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Live, attenuated influenza virus (LAIV) vehicles are strong inducers of immunity toward influenza B virus

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Abstract

Historically, vaccines developed toward influenza viruses of the B type using methodologies developed for influenza A viruses as a blueprint have not been equally efficacious or effective. Because most influenza research and public attention concerns influenza A viruses, these shortcomings have not been adequately addressed. In this manuscript, we utilized different influenza vaccine vehicles to compare immunogenicity and protection in mice and ferrets after vaccination against an influenza B virus. We report that plasmid DNA vaccines demonstrate low immunogenicity profiles and poor protection compared to either whole, inactivated influenza virus (IIV) or, live, attenuated influenza virus (LAIV) vaccines. When mixed prime:boost regimens using LAIV and IIV were studied, we observed a boosting effect in mice after priming with LAIV that was not seen when IIV was used as the prime. In ferrets LAIV induced high antibody titers after a single dose and provided a boost in IIV-primed animals. Regimens including LAIV as a prime demonstrated enhanced protection, and adjuvantation was required for efficacy using the IIV preparation. Our results differ from generally accepted influenza A virus vaccine models, and argue that strategies for control of influenza B virus should be considered separately from those for influenza A virus.

Keywords

Influenza; DNA vaccine; Hemagglutinin

1. Introduction

Approximately 36,000 deaths and 200,000 hospitalizations are attributed to influenza illness in the US on an annual basis [1;2]. Vaccination represents the most promising method for controlling influenza, and vaccines against this virus have a history of moderate efficacy [3-5]. The major factor affecting influenza vaccine efficacy and effectiveness is the ability to accurately predict representative isolates of the circulating subtypes for inclusion in annual vaccines [3-7]. Incorrect predictions can result in vaccines that have significantly reduced effectiveness, a problematic situation that most recently occurred during the 2007-08 influenza season [8;9].

Seasons where influenza B viruses represent the dominant circulating strain are infrequent, and the resulting excess mortality attributed to influenza B has typically been less than that due to influenza A viruses, specifically those of the H2N2 or H3N2 subtypes [10-12]. In addition, influenza B viruses exhibit a limited host range [13], thus precluding development

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of pandemic-type disease. These factors combined have led to less interest in influenza B viruses than in influenza A viruses, and less study has been directed toward this type. Nevertheless, the clinical symptoms of seasonal influenza A and B infections are indistinguishable [14-16], and significant morbidity and mortality [1;2] as well as other complications [17-19], are recorded during influenza B virus epidemics.

Current influenza vaccine strategies were designed with influenza A viruses as model antigens [20-22], and it has been assumed that what works for influenza A will also work for influenza B [23]. However, the epidemiology and evolution of influenza A and B viruses differ in several key areas, calling this implied assumption into question. The HA of influenza B viruses has evolved differently and more gradually than that of influenza A viruses circulating in humans, and the resulting slower rate of antigenic drift has led to less frequent seasonal changes in antigenicity [24-27]. In addition, multiple strains from different lineages of influenza B virus are known to co-circulate, often with different temporal and geographic patterns [28;29]. In the last decade, strains expressing HAs from lineages II and III (generally represented by strains B/Yamagata/16/88 and B/Victoria/2/87, respectively) have co-circulated in many parts of the world [28], making prediction of the best strain for inclusion in the vaccine difficult for any particular region or season. This was problematic in 2007-2008, when 98% of circulating influenza B virus strains were from lineage II, while the vaccine recommended for the Northern hemisphere was derived from a lineage III virus [8]. Further complicating matters, inactivated and live attenuated influenza virus (IIV and LAIV, respectively) vaccines in current use show consistently reduced immunogenicity for influenza B virus, compared to influenza A virus [30-32].

Based on these differences in evolution and vaccine effectiveness between influenza A and B viruses, we hypothesized that the most effective vaccines for influenza A viruses are not necessarily the ideal vaccines for influenza B viruses. We therefore decided to test multiple routes and vehicles for delivery of influenza B virus HA to directly compare regimens for eliciting immunity against this type of influenza. We compared vaccine vehicles that would induce antibodies exclusively toward the HA component of influenza (DNA) to whole virus preparations that incorporate additional viral proteins (IIV and LAIV). Furthermore, we included alum as an adjuvant for relevant groups in order to address immunogenicity in the presence of this adjuvant, which is the only adjuvant currently approved for use by the FDA [33]. Our data demonstrate that differences exist between these vehicles in relevant animal models of influenza infection. These data are discussed in the context of rational design for vaccines against influenza B virus.

