The Magnitude of Local Immunity in the Lungs of Mice Induced by Live Attenuated Influenza Vaccines Is Determined by Local Viral Replication and Induction of Cytokines^{∇†}

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While live attenuated influenza vaccines (LAIVs) have been shown to be efficacious and have been licensed for human use, the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) have to be updated for optimal protective efficacy. Little is known about the effect of different HA and NA proteins on the immunogenicity of LAIVs developed using the same backbone. A panel of LAIVs that share the internal protein genes, with unique HA and NA gene segments from different influenza subtypes, was rescued by reverse genetics, and a comparative study of immune responses induced by these vaccines was conducted in mice. The results suggest that the magnitude of lung immunity, including pulmonary IgA antibody and memory CD8⁺ T lymphocytes, induced by the vaccines depends on the replication efficiency of the LAIVs, as well as the induction of cytokines/chemokines in the lungs. However, these factors are not important in determining systemic immunity such as serum antibody titers and memory CD8⁺ T cells in the spleen. A qualitative analysis of immune responses induced by a single dose of an H5N1 LAIV revealed that the vaccine induced robust systemic and mucosal immunity in mice. In addition, antibodies and memory lymphocytes established in the lungs following vaccination were required for protection against lethal challenge with homologous and heterologous H5N1 viruses. Our results highlight the different requirements for inducing systemic and lung immunity that can be explored for the development of pulmonary immunity for protection against respiratory pathogens.

Vaccination remains the most cost-effective intervention to prevent influenza virus infection (13, 35). Currently, two different forms of licensed influenza vaccines are available in the United States. The trivalent inactivated influenza virus vaccine (TIV) has been used since 1945, and the live attenuated influenza virus vaccine (LAIV) was licensed in the United States in 2003 and is currently used in healthy children and adults (2 to 49 years of age) (19). The LAIV are 6:2 genetic reassortants that are currently produced using reverse genetics, in which the six internal protein gene segments (PB2, PB1, PA, NP, M, and NS) are derived from the vaccine donor strains (A/Ann Arbor/6/60 [AA60] H2N2 or B/Ann Arbor/1/66), reassorted with the hemagglutinin (HA) and the neuraminidase (NA) gene segments from the appropriate contemporary wild-type (wt) viruses. The influenza A virus component has five loci in three gene segments that are responsible for cold-adapted (ca), temperature sensitive, and attenuation phenotypes in ferrets (9, 25). A number of avian influenza viruses have crossed the species barrier and caused infections in humans (21, 36, 40). In recognition of the potential of avian viruses to cause a pandemic and to alleviate the possible impact, a number of LAIVs have been developed for these avian influenza viruses, and their protective efficacy against challenge with the

corresponding wt influenza viruses was evaluated in animal models (7, 27, 41). While a number of LAIVs, such as those developed for H9N2 and H7N3 subtypes, have been shown to provide complete protection from wt virus challenge following a single dose of vaccine in mice (7, 27), others, such as the H5N1 LAIVs, required two doses for complete protection (41). Although the difference in virulence of the avian influenza wt viruses in mice might contribute to the observed difference (the 50% mouse lethal dose [MLD₅₀] for the A/chicken/HK/G9/97 [H9N2] and A/Vietnam/1203/2004 [H5N1] viruses are >10⁵ and 10^{0.4} 50% tissue culture infective doses [TCID₅₀], respectively), we had not compared the magnitude of the immune responses induced by these LAIVs, especially the induction of cellular immunity. In addition, while the correlate of protection for inactivated influenza vaccine has been established and a serum hemagglutination inhibition titer of 1:40 or greater is considered protective, the correlates of protection for LAIV have not been identified. We evaluated the humoral and cellular immune responses and the distribution of immune effectors induced by eight different LAIVs at mucosal and systemic sites. Our results show that while all LAIVs tested induced serum antibodies and CD8⁺ cytotoxic T cells in the spleen, the induction and distribution of immune effectors in the respiratory tract are dependent on the ability of the LAIVs to replicate and induce proinflammatory cytokines in the lungs. We also evaluated the protective efficacy of two LAIVs (H3N2 and H5N1) that differed in their abilities to replicate in the lungs in order to determine the functional consequences of these observations.

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MATERIALS AND METHODS

Mice. Six- to 8-week-old female BALB/c mice (Taconic Farms, Inc., Germantown, NY) were used in all mouse experiments. The animal study protocols used were approved by the National Institutes of Health Animal Care and Use Committee and were conducted at NIH.

Medium. T-cell medium (TCM) containing RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS), glutamine (2 mM), sodium pyruvate (2 mM), 0.1 mM 2-mercaptoethanol, and antibiotics was used.

Immunization protocol. Mice were lightly anesthetized with 4% isoflurane, followed by intranasal (i.n.) administration of LAIVs in 50 μ l.

The generation of vaccine seeds. The vaccines used in this study were derived using plasmid-based reverse genetics as previously described (41). The vaccines used in this study were generated in collaboration with the Centers for Disease Control and Prevention or under a Cooperative Research and Development Agreement (CRADA) with MedImmune (Mountain View, CA) (6, 7, 10, 33, 41).

Sample collection for determining antibody titers. For selected experiments, nasal wash, nasal turbinates (NT), and lungs were collected as previously described (4). Bergquist et al. showed that this method can minimize tissue contamination by serum antibody. Clarified samples were stored at -20°C until used.

