The Contribution of Systemic and Pulmonary Immune Effectors to Vaccine-Induced Protection from H5N1 Influenza Virus Infection

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Live attenuated influenza vaccines (LAIVs) are effective in providing protection against influenza challenge in animal models and in preventing disease in humans. We previously showed that LAIVs elicit a range of immune effectors and that successful induction of pulmonary cellular and humoral immunity in mice requires pulmonary replication of the vaccine virus. An upper respiratory tract immunization (URTI) model was developed in mice to mimic the human situation, in which the vaccine virus does not replicate in the lower respiratory tract, allowing us to assess the protective efficacy of an H5N1 LAIV against highly pathogenic H5N1 virus challenge in the absence of significant pulmonary immunity. Our results show that, after one dose of an H5N1 LAIV, pulmonary influenza-specific lymphocytes are the main contributors to clearance of challenge virus from the lungs and that contributions of influenza-specific enzyme-linked immunosorbent assay (ELISA) antibodies in serum and splenic CD8⁺ T cells were negligible. Complete protection from H5N1 challenge was achieved after two doses of H5N1 LAIV and was associated with maturation of the antibody response. Although passive transfer of sera from mice that received two doses of vaccine prevented lethality in naive recipients following challenge, the mice showed significant weight loss, with high pulmonary titers of the H5N1 virus. These data highlight the importance of mucosal immunity in mediating optimal protection against H5N1 infection. Understanding the requirements for effective induction and establishment of these protective immune effectors in the respiratory tract paves the way for a more rational and effective vaccine approach in the future.

Acute respiratory tract infection is a significant cause of morbidity and mortality worldwide. Live attenuated vaccines are being developed for a number of respiratory viruses, including respiratory syncytial virus, human parainfluenza viruses, and human metapneumovirus. A live attenuated influenza vaccine (LAIV) is currently licensed for use in the form of a nasal spray for healthy children and adults in several countries (12), and vaccine efficacy has been demonstrated in a number of clinical studies (1–3, 24, 34, 47, 51). In addition, previous studies demonstrated that a single dose of LAIV induces a wide range of systemic and mucosal immune effectors in mice (25) and that vaccine-induced immunity is protective against wild-type (wt) virus challenge in different animal models, including mice, ferrets, nonhuman primates, and humans (4, 6, 7, 23, 31, 45, 47). In humans, LAIV induces humoral and cellular immunity, including influenza-specific CD8⁺ cytotoxic T lymphocytes (CTLs) in the peripheral blood (20), but direct evidence of the importance of CTLs in mediating protection against influenza infection and their establishment in the lower respiratory tract after immunization with LAIV is lacking.

Using LAIVs with hemagglutinin (HA) and neuraminidase (NA) from 8 different subtypes of wt influenza viruses, we previously demonstrated that the induction of pulmonary immunity, but not systemic immunity, requires pulmonary replication of the vaccine virus and induction of cytokines (25). Given that LAIVs are intended to be administered intranasally without significant replication in the lower respiratory tract in humans, protective efficacy of LAIV without the induction of pulmonary immunity would be relevant especially for viruses, such as the highly pathogenic avian influenza (HPAI) H5N1 viruses, which have tropism for the lower respiratory tract and the ability to cause systemic infection (10, 43). Additional pulmonary immune effectors might be required to protect the host from an H5N1 infection. To ad-

dress this question, we developed an upper respiratory tract immunization (URTI) model to address the relationship between lung immunity and protection against wt virus challenge using the A/Vietnam/1203/2004 (VN04) (H5N1) LAIV. We significantly extend our previous observations by showing that cellular immunity in the lungs is essential for protection against lethal wt H5N1 challenge, whereas influenza-specific serum enzyme-linked immunosorbent assay (ELISA) antibodies and splenic influenza-specific CD8⁺ CTLs make little contribution to this protection. Optimal protection against wt virus challenge requires maturation of humoral responses, with the development of neutralizing activity. Finally, passive transfer of postvaccination serum to naive mice demonstrates that the magnitude of the humoral response and access of antibodies to the respiratory tract are equally important determinants of protection.

MATERIALS AND METHODS

H5N1 LAIV. The VN04 H5N1 vaccine used in this study was derived using plasmid-based reverse genetics as previously described (45). The virus was generated in collaboration with Hong Jin and George Kemble from MedImmune (Mountain View, CA) under a Cooperative Research and Development Agreement (CRADA).

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Vaccination protocol. For total respiratory tract immunization (TRTI) with LAIV, mice were lightly anesthetized with 4% isoflurane, followed by intranasal (i.n.) administration of the H5N1 LAIV in a 50- μ l volume. For upper respiratory tract immunization (URTI), unanesthetized mice were given the VN04 H5N1 LAIV in 5 μ l. The inactivated subunit vaccine was administered subcutaneously (s.c.) in a volume of 100 μ l at the base of the tail and 50 μ l on each side of the tail. The animal study protocols used were approved by the National Institutes of Health Animal Care and Use Committee and were conducted at the NIH.

