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H5N1 vaccines in humans

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Abstract

The spread of highly pathogenic avian H5N1 influenza viruses since 1997 and their virulence for poultry and humans has raised concerns about their potential to cause an influenza pandemic. Vaccines offer the most viable means to combat a pandemic threat. However, it will be a challenge to produce, distribute and implement a new vaccine if a pandemic spreads rapidly. Therefore, efforts are being undertaken to develop pandemic vaccines that use less antigen and induce cross-protective and long-lasting responses, that can be administered as soon as a pandemic is declared or possibly even before, in order to prime the population and allow for a rapid and protective antibody response. In the last few years, several vaccine manufacturers have developed candidate pandemic and pre-pandemic vaccines, based on reverse genetics and have improved the immunogenicity by formulating these vaccines with different adjuvants. Some of the important and consistent observations from clinical studies with H5N1 vaccines are as follows: two doses of inactivated vaccine are generally necessary to elicit the level of immunity required to meet licensure criteria, less antigen can be used if an oil-in-water adjuvant is included, in general antibody titers decline rapidly but can be boosted with additional doses of vaccine and if high titers of antibody are elicited, cross-reactivity against other clades is observed. Prime-boost strategies elicit a more robust immune response. In this review, we discuss data from clinical trials with a variety of H5N1 influenza vaccines. We also describe studies conducted in animal models to explore the possibility of reassortment between pandemic live attenuated vaccine candidates and seasonal influenza viruses, since this is an important consideration for the use of live vaccines in a pandemic setting.

Keywords

H5N1; vaccine strategies; pandemic; adjuvants; clinical trials

1. Introduction

Since the first recorded direct bird-to-human transmission of highly pathogenic avian influenza (HPAI) H5N1 in Hong Kong in 1997, these viruses have spread to countries in Asia, the Middle East, Africa, and Europe. They have caused widespread death and illness in domestic and migratory birds, as well as human infections and fatalities (OIE, 2008; Subbarao et al., 1998). As of August 2012, the World Health Organization (WHO) had

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recorded 608 confirmed H5N1 cases with 359 fatalities since 2003 in 15 countries (WHO, 2012b), which represents a 59% case-fatality rate. This rate is computed from cases reported to the WHO since 2003. Because the WHO case definition (WHO, 2006) identifies severe illness associated with radiographic changes of pneumonia and most of the H5N1 cases have been reported from countries with limited resources for identifying milder cases, there may be an overestimation of case-fatality by over-counting of severe cases and under-counting of mild cases (Morens et al., 2012; Palese and Wang, 2012).

Since 2003, the H5N1 virus has diverged into ten clades (numbered 0–9), but only four clades (0, 1, 2 and 7) have been isolated from humans, including 3 subclades of clade 2 (Abdel-Ghaffar et al., 2008; WHO, 2012a), with the majority belonging to clade 0 and 2 (Chen et al., 2006; Wan et al., 2008). Sequence analysis of the clade 2 viruses shows that the circulating strains are evolving and have further diverged into five subclades (2.1–2.5); however, only subclades 2.1, 2.2, and 2.3 contain viruses isolated from humans. Although limited, non-sustained human-to-human transmission has been documented in several countries, particularly among blood relatives, H5N1 viruses have not yet acquired efficient transmissibility among humans (Kandun et al., 2006; Ungchusak et al., 2005; Wang et al., 2008; WHO, 2008).

Vaccination is the most effective public health intervention strategy and this must be supported by enhanced surveillance. Although the use of antiviral drugs is also an important public health countermeasure for preventing and treating influenza, the emergence of drug-resistant strains of avian H5N1 viruses (de Jong et al., 2005; Le et al., 2005; Puthavathana et al., 2005) underscores the need for new drugs and other novel preventive and therapeutic strategies. While vaccines are the most effective tool for the control of influenza (Cox and Subbarao, 1999; Nichol and Treanor, 2006), the combined production capacity of global vaccine suppliers is not sufficient to meet the demand during a pandemic, so a vaccine shortage is expected. Any strategy that can maximize vaccine coverage will be valuable in a pandemic.

The initial development of H5N1 vaccines began using the principles established with seasonal influenza virus vaccines. However, the development of effective vaccines against HPAI H5N1 viruses presented several new challenges. Due to their high pathogenicity for poultry, handling of the wild-type (wt) viruses requires enhanced biosafety level 3 (BSL-3) containment. In addition, these viruses are embryo-lethal so it is difficult to propagate the wt viruses efficiently and safely in eggs. Several strategies have been explored in attempts to overcome these obstacles including the use of a low pathogenicity H5N3 avian influenza virus that is antigenically related to circulating strains (Nicholson et al., 2001), the use of recombinant H5 HA expressed in baculovirus (Treanor et al., 2001), the use of recombinant adenoviruses expressing the H5 HA (Gao et al., 2006; Hoelscher et al., 2006), and the production of attenuated seed viruses with the H5 HA modified by reverse genetics (Subbarao et al., 2003; Webby et al., 2004).

In this chapter we review the published data on the evaluation of different H5N1 vaccines in clinical trials and the H5N1 vaccines that have been licensed.

1.1. Assessment of the immune response

Virus-specific neutralizing antibodies directed against the influenza virus HA are the major mediators of protection against subsequent infection with influenza viruses and the induction of anti-HA antibodies is the principle underlying licensed influenza vaccines. Thus, the immunogenicity of influenza vaccines is evaluated by serological hemagglutination inhibition (HAI) and microneutralization (MN) assays that measure influenza-specific antibodies. The HAI assay measures antibodies that specifically block the attachment of the

virus to red blood cells. An HAI titer of 1:40 is often referred to as a “protective titer” but it actually represents the titer at which approximately 50% of individuals in a population would be protected from infection with or disease from seasonal influenza viruses (de Jong et al., 2003; Potter and Oxford, 1979). However, because the quality and quantity of antibodies are important, there is no absolute titer at which protection from infection is guaranteed. Coudeville et al. developed a model that estimates the level of clinical protection against influenza at any given HAI titer (Coudeville et al., 2010); they propose that HAI titers associated with protection are better described as a curve rather than a single threshold titer such as a titer of 1:40 as a surrogate of protection. They found that the relationship between HAI antibody titers and clinical protection from influenza is consistently positive, irrespective of vaccination status or virus type (influenza A or B) and can be used to predict the efficacy of inactivated influenza vaccines when only immunogenicity data are available. However, the direct application of this model to pandemic influenza vaccines will require careful consideration.

Neutralization assays measure biologically functional antibodies that can inhibit attachment and entry of virus in tissue culture and are more sensitive than HAI assays. However, MN assays are not well standardized (Stephenson et al., 2009) and the titer that corresponds to protection is not known.

Antibodies that neutralize viral infectivity but do not inhibit hemagglutination, target conserved portions of the stem of the HA molecule and may play a role in protection (Ekiert et al., 2009; Sui et al., 2009). Some such antibodies act by blocking fusion. Non-neutralizing anti-HA antibodies can target epitopes on virus-infected cells through antibody-dependent cellular cytotoxicity. The extent to which stem and non-neutralizing antibodies are induced by influenza vaccines, and in particular H5N1 vaccines is unknown.

In addition to high affinity neutralizing antibodies directed against the HA, CD4⁺ T-cells also play an important role in protection (Droebner et al., 2008; Jelley-Gibbs et al., 2008). Although CTLs do not prevent the establishment of infection, CD8⁺ T cells play a critical role in viral clearance in mice (McKinstry et al., 2012; Yap et al., 1978) and humans (McMichael et al., 1983) and recent studies showed that preexisting influenza specific CD4⁺ cell immunity correlates with protection from influenza (Wilkinson et al., 2012). In addition, influenza-specific CTLs can provide partial protection across heterologous strains by targeting such conserved regions (Taylor and Askonas, 1986).

The European Committee for Medicinal Products (CHMP) (EMA, 2008) and the United States Food and Drug Administration (US FDA) (FDA, 2007b) have developed guidelines for the evaluation of pandemic influenza vaccines that are based on experience from studies with seasonal influenza vaccines. These criteria are listed in Table 1. Seroconversion rate is defined as the percentage of subjects or sera with at least a four-fold increase in HAI titer following vaccination or a post-vaccination titer of at least 1:40 if HAI antibodies were undetectable in pre-vaccination sera. Seroprotection rate is defined as the percentage of subjects achieving a post-vaccination HAI titer of at least 1:40. This term is somewhat controversial, particularly for pandemic influenza virus vaccines because the HAI antibody titer that correlates with protection is not known. In this review, the term ‘seroprotection’ is used as reported in the primary publications, but with the stated caveat. Although the MN results are not strictly required to obtain CHMP or FDA approval, the European Medicine Agency (EMA) recommends the quantification of neutralizing antibodies.

Although clinical trial data provide valuable information on safety and immunogenicity of pandemic vaccines, the protective efficacy of H5N1 vaccines can only be ascertained from

studies in animal models using formulations similar to those that have been approved or are awaiting regulatory approval for human use.

2. Influenza vaccines strategies

2.1. Inactivated influenza virus vaccines

General—In 1948, Salk et al. reported the use of an inactivated influenza vaccine prepared from egg-grown virus (Salk, 1948). This vaccine contained large amounts of egg proteins and endotoxin and was associated with substantial vaccine reactogenicity, including local reactions at the injection site and febrile illness, particularly among children (Nicholson et al., 1979; Wright et al., 1977). In the mid 1960s, the introduction of zonal ultracentrifugation reduced the nonviral impurities and with this, reactogenicity was reduced. Later, treatment of whole virus particles with detergents or organic solvents, like ether, allowed the production of disrupted or split-virus vaccines and in the mid 1970s, sub-unit vaccines were produced using techniques to separate viral membrane proteins from the viral core. These vaccines contain only the viral surface proteins, HA and NA, without internal components of the virus and were less reactogenic but also less immunogenic than whole virus vaccines (Levine et al., 1977; Wright et al., 1977).

Most inactivated vaccines are poorly immunogenic in naïve subjects and at least two doses of vaccine are required to elicit a robust immune response to a novel HA. In order to generate a strong immune response to the vaccine it is usually necessary to add an adjuvant to enhance the immunogenicity of antigens *in vivo*.

Aluminum hydroxide or phosphate, commonly referred to as alum, was introduced as an adjuvant in the 1920s and remains the most widely used adjuvant around the world. The exact mechanism of action is an area of ongoing research but the major contribution to adjuvanticity is the depot effect of antigen (Ag) adsorbed to the adjuvant, that is stored for an extended time at the site of injection and is thus available at comparatively high concentrations for uptake by phagocytic cells (Guy, 2007; Harrison, 1935). Activation and recruitment of antigen-presenting cells, which play a key role in the amplification and differentiation of antigen-specific T-cells, also explain the immunological phenomena triggered by alum (Levesque et al., 2006).

In 1997, MF59, an oil-in-water microemulsion composed of 5% v/v squalene, 0.5% v/v polysorbate 80 (Tween™ 80) and 0.5% v/v sorbitan trioleate (Span® 85) was licensed by Novartis Vaccines and Diagnostics and used in several vaccine formulations including influenza vaccines, in Europe (Cambridge, MA) (Podda and Del Giudice, 2003). Administration of MF59 induces an influx of macrophages and triggers the production of chemokines in cells resident at the site of injection (Schultze et al., 2008). These recruited cells can take up the adjuvant and antigen and transport them to the draining lymph nodes. Thus, MF59 leads to an influx of antigen presenting cells in the lymph nodes where they can interact with Ag-specific T cells to induce adaptive immunity. Efficient activation of large numbers of different target cells translates to higher numbers of Ag-specific effector and memory T cells, of antibody-secreting plasma cells and finally to greater vaccine potency (O'Hagan et al., 2012). MF59 was also shown to enhance the affinity and breadth of the antibody response to influenza vaccines (Khurana et al., 2010).

More recently, AS03, another oil-in-water emulsion, was approved as a component of pandemic H1N1 2009 vaccines (Garçon et al., 2012). AS03 contains a surfactant polysorbate 80 and two biodegradable oils, squalene and DL- α -tocopherol, in phosphate-buffered saline as the aqueous carrier (Aucouturier et al., 2001). The local effect of this adjuvant on the immune response has been studied in a mouse model (Morel et al., 2011).

The mode of action of AS03 is strictly dependent on spatial and temporal co-localization with the antigen. The adjuvant induces a transient NF- κ B-dependent cytokine and chemokine response and an increased recruitment of innate immune cells to the site of infection. AS03-adjuvanted vaccines have been shown to be well tolerated and safe (Langley et al., 2011; Rumke et al., 2008), although a statistically significantly greater risk of developing narcolepsy with the Pandemrix 2009 pandemic H1N1 vaccine was observed in children aged 4 to 19 years in Sweden, Finland and the UK where this vaccine was widely used (Dauvilliers et al., 2010; Miller et al., 2013; Nohynek et al., 2012; Wijnans et al., 2013).

In this section, we describe the evaluation of inactivated split-virus, sub-unit and whole virus H5N1 vaccines with or without adjuvant (see Tables 2 and 3). The seed viruses for the H5N1 vaccines were generated by reverse genetics; the HA gene was modified to remove the multibasic cleavage site for safety reasons and the internal protein genes were derived from influenza A/Puerto Rico/8/34 (PR8), the backbone of the licensed inactivated influenza virus vaccines (Chen et al., 2003; Suguitan et al., 2006). The HA and NA genes were derived from H5N1 isolates from different clades (WHO, 2009).

2.1.1. Unadjuvanted inactivated split-virus and sub-unit H5N1 vaccines—One of the first studies in which the safety and immunogenicity of an unadjuvanted inactivated subvirion H5N1 vaccine was evaluated was reported in 2006 (Treanor et al., 2006). The authors conducted a multicenter, randomized, double-blind, placebo-controlled clinical trial involving 451 healthy adults 18 to 64 years of age who were vaccinated with two intramuscular doses containing 90, 45, 15, or 7.5 μ g of HA antigen or placebo. This vaccine was produced from a clade 1 human influenza isolate, A/Vietnam/1203/2004 (H5N1). Although this vaccine was safe, only 58% of subjects who received two doses of 90 μ g each developed neutralizing antibody responses that were predicted to be associated with protection. The need for such a large dose of vaccine would pose a considerable barrier to production of an adequate supply to meet global needs. Nonetheless, this vaccine was approved by the FDA for use in first responders and individuals at risk for H5N1 infection and was the first avian influenza vaccine licensed in the USA (FDA, 2007a).

The antibody titers in vaccine recipients decreased substantially approximately 6 months after the second dose. Therefore, the authors offered a third dose of the vaccine to 337 participants, six months after the second dose. Antibody titers were boosted and protective titers were found in 78% and 67% of the subjects who had previously received two doses of 90 or 45 μ g, respectively (Zangwill et al., 2008). Although neutralizing antibody levels remained significantly higher five months after the third dose than after the second dose, the increased amount of antigen and the number of doses is not an optimal strategy for pandemic preparedness in terms of time and economics. Therefore, dose-sparing using adjuvants is of interest.

2.1.2. Adjuvanted inactivated split-virus and sub-unit H5N1 vaccines

Alum: In the United States, alum (aluminum hydroxide or phosphate) is the only adjuvant approved for use in human vaccines. However, alum-adjuvanted inactivated influenza vaccines have demonstrated variable results, with effects ranging from moderate to none. A randomized, open-label, phase I trial in 300 subjects aged 18-40 years, evaluated the effect of a two-dose regimen of six inactivated split H5N1 (A/Vietnam/1194/2004, NIBRG-14) influenza vaccine formulations containing 7.5, 15, or 30 μ g of HA, with or without alum. All vaccine formulations tested were well tolerated and after two doses, at least one of the CHMP requirements for seasonal vaccines was met but only the highest adjuvanted dose (30 μ g) elicited a 67% seroconversion rate and this was not statistically significant (Bresson et al., 2006). Bernstein et al. tested the same vaccine in 394 healthy adults aged 18-64 years.

The addition of aluminum hydroxide did not improve the antibody response at any antigen dose tested (15, 30, or 45 µg per dose) (Bernstein et al., 2008).

In two randomized, dose-comparison, parallel assignment, multicenter trials, healthy 18-64 year old subjects received two doses of 7.5 µg or 15 µg HA (A/Vietnam/1194/2004) vaccine with or without alum (phase I trial; N= 400) or two doses of 30 µg or 45 µg HA with aluminum adjuvant (phase II trial; N= 400) (Nolan et al., 2008b). In the phase I study, the vaccine formulations were poorly immunogenic and the immune response decreased to near pre-vaccination levels 6 months after primary vaccination, though titers were restored to post-dose 2 levels following a third dose. However, in the phase II study, immune responses persisted for up to 6 months after a primary vaccination. In a pandemic, antibody persistence will be important in reducing the impact of secondary and tertiary waves of disease. Of note, these vaccine formulations elicited modest levels of cross-protective neutralizing antibodies against antigenically variant clade 2 H5N1 strains.

This vaccine also showed promising results in infants and children aged 6 months to <9 years of age (Nolan et al., 2008a). In contrast to the immune response observed in adults, 30 µg or 45 µg of HA elicited strong immune responses by both MN (98-99% of subjects developed titers 1:20) and HAI assays (95-100% of subjects developed titers 1:32), and 85% of children had persistent MN antibody titers 1:20 for up to 6 months post-vaccination. Of note, in this study the MN threshold was 1:20. In addition, robust cross-reactive HAI antibody responses to clade 2.1 were elicited following two doses of the clade 1 vaccine. Similarly, a Phase II clinical trial in 180 children six months to 17 years of age given two 30 µg doses of an aluminum-hydroxide adjuvanted H5N1 influenza vaccine (NIBRG-14) showed sero-responses in 79% of the subjects (Chotpitayasunondh et al., 2008). Although the dose of vaccine was high, aluminum salts were safe in infants and children; these data are important because children must be included in plans for influenza vaccination programs.

Although alum has been shown to confer a significant antigen-sparing effect in mice, ferrets and macaques (Layton et al., 2011; Ninomiya et al., 2007; Ruat et al., 2008), clinical trials indicate that, although safe, the presence of alum did not greatly or consistently improve the immune response to split-virus influenza vaccines in humans. The reasons for the failure of alum to enhance immune responses to influenza virus HA in humans are not clear, but may be related to a number of factors, including the strength of adsorption of the HA to the adjuvant, the ratio of antigen to adjuvant, and the interactions of the adjuvanted preparation with interstitial fluid (Hem and Hogenesch, 2007).

