RESEARCH ARTICLE

An Intranasal Virus-Like Particle Vaccine Broadly Protects Mice from Multiple Subtypes of Influenza A Virus

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ABSTRACT **Influenza virus infections are a global public health problem, with a significant impact of morbidity and mortality from both annual epidemics and pandemics. The current strategy for preventing annual influenza is to develop a new vaccine each year against specific circulating virus strains. Because these vaccines are unlikely to protect against an antigenically divergent strain or a new pandemic virus with a novel hemagglutinin (HA) subtype, there is a critical need for vaccines that protect against all influenza A viruses, a so-called "universal" vaccine. Here we show that mice were broadly protected against challenge with a wide variety of lethal influenza A virus infections (94% aggregate survival following vaccination) with a virus-like particle (VLP) vaccine cocktail. The vaccine consisted of a mixture of VLPs individually displaying H1, H3, H5, or H7 HAs, and vaccinated mice showed significant protection following challenge with influenza viruses expressing 1918 H1, 1957 H2, and avian H5, H6, H7, H10, and H11 hemagglutinin subtypes. These experiments suggest a promising and practical strategy for developing a broadly protective "universal" influenza vaccine.**

IMPORTANCE **The rapid and unpredictable nature of influenza A virus evolution requires new vaccines to be produced annually to match circulating strains. Human infections with influenza viruses derived from animals can cause outbreaks that may be associated with high mortality, and such strains may also adapt to humans to cause a future pandemic. Thus, there is a large public health need to create broadly protective, or "universal," influenza vaccines that could prevent disease from a wide variety of human and animal influenza A viruses. In this study, a noninfectious virus-like particle (VLP) vaccine was shown to offer significant protection against a variety of influenza A viruses in mice, suggesting a practical strategy to develop a universal influenza vaccine.**

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Influenza viruses pose a major public health threat. Globally, influenza results in 3 to 5 million severe illnesses and up to 500,000 deaths annually (1). Influenza pandemics, in which novel influenza A viruses (IAVs) unpredictably emerge, and against which most humans lack protective immunity, can have even larger global impacts (2): e.g., the 1918 influenza pandemic resulted in 50 million deaths (3). The current strategy for preventing annual influenza is to develop a new vaccine each year against specific circulating virus strains. Because these vaccines are unlikely to protect against an antigenically divergent strain or a new pandemic virus with a novel hemagglutinin (HA) subtype (4), there is a critical need for influenza vaccines that protect against all IAVs, a so-called "universal" vaccine (5).

Influenza A viruses (IAVs) are enveloped, negative-sense, single-stranded RNA viruses with segmented genomes (6). In addition to humans, IAVs infect large numbers of warm-blooded animal hosts, including over 100 avian species (7) and many mammalian species, with numerous species of wild aquatic birds serving as the major natural reservoir. IAVs express three surface proteins—hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2). IAVs are subtyped by antigenic characterization of the HA and NA glycoproteins. Sixteen HA and 9 NA subtypes are consistently found in avian hosts in various combinations (e.g., H1N1 or H3N2), and these wild bird viruses are thought to be the ultimate source of human pandemic influenza viruses (8). IAV genome segmentation allows for viral reassortment, and since HA and NA are encoded on separate gene segments, novel IAVs of any subtype can be generated following mixed infections in any host, a process that has been termed "antigenic shift." IAVs are also evolutionarily dynamic RNA viruses with high mutation rates. Mutations that change amino acids in the antigenic portions of HA and NA proteins may allow strains to evade population immunity ("antigenic drift"). Unfortunately, despite enhanced surveillance and research on host switch events, future pandemics cannot be

TABLE 1 Properties of challenge viruses used and survival postchallenge

Challenge virus[a]	MLD_{50}	VLP vaccinated			Mock vaccinated		
		Total no.	Wt loss nadir[b]	% survival	Total no.	Wt loss nadir[b]	% survival
1918 H1N1	$10^{3.25}$	5	99.4	100	5	74.0	0
1957 H2N1	$10^{3.5}$	10	82.8	100	10	73.1	0
H5N1	$10^{0.7}$	10	89.6	90	10	74.4	10
H6N1	$10^{2.5}$	24	93.3	83.3	15	73	0
H7N9	$10^{1.7}$	15	96.3	100	15	73.2	0
H7N1	$10^{2.5}$	5	97.0	100	5	71.8	0
H10N1	$10^{1.6}$	10	88.3	100	10	73.6	20
H11N1	$10^{2.65}$	10	95.3	100	5	72.6	20
Overall		89		94.4	75		5.3
Duration of protection study							
H7N9	$10^{1.7}$	5	98.4	100	5	73.8	20
H10N1	$10^{1.6}$	5	79.2	80	5	74.0	20
Aged mouse study H10N1	$10^{1.6}$	6	79.8	66.7	5	73.9	0

[a] Chimeric viruses were rescued using the PB1, $PB2^{E627K}$, PA, NP, NA, M, and NS gene segments from influenza A/Green Wing Teal/Ohio/175/1986 (H2N1), along with the H6, H7, or H10 HA segments as previously described (42). Chimeras were also produced using HA segments from A/South Carolina/1/1918 (H1N1) and A/Japan/305/1957 (H2N2), as well as a laboratory variant of A/Green Wing Teal/Ohio/340/1987 (H11N9). (42). Wild-type (WT) influenza viruses A/Anhui/1/2013 (H7N9) and A/Vietnam/1203/1204 (H5N1) were also used as challenge viruses. MLD_{50}s were determined as previously described (42) using standard methods (45). See Materials and Methods for additional details.
[b] The weight loss nadir represents the lowest weight, measured as a percentage of the starting weight at day 0.

