV given via the s.l. (dark gray bars) or the i.m. (light gray bars) route and boosted with NIBRG-14 WIV via the i.m. route on day 56. IgG titers were measured in blood collected on day 28 and 84 against NIBRG-14 (left part) and NIBRG-23 (right part). (b) Mice were primed and boosted as outlined in the legend to Fig. 2b. IgG in sera of individual mice was determined on day 84.*

Figure 4. *NIBRG-14 and NIBRG-23 specific IgG1 and IgG2a titers of sera after one or two immunizations with H5N1 WIV. (a) Mice were primed on day 0 with NIBRG-14 WIV given via the s.l. (dark gray bars) or the i.m. (light gray bars) route and boosted with NIBRG-14 WIV via the i.m. route on day 56. IgG1 and IgG2a titers were measured in blood collected on day 84 against NIBRG-14 (left part) and NIBRG-23 (right part). (b) Mice were primed and boosted as outlined in the legend to Fig. 2b. IgG1 and IgG2a in sera of individual mice was determined on day 84.*

increased the IgG titers in s.l. primed mice **(Figure 3a and 3b, compare day 28 day 84 titers)**, and had an even greater effect in i.m. primed animals **(Figure 3a and 3b, compare dark with light gray bars)**. IgG titers in s.l. primed and i.m. boosted animals were significantly larger (p = 0.0008) than in animals which received a single i.m. immunization **(Figure 3b, day 84, compare dark grey bars with black bars)**. However, the difference was by far not as large as that between the i.m. primed and boosted and the i.m. immunized group **(Figure 3b, day 84, compare light grey bars with black bars)**.

IgG subtypes (IgG1 and IgG2a) were determined on day 84 to elucidate which type of T-helper cell response, i.e. Th1 (IgG2a) or Th2 (IgG1) was predominantly induced by the different vaccination regimens **(Figure 3c and 3d)**. In line with the IgG titers, IgG1 and IgG2a titers were lower for s.l. primed mice than for i.m. primed mice. For the NIBRG14/NIBRG14 scenarios **(Figure 4a)** both regimens induced balanced responses with equal amounts of IgG1 and IgG2a. In contrast, for the heterologous NIBRG-14 prime/NIBRG-23 boost scenarios IgG2a responses were clearly lower than IgG1 responses especially when tested against NIBRG-14 **(Figure 4b)**. This effect was more pronounced for s.l. primed mice than for i.m. primed mice indicating that s.l. priming of IgG2a is somewhat less effective than i.m. priming. Still, IgG1 and IgG2a responses were higher for the mice immunized following a prime/boost regimen than for mice immunized only once i.m. with NIBRG-23 (except for NIBRG-23-specific IgG2a).
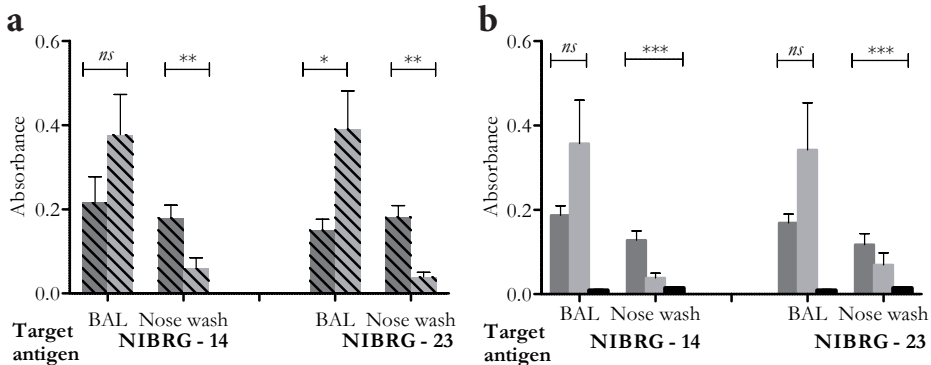
Figure 5. NIBRG-14 and NIBRG23 specific IgA responses in BAL and nasal washes (a) Mice were primed and boosted as outlined in the legend to Fig. 2a. IgA in BAL and nose of individual mice was determined on day 84. (b) Mice were primed and boosted as outlined in the legend to Fig. 2b. IgA in BAL and nose of individual mice was determined on day 84.

3.3 Mucosal immune response

The mucosal immune responses on day 84 were determined by measuring IgA levels against NIBRG-14 and NIBRG-23 in BAL and nasal washes. IgA reactive against both viruses was observed in the BAL of all experimental groups except the group which received a single i.m. immunization with NIBRG-23 vaccine **(Figure 5a and 5b)**. There was a trend towards higher levels of lung IgA in the i.m. prime/i.m. boost groups as compared to the s.l. prime/i.m. boost groups but differences were not statistically significant (except for NIBRG-23-specific lung IgA in the groups primed and boosted with NIBRG-14 **(Figure 5a)**). In contrast to lung IgA, nose IgA, important for virus neutralization at the port of entry, was significantly better induced by the s.l. prime/i.m. boost regimens than by the i.m. prime/i.m. boost regimens. As expected, no IgA was detected in the nose of mice that had received a single i.m. immunization with NIBRG-23.

These results indicate that s.l. priming is very effective in inducing antibody responses in the upper respiratory tract, the port of entry of the virus. Moreover, the induced IgA responses are cross-reactive across different clades and vaccine derived from one clade can prime for immunization with another clade.