Passive transfer of PVS. Postvaccination sera (PVS) were collected from mice that received either one or two doses of 10^6 50% tissue culture infective doses (TCID₅₀) of the H5N1 LAIV by TRTI. When two doses were administered, they were separated by 28 days. The mice were exsanguinated at the time points indicated in the legend to Fig. 6, and the sera were collected and pooled. The neutralizing (MN) antibody titers in sera pooled from day 28 (d28) and d56 were 32 and 512, respectively. For passive transfer, naïve mice received 500 μ l of PVS intraperitoneally (i.p.).

In vivo depletion of specific lymphocytes. The following monoclonal antibodies (MAb) were used for *in vivo* depletion of specific subsets of lymphocytes: GK1.5, specific for mouse CD4; 2.43, specific for mouse CD8; and SFR3-DR5, specific for human leukocyte antigen as an isotype control. All MAbs are rat IgG2b and were obtained from the National Cell Culture Center (Biovest International). The antibodies were injected i.p. in a volume of 1 ml 2 days before and after challenge. The completeness of the depletion was evaluated by staining single-cell suspensions from lungs with noncompeting CD4 and CD8 MAbs *ex vivo* (RM4-4 and 53.6.7). Cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA) and subsequently by Flow Jo (Tree Star, Inc., Ashland, OR).

For more detailed descriptions of the experimental procedures, see the supplemental Materials and Methods.

RESULTS

The URTI model in mice. In an earlier study of LAIVs representing several influenza virus subtypes, we found that the A/Panama/2007/99 (H3N2) LAIV did not replicate efficiently in the lungs of mice and induced poor local immune responses (25); we concluded that the magnitude of pulmonary immunity in the lungs of mice is determined by local replication of the vaccine virus (25). However, we could not determine the significance of the ineffective induction of pulmonary immunity on protective efficacy against wt virus challenge, because the corresponding H3N2 wt virus also failed to replicate in the lungs of mice. Since the VN04 H5N1 wt virus replicates well in the lungs of mice and the VN04 (H5N1) LAIV induced robust local and systemic immunity when given by TRTI (25, 45), we sought to evaluate the importance of pulmonary immunity in providing protection against viral challenge by using the URTI model to restrict the induction of pulmonary immunity. We developed the URTI model based on the work of Iida and Bang, who suggested that by administering a small volume of inoculum to awake mice, it is possible to establish an infection in the nasal epithelium without the involvement of the rest of the respiratory tract (22). In our model, groups of 5 unanesthetized BALB/c mice were given 1×10^6 TCID₅₀ or 1×10^7 TCID₅₀ of the VN04 H5N1 LAIV in a volume of 5 μ l intranasally (i.n.) and were euthanized 4 days later to determine the level of viral replication in various parts of the respiratory tract. As shown in Fig. 1A and B, at either dose, 3 or 4 of 5 mice had virus detected in the nasal turbinates (NT), but none of the mice had virus detected in the lungs. The results suggest that VN04 (H5N1) LAIV lacks the ability to spread to the lower respiratory tract in the URTI model, which is different from the mouse-adapted A/PR/8/34 H1N1 virus when tested in a similar model (50). Although some mice had no detectable virus in the lungs or NT on d4, in a sepa-

rate experiment we demonstrated that all URTI-immunized mice developed robust ELISA antibody responses 28 days following priming (Fig. 1C). Thus, we proceeded to determine whether there were any differences in the immunity induced by URTI and TRTI.

Administration of VN04 H5N1 LAIV by URTI led to a selective reduction of the pulmonary IgA response without affecting the systemic humoral response. Groups of 5 mice were immunized by TRTI (10^6 TCID₅₀) or URTI (10^7 or 10^6 TCID₅₀), and the magnitude of humoral responses at various sites was determined 28 days later. Since mice inoculated with VN04 H5N1 LAIV by TRTI show only low levels of hemagglutination-inhibiting (HI)/neutralizing (MN) activity in serum 28 days after vaccination (45), ELISA was used to detect the presence of influenza-specific humoral responses in serum. Mice that were inoculated by URTI developed serum ELISA antibody titers comparable to those achieved by TRTI, regardless of the dose of vaccine virus (Fig. 1D), which is consistent with the results of Nguyen et al. using A/Udorn/1972 (H3N2) (33). We further demonstrate that there was also no statistical difference in the ELISA IgA titers in the NT achieved by the two immunization protocols (Fig. 1E). However, there were significantly lower ELISA IgA titers in the lungs of mice that were immunized by URTI than in those immunized by TRTI ($P < 0.05$) (Fig. 1F). We also determined the frequency of antibody-secreting cells (ASCs) and influenza-specific CD8⁺ T cells in various organs following the vaccination protocol described in Fig. 1 and compared mice that received the VN04 H5N1 LAIV by URTI and TRTI; we found significantly reduced frequency of ASCs in the lungs of mice that received the LAIV by URTI, regardless of the dose given (Fig. 2A). Such a difference was not found in the spleen (Fig. 2B). Furthermore, compared with mice vaccinated by TRTI, a significant reduction in the frequency of NP₁₄₇-specific CD8⁺ T cells was found in the lungs ($P < 0.05$) (Fig. 2C) but not in the spleen (Fig. 2D) of mice that were vaccinated by URTI 28 days earlier.