Oil-in-water adjuvants

MF-59: In contrast to aluminum salts, oil-in-water emulsions have conferred significant adjuvant effects on inactivated H5N1 vaccines in humans. Stephenson et al. evaluated the immunogenicity of a low pathogenicity H5N3 virus, A/duck/Singapore/1997, which is antigenically similar to the 1997 HPAI H5N1 viruses adjuvanted with MF59. Healthy subjects aged 18-40 years received two doses of 7.5, 15 or 30 µg HA, with or without adjuvant. Although well tolerated, all doses of the unadjuvanted vaccine were poorly immunogenic, suggesting that this vaccine would be poorly protective against the A/Hong Kong/97 H5N1 influenza viruses. However, the MF59-adjuvanted formulation met the three CHMP criteria for immunogenicity (Nicholson et al., 2001). On follow-up 16 months later, antibody titers associated with protection were not detected but a booster dose of the MF59-adjuvanted vaccine (7.5-30 µg) increased antibody responses to levels associated with protection (Stephenson et al., 2003). This is an important finding because secondary and tertiary waves of infection may occur after the first outbreak of a pandemic virus. The sera from these study subjects were also tested against antigenically distinct H5N1 influenza

viruses from clades that have appeared after 1997 and cross-reactive neutralizing antibody titers to influenza A/Hong Kong/213/03, A/Thailand/16/04, and A/Vietnam/1203/04 viruses were detected (Stephenson et al., 2005). The induction of cross-reactivity could be of crucial importance in the early response to an emerging pandemic because we cannot predict which strain will cause a pandemic. Stephenson and colleagues also evaluated the effect of a booster immunization with an H5N1 vaccine after priming with an H5N3 vaccine. Two 7.5 µg doses of an inactivated MF59-adjuvanted clade 1 NIBRG-14 vaccine were administered to subjects who had been primed at least six years earlier with two doses of MF59-adjuvanted or non-adjuvanted A/duck/ Singapore/1997 (H5N3) vaccine containing 7.5-30 µg of HA (Stephenson et al., 2008). The clade 1 vaccine induced a significantly better response in terms of early induction and persistence of cross-reactive antibody for six months in subjects who had been previously primed with the MF59-adjuvanted H5N3 vaccine than in unprimed subjects or in those who had been primed with unadjuvanted H5N3 vaccine. As early as 7 days after administration of the clade 1 vaccine, at least 90% of MF59-primed subjects exhibited high titers of neutralizing antibodies against different H5N1 clades, as well as to the homologous H5N3 priming virus, suggesting that priming with MF59-adjuvanted H5 vaccine will allow dose-sparing by limiting the amount of antigen and number of doses required to achieve 'seroprotective' titers against a genetically distinct H5 virus (Stephenson et al., 2008). The authors hypothesized that this might be due to the induction of an immune memory B cell pool that expands more rapidly and efficiently with booster immunization (Galli et al., 2009). This prime-boost strategy that may induce long-lasting immune memory and allow a single booster dose to induce protection, is likely to be an effective way to protect populations against future influenza pandemics (Jennings et al., 2008).

MF-59 adjuvanted vaccines have been evaluated in different age groups. In a clinical trial in 486 healthy adults aged 18–60 years and elderly individuals over 60 years of age, an inactivated MF59-adjuvanted clade 1 sub-unit H5N1 vaccine demonstrated comparable 'seroprotection' rates assessed by HAI in 72-73% of adults and 75-76% of elderly after two doses of 7.5 or 15 µg of vaccine, respectively (Banzhoff et al., 2009). There was a significant reduction in antibody titers after six months in both adults and the elderly. However, the groups that received a third dose of vaccine (7.5 or 15 µg) showed 'seroprotection' rates 83-76 % and 92-96% in adults and the elderly, respectively. This vaccine also elicited cross-neutralizing antibodies to the clade 2 A/turkey/Turkey/1/2005 virus, suggesting that it can be used in a pre-pandemic scenario in both of these age groups (Banzhoff et al., 2009). Subsequently, the immunogenicity and safety of a booster dose of an MF59-adjuvanted H5N1 vaccine containing only 7.5 µg of the heterologous clade 2.2 H5N1 HA, given approximately 18 months after primary vaccination was evaluated (Fragapane et al., 2010). Seroconversion rate criteria were met 3 weeks after the booster dose for both the clade 1 and clade 2.2 strains in adult and elderly subjects. This study illustrated how pre-pandemic vaccination can prime a population, providing initial protection against an influenza virus that can be boosted with a different strain. Finally, when 7.5 µg of NIBRG-14 HA vaccine was evaluated in healthy children 6 months to 17 years of age, the vaccine was highly immunogenic with a 'seroprotection' rate that ranged from 89 to 97% and the vaccine was safe in all age groups (Vesikari et al., 2010).

AS03: Leroux-Roels et al. undertook an observer-blind randomized trial of a two-dose H5N1 inactivated, split-virion clade 1 H5N1 influenza vaccine derived from NIBRG-14 in 400 healthy subjects aged between 18 and 60 years (Leroux-Roels et al., 2007). Vaccines contained 3.8, 7.5, 15, or 30 µg of H5 HA antigen with or without a proprietary oil-in-water adjuvant (AS03). All eight vaccine formulations had a good safety profile across the wide age range of study subjects. The adjuvanted formulations were significantly more immunogenic than the non-adjuvanted vaccine at all antigen doses. Two 30 µg doses of non-

adjuvanted split-virion vaccine were needed to induce an HAI response that met at least two of the three CHMP criteria but neither of the proposed FDA criteria were met (Table 1). Interestingly, at the lowest antigen dose of 3.8- μ g of HA, the adjuvanted vaccine elicited HAI titers against the homologous vaccine strain (NIBRG-14) that met or exceeded all FDA and CHMP licensure criteria but also induced seroconversion rates for cross-reactive neutralizing antibody responses in 75, 85 and 75% of the subjects against clade 2.1, 2.2 and clade 2.3 viruses, respectively (Leroux-Roels et al., 2008). Based on these results, a dose of 3.8 μ g was selected for further development of the vaccine and was licensed by GlaxoSmithKline Biologicals, Rixensart, Belgium, in Europe, the Middle East and Africa, Malaysia and Hong Kong under the trade name Prepandrix™. This vaccine was approved in the European Union (EU) for use for active immunization against H5N1 subtype influenza virus in adults aged 18–60 years (SAGE, 2009).

In a follow-up study, the B- and T-cell responses elicited by this vaccine were evaluated in a subset of 50 study subjects. Compared with adjuvant alone, the vaccine enhanced persistence of neutralizing and HAI antibodies, promoted stronger B-cell and CD4 T-cell responses and induced polyfunctional cross-clade T-cell responses to two heterologous clade 2 H5N1 viruses, A/Indonesia/5/2005 (clade 2.1) and A/Anhui/1/2005 (clade 2.3) (Moris et al., 2011). Two doses of AS03-adjuvanted vaccine containing 1.9 μ g of NIBRG-14 HA elicited serum HAI titers 1:32 in 76% of study subjects; this dose of antigen is at the limit of dosing that can be achieved with influenza vaccines (Levie et al., 2008).

Diez-Domingo et al. evaluated AS03 adjuvanted vaccines with 1.9 μ g or 3.75 μ g of HA (NIBRG-14) and 11.86 mg of AS03A or 5.93 mg of AS03B tocopherol in a phase II, randomized, open-label study in children aged 3 to 9 years. The vaccine was immunogenic, fulfilling licensure criteria for influenza vaccines even at a dose of 1.9 μ g HA, and elicited cross-reactive antibodies against homologous and variant viruses (Diez-Domingo et al., 2010).

The efficacy of the AS03 adjuvanted H5N1 vaccine was tested in ferrets since the protective efficacy of pandemic influenza vaccines in humans will only be demonstrated during a pandemic (Baras et al., 2008). In this pre-clinical study, ferrets were vaccinated with two doses of 15, 7.5, 3.8 or 1.7 μ g HA of the NIBRG-14 clade 1 vaccine adjuvanted with AS03 and were challenged with a lethal dose of the heterologous wt clade 2.1 virus delivered intra-tracheally on day 49. All vaccine formulations induced neutralizing antibodies in the majority of ferrets to both clade 1 (74%) and clade 2.1 viruses (61%). The ferrets that received two doses of 3.8 μ g or more of the adjuvanted vaccine and 96% of the animals that received 1.7 μ g HA survived lethal challenge with the heterologous clade 2 virus.

Taken together the clinical and preclinical studies indicate that split-virion adjuvanted influenza vaccines are safe and immunogenic in humans. Doses as low as 3.8 μ g of HA elicited neutralizing antibodies specific for the vaccine strain and cross-reactive antibodies to heterologous H5N1 viruses from other clades, induced memory B-cell and CD4 T-cell responses in humans and was efficacious against challenge in ferrets. These data are encouraging and suggest that, in the event of an H5N1 pandemic, vaccines with oil-in-water adjuvants could have a major impact on the number of doses of vaccine available and therefore on the proportion of the population that can be vaccinated.

2.1.3. Egg-derived whole virus vaccines—Inactivation of infectivity for whole virus vaccines has been achieved by treatment with either formalin or betapropiolactone. The first clinical study of an egg-derived whole-virus vaccine against a clade 1 (NIBRG-14) avian influenza H5N1 virus was performed by Lin and colleagues (Lin et al., 2006). A two-dose regimen of 10 μ g of an alum-adjuvanted inactivated whole-virus H5N1 vaccine in 120

healthy adults aged 18–60 years resulted in 78% seropositivity by day 42. The vaccine was well tolerated and met all three criteria that the CHMP required for annual licensing of seasonal vaccines (CHMP, 1997). In a follow-up study one year later, the antibody titers declined significantly and a third dose of vaccine boosted the titers (Lin et al., 2009). In a phase II, double-blind, randomized trial, two doses of 10 µg of vaccine showed good immunogenicity and safety, and induced cross-reactive antibodies in 98% and 87% of subjects against heterologous clade 2 A/Indonesia/5/2005 and A/Anhui/1/2005 viruses, respectively (Wu et al., 2009).

A clade 1 H5N1 whole virion vaccine (NIBRG-14) formulated with an aluminum phosphate adjuvant administered as a single dose of 6 µg HA to 146 healthy subjects 18 years of age and older, was safe and immunogenic, meeting two independent criteria for immunogenicity 21 and 90 days after vaccination according to European Medicines Agency (EMA) requirements: GMT ratio >2.5 and seroconversion rate >40%. This study identified the lowest effective dose needed to induce an immune response (Vajo et al., 2007). Subsequently, this vaccine was tested in 480 healthy subjects who were 18–60 or >60 years of age. Subjects were vaccinated intramuscularly (i.m.) with two doses of 3.5 µg 21 days apart or one dose of vaccine containing 3.5, 6 or 12 µg of HA. With the exception of a single dose of 3.5 µg, all doses and age groups met CHMP, ECDC and FDA immunogenicity criteria by HAI. The authors concluded that the optimal dose was 6 µg, because similar responses were seen in subjects receiving two doses of 3.5-µg or one dose of 6 or 12 µg and thus, doses exceeding 6 µg or two injections seemed to be unnecessary (Vajo et al., 2010). This vaccine was also shown to be safe and immunogenic in children, and 8 of 12 healthy children who received a single dose of 6 µg of vaccine seroconverted after vaccination (Vajo et al., 2008). In addition, 44 adults (19–60 years old) and 44 elderly (60–83 years old) received a single 6 µg dose of the vaccine; seroconversion was demonstrated in 90% and 68%, respectively, against the homologous virus and both groups of subjects also showed significant cross-reactive antibody responses against clade 2 H5N1 viruses. Interestingly, higher titers of post-vaccination neutralizing antibodies and cross-reactive antibodies were detected in the elderly, and a surprising 56% of the elderly subjects also seroconverted against an H1N1 (A/Solomon Islands/13/2006-like) virus, compared with only 18% of adults, suggesting the potential importance of prior vaccination with seasonal influenza vaccines in developing cross-reactive immunity against pandemic viruses (Fazekas et al., 2009). This whole-virion vaccine containing 6µg of HA antigen adjuvanted with alum, was licensed by Omninvest Ltd. in Hungary in June 2007 under the name of Fluval H5N1 (SAGE, 2009).

Cell culture grown whole virion vaccine: Ehrlich and colleagues (Ehrlich et al., 2008) investigated the safety of a clade 1 Vero cell culture grown H5N1 whole-virus vaccine administered in two doses 21 days apart to 275 subjects 18–45 years of age. Vaccine doses contained 3.75, 7.5, 15, or 30 µg of HA antigen with alum or 7.5 or 15 of HA antigen without alum. The study showed that the 7.5 µg non-adjuvanted formulation was the optimal dose with respect to development of specific neutralizing antibody 21 days after two doses of vaccine. This vaccine also induced a cross-reactive immune response to clade 2.1 and clade 0 H5N1 viruses. A subsequent study (Ehrlich et al., 2009) investigated antibody persistence in 77 subjects after 2 doses of the H5N1 clade 1 vaccine administered at doses of 3.75, 7.5, 15, or 30 µg of HA with alum or 7.5 or 15 µg of HA without alum. Seven and 21 days after vaccination, antibody titers were measured against clade 1, clade 2.1, clade 2.2, and clade 2.3 viruses. Vaccination resulted in substantial antibody responses, not only to the homologous clade 1 vaccine virus, but also to the antigenically divergent clades 2.1, 2.2 2.3. However, 12–17 months after primary vaccination, only low titers of antibody were detected against all of the H5N1 virus clades. The investigators assessed the effect of a booster vaccination with a single dose of 7.5 µg of HA of a clade 2.1 H5N1 vaccine without alum in

recipients who were primed with the unadjuvanted clade 1 whole virion vaccine. Interestingly, by day 21 after boosting, almost 100% of the subjects had high antibody titers against all four of the H5N1 viruses. This study supports the idea that prime-boost strategies are of value in inducing rapid immunity during a pandemic and is in agreement with studies with H5N3 and H5N1 vaccines discussed in section 2.2.1. (Stephenson et al., 2005; Stephenson et al., 2003; Stephenson et al., 2008).

Homologous and heterologous cross-strain CD4⁺ responses of 40 subjects in two age groups of 18-59 and 60 years, were evaluated following two doses of the 7.5- μ g non-adjuvanted inactivated cell grown clade 1 whole virus vaccine administered 21 days apart (Crowe et al., 2010). The ability of this vaccine to induce memory B cell responses was also investigated as a determinant of long-term protection against influenza (Crotty and Ahmed, 2004; Kelly et al., 2005; Takahashi, 2007). The cross-reactivity of the response to seasonal influenza strains of different types and subtypes was also evaluated. The H5N1 vaccine induced a robust homologous CD4⁺ T cell response, which was maintained over 6 months, and a cross-clade (A/Indonesia/05/2005) cellular immune response. The CD4⁺ T-cells also cross-reacted with seasonal influenza A/New Caledonia/20/1999 (H1N1), but not with A/New York/55/2004 (H3N2) or B/Jiangsu/10/2003 viruses (Crowe et al., 2010).

Taken together, two doses of 7.5 μ g of an inactivated non-adjuvanted cell culture grown whole virus H5N1 vaccine shows promise because it was well tolerated and safe in adults and the elderly, and induced homologous and heterologous type and subtype-specific humoral and cellular immune responses.

2.1.4. Comparison between alum adjuvanted inactivated split-virus and egg-derived whole virus vaccines—Wu et al. conducted a randomized phase II study comparing the safety and immunogenicity of an adjuvanted split-virion and a whole-virion H5N1 vaccine in 280 children aged 12-17 years (Wu et al., 2010) who received 10, 15 or 30 μ g of a split-virion or 5 μ g of a whole-virion clade 1 H5N1 vaccine (NIBRG-14); both were formulated with alum. The 30 μ g split-virion vaccine formulation induced the highest immune response after two doses, meeting the CHMP licensure criteria with 71% seroconversion and 'seroprotection' rates by HAI. Five μ g of the whole-virion vaccine induced a higher response than 10 μ g of the split-virion vaccine. These results are consistent with those seen in adults (see section 2.1.2), where the whole-virion vaccine was more immunogenic than the split-virion vaccine. The 30 μ g split-virion vaccine in 3-11 year old children and 10 μ g and 15 μ g whole-virion vaccine in 12-17 year old children caused a higher incidence of adverse reactions (total incidence of 80%, 60% and 62.5%, respectively); fever was most commonly reported, in more than 50% of subjects but no serious adverse events were reported. The authors proposed that 5 μ g of a whole-virion vaccine could be considered for use in children aged 12-17 years. Of note, the use of inactivated whole virus influenza vaccines were previously noted to cause a relatively high incidence of adverse events, including local reactions at the site of injection and febrile illness, particularly among children (Wright et al., 1977).

2.2. Live attenuated vaccines

Live attenuated influenza vaccines (LAIV) are attractive as pandemic vaccines because they mimic natural infection by replicating primarily in the upper respiratory tract and inducing immune responses comprising mucosal IgA (Clements and Murphy, 1986), serum IgG antibodies and cell-mediated immunity (Gorse et al., 1995). In addition, LAIV are potentially cheaper and quicker to manufacture than inactivated subunit or split vaccines, because of higher yields per egg, no HA protein purification is required, and LAIV are administered by intranasal drops or spray. On the other hand, a potential disadvantage of

LAIV is that it is not approved for use in all age groups. Also, theoretical risks of reassortment with circulating influenza viruses (discussed below), and potential of vaccine virus transmission exist. In addition, as with any live attenuated vaccine, there is the hypothetical concern of reversion to virulence. A pandemic LAIV (pLAIV) would only be used after an emerging virus is known to be circulating widely in humans and thus this vaccine strategy would not be used prior to a pandemic.

The LAIVs that are currently licensed for seasonal influenza A vaccines were derived from two different master donor viruses (MDVs) through serial passage of influenza A/Ann Arbor/6/60 (H2N2) in the United States, and A/Leningrad/134/17/57 (H2N2) in Russia, at sequentially lower temperatures in specific pathogen-free primary chick kidney cells and embryonated chicken eggs (Kendal, 1981; Maassab, 1968). During this process, the viruses acquired multiple mutations in internal protein gene segments that resulted in the cold-adapted (*ca*), temperature-sensitive (*ts*), and attenuated (*att*) phenotypes of the MDVs. Temperature-sensitive viruses do not replicate well at the warmer temperatures in the lower respiratory tract (Gruber, 2002; Murphy and Coelingh, 2002). pLAIV contain the HA and NA gene segments from an animal influenza virus and internal protein genes from one of the two MDVs.