2. Materials and methods

2.1. Animals

Adult (6-8-week-old) female BALB/cJ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in groups of four to five mice as described previously [34]. Young adult ferrets were obtained from the St Jude Children's Research Hospital breeding program. All animal experiments were performed following guidelines established by the Animal Care and Use Committee at St. Jude Children's Research Hospital (Memphis, TN).

2.2. Construction of Reassortant Viruses

Influenza B virus genes cloned into plasmid pHW2000 were provided by Drs. Erich Hoffman and Robert G. Webster (St Jude Children's Research Hospital, Memphis, TN). The B/Yamanashi/166/98 (BYam98; lineage II with an HA related to B/Yamagata/16/88) HA (GenBank accession number AF100355) differed from the published sequence at position N196D (A586G). Viruses were created using reverse genetics as described previously using

all eight gene segments from BYam98 [35;36]. Influenza virus, rescued from MDCK:293T co-culture, was propagated in the presence of TPCK-trypsin using confluent MDCK monolayers, and sequencing was performed to confirm appropriate genotypes for rescued virus (Hartwell Center, St Jude Children's Research Hospital). Phenotypes of rescued viruses were characterized in MDCK cells (TCID₅₀) and in mice (MLD₅₀) as described below.

As an additional vehicle for the delivery of influenza HA to mice, viruses demonstrating an LAIV phenotype were created using plasmids mutated at specific sites in three BYam98 internal genes (NP; CY019534, PA; AF102024, and M; AF100392). These previously identified mutations in NP (V114A, T341C and G342A and P410H, C1229A), PA (V431M, G1291A and Y497H, T1489C), and M (H159Q, C477A and M183V, A547G and G549C) [37] were inserted using site-directed mutagenesis (Stratagene, La Jolla, CA). Genetic analyses confirmed the genetic makeup of the virus, the *ts* phenotype was confirmed in MDCK cells, and the *attenuated* phenotype was confirmed in ferrets (Table 1).

An influenza B virus that is lethal in mice has recently been described [36]. The lethality of this virus is due to a single mutation in the BYam98 M gene (AF100392) of the virus (N221S, A662G and T663C), which we mutated using site-directed mutagenesis (Stratagene), and the virus was created using reverse genetics as described above. This virus stock had a titer of $10^{8.25}$ TCID₅₀/mL in MDCK cells at 33°C, and had an LD₅₀ of $10^{6.375}$ TCID₅₀ in mice.

2.3. Plasmid DNA inoculation

Plasmid DNA expressing BYam98 HA was maxi-prepped (Qiagen Inc., Valencia, CA) and bound to 1 micron gold beads (Bio-Rad, Hercules, CA) as described [34]. When vector DNA (pHW2000) was delivered as a control, 2.4 µg vector DNA was bound per mg gold. When BYam HA-DNA was delivered, 1.6 µg vector DNA per mg gold was mixed with 0.8 µg of the BYam HA-DNA to maintain a total of 2.4 µg DNA per mg gold. In all instances, the individual DNA components were mixed thoroughly prior to addition to gold particles. DNA-coated gold particles were propelled onto the bare abdomen of anesthetized BALB/c mice using a Helios gene gun (Bio-Rad). Two non-overlapping shots of 0.5 mg gold from the gene gun (2.4 µg DNA on 1 mg gold) were administered twice at four-week intervals. When plasmid DNA was delivered i.m., vector DNA (100 µg per mouse) or BYam HA-DNA (100 µg per mouse) was injected into the left rear quadriceps in a 100 µL volume twice at four-week intervals.

2.4 LAIV inoculation

Mice were inoculated with 5×10^5 TCID₅₀ (33°C) BYam98 HA-expressing viruses containing *ts*- and *attenuated* phenotypes (Table 1), created as described above. LAIV were delivered in a 50 µL volume (25 µL per nostril) twice at four-week intervals. Ferrets were inoculated with 1×10^7 TCID₅₀ (33°C) BYam98 HA-expressing viruses containing *ts*- and *attenuated* phenotypes, created as described above, delivered twice in a 1 mL volume (500 µL per nostril).

