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Kinetics of Immune Responses to Influenza Virus-Like Particles and Dose-Dependence of Protection with a Single Vaccination▽

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The format of influenza virus-like particles (VLPs) as a nonreplicating particulate vaccine candidate is a promising alternative to conventional egg-based vaccines. In this study, we have investigated the detailed kinetics of immune responses and protective efficacy after a single intranasal immunization with different doses of VLPs alone or in the presence of an *Escherichia coli* mutant heat-labile enterotoxin [mLT(R192G)] or cholera toxin subunit B as adjuvants. Analysis of immune responses showed differential kinetics in a VLP antigen dose-dependent manner and dynamic changes in the ratios of antibody immunoglobulin G isotypes over the time course. Protection against lethal challenge was observed with a single immunization with influenza VLPs even without adjuvant. The addition of adjuvant showed significant antigen-sparing effects with improved protective efficacy. The protective immune responses, efficacies of protection, and antigen-sparing effects were significantly improved by a second immunization as determined by the levels of neutralizing antibodies, morbidity postchallenge, lung viral titers, and inflammatory cytokines. Our results are informative for a better understanding of the protective immunity induced by a single dose or two doses of influenza VLPs, which is dependent on antigen dosage and the presence of adjuvant, and will provide insights into designing effective vaccines based on VLPs.

Influenza, caused by a lipid-enveloped RNA virus, is among the most devastating human and animal diseases due to the ease that it is spread as an aerosol and its ability to cause severe mortality in a susceptible host. Vaccination is a potent and cost-effective means of controlling and preventing influenza infections. Licensed influenza vaccines are chemically inactivated whole virus or detergent-treated split forms of the viral surface antigens or a live, attenuated influenza virus vaccine (FluMist).

Currently licensed influenza vaccines produced using fertilized chicken egg substrates are partially protective, particularly in the very young and the elderly populations. Regarding the live attenuated vaccine, there are concerns related to the reversion of attenuated vaccine strains or recombination and the uncertainties of their pathogenic characteristics, particularly when used for highly pathogenic avian influenza viruses with pandemic potential. In addition, immunization with a live influenza vaccine is restricted to healthy individuals and not recommended for high-risk populations such as very young children or the elderly. Local or systemic allergic reactions to vaccine components can occur in some individuals due to residual egg proteins incorporated into the vaccines (9, 13).

Influenza virus-like particles (VLPs) have been demonstrated to be a promising alternative candidate to egg-based influenza vaccines. The noninfectious nature of VLPs and their lack of viral genomic material are attractive safety features that can be suitable for repeated administrations and for use in diverse populations, including high-risk groups. The self-assembled macrostructure of VLPs can present conformational epitopes of surface proteins to the immune system comparable to those of live virions. Recent studies demonstrated that intranasal or intramuscular immunizations of mice with influenza VLPs containing hemagglutinin (HA) or HA and neuraminidase induced antibodies specific to the vaccine strains and provided immunized animals with protection against lethal infections (2, 3, 7, 17, 22, 23, 25, 26).

Immunization with influenza VLPs via the respiratory route may directly stimulate the mucosal immune response at the site of pathogen entry where it is most needed to impede viral infection. It is also suggested that intranasal delivery is superior to systemic immunization in inducing cross protection (31–33). Studies of influenza VLPs as a vaccine candidate are still in an early developmental stage, and there is no detailed study of the kinetics of inducing virus-specific immune responses and protective efficacy after intranasal immunization with a single dose or two doses of influenza VLPs. Although limited previous studies demonstrated immune responses after one or two systemic vaccinations with inactivated whole virus or split vaccines (11, 12, 30), the HA dose-sparing effects on the kinetics of immune responses including isotypes of antibodies, functional antibodies, and protective efficacy, including lung viral titers and inflammation after lethal infection, remain largely unknown after mucosal vaccination. VLPs containing influenza M1 alone did not induce protective immune responses (25). Thus, influenza VLPs provide a unique tool to

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TABLE 1. HA content in influenza VLPs[a]

Vaccine	Protein (mg/ml)	HA titers (±SD) in VLPs or virus	Ratio of HA titers/ μg protein (±SD)	Ratio of HA/total protein (μg) (±SD)[b]
Influenza VLPs	3.5	40,960 ± 8,865	234 ± 52	0.10 ± 0.02
Inactivated A/PR8	4	138,600	693	0.29

[a] HA titers are shown in geometric mean values from three independent experiments of influenza VLPs (3.5 mg/ml).
[b] HA content was estimated based on the observation that influenza virus HA makes up approximately 29% of the total protein of purified virus (33).

study the HA dosage effects on inducing protective immunity since HA is the major protective antigen in VLP vaccines. It is hypothesized that a single dose of nonreplicating influenza VLPs containing HA as a major antigen induces functional antibodies contributing to protective immunity against lethal infection in an HA dose-dependent manner.

We have investigated the kinetics of antibody induction after priming with influenza VLPs, the protective immunity after a single immunization, the effects of VLP antigen dose on inducing protective immunity in the absence or presence of an adjuvant, and the impact of a second immunization in improving the quality of protection. Protection (100%) was observed with a single dose of influenza VLPs even in the absence of adjuvant. We found differential kinetics of antibody induction, dynamic changes in antibody isotypes, and qualitative differences in efficacies of protection in an HA dose- and adjuvant-dependent manner. Our results are very informative for a better understanding of the quality of protection by a single dose versus two intranasal immunizations with influenza VLPs.

MATERIALS AND METHODS

Virus and cells. Influenza virus A/PR/8/1934 (H1N1, abbreviated as A/PR8) was grown in 10-day-old embryonated hen's eggs and purified from allantoic fluid by using discontinuous sucrose gradient (15%, 30%, and 60%) layers. The purified virus was inactivated by mixing the virus with formalin at a final concentration of 1:4,000 (vol/vol) as described previously (19, 24, 27). Inactivation of the virus was confirmed by plaque assay on confluent monolayers of Madin-Darby canine kidney (MDCK) cells and inoculation of the virus into 10-day-old embryonated hen's eggs. For use in challenge experiments, mouse-adapted A/PR8 was prepared as lung homogenates of infected mice. *Spodoptera frugiperda* Sf9 cells were maintained in suspension in serum-free SF900II medium (Gibco-BRL) at 27°C in spinner flasks at a speed of 70 to 80 rpm. MDCK cells were grown and maintained in Dulbecco's modified Eagle's medium.