In the United States, seasonal LAIV are currently licensed for use in eligible patient populations and represent an injection-free and adjuvant-free approach to induce immunity that may protect against antigenically divergent viruses. However, the development of an immunogenic H5N1 LAIV has been challenging. Although a clade 1 (A/Vietnam/1203/2004) H5N1 LAIV candidate vaccine based on the A/Ann Arbor/6/60 MDV (AAca LAIV) was immunogenic and cross-protective in mice, two doses of the vaccine elicited only modest homologous serum antibody responses in ferrets. Nevertheless, ferrets were protected from pulmonary replication of homologous and heterologous H5N1 viruses (Suguitan et al., 2006). However, the H5N1 AAca LAIV was highly restricted in replication in humans and induced infrequent and low titer HAI and neutralizing serum antibody responses, even following two doses of $10^{7.5}$ 50% tissue culture infectious doses (TCID₅₀) administered by nasal spray (Karron et al., 2009b). Further analysis of the H5N1 AAca LAIV in mice suggested that in the context of the internal gene constellation of the AA *ca* virus, removal of the polybasic cleavage site of the HA for safety reasons contributed to the over-attenuation of vaccine (Suguitan et al., 2009).

An H5N2 LAIV using the A/Leningrad/134/17/57 *ca/ts* virus as the MDV, with an HA derived from low pathogenicity A/duck/Potsdam/1402-6/86 (H5N2) virus was evaluated in phase I-II clinical trials (Rudenko et al., 2008) and was licensed for use in Russia. A two-dose regimen induced seroresponses (4-fold antibody rise) to the homologous virus in 47 to 55% of subjects and evidence of cross-reactivity to the reverse genetics reassortant A/Indonesia/05/2005 × PR8 IBCDC-RG (H5N1) was observed in 29 to 31% of the subjects. This vaccine also induced significant antibody titers in nasal secretions and was well-tolerated and safe. Chirkova et al. demonstrated that immunization of healthy young adults with two doses of this vaccine induced an increase in influenza-specific CD4 and CD8 memory/effector T-cell levels in the peripheral blood of the subjects (Chirkova et al., 2011).

2.2.1. Characterization of reassortant influenza viruses generated from live attenuated pandemic influenza vaccine and wild-type human influenza viruses

—The segmented nature of the influenza A virus genome permits reassortment of gene segments during co-infection with two different influenza viruses to produce progeny viruses of different genotypes. Reassortment is of concern since this process can lead to the emergence of novel influenza viruses in the population. Although LAIVs are an attractive option for the prevention of disease in the event of a pandemic, there is concern that a

pLAIV virus could reassort with circulating human influenza viruses, resulting in viruses with a novel HA that may be able to replicate and transmit efficiently in an immunologically naïve human population. Investigational candidate pLAIV are currently evaluated in carefully planned inpatient studies (Karron et al., 2009a; Karron et al., 2009b; Talaat et al., 2012; Talaat et al., 2011), during the time of the year when human influenza viruses are not circulating in the community. Although such vaccines would not be used until a pandemic virus has emerged, the theoretical risk of a reassortment event between a live vaccine virus with a novel HA and circulating wt human influenza viruses exists, both for vaccines being evaluated in the inter-pandemic period and with their widespread use early in a nascent influenza pandemic.

The question of novel reassortant viruses arising as a result of the use of live attenuated vaccines for seasonal influenza has been addressed experimentally. Parks et al. (Parks et al., 2007) reported that reassortant viruses, generated by reverse genetics, derived from a wt H3N2 influenza virus and the corresponding LAIV, were generally no more virulent than the wt H3N2 virus in the ferret model, and, in fact, the data suggested that it is likely that reassortment between the wt and vaccine viruses would result in progeny viruses that would be attenuated compared to the wt virus. The concerns of reassortment with widespread use of a pLAIV prompted us to investigate whether reassortant viruses between candidate pLAIV viruses and contemporary human influenza viruses were viable, and, if so, whether they exhibited a growth advantage and increased virulence in animal models compared to the parent wt human influenza viruses and the candidate pLAIV. Theoretically there are 254 possible combinations of gene segments when two influenza viruses reassort. In our investigation, we generated only the 6:2 reassortant viruses bearing the novel HA and NA from the pLAIV viruses, and the full complement of internal protein genes from the human influenza viruses. Using reverse genetics technology, reassortant viruses bearing the modified HA and NA from the H5N1 cold-adapted (*ca*) candidate vaccine virus derived from A/Vietnam/1203/2004 (H5N1) [H5N1 *ca*] (Suguitan et al., 2006) and the internal protein genes from either a contemporary human H1N1 or H3N2 influenza virus were generated. We also generated reassortants between an H9N2 *ca* pLAIV vaccine candidate and human H1N1 or H3N2 influenza viruses to compare the behavior of a vaccine virus derived from a low pathogenicity avian influenza virus of a different subtype with one derived from an HPAI H5N1 virus. Growth characteristics of the reassortant viruses were compared with the parent vaccine and human influenza viruses *in vitro* and *in vivo* in mice and ferrets to determine whether such reassortants would pose a public health risk. Recombinant wt A/Solomon Islands/3/2006 (H1N1) and A/California/7/2004 (H3N2) viruses were generated by plasmid-based reverse genetics and used for comparison because the H5N1 *ca* and H9N2 *ca* pLAIV viruses and the reassortant viruses in this study were generated by reverse genetics.

Recombinant wt A/Solomon Islands/3/2006 (H1N1) [rSol] and A/California/7/2004 (H3N2) [rCal] (Cheng et al., 2012; Xu et al., 2010) and 6:2 reassortant viruses bearing the 6 internal protein genes from rSol or rCal viruses and HA and NA from either the H5N1 *ca* or H9N2 *ca* vaccine viruses, referred to as VN/Sol, VN/Cal, G9/Sol, G9/Cal, respectively were generated by plasmid-based reverse genetics. The viruses used in these studies are summarized in Table 4. The genome sequences of the recombinant wt and reassortant viruses bearing A/Solomon Islands/3/2006 (H1N1) genes were identical to the parental virus sequences that were introduced into the plasmid for virus rescue, with the exception of a single silent change in the HA sequence of rSol at nucleotide position 794 (C to T; silent change). The changes in sequence from the parental viruses were not expected to affect the biological properties of the viruses. When the plasmids were constructed for the rCal virus rescue, we found that plasmids encoding the PB1 and PA genes were not functional, therefore, the PB1 and PA sequences from an antigenically related virus, A/New York/

55/2004 (H3N2), were used. The silent changes between the sequences of these gene segments of the two viruses were not corrected. A change in the plus sense sequence of the A/California/7/2004 NP (DN to DD at amino acid residues 497 and 498) that was introduced into the plasmid reverted back to the wt sequence in the rCal virus, and changed to ND in both 6:2 reassortant viruses bearing the A/California/7/2004 internal protein genes.

Replication *in vitro*: The recombinant wt and 6:2 reassortant viruses achieved similar titers ranging from $10^{7.0}$ to $10^{8.0}$ TCID₅₀/mL in eggs (data not shown). Multicycle replication of the viruses was evaluated in Madin Darby canine kidney (MDCK) cells inoculated at a multiplicity of infection of 0.001. The replication kinetics are shown in Figure 1. The G9/Sol, G9/Cal and H9N2 G9 97 wt viruses, bearing the H9 HA, replicated at a slower rate initially than the viruses with H1, H3 or H5 HAs, but achieved similar titers as the other viruses by 65 hours post-infection. No obvious defects in replication *in vitro* were observed in any of the viruses.

Evaluation in the mouse model: The replication of the recombinant wt and reassortant viruses was assessed in mice. The mouse model is a convenient animal model to study influenza viruses, however, some human influenza viruses require adaptation to this species in order to replicate efficiently (Andrewes et al., 1934; Brown, 1990). In contrast, many avian influenza viruses, including isolates of the H5 and H9 subtypes, replicate efficiently in mice without prior adaptation (Chen et al., 2010a; Dybing et al., 2000; Gao et al., 1999; Gillim-Ross et al., 2008; Joseph et al., 2007). Groups of 12 anesthetized 6-8-week-old female BALB/c mice were inoculated intranasally (i.n.) with 10^5 TCID₅₀ of each virus in a volume of 50 μ L. Four mice per group were euthanized on days 2, 3 and 4 post-inoculation. Nasal turbinates and right lungs were collected and homogenized for virus titration in MDCK cells. Left lungs were inflated with and fixed in 10% neutral buffered formalin for histopathological analysis. All of the viruses except the rCal virus replicated efficiently in the upper and lower respiratory tract of mice (Figures 2A and 2B). The failure of the rCal H3N2 virus to replicate in mice was not surprising, since wt human H3N2 viruses in general do not replicate in mice without prior adaptation. In contrast, the rSol H1N1 virus replicated efficiently in the respiratory tract of mice, achieving a mean peak titer of $10^{5.5}$ TCID₅₀/g on day 2 post-infection (p.i.) in the nasal turbinates and a mean peak titer of $10^{5.4}$ TCID₅₀/g on day 4 p.i. in the lungs. The 6:2 reassortant viruses bearing the H1N1 internal protein genes replicated to slightly higher titers than the rSol virus in the nasal turbinates of mice on days 2, 3 and 4 p.i. but the differences were not statistically significant. In the lower respiratory tract, both 6:2 reassortant viruses replicated to modestly higher titers than the rSol virus. The peak of replication of the VN/Sol reassortant virus occurred on day 2 p.i. in both the upper and lower respiratory tract of mice, and peak replication of the G9/Sol reassortant virus occurred on day 3 p.i. in the upper respiratory tract and on day 2 p.i. in the lower respiratory tract.

Despite the failure of the rCal H3N2 virus to replicate in the respiratory tract of mice, the 6:2 reassortant viruses with the internal protein genes from the A/California/7/2004 (H3N2) virus replicated efficiently. The VN/Cal and G9/Cal viruses replicated to similar titers in both the upper and lower respiratory tract of mice although the G9/Cal virus was cleared more rapidly from the lower respiratory tract.

The G9/Cal virus replicated to similar peak titers in the nasal turbinates and lungs of mice as the H9N2 G9 wt virus. The H9N2 *ca* vaccine virus replicated efficiently in the upper respiratory tract of mice, but was not detected in the lungs of mice, consistent with previous reports (Chen et al., 2003).

The two 6:2 reassortant viruses bearing the HA and NA from H5N1 *ca* (VN/Sol and VN/Cal), replicated efficiently and to similar titers in the lower respiratory tract of mice, although the mean titers were approximately 100-fold lower than those observed for the H5N1 VN 04 wt virus. In the upper respiratory tract, the peak titer of the VN/Cal virus was significantly lower than that of the H5N1 VN 04 wt virus. The H5N1 *ca* vaccine virus replicated efficiently in the upper and lower respiratory tract of mice and the virus replicated to approximately 100-fold higher titer in the upper respiratory tract on day 3 p.i. in this study than was previously reported (Suguitan et al., 2006).

Thus, compared with the H9N2 *ca* virus, the G9/Cal virus showed a clear growth advantage *in vivo*. The VN/Sol and VN/Cal viruses did not demonstrate a growth advantage in the respiratory tract of mice compared to the H5N1 *ca* virus.

As a measure of virulence, we evaluated lung samples collected on day 3 p.i. from mice inoculated with the recombinant wt or 6:2 reassortant viruses for histopathological changes. The histopathological findings and replication of the various viruses in the lungs of mice are presented in Table 5. In general, inflammatory changes were observed in the larger airways of mice. Reassortant viruses with the HA and NA from the H5N1 *ca* vaccine virus caused more severe inflammation in the lungs (scores of 3 and 4) than those with the HA and NA from the H9N2 *ca* vaccine virus (scores of 0, 1 and 2). Inflammatory changes and viral antigen were not observed in the lung section from a mock-infected mouse.

A lung section from one mouse from each group inoculated with virus bearing the H5 HA was evaluated for viral antigen distribution by immunohistochemistry (IHC) using an influenza A H5-specific antibody, and for inflammatory changes by hematoxylin and eosin (H&E) staining (Figure 4). Histologically, the lungs from the mice that received the VN/Sol or the H5N1 VN 04 wt virus had the most severe pathology, with comparable diffuse, necrotizing bronchitis and bronchiolitis. In the lung section from the mouse inoculated with VN/Sol, the bronchus and bronchioles were necrotic and infiltrated by numerous inflammatory cells (macrophages, lymphocytes, plasma cells and scattered neutrophils). Throughout the section, there were rare mononuclear perivascular cuffs. The distribution of viral antigen was mostly restricted to the bronchial and bronchiolar epithelium. Focally, there was immunostaining in the alveolar epithelium and luminal karyorrhectic debris (mixture of mucus, dead cells, and inflammatory cells in the alveolar spaces). The lung section from the mouse that received the H5N1 VN 04 wt virus showed a bronchus and bronchioles infiltrated by moderate numbers of macrophages, lymphocytes, plasma cells, and scattered neutrophils. Most of the bronchus was denuded of its epithelium and in the few areas where the epithelium was intact, it was mostly necrotic. Most of the bronchioles were necrotic and the alveolar lumina were filled with numerous inflammatory cells (macrophages and neutrophils), and karyorrhectic debris. Perivascular edema was prominent throughout this section, and viral antigen was widely distributed in the bronchiolar and alveolar epithelia and luminal debris.

VN/Cal virus infection resulted in a different type of pathology in the mouse lung, and of lesser severity than that observed with the VN/Sol and H5N1 VN 04 wt viruses. The section of the lung from the mouse that received VN/Cal was characterized by focally extensive interstitial pneumonia. The interstitium (alveolar walls) were moderately expanded by inflammatory cells (macrophages, lymphocytes, a few plasma cells, and neutrophils). Mononuclear cells (macrophages, lymphocytes and rare plasma cells) were also observed expanding to the adjacent pleura. Often, the lumina of the alveoli were filled with mononuclear cells, neutrophils and karyorrhectic debris. Two bronchioles were also filled with mucus and debris. Throughout this section there were rare mononuclear perivascular cuffs. The presence of antigen was very limited – only scattered foci were

observed – and was seen mostly in the alveolar epithelium and rarely in the bronchiolar epithelium. The luminal cellular debris was also immunopositive.

Multifocal, mild bronchitis and bronchiolitis were observed in the lung section from the mouse inoculated with the H5N1 *ca* vaccine virus. The bronchus and bronchioles were moderately infiltrated, and often cuffed, by mononuclear cells and scattered neutrophils. In the inflamed airways there were scattered necrotic epithelial cells and involvement of the surrounding interstitium (mononuclear and neutrophilic infiltration with luminal debris and alveolar macrophages). Throughout the section, perivascular edema and rare mononuclear perivascular cuffs were observed. Epithelial cells of the bronchus, bronchioles and alveoli, and the luminal debris, were strongly immunopositive.

Reassortant viruses with the HA and NA from the H5N1 *ca* vaccine virus caused more severe inflammation in the lungs than their H9N2 counterparts. The H5N1 *ca* vaccine virus also caused moderate to marked inflammation in the lungs of mice. Whether or not this is an inherent property of the H5 HA remains to be determined. The HA from the H5N1 *ca* does not have the polybasic cleavage motif that is associated with virulence in poultry (Bosch et al., 1979; Hatta et al., 2001; Kawaoka and Webster, 1988) and removal of this motif was found to render the H5N1 VN 04 wt virus non-lethal in mice and chickens, and also restricted replication of the virus to the respiratory tract in mice (Chen et al., 2010b). Suguitan and colleagues also demonstrated that the multibasic cleavage motif of the HA of the H5N1 VN 04 wt virus contributes to virulence to differing degrees depending on the mammalian host species, more dramatically affecting virulence in mice and ferrets than in African green monkeys (Suguitan et al., 2012). Removal of this motif alone does not explain the less severe lung inflammation observed in mice infected with the H5N1 *ca* vaccine virus in this study, since the VN/Sol and VN/Cal viruses also bear the modified HA, and yet they caused marked to severe inflammation in the lungs of mice. The internal protein genes from the AA *ca* donor virus also contribute to the attenuation of the vaccine virus. Although the reassortant viruses with the H9 and N2 HA and NA genes replicated efficiently in the mouse respiratory tract, they did not cause severe inflammation in the lungs of mice.

Evaluation in ferrets: We also evaluated the replication of the recombinant wt and reassortant viruses in ferrets. The ferret is a popular model for the study of influenza viruses because they are exquisitely sensitive to infection by human influenza viruses, and they exhibit clinical signs that resemble human influenza, such as fever, rhinorrhea and sneezing (reviewed in (Luke and Subbarao, 2011). Avian influenza viruses also replicate efficiently in ferrets (Govorkova et al., 2005; Hinshaw et al., 1981; Maines et al., 2005; Zitzow et al., 2002). None of the ferrets exhibited clinical signs of illness. Groups of three 7-week-old male ferrets that were seronegative to circulating seasonal H1N1 and H3N2 influenza viruses were lightly anesthetized and inoculated i.n. with 10^6 TCID₅₀ of each virus in a volume of 0.2 mL. Ferrets were monitored for clinical signs of influenza infection and body temperature was recorded twice daily. Three days post-inoculation, ferrets were euthanized and nasal turbinates and the lower left lung lobe were harvested and homogenized for virus titration. Virus titers in lung homogenates were determined in eggs and those in nasal turbinates were determined in MDCK cells. The lower right lung lobe from each ferret was inflated with and fixed in 10% neutral buffered formalin for histopathological analysis. As was observed in the mouse model, all recombinant wt and 6:2 reassortant viruses replicated efficiently in the upper respiratory tract of ferrets (Figure 3). The pLAIV viruses replicated efficiently in the ferret upper respiratory tract, and, as expected for temperature sensitive (ts) viruses, they were not detected in the lower respiratory tract of ferrets (Suguitan et al., 2006), and as in mice, the rCal H3N2 virus was not detected in the lungs of ferrets. However, the rSol H1N1 virus also failed to replicate in the lungs of ferrets. This was an

unexpected observation, since the biological wt virus, A/Solomon Islands/3/2006 (H1N1) replicates efficiently in the upper and lower respiratory tract of ferrets. However, it has since been demonstrated that an egg-adaptation mutation in the HA of A/Solomon Islands/3/2006 (H1N1) abrogated the ability of the virus to replicate in the lungs of ferrets (Xu et al., 2010). It is possible that replication of the recombinant wt viruses occurred in the lungs of ferrets at an earlier or later timepoint than on day 3, when the lungs were harvested. It was also surprising that none of the ferrets exhibited clinical signs of influenza in this study, given the efficient replication of all of the viruses in the upper respiratory tract. These observations demonstrate that while the ferret has several advantages for the study of influenza, not all influenza viruses behave uniformly in this species.