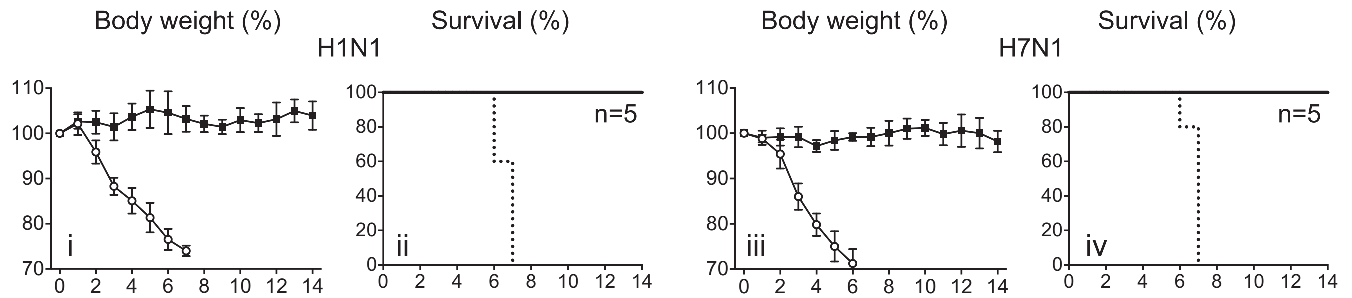
predicted, including when and where a pandemic virus strain will emerge, what the viral subtype will be, or how pathogenic it will be in humans. Severe human infections with animal-origin IAVs have also been observed, including recent human infections with avian H5N1 and 7N9 viruses (9, 10).

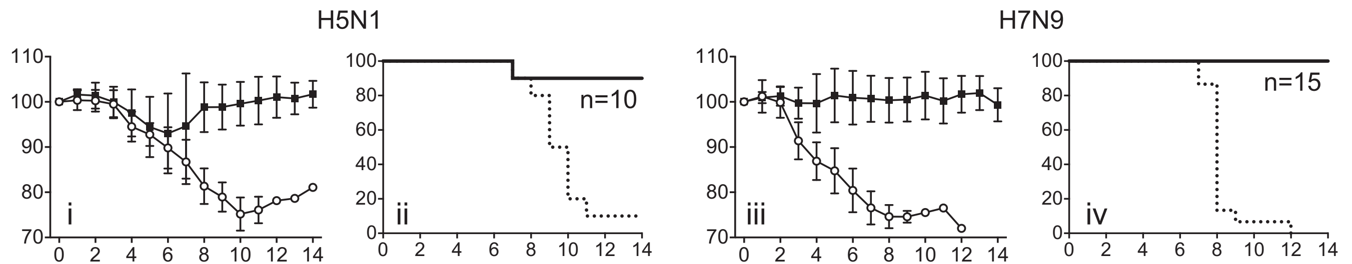
While antiviral drugs to treat influenza are available, vaccination remains the best public health approach to its control. Current annual inactivated and live attenuated vaccines are intended to protect against circulating IAV and influenza B virus (IBV) strains (11) but require a close antigenic match with circulating strains. Rapid antigenic drift can lead to mismatches, lowering the vaccine's protective efficacy (4). The unpredictable nature of pandemic virus emergence complicates vaccination strategies even further (12, 13). An effective prepandemic vaccine would ideally provide broad protection against all IAV subtypes. Efforts to develop such broadly protective vaccines have been under way for decades (14) and have included experimental vaccines specifically targeting the M2 ectodomain (15, 16) or NA (17, 18) proteins to stimulate the development of protective antibody responses, vaccines based on antigens that stimulate development of T-cell responses (19, 20), and most recently, a variety of vaccine approaches targeting antigenically conserved epitopes on the HA head and stalk (4, 5, 19–22). Various virus-like particle (VLP)-based vaccines have also been investigated (18, 23–31), but a practical vaccine inducing broad heterosubtypic or "universal" protection has not been previously demonstrated with any of the above approaches.