Preparation of influenza VLPs. Influenza VLPs containing A/PR8 H1 HA and M1 were prepared as described previously (25). A baculovirus (BV) transfer vector, pFastBac plasmid, and recombinant Bacmid BV DNAs (rAcNPV) containing PR8 HA isolated from transformed DH10Bac cells were used to transfect the Sf9 insect cells. Sf9 insect cells were coinfected with recombinant BVs expressing HA and M1 at a multiplication of infection of 2 and 1, respectively, and culture supernatants containing released VLPs were harvested after 2 to 3 days of culture postinfection. After removing cell debris by low-speed centrifugation (2,000 × *g* for 20 min at 4°C), VLPs in cleared culture supernatants were purified by 20%, 30%, and 60% sucrose layer-gradient ultracentrifugation. Characterization of influenza VLPs containing A/PR8 HA was performed by Western blot analysis using mouse polyclonal antibodies against A/PR8 virus as previously described (25). HA contents in purified influenza VLPs were estimated by a hemagglutination activity assay in comparison with inactivated A/PR8 virus (Table 1). Influenza VLPs used in this study contained approximately 0.1 μg HA per 1 μg of total protein of VLPs (~10%), which is a level similar to that previously described (26).

Immunization and challenge. Female inbred BALB/c mice (Charles River), 6 to 8 weeks old, were used. Groups of mice (12 mice per group) were intranasally immunized with VLPs containing 0.02 μg, 0.1 μg, or 0.5 μg HA or in combination with adjuvant (3 μg) as a single-dose regimen. Either *Escherichia coli* mutant heat-labile enterotoxin [mLT(R192G)] as described previously (33) or cholera toxin subunit B (CTB; Sigma-Aldrich) was used as adjuvant (3 μg). Another set of mice was immunized two times with the same dose of VLPs in the presence or absence of adjuvant (weeks 0 and 4) in 50 μl of phosphate-buffered saline (PBS) buffer. For virus challenge, isoflurane-anesthetized mice were intranasally infected with 1,000 PFU of A/PR8 virus (20× the 50% lethal concentration [LD_{50}]), in 50 μl of PBS per mouse at week 4 after the first or second immunization. For measurement of immune response parameters, six mice from each group were sacrificed prior to challenge or at day 4 postchallenge. Mice were observed daily to monitor changes in body weight and to record mortality (25% loss in body weight). We followed an approved Emory IACUC protocol for this study.

Antibody responses and virus neutralizing activities. Blood samples were collected by retro-orbital plexus puncture before immunization, at days 2, 4, 7, 10, 14, 20, and 28 postimmunization for a single immunization schedule, and at 2 weeks after the second (boost) immunization for the two-dose immunization schedule. Influenza virus-specific immunoglobulin G (IgG), IgG1, IgG2a, and IgA antibodies (isotypes) were determined in sera by enzyme-linked immunosorbent assay (ELISA) as described previously (25). As coating antigens to measure virus-specific antibodies, egg-grown inactivated influenza virus (A/PR8) was coated onto 96-well microtiter plates (Nunc Life Technologies, Rochester, NY) with 100 μl in coating buffer (0.1 M sodium carbonate, pH 9.5, 4 μg inactivated A/PR8 virus per milliliter) at 4°C overnight. Serum neutralizing activities were also determined in immunized mouse sera collected from a single immunization and two immunizations following a procedure previously described (25).

HAI. For determination of hemagglutination inhibition (HAI) titers, serum samples were first treated with receptor-destroying enzyme (Denka Seiken Co., LTD, Tokyo, Japan) by incubation overnight at 37°C and then incubated 30 min at 56°C. Sera were serially diluted twofold in 25 μl PBS, and 4 hemagglutination units of influenza A/PR8 virus was used in a volume of 25 μl. The contents of each well were gently mixed with a micropipettor, and then the plates were incubated for 30 min at room temperature. Finally, 50 μl of a 0.5% chicken erythrocyte suspension was added to each well. The highest serum dilution capable of preventing hemagglutination was scored as the HAI titer. Presented data are the geometric means with standard deviation from three independent replicate experiments.

Lung viral titers and cytokine assays. The whole-lung extracts prepared as homogenates using frosted glass slides were centrifuged at 1,000 rpm for 10 min to collect supernatants. Determination of viral titers in lung extracts was performed using MDCK cells as previously described (25). The cytokine ELISA was performed as described previously (25). Ready-Set-Go interleukin 6 (IL-6) and gamma interferon kits (eBioscience, San Diego, CA) were used for detecting cytokine levels in lung extracts following the manufacturer's procedures.

Statistics. All parameters were recorded for individuals within all groups. Statistical comparisons of data were carried out using the correlation and regression test of the PC-SAS system (SAS Institute Inc., Cary, NC). A P value of less than 0.05 was considered significant.

RESULTS

Kinetics of virus-specific IgG antibody responses after a single immunization. Most previous vaccine studies demonstrated the immune responses and/or protection of vaccine candidates including influenza VLPs after a two-dose prime-boost immunization scheme (2, 3, 7, 17, 22, 25, 26). Particularly, influenza VLPs were suggested to be a promising candidate as a new generation of vaccine format. However, the immune kinetics and protective immunity after a single immunization remain largely unknown. To determine the effects of influenza VLP dosage on inducing protective immunity and effects of adjuvants on antigen sparing, groups of mice (n = 12) were intranasally immunized with different doses of influenza VLPs alone (0.02 μg, 0.1 μg, 0.5 μg in terms of HA amounts) or in the presence of an *E. coli* mLT(R192G) or CTB adjuvant. We determined the kinetics of inducing virus-specific antibody responses in immune sera collected at different time points