2.5. IIV preparation and inoculation

MDCK-grown wild-type BYam98 virus was concentrated, purified over a sucrose gradient, inactivated with formalin, and HA content was quantitated as described previously [34]. Mice were vaccinated with 3 µg HA in 100 µL volume i.m. in the right rear quadriceps twice at four-week intervals. When Alum (Reheis, Berkeley Heights, NJ) was included as an adjuvant, it was added at a concentration of 2 mg/mL. Ferrets were inoculated with 15 µg HA in 250 µL volume in the right rear quadriceps twice at four-week intervals.

2.6. Serum Collection and Treatment

Clotted blood collected from either the retro-orbital plexus of anesthetized mice or the internal mammary vein of anesthetized ferrets 21 days after each vaccination were centrifuged for 10 minutes at 6000×g. Serum (100 µL) was treated with 300 µL receptor-destroying enzyme (RDE) as described by the manufacturer (Accurate Chemical & Scientific Corp., Westbury, NY). After addition of equal volumes (300 µL) of 2.5% (v/v) sodium citrate and PBS, sera were used in assays to determine vaccine efficacy.

2.7. ELISA

96-well plates (Becton Dickinson and Company, Franklin Lakes, NJ) were coated with concentrated BYam98 wild-type virus ($1 \mu\text{g HA mL}^{-1}$) and incubated overnight at 4°C. Plates were washed with PBS containing 0.05% (v/v) Tween-20 (Sigma) (PBST) and blocked with 10% FBS in PBST (FBS-PBST) for two hours at RT. RDE-treated sera was serially diluted in FBS-PBST and incubated overnight at 4°C. Plates were washed and alkaline phosphatase-conjugated goat anti-mouse IgG (γ -specific), IgG1, and IgG2a (Southern Biotech, Birmingham, AL) antibodies diluted in FBS-PBST were added to the plates and incubated for 2h. Plates were washed, and 1 mg mL^{-1} *p*-nitrophenyl phosphate substrate (Sigma) in diethanolamine buffer was added. 1 h after substrate addition, OD was read at 405 nm using a Multiskan Ascent[®] plate reader (Labsystems, Helsinki, Finland). Reactivity of ferret sera was determined similarly, using individual two hour incubations with unconjugated goat anti-ferret IgG (H + L) (Bethyl Laboratories, Inc., Montgomery, TX) and alkaline-phosphatase-conjugated rabbit anti-goat IgG (H + L) (Bethyl Laboratories, Inc.), both diluted in FBS-PBST, prior to substrate addition as described above. Reciprocal serum antibody titers were calculated at 50% maximal binding.

2.8. Virus challenge

Four weeks after receiving the final dose of respective vaccine, mice were challenged i.n. with 7.5 MLD₅₀ ($10^{7.25}$ TCID₅₀) in a 100 µL volume (50 µL per nostril) reassortant BYam98 viruses containing the M1 mutation (N221S) created as described above. Four weeks after final dose of respective vaccine, ferrets were similarly challenged i.n. with $10^{5.00}$ TCID₅₀ in 1 mL (500 µL per nostril). Ferrets were monitored daily for signs of clinical illness as described previously [38] and mice were monitored daily for morbidity (weight loss) and mortality (survival). Mice that lost more than 30% of their initial body weight were euthanized and recorded as dying on the following day.

2.9. Nasal wash viral titers

Nasal wash was collected from LAIV-inoculated ferrets on days 1, 3, 5, and 12 after primary LAIV inoculation and on days 1, 3, and 5 after secondary LAIV inoculation. Nasal wash was also collected from all ferrets daily on days 1-6, and on day 9 after challenge with BYam. Nasal wash was collected as effluvium into 50-mL tubes (Corning) after instillation of 500 µL PBS per nostril, and exposed to MDCK cells for virus propagation as described previously [38]. Briefly, nasal wash was diluted 10-fold in MDCK infection media, and MDCK monolayers (washed twice with sterile PBS) were incubated with diluted nasal wash samples for 1 h. The inoculum was then removed, MDCK infection media containing $1 \mu\text{g mL}^{-1}$ TPCK-trypsin (Worthington) was added, and cells were incubated for four days at 33°C, 5% CO₂. Cells were observed for cytopathic effect, and infection within individual wells was confirmed using a standard HA assay. Lung viral titers were calculated using the Reed-Muench method. Nasal wash was plated beginning with 100 µL undiluted effluvium through a 1:100,000 dilution (10^{-1} through 10^{-6} TCID₅₀/mL). The minimum titer detectable by this assay was 10^1 , and the maximum titer was $10^{6.50}$.