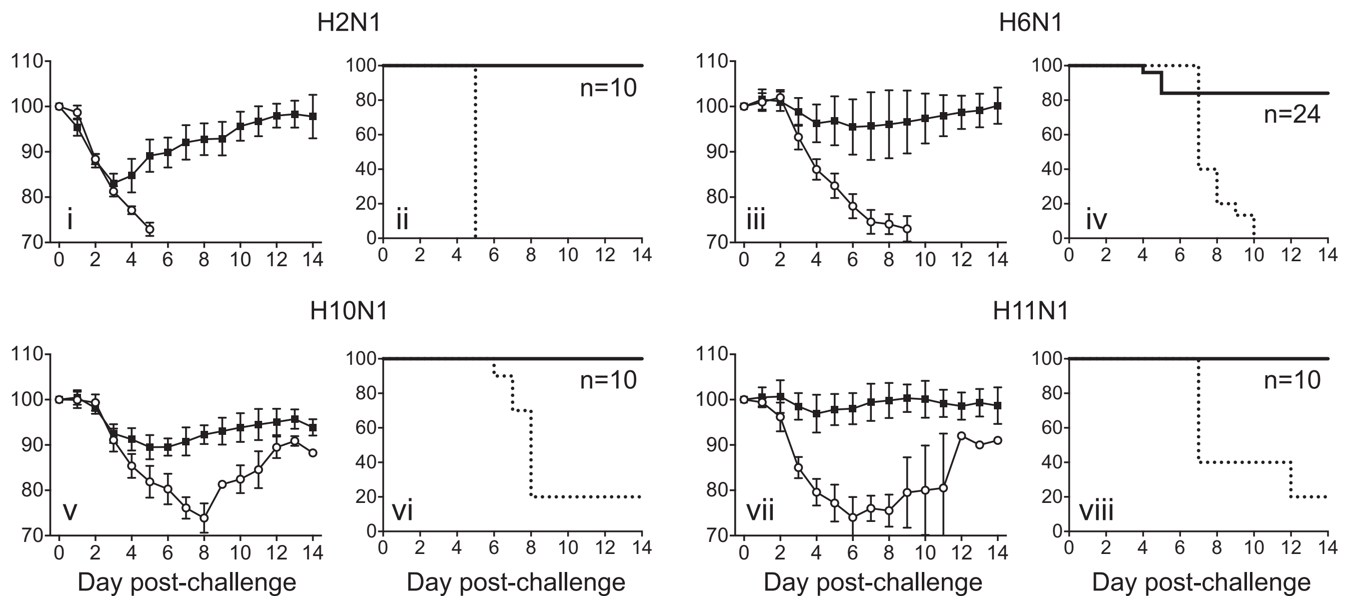
In the present study, protection afforded by immunization with a prepandemic VLP vaccine was assessed in a lethal IAV murine challenge model. The vaccine cocktail included four VLPs displaying either the 1918 H1 subtype or low-pathogenicity avian influenza H3, H5, or H7 HA subtypes. The VLPs were produced in baculovirus utilizing one of the four different HA genes along with a shared matrix 1 protein. These subtypes were chosen to reflect the subtypes of currently circulating annual IAV strains (H1 and H3) or recent epizootic IAV infections (H5 and H7) and represent both major phylogenetic HA groupings—clade 1 (H1 and H5) and clade 2 (H3 and H7) (6). Recent data have supported the idea that conserved antigens (e.g., on the HA stalk) stimulate cross-protective immunity (4, 5), with the implication that a universal vaccine strategy could be developed that would induce such protection. In this study, we hypothesized that a cocktail of different VLPs, each displaying an individual HA subtype, could uniquely induce broadly protective immunity without the need for antigenic matching of vaccine and challenge virus strains. To test this hypothesis, groups of 8-week-old BALB/c mice were vaccinated intranasally (i.n.) with this VLP cocktail (1.5 μg total protein for each VLP) or were mock vaccinated with phosphate-buffered saline (PBS) on day 0 and boosted i.n. on day 21; cohorts of mice were then challenged on day 50 with different IAV strains (Table 1), each at a 10× mouse 50% lethal dose (MLD_{50}). We found that mice vaccinated with the HA-VLP mixture were protected against lethal challenge with influenza viruses expressing diverse HA subtypes and that this protection is partially antibody mediated.

RESULTS AND DISCUSSION

Mice vaccinated with the H1-H3-H5-H7 VLP cocktail were protected against lethal challenge with eight different IAV strains expressing 7 different HA subtypes (Table 1); in aggregate, 94.4% (84/89 animals) of vaccinated mice survived challenge compared to 5.3% (4/75 animals) of mock-vaccinated mice ($P < 0.001$). When challenged with viruses that expressed HAs identical to those contained in the vaccine (homologous challenge with 1918 H1N1 and avian H7N1) (Fig. 1A), vaccinated mice showed 100% survival with only minimal weight loss nadirs (0.6% for 1918 H1N1 and 3.0% for H7). The mock-vaccinated animals all died following challenge. In the second group of experiments, two of the challenge viruses expressed HAs from different strains of the same subtype but were not antigenically matched to the vaccine HAs (intrasubtypic heterologous challenge with H5N1 and H7N9) (Fig. 1B). Here vaccinated mice also showed 100% survival following A/Anhui/1/2013 (H7N9) virus challenge (weight loss nadir, 3.7%) and 90% survival following A/Vietnam/1203/2004







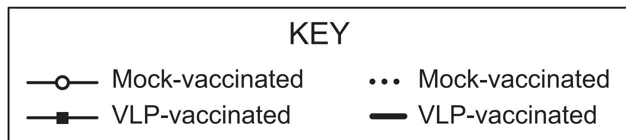


FIG 1 VLP vaccination protects mice from lethal influenza challenge across HA subtypes. Eight- to 9-week-old female BALB/c mice were vaccinated with 1.5 μg each of H1, H3, H5, and H7 VLPs (6 μg total) i.n. or with PBS for mock vaccination. Mice were boosted at 21 days postimmunization. At 50 days post-initial immunization, mice were challenged with a lethal dose (10× MLD_{50} [Table 1]) of various challenge influenza A viruses and weighed daily. Weight loss (i, iii, v, and vii) of VLP-vaccinated (solid squares) and mock-vaccinated (open circles) mice is expressed as mean percentage of initial weight ± standard deviation (SD). Survival rates (ii, iv, vi, and viii) of VLP-vaccinated (solid lines) and mock-vaccinated (dotted lines) mice were assessed for 14 days postchallenge. (A) Polyvalent vaccination completely protected against challenge with influenza viruses expressing identical (homologous) HA proteins. Mice were challenged with 1918 H1N1 (i and ii; $n = 5$) or H7N1 (iii and iv; $n = 5$). (B) Vaccinated mice were protected from intrasubtypic (heterologous) challenge with H5N1 (i and ii; $n = 10$) or H7N9 (iii and iv; $n = 15$) virus. (C) Vaccinated mice were challenged with heterosubtypic influenza A viruses (H2N1 [i and ii; $n = 10$], H6N1 [iii and iv; VLP, $n = 24$, and mock, $n = 15$], H10N1 [v and vi; $n = 10$], or H11N1 [vii and viii; VLP, $n = 10$, and mock, $n = 5$]). For all challenge groups, vaccinated mice had significantly reduced weight loss compared to mock-vaccinated mice (Mann-Whitney test, $P < 0.01$).