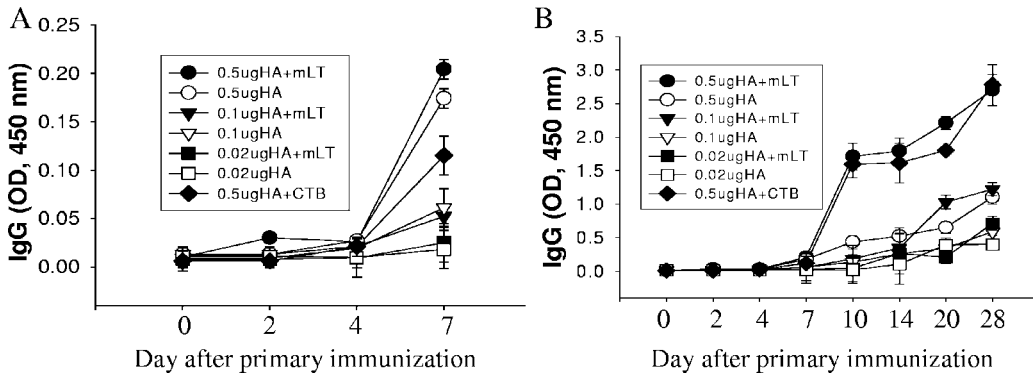

FIG. 1. Kinetics of virus-specific IgG responses upon primary intranasal immunization. (A) Kinetics of IgG responses at days 2, 4, and 7 in an expanded optical density scale. (B) Kinetics of IgG responses at days 2, 4, 7, 10, 14, 20, and 28. Mice (12 per group) were intranasally immunized with VLPs containing 0.02 μg, 0.1 μg, and 0.5 μg HA or in combination with mLT(R192G) (3 μg) or CTB (3 μg). Blood samples were collected at day 2, 4, 7, 10, 14, 20, and 28 postimmunization and diluted 100-fold before analysis. Optical densities were read at 450 nm (OD_{450}), results are expressed as the arithmetic mean (OD_{450}), and error bars indicate the standard deviation. Significant differences were observed between groups depending on doses and on adjuvant. Comparisons by dosage are as follows: with mLT(R192G), 0.5 μg HA versus 0.1 μg HA ($P < 0.0001$) and 0.1 μg HA versus 0.02 μg HA ($P = 0.02$); without mLT(R192G), 0.5 μg HA versus 0.1 μg HA ($P < 0.0001$) and 0.1 μg HA versus 0.02 μg HA ($P = 0.004$). Comparisons by adjuvant are as follows: 0.5 μg HA plus mLT(R192G) versus 0.5 μg HA ($P = 0.0002$), 0.1 μg HA plus mLT(R192G) versus 0.1 μg HA ($P = 0.003$), and 0.02 μg HA plus mLT(R192G) versus 0.02 μg HA ($P = 0.028$). mLT in the legend indicates mLT(R192G).

from mice that received a single immunization with influenza VLPs using ELISA plates coated with inactivated A/PR8 virus. At day 7 postimmunization, virus-specific IgG antibodies were clearly detectable in mice from the 0.5-μg-HA group containing VLP with or without adjuvant (Fig. 1A). IgG titers in low-dose groups (0.1 μg, 0.02 μg) were not evident at this early day 7 time point. By day 10, the 0.5-μg-HA VLPs alone or 0.5-μg-HA VLPs plus adjuvant [mLT(R192G) or CTB] groups showed significantly enhanced levels of virus-specific IgG antibodies (Fig. 1B). At 3 and 4 weeks after a single immunization, all low-dose groups induced detectable levels of antibodies specific to the virus; the 0.1-μg-HA group exhibited higher levels than the 0.02-μg-HA group. The mLT(R192G) adjuvant induced approximately fivefold or higher increases in IgG antibody levels in the 0.5-μg-HA group by day 7, in the 0.1-μg-HA group by day 20, and in the 0.02-μg-HA group by day 28, respectively (Fig. 1B).

We also found that there were good correlations between VLP dosage and PR8-specific IgG responses (Fig. 2). It is interesting to note that the correlation slope for the adjuvant groups was higher (0.244; $P = 0.02$) than that for the groups without adjuvant (0.162; $P = 0.01$), which indicates that dosage responses were greater for adjuvant groups. Supporting this correlation slope, as shown in Table 2, are higher fold increases in antibodies induced in the groups containing adjuvant after the boost immunization. Overall, these results provide evidence that immune responses with differential kinetics were induced after a single immunization with nonreplicating influenza VLPs, depending on both the antigen dosage and the presence of an adjuvant in a mouse model.

Dynamic changes in the ratios of IgG1 and IgG2a isotypes. Types of $CD4^+$ T helper cell responses are known to determine the pattern of antibody isotypes. However, it has not been investigated whether there are kinetic differences among isotypes induced by intranasal immunization with nonreplicating influenza VLPs. IgG2a isotype antibodies in mice intranasally immunized with influenza VLPs appeared at an earlier time (day 10 to 14) than IgG1 antibodies that were observed on day 28 at detectable levels (Fig. 3A). Therefore, the ratio of IgG2a/IgG1 was dynamic reaching a peak at day 14 and then slightly decreased thereafter (Fig. 3D). The delayed induction of IgG1 antibodies was similar to that observed in mice intramuscularly immunized with a single dose of 15 μg of split influenza vaccine (11, 30). The addition of either mLT(R192G) or CTB significantly increased the levels of IgG2a by fourfold (Fig. 3B and C). The mLT(R192G) adjuvant showed a 3- to 7-day lag time, whereas CTB extended the lag time for inducing IgG1 antibodies to approximately 20 days, suggesting that mLT(R192G) stimulates the production of IgG1 antibody compared to CTB. These findings are significant for elucidat-

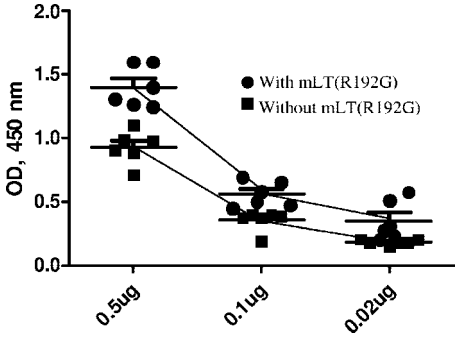

FIG. 2. Correlation between the VLP antigen dosage and virus-specific immune responses. Blood samples at week 2 after a single immunization were used for analysis of dose-dependent virus-specific IgG responses with or without mLT(R192G) adjuvant. Linear regression of dose-dependent IgG responses showed significant correlations between the VLP antigen dosage and virus-specific immune responses as represented by optical densities at 450 nm (OD_{450}). With mLT(R192G) adjuvant, IgG (OD_{450}) equals 0.2145 plus 0.244 times the Ag dose (μg) ($P = 0.0225$). Without adjuvant, IgG (OD_{450}) equals 0.132 plus 0.162 times the Ag dose (μg) ($P = 0.0115$).