4. Discussion and conclusion

In this study, we found that s.l. administration of NIBRG-14 WIV vaccine can prime for a subsequent i.m. boost. Priming was not only effective for providing a boost with homologous NIBRG-14 but also with heterologous NIBRG-23 WIV vaccine. This prime-boost regimen induced much better immune responses than an i.m. administration of NIBRG-14 or NIBRG-23 alone and resulted in HI titers of more than 40, considered as protective in humans. Moreover, mice immunized by s.l. priming followed by i.m. boosting showed significantly higher nasal IgA antibody levels than mice vaccinated by i.m. priming and boosting. Furthermore, we have shown that WIV vaccine can be formulated as a stable tablet with sufficient mechanical strength and a short disintegration time suitable for sublingual application.

Humans are naïve to H5 antigen. Hence, a single immunization with H5N1 vaccines appeared insufficient to induce protective antibody levels [39] Therefore, a multi dose vaccination strategy is required to achieve robust immune responses. Indeed, the necessity of a 2-dose vaccination strategy for H5 vaccines has been proven in several clinical trials [40,41]. In 2008, it was shown that an immune response primed by one type of H5N1 vaccine can be boosted with an antigenically distinct H5N1 vaccine for the induction of cross-clade reactive, protective antibodies [12,42,43]. This observation opened the way for a pandemic vaccination strategy involving priming with a stockpiled H5N1 vaccine (derived for example from clade 1) followed by boosting with an H5N1 vaccine derived from the pandemic strain (which might be a clade 2 virus). So far, prime-boost strategies have been tested with inactivated virus administered via the i.m. route and with live attenuated virus given by the i.n. route. In this study, we provide evidence that priming is also possible when

vaccine is administered via the s.l. route. Moreover, we show that s.l. priming not only enhances antibody titers upon i.m. boosting with a homologous virus strain but also effective in priming for boosting with a heterologous strain.

In addition to enhancing serum IgG titers, s.l. priming also induced IgA responses in the upper respiratory tract. This in contrast to i.m. priming which could induce IgA in the lungs but not in the nose. IgA in the upper respiratory tract is very important for neutralization of influenza virus before an infection can get established and IgA was shown to be much more effective in this respect than IgG [44]. Thus, next to the advantage of ease of immunization, s.l. antigen delivery has the additional advantage of inducing local immunity at the port of entry of influenza virus.

Sublingual immunization has so far been performed mainly in the context of s.l. immunotherapy (SLIT) in which allergens are administered via the s.l. route to diminish an allergenic immune response. Administration of allergens via the s.l. route has been in use for several years and has been very successful. More recently, the sublingual route has also been used for the induction of immune responses. Generally, s.l. vaccination with influenza vaccine required high doses of antigen and/or use of an adjuvant to elicit robust immune response [24]. In this study, we tested s.l. vaccine administration as a priming strategy. Our results indicate that s.l. priming of immune responses can work even without an adjuvant, at least when performed with WIV which is known to be highly immunogenic due to the presence of single stranded viral RNA [45]. The effectiveness of s.l. priming can possibly be further enhanced by addition of suitable adjuvants to the vaccine. Recent studies of influenza vaccines with adjuvants including detoxified cholera toxin and c-di-GMP demonstrate promising results [27,29,46]. Although in the animal study reported here liquid vaccine was given sublingually, we envisage that the WIV vaccine should ideally be converted into a dry and stable product and formulated into a s.l. tablet. Such tablets have two major advantages over the conventional liquid influenza vaccines. First, stockpiling is much easier because no refrigerated conditions are required. Secondly, administration of a s.l. tablet is much more easy than the conventional parenteral administration facilitating a rapid vaccination of the population which is imperative during a pandemic outbreak. In the present study, the NIBRG-14 WIV vaccine was incorporated in an amorphous inulin glass by freeze-drying and then compressed into tablets using appropriate excipients. The antigen stability after tableting was confirmed by SDS-PAGE and the HA assay. In previous studies, we have shown that WIV incorporated in inulin glasses shows an excellent storage stability [22,45]. Therefore, although not investigated in the present study, it can be assumed that the WIV vaccine incorporated in the s.l. tablet can be stored at ambient temperatures for prolonged periods of time (years) without loss of antigenicity, The freeze-dried WIV influenza vaccine was formulated with Avicel pH 102, mannitol and Ac-Di-Sol to fulfill the requirements of the s.l. tablet. The crushing strength (> 40 N) and disintegration time (< 3 seconds) of the formulated s.l. tablets were well within the limits [47]. The fast disintegration guarantees rapid delivery of antigen for effective immunization [38].

Taken together, our results show that s.l. administration of WIV influenza vaccine can successfully prime for a later i.m. booster with a vaccine derived from a homologous but also a heterologous influenza strain. This vaccination strategy results in protective levels of serum antibodies as well as IgA in the upper respiratory tract. When incorporated into a polysaccharide matrix the vaccine can be formulated as a stable tablet, enabling long-term storage and easy vaccine administration. The results indicate that s.l. priming of influenza-specific immune response with s.l. tablets vaccine is an ideal strategy for pandemic preparedness with respect to H5N1 a concept that may also be suitable for other influenza strains.

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7

Chapter 8

Summary and concluding remarks

Senthil Murugappan, Wouter L.J. Hinrichs

Summary

Vaccination against influenza is the most important strategy to control the virus spread during seasonal epidemics and pandemics. However, there are several shortcomings related to current vaccines, i.e. they have to be administered parenterally (except for the live attenuated virus vaccine Flumist®), their production capacities are limited, the immune response they elicit is sub-optimal, and they are unstable.