The VN/Sol virus was not detected in the lungs of ferrets, whereas the corresponding G9/Sol replicated to low titers in 2 out of the 3 ferrets. The VN/Cal virus replicated in the lungs of 2 out of 3 ferrets, although again the titers achieved were low (mean titer $10^{1.9}$ 50% egg infectious doses (EID₅₀)/g). The G9/Cal virus replicated in the lungs of 3 out of 3 ferrets (mean titer $10^{2.9}$ EID₅₀/g). The observations in ferrets were quite different from those in mice, although they also suggest a slight growth advantage of the G9/Sol, G9/Cal and VN/Cal viruses in the lower respiratory tract compared to the recombinant wt viruses.

The inflammation observed in the ferret lungs was generally less severe than that seen in the mice; this correlates with the lack of replication of the reassortant and recombinant wt viruses in the lungs of ferrets. Inflammation in the ferret lungs varied from multifocal scattered individual macrophages within alveolar spaces to more prominent localized areas of inflammation characterized by neutrophils, lymphocytes and macrophages with areas of mild necrosis. The more severe inflammation was most often peribronchiolar or locally extensive in alveolar spaces and rarely larger airways (bronchi). In a few animals, there was necrosis of the glands surrounding the bronchi.

The histopathological findings in the ferrets were discordant with the observed patterns of replication of the viruses in the lungs of ferrets and clinical signs of illness were not observed, so it is difficult to draw conclusions regarding virulence in ferrets (Table 5). Marked inflammation was observed in the lungs of ferrets inoculated with rSol H1N1, in the absence of detectable virus. Similarly, moderate inflammatory changes were noted in ferrets that received rCal H3N2 or VN/Sol, even though virus was not detected in the lungs of ferrets from either group.

Summary: In this study, we focused on 6:2 reassortants that derived their surface glycoproteins from candidate pLAIV viruses, with the full complement of internal protein genes from human influenza viruses because these viruses bear novel surface glycoproteins, and may have the greatest potential for replication and spread in the human population, and, as such, would present the greatest threat to public health. However, it cannot be predicted how individual gene constellations will behave. It appears that reassortment *in vivo* may result in fewer viable genotypes than reassortment by transfection of selected plasmids *in vitro*; when ferrets were co-infected with an H5N1 AI virus and an H3N2 human influenza virus, 9% of viruses recovered had gene segments from both parental viruses (Jackson et al., 2009), and of these, approximately one third had the H5 HA.

The findings from our study underscore that influenza viruses behave quite differently in these two mammalian model systems, and that observations from such studies should be interpreted with caution. Our observations suggest that if reassortment were to occur following co-infection with live pandemic vaccine candidates and wt human seasonal influenza viruses, viable reassortant viruses could arise that may be more virulent than the parent human influenza viruses. The behavior of influenza viruses is influenced by many

complex factors, including the particular host, the virus subtype and gene constellation and replication levels *in vivo*. The focused studies described here do not address the issues of whether or not such reassortant viruses could result from co-infection *in vivo*, or whether they would be efficiently transmitted between hosts. However, our findings suggest that care is warranted in the evaluation of live attenuated vaccines for pandemic influenza, and that such vaccines should only be used when an influenza pandemic has materialized.

2.3. Recombinant protein-based vaccines

Baculoviruses are characterized by their narrow host range (Tinsley, 1978) and cause fatal disease in specific insect species but they are unable to replicate in mammalian cells. However, they can efficiently transduce a variety of mammalian cells (Kost et al., 2005). The expression of proteins by recombinant baculoviruses in insect cells is a convenient and rapid method to generate large amounts of HA protein for influenza vaccines and an alternative to the current egg-based inactivated influenza vaccines.

Several clinical trials have demonstrated the safety and immunogenicity of baculovirus-expressed recombinant seasonal HA vaccines (Lahey et al., 1996; Powers et al., 1997; Roos, 2007; Treanor et al., 1996). Treanor et al. evaluated the immunogenicity, dosage and timing of administration of a recombinant baculovirus-expressed H5 HA (rH5) in 147 healthy adults (Treanor et al., 2001). Subjects received two doses of 25, 45, or 90 µg rHA derived from a clade 0 (A/Hong Kong/156/1997) virus or one dose of 90 µg followed by a dose of 10 µg, or two doses of placebo, by i.m. injection at intervals of 21, 28 or 42 days. The vaccine was well tolerated but only 52% of the subjects developed neutralizing antibodies after two doses of 90 µg of rHA. In a follow-up study, 37 subjects who had previously received the baculovirus expressed clade 0 rH5 HA were re-vaccinated 8 years later with a single 90 µg dose of an inactivated subvirion clade 1 vaccine. Serum samples were tested by HAI assay against clade 0, clade 1 and clade 2 viruses. Seventy to 76% of the subjects who had been primed 8 years earlier with the clade 0 rHA developed an HAI or MN titer 1:40 against the antigenically distinct clade 1 virus compared to only 44 to 42% of the subjects who were previously H5 naïve, respectively. Twenty-four percent of the subjects primed with clade 0 developed HAI antibodies against clade 2. Importantly, 63%–67% of non-responders to the clade 0 rH5 vaccine, subsequently developed HAI and MN titers 1:40 after a booster dose of the A/Vietnam/1203/2004 split-virion vaccine (Goji et al., 2008).

Taken together, these results demonstrated that the baculovirus-expressed rH5 HA induced functional antibodies in individuals who had not had prior exposure to H5 viruses but also showed evidence for a prime-boost phenomenon, with higher antibody titers among those who were primed 8 years earlier.

2.4. Virus-like particle vaccines

Virus-like particle (VLP) vaccines are highly organized particles derived from the self-assembly of viral structural proteins that are non-infectious because they lack the viral genome and full complement of viral proteins. Immunologically VLPs mimic live viruses and can induce robust humoral and cellular immune responses (Grgacic and Anderson, 2006), and they can be manufactured in bulk using a baculovirus expression system and insect cells (Ludwig and Wagner, 2007). Recombinant influenza VLPs have been generated in insect cells infected with recombinant baculoviruses expressing three (HA, NA and M1) or four (HA, NA, M1 and M2) (Latham and Galarza, 2001) influenza structural proteins cloned into a single baculovirus construct (Bright et al., 2007; Pushko et al., 2007).

Several pre-clinical studies in mice and ferrets have evaluated the safety and protective efficacy of this approach for the development of pandemic influenza vaccines (Bright et al.,

2008; Kang et al., 2009; Mahmood et al., 2008; Perrone et al., 2009) but only a few studies have been done in humans. Recently, Khurana et al. reported a phase I/IIa randomized, placebo-controlled study to evaluate the safety and immunogenicity of an A/Indonesia/05/2005 (H5N1) influenza virus VLP vaccine in subjects 18 to 40 years of age (Khurana et al., 2011). VLPs were produced with recombinant baculovirus AcMNPV expressed HA, NA, and M1 proteins from A/Indonesia/05/2005 (H5N1). The vaccine was formulated to contain 15, 45, or 90 µg of HA. In general, the vaccine was well tolerated and after the second dose, HAI seroconversion rates of 40%, 57%, and 61% and MN of 39%, 52%, and 76% were achieved in recipients of 15, 45, and 90 µg, respectively. Cross-reactivity against clade 2 (A/Turkey/1/05 (clade 2.2), and A/Anhui/1/05 (clade 2.3.4) reassortant H5N1 viruses was observed in subjects vaccinated with 45 and 90 µg of HA. These results are promising, although efforts are needed to improve the immunogenicity of this vaccine.

2.4.1. Plant-made virus-like particle vaccine—D'Aoust et al. described an interesting plant-based manufacturing technology from which vaccines can be produced within one month (D'Aoust et al., 2010). This approach has been used for the production of influenza VLPs expressing the HA from different influenza viruses, by cloning the HA gene(s) into a well-characterized vector followed by transient expression in *Nicotina benthamiana* plants (Shoji et al., 2008). The HA expressed in plant cells remains in the form of uncleaved HA₀ molecules that spontaneously assemble into VLPs which accumulate between the plasma membrane and the cell wall and must be released by biochemical means before being purified. Plants are harvested five to six days after infection for purification of the VLPs.

HA-based influenza VLPs have been tested for immunogenicity in mice and ferrets. In mice, two doses of 5 or 1 µg H5-VLPs (A/Indonesia/05/2005) were administered by the i.m. or intranasal (i.n.) routes, respectively, with or without adjuvant, which was alum for i.m. and chitosan for i.n. use. The VLPs induced a stronger HAI antibody response than the same antigen that was not assembled into VLPs (D'Aoust, 2009). In another study, two i.m. doses of 0.1 µg of H5-VLPs induced an HAI antibody response with mean titer >1:40 but increasing the antigen dose up to 12 µg did not result in a dose-response. Two doses of 0.5 µg of H5-VLPs protected against heterologous lethal challenge with A/Vietnam/1194/04 (D'Aoust et al., 2008) and 1 µg protected mice against challenge with A/Turkey/582/06 (D'Aoust, 2009). In ferrets, a single dose of 5 µg or two doses of 1 µg of alum-adsorbed H5-VLPs induced a strong HAI antibody response against the homologous H5N1 virus (A/Indonesia/5/05) and elicited cross-reactivity against heterologous H5N1 viruses (D'Aoust et al., 2010).

Landry et al. evaluated this vaccine in ferrets and in humans. At doses as low as 1.8 and 3.7 µg, the vaccine induced cross-reactive HAI antibodies in ferrets, prevented pathology and reduced viral load on challenge (Landry et al., 2010). In a phase I clinical trial, 48 healthy adults aged 18-60 years received 2 i.m. doses of 5, 10 or 20 µg of alum-adsorbed A/Indonesia/05/2005 VLP vaccine or placebo consisting of alum alone. The vaccine was well tolerated and after two doses of 20 µg, 75 and 91.7% of the subjects mounted a detectable response by HAI and MN assays, respectively.

2.5. DNA vaccines

Plasmid DNA vaccines represent an alternative to conventional vaccine approaches. Their usefulness lies in the fact that they are non-infectious and non-replicating, thereby mitigating safety concerns associated with live viruses. They are very stable and can be produced in large quantities using *Escherichia coli* fermentation procedures. The method consists of injecting plasmid DNA encoding antigen directly into the muscle or dermis. At the site of immunization, cells transfected with plasmid DNA express the protein of interest, which is

processed and presented to the immune system in the context of MHC class I and/or II to activate antigen-specific CD8⁺ and CD4⁺ T cells. DNA vaccines are also capable of inducing long-term memory, an important requirement for a successful vaccine (Kim and Jacob, 2009).

DNA vaccines have shown promising results in mice, ferrets and non-human primates (Epstein et al., 2002; Laddy et al., 2008) and in clinical trials for seasonal and pandemic influenza virus (Jones et al., 2009; Smith et al., 2010). However, as is true for inactivated and subunit protein-based vaccines, adjuvants may be required to potentiate the immune response to these vaccines, although the adjuvants that are commonly used with protein-based vaccines may not be suitable for use with plasmid DNA vaccines. Vaxfectin® (Vical, CA, United States) is a second-generation cationic lipid-based suspension that has been shown to adjuvant both plasmid DNA and protein-based vaccines effectively. This adjuvant presents many desired features: simplicity, ease of formulation and stability, low manufacturing costs, ability to improve and favor specific humoral and cellular immune responses, provide a dose-sparing effect, and promising safety profile (Sullivan et al., 2010).

Smith et al. conducted two double-blind, placebo-controlled phase I studies involving 103 healthy adults aged 18-45 years, who received two i.m. injections of Vaxfectin®-adjuvanted plasmid H5 DNA vaccine or placebo 21 days apart. Subjects received either a monovalent vaccine containing an A/Vietnam/1203/04 H5 HA-encoding plasmid or a three-component vaccine with plasmids encoding full length H5 HA, nucleoprotein (NP), and M2 proteins in doses from 0.1 to 1 mg of DNA/injection by needle or needle-free device (Smith et al., 2010). NP and M2, which are highly conserved among influenza subtypes, were included to increase the breadth of the antiviral immune response as it was previously shown that they protected mice against lethal challenge with different influenza A viruses in the absence of an HA vaccine component (Epstein et al., 2002; Lalor et al., 2008). The vaccine was well tolerated when 1 mg of vaccine was administered i.m. using a syringe or 0.5 or 1 mg of vaccine was administered using a needle-free device. The vaccine containing H5 HA alone elicited HAI titers 40 in 50, 67 or 47 % of the subjects, respectively. By day 56, sera from 47% and 27% of the among the 15 HAI 'responder' subjects cross-reacted with heterologous clade 0 and clade 2.2 H5N1 viruses, A/Hong Kong/156/97 and A/turkey/Turkey/1/05, respectively. Interferon- γ -producing H5-specific T-cells were elicited in 75 to 100% of the subjects 182 days after immunization (Smith et al., 2010).

The study subjects that received the HA+NP+M2 vaccine had lower HAI response rates (20%), but regardless of dose or delivery device, 72% (23/32) of the subjects mounted antibody and/or T-cell responses to one or more antigens by day 56. Although the frequency of responses to any individual antigen did not exceed 57%, HAI antibody responses in particular were considerably lower in the HA+NP+M2 vaccinees than in the HA alone vaccine cohorts. This was the first study to report immune response elicited by an H5-based DNA vaccine in the range reported with inactivated protein-based H5 vaccines, suggesting that adjuvanted DNA vaccines could be useful for pandemic influenza.

Ledgerwood et al. conducted two sequential phase I, open-label, randomized clinical trials, in healthy adults aged 18-60 years, to assess the potential of DNA to prime for a boost with 90 μ g of an unadjuvanted monovalent inactivated sub-virion vaccine (MIV) (Ledgerwood et al., 2011). Sixty subjects were primed with 4 mg of plasmid DNA expressing influenza A/Indonesia/5/05 HA, once at day 0 or twice, at day 0 and week 4, followed by H5N1 MIV at 4 or 24 weeks. This was compared with a two-dose regimen of H5N1 MIV with a 4 week or 24 week interval. In the second study, 21 subjects received 90 μ g of H5N1 MIV at day 0 and week 24 or 4 mg of H5 DNA given with a needle-free device at day 0 followed by a 90 μ g H5N1 MIV boost at 24 weeks. All vaccine formulations were well tolerated. In both studies,

81% of the subjects who were primed at day 0 followed by an MIV boost at week 24 developed protective HAI titers (>40). MIV given twice induced a protective HAI titer in 40-44% of the individuals in study 1 and 2, respectively. These studies showed the relatively weak immunogenicity of the H5 HA MIV antigen and demonstrated that priming with one dose of DNA greatly improved the antibody response. Interestingly, the longer interval between prime and boost (24 vs. 4 weeks) significantly improved the response in the DNA primed groups (post-vaccination HAI titer >40 in 73% versus 27% of individuals, respectively), but did not significantly improve the responses in individuals who received two doses of MIV (post-vaccination titer HAI >40 in 40% of individuals). Additionally, the prime-boost regimen induced neutralizing antibodies directed to the conserved stem region of the HA that were able to neutralize a range of influenza virus subtypes, including H5N1, H5N2, and H9N2 viruses in several individuals.

Overall, these two studies suggest that DNA priming could substantially augment the responses to inactivated protein vaccines, and that gene-based regimens may also provide an opportunity to induce broader and potentially more potent neutralizing antibodies directed against conserved influenza epitopes such as on the stem of the HA.

2.6. Adenovirus-vectored vaccines

Adenoviruses possess several attributes that make them suitable vaccine vectors. Selected adenoviruses are noncontagious and are capable of infecting both dividing and nondividing cells and providing a high level of expression of a gene of interest in eukaryotic target cells without integration into the host genome. They are stable and importantly, can be grown to high titers in qualified cell lines (Tutykhina et al., 2011). The efficacy of nasal vaccines based on adenoviral vectors (AdVs) has been proven in series of studies focused on development of vaccines against a variety of influenza subtypes, including H5N1 viruses (Park et al., 2009; Price et al., 2009). Vaccines based on AdVs are able to effectively induce both humoral and cell-mediated immunity against expressed foreign proteins (Hoelscher et al., 2006; Hoelscher et al., 2007; Park et al., 2009; Price et al., 2009). Intranasal immunization with an Ad5-H5HA expressing the HA of influenza A/Hong Kong/156/197 induced both humoral and cell-mediated immunity against the H5N1 virus (Hoelscher et al., 2006). Importantly, even a single-dose of vaccine elicited protective immunity against different strains of influenza in mice and ferrets (Hoelscher et al., 2006; Hoelscher et al., 2007; Park et al., 2009; Price et al., 2009).

In a different approach using adenoviruses, Alexander et al. designed, produced, and evaluated the safety, immunogenicity and efficacy of a prototype recombinant replicating Ad4 virus vaccine encoding the H5 HA gene from A/Vietnam/1194/2004 (Alexander et al., 2012). This vaccine induced H5 specific HAI antibody and cellular immune responses after a single i.n. immunization in mice, even in the presence of pre-existing Ad4-specific neutralizing antibody. This vaccine was evaluated in a Phase I double-blind, placebo-controlled ascending dosage study (Gurwith et al., 2013). Healthy subjects, aged 18-40 years, received escalating dosages of the Ad4-H5-vaccine from 10^7 to 10^{11} viral particles in an oral formulation on days 1, 56 and 114 followed 3 to 12 months later by a booster with 90 µg of an inactivated split virion H5N1 vaccine. The Ad4-H5-vaccine was safe, showed low transmissibility among household contacts and induced a cellular immune response but only minimum antibody response. However, boost with one dose of the inactivated split virion vaccine elicited HAI antibodies 40 in 69% and 78% of the subjects who received 10^7 or 10^8 viral particles per dose, respectively (Gurwith et al., 2013).

2.7. Universal vaccines

A vaccine that is able to protect against all subtypes of influenza would mean that a vaccine could be produced, manufactured and implemented prior to the emergence of a virus, potentially saving many lives. Due to the inherent variation of the influenza HA, a number of attempts have been made to design influenza vaccines based on more conserved viral proteins. One of the most studied and developed of these “universal target” antigen vaccines are those that target the extracellular portion of the M2 protein (M2e) (Schotsaert et al., 2009). The M2 protein is a transmembrane ion channel and in appropriate configurations and in certain models, antibodies to the M2e domain can be protective. Vaccination of mice with M2e protects against a range of influenza viruses. However, the potency of the immunity in mice has been questioned and the data is less promising in other animal models (Fan et al., 2004; Jegerlehner et al., 2004; Slepishkin et al., 1995) and M2e antibodies are not produced after natural infection in humans. Nevertheless, clinical trials have been initiated with some M2e vaccines (Schotsaert et al., 2009).