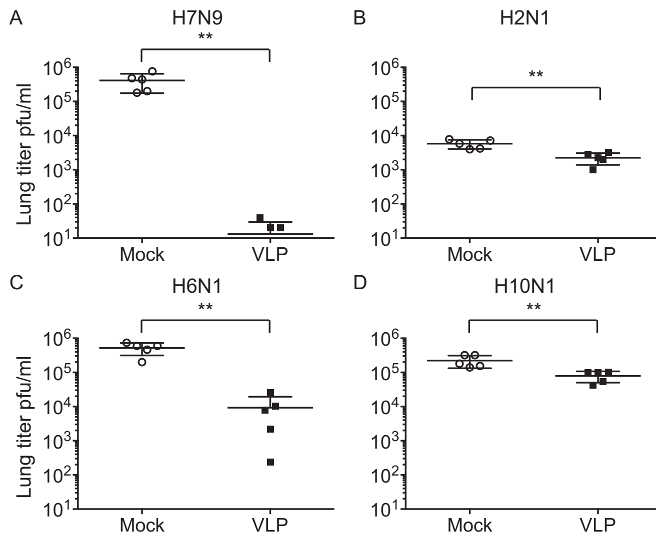


FIG 2 Viral replication is reduced in the lungs of VLP-vaccinated mice. Mice were vaccinated and challenged with H7N9 (A), H2N1 (B), H6N1 (C), or H10N1 (D) as described in the legend to Fig. 1. At 3 days postinfection, lungs were harvested, and the titer was determined by plaque assay. Vaccinated mice had significantly reduced titers compared to mock-vaccinated mice for each of the four viruses (each group, $n = 5$; Mann-Whitney test, $P < 0.01$). Brackets joining mock-vaccinated and VLP-vaccinated groups indicate statistically significant differences in lung titers.

highly pathogenic H5N1 virus challenge (weight loss nadir, 10.4%). Mock-vaccinated animals showed rapid weight loss and 96% fatality (24/25) in aggregate. In the third group of experiments, four of the challenge viruses expressed HA subtypes not contained in the vaccine (heterosubtypic challenge): viruses expressing the 1957 pandemic H2 (H2N1) and avian H6N1, H10N1, and H11N1 IAVs (Fig. 1C). These vaccinated animals all showed 100% survival following challenge with H2N1 (weight loss nadir, 17.2%), H10N1 (weight loss nadir, 11.7%), and H11N1 (weight loss nadir, 4.7%) and 83.3% survival following challenge with H6N1 (weight loss nadir, 6.7%). Mock-vaccinated animals showed rapid weight loss and 92.5% (37/40) fatality in aggregate against these four challenge viruses.

Viral titers in lung homogenates at day 3 postchallenge were significantly reduced in VLP-vaccinated compared to mock-vaccinated mice in all cases studied ($P < 0.01$). Heterologous (intrasubtypic) challenge with H7N9 showed viral lung titers reduced ~5 $\log_{10}$ PFU; heterosubtypic challenge with H2N1, H6N1, or H10N1 viruses showed lung titers reduced ~0.5 to 1.5 $\log_{10}$ PFU (Fig. 2). Comparison of titers between VLP-vaccinated mice showed a significant difference between intrasubtypic challenge and heterosubtypic challenge groups ($P < 0.01$); however, there was no statistical difference in survival rates between these groups (Fig. 1).

To evaluate the duration of VLP vaccine cocktail-induced protection, groups of mice were vaccinated and boosted as described above and challenged 6 months later. Mice showed 100% survival following heterologous (intrasubtypic) challenge with H7N9 and 80% survival following heterosubtypic challenge with H10N1 (Fig. 3A). In each case, mock-vaccinated animals showed 80% fatality following challenge.

Older adults are particularly susceptible to severe disease following IAV infection, and in this group, a vaccine is less efficacious than in younger adults (32). To evaluate the VLP cocktail in older animals, 8-month-old mice were vaccinated and boosted as described above and then heterosubtypically challenged with H10N1. A total of 66.7% of VLP-vaccinated aged mice survived lethal H10N1 virus challenge, compared with no survival of mock-vaccinated aged mice, all of whom met endpoint weight loss criteria of 25% (Fig. 3B); viral lung titers were significantly reduced by approximately 1 $\log_{10}$ PFU compared to those of mock-vaccinated animals ($P < 0.01$).

To investigate the correlates of protection, antibody production in vaccinated mice was evaluated. Levels of HA-specific IgG or IgA antibodies were measured by enzyme-linked immunosorbent assay (ELISA) in serum or bronchoalveolar lavage (BAL) fluid after boost (Fig. 4A). Mice showed seroconversion with detectable IgG antibodies against both homologous (H1, top panels) and intrasubtypic heterologous (H7, bottom panels) HA proteins, as well as IgA antibodies in the BAL fluid. Functional binding of vaccine-induced HA antibodies was evaluated by hemagglutination inhibition (HAI) using both vaccine VLP antigens and viruses (Fig. 4B) and microneutralization (MN) activity against a variety of influenza viruses (Fig. 4C). Sera from vaccinated mice showed high neutralizing titers against the homologous H1, with intermediate titers against the other homosubtypic HA proteins contained in the VLP vaccine cocktail. No HAI or MN activity was detected against heterosubtypic viruses. Protective efficacy of the vaccine-induced antibodies was evaluated by passive transfer of serum from PBS- or VLP-vaccinated mice to naive mice 1 day prior to lethal challenge (Fig. 4D). Mice challenged with homologous (1918 H1N1) or heterologous intrasubtypic (H7N9) viruses were completely protected by passive transfer alone. Conversely, no protection against heterosubtypic virus (H10N1) was afforded by serum transfer. These results demonstrate that a protective antibody response was generated against homologous and intrasubtypic heterologous influenza viruses but suggest that other vaccine-induced immune mechanisms must be involved in protection against lethal challenge with heterosubtypic viruses.