Virus titration assay. Lungs, NT, and brains were harvested, weighed, and homogenized in L-15 medium to prepare a 10% (wt/vol) tissue homogenate. The homogenates were clarified by low-speed centrifugation, and the viral titers determined on MDCK monolayers were expressed as $\log_{10}$ TCID₅₀/g of tissue (22). The lower limit of detection was $10^{1.5}$ TCID₅₀ per g of tissue.

Cytotoxic T-lymphocyte (CTL) epitopes. Peptides comprised of residues 518 to 526 of HA (HA_{518–526}), YSTVASSL, and residues 147 to 155 of nucleocapsid protein NP (NP_{147–155}), TYQRTRALV, were synthesized by GenScript USA Inc. (Piscataway, NJ). Each was dissolved in phosphate-buffered saline (PBS) and stored at -20°C .

ICS. An intracellular cytokine staining (ICS) assay was performed as previously described (30). In brief, single cell suspensions from various organs were cultured for 5 h in 96-well round-bottom plates in 200 μ l of TCM containing 50 IU of human recombinant interleukin-2 (IL-2; Roche Diagnostics GmbH) and 1 μ l of Golgi Plug, in the presence or absence of 1 μ M CTL epitopes. Epitope-specific gamma interferon (IFN- γ)-secreting cells were detected using a Cytofix/Cytoperm kit. Anti-mouse CD8 α monoclonal antibody ([MAb] 53–6.7) and anti-mouse IFN- γ MAb (XMG 1.2) were obtained from BD Biosciences (San Diego, CA).

In vivo cytotoxicity assay. An *in vivo* cytotoxicity assay was performed as previously described (24). Splenocytes from naïve BALB/c mice were used as target cells and were pulsed with 9 μ M of the respective CTL epitopes at 37°C for 90 min or incubated without peptide. After incubation, cells were washed two times with plain RPMI 1640 medium before carboxyfluorescein succinimidyl ester (CFSE) labeling. The peptide-pulsed and the unpulsed populations were labeled with 3 μ M or 0.5 μ M CFSE, respectively. After 10 min at 37°C , the cells were washed three times with ice-cold TCM, and the peptide-pulsed and the unpulsed populations were mixed together at a 1:1 ratio and inoculated into immunized or naïve mice. The mice were killed 16 h later, and spleen cell suspensions were analyzed by flow cytometry. The following formula was used to calculate specific lysis: percentage of specific lysis = $[1 - (\text{ratio for naïve mice/ratio for vaccinated mice})] \times 100$, where the ratio is defined as the percentage of population with low CFSE fluorescence/percentage of population with high CFSE fluorescence.

ELISA. β -Propiolactone (BPL)-inactivated whole virion influenza vaccines (HA and NA gene from various wt viruses, with internal genes from the A/Ann Arbor/6/60 ca virus; 50 HA units/well) were used for enzyme-linked immunosorbent assay (ELISA). The sera were serially diluted in half-log dilutions and incubated on plates at 4°C overnight. Bound antibody (Ab) was detected with polyclonal goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (HRP; Dako, Glostrup, Denmark). ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; Sigma-Aldrich, St. Louis, MO)] was used as a substrate; the reaction was stopped with 1% SDS solution after 15 min, and the color intensity was measured with a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA) at a wavelength of 405 nm and reference wavelength of 450 nm. An optical density (OD) of >0.2 was considered to be positive. For isotyping experiments, based on the total influenza virus-specific Ab titers, the samples were optimally diluted to ensure that the reaction would be in the linear range. Isotype-specific MAbs (IgG1, LO-MG-1-2; IgG2a, LO-MG2a-7; IgG2b, LO-MG2b-2) were used; all were purchased from Abcam Inc., Cambridge, MA. The

IgA-specific MAb (C10-1) was from BD Bioscience. Apart from the isotype-specific MAbs, the other reagents were used under identical conditions.

B-cell ELISPOT assays. A B-cell enzyme-linked immunospot assay (ELISPOT) was performed as previously described (11, 14). Wells were coated with 5,000 HA units per ml of BPL-inactivated vaccine virus (50 μ l per well), and cells from various organs were subjected to serial 2-fold dilutions and incubated at 37°C overnight. The total number of antibody-secreting cells (ASCs) was enumerated using horseradish peroxidase-conjugated goat anti-mouse Ig (1:1,000 dilution; Dako). Wells coated with plain medium without influenza virus antigens were used to determine the level of nonspecific binding. Spots were developed using aminoethylcarbazole (AEC) peroxidase substrate according to the manufacturer's instructions (Vector Labs, Burlingame, CA). Wells were rinsed extensively with water and allowed to dry completely before spots were counted using an ImmunoSpot plate reader (Cellular Technologies, Ltd., Cleveland, OH). For isotyping experiments, the above-mentioned biotinylated isotype-specific MAbs were used at concentrations recommended by the manufacturers, followed by streptavidin-alkaline phosphatase. Spots were developed using Vector Blue substrate (Vector Labs).

Cytokine analysis using Bio-Plex. The concentration of various cytokines or chemokines in clarified lung homogenates was measured by a Bio-Plex Protein Array system (Bio-Rad, Hercules CA). The assay was performed according to the manufacturer's instructions, and the results were analyzed using the Bio-Plex manager software.

Histopathological evaluation. Tissue sections (4 to 7 μ m) from formalin-fixed paraffin-embedded lungs were stained with hematoxylin and eosin (H&E) for histopathological evaluation. Lung sections were evaluated blindly, and a scoring system was developed to quantify the spectrum of observed pathological changes.