3. Results

3.1. Comparison of vehicles for HA delivery administered using two-dose identical or mixed vaccination regimens

To directly compare immunogenicity of different B HA delivery vehicles in a naïve population, we administered combinations of five vaccines twice either using identical vaccines (Fig 1A) or mixing delivery vehicles (Fig 1B). The choice to expose animals to HA twice was based on the current recommendations for vaccination of the naïve human population (<9 years of age) [39]. Delivery of influenza B virus HA using identical vehicles demonstrated LAIV and whole virus IIV vaccines elicited similar influenza-specific IgG antibody titers. Low or negative IgG responses were detected after vaccination with DNA vaccines delivered by two different routes. Addition of adjuvant (Alum) to IIV preparations enhanced immunogenicity compared to whole virus vaccine without alum. IgG1 and IgG2a titers from the IIV + Alum group were of a similar magnitude, with no evidence of skewing toward IgG1 as has been reported for the influenza A virus HA [40]. However, delivery of IIV without Alum was skewed toward IgG2a, with lower levels of IgG1 detected. Similar to IIV + Alum, LAIV induced both IgG1 and IgG2a antibodies, whereas HA-DNA delivered via the gene gun (HA-DNA (GG)) demonstrated low levels of antibodies, mostly of the IgG1 isotype. HA-DNA delivered i.m. (HA-DNA(IM)) did not induce a significant IgG antibody response (Fig 1A), even in the presence of Alum (data not shown). Using a mixed delivery regimen (Fig 1B), IIV followed by LAIV resulted in modest titers, similar to those seen with 2 doses of IIV alone, while LAIV priming before IIV delivery engendered a more robust response. LAIV demonstrated an ability to prime the immune response for a secondary boost by IIV, evidenced as increases in IgG (γ -specific), IgG1, and IgG2a titers, while priming with IIV did not allow for a significant boost of the IgG isotypes assayed.

3.2. Protection of vaccinated mice from lethal influenza virus challenge

To directly assess the fitness of these immune responses in controlling a lethal influenza infection, mice were challenged with 7.5 MLD₅₀ of a lethal influenza B virus expressing the BYam98 HA on its surface (Figure 2) [36]. The groups most greatly affected by this challenge dose were those that received either vehicle (PBS or Alum) or HA-DNA (IM), even when Alum was added to the HA-DNA preparation (data not shown). None of these groups had demonstrated high antibody titers after inoculation (Fig 1A). Seven of the eight mice inoculated with Alum exhibited weight loss identical to the group inoculated with PBS, and these seven mice succumbed to the infection. The remaining mouse in this group did not exhibit morbidity or mortality after inoculation, and is not believed to have received the entire inoculation dose. In agreement with the antibody responses observed, mice that were vaccinated with LAIV as a prime, followed by either LAIV or IIV, achieved maximal survival (100%). Alternatively, the groups that were primed with IIV (and boosted with either IIV or LAIV) demonstrated 50-60% survival, corresponding with the moderate humoral immunity induced before challenge, in particular the lower IgG1 titers observed (Fig 1). Addition of Alum to the IIV preparation led to enhanced survival (100%). The group that received HA-DNA (GG) showed a low level of protection after challenge (25%), which corresponded with the low level of immunity (mostly IgG1) demonstrated after vaccination. The poor protective efficacy of DNA vaccines in mice is similar to previous data from our lab studying influenza A virus HA-DNA vaccination by gene gun [34].

3.3. Comparison of LAIV shedding between naïve ferrets and ferrets primed with IIV

The finding that IIV priming for LAIV resulted in lower secondary titers in mice than LAIV priming was surprising, and potentially has important implications for humans, since ACIP recommendations currently support the use of the two vaccine vehicles in any order [41]. Therefore, we decided to test this important point in the ferret model, which may be more

representative of human responses [42;43]. We first assessed whether IIV priming limited the growth of subsequent LAIV exposure (Table 2). When LAIV was administered to naïve animals, virus shedding was observed on days 1, 3, and 5 after inoculation. All ferrets were negative when tested 12 days after LAIV delivery. If the animals had been exposed to IIV vehicle as a prime, shedding of LAIV after secondary exposure was limited to days 1 and 3, with no detectable virus at day 5. This finding indicates immunity induced after IIV inoculation limits LAIV, but does not completely prevent LAIV shedding. Therefore, neutralization of virus by pre-existing antibody is unlikely to account for any differences in secondary antibody titer.