In summary, when an LAIV that could replicate in lungs of mice and induce robust pulmonary immunity when given by TRTI was administered by URTI, replication of the vaccine virus was confined to the upper respiratory tract, and there was a significant reduction in the magnitude of induced pulmonary immunity. These results suggest a cause-and-effect relationship between local replication of the vaccine virus and local immunity. In addition, the data demonstrate that viral replication in the upper respiratory tract is sufficient to induce robust systemic immunity.

Mice immunized by URTI were not protected from challenge with homologous and heterologous H5N1 wt viruses. We had previously demonstrated that mice vaccinated with a single dose of VN04 H5N1 LAIV by TRTI were protected from lethal challenge with H5N1 wt viruses without a high level of neutralizing antibodies in serum (25, 45), but the immune effectors that were responsible for this protection remained unclear. Since mice vaccinated with the VN04 H5N1 LAIV by URTI developed influenza-specific immune effectors in serum and spleen but not in the lungs (Fig. 1D and F and 2), we had an opportunity to determine the importance of pulmonary immunity in mediating protection from challenge with the wt H5N1 virus. Groups of 5 mice vaccinated with 10^6 or 10^7 TCID₅₀ of the VN04 H5N1 LAIV by TRTI or URTI were challenged with 10^5 TCID₅₀ of VN04 H5N1 wt virus 28 days later and were monitored for survival for 3 weeks. As shown in Fig. 3A, 4 out of 5 mice vaccinated with 10^6 TCID₅₀ VN04 H5N1

FIG 1 Restricted replication of VN04 (H5N1) LAIV by URTI is associated with diminished pulmonary humoral responses but does not affect serum antibody titers. (A and B) Groups of five mice were inoculated with the indicated doses of the VN04 H5N1 LAIV by URTI. Viral titers in the NT (A) and lungs (B) were determined 4 days pi and are expressed as $\log_{10}$ TCID₅₀/g of tissues. The dotted lines represent the lower limit of detection. (C) BALB/c mice received 1×10^6 or 1×10^7 TCID₅₀ of VN04 H5N1 LAIV by URTI. The magnitudes of the serum antibody responses detected 28 days later by ELISA are presented as $\log_{10}$ antibody titers. (D to F) Groups of 5 mice were vaccinated with the indicated doses of the VN04 H5N1 LAIV by URTI or TRTI. Serum samples (D) and homogenates of NT (E) and lung (F) were collected 28 days later. The titers of total influenza-specific antibodies in serum and influenza-specific IgA in the homogenates of NT and lungs were determined by ELISA. The bars and error bars represent the means and standard deviations of the group. *, $P < 0.05$.

LAIV by TRTI survived the challenge. On the other hand, regardless of the dose of vaccine, all mice that were vaccinated by URTI succumbed to lethal challenge. Mice vaccinated with 10^6 TCID₅₀ VN04 H5N1 LAIV by URTI had no significant survival advantage over the mock-vaccinated control group in which all mice succumbed to the infection within 5 days after challenge (Fig. 3A). All of the mice that were vaccinated with 10^7 TCID₅₀ VN04 H5N1 LAIV by URTI succumbed by d8. Similar observations were made following challenge with a heterologous clade 2.1 H5N1 virus (A/Indonesia/5/2005) (data not shown).

To determine whether there was a benefit of TRTI over URTI in terms of a virologic outcome, mice that were vaccinated and challenged as described in Fig. 3A were euthanized on d1 and d3 postinfection (pi), and organs were harvested for viral titration. As shown in Fig. 3B, on d1 pi, mice that were vaccinated by TRTI had significantly lower titers of VN04 H5N1 wt virus in the NT than the mock-vaccinated (with Leibovitz-15 [L-15] medium) controls ($P = 0.0073$), while among mice that were vaccinated by URTI, only the group that received 10^7 TCID₅₀ showed a significant reduction compared with the mock-vaccinated controls ($P = 0.0114$). By d3 pi, all vaccinated mice had significantly lower viral titers in the NT than the mock-vaccinated controls ($P < 0.05$). Additionally, TRTI-vaccinated mice had significantly lower viral titers than those that received vaccine by URTI ($P = 0.0104$ and 0.0214 for 10^6 and 10^7 TCID₅₀ groups, respectively), and 2 of 5