Other targets for universal vaccines include the nucleoprotein, more conserved domains of HA and polymerase proteins, which would provide T cell mediated protection (Chen et al., 2009; Epstein et al., 2005; Thomas et al., 2006). A novel Modified Vaccinia virus Ankara (MVA) vectored vaccine designed to boost cross-reactive T-cell responses by expressing the nucleoprotein and matrix protein, that are conserved across all subtypes (MVA-NP+M1) provided protection against influenza disease and virus shedding in a phase I clinical trial (Berthoud et al., 2011). Following this study, a phase 2a vaccination and influenza challenge study was conducted in a small number of healthy adult subjects (Lillie et al., 2012). A significantly increased IFN- γ producing T-cell response to NP and M1 vaccine antigens was seen in subjects following a single dose of the vaccine. The results of this clinical study are encouraging though further studies are needed to characterize safety and efficacy in a large number of individuals and to assess vaccine immunogenicity in both older and younger age groups.

Studies with monoclonal antibodies have shown that a domain in the stalk region of the HA is conserved across a number of HA subtypes, and these results have led to speculation about using the epitope as an immunogen (Corti et al., 2011; Ekiert et al., 2009; Ekiert et al., 2011; Sui et al., 2009; Throsby et al., 2008; Wang et al., 2010; Yoshida et al., 2009). A novel class of human antibodies of which CR6261 (Ekiert et al., 2009; Throsby et al., 2008) and F10 (Sui et al., 2009) are the best studied, share similar patterns of cross-reactivity and neutralization of a large number of group 1 HAs, including the H1, H2, H5, and H9 subtypes. CR6261 and F10, which both recognize the same, conserved epitope in the stem, are protective in mouse models. Along similar lines, a human antibody called CR8020 was identified with broad activity against group 2 HAs that also binds an epitope on the HA stem (Ekiert et al., 2011). This antibody bound HAs from all six subtypes of group 2 HAs and neutralized H3, H7, and H10 viruses in vitro and protected mice from lethal challenge with H3 and H7 viruses. The first antibody that was reported to neutralize both group 1 and group 2 viruses was S139/1 (Yoshida et al., 2009). S139/1 interferes with virus attachment, suggesting that it binds the HA1 head. Also, FI6, a neutralizing antibody isolated from plasma cells, was found to recognize the HA of all 16 subtypes, neutralize both group 1 and group 2 influenza A viruses, and confer protection in mice and ferrets (Corti et al., 2011). The protective efficacy data from animal models suggest that if immunogens can be engineered to elicit similar antibodies, they may result in broader immunity than current vaccines.

3. Concluding remarks

Because H5N1 infection of humans is quite rare, the population has not been primed by previous exposure. This lack of prior exposure makes the population more vulnerable to spread of an H5N1 virus and makes immunization more challenging. Because avian influenza HAs appear to be less immunogenic than human influenza HAs, a higher antigen content, multiple doses and the use of adjuvants are needed to elicit sufficient antibody titers. It is entirely possible that the virus that causes an H5 pandemic will have drifted from the vaccine strain and may even be from a different clade. Cross-protection between clades would therefore be highly beneficial. The degree of protection achieved may depend on the type of vaccine used but protection from severe illness and death would be a more realistic goal than prevention of all forms of illness, including mild disease.

As mentioned before, although the current CHMP criteria have been adopted by the EMEA and FDA for the assessment of pandemic vaccines, these criteria were originally designed for the assessment of seasonal influenza vaccines and may not be entirely relevant or appropriate for assessment of H5 vaccines.

The development of representative pre-pandemic H5N1 candidate vaccine viruses by the WHO Global Influenza Programme is intended as guidance for national authorities on the production of pre-pandemic vaccine. The H5N1 viruses chosen for development of pre-pandemic candidate vaccine viruses are representative of antigenically and genetically distinct groups of viruses that have infected humans through contact with ill or dead H5N1-infected birds. These representative candidate H5N1 vaccine viruses have been prepared by reverse genetics, and safety tested prior to release for the production of pilot vaccine lots that may be used for experimental studies and for stockpiling by governments in advance of a possible H5N1 pandemic (Lillie et al., 2012). Mathematical modeling suggests that implementation and administration of stockpiled pre-pandemic influenza vaccines could markedly reduce attack rates, even if the vaccine had relatively low efficacy against the pandemic strain (Ferguson et al., 2006). The WHO pandemic influenza preparedness (PIP) framework includes provision for a stockpile of 150 million doses of pre-pandemic H5N1 vaccine, and involves distribution of the vaccine to affected countries to assist in containing the first outbreak(s) of an emerging pandemic, and to developing countries once a pandemic begins (WHO, 2011).

As both the vaccine stockpile size and the rate at which vaccination can be carried out are potentially limited, choices will have to be made to decide which individuals would be the first to be vaccinated. In accordance with current pandemic planning (HHS, 2006; UKDH, 2011), it is assumed that a fixed cohort of individuals would have the highest priority for vaccine. The prioritization order for this strategy is hospital staff, followed by infants in child-care together with child-care staff, followed by school children and staff, followed by adults. There are few data on the shelf-life of currently licensed H5N1 vaccines. The main criterion for assessing stability is the retention of HA content in the antigen preparation. In general, the stability has usually been demonstrated to be up to 24 months for antigen and up to 36 months for adjuvant.

Although there are several reasons why LAIVs are appealing for responding to a pandemic, there remains the concern that a vaccine based on an H5N1 virus used prior to a pandemic could reassort with a seasonal human virus resulting in the creation of a virus with a novel HA on the backbone of an epidemiologically successful virus. Although the overall probability of such an event is low, an element of risk remains. So the use of a live attenuated H5N1 vaccine prior to the emergence of a pandemic would not be prudent.

There are insufficient data about the duration of immunological memory but from first principles an assumption can be made that priming will produce some degree of immune memory and is likely to be worthwhile (Goji et al., 2008). The question will remain difficult to answer until the immune correlates of protection are better understood. Adjuvant appears to prolong the duration and to alter the quality of the immune response.

A variety of novel platforms are being tested to facilitate timely production of influenza vaccines. Reactogenicity and immunogenicity of these novel vaccines can be demonstrated in a variety of animal models; however, studies of vaccine effectiveness are best performed in two animal models, typically mice and ferrets, because they can be challenged with wt human influenza virus strains. Regulatory requirements of new vaccines include demonstration of consistency in the manufacturing process and testing for contaminants. This includes *in vivo* and *in vitro* tests to support product licensure and, following licensure, testing of each manufactured lot prior to distribution to ensure the absence of adventitious agents.

In the last decade there has been tremendous progress in the field of influenza vaccines and several of the advances have come from experience with H5 vaccine development. These include the use of larger doses of antigen, the absence of a reproducible effect of alum, the implementation of oil-in-water adjuvants for split virion vaccines, re-consideration of whole virion vaccines, awareness of the shortcomings of the conventional assays for HAI antibodies, the utility of prime-boost, the use of novel platforms and a discussion of ‘pre-pandemic’ vaccine usage, among others. The lessons learned from the H5 vaccine development efforts have informed vaccine development for the 2009 pandemic influenza vaccine and seasonal influenza vaccines and vice versa.

However, several questions remain. It is not clear why the H5 HA is a poor immunogen and why the antibody response decays rapidly. It is also not known whether prior exposure to non-H5 influenza A viruses will prime for responses to H5 vaccines as was seen with the 2009 pandemic influenza virus. The WHO Global Influenza Programme recommends the development of vaccine seed viruses for each representative genetically and antigenically distinct clade of H5N1 viruses. Research is needed to determine the extent of cross-reactive immunity and cross-protection conferred by vaccines based on heterologous clades of H5N1 viruses.

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References

- Abdel-Ghaffar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soerose S, Uyeki TM. Update on avian influenza A (H5N1) virus infection in humans. *N Engl J Med*. 2008; 358(3):261–73. [PubMed: 18199865]
- Alexander J, Ward S, Mendy J, Manayani DJ, Farness P, Avanzini JB, Guenther B, Garduno F, Jow L, Snarsky V, Ishioka G, Dong X, Vang L, Newman MJ, Mayall T. Pre-clinical evaluation of a replication-competent recombinant adenovirus serotype 4 vaccine expressing influenza H5 hemagglutinin. *PLoS One*. 2012; 7(2):e31177. [PubMed: 22363572]

- Andrewes CH, Laidlaw PP, Smith W. The susceptibility of mice to the viruses of human and swine influenza. *Lancet*. 1934; 224:859–862.
- Aucouturier J, Dupuis L, Ganne V. Adjuvants designed for veterinary and human vaccines. *Vaccine*. 2001; 19(17-19):2666–72. [PubMed: 11257407]
- Banzhoff A, Gasparini R, Laghi-Pasini F, Staniscia T, Durando P, Montomoli E, Capecchi PL, di Giovanni P, Sticchi L, Gentile C, Hilbert A, Brauer V, Tilman S, Podda A. MF59-adjuvanted H5N1 vaccine induces immunologic memory and heterotypic antibody responses in non-elderly and elderly adults. *PLoS One*. 2009; 4(2):e4384. [PubMed: 19197383]
- Baras B, Stittelaar KJ, Simon JH, Thoolen RJ, Mossman SP, Pistor FH, van Amerongen G, Wettendorff MA, Hanon E, Osterhaus AD. Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine. *PLoS One*. 2008; 3(1):e1401. [PubMed: 18167560]
- Bernstein DI, Edwards KM, Dekker CL, Belshe R, Talbot HK, Graham IL, Noah DL, He F, Hill H. Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults. *J Infect Dis*. 2008; 197(5):667–75. [PubMed: 18260764]
- Berthoud TK, Hamill M, Lillie PJ, Hwenda L, Collins KA, Ewer KJ, Milicic A, Poyntz HC, Lambe T, Fletcher HA, Hill AV, Gilbert SC. Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. *Clin Infect Dis*. 2011; 52(1):1–7. [PubMed: 21148512]
- Bosch FX, Orlich M, Klenk HD, Rott R. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. *Virology*. 1979; 95:197–207. [PubMed: 442540]
- Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, Hoschler K, Zambon MC. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. *Lancet*. 2006; 367(9523):1657–64. [PubMed: 16714186]
- Bright RA, Carter DM, Crevar CJ, Toapanta FR, Steckbeck JD, Cole KS, Kumar NM, Pushko P, Smith G, Tumpey TM, Ross TM. Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. *PLoS One*. 2008; 3(1):e1501. [PubMed: 18231588]
- Bright RA, Carter DM, Daniluk S, Toapanta FR, Ahmad A, Gavrillo V, Massare M, Pushko P, Mytle N, Rowe T, Smith G, Ross TM. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. *Vaccine*. 2007; 25(19):3871–8. [PubMed: 17337102]
- Brown EG. Increased virulence of a mouse-adapted variant of Influenza A/FM/1/47 virus is controlled by mutations in genome segments 4, 5, 7 and 8. *Journal of Virology*. 1990; 64:4523–4533. [PubMed: 2117072]
- Chen GL, Lamirande EW, Yang CF, Jin H, Kemble G, Subbarao K. Evaluation of replication and cross-reactive antibody responses of H2 subtype influenza viruses in mice and ferrets. *J Virol*. 2010a; 84(15):7695–702. [PubMed: 20504935]
- Chen H, Matsuoka Y, Chen Q, Cox NJ, Murphy BR, Subbarao K. Generation and characterization of an H9N2 cold-adapted reassortant as a vaccine candidate. *Avian Dis*. 2003; 47(3 Suppl):1127–30. [PubMed: 14575127]
- Chen H, Smith GJ, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, Zhang JX, Zhang LJ, Guo CT, Cheung CL, Xu KM, Duan L, Huang K, Qin K, Leung YH, Wu WL, Lu HR, Chen Y, Xia NS, Naipospos TS, Yuen KY, Hassan SS, Bahri S, Nguyen TD, Webster RG, Peiris JS, Guan Y. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proc Natl Acad Sci U S A*. 2006; 103(8):2845–50. [PubMed: 16473931]
- Chen Y, Qin K, Wu WL, Li G, Zhang J, Du H, Ng MH, Shih JW, Peiris JS, Guan Y, Chen H, Xia N. Broad cross-protection against H5N1 avian influenza virus infection by means of monoclonal antibodies that map to conserved viral epitopes. *J Infect Dis*. 2009; 199(1):49–58. [PubMed: 19032063]
- Chen Z, Wang W, Zhou H, Suguitan AL Jr, Shambaugh C, Kim L, Zhao J, Kemble G, Jin H. Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. *J Virol*. 2010b; 84(1):44–51. [PubMed: 19864389]

- Cheng X, Zengel JR, Xu Q, Jin H. Surface glycoproteins of influenza A H3N2 virus modulate virus replication in the respiratory tract of ferrets. *Virology*. 2012; 432(1):91–8. [PubMed: 22743127]
- Chirkova TV, Naykhin AN, Petukhova GD, Korenkov DA, Donina SA, Mironov AN, Rudenko LG. Memory T-cell immune response in healthy young adults vaccinated with live attenuated influenza A (H5N2) vaccine. *Clin Vaccine Immunol*. 2011; 18(10):1710–8. [PubMed: 21813657]
- CHMP. Committee for Proprietary Medicinal Products. Note for guidance on harmonisation of requirements for influenza vaccines (CPMP/BWP 214/96). European Agency for the Evaluation of Medicinal Products; London: 1997.
- Chotpitayasunondh T, Thisyakorn U, Pancharoen C, Pepin S, Nougarede N. Safety, humoral and cell mediated immune responses to two formulations of an inactivated, split-virion influenza A/H5N1 vaccine in children. *PLoS One*. 2008; 3(12):e4028. [PubMed: 19112513]
- Clements ML, Murphy BR. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. *J Clin Microbiol*. 1986; 23(1):66–72. [PubMed: 3700610]
- Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, Vachieri SG, Pinna D, Minola A, Vanzetta F, Silacci C, Fernandez-Rodriguez BM, Agatic G, Bianchi S, Giacchetto-Sasselli I, Calder L, Sallusto F, Collins P, Haire LF, Temperton N, Langedijk JP, Skehel JJ, Lanzavecchia A. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*. 2011; 333(6044):850–6. [PubMed: 21798894]
- Coudeville L, Bailleux F, Riche B, Megas F, Andre P, Ecochard R. Relationship between haemagglutination-inhibiting antibody titres and clinical protection against influenza: development and application of a bayesian random-effects model. *BMC Med Res Methodol*. 2010; 10:18. [PubMed: 20210985]
- Cox NJ, Subbarao K. Influenza. *Lancet*. 1999; 354(9186):1277–82. [PubMed: 10520648]
- Crotty S, Ahmed R. Immunological memory in humans. *Semin Immunol*. 2004; 16(3):197–203. [PubMed: 15130504]
- Crowe BA, Bruhl P, Gerencer M, Schwendinger MG, Pilz A, Kistner O, Koelling-Schlebusch K, Aichinger G, Singer J, Zeitlinger M, Muller M, Ehrlich H, Barrett PN. Evaluation of the cellular immune responses induced by a non-adjuvanted inactivated whole virus A/H5N1/VN/1203 pandemic influenza vaccine in humans. *Vaccine*. 2010; 29(2):166–73. [PubMed: 21055500]
- D'Aoust MA, Couture MM, Charland N, Trepanier S, Landry N, Ors F, Vezina LP. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. *Plant Biotechnol J*. 2010; 8(5):607–19. [PubMed: 20199612]
- D'Aoust MA, Lavoie PO, Couture MM, Trepanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vezina LP. Influenza virus-like particles produced by transient expression in *Nicotiana benthamiana* induce a protective immune response against a lethal viral challenge in mice. *Plant Biotechnol J*. 2008; 6(9):930–40. [PubMed: 19076615]
- D'Aoust, M-A.; Couture, M.; Ors, F.; Trepanier, S.; Lavoie, P-O.; Dargis, M.; Vezina, L-P. Recombinant influenza virus-like particles (VLPs) produced in transgenic plants expressing hemagglutinin.. 2009. International Patent application WO2009/076778
- Dauvilliers Y, Montplaisir J, Cochen V, Desautels A, Einen M, Lin L, Kawashima M, Bayard S, Monaca C, Tiberge M, Filipini D, Tripathy A, Nguyen BH, Kotagal S, Mignot E. Post-H1N1 narcolepsy cataplexy. *Sleep*. 2010; 33(11):1428–30. [PubMed: 21102981]
- de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD. Haemagglutination-inhibiting antibody to influenza virus. *Dev Biol (Basel)*. 2003; 115:63–73. [PubMed: 15088777]
- de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, Bach VC, Phan TQ, Do QH, Guan Y, Peiris JS, Tran TH, Farrar J. Oseltamivir resistance during treatment of influenza A (H5N1) infection. *N Engl J Med*. 2005; 353(25):2667–72. [PubMed: 16371632]
- Diez-Domingo J, Garces-Sanchez M, Baldo JM, Planelles MV, Ubada I, JuBert A, Mares J, Moris P, Garcia-Corbeira P, Drame M, Gillard P. Immunogenicity and Safety of H5N1 A/Vietnam/1194/2004 (Clade 1) AS03-adjuvanted prepandemic candidate influenza vaccines in children aged 3 to 9 years: a phase ii, randomized, open, controlled study. *Pediatr Infect Dis J*. 2010; 29(6):e35–46. [PubMed: 20375709]