3.4. Humoral immunity induced in ferrets using a mixed vaccine delivery regimen

Three weeks after either primary or secondary inoculation, ferret sera were analyzed for antibody expression (Figure 3). An ELISA assay that was used to detect IgG (H + L) demonstrated no HA-specific antibody detected prior to vaccination. After priming with LAIV, all four ferrets demonstrated high IgG titers, while two of the four ferrets in the IIV-primed group had low antibody titers, with the other two demonstrating antibody that was below the level of detection for the assay. Boosting with LAIV enhanced antibody titers to levels similar to those seen with primary LAIV exposure. Boosting with IIV after the LAIV prime did not induce a further increase in titers. Thus, LAIV appears to induce stronger immunity against influenza B viruses in the ferret model, as high antibody titers are elicited after a single LAIV exposure, and similar antibody levels are not achieved in IIV primed animals until the animals are boosted with LAIV. Of note, IIV priming did not diminish the boosting effect of LAIV as was seen in the mouse model. Similar patterns of serum antibody levels were detected using the standard HI assay (data not shown). In addition, assays used to detect influenza-specific IgA antibodies in sera and nasal wash, as well as influenza-specific IgG (H + L) antibodies in nasal wash did not detect significant stimulation of antibodies of these isotypes (data not shown).

3.5. Shedding of virus by ferrets after vaccination using a mixed delivery regimen

As a direct correlate of protective immunity induced after vaccination, ferrets were challenged with BYam98-expressing virus as described above for the murine model. Using clinical score (combined activity and sneezing ratings), mean body temperature, percent initial body weight, and protein levels in nasal wash fluid as readouts of illness [38], challenging either group of ferrets with this virus did not result in significant clinical illness (data not shown). However, when nasal washes were collected to assess virus shedding after challenge (Figure 4), the group that was inoculated with PBS shed virus at moderately high titers for at least 6 days after challenge. The ferrets that received IIV followed by LAIV did not shed detectable levels of virus at any timepoint after challenge, whereas ferrets that were in the group primed with LAIV and boosted with IIV shed only minimal amounts of virus between days 1 and 5 after challenge. All immunized ferrets were negative at day 6 after challenge with this virus. Although the antibody data suggest that LAIV is a superior vaccine vehicle to IIV, both were equally efficacious in the challenge model. Taken together, these data suggest that if a mixed prime:boost regimen is used for influenza B virus IIV and LAIV, an LAIV prime would be favorable in a naïve population (i.e. first-time vaccine recipients or new vaccine formulation), and LAIV would be better suited for boosting individuals that had already been primed by either vaccination or natural infection.

4. Discussion

Current vaccines have compromised effectiveness for influenza B virus compared to influenza A virus [30-32]. When coupled with difficulties in strain selection [29], it appears that alternate strategies for prevention of influenza B virus should be considered. Our data demonstrate that

vaccines based on the LAIV system are more effective against influenza B virus in animal models than other potential alternatives. LAIV produced a balanced IgG1 and IgG2a response, a factor shown to be important for neutralization of virus, clearance of virus from the lungs, and survival in the influenza A virus model [34]. In fact, despite our utilization of a whole-virus preparation for IIV, which is generally more immunogenic than the purified, split product preparations delivered to humans annually [44;45], adjuvantation was necessary to achieve similar protection to that demonstrated with LAIV. Furthermore, we show that administering LAIV as a prime enhances primary immunity, as well as the capacity to respond to either an identical (LAIV) or mixed (IIV) boost, and we report similar findings in both mouse and ferret models of influenza immunity.

Beginning in the 1960's, vaccine technologies that utilized detergents to split or purify virus surface proteins from inactivated viral particles were employed to reduce the reactogenicity seen after inactivated, whole virus vaccine delivery [22;46-49]. Unfortunately, this reduced reactogenicity was associated with reduced immunogenicity of split product preparations [49-51]. Efforts were made to enhance immunogenicity using adjuvants [52-56], but incorporation of adjuvants was hindered either by a perceived lack of benefit (Alum [57]) or development of sterile abscesses at the injection site (Arlacel A [58]). Recently the use of Alum as an adjuvant has been revisited for influenza HAs with pandemic potential that have moderate to low immunogenicity in humans [59;60]. In addition to Alum, adjuvants based on oil-in-water formulations have shown success in clinical trials (AS [61;62]) and have achieved clinical approval in elderly populations in Europe (MF59 [63]). The data reported here, while using vaccine production technologies that differ from those used in humans, suggest that adjuvantation for influenza B virus may help achieve optimal immunity against this type of influenza virus.