Statistical analysis. The significance of difference between any two different groups was assessed by a Mann-Whitney test using Prism, version 5 (GraphPad Software, CA). The Mann-Whitney test lacks the power to detect significance at a P value of 0.05 in small samples. Therefore, in selected experiments, where the sample size of each group was less than 5, an unpaired t test was used with the assumption that the data fit a normal distribution. P values of <0.05 are considered significantly different.

RESULTS

Different LAIVs differed in their abilities to replicate and activate the innate immune system in mice. A total of eight LAIVs were generated using reverse genetics including an H1N1 virus (A/Puerto Rico/8/34 [PR8 (H1N1) ca]), an H2N2 virus (A/Ann Arbor/6/60 [AA60 (H2N2) ca]), an H3N2 virus (A/Panama/2007/99 [Panama99 (H3N2) ca]), two H5N1 viruses (A/Vietnam/1203/04 [VN04 (H5N1) ca]) and (A/Hong Kong/213/03 [HK03 (H5N1) ca]), an H6N1 virus (A/teal/Hong Kong/W312/97 [HK97 (H6N1) ca]), an H7N7 virus (A/Netherlands/219/03 [NL03 (H7N7) ca]), and an H9N2 virus (A/chicken/Hong Kong/G9/97 [HK97 (H9N2) ca]). These LAIVs share the internal protein genes that are derived from the vaccine donor strain, AA60 [H2N2] ca) and the HA and the NA gene segments from the indicated wt viruses.

Because the replication efficiency of a LAIV could have an impact on its immunogenicity, we first examined the replication efficiency of selected LAIVs in mice. As shown in Fig. 1, all LAIVs except Panama99 (H3N2) ca replicated to moderately high titers in the NT and lungs on days 2 and 4 following intranasal (i.n.) infection with 10^6 TCID₅₀. The replication efficiency of the Panama99 (H3N2) ca vaccine was significantly lower than that of the other groups; on day 2 postvaccination (p.v.), the titers in NT and lungs were ~ 100 - and 1,000-fold lower, respectively. The difference continued through day 4, when the titer in the NT was still significantly lower than titers of the other groups ($P < 0.05$) and was below the detection limit in the lungs. Verhoeven et al. showed that mice challenged with 10 or 100 TCID₅₀ of PR8 wt virus had average lung viral titers of 5×10^5 and 1×10^7 TCID₅₀ on day 5 postinfection.

FIG. 1. Different LAIVs differ in their replication in mice. Groups of five mice each were inoculated with 1×10^6 TCID₅₀ of various LAIVs i.n. Lungs and NT were harvested on the indicated days p.v. Virus titers in the NT (A) and lungs (B) were determined on MDCK monolayers and are expressed as log₁₀ TCID₅₀/g of tissue. The lower limit of detection is represented by the dotted line. The bars and error bars represent the means and standard deviations, respectively, of the group. d, day.

tion, respectively (44), and the wt avian influenza viruses and their ca vaccine virus counterparts infected mice and induced antibodies in the serum (6, 7, 10, 33, 41).

We selected the H5N1 LAIV that replicated well in the respiratory tract of mice and the H3N2 LAIV that was restricted in replication in the respiratory tract to determine how the difference in replication influenced the ability of the vaccines to activate the innate immune system. Groups of three mice received 10^6 TCID₅₀ of VN04 (H5N1) ca, Panama99 (H3N2) ca, or L15 (mock immunization) i.n., and lung homogenates obtained at different time points p.v. were tested for cytokines and chemokines. The Panama99 (H3N2) ca vaccine induced significantly lower cytokine/chemokine levels for a shorter duration than the H5N1 ca vaccine (see Fig. S1 in the supplemental material). In addition, a biphasic increase of chemokines MIG (monokine induced by gamma interferon) and RANTES were observed in mice that were vaccinated with the VN04 (H5N1) ca vaccine but not the Panama99 (H3N2) ca vaccine. Finally, a significant amount of IL-10 that could be produced by infiltrating lymphocytes was detected on day 6 p.v.

in VN04 (H5N1) ca virus-vaccinated mice that was not detected in Panama99 (H3N2) ca virus-vaccinated mice. These results indicate that LAIVs with different surface glycoproteins differ in their abilities to replicate and activate the innate immune system in mice; this could affect the magnitude and the quality of the adaptive immune response elicited by these vaccines.

LAIVs induced robust serum ELISA and variable mucosal antibody responses. To compare the immunogenicity of different LAIVs, groups of three BALB/c mice were given 10^6 TCID₅₀ of different LAIVs i.n. The PR8 wt virus, which is a mouse-adapted strain of influenza virus, was given at 50 TCID₅₀ i.n. as a positive control. Mice that were infected with this dose of PR8 wt virus are reported to have high titers of virus in the lungs 5 days after challenge (44). Serum samples were collected 28 days later, and the levels of influenza virus-specific antibodies in the samples were determined by a number of serological assays. As shown in Fig. 2A, serum ELISA antibody titers in mice that were vaccinated with LAIVs were comparable to those of PR8 wt virus-infected mice on day

FIG. 2. The induction of systemic and mucosal humoral immune responses by various LAIVs in mice. (A) Groups of three mice were vaccinated i.n. with various LAIVs as previously described, and serum samples were collected 28 days later. Influenza virus-specific serum antibody titers were determined by ELISA, using homologous BPL-inactivated whole virion preparations as the coating antigen. (B) The isotype distribution of influenza virus-specific antibodies in serum. The lower limit of detection is represented by the dotted line. (C) The serum neutralizing antibody titer on day 28 p.v. against the homologous LAIV virus. Plates were scored for cytopathic effect after 6 days of incubation at 33°C. The bars and error bars represent the means and standard deviations, respectively, for the groups. (D and E) Influenza virus-specific antibodies in lungs (D) and NT (E) determined by ELISA. PR8 wt virus was used as a positive control ($n = 5$).