- Droebner K, Haasbach E, Fuchs C, Weinzierl AO, Stevanovic S, Buttner M, Planz O. Antibodies and CD4(+) T-cells mediate cross-protection against H5N1 influenza virus infection in mice after vaccination with a low pathogenic H5N2 strain. *Vaccine*. 2008; 26(52):6965–74. [PubMed: 18848593]
- Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML. Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. *J Virol*. 2000; 74:1443–1450. [PubMed: 10627555]
- Ehrlich HJ, Muller M, Fritsch S, Zeitlinger M, Berezuk G, Low-Baselli A, van der Velden MV, Pollabauer EM, Maritsch F, Pavlova BG, Tambyah PA, Oh HM, Montomoli E, Kistner O, Noel Barrett P. A cell culture (Vero)-derived H5N1 whole-virus vaccine induces cross-reactive memory responses. *J Infect Dis*. 2009; 200(7):1113–8. [PubMed: 19712040]
- Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, Fisher D, Berezuk G, Fritsch S, Low-Baselli A, Vartian N, Bobrovsky R, Pavlova BG, Pollabauer EM, Kistner O, Barrett PN. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *N Engl J Med*. 2008; 358(24):2573–84. [PubMed: 18550874]
- Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. *Science*. 2009; 324(5924):246–51. [PubMed: 19251591]
- Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, Vogels R, Brakenhoff JP, Kompier R, Koldijk MH, Cornelissen LA, Poon LL, Peiris M, Koudstaal W, Wilson IA, Goudsmit J. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science*. 2011; 333(6044):843–50. [PubMed: 21737702]
- EMA. Guideline on dossier structure and content for pandemic influenza vaccine marketing authorisation application (CPMP/VEG/4717/03). 2008. <http://www.emea.europa.eu/pdfs/human/vwp/471703en.pdf>
- Epstein SL, Kong WP, Mispion JA, Lo CY, Tumpey TM, Xu L, Nabel GJ. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine*. 2005; 23(46-47):5404–10. [PubMed: 16011865]
- Epstein SL, Tumpey TM, Mispion JA, Lo CY, Cooper LA, Subbarao K, Renshaw M, Sambhara S, Katz JM. DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. *Emerg Infect Dis*. 2002; 8(8):796–801. [PubMed: 12141964]
- Fan J, Liang X, Horton MS, Perry HC, Citron MP, Heidecker GJ, Fu TM, Joyce J, Przysiecki CT, Keller PM, Garsky VM, Ionescu R, Rippeon Y, Shi L, Chastain MA, Condra JH, Davies ME, Liao J, Emini EA, Shiver JW. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. *Vaccine*. 2004; 22(23-24):2993–3003. [PubMed: 15297047]
- Fazekas G, Martosne-Mendi R, Jankovics I, Szilvasy I, Vajo Z. Cross-reactive immunity to clade 2 strains of influenza virus A subtype H5N1 induced in adults and elderly patients by Fluval, a prototype pandemic influenza virus vaccine derived by reverse genetics, formulated with a phosphate adjuvant, and directed to clade 1 strains. *Clin Vaccine Immunol*. 2009; 16(4):437–43. [PubMed: 19020111]
- FDA. U.S. FDA approves first U.S. vaccine for humans against the avian influenza virus H5N1. 2007a. <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2007/ucm108892.htm>
- FDA. U.. Guidance for industry. Clinical data needed to support the licensure of pandemic influenza vaccines. 2007b. <http://www.fda.gov/cber/gdlns/panfluvac.htm>
- Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS. Strategies for mitigating an influenza pandemic. *Nature*. 2006; 442(7101):448–52. [PubMed: 16642006]
- Fragapane E, Gasparini R, Schioppa F, Laghi-Pasini F, Montomoli E, Banzhoff A. A heterologous MF59-adjuvanted H5N1 prepandemic influenza booster vaccine induces a robust, cross-reactive immune response in adults and the elderly. *Clin Vaccine Immunol*. 2010; 17(11):1817–9. [PubMed: 20810680]
- Galli G, Hancock K, Hoschler K, DeVos J, Praus M, Bardelli M, Malzone C, Castellino F, Gentile C, McNally T, Del Giudice G, Banzhoff A, Brauer V, Montomoli E, Zambon M, Katz J, Nicholson K, Stephenson I. Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine. *Proc Natl Acad Sci U S A*. 2009; 106(19):7962–7. [PubMed: 19416838]

- Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *J Virol.* 1999; 73:3184–3189. [PubMed: 10074171]
- Gao W, Soloff AC, Lu X, Montecalvo A, Nguyen DC, Matsuoka Y, Robbins PD, Swayne DE, Donis RO, Katz JM, Barratt-Boyes SM, Gambotto A. Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *J Virol.* 2006; 80(4):1959–64. [PubMed: 16439551]
- Garçon N, Vaughn DW, Didierlaurent AM. Development and evaluation of AS03, an Adjuvant System containing alpha-tocopherol and squalene in an oil-in-water emulsion. *Expert Rev Vaccines.* 2012; 11(3):349–66. [PubMed: 22380826]
- Gillim-Ross L, Santos C, Chen Z, Aspelund A, Yang CF, Ye D, Jin H, Kemble G, Subbarao K. Avian influenza h6 viruses productively infect and cause illness in mice and ferrets. *J Virol.* 2008; 82(21):10854–63. [PubMed: 18715930]
- Goji NA, Nolan C, Hill H, Wolff M, Noah DL, Williams TB, Rowe T, Treanor JJ. Immune responses of healthy subjects to a single dose of intramuscular inactivated influenza A/Vietnam/1203/2004 (H5N1) vaccine after priming with an antigenic variant. *J Infect Dis.* 2008; 198(5):635–41. [PubMed: 18694338]
- Gorse GJ, Campbell MJ, Otto EE, Powers DC, Chambers GW, Newman FK. Increased anti-influenza A virus cytotoxic T cell activity following vaccination of the chronically ill elderly with live attenuated or inactivated influenza virus vaccine. *J Infect Dis.* 1995; 172(1):1–10. [PubMed: 7797897]
- Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hahn TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J Virol.* 2005; 79:2191–2198. [PubMed: 15681421]
- Grgacic EV, Anderson DA. Virus-like particles: passport to immune recognition. *Methods.* 2006; 40(1):60–5. [PubMed: 16997714]
- Gruber WC. The role of live influenza vaccines in children. *Vaccine.* 2002; 20(Suppl 2):S66–73. [PubMed: 12110263]
- Gurwith M, Lock M, Taylor EM, Ishioka G, Alexander J, Mayall T, Ervin JE, Greenberg RN, Strout C, Treanor JJ, Webby R, Wright PF. Safety and immunogenicity of an oral, replicating adenovirus serotype 4 vector vaccine for H5N1 influenza: a randomised, double-blind, placebo-controlled, phase 1 study. *Lancet Infect Dis.* 2013; 13(3):238–50. [PubMed: 23369412]
- Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol.* 2007; 5(7):505–17. [PubMed: 17558426]
- Harrison WT. Some Observations on the Use of Alum Precipitated Diphtheria Toxoid. *Am J Public Health Nations Health.* 1935; 25(3):298–300. [PubMed: 18014174]
- Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science.* 2001; 293:1840–1842. [PubMed: 11546875]
- Hem SL, Hogenesch H. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiality. *Expert Rev Vaccines.* 2007; 6(5):685–98. [PubMed: 17931150]
- HHS. U.. US Department of Health and Human Services 2006 Pandemic influenza plan. 2006. See <http://www.hhs.gov/pandemicflu/plan/>
- Hinshaw VS, Webster RG, Easterday BC, Bean WJ. Replication of avian influenza A viruses in mammals. *Infect Immun.* 1981; 34:354–361. [PubMed: 7309229]
- Hoelscher MA, Garg S, Bangari DS, Belser JA, Lu X, Stephenson I, Bright RA, Katz JM, Mittal SK, Sambhara S. Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. *Lancet.* 2006; 367(9509):475–81. [PubMed: 16473124]
- Hoelscher MA, Jayashankar L, Garg S, Veguilla V, Lu X, Singh N, Katz JM, Mittal SK, Sambhara S. New pre-pandemic influenza vaccines: an egg- and adjuvant-independent human adenoviral vector strategy induces long-lasting protective immune responses in mice. *Clin Pharmacol Ther.* 2007; 82(6):665–71. [PubMed: 17957181]

- Jackson S, Van Hoven N, Chen LM, Maines TR, Cox NJ, Katz JM, Donis RO. Reassortment between avian H5N1 and human H3N2 influenza viruses in ferrets: a public health risk assessment. *J Virol*. 2009; 83(16):8131–40. [PubMed: 19493997]
- Jegerlehner A, Schmitz N, Storni T, Bachmann MF. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *J Immunol*. 2004; 172(9):5598–605. [PubMed: 15100303]
- Jelley-Gibbs DM, Strutt TM, McKinsty KK, Swain SL. Influencing the fates of CD4 T cells on the path to memory: lessons from influenza. *Immunol Cell Biol*. 2008; 86(4):343–52. [PubMed: 18362946]
- Jennings LC, Monto AS, Chan PK, Szucs TD, Nicholson KG. Stockpiling prepandemic influenza vaccines: a new cornerstone of pandemic preparedness plans. *Lancet Infect Dis*. 2008; 8(10):650–8. [PubMed: 18922487]
- Jones S, Evans K, McElwaine-John H, Sharpe M, Oxford J, Lambkin-Williams R, Mant T, Nolan A, Zambon M, Ellis J, Beadle J, Loudon PT. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial. *Vaccine*. 2009; 27(18):2506–12. [PubMed: 19368793]
- Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, Subbarao K. Evaluation of replication and pathogenicity of avian influenza A H7 subtype viruses in a mouse model. *J Virol*. 2007; 81(19):10558–66. [PubMed: 17634234]
- Kandun IN, Wibisono H, Sedyaningsih ER, Yusharmen, Hadisoedarsuno W, Purba W, Santoso H, Septiawati C, Tresnaningsih E, Heriyanto B, Yuwono D, Harun S, Soeroso S, Giriputra S, Blair PJ, Jeremijenko A, Kosasih H, Putnam SD, Samaan G, Silitonga M, Chan KH, Poon LL, Lim W, Klimov A, Lindstrom S, Guan Y, Donis R, Katz J, Cox N, Peiris M, Uyeki TM. Three Indonesian clusters of H5N1 virus infection in 2005. *N Engl J Med*. 2006; 355(21):2186–94. [PubMed: 17124016]
- Kang SM, Yoo DG, Lipatov AS, Song JM, Davis CT, Quan FS, Chen LM, Donis RO, Compans RW. Induction of long-term protective immune responses by influenza H5N1 virus-like particles. *PLoS One*. 2009; 4(3):e4667. [PubMed: 19252744]
- Karron RA, Callahan K, Luke C, Thumar B, McAuliffe J, Schappell E, Joseph T, Coelingh K, Jin H, Kemble G, Murphy BR, Subbarao K. A live attenuated H9N2 influenza vaccine is well tolerated and immunogenic in healthy adults. *J Infect Dis*. 2009a; 199(5):711–6. [PubMed: 19210163]
- Karron RA, Talaat K, Luke C, Callahan K, Thumar B, Dilorenzo S, McAuliffe J, Schappell E, Suguitan A, Mills K, Chen G, Lamirande E, Coelingh K, Jin H, Murphy BR, Kemble G, Subbarao K. Evaluation of two live attenuated cold-adapted H5N1 influenza virus vaccines in healthy adults. *Vaccine*. 2009b; 27(36):4953–60. [PubMed: 19540952]
- Kawaoka Y, Webster RG. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. *Proc Natl Acad Sci U S A*. 1988; 85:324–328. [PubMed: 2829180]
- Kelly DF, Pollard AJ, Moxon ER. Immunological memory: the role of B cells in long-term protection against invasive bacterial pathogens. *JAMA*. 2005; 294(23):3019–23. [PubMed: 16414950]
- Kendal AP, Maassab HF, Alexandrova GI, Ghendon YZ. Development of cold-adapted recombinant live, attenuated influenza A vaccines in the U.S.A. and U.S.S.R. *Antiviral Res*. 1981; 1:339–65.
- Khurana S, Chearwae W, Castellino F, Manischewitz J, King LR, Honorkiewicz A, Rock MT, Edwards KM, Del Giudice G, Rappuoli R, Golding H. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. *Sci Transl Med*. 2010; 2(15):15ra5.
- Khurana S, Wu J, Verma N, Verma S, Raghunandan R, Manischewitz J, King LR, Kpamegan E, Pincus S, Smith G, Glenn G, Golding H. H5N1 virus-like particle vaccine elicits cross-reactive neutralizing antibodies that preferentially bind to the oligomeric form of influenza virus hemagglutinin in humans. *J Virol*. 2011; 85(21):10945–54. [PubMed: 21865396]
- Kim JH, Jacob J. DNA vaccines against influenza viruses. *Curr Top Microbiol Immunol*. 2009; 333:197–210. [PubMed: 19768407]
- Kost TA, Condreay JP, Jarvis DL. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol*. 2005; 23(5):567–75. [PubMed: 15877075]

- Laddy DJ, Yan J, Kutzler M, Kobasa D, Kobinger GP, Khan AS, Greenhouse J, Sardesai NY, Draghia-Akli R, Weiner DB. Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens. *PLoS One*. 2008; 3(6):e2517. [PubMed: 18575608]
- Lakey DL, Treanor JJ, Betts RF, Smith GE, Thompson J, Sannella E, Reed G, Wilkinson BE, Wright PF. Recombinant baculovirus influenza A hemagglutinin vaccines are well tolerated and immunogenic in healthy adults. *J Infect Dis*. 1996; 174(4):838–41. [PubMed: 8843225]
- Lalor PA, Webby RJ, Morrow J, Rusalov D, Kaslow DC, Rolland A, Smith LR. Plasmid DNA-based vaccines protect mice and ferrets against lethal challenge with A/Vietnam/1203/04 (H5N1) influenza virus. *J Infect Dis*. 2008; 197(12):1643–52. [PubMed: 18513153]
- Landry N, Ward BJ, Trepanier S, Montomoli E, Dargis M, Lapini G, Vezina LP. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PLoS One*. 2010; 5(12):e15559. [PubMed: 21203523]
- Langley JM, Risi G, Caldwell M, Gilderman L, Berwald B, Fogarty C, Poling T, Riff D, Baron M, Frenette L, Sheldon E, Collins H, Shepard M, Dionne M, Brune D, Ferguson L, Vaughn D, Li P, Fries L. Dose-sparing H5N1 A/Indonesia/05/2005 pre-pandemic influenza vaccine in adults and elderly adults: a phase III, placebo-controlled, randomized study. *J Infect Dis*. 2011; 203(12):1729–38. [PubMed: 21606531]
- Latham T, Galarza JM. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. *J Virol*. 2001; 75(13):6154–65. [PubMed: 11390617]
- Layton RC, Gigliotti A, Armijo P, Myers L, Knight J, Donart N, Pyles J, Vaughan S, Plourde J, Fomukong N, Harrod KS, Gao P, Koster F. Enhanced immunogenicity, mortality protection, and reduced viral brain invasion by alum adjuvant with an H5N1 split-virion vaccine in the ferret. *PLoS One*. 2011; 6(6):e20641. [PubMed: 21687736]
- Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y. Avian flu: isolation of drug-resistant H5N1 virus. *Nature*. 2005; 437(7062):1108. [PubMed: 16228009]
- Ledgerwood JE, Wei CJ, Hu Z, Gordon IJ, Enama ME, Hendel CS, McTamney PM, Pearce MB, Yassine HM, Boyington JC, Bailer R, Tumpey TM, Koup RA, Mascola JR, Nabel GJ, Graham BS. DNA priming and influenza vaccine immunogenicity: two phase 1 open label randomised clinical trials. *Lancet Infect Dis*. 2011; 11(12):916–24. [PubMed: 21975270]
- Leroux-Roels I, Bernhard R, Gerard P, Drame M, Hanon E, Leroux-Roels G. Broad Clade 2 cross-reactive immunity induced by an adjuvanted clade 1 rH5N1 pandemic influenza vaccine. *PLoS One*. 2008; 3(2):e1665. [PubMed: 18301743]
- Leroux-Roels I, Borkowski A, Vanwolleghem T, Drame M, Clement F, Hons E, Devaster JM, Leroux-Roels G. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. *Lancet*. 2007; 370(9587):580–9. [PubMed: 17707753]
- Levesque PM, Foster K, de Alwis U. Association between immunogenicity and adsorption of a recombinant *Streptococcus pneumoniae* vaccine antigen by an aluminum adjuvant. *Hum Vaccin*. 2006; 2(2):74–7. [PubMed: 17012880]
- Levie K, Leroux-Roels I, Hoppenbrouwers K, Kervyn AD, Vandermeulen C, Forgue S, Leroux-Roels G, Pichon S, Kusters I. An adjuvanted, low-dose, pandemic influenza A (H5N1) vaccine candidate is safe, immunogenic, and induces cross-reactive immune responses in healthy adults. *J Infect Dis*. 2008; 198(5):642–9. [PubMed: 18576945]
- Levine MM, Hughes TP, Simon P, O'Donnell S, Grauel S, Levine SG. Monovalent inactivated A/New Jersey/8/76 (Hsw1N1) vaccine in healthy children aged three to five years. *J Infect Dis*. 1977; 136(Suppl):S571–4. [PubMed: 342629]
- Lillie PJ, Berthoud TK, Powell TJ, Lambe T, Mullarkey C, Spencer AJ, Hamill M, Peng Y, Blais ME, Duncan CJ, Sheehy SH, Havelock T, Faust SN, Williams RL, Gilbert A, Oxford J, Dong T, Hill AV, Gilbert SC. Preliminary assessment of the efficacy of a T-cell-based influenza vaccine, MVA-NP+M1, in humans. *Clin Infect Dis*. 2012; 55(1):19–25. [PubMed: 22441650]
- Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, Gao Q, Zhang Z, Liu Y, Wang Z, Yang M, Sun R, Li C, Lin S, Ji M, Liu Y, Wang X, Wood J, Feng Z, Wang Y, Yin W. Safety and immunogenicity of