In this thesis, we explored two different strategies to avoid these shortcomings. In the first strategy, a dry and stable adjuvanted vaccine powder has been developed that can be administered through the pulmonary route. In the second strategy a sublingual (s.l.) tablet containing a stabilized vaccine of a previous strain was developed that can be used to prime the immune response for a booster of a vaccine prepared from the current drifted strain.

Hence, in this thesis we evaluated,

1. The storage stability of powdered pulmonary vaccines.

2. The preservation of immunogenicity of adjuvanted pulmonary influenza vaccines during spray-freeze drying process, we also evaluated the adjuvant effect of adjuvanted vaccines.

3. The sublingual priming (with stabilized vaccine) for subsequent intra muscular (i.m.) boosting of immune response with heterologous influenza vaccine

In chapter 2, a literature review is given on particulate influenza vaccines. The particulate influenza vaccines resemble the intact influenza virus. The particulate nature of the vaccines holds great promise to induce better immune response than subunit or split vaccine. Furthermore, the review discusses about the regulatory challenges and perspectives of particulate influenza vaccines.

In chapter 3, we evaluated the physical and immunogenic stability of powdered WIV for pulmonary vaccination. WIV was spray freeze-dried in the presence of inulin, dextran and mixture of dextran and trehalose. The properties of spray freeze dried powders were found to be suitable for pulmonary immunization. Furthermore, during spray freeze drying, WIV retained its particulate nature and its antigenicity was maintained. The powdered vaccines were stored at -20, 2-8, 30 and 40 °C for 3 months. The physical properties of all three vaccine powders, i.e. the particle size and specific surface area were maintained when stored at temperatures up to 30°C. The powdered vaccines stored at 30 °C for 3 months were used to evaluate their receptor binding capacity and immunogenicity. It was found that the receptor binding capacity of dry powder vaccines was preserved which was confirmed by the maintenance of the hemagglutination titers. The immunogenic stability of the formulated powders was evaluated in mice. The results revealed that the immunopotentiating effects of dry powder vaccines were maintained as the immune responses elicited by the stored vaccines were comparable to freshly prepared spray freeze-dried vaccines. During

storage, however, most likely some particle-particle interaction occurred which interfered with a proper vaccine delivery to the lungs when using the dry powder insufflator. In contrast, this problem was not observed when the RODOS powder disperser was used. Hence, it should be noted that the stored vaccines require an efficient disperser for proper delivering the powder to lungs.

In chapter 4, the adjuvant effect of δ-inulin with liquid pulmonary vaccine was evaluated. δ-inulin was mixed with influenza vaccine and then administered to mice either via i.m. injection or the pulmonary route. The immunological readouts including serum IgG, IgG subtypes, nose IgA and serum hemagglutination titers were evaluated. In terms of serum IgG and nasal IgA antibody levels, pulmonary vaccination induced a better immune response than i.m. vaccination. However, pulmonary vaccination of vaccine alone induced a Th2 dominant immune response. The addition of δ-inulin to vaccine induced a more balanced Th1/Th2 immune response without compromising the Th2 immune response. Furthermore, the δ-inulin adjuvanted vaccine enhanced the nasal IgA antibody levels compared to pulmonary vaccine alone. The adjuvanted pulmonary vaccine induced hemagglutination titers of > 40 which is generally considered to be protective. Thus, the addition of δ-inulin to the pulmonary influenza vaccine has an advantage over both the non-adjuvanted pulmonary vaccine and the adjuvanted i.m. vaccine.

In chapter 5, we incorporated the adjuvant monophosphoryl lipid A (MPLA) together with WIV in sugar glass matrix of inulin by spray freeze-drying technique. The physical and immunological properties of the vaccine powder for pulmonary immunization were evaluated. Maintenance of the adjuvant activity of MPLA during spray freeze drying was confirmed by NFkB activation with RAW-Blue™ cells. The particle size of the vaccine powder appeared to be within the acceptable range for inhalation. The maintenance of the receptor binding efficacy of WIV during spray freeze drying was confirmed by the hemagglutination titer. The immunogenicity of WIV in mice was confirmed by antibody immune responses. The MPLA adjuvanted pulmonary vaccine induced high IgA antibody levels compared to vaccine alone. The incorporation of MPLA in influenza vaccines induced a more balanced Th1/ Th2 immune responses than pulmonary vaccine alone. Moreover, the adjuvanted pulmonary influenza vaccine equally neutralized the influenza virus as seen in the control (i.m.). Overall, the MPLA adjuvanted pulmonary influenza vaccine induced better mucosal and systemic immune responses than pulmonary vaccine alone.

In chapter 6, various adjuvants were evaluated for pulmonary vaccine powders. The adjuvants include palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4, TLR 1 and TLR 2 ligand), MPLA (TLR 4 ligand), CpG oligodeoxynucleotide (CpG-ODN-1826, TLR 9 ligand) and GPI-0100 (a saponin based, non-pattern recognizing receptor binding compound). The adjuvants were incorporated in the vaccine powders by spray freeze-drying a mixture of the adjuvant, WIV and inulin as lyoprotectant. The NFκB reporter cell line studies confirmed the preservation of immunostimulating properties of these vaccine powders. All the TLR ligands were capable of inducing

systemic immune responses in mice, but they failed to induce potent mucosal immune responses. However, GPI-0100 induced both potent mucosal and systemic immune responses compared to the vaccine alone. The GPI-0100 also protected the mice from a lethal challenge of a heterologous influenza strain. Thus, GPI-0100 holds great promise to act as a potent adjuvant for pulmonary influenza vaccines.

