Seasonal influenza infection and live vaccine prime for a response to the 2009 pandemic H1N1 vaccine

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The robust immune response to a single dose of pandemic 2009 H1N1 vaccine suggests that a large segment of the population has been previously primed. We evaluated the effect of seasonal (s) H1N1 infection, s-trivalent inactivated vaccine (s-TIV), and trivalent s-live attenuated influenza vaccine (s-LAIV) before immunization with a pandemic live attenuated influenza vaccine (p-LAIV) in mice. We compared serum and mucosal antibody and pulmonary CD8 and CD4 responses and the virologic response to challenge with a wild-type 2009 pandemic H1N1 (p-H1N1) virus. Two doses of p-LAIV induced cellular immune and robust ELISA and neutralizing antibody responses that were associated with complete protection from p-H1N1 challenge. A single dose of p-LAIV induced a cellular response and ELISA but not a neutralizing antibody response, and incomplete protection from p-H1N1 virus challenge. Primary infection with s-H1N1 influenza virus followed by a dose of p-LAIV resulted in cross-reactive ELISA antibodies and a robust cellular immune response that was also associated with complete protection from p-H1N1 virus challenge. A lower-magnitude but similar response associated with partial protection was seen in mice that received a dose of s-LAIV followed by p-LAIV. Mice that received a dose of s-TIV followed by p-LAIV did not show any evidence of priming. In summary, prior infection with a seasonal influenza virus or s-LAIV primed mice for a robust response to a single dose of p-LAIV that was associated with protection equivalent to two doses of the matched pandemic vaccine.

The factors underlying the epidemiology of the 2009 H1N1 influenza pandemic remain undefined. Although the virus is genetically and antigenically distinct from seasonal human H1N1 viruses (1, 2), clinical data from the pandemic suggest that prior exposure to influenza played a significant role in susceptibility to disease and immune response to the pandemic virus. Individuals over 50 y of age have antibodies that cross-react with and appear to be less susceptible to infection with the pandemic H1N1 (p-H1N1) virus, presumably due to prior exposure to an antigenically related H1N1 influenza virus (3–6). In addition, data from recent p-H1N1 vaccine trials suggest that a large segment of the population has been exposed to an influenza virus that “primed” individuals such that only one dose of the novel pandemic vaccine is sufficient to elicit a protective antibody titer (7–10). This observation was unexpected, because studies conducted in the 1970s had shown that two doses of vaccine were needed to immunize a naïve population (11).

As the priming effect was observed in all age groups in the vaccine trials, it is likely that exposure to seasonal influenza infection or vaccination plays a role in modulating the immune response to the p-H1N1 vaccine. Although several retrospective studies have examined the impact of prior seasonal influenza exposure on the susceptibility to and morbidity from p-H1N1 infection, the observed effects have differed. In two studies conducted in the United States and Australia, prior seasonal influenza vaccination did not have a significant effect on the incidence of p-H1N1 infection (6, 12). In contrast, in a small retrospective case–control study in Mexico, more severe clinical outcomes were noted among individuals infected with the p-H1N1 virus who had not been previously vaccinated with the 2008–2009 seasonal influenza vaccine (13). Another retrospective analysis in Mexico also noted a lowered risk of p-H1N1 infection among individuals who had been vaccinated with seasonal influenza vaccine (14). Most recently, observational studies conducted in Canada and the United States have reported an association between receipt of seasonal influenza vaccine and an increased incidence of p-H1N1 infection (15, 16).

In studies conducted in animals, the observed effects of seasonal influenza on the response to the p-H1N1 virus have also differed. The transmission of the p-H1N1 virus was reduced when guinea pigs were previously infected with seasonal H1N1 or H3N2 influenza viruses (17). Recent studies examining the molecular basis for preexisting immunity to the p-H1N1 virus have demonstrated that a number of CD4 and CD8 T epitopes (18–20) are shared between the pandemic and seasonal H1N1 viruses. Studies on the effect of seasonal influenza vaccination have had discrepant results: Ferrets that were vaccinated with seasonal trivalent inactivated vaccine (s-TIV) before receipt of an adjuvanted p-H1N1 vaccine developed higher antibody titers than animals that were not primed; however, despite the difference in antibody titer, no difference in protective efficacy was observed (21). In another study, immunization of ferrets with an s-TIV alone did not affect morbidity or mortality from subsequent p-H1N1 infection and, although lung virus titers were similar, higher mortality following p-H1N1 infection was observed in animals that had received live attenuated seasonal influenza vaccine (22).