Historical analysis of influenza B virus vaccines in IIV form delivered either alone [16;64] or as part of a trivalent preparation [3;6;30;65;66] have demonstrated reduced immunity compared to similar formulations of influenza A virus components. Furthermore, Ohmit et al. [32] recently demonstrated reduced efficacy and effectiveness toward the B virus component of a trivalent vaccine delivered in LAIV form. Strains from both dominant B lineages circulated in the United States during the study period, but the strain(s) infecting subjects in this study were not reported, and only one influenza B virus strain was subtyped in a follow-up study in 2005-2006 (it was mis-matched to the vaccine) [67]. Thus, it is difficult from the results reported thus far from these studies to decide how much of the perceived poor efficacy of vaccines for B vs. A is related to strain mis-match and how much is due to decreased immunogenicity. An added confounder is that protection against influenza B virus can be achieved with less than the 1:32 HI titer currently used to correlate with effective protection against influenza A viruses of the H1N1 and H3N2 subtypes [64;66,6870]. These factors and our data suggest that, contrary to current practice, utilizing analogous production techniques and correlates of immunity for both influenza A and B virus vaccines, as well as incorporating only a single strain each year, yields less than optimal immunity. It is clear that further study of these issues is needed.

Direct comparison of DNA, IIV, and LAIV vehicles for B HA delivery, in unadjuvanted form demonstrated the following immunogenicity profile for the different vehicles delivered twice: LAIV $\geq$ IIV > HA-DNA (GG) > HA-DNA (IM). Surprisingly, an immune response to HA-DNA delivered using the IM route did not induce an immune response toward the HA [71], whereas HA-DNA delivered via the gene gun revealed humoral and protective immunity similar to results we have reported for influenza A virus [34]. With regard to HA-DNA delivered IM, literature supports the induction of antibody responses of the IgG2a isotype after HA-DNA delivery [72], which would predict enhanced ability to clear the virus after infection [34;73]. However, the sensitive ELISA assays employed here [34;74] were unable to detect

significant humoral immunity (including IgG2a) or protective responses after HA-DNA (IM) inoculation with a dose of 100 µg per mouse (compared to 0.8 µg HA-DNA (GG) per mouse) [75], even in the presence of Alum. The lack of a response in mice inoculated with influenza B HA via the IM route may be associated with either the route/method of delivery [75-78] or reduced antigenicity for influenza B HA [30;32] expressed by the plasmid DNA.

In the current study, high IgG (γ-specific and IgG1 for mice and (H + L) for ferrets) expression was a strong correlate of protective immunity. In addition, groups of mice that demonstrated maximal survival were those that expressed antibodies of both the IgG1 and IgG2a isotypes simultaneously. Corroborating previous data from our group [34], increased IgG2a expression in mice vaccinated with IIV alone yielded better protection from lethal challenge over a group that predominantly expressed IgG1 after vaccination (HA-DNA (GG)), even when IgG1 levels were identical for the two groups at the time of challenge. Lower IgG1 titers in the IIV group, however, correlated with poor outcomes compared to adjuvanted IIV and LAIV despite similar IgG2a titers. A recent study by Bungener et al. [40] reported skewing toward an IgG2a response after vaccination with an unadjuvanted, whole, inactivated influenza A vaccine, and reported an alternate skewing toward IgG1 when Alum was included in the vaccine preparation. Our vaccine did not exhibit a similar trend, thus hinting at differences in the type of immunity induced toward influenza A and B viruses.