In chapter 7, it was investigated whether s.l. administration of influenza vaccine can prime the immune system for a later i.m. boost with a heterologous influenza vaccine, the priming efficacy of a stabilized influenza vaccine through s.l. route for a heterologous i.m. booster vaccine. Ideally, the vaccine for s.l. applications should be administered as a tablet in which the vaccine is stable. Therefore, WIV was freeze-dried with in the presence of inulin and then formulated as a s.l. tablet. During processing, the vaccine stabilized with in the sugar glass matrix remained intact, which was confirmed by SDS-PAGE and hemagglutination titers. The sugar-glass stabilized vaccine was successfully formulated into a s.l. tablet. For animal studies, however, we reconstituted the freeze-dried vaccine to test the priming efficacy of vaccine because of practical difficulties related to s.l. administration of tablets to mice. The i.m. boosting efficacy of heterologous vaccine was tested. The i.m. booster vaccine of a heterologous strain resulted in the induction of serum IgG and nasal IgA antibody immune responses against both new and old strains. Therefore, it was concluded that the s.l. vaccination can indeed prime the immune response for a subsequent i.m. booster vaccine.

Concluding remarks and perspectives

In this thesis, two different strategies we explored to overcome / minimize the shortcomings of conventional influenza vaccines. First, a stable influenza vaccine powder for pulmonary administration was developed and the possibilities of adding adjuvants to pulmonary vaccines were evaluated. Second, as a pre-pandemic measure, we tested the priming efficacy of stable s.l. vaccine for a subsequent heterologous i.m. booster vaccine, especially with H5N1 strains, which pose a threat for future pandemic.

To prepare a dry powder vaccine formulation for pulmonary administration, WIV was spray freeze-dried in the presence of inulin, dextran or a mixture of dextran and trehalose (dex/tre). Spray freeze-drying was selected as a technique to dry the vaccine as it can yield powder particles with proper characteristics for pulmonary administration. It has been shown before that WIV can be spray freeze-dried in the presence of inulin without loss of its antigenicity [1]. In this thesis, it has been shown that both dextran and dex/tre can be used as stabilizing excipients. Furthermore, it was found that the antigenicity of WIV incorporated in these sugars was maintained during storage for atleast three months at temperatures up to 40 ˚C Also the powder characteristics during storage were evaluated, as a change of these characteristics can be highly detrimental for pulmonary deposition. It was found that the particle size distribution and specific surface area of the inulin and dex/tre based formulations

did not change during storage for three months at a temperature up to 30 ˚C. Interestingly, the powder characteristics of the dextran based formulation was even stable at 40 ˚C. Hence, the inulin and dext/tre stabilized influenza vaccines have a potential for an excellent long term storage stability at ambient temperatures while the dextran based formulation even has a superior stability profile (**Chapter 3**). Therefore, stockpiling of these dry powder vaccine formulations are not or less dependent on refrigerated conditions. Inulin, dextran and trehalose are approved for parenteral and oral use in humans, but not for pulmonary administration. In studies of Zijlstra et al. and Audouy et al. it was shown that pulmonary administration of inulin to the lungs of rats and mice respectively, only induced a mild inflammatory response [1,2]. However, further research towards the safety of administering inulin and the other two sugars to the lungs have to be tested in more detail in appropriate animal models as well as in humans.

Previous studies indicate that pulmonary administration of WIV elicits comparable or higher immune responses than conventional i.m. vaccines [1,3]. However, it induces a Th2 skewed immune response while a balanced Th1/Th2 immune response is preferred [4]. In addition, the induction of nasal IgA antibodies is poor. To improve the quality of immune response, a range of potential vaccine adjuvants was incorporated by spray freeze-drying in inulin glasses together with WIV. It was found that the co-incorporation of adjuvants PAM3CSK, CpG ODN, monophosphoryl lipid A and GPI-0100 in inulin glasses did not affect their adjuvant activity. Pulmonary administration of these powders to mice indicated that in particular monophosphoryl lipid A and GPI-0100 elicit improved immune responses (**Chapter 5 and 6**). Both the cellular immune response and the local immune response in nose were improved when compared with the vaccine alone. After a challenge with a heterologous influenza strain, the formulation with GPI-0100 even reduced the lung virus titer 10 times more than the vaccine alone. Furthermore, in a preliminary study δ-inulin was evaluated as adjuvant for pulmonary immunization. In this study, the adjuvant was ad-mixed with WIV dispersion and pulmonary administered to mice. This study showed that also δ-inulin improved the immune response with respect to cellular and local immune responses when compared to the vaccine alone (**chapter 4**). Therefore, these studies clearly indicate that various adjuvants can be used to potentiate pulmonary influenza vaccines. The studies described in this thesis were performed at a fixed adjuvant dose. Thus, to further improve the immune response the adjuvant dose should be optimized. In these studies, also the level of protection by these immunizations against a virus challenge should be investigated more extensively, not only in mice but also in more appropriate animal models such as cotton rats and ferrets. More interestingly, the possibilities of dose sparing for the vaccine should be investigated. The δ-inulin adjuvant was evaluated as a liquid formulation. From previous studies it is well known that the pulmonary administration of influenza vaccine in dry powder state induces a more potent immune response than in liquid state [1]. Thus, it would be interesting to investigate the pulmonary administration of dry powder formulation of a spray freeze-dried formulation containing δ-inulin

and WIV. Furthermore, similar to the safety aspects described above for the applied sugars, also the toxicity of the adjuvants in the lungs should be studied in detail before application in human.