mice had cleared the virus. In the lungs, all of the mice had significant amounts of virus on d1 pi; only the TRTI-vaccinated group and the 10^7 TCID₅₀ URTI groups showed a significant reduction in viral titer compared with that of the mock-vaccinated group ($P = 0.0109$ and 0.0452). By d3 pi, viral titers in the TRTI-vaccinated mice were significantly lower than in the URTI and mock-vaccinated groups ($P < 0.05$); mice that received 10^6 TCID₅₀ by URTI had viral titers similar to those of the mock-vaccinated group ($P = 0.0634$). Similar results were obtained when VN04 LAIV-vaccinated mice were challenged with the clade 2.1 Indo 05 H5N1 virus (data not shown).

Cellular immunity is important for pulmonary viral clearance following vaccination by TRTI. Results from Fig. 3 suggest that pulmonary immunity, rather than systemic immunity, induced by TRTI is important for accelerated clearance of the H5N1 wt challenge virus. Therefore, specific depleting antibodies were used to gain insights into the cell population(s) responsible for viral clearance. Groups of 5 mice were vaccinated with 10^6 TCID₅₀ of VN04 H5N1 LAIV by TRTI, and T cell subsets were depleted using monoclonal antibodies (MAb) around the time of wt virus challenge. NT and lungs were harvested on d2 and d4 pi. The completeness of the depletion of specific T cell subsets in the lungs was confirmed by flow cytometry (Fig. 4A). As shown in Fig. 4B, all mice that were vaccinated with the VN04 H5N1 LAIV showed significant reduction in viral titers in the NT ($P < 0.05$) on d2 pi

FIG 2 Mice vaccinated by URTI showed diminished cellular responses in the lungs but not in the spleen. Groups of 5 mice were vaccinated as described in the legend to Fig. 1. Single-cell suspensions were obtained from lungs and spleens 28 days after vaccination. (A and B) The frequency of ASC in lungs (A) and spleen (B) was determined by B-cell enzyme-linked immunosorbent spot assay (ELISPOT). Due to the difference in frequencies, note that the scales of the y axes are different. (C and D) The frequency of NP₁₄₇-specific CD8⁺ T cells in lungs (C) and spleen (D) was determined by gamma interferon (IFN- γ) ELISPOT assay. The bars and error bars represent the means and standard deviations of the group. *, $P < 0.05$; N.D., not detected.

compared with those of the mock-vaccinated controls. By d4 pi, vaccinated mice that received isotype control or CD8-depleting antibody cleared the infection in NT (4/5 and 5/5 mice, respectively) (Fig. 4D). Interestingly, more mice that were depleted of CD4⁺ T cells had virus in the NT on d4 pi (3/5 and 4/5 mice for CD4⁺ and CD4⁺/CD8⁺, respectively). In the lungs, all vaccinated groups had significantly less virus than the mock-vaccinated controls on d2 ($P < 0.05$) (Fig. 4C); however, depletion of both CD4⁺ and CD8⁺ populations led to a smaller reduction that was statistically different from mice that did not have any T cells depleted (isotype control; $P = 0.0157$). The pulmonary viral titers were lower by d4 (Fig. 4E), and complete viral clearance was observed in vaccinated mice that received the isotype control MAb. A larger number of vaccinated mice that were depleted of CD8⁺ or CD4⁺ T cells had detectable virus in the lungs (4/5 and 3/5 mice, respectively), and the difference reached statistical significance for the group depleted of CD8⁺ T cells ($P = 0.0248$). Depletion of both cell populations led to a delay in viral clearance with an average

titer of 10^4 TCID₅₀/g of tissue that was significantly higher than the isotype group ($P = 0.0073$). Therefore, this experiment suggests that cellular immunity is important for mediating clearance in the lungs but not in the upper respiratory tract.

The immunogenicity and protective efficacy of URTI can be improved by boosting and lengthening the duration between priming and viral challenge. We have previously demonstrated that 2 doses of VN04 H5N1 LAIV by TRTI prevented replication of challenge VN04 H5N1 wt virus in mice (45). Therefore, we now evaluated whether an additional dose of VN04 H5N1 LAIV by URTI would improve its immunogenicity and protective efficacy against lethal challenge. Groups of 5 mice were vaccinated with 1 or 2 doses of VN04 H5N1 LAIV by TRTI (10^6 TCID₅₀) or URTI (10^7 TCID₅₀) 28 days apart. On day 56, all mice developed high levels of serum ELISA antibodies, regardless of the vaccination route and schedule (Fig. 5A). Mice that were vaccinated by URTI failed to develop high levels of pulmonary IgA antibodies even after boosting, whereas mice that were vaccinated by TRTI had significant levels of pulmonary IgA, especially after 2 doses of vaccine (Fig. 5B). Comparable levels of IgA were detected in the NT regardless of the vaccination route and schedule (Fig. 5C). MN antibody titers were lowest in sera collected 28 days after priming by TRTI or URTI. The MN titers continued to increase over the next 28 days (Fig. 5D) ($P = 0.0196$ and 0.0254 for TRTI and URTI, respectively), and a second dose of vaccine by TRTI but not URTI led to an increase in serum MN titers ($P = 0.0273$ and 0.6733 , respectively).