TABLE 2. IgG and IgA antibody titers from the first or second immune serum[a]

Vaccine group (μg)[b]	Titers (10^3) (±SD) of first immune serum		Titers (10^3) (±SD) of second immune serum (fold increase)[c]	
	IgG	IgA	IgG	IgA
HA (0.5)+ mLT	26 ± 2	1.6 ± 0.2	410 ± 50 (16)	52 ± 6 (31)
HA (0.5)	13 ± 1	0.4 ± 0.05	102 ± 20 (8)	3.2 ± 0.4 (8)
HA (0.1)+ mLT	6.4 ± 0.5	0.4 ± 0.04	154 ± 20 (24)	20 ± 2 (47)
HA (0.1)	1 ± 0.4	0.2 ± 0.02	6.4 ± 0.5 (6)	0.4 ± 0.05 (2)
HA (0.02)+ mLT	1.6 ± 0.1	0.6 ± 0.03	51 ± 6 (32)	3.2 ± 0.4 (5)
HA (0.02)	0.4 ± 0.05	0.2 ± 0.02	6 ± 0.5 (16)	0.4 ± 0.05 (2)
HA (0.5)+ CTB	26 ± 3	0.8 ± 0.05	205 ± 15 (8)	25 ± 3 (31)
Naïve	0.1 ± 0.02	0.2 ± 0.02		

[a] IgG and IgA antibody titers specific to influenza virus A/PR8 were determined at week 2 after a single immunization and after two immunizations. Titers are expressed as the highest dilution of serum having a mean optical density at 450 nm greater than the mean plus two standard deviations of similarly diluted naïve serum samples.
[b] Groups of mice are as described in the legend of Fig. 1. mLT, mutant LT(R192G).
[c] Fold increase in antibody titers after the second immunization compared to that after the single-dose immunization.

TABLE 3. HAI titers against A/PR8/34 from a single immunization[a]

Vaccine group (μg)[b]	Wk 2 after priming		Day 4 after challenge	
	HAI titers (±SD)	Log_2 (±SD)	HAI titers (±SD) (fold increase)	Log_2 (±SD)
HA (0.5)+mLT	24 ± 2	4.6 ± 1	100 ± 2 (4.2)	6.6 ± 1
HA (0.5)	24 ± 2	4.6 ± 1	72 ± 2 (3)	6.2 ± 1.5
HA (0.1)+mLT	24 ± 1.5	4.6 ± 0.6	48 ± 2 (2)	5.6 ± 1
HA (0.1)	18 ± 2	4.2 ± 1	36 ± 3 (2)	5.2 ± 1
HA 0.02)+mLT	18 ± 2	4.2 ± 1	40 ± 4 (2.2)	5.3 ± 1
HA (0.02)	12 ± 1.5	3.6 ± 0.6	18 ± 2 (1.5)	4.2 ± 1
HA (0.5)+CTB	24 ± 2	4.6 ± 1	96 ± 2 (4)	6.6 ± 1
Naïve	12 ± 1	4.2 ± 0		

[a] HAI titers against A/PR8/34 virus at week 2 after a single immunization or at day 4 after challenge infection. Data are from three different experiments.
[b] Groups of mice are as described in the legend of Fig. 1. mLT, mutant LT(R192G).

ing the dynamic features of antibody isotypes and indicate that VLP antigens are likely to induce IgG2a isotype antibodies, an indication for T helper type 1 (Th1) responses.

Induction of HAI and neutralizing activities. Induction of functional antibodies such as HAI and neutralizing antibodies in immune sera were determined since these tests are widely accepted as indicators for protection. Low HAI titers were detected in immune sera after priming (Table 3) and were maintained over the time points examined, with titers ranging from 24 [0.5 μg HA with or without adjuvant, 0.1 μg HA plus mLT(R192G)] to 18 [0.1 μg HA, 0.02 μg HA plus mLT(R192G)]. Importantly, within day 4 postchallenge, HAI titers rapidly increased by two- to fourfold except for the lowest group (0.02 μg HA containing VLPs) (data not