FIG. 3. The induction of antibody-secreting cells in lungs of mice. (A to C) Groups of three LAIV-vaccinated mice were killed on day 28 p.v., and the numbers of influenza virus-specific antibody-secreting cells in lungs (A), spleen (B), and bone marrow (C) were determined by B-cell ELISPOT assay. (D to F) The isotype distribution of ASCs in lungs: IgA-specific (D), IgG_{2a}-specific (E), and IgG1-specific (F). The bars and error bars represent the means and standard deviations, respectively, for the groups. (G and H) Groups of five mice were vaccinated with the VN04 (H5N1) ca vaccine i.n., and the isotype distribution of influenza virus-specific antibodies in the nasal wash (G) and lung homogenates (H) was determined by ELISA. Samples were diluted appropriately and added in three sets of wells that were coated with BPL-inactivated VN04 (H5N1) ca. Different isotype-specific antibodies were added in indicated sets and developed as mentioned in Materials and Methods. The optical density at 405 nm was measured. (I) IgA antibody titers in the lungs of mice 28 days after receiving various LAIV. *, the difference between the groups was statistically significant ($P < 0.05$). PR8 wt virus was used as a positive control.

28 p.v. Most of the influenza virus-specific antibodies in the serum belonged to the IgG1 and IgG2a subclasses, with no isotype bias (Fig. 2B). Although comparable ELISA antibody titers were detected in the sera, different levels of neutralizing activity against homologous LAIVs were observed (Fig. 2C). The AA60 (H2N2) ca, Panama99 (H3N2) ca, HK03 (H5N1) ca, and VN04 (H5N1) ca vaccines induced approximately 4-fold lower neutralizing antibody responses than other LAIVs, suggesting that the HA proteins play a role in determining the magnitude of neutralizing activity of the humoral responses. In addition to serum antibody responses, we examined the mucosal immunity induced by the LAIVs. Vaccinated mice had significant antibody responses in the lungs and NT (Fig. 2D and E), and the titers at both sites correlated well with each other. The group that received the Panama99 (H3N2) ca virus had the lowest antibody titers at both sites.

LAIVs induced antibody-secreting cells in the lungs, spleen, and bone marrow. Because Joo et al. showed that lungs are the major site for the establishment of antibody-secreting cells

(ASCs) following a wt influenza virus infection (26), the numbers of ASCs in the lungs and spleen of groups of three mice were determined by a B-cell ELISPOT assay 4 weeks p.v. to determine if there was a correlation between the number of ASCs in the lungs and the antibody titers at mucosal sites. As shown in Fig. 3A, with the exception of the Panama99 (H3N2) ca vaccine, mice that were vaccinated with LAIVs had substantial numbers of ASCs in their lungs, and the numbers were similar to or exceeded the number of ASCs seen in mice that were infected with the PR8 wt virus. Some ASCs were also found in the spleen and bone marrow (Fig. 3B and C). The majority of the ASCs in the lungs (~50%) were IgA-secreting cells, followed in frequency by IgG2a and IgG1 subtypes (Fig. 3D to F). The isotype distribution of influenza virus-specific ASCs matched well with the antibody isotype profile in the nasal wash (Fig. 3G) but not in the lung homogenates (Fig. 3H). Because mice that were vaccinated with the Panama99 (H3N2) ca virus had significantly fewer IgA-secreting ASCs in the lungs than mice that received the other vaccines, we de-

terminated the IgA titer in the lungs of mice vaccinated with the different LAIVs. As shown in Fig. 3I, mice that received the Panama99 (H3N2) ca vaccine had significantly lower IgA titers in the lungs than mice that received other LAIVs ($P < 0.05$).

In summary, although all LAIVs tested in this study were capable of inducing robust serum antibody responses, the induction of pulmonary immunity varied, as did the replication efficiency and ability of the LAIVs to activate innate immune responses.

LAIVs differed in their ability to induce functional cytotoxic CD8⁺ T lymphocytes in the lungs of mice. In addition to humoral responses, LAIVs also induce cytotoxic CD8⁺ T-cell (CTL) responses in mice and humans (23, 37). Using the VN04 (H5N1) ca virus, we observed an infiltration of both NP₁₄₇- and HA₅₁₈-specific CD8⁺ CTLs in the lungs of vaccinated mice, starting from day 5 p.v.; the response peaked on day 8 and returned to a basal level by day 12 (Fig. 4A). The magnitude of the CTL response was dose dependent (Fig. 4B). With direct *ex vivo* stimulation with the appropriate CTL epitopes, an increase in the surface expression of CD107a/b, which is a surrogate marker for lytic activity (42), was observed, suggesting that these cells can be cytolytic *in vivo* (Fig. 4C and D). This was supported by *in vivo* epitope-specific cytotoxic activity observed in VN04 (H5N1) ca virus-vaccinated mice but not in mock-infected mice (Fig. 4E). To determine the magnitude of the CTL responses induced by different LAIVs, groups of three mice were administered 10⁶ TCID₅₀ of various LAIVs and were sacrificed 8 days later to quantify the frequency of epitope-specific CD8⁺ T cells in the lungs by intracellular cytokine production. As shown in Fig. 4F, with the exception of the Panama99 (H3N2) ca vaccine, all LAIVs were able to elicit NP₁₄₇-specific CTL responses in mice at a magnitude that was comparable with the PR8 wt virus. The HA₅₁₈ epitope was not conserved among all the eight LAIVs. The PR8 (H1N1) ca, HK03 (H5N1) ca, VN04 (H5N1) ca, and HK97 (H9N2) ca viruses contained the conserved epitope, and the magnitudes of the CTL responses induced by the PR8 (H1N1) ca, HK03 (H5N1) ca, and VN04 (H5N1) ca viruses were comparable to the response induced by the PR8 wt virus. The group that received the HK97 (H9N2) ca vaccine had a lower frequency of CTLs than the H1N1 and H5N1 groups (Fig. 4G). The LAIVs that had altered sequences in the HA₅₁₈ epitope, except the AA60 (H2N2) ca, induced lower HA₅₁₈-specific CTL responses (Fig. 4H) than the viruses that contained the conserved epitope.