The mice were challenged with VN04 H5N1 wt virus to evaluate the biological significance of the improvement in the humoral responses after boosting. Since we had previously shown that mice that received two doses of VN04 H5N1 LAIV by TRTI survived challenge with minimal weight loss (45), we conducted this experiment with URTI only. As shown in Fig. 5E and F, consistent with data from previous experiments, all the mice that received 1 dose of vaccine by URTI and were challenged 28 days later displayed significant weight loss and succumbed to infection. When mice were challenged 56 days after 1 dose of vaccine, all mice survived the challenge, but they experienced significant weight loss that started on d4 pi and lasted for 8 days. Mice that received 2 doses of vaccine by URTI survived challenge without significant weight loss. Virologic data revealed that mice that received the vaccine by URTI had significantly less virus in the NT compared with the mock-vaccinated control group (Fig. 5G) ($P < 0.05$), and NT viral titers were comparable among the vaccinated groups, regardless of whether they received a boost or not. However, among URTI-vaccinated mice, only those that received a boost cleared the virus from the lungs by d4 pi (Fig. 5H). All the mice that were challenged on day 28 or 56 following one dose of vaccine had significant amounts of challenge virus in the lungs on d4 pi. The mice that were challenged 56 days postvaccination had significantly less virus in the lungs compared with the group that were challenged 28 days after one dose of vaccine ($P = 0.0181$).

Transfer of postvaccination serum protects mice from lethality but not from pulmonary replication of the VN04 H5N1 wt virus. Since mice vaccinated with VN04 H5N1 LAIV by TRTI survived lethal viral challenge, we proceeded to evaluate whether passive transfer of serum from VN04 H5N1 LAIV-immunized mice would protect naïve mice from lethal VN04 H5N1 infection. Sera were collected on day 28 (denoted as d28-1 dose sample)

FIG 3 Mice vaccinated by URTI succumbed to lethal challenge with H5N1 wt virus. (A) Groups of 5 mice were vaccinated as described in Fig. 1 and were challenged with 1×10^5 TCID₅₀ of VN04 (H5N1, clade 1) wt virus i.n. 28 days later. Survival was monitored for 3 weeks after challenge. The symbol for each group is as follows: ●, 10^6 TCID₅₀ of VN04 H5N1 LAIV by TRTI; x, 10^6 TCID₅₀ by URTI; ◆, 10^7 TCID₅₀ by URTI; ▼, mock-vaccinated group. As the mortality rates for the 10^6 TCID₅₀ by URTI and mock-vaccinated groups were identical, the symbols for these groups are superimposed. (B and C) Mice were vaccinated and challenged as described in panel A. Viral titers in NT (B) and lungs (C) at the indicated time points were determined on MDCK monolayers. The dotted lines represent the lower limit of detection. *, $P < 0.05$; #, there was a significant difference between this group and the mock-vaccinated group.

from mice that were vaccinated with a single dose and on day 56 (denoted as d56-2 dose sample) from mice that were vaccinated with 2 doses of 10^6 TCID₅₀ VN04 H5N1 LAIV by TRTI. Postvaccination sera (PVS) were administered to naïve mice, and a day later the recipient mice were challenged with a lethal dose of VN04 H5N1 wt virus. For comparison, groups of 5 mice were vaccinated subcutaneously with 2 doses of 500 ng of an adjuvanted subunit VN04 H5N1 vaccine. This vaccine formulation had previously been demonstrated to elicit good humoral immunity and was effective in protecting mice from VN04 H5N1 wt virus challenge (26). As shown in Fig. 6A and B, passive transfer of d28-1 dose sera, which had low neutralizing activity (MN titer = 32) against the VN04 H5N1 wt virus, failed to protect the recipient mice from lethal challenge. The mice had significant weight loss and succumbed to infection by day 8 pi. Passive transfer of d56-2 dose sera, which had high neutralizing activity (MN titer = 512) against the VN04 H5N1 wt virus, protected recipient mice from lethality, though the mice experienced significant weight loss. Active immunization by the s.c. route completely protected mice from challenge without significant weight loss. The virologic data were consistent with the survival data; mice that received the adjuvanted subunit vaccine had no detectable virus in the NT and lungs (Fig. 6C and D). On the other hand, passive transfer of PVS had little effect on preventing viral replication in the NT (Fig. 6C). Al-

though there was still a significant amount of virus in the lungs of mice that received passive transfer of PVS on day 4 pi, the administration of PVS significantly reduced the pulmonary viral titers compared to those in mice that were mock vaccinated ($P = 0.0107$). Mice that received the d56-2 dose sera had lower pulmonary virus titers than those that received the d28-1 dose sera, but the difference did not reach statistical significance ($P = 0.1388$).