- an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. *Lancet*. 2006; 368(9540):991–7. [PubMed: 16980114]
- Lin JT, Li CG, Wang X, Su N, Liu Y, Qiu YZ, Yang M, Chen JT, Fang HH, Dong XP, Yin WD, Feng ZJ. Antibody persistence after 2-dose priming and booster response to a third dose of an inactivated, adjuvanted, whole-virion H5N1 vaccine. *J Infect Dis*. 2009; 199(2):184–7. [PubMed: 19067606]
- Ludwig C, Wagner R. Virus-like particles-universal molecular toolboxes. *Curr Opin Biotechnol*. 2007; 18(6):537–45. [PubMed: 18083549]
- Luke, CJ.; Subbarao, K. The role of animal models in influenza vaccine research.. In: Rappuoli, R.; Del Giudice, G., editors. *Influenza Vaccines for the Future*. 2nd ed.. Springer; Basel: 2011. p. 223-272.
- Maassab HF. Plaque formation of influenza virus at 25 degrees C. *Nature*. 1968; 219(5154):645–6. [PubMed: 5665725]
- Mahmood K, Bright RA, Mytle N, Carter DM, Crevar CJ, Achenbach JE, Heaton PM, Tumpey TM, Ross TM. H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses. *Vaccine*. 2008; 26(42):5393–9. [PubMed: 18706956]
- Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P, Chittaganpitch M, Waicharoen S, Nguyen DT, Nguyen TH, Nguyen HH, Kim JH, Hoang LT, Kang C, Phuong LS, Lim W, Zaki S, Donis RO, Cox NJ, Katz JM, Tumpey TM. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *J Virol*. 2005; 79:11788–11800. [PubMed: 16140756]
- McKinstry KK, Strutt TM, Kuang Y, Brown DM, Sell S, Dutton RW, Swain SL. Memory CD4+ T cells protect against influenza through multiple synergizing mechanisms. *J Clin Invest*. 2012; 122(8):2847–56. [PubMed: 22820287]
- McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *N Engl J Med*. 1983; 309(1):13–7. [PubMed: 6602294]
- Miller E, Andrews N, Stelitano L, Stowe J, Winstone AM, Shneerson J, Verity C. Risk of narcolepsy in children and young people receiving AS03 adjuvanted pandemic A/H1N1 2009 influenza vaccine: retrospective analysis. *BMJ*. 2013; 346:f794. [PubMed: 23444425]
- Morel S, Didierlaurent A, Bourguignon P, Delhay S, Baras B, Jacob V, Planty C, Elouahabi A, Harvengt P, Carlsen H, Kielland A, Chomez P, Garcon N, Van Mechelen M. Adjuvant System AS03 containing alpha-tocopherol modulates innate immune response and leads to improved adaptive immunity. *Vaccine*. 2011; 29(13):2461–73. [PubMed: 21256188]
- Morens DM, Subbarao K, Taubenberger JK. Engineering H5N1 avian influenza viruses to study human adaptation. *Nature*. 2012; 486(7403):335–40. [PubMed: 22722191]
- Moris P, van der Most R, Leroux-Roels I, Clement F, Drame M, Hanon E, Leroux-Roels GG, Van Mechelen M. H5N1 influenza vaccine formulated with AS03 A induces strong cross-reactive and polyfunctional CD4 T-cell responses. *J Clin Immunol*. 2011; 31(3):443–54. [PubMed: 21174144]
- Murphy BR, Coelingh K. Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. *Viral Immunol*. 2002; 15(2):295–323. [PubMed: 12081014]
- Nichol KL, Treanor JJ. Vaccines for seasonal and pandemic influenza. *J Infect Dis*. 2006; 194(Suppl 2):S111–8. [PubMed: 17163383]
- Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, Zambon MC. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet*. 2001; 357(9272):1937–43. [PubMed: 11425416]
- Nicholson KG, Tyrrell DA, Harrison P, Potter CW, Jennings R, Clark A, Schild GC, Wood JM, Yetts R, Seagroatt V, Huggins A, Anderson SG. Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. *J Biol Stand*. 1979; 7(2):123–36. [PubMed: 479199]
- Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal

challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. *Vaccine*. 2007; 25(18):3554–60. [PubMed: 17293015]

Nohynek H, Jokinen J, Partinen M, Vaarala O, Kirjavainen T, Sundman J, Himanen SL, Hublin C, Julkunen I, Olsen P, Saarenpaa-Heikkila O, Kilpi T. AS03 adjuvanted AH1N1 vaccine associated with an abrupt increase in the incidence of childhood narcolepsy in Finland. *PLoS One*. 2012; 7(3):e33536. [PubMed: 22470453]

Nolan T, Richmond PC, Formica NT, Hoschler K, Skeljo MV, Stoney T, McVernon J, Hartel G, Sawlwin DC, Bennet J, Ryan D, Bassier RL, Zambon MC. Safety and immunogenicity of a prototype adjuvanted inactivated split-virus influenza A (H5N1) vaccine in infants and children. *Vaccine*. 2008a; 26(50):6383–91. [PubMed: 18801398]

Nolan TM, Richmond PC, Skeljo MV, Pearce G, Hartel G, Formica NT, Hoschler K, Bennet J, Ryan D, Papanou K, Bassier RL, Zambon MC. Phase I and II randomised trials of the safety and immunogenicity of a prototype adjuvanted inactivated split-virus influenza A (H5N1) vaccine in healthy adults. *Vaccine*. 2008b; 26(33):4160–7. [PubMed: 18599164]

O'Hagan DT, Ott GS, De Gregorio E, Seubert A. The mechanism of action of MF59 - An innately attractive adjuvant formulation. *Vaccine*. 2012; 30(29):4341–8. [PubMed: 22682289]

OIE. Update on avian influenza in animals (type H5). 2008. http://www.oie.int/download/AVIANINFLUENZA/A_AI-Asia.htm

Palese P, Wang TT. H5N1 influenza viruses: facts, not fear. *Proc Natl Acad Sci U S A*. 2012; 109(7):2211–3. [PubMed: 22308474]

Park KS, Lee J, Ahn SS, Byun YH, Seong BL, Baek YH, Song MS, Choi YK, Na YJ, Hwang I, Sung YC, Lee CG. Mucosal immunity induced by adenovirus-based H5N1 HPAI vaccine confers protection against a lethal H5N2 avian influenza virus challenge. *Virology*. 2009; 395(2):182–9. [PubMed: 19836045]

Parks CL, Latham T, Cahill A, O'Neill R E, Passarotti CJ, Buonagurio DA, Bechert TM, D'Arco GA, Neumann G, Destefano J, Arendt HE, Obregon J, Shutyak L, Hamm S, Sidhu MS, Zamb TJ, Udem SA. Phenotypic properties resulting from directed gene segment reassortment between wild-type A/Sydney/5/97 influenza virus and the live attenuated vaccine strain. *Virology*. 2007; 367(2):275–87. [PubMed: 17599381]

Perrone LA, Ahmad A, Veguilla V, Lu X, Smith G, Katz JM, Pushko P, Tumpey TM. Intranasal vaccination with 1918 influenza virus-like particles protects mice and ferrets from lethal 1918 and H5N1 influenza virus challenge. *J Virol*. 2009; 83(11):5726–34. [PubMed: 19321609]

Podda A, Del Giudice G. MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. *Expert Rev Vaccines*. 2003; 2(2):197–203. [PubMed: 12899571]

Potter CW, Oxford JS. Determinants of immunity to influenza infection in man. *Br Med Bull*. 1979; 35(1):69–75. [PubMed: 367490]

Powers DC, McElhaney JE, Florendo OA Jr, Manning MC, Upshaw CM, Bentley DW, Wilkinson BE. Humoral and cellular immune responses following vaccination with purified recombinant hemagglutinin from influenza A (H3N2) virus. *J Infect Dis*. 1997; 175(2):342–51. [PubMed: 9203655]

Price GE, Soboleski MR, Lo CY, Misplon JA, Pappas C, Houser KV, Tumpey TM, Epstein SL. Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. *Vaccine*. 2009; 27(47):6512–21. [PubMed: 19729082]

Pushko P, Tumpey TM, Van Hoven N, Belser JA, Robinson R, Nathan M, Smith G, Wright DC, Bright RA. Evaluation of influenza virus-like particles and Novasome adjuvant as candidate vaccine for avian influenza. *Vaccine*. 2007; 25(21):4283–90. [PubMed: 17403562]

Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, Khanyok R, Thawachsupha P, Kijphati R, Sawanpanyalert P. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. *J Gen Virol*. 2005; 86(Pt 2):423–33. [PubMed: 15659762]

Roos, R. Insect-cell-based flu vaccine looks good in clinical trial. 2007. <http://www.cidrap.umn.edu/cidrap/content/influenza/panflu/news/apr1207cell.html>

- Ruat C, Caillet C, Bidaut A, Simon J, Osterhaus AD. Vaccination of macaques with adjuvanted formalin-inactivated influenza A virus (H5N1) vaccines: protection against H5N1 challenge without disease enhancement. *J Virol*. 2008; 82(5):2565–9. [PubMed: 18094159]
- Rudenko L, Desheva J, Korovkin S, Mironov A, Rekestin A, Grigorieva E, Donina S, Gambaryan A, Katlinsky A. Safety and immunogenicity of live attenuated influenza reassortant H5 vaccine (phase I-II clinical trials). *Influenza Other Respi Viruses*. 2008; 2(6):203–9. [PubMed: 19453396]
- Rumke HC, Bayas JM, de Juanes JR, Caso C, Richardus JH, Campins M, Rombo L, Duval X, Romanenko V, Schwarz TF, Fassakhov R, Abad-Santos F, von Sonnenburg F, Drame M, Sanger R, Ballou WR. Safety and reactogenicity profile of an adjuvanted H5N1 pandemic candidate vaccine in adults within a phase III safety trial. *Vaccine*. 2008; 26(19):2378–88. [PubMed: 18407382]
- SAGE. Use of licensed H5N1 influenza vaccines in the interpandemic period. Report of the H5N1 SAGE Working Group to the April 2009 meeting of the Strategic Advisory Group of Experts. 2009. http://www.who.int/immunization/sage/SAGE_H5N1_26Mayb.pdf
- Salk JE. Reactions to concentrated influenza virus vaccines. *J Immunol*. 1948; 58(4):369–95. [PubMed: 18864082]
- Schotsaert M, De Filette M, Fiers W, Saelens X. Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. *Expert Rev Vaccines*. 2009; 8(4):499–508. [PubMed: 19348565]
- Schultze V, D'Agosto V, Wack A, Novicki D, Zorn J, Hennig R. Safety of MF59 adjuvant. *Vaccine*. 2008; 26(26):3209–22. [PubMed: 18462843]
- Shoji Y, Chichester JA, Bi H, Musiyuchuk K, de la Rosa P, Goldschmidt L, Horsey A, Ugulava N, Palmer GA, Mett V, Yusibov V. Plant-expressed HA as a seasonal influenza vaccine candidate. *Vaccine*. 2008; 26(23):2930–4. [PubMed: 18440103]
- Slepushkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. *Vaccine*. 1995; 13(15):1399–402. [PubMed: 8578816]
- Smith LR, Wloch MK, Ye M, Reyes LR, Boutsaboualoy S, Dunne CE, Chaplin JA, Rusalov D, Rolland AP, Fisher CL, Al-Ibrahim MS, Kabongo ML, Steigbigel R, Belshe RB, Kitt ER, Chu AH, Moss RB. Phase 1 clinical trials of the safety and immunogenicity of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin. *Vaccine*. 2010; 28(13):2565–72. [PubMed: 20117262]
- Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood JM, Zambon MC, Katz JM. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. *J Infect Dis*. 2005; 191(8):1210–5. [PubMed: 15776364]
- Stephenson I, Heath A, Major D, Newman RW, Hoschler K, Junzi W, Katz JM, Weir JP, Zambon MC, Wood JM. Reproducibility of serologic assays for influenza virus A (H5N1). *Emerg Infect Dis*. 2009; 15(8):1252–9. [PubMed: 19751587]
- Stephenson I, Nicholson KG, Colegate A, Podda A, Wood J, Ypma E, Zambon M. Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine*. 2003; 21(15):1687–93. [PubMed: 12639491]
- Stephenson I, Nicholson KG, Hoschler K, Zambon MC, Hancock K, DeVos J, Katz JM, Praus M, Banzhoff A. Antigenically distinct MF59-adjuvanted vaccine to boost immunity to H5N1. *N Engl J Med*. 2008; 359(15):1631–3. [PubMed: 18843132]
- Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, Xu X, Lu X, Katz J, Cox N, Matsuoka Y. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology*. 2003; 305(1):192–200. [PubMed: 12504552]
- Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science*. 1998; 279(5349):393–6. [PubMed: 9430591]

- Suguitan AL Jr, Marino MP, Desai PD, Chen LM, Matsuoka Y, Donis RO, Jin H, Swayne DE, Kemble G, Subbarao K. The influence of the multi-basic cleavage site of the H5 hemagglutinin on the attenuation, immunogenicity and efficacy of a live attenuated influenza A H5N1 cold-adapted vaccine virus. *Virology*. 2009; 395(2):280–8. [PubMed: 19833372]
- Suguitan AL Jr, Matsuoka Y, Lau YF, Santos CP, Vogel L, Cheng LI, Orandle M, Subbarao K. The multibasic cleavage site of the hemagglutinin of highly pathogenic A/Vietnam/1203/2004 (H5N1) avian influenza virus acts as a virulence factor in a host-specific manner in mammals. *J Virol*. 2012; 86(5):2706–14. [PubMed: 22205751]
- Suguitan AL Jr, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, Luke CJ, Murphy B, Swayne DE, Kemble G, Subbarao K. Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med*. 2006; 3(9):e360. [PubMed: 16968127]
- Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol*. 2009; 16(3):265–73. [PubMed: 19234466]
- Sullivan SM, Doukas J, Hartikka J, Smith L, Rolland A. Vaxfectin: a versatile adjuvant for plasmid DNA- and protein-based vaccines. *Expert Opin Drug Deliv*. 2010; 7(12):1433–46. [PubMed: 21118032]
- Takahashi Y. Memory B cells in systemic and mucosal immune response: implications for successful vaccination. *Biosci Biotechnol Biochem*. 2007; 71(10):2358–66. [PubMed: 17928717]
- Talaat KR, Karron RA, Liang PH, McMahon BA, Luke CJ, Thumar B, Chen GL, Min JY, Lamirande EW, Jin H, Coelingh KL, Kemble GW, Subbarao K. An open-label phase I trial of a live attenuated H2N2 influenza virus vaccine in healthy adults. *Influenza Other Respi Viruses*. 2012
- Talaat KR, Karron RA, Luke CJ, Thumar B, McMahon BA, Chen GL, Lamirande EW, Jin H, Coelingh KL, Kemble G, Subbarao K. An open label Phase I trial of a live attenuated H6N1 influenza virus vaccine in healthy adults. *Vaccine*. 2011; 29(17):3144–8. [PubMed: 21377509]
- Taylor PM, Askonas BA. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. *Immunology*. 1986; 58(3):417–20. [PubMed: 2426185]
- Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. *Emerg Infect Dis*. 2006; 12(1):48–54. [PubMed: 16494717]
- Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L, Bakker A, Cox F, van Deventer E, Guan Y, Cinatl J, ter Meulen J, Lasters I, Carsetti R, Peiris M, de Kruijff J, Goudsmit J. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS One*. 2008; 3(12):e3942. [PubMed: 19079604]
- Tinsley, T.; Harrap, KA. Viruses of invertebrates. In: Fraenkel-Conrat, H.; Wagner, RR., editors. *Comprehensive Virology*. Vol. 12. Ienum Press; New York: 1978. p. 1-101.
- Treanor JJ, Betts RF, Smith GE, Anderson EL, Hackett CS, Wilkinson BE, Belshe RB, Powers DC. Evaluation of a recombinant hemagglutinin expressed in insect cells as an influenza vaccine in young and elderly adults. *J Infect Dis*. 1996; 173(6):1467–70. [PubMed: 8648221]
- Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med*. 2006; 354(13):1343–51. [PubMed: 16571878]
- Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D, Wolff M, Rabinovich G, Blackwelder W, Katz JM. Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. *Vaccine*. 2001; 19(13-14):1732–7. [PubMed: 11166898]
- Tutykhina IL, Logunov DY, Shcherbinin DN, Shmarov MM, Tukhvatulin AI, Naroditsky BS, Gintsburg AL. Development of adenoviral vector-based mucosal vaccine against influenza. *J Mol Med (Berl)*. 2011; 89(4):331–41. [PubMed: 21104066]
- UKDH. UK Department of Health. Pandemic flu: a national framework for responding to an influenza pandemic. 2011. See http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_080734
- Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, Uiprasertkul M, Boonnak K, Pittayawonganon C, Cox NJ, Zaki SR, Thawatsupha P, Chittaganpitch M, Khontong

- R, Simmerman JM, Chunsutthiwat S. Probable person-to-person transmission of avian influenza A (H5N1). *N Engl J Med*. 2005; 352(4):333–40. [PubMed: 15668219]
- Vajo Z, Kosa L, Szilvasy I, Pauliny Z, Bartha K, Visontay I, Kis A, Tarjan I, Rozsa N, Jankovics I. Safety and immunogenicity of a prepandemic influenza A (H5N1) vaccine in children. *Pediatr Infect Dis J*. 2008; 27(12):1052–6. [PubMed: 18978514]
- Vajo Z, Kosa L, Visontay I, Jankovics M, Jankovics I. Inactivated whole virus influenza A (H5N1) vaccine. *Emerg Infect Dis*. 2007; 13(5):807–8. [PubMed: 18044056]
- Vajo Z, Wood J, Kosa L, Szilvasy I, Paragh G, Pauliny Z, Bartha K, Visontay I, Kis A, Jankovics I. A single-dose influenza A (H5N1) vaccine safe and immunogenic in adult and elderly patients: an approach to pandemic vaccine development. *J Virol*. 2010; 84(3):1237–42. [PubMed: 19906909]
- Vesikari T, Karvonen A, Tilman S, Borkowski A, Montomoli E, Banzhoff A, Clemens R. Immunogenicity and safety of MF59-adjuvanted H5N1 influenza vaccine from infancy to adolescence. *Pediatrics*. 2010; 126(4):e762–70. [PubMed: 20819892]
- Wan XF, Nguyen T, Davis CT, Smith CB, Zhao ZM, Carrel M, Inui K, Do HT, Mai DT, Jadhao S, Balish A, Shu B, Luo F, Emch M, Matsuoka Y, Lindstrom SE, Cox NJ, Nguyen CV, Klimov A, Donis RO. Evolution of highly pathogenic H5N1 avian influenza viruses in Vietnam between 2001 and 2007. *PLoS One*. 2008; 3(10):e3462. [PubMed: 18941631]
- Wang H, Feng Z, Shu Y, Yu H, Zhou L, Zu R, Huai Y, Dong J, Bao C, Wen L, Wang H, Yang P, Zhao W, Dong L, Zhou M, Liao Q, Yang H, Wang M, Lu X, Shi Z, Wang W, Gu L, Zhu F, Li Q, Yin W, Yang W, Li D, Uyeki TM, Wang Y. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. *Lancet*. 2008; 371(9622):1427–34. [PubMed: 18400288]
- Wang TT, Tan GS, Hai R, Pica N, Petersen E, Moran TM, Palese P. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog*. 2010; 6(2):e1000796. [PubMed: 20195520]
- Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, McClain-Moss LR, Peiris JS, Rehg JE, Tuomanen EI, Webster RG. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet*. 2004; 363(9415):1099–103. [PubMed: 15064027]
- WHO. WHO case definitions for human infections with influenza A(H5N1) virus. 2006. http://www.who.int/influenza/resources/documents/case_definition2006_08_29/en/index.html
- WHO. Human cases of avian influenza A (H5N1) in North-West Frontier Province, Pakistan, October–November 2007. *Wkly Epidemiol Rec*. 2008; 83(40):359–64. [PubMed: 18833663]
- WHO. Antigenic and genetic characteristics of influenza A(H5N1) viruses and candidate vaccine viruses developed for potential use in human vaccines. *Wkly Epidemiol Rec*. 2009; 84(41):432–6. [PubMed: 19817020]
- WHO. World Health Organization. Pandemic influenza preparedness framework for the sharing of influenza viruses and access to vaccines and other benefits. 2011. http://whqlibdoc.who.int/publications/2011/9789241503082_eng.pdf
- WHO. Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness. *Wkly Epidemiol Rec*. 2012a; 87(11):97–108. [PubMed: 22462204]
- WHO. Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO. WHO; Geneva: 2012b. Available from URL: http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html [2012 June 7]
- Wijnans L, Lecomte C, de Vries C, Weibel D, Sammon C, Hviid A, Svanstrom H, Molgaard-Nielsen D, Heijbel H, Dahlstrom LA, Hallgren J, Sparen P, Jennum P, Mosseveld M, Schuemie M, van der Maas N, Partinen M, Romio S, Trotta F, Santuccio C, Menna A, Plazzi G, Moghadam KK, Ferro S, Lammers GJ, Overeem S, Johansen K, Kramarz P, Bonhoeffer J, Sturkenboom MC. The incidence of narcolepsy in Europe: before, during, and after the influenza A(H1N1)pdm09 pandemic and vaccination campaigns. *Vaccine*. 2013; 31(8):1246–54. [PubMed: 23246544]
- Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, Lambkin-Williams R, Gilbert A, Oxford J, Nicholas B, Staples KJ, Dong T, Douek DC, McMichael AJ, Xu XN. Preexisting