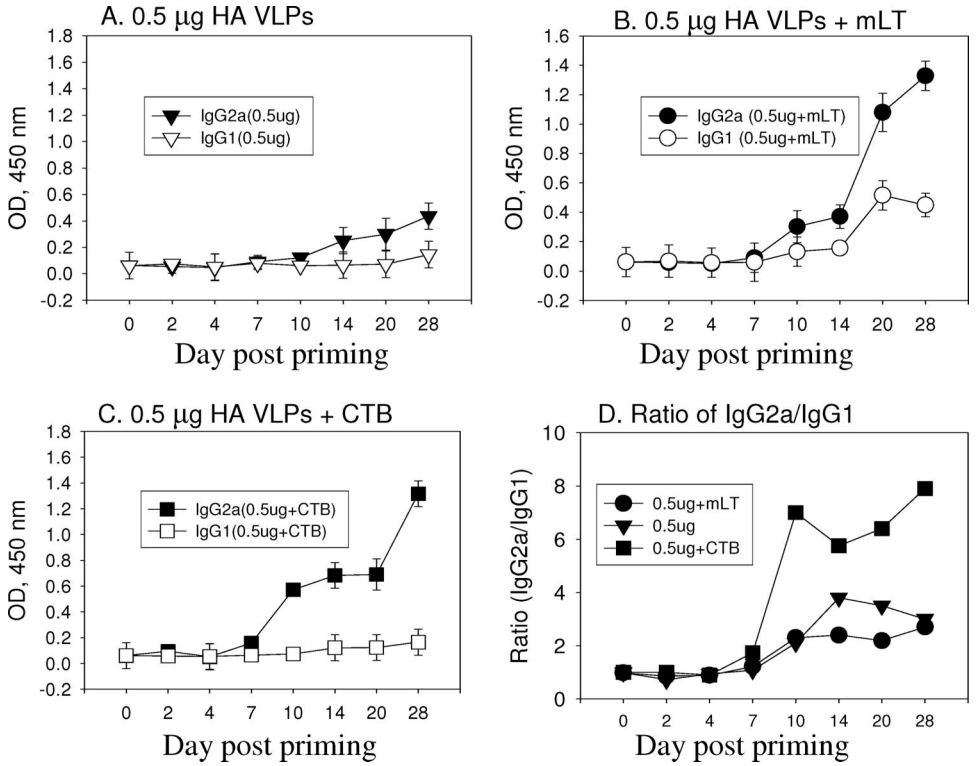

FIG. 3. Kinetics of IgG2a and IgG1 responses and dynamic changes in IgG2a/IgG1 ratios after a single immunization. IgG2a and IgG1 titers after immunization with 0.5 μg HA containing VLPs (A), 0.5 μg HA plus mLT(R192G) (B), and 0.5 μg HA plus CTB (C), respectively. (D) Ratio of IgG2a/IgG1 from groups with 0.5 μg HA and 0.5 μg HA plus mLT(R192G) or CTB. Serum samples from the groups with 0.5 μg HA and 0.5 μg HA plus mLT(R192G) or CTB were used for determination of kinetics of IgG2a, IgG1, and the ratio of IgG2a/IgG1 at days 2, 4, 7, 10, 14, 20, and 28. Optical densities were read at 450 nm (OD_{450}), results are expressed as the arithmetic mean (OD_{450}), and error bars indicate standard deviation.

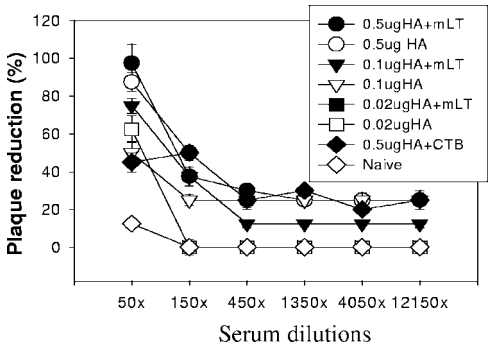

FIG. 4. Neutralizing activities against influenza virus A/PR8/34 after a single immunization. Sera at day 14 after a single immunization were used to determine neutralizing activities. Neutralizing activities were expressed as percentage of plaque reduction compared to that of naïve serum control. mLT(R192G) is denoted as mLT in the legend.

shown), indicating effective priming in VLP-immunized groups.

For neutralizing activity analysis, dilutions giving 40% plaque reduction were compared since 40% reduction in plaque formation was considered to be significant compared to 0% plaque reduction in naïve control sera (Fig. 4). Groups of mice that received 0.5 μg HA with or without adjuvant or 0.1 μg HA plus mLT(R192G) showed 40% neutralizing titers at a dilution of 1:150, whereas we observed neutralizing titers at a dilution of 1:50 in the low-dose groups of mice (0.1 μg HA alone, 0.02 μg HA with or without adjuvant) after a single-dose delivery via the intranasal route. These results suggest that functional antibodies were induced by a single immunization with a nonreplicating VLP vaccine, depending on the HA dose in VLPs and the presence of adjuvant.

Protective efficacy with a single immunization. The protective efficacy after a single intranasal immunization with a nonreplicating influenza VLP vaccine in a naïve host is not well known. To determine the protective efficacy of a single immunization, immunized mice were challenged with a lethal dose of A/PR/834 (20× LD_{50}) at 4 weeks postimmunization (Fig. 5A and B). Groups of mice immunized with 0.5 μg HA with or without adjuvant or 0.1 μg HA plus mLT(R192G) were completely protected against a lethal challenge infection with no or minimal clinical illness, showing a fivefold antigen-sparing effect by the addition of the adjuvant. Approximately 5 to 10% losses in body weight were transiently observed in the group with 0.1 μg HA plus mLT(R192G). All naïve mice died by day 7 postinfection. Mice in the low-dose groups with 0.02 μg HA plus mLT(R192G) and 0.1 μg HA showed 66% and 33% protection, respectively, and surviving mice experienced a transient body weight loss of 10 to 20%. Mice in the 0.02-μg-HA group displayed severe illness, resulting in over 25% body weight loss, and had to be euthanized. Significant levels of virus-specific IgA antibodies were induced in the lungs of mice in all VLP-immunized groups at day 4 postchallenge (data not shown). Particularly, the 100% protected groups [0.5 μg HA with or without adjuvant, 0.1 μg HA plus mLT(R192G)] showed 5- to 50-fold-higher lung IgG antibody titers than those in partially protected groups (0.02 μg HA with or without adjuvant, 0.1 μg HA) (data not shown). These results from challenge studies indicate that a single dose of 0.5 μg HA containing VLPs alone or 0.1 μg HA containing VLPs plus mLT(R192G) adjuvant could provide complete protection against lethal infection and that protection had a moderate correlation with the induction of HAI and neutralizing antibodies even at low levels.