In this study, an intranasally inoculated VLP vaccine cocktail comprised of four representative IAV HA proteins was evaluated by experimental postvaccination lethal viral challenge. Vaccinated mice demonstrated broad protection against a variety of lethal IAV challenge strains, including heterosubtypic protection from challenge viruses expressing HA subtypes not contained in the vaccine cocktail (1957 H2 and avian H6, H10, and H11 IAV strains). The protection afforded was durable, in that mice challenged 6 months after vaccination were protected against lethal heterosubtypic H10N1 virus challenge. Vaccinated aged mice also showed very substantial protection following H10N1 challenge.

The VLP cocktail chosen, a mixture of four HA VLPs (H1, H3, H5, and H7), afforded broad protection; further experiments will evaluate other VLP combinations, including mixtures of both HA- and NA-expressing VLPs with and without adjuvants. Previous studies have shown the importance of NA immunity in preventing or mitigating influenza virus infections in humans and experimental animals; studies from our laboratory have shown that NA-only VLP vaccines can also completely protect against lethal viral challenge (18).

In the set of murine challenge experiments reported here, we observed near complete protection in vaccinated mice (Fig. 1). The exceptions were lethal challenges with the highly pathogenic avian H5N1 virus strain (90% protection) and challenge with

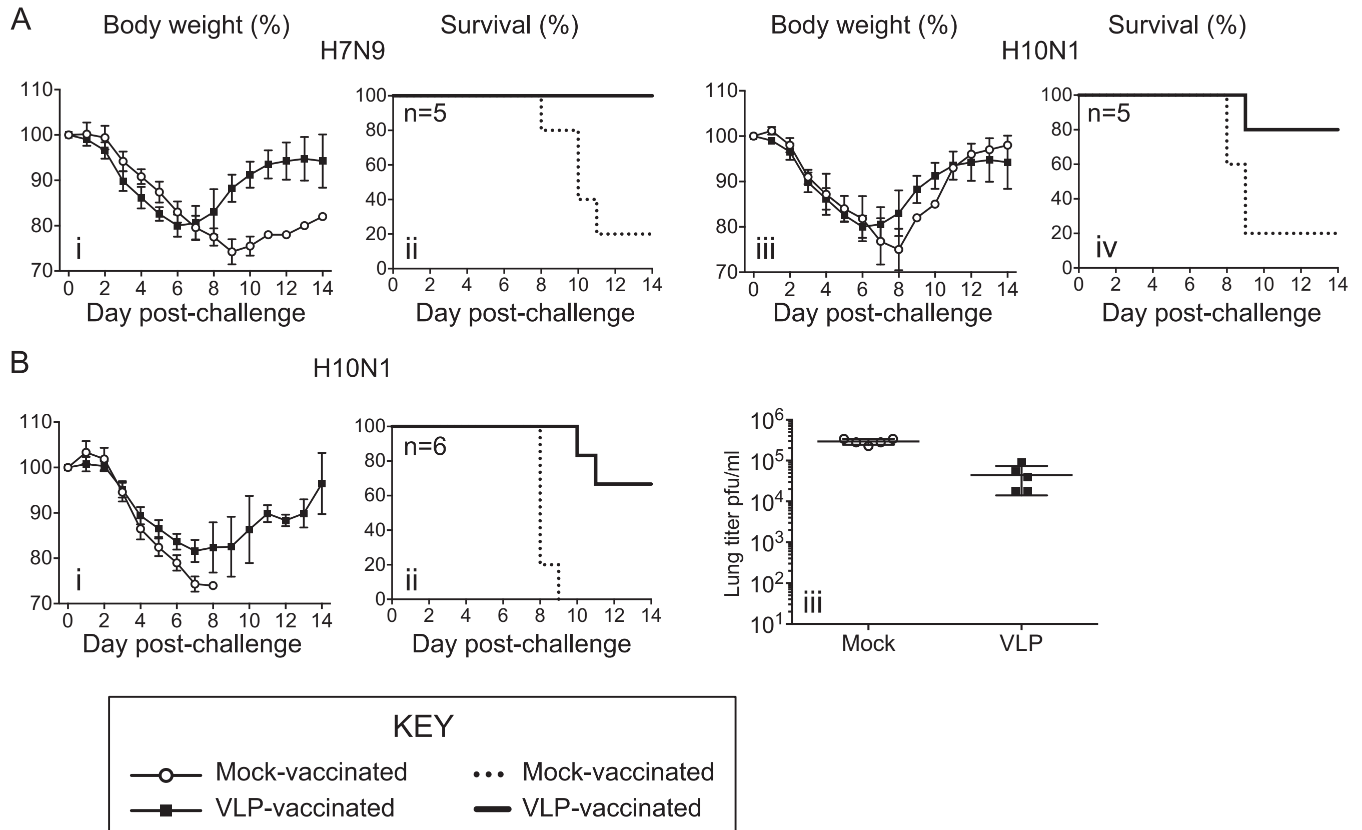


FIG 3 Protective efficacy of VLP vaccination. (A) VLP vaccination protects mice from lethal challenge 6 months postvaccination. Eight-week-old mice were VLP vaccinated (solid squares and solid lines) or mock vaccinated (open circles and dotted lines) as described in the legend to Fig. 1. At 6 months post-initial vaccination, mice were challenged intrasubtypically with H7N9 (i and ii; $n = 5$) or challenged heterosubtypically with H10N1 (iii and iv; $n = 5$), and weights were recorded for 14 days following challenge. (B) Vaccination protects aged mice from lethal challenge. Eight-month-old mice were vaccinated as described in the legend to Fig. 1. Mice were challenged heterosubtypically with H10N1 chimeric influenza virus 50 days postvaccination. Weight loss (i) and survival (ii) were monitored for 14 days following infection ($n = 6$). Lungs were harvested at 3 days postchallenge (iii), and the titer was determined by plaque assay ($n = 5$). Lung titers (Biii) were significantly reduced in vaccinated mice compared to those in mock-vaccinated mice (Mann-Whitney test, $P < 0.01$).

avian H6N1 virus (83.3% protection). Since highly pathogenic H5N1 viruses can replicate systemically in mice, including replication in the central nervous system (33), it is possible that some mice will succumb to lethal challenge following inoculation, even in the presence of a strong immune response. The cause of incomplete protection against H6N1 challenge is currently being investigated.