We designed a study to evaluate the effect of priming with seasonal influenza vaccine or infection on the immunogenicity and efficacy of a live attenuated 2009 p-H1N1 vaccine in mice and examined the components of the immune response.

Results

Efficacy Against p-H1N1 Wild-Type Virus Challenge. Two doses of pandemic live attenuated influenza vaccine (p-LAIV) conferred complete protection from challenge with the p-H1N1 virus (Fig. 1). Notably, prior exposure to seasonal (s)-WT virus infection followed by p-LAIV also conferred complete protection in the upper and lower respiratory tracts of mice.

One dose of p-LAIV provided partial protection in the upper (400- and 9,000-fold reduction on days 2 and 4, respectively) and lower respiratory tracts (10- and 200-fold reduction on days 2 and 4, respectively) of mice compared with mock-immunized animals.

Administration of s-TIV before p-LAIV induced protection that was comparable to one dose of p-LAIV alone. In contrast, administration of one dose of seasonal live attenuated influenza vaccine (s-LAIV) before p-LAIV led to complete protection in the upper respiratory tract and a 25,000-fold reduction of challenge virus titer in the lower respiratory tract on day 2 postchallenge compared with mock-immunized animals; viral titers
on day 4 postchallenge were not obtained because 9 of the 26 mice inoculated with s-LAIV died on days 3–7 following primary vaccination with s-LAIV, an indication of the virulence of the trivalent s-LAIV for mice at the dose used.

ELISA Antibody Response. An ELISA antibody response against the p-H1N1 virus was detected in each of the groups in which protection from challenge virus replication was observed. Cross-reactive antibodies that bound to β-propiolactone (BPL)-inactivated p-H1N1 virus were detected in mice after a single dose of s-LAIV or s-WT virus (Fig. 2A). These titers were comparable to those that were achieved by a single dose of p-LAIV. Cross-reacting antibodies were not detected in mice that received a single dose of s-TIV. To determine whether the cross-reacting ELISA antibodies were targeting the HA protein, baculovirus-expressed recombinant p-H1N1 HA was used as the coating antigen in ELISA (Fig. S1). Notably, we observed that only antibody elicited by the p-LAIV and the s-WT (H1N1) virus cross-reacted with the p-H1N1 HA.

Antibody titers against s-H1N1 virus were also determined using BPL-inactivated s-WT virus. Titers against BPL-inactivated s-WT virus were comparable in the mice that received s-LAIV, s-WT, and p-LAIV (Fig. 2B), but only mice in the s-WT group (2/3 mice) had antibody that bound to the recombinant s-WT HA (Fig. S1). These data indicate that a portion of the ELISA antibody response elicited by s-LAIV and p-LAIV is directed at influenza proteins other than HA.

There was an ∼10-fold rise in antibody titers against the BPL-inactivated p-H1N1 virus following secondary immunization with p-LAIV in groups that received s-LAIV, s-WT, or p-LAIV for primary vaccination. Lower antibody titers were seen following secondary immunization with p-LAIV in mice that received s-TIV or Leibovitz-15 (L-15) medium alone for primary vaccination. A significant boost in antibody titers against the baculovirus-expressed recombinant p-H1N1 HA was also noted in groups that received s-LAIV or s-WT before p-LAIV; these titers were comparable to those achieved by two doses of p-LAIV (Fig. S1). In addition, the antibody titers following p-LAIV were significantly higher in the mice that were primed with s-LAIV or s-WT infection than those that only received a single dose of p-LAIV (P < 0.05, unpaired t test). These results suggest that the humoral immunity induced by s-LAIV immunization or s-WT infection primes the host for stronger responses to the p-H1N1 recombinant HA on subsequent vaccination with p-LAIV. This effect was not observed in the group that received s-TIV as primary vaccination.