The priming efficacy of s.l. vaccine was tested for a subsequent heterologous i.m. booster vaccine in an animal study using mice. The immune response was primed by s.l. administration of WIV prepared from NIBRG-14 and booster by i.m. injection of WIV prepared from and NIBRG-23 to test the prime-heterologous boost strategy for H5N1 bird flu vaccines. The results showed that indeed s.l. immunization with NIBRG-14 primed for a subsequent heterologous i.m. booster immunization with NIBRG-23 vaccine as the serum HI titers and nasal IgA antibody titers against NIBRG-23 were much higher than after i,m. immunization with NIBRG-23 alone. In this study, WIV was also freeze-dried together with inulin as lyoprotectant and then formulated using appropriate excipients into a stable tablet suitable for s.l. administration (**Chapter 7**). Such tablets holds great promise for a future pandemic as a number of advantages: **1)** They can be prepared from a previous influenza strain, thus reducing the risk for vaccine shortage of vaccine prepared from the virus spreading at that moment. **2)** They are stable and can thus be stockpiled under no or less stringent refrigerated conditions. **3)** They are easy to distribute and administered. Therefore, priming of the immune response with a s.l. tablet for a booster vaccine prepared from heterologous drifted strain can be considered as a step forward in pandemic preparedness. In this thesis, the s.l. prime was administered as a liquid formulation due to technical problems associated with the s.l. administration of tablets to mice. Therefore, the s.l. tablet primer and heterologous i.m. booster strategy should be investigated in bigger animals e.g. cotton rats or ferrets. Furthermore, the protective immunity should be evaluated in challenge models. To improve the priming efficacy, potent adjuvants could be incorporated into the s.l. vaccine tablets. The mucosal adjuvants discussed in this thesis like MPLA, GPI-0100 or other potent adjuvants may serve as future adjuvant candidates for s.l. vaccines. Moreover, adjuvation of s.l. vaccines might be a step forward to further facilitate vaccination e.g. by replacing the s.l. prime and i.m. boost strategy with non-invasive s.l. prime and s.l. boost strategy. By eliminating the need for invasive (i.m.) booster vaccination, the reach of vaccine could be improved.

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Annexure I

Nederlandse Samenvatting

Samenvatting

Vaccinatie tegen griep is de belangrijkste strategie om verspreiding van het griepvirus te beheersen, niet alleen tijdens de jaarlijks terugkerende epidemieën maar ook tijdens een eventuele pandemie. De huidige vaccins hebben echter een aantal tekortkomingen. Ze moeten per injectie worden toegediend (met uitzondering van het verzwakte levende griepvirus Flumist®), de productiecapaciteit is beperkt, de immuunreactie die ze opwekken is niet optimaal en ze zijn niet stabiel tijdens opslag.

In dit proefschrift zijn twee verschillende strategieën onderzocht om deze tekortkomingen op te lossen. De eerste strategie behelst de ontwikkeling van een droog en stabiel poeder voor pulmonale toediening waarin behalve het vaccin ook een adjuvans is verwerkt. De tweede strategie richtte zich op de ontwikkeling van een sublinguale (s.l.) tablet waarin een gestabiliseerd vaccin is verwerkt dat gemaakt is van een pandemische influenzastam die eerder heeft gecirculeerd. Een dergelijke s.l. tablet zou mogelijk gebruikt kunnen worden als een "primer" voor een daaropvolgende intramusculaire (i.m.) injectie met een vaccin vervaardigd van de pandemische influenza stam die op dat moment circuleert (heterologe stam). Deze vaccinatiestrategie is aantrekkelijk omdat de s.l. tablet stabiel is en een vaccin bevat wat gebaseerd is op een al bekend en dus beschikbaar virus. Het product kan dus op voorraad geproduceerd worden en nadien tijdens een pandemie worden ingezet.

Belangrijke aandachtspunten van het onderzoek dat beschreven is in dit proefschrift zijn:

1. De fysische en immunologische stabiliteit gedurende opslag van gesproeivriesdroogde vaccinpoeders voor pulmonale toediening.

2. Het behoud van de immunogeniciteit van het adjuvans tijdens het sproeivriesdrogen van een waterige dispersie van het adjuvans samen met het influenzavaccin en stabiliserende hulpstoffen.

3. Het behoud van het vermogen om immuunreacties op te roepen na pulmonale toediening van deze gesproeivriesdroogde poeders.

4. De sublinguale "priming" van het immuunsysteem (met gestabiliseerd vaccin) voorafgaande aan een intramusculaire vaccinatie met een heteroloog vaccin.

Hoofdstuk 1 is algemene inleiding op het werk dat in dit proefschrift is beschreven.

In hoofdstuk 2 wordt een literatuuroverzicht gepresenteerd over griepvaccins waarin het antigeen is geformuleerd tot een deeltjesstructuur. Geconcludeerd werd dat deze vaccins de potentie hebben om een veel betere immuunreactie te induceren dan de conventionele "subunit"- of "split"-vaccins omdat ze veel meer op het intacte influenzavirus lijken. Daarnaast zijn de uitdagingen besproken met betrekking tot regelgeving en registratie die verbonden zijn aan de implementatie van deze vaccines.