DISCUSSION

An ideal vaccine should elicit protective immunity rapidly. We have demonstrated that there are a number of immune effectors that can provide complete protection against wt H5N1 challenge and have defined the requirements for effective induction of these effectors by LAIV (summarized in Table 1). We demonstrated that induction of pulmonary immunity with a VN04 H5N1 LAIV protects against replication of wt challenge virus. This conclusion is based on the results from the URTI model and lymphocyte depletion study. The diminished pulmonary immunity in the URTI model (Fig. 1 and 2) coincided with loss of protection against H5N1 virus challenge, suggesting that pulmonary immunity was responsible for protection. This observation is consistent with our previous observation of prominent perivascular and peribronchial lymphoid cuffs and general lymphoid hyperplasia in VN04 H5N1 LAIV-vaccinated mice 2 days after wt virus challenge that

FIG 4 CD4⁺ and CD8⁺ lymphocytes are important in mediating accelerated viral clearance from the lungs. Groups of 5 mice were vaccinated with 1×10^6 TCID₅₀ of VN04 (H5N1) LAIV by TRTI and were challenged with wt VN04 (H5N1) 28 days later. Specific monoclonal antibodies were injected i.p. 2 days before and after challenge to deplete specific subsets of lymphocytes. Lungs were harvested on day 4 pi. (A) Single-cell suspensions were prepared to verify the depletion of the indicated cell subsets in the lungs. (B to E) Vaccinated mice were euthanized 2 (B, C) or 4 (D, E) days postchallenge. Viral titers in NT (B, D) and lungs (C, E) were determined on MDCK monolayers. *, $P < 0.05$; #, there was a significant difference between this group and the mock-vaccinated group. N.A. indicates that all mice succumbed to the infection prior to sample collection. From an independent study (25), the average viral titers in NT and lungs of mock-infected mice on d4 postinfection were 10^6 and $10^{7.2}$ TCID₅₀/g of tissues, respectively.

coincided with pulmonary viral clearance by d4 postinfection (25). Furthermore, the role of cellular immunity in mediating pulmonary viral clearance is strengthened by the lymphocyte depletion study in which TRTI-vaccinated mice depleted of CD4⁺ and CD8⁺ lymphocytes showed delayed pulmonary viral clearance (Fig. 4E). CD4⁺ Th cells and CD8⁺ CTLs independently contribute to 1,000-fold reduction in pulmonary viral titers (Fig. 4C). Our results agree with a recent study by Guo et al. which shows that cross-reactive CD4⁺ and CD8⁺ T cells contribute to *in vivo* protection against H1N1 virus infection (18). Our results are consistent with the report of Liang et al. that showed that *in vivo* depletion of CD8⁺ T cells led to partial but not complete reduction of cellular immunity against influenza infection (29). In addition, cross-reactive, cell-mediated protection has been reported in animals (21, 41, 42), and memory T cells established by seasonal influenza infection in humans can recognize immunological epitopes from H5N1 viruses (28). Apart from CTL induction, the induction of CD4⁺ Th cells by vaccination could be an attractive target, given that memory CD4⁺ T cell responses are more diverse in humans (39), they exhibit a high level of cross-reactivity (16), and they can persist for a long time. The results from the URTI model also suggest that, for a CTL-based vaccine to provide optimal protection against an acute infection, these effectors will have to be maintained at the site of infection for an extended period of time. A similar conclusion about the importance of the location of CTLs was drawn by Nguyen et al. (33). However, the correlation

with survival was related to cytolytic activity in the mediastinal lymph nodes rather than in lungs, because significant cytolytic activity was not detected in lungs in their study. This contrasts with our previous study in which ~4% of pulmonary CD8⁺ T cells were specific for a nucleoprotein CTL epitope with immediate effector function (25). This difference is likely due to the sensitivity of the assays used in the studies. Although cellular immunity does not provide sterilizing immunity, it can lead to early viral clearance (13, 36) and heterosubtypic immunity (11, 33). Our studies show that local inflammation is required for the establishment of pulmonary immunity and optimal protection. Therefore, for a successful vaccine based on cellular immunity, we will need to find a way to guide these immune effectors to the lungs without causing pneumonitis.