All LAIVs elicited similar frequencies of memory CD8⁺ CTLs in the spleen but not in the lungs. To examine the induction of CD8⁺ memory T cells, groups of three mice were vaccinated as previously described and were sacrificed 28 days later. The frequency of epitope-specific CD8⁺ CTLs in the lungs and spleen was determined by ICS assay. As shown in Fig. 5A, the frequency of CTLs in the lungs on day 28 p.v. was lower than at day 8 (average, 3- to 5-fold reduction) (Fig. 4A). Furthermore, the group that received the Panama99 (H3N2) ca virus had the lowest frequency of NP₁₄₇-specific T cells, consistent with the lower primary response. All LAIVs, including the Panama99 (H3N2) ca, induced a similar frequency of NP₁₄₇-specific CTLs in the spleen (0.5% of the splenic CD8⁺ population) as the PR8 wt virus (Fig. 5B). In addition, the function of these cells was evaluated by measuring the amount

of IFN- γ produced by these epitope-specific CD8⁺ T cells in response to antigenic stimulation. With the exception of the VN04 (H5N1) ca vaccine, which induced a larger amount of IFN- γ ($P = 0.0205$) (Fig. 5C), the amount of IFN- γ produced by Panama 99 (H3N2) ca virus-induced CTLs was not statistically different from levels of other groups. Finally, significant cytotoxic activity could still be detected *in vivo* up to 10 months after a single dose of the VN04 (H5N1) ca vaccine, indicating that the CTLs are long-lived (Fig. 5D).

Panama99 (H3N2) ca and VN04 (H5N1) ca vaccines use different immune effectors to protect mice from homologous wt virus infection. Two LAIVs (Panama99 H3N2 and VN04 H5N1) that differed in replication efficiencies and induction of local immunity were selected to evaluate the degree of protection against wt influenza virus challenge in mice. Groups of Panama99 (H3N2) ca virus-vaccinated mice were challenged i.n. with 10⁶ TCID₅₀ of the H3N2 wt virus on day 28 p.v. NT and lungs were harvested 2 and 4 days later for virus titration. As shown in Fig. 6A, viral replication in the NT was significantly restricted in the vaccinated mice ($P = 0.0097$ and 0.0071 for day 2 and day 4, respectively). The efficacy of the H3N2 LAIV in preventing replication of the wt virus in the lungs could not be determined because the virus did not replicate efficiently even in the mock-vaccinated control mice (Fig. 6B).

Groups of 10 VN04 (H5N1) ca virus-vaccinated mice were challenged i.n. with 10⁵ TCID₅₀ of VN04 wt virus ($\sim 4 \times 10^4$ MLD₅₀s) on day 28 p.v. to assess the effectiveness of the immune responses induced by the H5N1 ca vaccine. NT, lungs, and brain were collected 1, 2, and 4 days later for virus titration. As shown in Fig. 7A, the H5N1 wt virus replicated in the NT of mock-vaccinated mice, and the titer continued to increase over 4 days. In vaccinated mice, the majority (8/10, 7/10, and 8/10 on day 1, day 2, and day 4, respectively) of the mice had no detectable virus in the NT, and in those that did, the titers were 10- to 1,000-fold lower than in the mock-vaccinated group. Most of the vaccinated mice had virus in the lungs on day 1 and day 2 postinfection (p.i.) (8/10 on each day), but the titer was significantly lower than in mock-vaccinated mice (Fig. 7B) ($P < 0.05$). On day 4 p.i., all vaccinated mice had cleared the virus from the lungs while the mock-vaccinated group had 10⁶ TCID₅₀/g of virus (Fig. 7B). The immune responses induced by the VN04 (H5N1) ca vaccine also prevented the dissemination of the virus to the brain. None of the vaccinated mice had virus detected in the brain while 10/10 mice in the control group did on day 4 p.i. (Fig. 7C). To evaluate the breadth of protection induced by the vaccine against a heterologous H5N1 virus, the H5N1 ca virus-vaccinated mice were challenged i.n. with 10⁵ TCID₅₀ of the Indo 05 (H5N1) wt virus (a clade 2.1 virus). As shown in Fig. 7D, only 4/10 and 1/10 of the vaccinated mice had detectable challenge virus in the NT on day 2 and day 4 p.i. Similar to the homologous virus challenge, the Indo 05 wt virus was recovered from the lungs of the vaccinated mice; however, the titer was significantly lower than in mock-vaccinated mice, and by day 4 p.i., there was an average 1,000-fold reduction in the pulmonary virus titer in the vaccinated mice (Fig. 7E). The vaccine also prevented the virus from disseminating to the brain; none of the mice in the vaccinated group had virus detected in the brain, compared with 10/10 in the mock-vaccinated control group (Fig. 7F).