Current vaccine recommendations call for administration of two influenza vaccine inoculations to naïve human populations (i.e. first time vaccine recipients under the age of 9) [39]. This is due to limited detection of a humoral response following a single inoculation with typical inactivated vaccine formulations, most notably for the H1N1 and B virus components [30, 31,79-81]. While humoral correlates of immunity after inoculation with trivalent vaccines are biased toward the H3N2 subtype [30-32,79-81], significant protective immunity toward influenza B after a single dose of LAIV has been reported [81], and is maintained even when humoral immune correlates have not achieved the desired level [82-84]. Current recommendations for influenza vaccination from the ACIP indicate that mixed delivery of IIV and LAIV can occur in any order [41]. Our data indicate that this assumption on the order of mixed vaccine administration requires more attention, at least for influenza B virus. Specifically, using both the mouse and the ferret model we show a difference in antibody kinetics after mixed IIV and LAIV delivery in both mice and ferrets. The reduced capacity for adequate boosting after IIV delivery is associated with adverse response to challenge in the mouse model, but not in ferrets. Thus, while the scope of this study does not allow for definition of a regimen for delivery of mixed vehicles, our data urge analyses of human influenza vaccine recipients to see what effects are seen under mixed vaccine conditions. If LAIV is the preferred prime, the earlier availability of LAIV compared to IIV (typically 1-2 months lead time) may facilitate efforts to administer 2 doses in naïve subjects, something that has been difficult to achieve with IIV formulations which are typically not available until October [85]. In addition, we support further attempts to define adjuvants that would boost immunity to IIV and/or LAIV, but caution against treating influenza B and influenza A virus subtypes similarly with regard to formulation, immunogenicity, and correlates of protective immunity.

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Figure 1.

Vaccination against influenza B virus with identical and mixed delivery vehicles induces IgG immunity in BALB/c mice. Sera taken from mice prior to vaccination (Pre), three weeks after primary (Pri), and three weeks after secondary (Sec) inoculation with influenza B virus HA expressed by identical (A) or mixed (B) vectors were analyzed for IgG isotypes (IgG (γ-specific), IgG1, or IgG2a) by ELISA. ELISA titers are reported as the reciprocal serum dilution representing 50% maximal binding on the titration curve. Titers are reported as the mean $\pm$ standard deviation for individual groups of mice at each time-point (Alum + Alum ($\diamond$), PBS + PBS (∇), $n = 11$; $n = 18$; HA-DNA (IM) + HA-DNA (IM) (Δ), $n = 18$; HA-DNA (GG) + HA-DNA (GG) ($\square$), $n = 18$; IIV (Alum) + IIV (Alum) ($\bullet$), $n = 17$; IIV + IIV ($\blacktriangledown$), $n = 11$; LAIV + LAIV ($\blacksquare$), $n = 18$; IIV + LAIV ($\blacktriangle$), $n = 11$; and LAIV + IIV ($\blacklozenge$), $n = 11$), except for Pre and Pri titers reported for the group receiving IIV + LAIV ($n = 14$).

Figure 2.

Vaccination against influenza B virus with identical and mixed delivery vehicles induces protective immunity in BALB/c mice. Four weeks after secondary exposure to HA antigen expressed in multiple delivery vehicles, mice were challenged with 7.5 MLD₅₀ BYam98 HA-expressing virus with a mouse lethal phenotype. Morbidity (% initial body weight) and mortality (% survival) are reported for individual groups of mice (Alum + Alum (◇), PBS + PBS (▽), n = 11; n = 18; HA-DNA (IM) + HA-DNA (IM) (Δ), n = 18; HA-DNA (GG) + HA-DNA (GG) (□), n = 18; IIV (Alum) + IIV (Alum) (●), n = 17; IIV + IIV (▼), n = 11; LAIV + LAIV (■), n = 18, IIV + LAIV (▲), n = 11; and LAIV + IIV (◆), n = 11),

Figure 3.

Vaccination against influenza B virus using mixed delivery vehicles induces disparate antibody kinetics. Sera taken from ferrets prior to vaccination (Pre), three weeks after primary (Pri) inoculation, and three weeks after secondary (Sec) inoculation with influenza B virus HA expressed by mixed vectors were analyzed for IgG (H + L) expression by ELISA. ELISA titers are reported as the reciprocal serum dilution representing 50% maximal binding on the titration curve. Titers for individual ferrets are reported as individual symbols, with the bar representing the mean for the (PBS (■), n = 2; IIV + LAIV (○), n = 4; and LAIV + IIV (◆), n = 4).

Figure 4.

Nasal wash viral titers in vaccinated ferrets after challenge with BYam98 HA-expressing influenza virus. Nasal wash collected daily after challenge was tested for presence of influenza virus. Titters are reported as the mean $\pm$ standard deviation for each group at time-points indicated by the symbols (PBS (■), $n = 2$; IIV + LAIV (○), $n = 4$; and LAIV + IIV (◆), $n = 4$).