Immune responses after the second immunization. Single immunizations with low-dose VLPs (0.02 μg HA with or without adjuvant, 0.1 μg HA) did not confer protection against a lethal challenge infection, and surviving mice suffered severe illness. Another group of primed mice was boosted intranasally with the same dose of influenza VLPs with or without adjuvant at 4 weeks after the first immunization. We determined virus-specific IgG and IgA antibody titers in serum samples at week 2 post boost immunization (Table 2). The group of mice immunized with 0.5 μg HA plus adjuvant [mLT(R192G) or CTB] showed the highest levels of IgG antibodies, increasing approximately 8- to 16-fold in IgG titers and 31-fold in IgA antibodies. The highest increases of 24- to 32-fold in IgG antibody

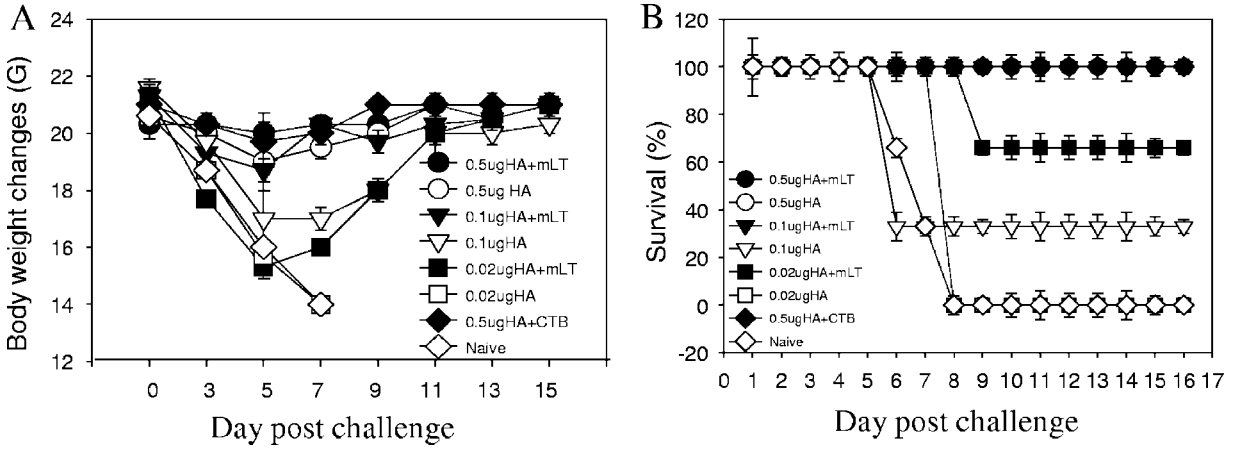

FIG. 5. Protection against lethal dose of influenza virus PR8 (H1N1) challenge after a single immunization with VLPs. (A) Body weight changes (grams [G]). (B) Survival rates (%). Groups of mice are as described in legend to Fig. 1. At week 4 after a single immunization, naïve and immunized mice were intranasally infected with a lethal dose of mouse-adapted A/PR8 (20× LD_{50}). Mice were monitored daily to determine the body weight changes as an indicator of morbidity and the percentage of mortality rates. mLT, mutant LT(R192G).

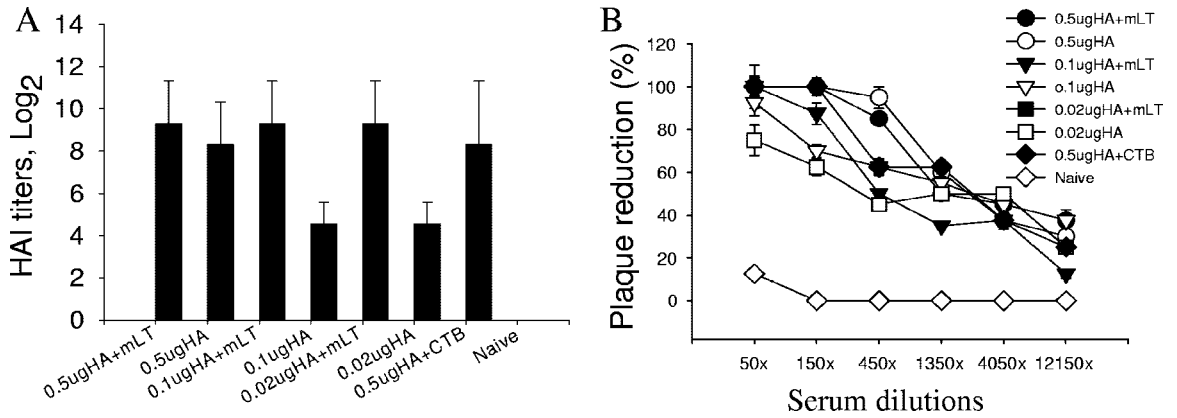

FIG. 6. HAI titers and neutralizing activity against influenza virus A/PR8/34 after the second immunization. (A) HAI titers. (B) Neutralizing activities. Sera at week 2 after the second immunization were used for HAI titers and neutralizing activity. HAI titers were expressed as $\log_2$, and neutralizing activities were expressed as the percentage of plaque reduction compared to that of naïve serum control. Groups of mice are as described in the legend to Fig. 1. mLT, mutant LT(R192G).

titers from the second immunization were found in the low-dose groups with adjuvant [0.02 μg and 0.1 μg HA plus mLT(R192G)]. Groups without adjuvant (0.02 μg, 0.1 μg, and 0.5 μg HA) also showed increased IgG antibody levels by 6- to 16-fold after the second immunization. Importantly, the groups with adjuvant showed the highest fold increases in virus-specific serum IgA antibody titers [31-fold in the group with 0.5 μg HA plus mLT(R192G) or CTB and 47-fold in the group with 0.1 μg HA plus mLT(R192G)]. These results indicate that prime-boost mucosal immunizations significantly increased antibody levels in a dose-dependent manner and that the addition of adjuvant enhanced both IgG and IgA serum antibodies.

Significant increases in functional antibodies after the second immunization. After the second immunization, mice exhibited significant increases in functional antibodies with both neutralizing and HAI activities (Fig. 6A and B). The low-dose groups [0.02 μg HA, 0.02 μg HA plus mLT(R192G), 0.1 μg HA] showed increased neutralizing titers (40% reduction in plaque formation) by approximately 27- to 81-fold, and groups with 0.5 μg HA with or without adjuvant [mLT(R192G) or CTB] and 0.1 μg HA plus mLT(R192G) showed increased neutralizing titers by 27-fold compared to those induced after the first immunization. Significant increases in HAI titers over 13- or 26-fold were observed in all groups with adjuvants mLT(R192G) or CTB, compared to those induced by a single immunization (Fig. 6A). In addition, as shown in Fig. 6A, we detected the induction of HAI titers of approximately 20 in the low-dose immunized groups even in the absence of adjuvant (0.02 μg HA, 0.1 μg HA). Therefore, the boost immunization significantly increased the functional antibody levels in all groups, and the inclusion of adjuvant in low-dose groups had augmenting effects on enhancing the functional antibodies.