To assess the histopathological changes following challenge,

FIG. 4. Cellular immunity elicited by LAIV in mice. (A) Groups of three mice were vaccinated with 10^6 TCID₅₀ of VN04 (H5N1) ca vaccine i.n. and sacrificed at the indicated time (x axis), and the frequency of epitope-specific CD8⁺ CTLs in lungs was determined by intracellular cytokine staining (y axis). The solid line indicates NP₁₄₇-specific and the dotted line indicates HA₅₁₈ specific data. The error bars are the standard deviation of the group. (B) Groups of three mice were vaccinated with the indicated dose of the H5N1 vaccine i.n. and were sacrificed 8 days later. The frequency of NP₁₄₇-specific CD8⁺ T cells in the lungs was determined by ICS. The bars and error bars are the means and standard deviations, respectively, of the groups. *, the difference between the groups was statistically significant ($P < 0.05$). (C and D) Upregulation of surface expression of CD107a/b on epitope-specific CD8⁺ CTL. Pulmonary lymphocytes were stimulated with NP₁₄₇ epitope *in vitro* for 5 h, and epitope-specific CD8⁺ CTLs were identified by IFN- γ production (area B). Monoclonal antibodies against CD107a/b were used to detect exocytosis of lytic granules, and the surface expression of CD107a/b on epitope-specific CD8⁺ CTLs is shown in panel D. (E) Groups of three vaccinated or mock-vaccinated mice received 5×10^6 NP₁₄₇-pulsed (hatched bar) or HA₅₁₈-pulsed cells (white bar) on day 8 p.v. by intravenous injection. An equal number of unpulsed targets was coinjected to measure nonspecific lysis. The degree of specific lysis was determined 16 h later by calculating the ratio between the epitope-pulsed and unpulsed targets in the spleen. The bars and error bars are the means and standard deviations, respectively, of the groups. ND, not detected. (F and G) The frequency of epitope-specific CD8⁺ CTLs induced by various LAIVs. Groups of three mice were vaccinated with 1×10^6 TCID₅₀ of different LAIVs i.n., and the frequency of NP₁₄₇-specific (F) and HA₅₁₈-specific (G) CD8⁺ T cells in lungs on day 8 p.v. was detected by ICS. The asterisk indicates that the LAIV strain has an altered HA₅₁₈ epitope. PR8 wt virus was used as a positive control.

tissue sections from lungs were prepared on day 2 and day 4 following challenge. Mock-vaccinated mice had a higher pathological score than vaccinated mice at both time points (see Table S1 in the supplemental material) after challenge with two different H5N1 viruses. Microscopic examination revealed that the bronchi and bronchioles of mock-vaccinated mice were necrotic, the lumens were filled with necrotic debris,

and inflammation often extended to the surrounding alveoli. Vaccinated mice had fewer necrotic airways and overall pathology than mock-vaccinated mice, with prominent perivascular and peribronchial lymphoid cuffs and general lymphoid hyperplasia at both day 2 and day 4 p.i. (see Fig. S2 in the supplemental material), consistent with the pulmonary virus titers shown in Fig. 7.

FIG. 5. The induction of cellular immune memory by LAIV. (A and B) Groups of three vaccinated mice were sacrificed on day 28 to examine the frequency of NP₁₄₇-specific CD8⁺ T cells in the lungs (A) and spleen (B). The bars and error bars are the means and standard deviations, respectively, of the groups. (C) The level of IFN-γ produced by splenic NP₁₄₇-specific CD8⁺ T cells based on the mean fluorescence (MFL) intensity in the detecting channel. (D) The degree of *in vivo* lytic activity in vaccinated mice at indicated time points (x axis) was determined by an *in vivo* cytotoxicity assay. Open symbols represent lytic activity detected in vaccinated mice. Filled symbols represent mock-vaccinated mice. The error bars represent the standard deviations of the means. In panels A and B, PR8 wt virus was used as a positive control.

DISCUSSION

As all the LAIVs used in this study share the same set of internal protein genes from the AA60 ca virus, we were able to determine the influence of the surface glycoproteins on the magnitude and distribution of immune effectors elicited by LAIVs in mice. Using eight different LAIVs, our data demonstrate that the LAIVs are able to induce robust serum antibody responses and memory CD8⁺ CTL responses in the spleen. Consistent with the findings reported by Powell et al., priming with LAIV can elicit T-cell-based immunity against influenza A viruses in mice (37). In addition, apart from the route of administration that can influence the quality of the immune response (46), we further demonstrate that the ability of LAIVs to induce local immunity in the lower respiratory tract is determined by the ability of the vaccine virus to replicate in the lungs. Although when incorporated into a trivalent formulation the A/Panama/2007/99 (H3N2) vaccine strain provided 64% to 90% protection in young children against antigenically similar A/H3N2 strains compared to placebo in three random-

ized controlled studies (20, 31, 45), Block and colleagues found that a higher proportion of recipients of seasonal trivalent LAIV shed the A/New Caledonia/20/99 (H1N1) vaccine virus than recipients of the A/Panama/2007/99 (H3N2) vaccine virus in a clinical trial and that the H1N1 LAIV was more immunogenic than the H3N2 strain, especially in young children 5 to 8 years of age (5). Consistent with these data, the Panama99 (H3N2) ca vaccine failed to replicate efficiently in the respiratory tract of mice, and this led to a significantly reduced magnitude of primary and memory CD8⁺ CTL responses and influenza virus-specific ASCs in the lungs. However, the systemic immune responses were not affected; the serum antibody titers and memory CD8⁺ CTL levels in the spleen were comparable with those induced by LAIVs that replicated efficiently. The presence of comparable splenic memory NP₁₄₇-specific responses in mice vaccinated with Panama99 (H3N2) ca also suggests that the epitope was processed and presented properly. This makes it unlikely that the diminished pulmonary CTL responses were caused by the presence of more immunodominant epitopes in the H3N2 glycoprotein that might have shifted the hierarchy of the CTL responses.