- influenza-specific CD4⁺ T cells correlate with disease protection against influenza challenge in humans. *Nat Med*. 2012; 18(2):274–80. [PubMed: 22286307]
- Wright PF, Thompson J, Vaughn WK, Folland DS, Sell SH, Karzon DT. Trials of influenza A/New Jersey/76 virus vaccine in normal children: an overview of age-related antigenicity and reactogenicity. *J Infect Dis*. 1977; 136(Suppl):S731–41. [PubMed: 606798]
- Wu J, Fang HH, Chen JT, Zhou JC, Feng ZJ, Li CG, Qiu YZ, Liu Y, Lu M, Liu LY, Dong SS, Gao Q, Zhang XM, Wang N, Yin WD, Dong XP. Immunogenicity, safety, and cross-reactivity of an inactivated, adjuvanted, prototype pandemic influenza (H5N1) vaccine: a phase II, double-blind, randomized trial. *Clin Infect Dis*. 2009; 48(8):1087–95. [PubMed: 19281330]
- Wu J, Liu SZ, Dong SS, Dong XP, Zhang WL, Lu M, Li CG, Zhou JC, Fang HH, Liu Y, Liu LY, Qiu YZ, Gao Q, Zhang XM, Chen JT, Zhong X, Yin WD, Feng ZJ. Safety and immunogenicity of adjuvanted inactivated split-virion and whole-virion influenza A (H5N1) vaccines in children: a phase I-II randomized trial. *Vaccine*. 2010; 28(38):6221–7. [PubMed: 20638454]
- Xu Q, Wang W, Cheng X, Zengel J, Jin H. Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. *J Virol*. 2010; 84(10):4936–45. [PubMed: 20200248]
- Yang PC, Yu CJ, Chang SC, Hsieh SM, Drame M, Walravens K, Roman F, Gillard P. Safety and immunogenicity of a split-virion AS03A-adjuvanted A/Indonesia/05/2005 (H5N1) vaccine in Taiwanese adults. *J Formos Med Assoc*. 2012; 111(6):333–9. [PubMed: 22748624]
- Yap KL, Ada GL, McKenzie IF. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature*. 1978; 273(5659):238–9. [PubMed: 306072]
- Yoshida R, Igarashi M, Ozaki H, Kishida N, Tomabechi D, Kida H, Ito K, Takada A. Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. *PLoS Pathog*. 2009; 5(3):e1000350. [PubMed: 19300497]
- Zangwill KM, Treanor JJ, Campbell JD, Noah DL, Ryea J. Evaluation of the safety and immunogenicity of a booster (third) dose of inactivated subvirion H5N1 influenza vaccine in humans. *J Infect Dis*. 2008; 197(4):580–3. [PubMed: 18237269]
- Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. *J Virol*. 2002; 76:4420–4429. [PubMed: 11932409]

Highlights

- 2 doses of unadjuvanted vaccine are necessary to elicit robust antibody titers.
- Oil-in-water adjuvants permit antigen sparing.
- Antibody titers decline rapidly but can be boosted with additional doses of vaccine.
- High titers of antibody are associated with cross-reactivity against other clades.
- Prime-boost strategies elicit a robust immune response.

Figure 1.

Multi-cycle replication of recombinant wt and seasonal influenza/pandemic vaccine virus reassortants in MDCK cells. MDCK cell monolayers were inoculated with each virus at an m.o.i. of 0.001. Aliquots of supernatant were removed from duplicate wells at the timepoints shown, and virus titers were determined in MDCK cells.

A

H5 groups nasal turbinates

B

H5 groups lungs

C

H9 groups nasal turbinates

D

H9 groups lungs

Figure 2.

Level of replication of recombinant wt and seasonal influenza/pLAIV virus reassortants in the upper respiratory tract (panels A and C) and lower respiratory tract (panels B and D) of mice, on days 2, 3 and 4 post-infection with 10^5TCID_{50} of each virus.

Figure 3. Level of replication of recombinant wt and seasonal influenza/pLAIV virus reassortants in the upper and lower respiratory tract of ferrets on day 3 post-infection with 10^6 TCID₅₀ of each virus.

H&E

IHC

 Δ VN/Sol Δ VN/Cal Δ H5N1 ca

H&E

IHC

H5N1 VN
2004 wt

Mock

Figure 4.
H&E staining and IHC of lung sections from mice inoculated with the indicated viruses bearing the H5 HA.

Table 1

Criteria applied by the CHMP and FDA to assess the antibody response to pandemic influenza vaccines[#].

Parameter	Adults 18–60 years	Adults ≥ 60 years
Seroconversion rate	40%	30%
Seroprotection rate	70%	60%
Geometric mean ratio	2.5	2.0

The FDA also requires:

For adults < 65 years of age and for children:

-The lower bound of the two-sided 95% confidence intervals (CI) for the percent of subjects achieving seroconversion for HAI antibody should meet or exceed 40%.

-The lower bound of the two-sided 95% CI for the percent of subjects achieving an HAI antibody titer ≥ 1:40 should meet or exceed 70%.

For adults ≥ 65 years of age and for children:

-The lower bound of the two-sided 95% confidence intervals (CI) for the percent of subjects achieving seroconversion for HAI antibody should meet or exceed 30%.

- The lower bound of the two-sided 95% CI for the percent of subjects achieving an HAI antibody titer ≥ 1:40 should meet or exceed 60%.

[#]For pre-pandemic vaccines, it is expected that all three criteria will be met.

Table 2

Selected clinical trials of inactivated split-virus and subunit influenza H5N1 vaccines

Serum antibody response after second dose unless indicated													
HAI										MN			
Virus	Clade	Age of subjects	Number of subjects	Dose of HA (µg) ^a	Adjuvant	% with titers 1:40 (95% CI)	% seroconversion ^b (95% CI)	GMT (95% CI)	% with titers 1:40 (95% CI)	% seroconversion (95% CI)	GMT (95% CI)	Cross-reactivity ^c	Reference
A/Vietnam/1203/2004	1	18-64 y	102	90	None	N/A	57(46-67)	56.3(41.2-76.8)	N/A	53(42-63)	45.9(36.0-58.5)	N/A	Treanor et al. 2006
A/Vietnam/1203/2004	1	18-64 y	85	90 (3 doses)	None	N/A	64(52-74)	69.8(49.2-99.1)	N/A	78(67-86)	96.0(74.2-124.2)	N/A	Zangwill et al. 2008
A/Vietnam/1194/2004	1	18-40 y	50	30	Alum	67(52-79)	66(51-79)	11.6(6.7-20.0)	N/A	41(28-56)	32.6(24.9-42.8)	N/A	Bresson et al. 2006
A/Vietnam/1194/2004	1	18-64 y	200	30	Alum	N/A	59(52-66)	29.4(23.7-36.5)	N/A	54(47-61)	43.3(37.3-50.3)	clade 2.2	Nolan et al. 2008b
A/Vietnam/1194/2004	1	6 m to 9 y	66	30	Alum	N/A	99(92-100)	432.8(337-556)	N/A	95(87-99)	396.9(282-560)	clade 2.1	Nolan et al. 2008a
A/duck/Singapore/1997	0	18-40 y	10	7.5	MF-59	N/A	100	4.12(0.16) ^d	N/A	N/A	N/A	N/A	Nicholson et al. 2001
A/duck/Singapore/1997	0	18-40 y	6	7.5 ^e	MF-59	67(N/A)	67	N/A	100	100	N/A	A/Hong Kong/213/200	Stephenson et al., 2003
A/Vietnam/1194/2004	1	6-36 m	134	7.5	MF-59	97(92-99)	97(92-99)	658(560-773)	99(96-100)	94(88-97)	519(454-594)	N/A	Vesikari et al. 2010
A/Vietnam/1194/2004	1	3-9 y	91	7.5	MF-59	97(91-99)	97(91-99)	585(484-708)	99(94-100)	92(85-97)	456(377-551)	N/A	Vesikari et al. 2010
A/Vietnam/1194/2004	1	9-18 y	89	7.5	MF-59	89(80-94)	89(80-94)	344(261-453)	99(94-100)	72(62-81)	313(267-367)	N/A	Vesikari et al. 2010
A/Vietnam/1194/2004	1	18-60 y	151	7.5	MF-59	73(65-80)	73(65-80)	82(62-110)	85(78-90)	N/A	117(98-140)	clade 2.2	Banzhoff et al. 2009
A/Vietnam/1194/2004	1	>60 y	81	7.5	MF-59	75(64-84)	67(55-77)	77(53-114)	79(68-87)	N/A	80(61-105)	clade 2.2	
A/Vietnam/1194/2004	1	18-60 y	51	3.8	AS03	82(68.6-91.4)	84(70.9-92.8)	27.9(17.2-45.2)	N/A	86(72.8-94.1)	314.7(243.1-407.3)	clade 2.1	Leroux-Roels et al. 2007
												clade 2.2	Leroux-Roels et al. 2008

Serum antibody response after second dose unless indicated													
Virus	Clade	Age of subjects	Number of subjects	Dose of HA (µg) ^a	Adjuvant	HAI			MN			Cross-reactivity ^c	Reference
						% with titers 1:40 (95% CI)	% seroconversion ^b (95% CI)	GMT (95% CI)	% with titers 1:40 (95% CI)	% seroconversion (95% CI)	GMT (95% CI)		
A/Vietnam/1194/2004	1	18-40 y	50	1.9	AS03	72(57.5-83.8)	72(57.5-83.8)	44(34.2-56.7)	N/A	92(80.8-97.8)	67(49.4-90.9)	clade 2.1	Levie et al. 2008
A/Vietnam/1194/2004	1	3-5 y	46	1.9	AS03	N/A	N/A	N/A	N/A	95.6(84.9-99.5)	1044.4(845.4-1290.3)	clade 2.1	Diez-Domingo et al. 2010
A/Vietnam/1194/2004	1	6-9 y	43	1.9	AS03	N/A	N/A	N/A	N/A	100(91.6-100)	1155.1(920.9-1448.7)	clade 2.1	Diez-Domingo et al. 2010
A/Indonesia/5/2005	2.1	18-60 y	160	3.5	AS03	100(97.7-100)	100(97.7-100)	739.5(667.8-818.8)	N/A	N/A	N/A	clade 1	Yang et al., 2012

N/A = not available. GMT = geometric mean titer. CI = confidence interval.

Clade 1: A/Vietnam/1194/2004; clade 2.1: A/Indonesia/5/2005; clade 2.2: A/Turkey/1/2005; clade 2.3: A/Anhui/1/2005

^aThe dose of HA shown elicited the highest HAI titers among the doses evaluated in the clinical trial.

^bSeroconversion rate is defined as the percentage of subjects or sera with at least a four-fold increase in HAI titer following vaccination or a post-vaccination titer of at least 1:40 if HAI antibodies were undetectable in pre-vaccination sera

^cClade 1: A/Vietnam/1194/2004; clade 2.1: A/Indonesia/5/2005; clade 2.2: A/Turkey/1/2005; clade 2.3: A/Anhui/1/2005

^dMean (standard deviation) increase in GMT.

^eThese subjects received 2 doses of 7.5 µg of HA (with MF-59) and a booster dose 16 months later.

Selected clinical trials of inactivated whole virus influenza H5N1 vaccines

Table 3

Serum antibody response after second dose unless indicated										
Virus	Clade	Age of subjects	Number of subjects	Dose of HA (µg) ^a	Adjuvant	HAI			MN	
						% with titers 1:40 (95% CI)	% seroconversion ^{b,d} (95% CI)	GMT (95% CI)	% with titers 1:40 (95% CI)	% seroconversion (95% CI)
A/Vietnam/1194/2004	1	18-60 y	23	10	Alum	78(56-92)	78(56-92)	57.4(36.9-89.4)	N/A	N/A
A/Vietnam/1194/2004	1	18-60 y	99	10	Alum	73.7(63.8-82.0)	73.7(63.8-82.0)	53.7(44.3-65.1)	93.9(86.8-97.9)	85.9(77.1-92)
A/Vietnam/1194/2004	1	9-17 y	12	6	Alum	75(N/A)	75(N/A)	16.95 ^c	N/A	66.7(N/A)
A/Vietnam/1194/2004	1	18-60 y	60	6 ^e	Alum	71.7(58.6-82.6)	37.7(25.6-51)	42.2(33-54.1)	N/A	42.7(30-55.9)
A/Vietnam/1194/2004	1	>60 y	54	6 ^e	Alum	64.8(50.6-77.3)	64.8(50.6-77.3)	29.6(20.3-43.2)	N/A	64.8(50.6-77.3)
A/Vietnam/1194/2004	1	19-60 y	44	6 ^e	Alum	79.5(N/A)	90.9(N/A)	18.73	N/A	54.5(N/A)
A/Vietnam/1194/2004	1	>60 y	44	6 ^e	Alum	61.4(N/A)	68.2(N/A)	10.96	N/A	47.7(N/A)
A/Vietnam/1194/2004	1	18-45 y	45	7.5	None	38.5(23.4-55.4)	35.9(21.2-52.8)	3.6(1.9-6.8)	76.2(60.5-87.9)	51.3(34.8-67.6)

N/A = not available.

GMT = geometric mean titer.

CI = confidence interval.

^aThe dose of HA shown is that which elicited the highest HAI titers among the doses evaluated in the clinical trial.

^bSeroconversion rate is defined as the percentage of subjects or sera with at least a four-fold increase in HAI titer following vaccination or a post-vaccination titer of at least 1:40 if HAI antibodies were undetectable in pre-vaccination sera.

^cClade 2.1: A/Indonesia/5/2005; clade 2.3: A/Anhui/1/2005

^dPost-vaccination:pre-vaccination GMT ratio.

^cThese subjects received 1 dose of 6 µg of HA (with Alum).

Table 4

Viruses used in the generation and evaluation of seasonal influenza:pLAIV reassortants.

Virus	Abbreviation	Source of HA and NA genes	Source of internal protein genes
Biological wt avian influenza viruses:			
A/VietNam/1203/2004 (H5N1)	H5N1 VN 04 wt	N/A ^a	N/A
A/chicken/G9/Hong Kong/1997 (H9N2)	H9N2 G9 97 wt	N/A	N/A
Recombinant wt seasonal influenza viruses:			
A/Solomon Islands/3/2006 (H1N1)	rSol	A/Solomon Islands/3/2006 (H1N1)	A/Solomon Islands/3/2006 (H1N1)
A/California/7/2004 (H3N2)	rCal	A/California/7/2004 (H3N2)	A/California/7/2004 (H3N2). PB1 and PA genes from A/New York/55/2004 (H3N2)
Pandemic live attenuated influenza vaccine (pLAIV) viruses:			
H5N1 VN 04/AA <i>ca</i>	H5N1 <i>ca</i> ^b	H5N1 VN 04 wt	A/Ann Arbor/6/60 <i>ca</i> MDV ^c
H9N2 G9 97/AA <i>ca</i>	H9N2 <i>ca</i>	H9N2 G9 97 wt	A/Ann Arbor/6/60 <i>ca</i> MDV
Reassortants between recombinant wt seasonal influenza viruses and pLAIV viruses:			
H5N1 VN 04/AA <i>ca</i> × A/Solomon Islands/3/2006 (H1N1)	VN/Sol	H5N1 <i>ca</i> ^b	A/Solomon Islands/3/2006 (H1N1)
H5N1 VN 04/AA <i>ca</i> × A/California/7/2004 (H3N2)	VN/Cal	H5N1 <i>ca</i> ^b	A/California/7/2004 (H3N2)
H9N2 G9 97/AA <i>ca</i> × A/Solomon Islands/3/2006 (H1N1)	G9/Sol	H9N2 <i>ca</i>	A/Solomon Islands/3/2006 (H1N1)
H9N2 G9 97/AA <i>ca</i> × A/California/7/2004 (H3N2)	G9/Cal	H9N2 <i>ca</i>	A/California/7/2004 (H3N2). PB1 and PA genes from A/New York/55/2004 (H3N2)

^a N/A=not applicable.^b HA modified to remove the multi-basic cleavage motif.^c MDV=Master Donor Virus

Table 5

Replication of seasonal influenza:pLAIV reassortant viruses in the lower respiratory tract and associated lung pathology in mice and ferrets on day 3 post-inoculation.

Inoculum	Mice		Ferrets	
	Mean lung virus titer (log ₁₀ TCID ₅₀ /g) ^a	Mean inflammation severity score ^b	Mean lung virus titer (log ₁₀ EID ₅₀ /g) ^c	Mean inflammation severity score
H5N1 VN 04 wt	8.0	4	ND ^d	ND
H9N2 G9 97 wt	5.5	0.5	ND	ND
rSol	5.1	0	1.5	3
rCal	1.5	0	1.5	2
H5N1 <i>ca</i>	6.1	2.5	1.5	0
H9N2 <i>ca</i>	1.5	0	1.5	0
VN/Sol	6.0	4	1.5	2
VN/Cal	5.6	2.5	1.9	1
G9/Sol	6.3	1.5	2.1	0
G9/Cal	5.3	1	2.9	0
Mock	1.5	0	1.5	0

^aLower limit of detection = 1.5 log₁₀TCID₅₀/g.

^bLung inflammation severity scores were as follows: 0 = within normal limits (WNL) to mild inflammation; 1 = mild inflammation; 2 = moderate inflammation; 3 = marked inflammation; 4 = severe inflammation.

^cVirus titers in ferret lung samples were determined in eggs.

^dND = not done.