While we were able to demonstrate broad and durable protection against lethal influenza challenge in these experiments, further studies are needed to characterize the humoral and/or cellular immune correlates of protection afforded by this vaccine strategy, particularly in the case of heterosubtypic protection. Passive immunization studies demonstrated complete protection in mice from lethal homologous (1918 H1N1) and intrasubtypic heterologous (H7N9) challenge, but serum transfer did not protect mice with heterosubtypic challenge (H10N1). These data suggest that intrasubtypic immunoprotection appears to be substantially humorally mediated, whereas heterosubtypic immunoprotection is more likely not predominantly antibody dependent. Possible mechanisms of heterotypic protection (34, 35) include cellular immunoprotection, involving memory CD4 T cells, cytotoxic T lymphocytes (CTLs), or nonneutralizing antibodies not detected in the ELISA, HAI, or MN assays performed here. Ongoing studies are aimed at elucidating the specific roles of immune cell subsets in VLP-afforded intrasubtypic and heterosubtypic immunoprotection. Additionally, experiments comparing intranasal versus intramuscular vaccination, each with and without adjuvants, will be performed. Experiments with ferrets will also be done to evaluate whether the VLP cocktail strategy will provide similar protection in a second relevant experimental animal, not only with challenge by candidate prepandemic IAV but also with a variety of annual human strains. These latter experiments will evaluate whether this vaccine strategy could also induce broad protection against annually drifting epidemic viruses.

VLP-based vaccines have been approved for human use against hepatitis B virus, human papillomavirus, and hepatitis E virus (in China). Experimental VLP vaccines are also being developed against human immunodeficiency, Ebola, chikungunya, and Nipah viruses (31). VLPs also appear to be a promising platform for influenza vaccines (18, 27). A variety of VLP approaches have been used for influenza vaccination, including VLPs bearing both HA and NA (29), VLPs bearing either HA or NA (18), or VLPs bearing more than one HA (36, 37). VLP vaccines have been administered by intramuscular injection or intranasally (29). Protection against homologous challenge has frequently been observed as well as some reports of intrasubtypic protection—e.g.,