We believe that the surface glycoproteins rather than the internal protein genes mediate the restriction of replication of the H3N2 viruses in the lungs of mice. Panama99 (H3N2) wt virus is not the only H3N2 influenza virus that replicates poorly in the lungs of mice. Another H3N2 wt virus, A/Wyoming/2/2003, also failed to replicate in the lungs of mice and was poorly pathogenic (MLD₅₀ of >10⁶ PFU) (8) while a reassortant virus with the HA and NA genes from A/Thailand/16/2004 (H5N1) was able to replicate to high titer (8). A similar observation was reported in pigs by Landolt et al.; a human H3N2 virus caused mild pulmonary lesions in pigs while a reassortant virus that possessed HA and NA genes from a swine H3N2 virus was significantly more pathogenic (29). The fact that the Panama99 (H3N2) wt virus replicates efficiently at 37°C *in vitro* makes it unlikely that the virus is unstable in the lungs. It will be important to establish whether other seasonal H3N2 LAIVs have phenotypes similar to the phenotype of the Panama 99 (H3N2) ca vaccine.

Although the Panama99 (H3N2) ca virus was restricted in

FIG. 6. The immunity induced by Panama99 (H3N2) ca vaccine provides significant protection in mice. Groups of five vaccinated or mock-vaccinated mice were challenged i.n. with 1×10^6 TCID₅₀ of Panama99 (H3N2) wt virus on day 28 p.v. Viral titers in NT (A) and lungs (B) at indicated time points were determined on MDCK monolayers. The short solid and dotted lines represent the means of the L15 and Panama99 (H3N2) ca groups, respectively. The lower limit of detection is denoted by a long dotted line. *, difference between the vaccinated and the mock-vaccinated group was statistically significant ($P < 0.05$).

FIG. 7. The immunity induced by the VN04 (H5N1) ca vaccine provides significant protection in the NT and accelerated viral clearance from the lungs following challenge with homologous and heterologous wt viruses. (A to C) Groups of 10 vaccinated or mock-vaccinated mice were challenged i.n. with 1×10^5 TCID₅₀ of VN04 (H5N1) wt virus on day 28 p.v. Viral titers in NT (A) and lungs (B) at indicated time points were determined on MDCK monolayers. (C) Viral titers in brain 4 days after the infection. (D to F) Vaccinated mice were challenged with 1×10^5 TCID₅₀ of Indo 05 (H5N1) wt virus. Viral titers in NT (D) and lungs (E) at indicated time points were determined on MDCK monolayers. (F) Viral titers in brain 4 days after the infection. The lower limit of detection is denoted by a long dotted line. The short solid and dotted lines represent the means of the L15 and VN04 (H5N1) ca groups, respectively. *, the difference between the two groups was statistically significant ($P < 0.05$). The results are the representative of two individual experiments.

replication and induced diminished IgA responses in the NT compared with the AA60 ca virus from which the internal genes were derived, the response was sufficient to prevent the replication of the wt challenge virus in the NT. We were unable to determine the consequences of the limited pulmonary replication of the Panama99 (H3N2) ca virus on protective efficacy in the lower respiratory tract because the challenge virus did not replicate in the lungs of mice. Our data also suggest that replication of LAIVs in the upper respiratory tract is sufficient to induce serum antibody responses. This requirement might provide an explanation for the discrepancy between the observations in animal models and humans. For example, the VN04 (H5N1) ca vaccine virus replicated efficiently in the NT of mice and ferrets, and the vaccine was efficacious against wt lethal challenge (41; also this study); but viral shedding was not detected in humans, and the immunogenicity of the vaccine was suboptimal in clinical trials (28). Strategies that enhance the replication of LAIV in the upper respiratory tract can potentially enhance the systemic immune response to the vaccine. Since the HA protein of the H5N1 viruses has a preference for α 2,3-linked sialic acid receptors and since cells in the upper respiratory tract of humans mainly have sialic acids with α 2,6-linkages (39), modifying the hemagglutinin protein of the virus for an α 2,6-linkage preference might enhance the replication and immunogenicity of the VN04 (H5N1) ca vaccine in humans. The LAIVs replicate in the lungs of mice because the shutoff temperature of the temperature-sensitive ca viruses is

higher than the core body temperature of mice. Additionally, when virus is administered intranasally to anesthetized mice, the virus is delivered to the lower respiratory tract as well as the upper respiratory tract. In contrast, LAIVs are delivered by nose drops or nasal spray in clinical use, and vaccine virus replication is restricted to the upper respiratory tract. Vaccine virus replication in the lungs is not necessary for seasonal LAIVs to be effective vaccines.