In the URTI model, although serum antibodies elicited by 1 dose of LAIV should be available to transude to tissues before virus challenge, these serum antibodies were not sufficient for protection against lethal H5N1 challenge. Similar results were obtained from passive transfer of postvaccination serum to naïve mice 1 day prior to challenge (Fig. 6A). Serum antibodies were able to reduce pulmonary viral titers only by 10-fold (Fig. 6D), and a similar level of reduction was observed in mice depleted of both CD4⁺ and CD8⁺ cells (Fig. 4C). Vaccination with a single dose of the VN04 H5N1 LAIV also showed an increase in serum MN antibody titers over time (d56) (Fig. 5D), and these mice were protected from lethal challenge but still lost weight and had challenge virus repli-

FIG 5 The effect of boosting on the immunogenicity of the VN04 (H5N1) LAIV given by URTI. Groups of 5 mice were given the VN04 (H5N1) LAIV by URTI or TRTI as indicated on days 0 and 28. (A to C) Serum samples (A), lung homogenates (B), and NT homogenates (C) were collected on day 56. The titers of total influenza-specific antibodies in serum and influenza-specific IgA in the homogenates of NT and lungs were determined by ELISA. (D) Serum-neutralizing antibody titers against 100 TCID₅₀ of VN04 H5N1 wt virus were determined in an MN assay. (E and F) Mice vaccinated as indicated by URTI were challenged with 1×10^5 TCID₅₀ of VN04 H5N1 wt virus. Survival (E) and weight loss (F) were monitored for 15 days. (G and H) Groups of 5 mice were vaccinated as indicated and challenged with 1×10^5 TCID₅₀ of VN04 H5N1 wt virus on day 56. Viral titers in NT (G) and lungs (H) on day 4 postchallenge were determined on MDCK monolayers. *, $P < 0.05$.

cation in the lungs. Improvement in the quality of antibody through affinity maturation and/or expansion of appropriate B lymphocyte clones are possible explanations that were not assessed in this study and would be of interest in future studies. Complete protection from pulmonary infection mediated by humoral immunity requires two doses of the VN04 H5N1 LAIV in mice (45). Mice vaccinated with two doses of vaccine administered either by URTI or TRTI showed complete protection from pulmonary viral replication without significant weight loss (Fig. 5E and F). The increase in protection was associated with an increase in serum MN antibody titers, although the increase in titer was more prominent in mice that received the vaccine by TRTI (Fig. 5D).

While mice that received 2 doses of the VN04 H5N1 LAIV were completely protected from challenge without weight loss or lethality and had no significant viral replication in the lungs (Fig. 5E, F, H), passive transfer of sera from these vaccinated animals into naïve mice protected the animals only from lethality; the recipient mice still had weight loss and significant virus load in the lungs (Fig. 6B and C). It is possible that although virus was detected in the lung homogenates, there was less virus in the parenchyma of the lungs following passive transfer, as was reported by Simmons et al. following passive transfer of an H5-specific monoclonal antibody (44). Unfortunately, we did not perform immunohistochemistry on lung tissue to determine the location of viral antigen in this study. It is also possible that the high challenge dose could be a factor in determining the outcome, because it was previously demonstrated that mice that received passive transfer of another monoclonal antibody were protected from weight loss when they

were challenged with a lower dose (10 50% minimal lethal doses [MLD₅₀]) of H5N1 wt virus (19). Our results underscore the importance of mucosal immunity for optimal protection against respiratory infections; high titers of serum antibodies are necessary for optimal protection at a mucosal surface (49).

As for protection of the upper respiratory tract from viral infection, significant reduction in viral titers was achieved in the presence or absence of CD4⁺ and CD8⁺ T cells (Fig. 4B). As the reduction was observed as early as d1 postchallenge (Fig. 3B) and was maintained over the observation period (from d2 to d4) (Fig. 4B and D and Fig. 3B), antibodies are likely the main protective mediator by preventing viral attachment. Our observation that more mice that lacked CD4⁺ T cells had virus detected in the NT than those with CD4⁺ T cells suggests that CD4⁺ cells might contribute to viral clearance in the NT. CD4⁺ T cells are important for antibody production (32, 40, 46). It is of interest to note the difference between NT and pulmonary viral titers in TRTI-vaccinated mice after viral challenge (Fig. 3B and C). The observation that antibody responses protected the NT suggests that the antibody repertoire in the NT was broad enough to interrupt viral replication. It was surprising that antibody responses were not able to prevent viral replication in the lungs and that high titers of virus were detected there. As H5N1 wt virus replicates more efficiently in lungs than in the upper respiratory tract (43), one possible explanation for the observation is that rapid viral replication might overwhelm pulmonary immunity. Alternatively, since IgA has been shown to be effective in protecting the upper respiratory tract (37, 38), it may be that IgA in the NT interrupts viral replication intracellularly (30), a mechanism that is not seen with IgG