I

In hoofdstuk 3 werd de fysische en immunogene stabiliteit van "influenza whole inactivated virus vaccine" (WIV) poeders voor pulmonale toediening geëvalueerd. WIV werd gesproeivriesdroogd in aanwezigheid van inuline, dextraan en een mengsel van dextraan en trehalose. Alle drie gesproeivriesdroogde poeders bleken geschikt te zijn voor pulmonale toediening. Bovendien bleef de antigeniciteit van het vaccin tijdens het sproeivriesdrogen volledig behouden. De vaccinpoeders werden gedurende drie maanden opgeslagen bij -20 , 2-8 , 30 en 40 °C. De deeltjesgrootteverdeling en het specifieke oppervlak van alle drie vaccinpoeders bleven onveranderd tijdens opslag bij temperaturen tot en met 30 °C. Ook het receptorbindend vermogen en de antigeniciteit van de vaccinpoeders bleven gedurende deze opslagcondities volledig behouden. De immunogene stabiliteit van de geformuleerde poeders werd geëvalueerd in muizen. Hieruit bleek dat ook de immunogeniciteit van de vaccinpoeders volledig intact bleef tijdens opslag gedurende drie maanden bij 30 °C. Wel werd in de dierstudies gevonden dat er tijdens opslag waarschijnlijk subtiele veranderingen in de poederstructuur waren opgetreden. Deze resulteerden in een niet optimale longdepositie wanneer er gebruik werd gemaakt van een disperser die het poeder niet krachtig genoeg kan dispergeren. Dit probleem deed zich echter niet voor met dispersers die wel in staat waren om de poeders krachtig te dispergeren.

In hoofdstuk 4 werd onderzocht of δ-inuline kan worden toepast als adjuvans voor pulmonale influenzavaccinatie. δ-inuline werd toegevoegd aan een vloeibare influenzavaccinformulering die vervolgens werd toegediend aan muizen, zowel via i.m. injectie als via de pulmonale route. Met betrekking tot IgG antilichaamtiters in het serum en nasale IgA antilichaamtiters, induceerde de pulmonale toediening zonder δ-inuline een betere immuunreactie dan i.m. vaccinatie. Pulmonale vaccinatie induceerde echter een (ongewenste) dominante Th2 immuunreactie. De toevoeging van δ-inuline aan het pulmonale vaccin voorkwam dit nadeel. Pulmonale toediening van het vaccin met δ-inuline resulteerde namelijk in een evenwichtige Th1/Th2 immuunreactie. Een ander voordeel van het δ-inuline geadjuvanteerde pulmonale vaccin was dat het veel hogere nasale IgA antilichaamtiters induceerde dan het pulmonale vaccin zonder δ-inuline. In alle gevallen werden hemagglutinatietiters van > 40 gevonden. Algemeen wordt aangenomen dat hemagglutinatietiters boven deze grens leiden tot een goede bescherming tegen het virus. Uit de resultaten kan geconcludeerd worden dat toevoeging van δ-inuline aan het pulmonale vaccin leidt tot een betere immuunreactie dan van het niet-geadjuvanteerde pulmonale vaccin of van het geadjuvanteerde i.m. vaccin.

In hoofdstuk 5 werd het adjuvans monophosphoryl lipide A (MPLA) samen met WIV ingesloten in een matrix van inulineglas door middel van sproeivriesdrogen. Vervolgens werden de fysische en immunologische eigenschappen van het vaccinpoeder voor pulmonale immunisatie onderzocht. Laserdiffractiemetingen toonden aan dat de deeltjesgrootteverdeling van het verkregen vaccinpoeder geschikt was voor inhalatie. Het behoud van de adjuvansactiviteit van MPLA

tijdens sproeivriesdrogen werd bevestigd in experimenten waarbij de NFkB activatie van RAW-Blue™ cellen werd bestudeerd. Daarnaast is met het bepalen van de hemagglutinatietiter aangetoond dat het receptorbindend vermogen van het vaccin tijdens sproeivriesdrogen niet was veranderd. Voorts werd het behoud van de immunogeniciteit van WIV bevestigd in muizenstudies. Het MPLA geadjuvanteerde pulmonale vaccinpoeder induceerde hogere IgA antilichaamtiters dan het vaccinpoeder zonder MPLA. Insluiting van MPLA in het vaccinpoeder induceerde ook een meer evenwichtige Th1/Th2 immuunreactie dan het pulmonale vaccinpoeder zonder MPLA. Ten slotte is gevonden dat muizen die gevaccineerd werden met het geadjuvanteerde vaccinepoeder via de pulmonale route even goed beschermd waren tegen een infectie met het virus als muizen die i.m. gevaccineerd waren met het "subunit" vaccin. Geconcludeerd werd dat het MPLA geadjuvanteerde pulmonale vaccinpoeder betere mucosale en systemische immuunreacties induceerde dan het pulmonale vaccin zonder MPLA.

In hoofdstuk 6 werden verschillende adjuvantia geëvalueerd voor pulmonale vaccinpoeders. De onderzochte adjuvantia zijn palmitoyl-3-cysteïne-serine-lysine-4 (Pam3CSK4 , een TLR-1 en TLR-2 ligand) , MPLA (een TLR-4 ligand) , CpG oligodeoxynucleotide (CpG- ODN-1826 , een TLR-9 ligand) en GPI-0100 (een saponine dat geen TRL receptorbinding vertoont). Door middel van sproeivriesdrogen werden poeders verkregen met daarin ingesloten de verschillende adjuvantia, WIV en inuline als stabilisator. Experimenten met de NFκB reporter cellijn toonden aan dat de immuunstimulerende eigenschappen van de TLR liganden en het WIV tijdens het sproeivriesdrogen behouden bleven. Alle TLR liganden induceerden systemische immuunreacties bij muizen, maar geen substantiële mucosale immuunreacties. GPI-0100 daarentegen induceerde zowel sterke systemische als mucosale immuunreacties in vergelijking tot het vaccin zonder adjuvans. Ook bleken muizen die pulmonaal waren gevaccineerd met de GPI-0100 formulering gedeeltelijk beschermd tegen een infectie met een virus van een heterologe stam. De conclusie was dat GPI-0100 een veelbelovend adjuvans is voor pulmonale griepvaccins.