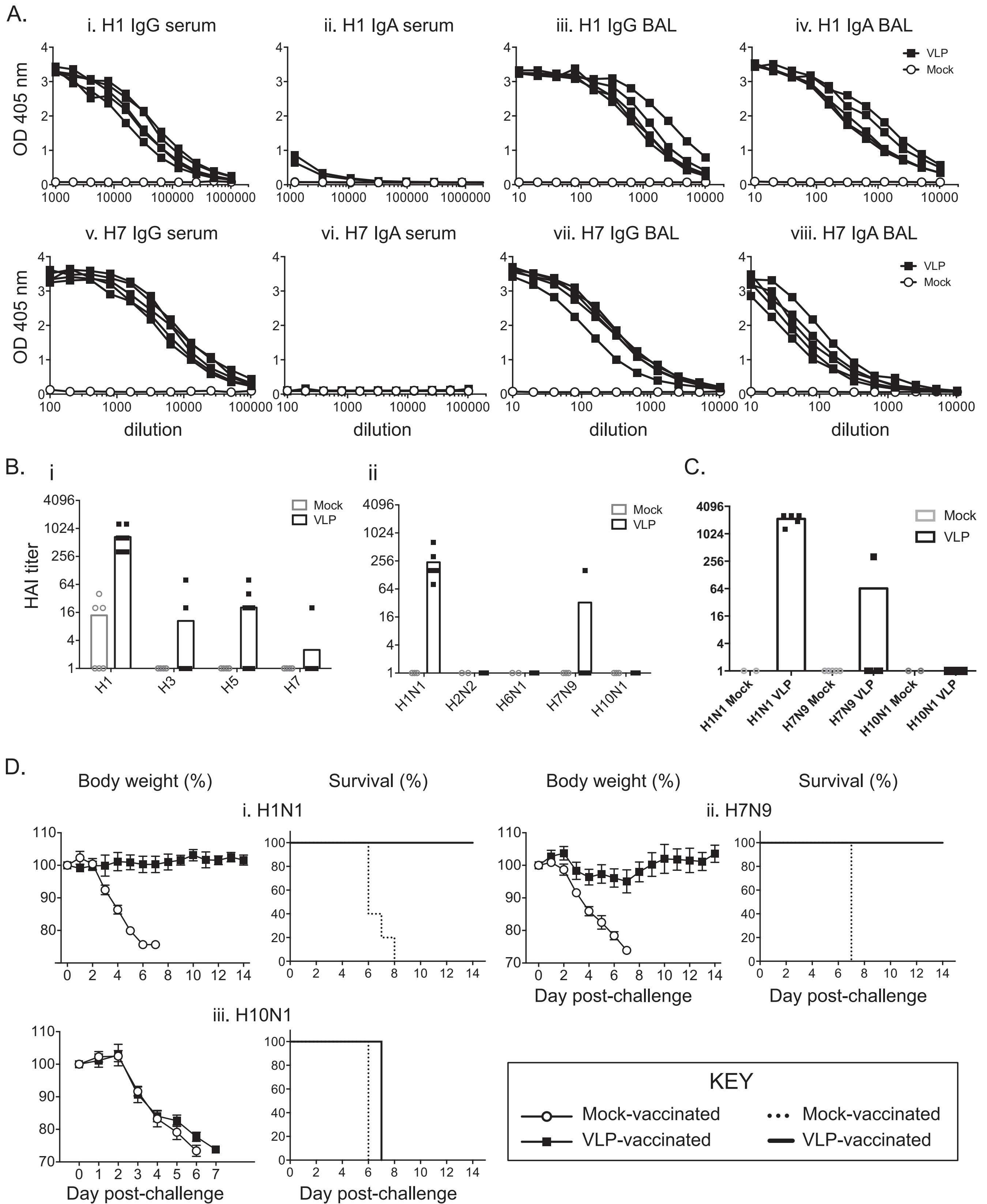


FIG 4 Induction of antibodies after VLP vaccination. (A) Antibody levels in serum from mock-vaccinated (open circles) or VLP-vaccinated (solid squares) mice were evaluated from serum or bronchoalveolar lavage (BAL) fluid. Antibody binding was measured by ELISA with serial 1:2 dilutions of serum (left two panels, i, ii, v, and vi) or BAL fluid (right two panels, iii, iv, vii, and viii). Influenza virus HA-VLP-specific IgG (i, iii, v, and vii) or IgA (ii, iv, vi, and viii) titers to either homologous H1 (top panels, i, ii, iii, and iv) or intrasubtypic heterologous H7 (bottom panels; v, vi, vii, and viii) HA was measured with VLP-coated plates.

(Continued)

different H5N1 clades (38). A few instances of heterosubtypic protection have been reported: for example, protection from H5N1 influenza challenge following 1918 H1N1 influenza VLP vaccine (expressing both the 1918 HA and NA glycoproteins) when administered intransally but not when administered intramuscularly (29).

If the preliminary findings described here with the VLP vaccine cocktail are confirmed in ferrets and subsequent human studies, a straightforward pathway to developing effective vaccines against both currently circulating influenza viruses and also future pandemic viruses can be envisioned. The VLP cocktail vaccination approach described here should allow rapid switching or updating of influenza virus antigens in vaccine cocktails. As noninfectious vaccines, they should have a good safety profile. Importantly, the vaccine cocktail approach should also offer relative freedom from antigenic competition in elicitation of immune responses. For example, HA immunogenicity has been shown to outcompete NA immunogenicity in the context of viral or VLP coexpression (18, 39). In future experiments, in which NA VLPs are added to the HA VLP cocktail, it is hypothesized that NA antibody responses will be enhanced compared to conventional vaccination strategies. These are all traits desirable in a "universal" influenza vaccine—that could be used for individual protection, in vaccine prevention efforts in large populations, and for stockpiling for future pandemic responses.

MATERIALS AND METHODS

VLP production. Hemagglutinin segments of A/South Carolina/1/1918 (H1N1), A/pintail/Ohio/339/1987 (H3N8), A/mallard/Maryland/802/2007 (H5N1), and A/Environment/Maryland/261/2006 (H7N3), along with the A/New York/312/2001 (H1N1) M1 gene, were cloned into the pFastBac expression vector immediately downstream of the polyhedron promoter. VLPs were produced by the NCI Protein Expression Laboratory (Frederick, MD) or by Medigen Laboratories (Frederick, MD) using standard baculovirus expression systems (29, 40, 41). Two separate recombinant baculoviruses containing HA and M1 genes were used to produce the H1 VLP. A single recombinant baculovirus containing both open reading frames of the HA and M1 genes was used to produce the H3, H5, and H7 VLPs. Cellular debris was removed by centrifugation of the supernatants at 2,000 relative centrifugal force (RCF) for 10 min. Clarified VLP supernatants were concentrated by ultracentrifugation at 100,000 RCF for 2 h, and total protein was purified using a discontinuous sucrose gradient from 20% to 60%. HA-containing fractions were confirmed by HA assay and centrifuged at 100,000 RCF for 2 h. The Bradford bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) was used to quantify total protein. VLP preparations were analyzed by negative-staining transmission electron microscopy (TEM) to verify that the produced VLPs had a morphology similar to that of influenza virions.

Challenge viruses. A standard reverse genetics-based system was utilized to produce influenza A viruses. Isogenic chimeric viruses were rescued using the PB1, $PB2^{E627K}$, PA, NP, NA, M, and NS gene segments from influenza A/Green Wing Teal/Ohio/175/1986 (H2N1), along with the H6, H7, or H10 HA segments as previously described (42). Chimeras were also produced using HA segments from A/South Carolina/1/1918 (H1N1) and A/Japan/305/1957 (H2N2), as well as a laboratory variant of A/Green Wing Teal/Ohio/340/1987 (H11N9) (42). Wild-type (WT) influenza viruses A/Anhui/1/2013 (H7N9) and A/Vietnam/1203/1204 (H5N1) were also used as challenge viruses. All WT and chimeric viruses were passaged 1 to 2 times in Madin-Darby canine kidney (MDCK) cells. Viruses were sequence verified, and titers were determined using plaque assays, as described previously (43). Both viruses and samples were handled under biosafety level 3 enhanced laboratory (BSL3+) conditions (42). Influenza A/H5N1 virus and infectious samples were handled under BSL3+ conditions in accordance with the Select Agent guidelines of the National Institutes of Health (NIH), the Centers for Disease Control and Prevention (CDC), and the United States Department of Agriculture (USDA).