Enhanced protection after the second immunization. After the second immunization, all VLP-immunized mice including the 0.02-μg-HA group were protected with only a slight transient loss of body weight occurring in the low-dose group (Fig. 7A and B). To better understand and compare the protective efficacies after the first and second immunizations, we determined the lung viral titers at day 4 postchallenge (Table 4). Virus was not detected in lung samples from the groups with adjuvant [0.5 μg HA plus mLT(R192G) or CTB, 0.1 μg HA plus mLT(R192G)], indicating the complete inhibition of viral replication by day 4 postchallenge. The 0.5-μg-HA group showed improved protection with a 52-fold decrease in lung viral titers compared to that after the first immunization. The 0.02-μg-HA group with mLT(R192G) adjuvant showed 100% protection and significantly lower lung viral titers by 250-fold compared to those groups with a single immunization. The 0.02-μg-HA and 0.1-μg-HA groups without adjuvant also showed 100% protection and decreased lung viral titers by 70- and 100-fold, respectively. These results indicate that a prime-boost immunization scheme induces effective protective immunity even with the lowest dose of VLPs containing 0.02 μg HA and that adjuvant-enhanced immunogenicity of VLP vaccines plays a role in completely inhibiting virus replication in lungs. As additional supportive evidence for this improved protection, inflammatory cytokines gamma interferon and IL-6 were not detected or were found at low levels close to the detection limit in lungs of mice after two immunizations; even the lowest-dose, 0.02-μg-HA group showed a fourfold reduction in levels of IL-6 compared to that of the naïve control (data not shown). Overall, these results suggest that two immunizations significantly improved protective efficacy even with the lowest dose of influenza VLPs tested.

DISCUSSION

Two doses of inactivated virus vaccines are usually recommended to immunize naïve individuals for adequate protection due to their relatively low immunogenicity (20). Therefore, development of a single-dose protective and safe vaccine would have significant advantages for application to humans. A single vaccine administration would be highly desirable, particularly in the case of a pandemic outbreak when there would not be sufficient time for booster vaccination. Significantly reduced vaccine cost and an increased coverage rate of vaccination would be expected with a single vaccination regimen. In addition, a single dose which induces protection would limit the spread of a virus and at the same time would provide protection from severe illness and death in humans.

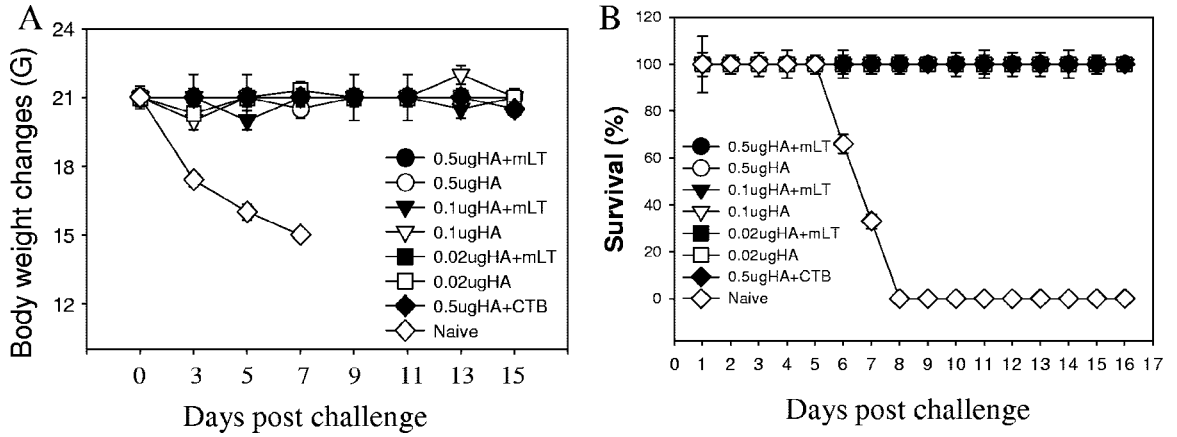

FIG. 7. Protection against lethal dose of influenza virus PR8 (H1N1) challenge after two immunizations with VLPs. (A) Body weight (grams [G]). (B) Survival rates (%). At week 4, after the second immunization, naïve and immunized mice were intranasally infected with a lethal dose of mouse-adapted A/PR8 (20× LD_{50}). Mice were monitored daily to determine the body weight changes and percentages of mortality rates. Groups of mice are as described in the legend to Fig. 1.

Mice immunized with a single dose of live attenuated influenza virus vaccine were protected from lethality despite undetectable or low levels of neutralizing antibodies (29). Limited previous studies reported immune responses or protection after a single intramuscular immunization of mice with inactivated influenza whole virus or split vaccines at higher doses (7.5 μg to 30 μg HA) (11, 12, 30). Viral titers in nasal wash samples in primed mice were lower than those in naïve mice after infection with a nonlethal dose (12). A recent study reported that a single intramuscular immunization of mice with influenza H5N1 VLPs containing 3 μg HA provided survival against lethal infection even in the absence of detectable HAI titers (2). We observed protection in mice immunized intranasally with a single dose of influenza A/PR8 HA VLPs containing 0.5 μg HA in the absence of adjuvant, or 0.1 μg of HA in the presence of mLT(R192G) adjuvant, with little or no body weight loss. The groups of mice that were protected from death and clinical illness of lethal infection showed low but significant levels of HAI and neutralizing activities compared to those in groups with partial protection and clinical illness after a single dose, indicating that these functional antibodies contribute to protection. Protection with a low dose of H1 HA VLPs may be due to the intrinsic property of VLPs presenting HA in native-like conformation. Although H5 HA is reported to have intrinsically lower immunogenicity than H1 HA (8, 15, 28), the dosage effects of H5 HA in VLPs on inducing protective immunity after a single vaccination of mice remain to be determined. A difference in route of delivery, intranasal versus intramuscular immunization, may also result in different responses. Our results support the possibility that a single-dose vaccine can be developed based on influenza VLPs.