In hoofdstuk 7 werd onderzocht of de s.l. toediening van het griepvaccin kan fungeren als "primer" voor een latere i.m. "boost" met een heteroloog griepvaccin. Het zou ideaal zijn als een dergelijke "primer" toegediend zou kunnen worden in de vorm van een stabiele s.l. tablet. Daarom werd WIV gevriesdroogd in de aanwezigheid van inuline als stabilisator. Met SDS-PAGE en het bepalen van de hemagglutinatietiters werd aangetoond dat het vaccin tijdens vriesdrogen intact bleef. Het met suikerglas gestabiliseerde vaccin kon vervolgens worden geformuleerd tot een s.l. tablet met gewenste eigenschappen met betrekking tot oplosnelheid en mechanische sterkte. Bij proeven met muizen werden echter geen tabletten s.l. toegediend maar werd het gevriesdroogde vaccin gereconstitueerd en werd de verkregen waterige dispersie vervolgens s.l. toegediend. De reden hiervoor was dat de s.l. toediening van tabletten aan muizen praktische problemen oplevert. Na s.l. vaccinatie kregen de muizen een heteroloog vaccin i.m. toegediend. Deze immunisatiestrategie leidde tot de inductie van serum IgG en nasale IgA antilichaamtiters tegen zowel homologe als heterologe

influenzastammen. De conclusie was dat de s.l. vaccinatie inderdaad kan dienen als een "primer" van de immuunreactie voor een daaropvolgende i.m. booster met een heteroloog vaccin.

In dit proefschrift is aangetoond dat het mogelijk is om door middel van sproeivriesdrogen een vaccinpoeder te produceren dat geschikt is voor pulmonale toediening en dat gedurende lange tijd buiten de koelkast bewaard kan worden. Pulmonale toediening van het griepvaccin heeft grote voordelen boven de gebruikelijke i.m. injectie onder andere omdat het een naaldvrije toedieningsvorm is en omdat het eenvoudig toe te dienen is. Daarnaast heeft pulmonale toediening de potentie om naast een immuunreactie in de algemene circulatie ook een immuunreactie in de luchtwegen te induceren. Dit zou tot een betere bescherming tegen griep kunnen leiden aangezien het virus ook via de luchtwegen het lichaam binnendringt. Muizenstudies toonden aan dat pulmonale toediening van het vaccin inderdaad lokale immuunreacties induceerden. De nasale immuunreacties waren echter niet optimaal en bovendien werd een dominante Th2 immuunreactie gevonden en niet een evenwichtige Th1/Th2 immuunreactie. Deze nadelen konden ondervangen worden door de toevoeging van verschillende adjuvantia. De verbetering van de immuunreactie door pulmonale griepvaccin formuleringen waarin ook geschikte adjuvantia zijn opgenomen zou kunnen leiden tot een verlaging van de vaccindosis. Dit vermindert de druk op de productiefaciliteiten van het griepvaccin tijdens een epidemie of pandemie. In inleidende experimenten werd aangetoond dat de pulmonale toediening van het inuline-gestabiliseerd vaccin een milde ontstekingsreactie induceerde. Uitgebreide toxiciteitstudies zullen nog moeten worden uitgevoerd om de veiligheid van de pulmonale vaccinpoeders (met adjuvantia) te kunnen garanderen. Voor de pulmonale toediening werden enkel muizenstudies uitgevoerd. Voordat de stap naar de mens kan worden gemaakt zullen eerst nog uitgebreide onderzoeken in grotere proefdiermodellen, bijvoorbeeld katoenratten en fretten, moeten worden uitgevoerd waarbij ook de bescherming tegen het griepvirus nader moet worden bestudeerd.

In dit proefschrift is verder aangetoond dat s.l. toediening van het griepvaccin (in tabletvorm) kan dienen als een "primer" voor een daaropvolgende i.m. injectie met een heteroloog vaccin. Een dergelijke vaccinatiestrategie zou met name grote voordelen bieden tijdens een pandemie. In tegenstelling tot een epidemie moeten tijdens een pandemie grote delen van de bevolking worden gevaccineerd. Daarnaast bestaat het vaccinatieregime meestal uit twee doses, een "primer" en een "booster". Hierdoor moet er binnen korte tijd zeer veel vaccin worden geproduceerd hetgeen gezien de beperkte productiefaciliteiten onmogelijk zou kunnen zijn. Met de hierboven beschreven strategie zou de "primer" kunnen worden vervangen door een vaccin dat gemaakt is van een pandemische influenzastam die eerder heeft gecirculeerd. Dit zou de druk op de productiefaciliteiten om twee redenen enorm verlichten. Ten eerste hoeft er veel minder vaccin op basis van het virus dat dan circuleert te worden geproduceerd aangezien alleen de "booster" van dit vaccin hoeft te worden toegediend. Ten tweede is er meer tijd om het vaccin te produceren omdat

het vaccin pas gereed hoeft te zijn op het moment dat de "booster" toegediend moet worden. Ook is aangetoond dat het "primer" vaccin verwerkt kan worden tot een stabiele s.l. tablet. Een dergelijke tablet heeft grote voordelen boven vloeibare formuleringen omdat het buiten de koelkast bewaard kan worden, gemakkelijk te distribueren is en eenvoudig toegediend kan worden. Ook bij dit onderzoek werden alleen muizenstudies uitgevoerd en er zal dus ook bij deze vaccinatiestrategie onderzoek bij grotere proefdieren moeten worden uitgevoerd voordat het klinisch kan worden getest in de mens. Verder moet nog worden onderzocht in hoeverre deze vaccinatiestrategie leidt tot een adequate bescherming tegen het heterologe virus. Verschillende adjuvantia zouden kunnen worden geëvalueerd om de immuunreactie verder te verbeteren. Ten slotte zou onderzocht kunnen worden of de immuunreactie verbeterd kan worden door een s.l. "primer" te combineren met een heterologe s.l. of pulmonale "booster".