Although serum hemagglutination-inhibiting (HI) antibody is a correlate of protection for inactivated influenza virus vaccines, it is not a good correlate of protection for LAIV (1, 16, 43). Nasal wash IgA induced by LAIV or natural infection is associated with resistance to reinfection. In this study, mice vaccinated with the VN04 (H5N1) ca virus were protected from replication of the H5N1 wt virus in the NT. We believe that the protection is likely to be mediated by humoral immunity at the local site because significant viral clearance in the NT was achieved 1 day after wt challenge virus infection (8/10 mice had no detectable virus) (Fig. 7), and IgA, rather than IgG, has been shown to be important for protection in the nasal compartment (38). Although a single dose of the H5N1 ca vaccine was not sufficient to prevent replication of the H5N1 wt challenge virus in the lungs, the pulmonary virus titer in vaccinated mice was significantly lower than in mock-vaccinated mice even at day 1 p.i. Vaccinated mice cleared the virus by day 4 p.i., and Suguitan et al. showed that vaccinated mice survived lethal challenge while mock-vaccinated mice showed

significant morbidity and succumbed to infection (41). Viral replication was observed in lungs 2 days after wt virus challenge in mice that received one dose of the H5N1 LAIV but not in mice that received two doses of the LAIV (41), suggesting that two different mechanisms are involved in viral clearance in these scenarios. Since neutralizing antibody titers in serum increased significantly after two doses of the H5N1 LAIV (41), it is reasonable to speculate that the humoral immunity elicited by two doses of the vaccine is robust enough to neutralize the wt H5N1 challenge virus completely and to prevent viral replication in the lungs. In the absence of a robust neutralizing antibody response, as was seen following a single dose of H5N1 LAIV, the wt challenge virus replicates in the lungs, and other immune effectors are responsible for the survival of the host. These include local pulmonary antibodies produced by ASCs that can limit viral attachment and CD8⁺ T lymphocytes that can eliminate virus-infected cells and accelerate viral clearance. This model is supported by our data on viral clearance and histological examination of the lungs. On day 2 following wt virus challenge, we observed a prominent increase in lymphocytes in perivascular cuffs and around bronchi and bronchioles in vaccinated mice. Our results suggest that LAIVs induce virus-specific memory CD8⁺ T cells in the lungs and that these cells produce antiviral cytokines and have cytolytic activity. We believe that the accelerated appearance of these CD8⁺ T cells in the lungs is induced by vaccine and not by the challenge virus because lymphoid hyperplasia was not observed in the control group of mock-vaccinated mice. An accelerated appearance of influenza virus-specific CD8⁺ CTLs was also seen when mice primed with a type A influenza virus were challenged with another type A influenza virus (3). Recently, Bannard et al. demonstrated that CD8⁺ effector T cells induced by influenza virus infection proliferate during secondary infection (2). Using a murine model of Sendai virus infection, Ely et al. also demonstrated that CD11a^{low} CD8⁺ T cells (expressing low levels of CD11a and CD8) that persist in the lung airways are not terminally differentiated and retain the potential to proliferate (17). In addition, Mrusek et al. showed that the recruitment and expansion of respiratory syncytial virus (RSV)-specific CD8⁺ T cells in the lungs were not affected by splenectomy, suggesting that memory T cells persisting in the lungs or local lymphoid tissue are the source of precursors for expansion and that they contribute to viral clearance by cytolytic activity on virus-infected cells (34). Nasal-associated lymphoid tissue (NALT) was shown to be the site of induction of mucosal responses (47). CD8⁺ T cells induced either by vaccination or adoptive transfer can mediate viral clearance in the respiratory tract in the absence of humoral immunity (12, 15, 24, 30, 32). Although the contribution of CD8⁺ T cells to protection from influenza virus infection in humans is less clear than in mice, the induction of CD8⁺ T cells by LAIVs has been demonstrated in children (23), and evidence suggestive of heterosubtypic immunity during the 1957 pandemic was presumed to be mediated by CD8⁺ T cells (18).

Joo et al. showed that the lungs are the major organ for the establishment of ASCs following an influenza virus infection (26). Our results provide further evidence that these ASCs contribute to local antibody production in the respiratory tract. First, the number of the lung ASCs elicited by various LAIVs

correlates with the magnitude of the antibody response. Second, the isotype distribution of the influenza virus-specific antibodies in the nasal and tracheal wash is consistent with the isotype distribution in the lungs. Although it has been shown that exsanguination followed by extensive perfusion of the circulatory system reduces contamination of organ samples by serum antibodies to below 2% (4), even low-level contamination by influenza virus-specific serum antibodies, which mainly belong to IgG1 and IgG2a subclasses, could significantly shift the isotype distribution in lung samples. This might explain the weaker correlation between isotype distribution of antibody and the number of isotype-specific ASCs in the lungs than in nasal and tracheal washes. In contrast, mucosal IgA was used to measure the magnitude of mucosal responses, and because the level of IgA in the serum was insignificant (data not shown), the presence of these serum antibodies in the lung samples would not be expected to have a significant impact on the mucosal responses.

In summary, the LAIV platform is capable of inducing a broad spectrum of immune effectors in the systemic and mucosal immune system. All LAIVs tested induced robust systemic immune responses but variable pulmonary immunity, which depended on the replication of the vaccine virus in the lungs. We believe that both cellular and humoral immunity likely contribute to the protection provided by LAIV; the relative contribution of the two effector arms in viral clearance depends on the location and the rate of replication of a particular vaccine virus. The results of this study highlight what is needed to induce these local immune effectors.

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