TABLE 4. Lung virus titers at day 4 postchallenge[a]

Vaccine group (μg)[b]	Lung virus titer (10^4 PFU/ml) (±SD)		
	Single immunization	Second immunization	Fold decrease[c]
HA (0.5)+mLT	6 ± 0.5	0	–[d]
HA (0.5)	50 ± 4	0.97 ± 0.1	52
HA (0.1)+mLT	6.5 ± 1	0	–[d]
HA (0.1)	100 ± 20	1 ± 0.2	100
HA (0.02)+mLT	75 ± 8	0.3 ± 0.04	250
HA (0.02)	280 ± 30	4 ± 0.5	70
HA (0.5)+CTB	20 ± 3	0	–[d]
Naïve	500 ± 60	500 ± 60	

[a] Lung samples from individual mice in each group ($n = 6$) were collected on day 4 postchallenge with a lethal dose of mouse-adapted A/PR/8/34. Each lung sample was diluted to 1 ml with Dulbecco's modified Eagle's medium.

[b] Groups of mice are as described in the legend of Fig. 1. mLT, mutant LT(R192G).

[c] Fold decrease in lung viral titers after the second immunization compared to that observed after the single immunization.

[d] –, No virus in lungs was detected after the second immunization, indicating a complete inhibition of viral replications.

From kinetic analysis of antibody induction by different influenza VLP doses, it is interesting to note that the time for reaching the peak antibody levels is more closely dependent on the dosage of VLPs rather than the presence of adjuvant. Immunization with a moderate dose of VLPs containing 0.5 μg HA induced significant levels of antibodies at the earlier days 7 and 10, and then high levels of antibodies were maintained up to week 4. Low doses of influenza VLPs showed much slower kinetics but with continued increases up to week 5. The lowest-dose group with VLPs containing 0.02 μg HA without adjuvant did not show significant increases in antibody levels with a single immunization over the time course tested. However, this group was completely protected after a second dose, suggesting effective priming with the first dose. This observation that intranasal immunization with low doses of VLPs shows delayed immune response kinetics has not been explored previously, and additional studies are needed to better understand the underlying mechanisms.

Cholera toxin (CT) and LT are known to be the most potent mucosal adjuvants. The CT or LT holotoxin consists of a toxigenic A subunit with ADP ribosyltransferase activity and a nontoxic pentameric B subunit, which is responsible for the whole-toxin binding to GM1 gangliosides present on most nucleated cells (4). The ribosyltransferase activity increases the intracellular cyclic AMP, acting on several GTP-binding proteins. CT or LT can induce maturation of dendritic cells (1, 6,

10), activate epithelial cells inducing the production of chemokines, and augment the priming of $CD4^+$ T cells and the antigen presentation by dendritic cells and B cells (5, 16). However, the toxicity of CT or LT precludes their application to humans as illustrated by the association of an LT-plus-adjuvant inactivated influenza vaccine with Bell's palsy or an adverse event of facial nerve palsy when given intranasally (18). In efforts to develop safe mucosal adjuvants, mutant derivatives of LT [mLT(R192G), LTK63] with negligible toxicity were developed and demonstrated to retain adjuvant properties and have been shown to be safe in animal and human studies, thus holding promise as a mucosal adjuvant (21). The nontoxic subunit CTB has also been safely administered to humans (14, 34). Both mLT(R192G) and CTB were found to exhibit potent mucosal adjuvanticity in the context of influenza VLPs, demonstrating the potential for use of these less-toxic forms of adjuvants for enhancing the immunogenicity of influenza VLPs. In contrast to recombinant mLT(R192G), CTB obtained commercially contains a trace of CTA of less than 0.5%, which might affect the CTB adjuvanticity observed.

Our analysis of IgG1/IgG2a isotype antibodies over the time course showed an interesting finding that the timing of different antibody isotype production does not follow the same kinetics and is dependent on the isotypes of antibodies. The fact that the ratios of IgG1/IgG2a isotype antibodies are not static implies that the timing of analysis is an important factor. After immunization with influenza VLPs, the induction of IgG2a was observed much earlier and its levels were higher than that of IgG1, which indicates that Th1-type immune responses are dominant. The earlier induction phenotype of IgG2a was also demonstrated to be more prominent after a single immunization of mice with inactivated whole virus than split vaccine containing the same high dose of 15 μg HA or infection with a sublethal dose (30). Influenza VLPs are likely to induce a similar pattern of immune responses as inactivated whole virus, although direct comparison was not possible because of differences in dosage and the route of vaccine delivery. The addition of CTB adjuvant significantly increased the levels of IgG2a but not IgG1. In contrast, mLT(R192G) enhanced the levels of IgG2a as well as IgG1, indicating that the mechanisms by which mLT(R192G) and CTB exert their adjuvanticity might be quite different.

The addition of an adjuvant plays an important role in enhancing the protective efficacy. When the protected mice were examined at day 4 postchallenge, we found differential efficacy in terms of lung viral titers and inflammatory cytokines although 100% protection was observed. The groups with adjuvant, including 0.1 μg HA plus mLT(R192G), showed improved protection as indicated by 10-fold-lower lung viral titers and lower inflammatory cytokine levels compared to a 0.5-μg-HA group without adjuvant, although these two groups exhibited similar levels of HAI and neutralizing activities. A possible explanation is that the addition of adjuvant might enhance innate immunity together with VLP vaccines, contributing to the inhibition of viral replication and resulting in reduced production of inflammatory cytokines. Another possibility is that more effective priming occurs by the presence of an effective mucosal adjuvant as indicated by rapid increases in HAI titers at day 4 postchallenge. Therefore, the use of a safe and effective mucosal adjuvant allows significant antigen-sparing effects and improved protective efficacy, particularly for developing single low-dose nonreplicating VLP vaccines.

Overall, the present studies provide new insights into the kinetics of immune responses, dynamics of antibody isotypes, and quantitative and qualitative differences in protective efficacy after primary and boost immunizations. The fact that influenza VLPs could induce protective immunity with a single-dose immunization even in the absence of adjuvant further provides evidence that the VLP format is highly immunogenic and is a promising approach for developing effective vaccines.

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