I

Annexure II

Acknowledgment

Kindness shown by those who weigh not what the return may be
when you ponder right its merit , 'Tis vaster than the sea.

Said the great Tamil Saint Thiruvalluvar about 2000 years ago in his collection of couplets known as Thirukural.

Yes, it is time to recollect, acknowledge, and express my sincere gratitude and thanks to everyone who have been with me all through the past four and half years who helped me in academic and personal life. This thesis would not have been possible without all of you.

I am very thankful to Prof. Erik Frijlink my PhD promoter for supporting me and my ideas during my tenure as a PhD student. To be honest, I have never seen a person like him before in my life, always supporting the novel ideas and motivating the students. "How can a person be energetic like this?", I wondered many times in this past four and half years. I am fortunate to have a Promoter like Erik.

My Supervisor, Dr. Wouter Hinrichs is the first person who I met in Groningen. You helped me in complicated calculations and also guided me in making my presentations in proper form. Now I am sure that I can manage complicated calculations in future. Personally, I admire you a lot. I am not very sure whether I would meet some personalities like you in future. Wouter, I would like to thank you for all your help and guidance during my tenure in our department.

Prof. Anke Huckeride, is my PhD Co-supervisor. We used to have our meetings once a month. However, they were productive and constructive for my PhD research. I would like to thank you for clarifying my doubts regarding logarithmic tables. As rightly said by Harshad, you always helped us to progress with our research by asking "What is your research question?" Thank you Anke, I will always try ask this question myself in future.

Dr. Harshad Patil, my fellow PhD, knows a lot about immunology. You helped me to understand the basics of immunology related to influenza and also guided me in designing new animal studies. I cannot forget those late evening lab works with you for our animal studies. It is quite hard to find a decent house in Groningen. It is because of you, I found one in Groningen. I would also like to thank Sneha, for keeping Priya busy with her Dutch lessons and daily chats.

I am honored to be Ubbo Emmius PhD scholar at University of Groningen. I would like to thank the Selection Committee for giving me an opportunity to carryout research in the University. I am also thankful to GSMS and GUIDE for providing me with informative courses as a part of training.

I would like to appreciate and thank Prof. Toos Daemen, Prof. Klaas Poelstra and Prof. Nikolai Petrovsky, members of my PhD thesis reading committee.

Annexure III

Curriculum vitae

SENTHIL MURUGAPPAN

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Education and research

2009 - 2014   Ph.D

University of Groningen, The Netherlands.

Formulated a novel influenza vaccine for pulmonary and sublingual route of vaccination (dry vaccine).

Formulated the influenza vaccine with potent adjuvants (dry vaccine).

2006-2008   Master of Pharmacy, Pharmaceutics

MGR Medical University (JSS College of Pharmacy, Ooty), India

Formulated and evaluated a liposomal delivery system for L-asparaginase.

2002 - 2006   Bachelor of Pharmacy

Sri Ramachandra Medical University (College of Pharmacy), India

Designed and evaluated a novel pharmaceutical powder blender.

Publications and presentations

Simplifying Influenza Vaccination during Pandemics - Sublingual Priming and Intramuscular Boosting of Immune Responses with Heterologous Whole Inactivated Influenza Vaccine

(The AAPS Journal - 2014)

Physical and immunogenic stability of spray freeze-dried influenza vaccine powder for pulmonary delivery

(European Journal of Pharmaceutics & Biopharmaceutics - 2013)

Development of spray freeze-dried monophosphoryl lipid A-adjuvanted whole inactivated virus influenza vaccine for pulmonary vaccination

(Journal of Controlled Release - 2014)

Development of a dry, stable and inhalable acyl-homoserine-lactone-acylase powder formulation for the treatment of pulmonary Pseudomonas aeruginosa infections

(European Journal of Pharmaceutical Sciences – 2013)

Enhanced Pulmonary Immunization with Aerosolized Inactivated Influenza Vaccine Containing Delta Inulin Adjuvant

(Submitted)

Comparison of adjuvants for pulmonary vaccination of whole inactivated virus influenza vaccine

(Under preparation)

Review on particulate influenza vaccines

(Under preparation)

" Is spray freeze-dried influenza vaccine a stable product suitable for inhalation" at Dutch medicine days 2013, Netherlands

"Sublingual Priming and Heterologous Intramuscular Boosting of Immune Response with Whole Inactivated Influenza Virus Vaccine" at Options VIII : Control of influenza 2013, South Africa

"Physical and Immunogenic Stability of Spray Freeze-dried Influenza Vaccine Powder for Pulmonary Delivery" at Options VIII : Control of influenza 2013, South Africa

"Priming the immune response by sublingual vaccination with whole inactivated influenza virus vaccine" at AAPS annual exposition 2012, USA

"Incorporation of enzyme into liposome" at the 12th APTI National Convention, India

III