Mouse vaccinations and infections. Mouse 50% lethal dose (MLD_{50}) determinations were performed on 8- to 9-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) to assess murine pathogenicity of chimeric and WT viruses as previously described (42). MLD_{50} determinations were also performed on 15-week-old female BALB/c mice for a subset of viruses and found to be very similar to values for 8- to 9-week-old mice. For vaccination studies, 8- to 9-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) ($n = 5$ to 10 per treatment group) were vaccinated intranasally (i.n.) with a cocktail of H1, H3, H5, and H7 VLPs (1.5 μg total protein per VLP, total of 6 μg protein) diluted in total in 50 μl of PBS at day 0. Mice were observed daily and boosted i.n. with an identical 6-μg cocktail dose at day 21. Cohorts of mock-vaccinated mice were vaccinated and boosted i.n. alongside vaccinated mice with 50 μl phosphate-buffered saline (PBS). For evaluation of vaccine efficacy in aged mice, 37-week-old BALB/c female mice were vaccinated and boosted i.n. at days 0 and 21. Mice were challenged on day 50 with 10× MLD_{50} of virus diluted in 50 μl of Dulbecco's modified Eagle's medium (DMEM) i.n. To assess longevity of protection, mice were housed for an additional 6 months prior to challenge. All experimental animal work was performed in accordance with United States Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals in an enhanced animal BSL3 (ABSL-3+) laboratory at the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) following approval of animal safety protocols by the NIAID Animal Care and Use Committee (42). Influenza A/H5N1 virus and infectious samples were handled under ABSL3+ conditions in accordance with the select agent guidelines of the NIH, CDC, and USDA. Survival and body weight were monitored for 14 days, and mice were humanely euthanized if more than 25% of initial body weight was lost. Survival and mean time to death were analyzed by Kaplan-Meier survival analysis (Graph Pad Prism, La Jolla, CA). Differences in the percentages of weight loss nadirs, lung virus titers, and antibody responses were assessed using the Mann-Whitney test. Mean differences were considered statistically significant if P was <0.05. Mouse lungs were collected for viral titrations from H6N1-, H10N1-, and Anhui H7N9-infected animals on day 3 postinfection. Viral titers were determined for each lung sample as previously described (42).

ELISA, hemagglutination inhibition, and microneutralization assays. Serum was obtained from cheek bleeds at day 30 or day 50 postvaccination. Bronchoalveolar lavage (BAL) fluid samples were obtained at day 30 postvaccination by flushing with 1 ml of 1× sterile PBS with EDTA.

Figure Legend Continued

Results are reported as absorbance (optical density [OD]) at 405 nm. Each curve represents the binding profile from one mouse. (B) Functional HA binding of antibodies in sera was measured by hemagglutination inhibition (HAI) assay against HA-VLPs (i) or a variety of influenza A viruses (ii). The titer is represented as the reciprocal of the highest dilution that resulted in inhibition of hemagglutination. (C) Neutralization titers in serum of mock- or VLP-vaccinated mice were measured by microneutralization (MN) assays against homologous H1N1 virus, intrasubtypic heterologous H7N9 virus, or heterosubtypic H10N1 virus. The titer is displayed as the reciprocal of the highest neutralizing dilution. (D) Passive transfer experiments, whereby serum was transferred intraperitoneally (i.p.) to naive mice 1 day prior to lethal challenge (10× MLD_{50}). Mice were challenged with the homologous 1918 H1N1 (i), intrasubtypic heterologous H7N9 (ii), or heterosubtypic H10N1 influenza virus (iii). Body weight (left panels) and survival (right panels) were monitored for 14 days postchallenge ($n = 5$ per group).

For the ELISAs, Maxisorp ELISA plates (Sigma-Aldrich, St. Louis, MO) were coated with 2 μg/ml H1 or H7 VLP and blocked in 1% bovine serum albumin (BSA) in PBS for 90 min each at 37°C. Plates were then incubated with diluted serum samples overnight at 4°C. Binding was visualized by incubation with goat anti-mouse IgG (Southern Biotech, Birmingham, AL) for 1 h at 37°C and addition of phosphatase substrate (Sigma-Aldrich). Color was read at 405 nm on a SynergyHT plate reader (BioTek, Winooski, VT). Between each step, plates were washed with 0.1% Tween 20 in PBS. Hemagglutination inhibition (HAI) titers were determined using standard methods as previously described (44). Briefly, receptor-destroying enzyme (RDE II; Denka Seiken, Tokyo, Japan)-treated serum was serially diluted and incubated with 8 HA units of virus for 15 min at room temperature. HAI titers were measured by addition of 0.5% turkey red blood cells and observing agglutination. Influenza microneutralization assays were performed as previously described (44). Briefly, diluted serum samples were incubated with $10^{3.3}$ tissue culture infective doses ($TCID_{50}$)/ml virus for 1 h at room temperature prior to incubation with MDCK cells supplemented with 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin. Cytopathic effect and HA titers were used to determine the neutralization titer at 96 h postinfection. All data are representative of two or more separate experiments.

Passive immunization. For passive serum transfer experiments, VLP- or mock-vaccinated mice were terminally bled on day 50 postvaccination. Serum was collected, and naive mice were given 100 to 300 μl serum intraperitoneally (i.p.) 1 day prior to challenge. Mice were challenged with lethal doses of viruses and observed for 14 days, as described above.

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J.K.T. conceived the project. L.M.S., A.L.C., L.M.P., L.Q., J.C.K., and J.K.T. performed the experiments. L.M.S., A.L.C., L.M.P., J.C.K., and J.K.T. analyzed data. J.K.T., L.M.S., and A.L.C. wrote the manuscript, with all authors contributing to the editing and providing advice.

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