Fig. 2. The impact of different priming strategies on the induction of humoral immunity by p-LAIV. (A) ELISA: For 1° immunization, groups of three mice were vaccinated i.m. with 300 ng of the 2008–2009 s-TIV or i.n. with L-15 medium, 10^6 TCID_50 of s-LAIV or p-LAIV, or 10^5 TCID_50 of A/Brisbane/57/2007 (H1N1) s-WT virus. Serum samples were collected 24 d later. The mice were then given 10^6 TCID_50 of p-LAIV (2° immunization) on day 31 and serum samples were collected again on day 52. Influenza-specific serum antibody titers (log_{10}) were determined by ELISA using BPL-inactivated whole virus. Each dot presents the titer of an individual mouse and the bars represent the mean for each group. The lower limit of detection is represented by the dotted line. Asterisks denote that the difference between the indicated group and the group that received one dose of p-LAIV was statistically significant (P < 0.05). (B) Neutralizing antibodies: The serum neutralizing antibody titer (log_{10}) against the p-H1N1 and s-WT viruses in mice immunized as indicated in A. Each dot represents the titer of an individual mouse and the bars represent the mean for the groups. (C) Mucosal antibody response: Influenza-specific ELISA and neutralizing antibodies in nasal turbinates (N.T.) and lungs (L.H.) of mice on day 52 in mice immunized as indicated in A. The left y axis represents ELISA titer (log_{10}) and black dots represent the titer of each mouse. The right y axis represents the neutralizing titer (log_{10}) and red squares represent the titer of each mouse.
We also evaluated the antibody response elicited by each of the vaccine regimens against the s-WT HA. Only mice that received s-WT and s-LAIV for primary vaccination had detectable antibodies against the s-WT HA. p-LAIV did not elicit cross-reactive antibodies against the seasonal H1N1 HA, suggesting that the antibody response to the p-H1N1 virus is narrower than the response to the s-H1N1 virus.

**Evaluation of the Serum Neutralizing Antibody Response.** Mice that received two doses of p-LAIV developed a robust neutralizing (neut) antibody response against the p-H1N1 virus (Fig. 2B). Interestingly, although mice that were primed with s-WT virus infection before p-LAIV were also fully protected from challenge virus replication, these mice did not develop a detectable neutralizing antibody response against the p-H1N1 virus. Administration of L-15, s-TIV, or s-LAIV before p-LAIV did not elicit a neutralizing antibody response to the p-H1N1 virus. Notably, the p-LAIV virus without the two amino acid changes (K119E and A186D) in the HA protein that enhanced vaccine virus yield in eggs (23) elicited a detectable neutralizing antibody response following one dose in mice. We confirmed that mice vaccinated with s-TIV, s-LAIV, or s-WT were appropriately primed, because these mice developed a detectable neutralizing antibody response against the s-WT virus (Fig. 2B). Mice that received p-LAIV, even after two doses, failed to develop a detectable neutralizing antibody response against the s-WT virus.

**Evaluation of the Mucosal Antibody Response.** In addition to serum antibodies, mucosal antibodies have also been shown to be important for protecting the respiratory tract against influenza virus infection (24). Therefore, we determined antibody titers by ELISA in the nasal turbinates and lungs to assess the mucosal antibody response (Fig. 2C). Comparable to the serum ELISA antibody response, mucosal ELISA antibodies against the p-H1N1 virus were detected in mice that received two doses of p-LAIV or one dose of s-WT or s-LAIV followed by p-LAIV vaccination. Following two doses of p-LAIV, a modest neutralizing antibody response against the p-H1N1 virus was noted in the upper respiratory tract of one mouse but neutralizing antibody was not detected in the lung homogenates. A neutralizing antibody response to the p-H1N1 virus was not detected in either the upper or lower respiratory tract among any of the other groups. Only mice that had prior infection with s-LAIV or s-WT virus had detectable anti-s-WT HA antibodies with neutralizing activity in the nasal turbinates and lungs.