FIG 6 Passive transfer of postvaccination sera (PVS) confers variable protection against lethal challenge but does not prevent pulmonary viral replication of the VN04 (H5N1) wt challenge virus. (A to C) Two groups of mice were given 1×10^6 TCID₅₀ of VN04 H5N1 LAIV by TRTI. One group was euthanized for serum (d28-1 dose PVS) on d28 and the other group received a boost, and sera were collected on d56 (d56-2 dose PVS). Groups of 5 naïve mice received 0.5 ml of the indicated PVS i.p. 1 day before wt virus challenge. For active immunization, a group of mice was vaccinated with two doses of 500 ng of an adjuvanted subunit VN04 H5N1 vaccine s.c. 28 days apart. All mice were challenged with 1×10^5 TCID₅₀ of VN04 H5N1 wt virus on day 56. Survival (A) and weight loss (B) were monitored for 15 days. (C and D) The viral titers in NT (C) and lungs (D) were determined on day 4 postchallenge. *, there was a significant difference between this group and the L15 mock-vaccinated group. The dotted line represents the lower limit of detection.

in the lungs. The trend between the number of pulmonary ASCs and IgA titers in lungs (Fig. 1E and 2A) suggests that local ASCs are responsible for local antibody production in the respiratory tract and that vaccine-induced inflammatory responses are required for the induction of local ASCs. This observation confirmed our previous results (25).

For mice vaccinated with 10^6 and 10^7 TICD₅₀ of VN04 LAIV by URTI, 2/5 and 1/5 mice had viral titers below the detection limit,

respectively (Fig. 1A). As the mice were not anesthetized during inoculation, the inoculum could be sneezed out, causing this variation. However, all of the mice had an immune response (Fig. 1C). We cannot exclude the possibility that part of the inoculum might be ingested during URTI; it is unlikely that this form of oral immunization would contribute significantly to the immune responses we observed for the following reasons. First, successful oral immunization usually requires inhibition of gastric acid by

TABLE 1 Immune effectors that play a role in protection from H5N1 wt virus challenge and their induction by VN04 H5N1 LAIV in mice

Immune effector	Location	Requirement for induction by LAIV		Functional consequence		
		Site of replication	No. of doses	Prevents infection?	Accelerated clearance from lungs?	Comments
Influenza-specific serum ELISA Ab without neutralizing activity	Systemic	Upper respiratory tract	1	No	No	Not protective against wt virus challenge
Influenza-specific serum ELISA Ab with neutralizing activity	Systemic	Upper respiratory tract	2	Yes in NT and lungs	Not applicable	Access to lung tissue is crucial for protection
Splenic CD4 ⁺ /CD8 ⁺ lymphocytes	Systemic	Upper respiratory tract	1	No	No	Not protective against wt virus challenge
Pulmonary influenza-specific IgA	Local	Lower respiratory tract	1	Yes in NT > lungs	Yes	Main effector in NT
Pulmonary CD8 ⁺ lymphocytes	Local	Lower respiratory tract	1	No	Yes	No role in clearance from NT
Pulmonary CD4 ⁺ lymphocytes	Local	Lower respiratory tract	1	No	Yes	No role in clearance from NT

measures such as preadministration of aluminum hydroxide and Tagamet before inoculation to protect the antigenicity of the immunogen (8). Second, repeat immunization with a high dose of vaccine (four consecutive daily doses of 43 μ g) was required for the induction of immune responses by the oral route (8). These factors lead us to believe that the site of induction for URTI is likely to be confined to the upper respiratory tract.

Two doses of H5N1 LAIV are required for complete protection from virus challenge when given by TRTI (45) or URTI (this study) in naïve mice. As most people are not immunologically naïve to influenza virus, fewer doses might be sufficient to achieve protection in humans, as was seen during the 2009 H1N1 pandemic. Even though the 2009 H1N1 virus was genetically and antigenically distinct from seasonal H1N1 viruses (9, 14), only one dose of the 2009 H1N1 vaccine was required to achieve a protective antibody titer in all except very young children (17, 35). Cross-reactive cellular immune effectors induced by prior seasonal vaccination or infection are proposed to have had a priming effect (5, 15, 16, 18, 48).

In summary, the broad range of immune effectors induced by the LAIV platform allowed us to evaluate the relative contribution of different effectors in mediating protection against wt H5N1 virus infection and to determine the immunological requirements for their induction by vaccination. Most importantly, we have demonstrated that the presence of both cellular and humoral effectors in the respiratory tract is critical for optimal protection. In addition to influenza, there is active research in the development of safe live attenuated vaccines using modern molecular techniques (27) for other human pathogens. The requirements for the induction of the different immune effectors presented in this study will inform vaccinologists on the use of live attenuated vaccines to induce appropriate immunity for optimal protection against a variety of respiratory pathogens.

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