Thus, in the absence of detectable neutralizing antibodies in the sera, we detected ELISA antibodies in the serum and mucosa in mice that were primed with s-WT or s-LAIV and these mice were completely or partially protected from viral replication, suggesting that ELISA (binding) antibodies or cellular immunity plays a role in mediating protection.

**Evaluation of the T-Cell Response.** We immunized mice with two doses of s-TIV intramuscularly (i.m.) or two doses of s-WT virus intranasally (i.n.) 28 d apart and enumerated influenza-specific CD8+ T cells in the lungs by intracellular cytokine staining (ICS) at various time points to determine whether preexisting immunity to homologous virus would interfere with the expansion of influenza-specific CD8+ T cells in the lungs upon secondary vaccination (Fig. S2). Because unadjuvanted s-TIV is generally not effective in inducing influenza-specific CD8+ T cells (25–27), not surprisingly, mice that received s-TIV did not have a significant number of influenza-specific CD8+ T cells in the lungs. On the other hand, in mice infected with s-WT virus, ~10% of the pulmonary CD8+ T cells on day 8 after primary infection were specifically NP147-specific cytotoxic T cells (CTL) epitope in the nucleoprotein. On day 36 postinfection, the NP147-specific CD8+ T cells were maintained as ~2% of the CD8+ T cell population in the lungs. When mice were rechallenged with the homologous WT virus, no expansion of the CD8+ population was detected, indicating that preexisting humoral immunity can inhibit reinfection and expansion of the CD8+ population, an observation that is consistent with those of Armerding and Lichl and Loosli et al. (28, 29).

We then examined the effect of priming with seasonal influenza infection or vaccines on secondary CD8+ T-cell responses to p-LAIV. Groups of three mice were vaccinated or infected with s-TIV, s-LAIV, s-WT, or p-LAIV followed by p-LAIV virus as previously described. Lungs and spleens were collected on days 8 and 36 following primary immunization and days 5 and 8 following secondary immunization to enumerate NP147-specific CD8+ T cells. Very few NP147-specific CD8+ T cells were detected on days 8 and 36 after s-TIV immunization (Fig. 3). Secondary immunization of s-TIV-primed mice with p-LAIV resulted in the infiltration of influenza-specific CD8+ T cells into the lungs on day 8, kinetics that indicated a primary response. On the other hand, in mice primed with s-LAIV, a small population of memory CD8+ T cells was established in the lungs (day 36 postprimary immunization) that underwent rapid recall and expansion in response to secondary p-LAIV vaccination. Significant numbers of NP147-specific T cells were found in the lungs on day 5 after secondary vaccination, and the magnitude was comparable to that observed on day 8 after primary vaccination. By day 8 after secondary vaccination, the NP147 population was three times larger than was observed following primary vaccination.

Similarly, accelerated infiltration of NP147-specific CD8+ T cells was observed in the lungs of mice that were primed with s-WT infection before p-LAIV vaccination. On day 8 following secondary vaccination, ~40% of the CD8+ T cells were VP147-specific. This was four times greater than the response following primary infection. In mice that received two doses of p-LAIV, there was an accelerated infiltration of CD8+ cells but the overall magnitude of the response was lower than in mice that received heterologous priming.

In addition to the expansion of the NP147-specific CD8+ T cells in the lungs, we also observed clonal expansion in the spleen at levels that mirrored the expansion in the lungs (Fig. S3). Our findings suggest that the cross-reactive ELISA antibodies induced by seasonal vaccines do not interfere with the induction (s-TIV) and expansion (s-LAIV and s-WT) of influenza-specific CD8+ T-cell responses in the lungs. Thus, we observed that s-LAIV immunization of mice with two doses of s-TIV induced a cross-reactive antibody response with neutralizing activity, which was not observed following vaccination with s-WT.
munization or s-WT infection elicited a primary influenza-specific CD8+ T-lymphocyte response on day 8 that expanded and was detected at day 5 after secondary vaccination with p-LAIV. Notably, mice in these two groups were significantly protected from challenge with p-H1N1 virus, suggesting that a robust pulmonary cellular recall response confers protection, even in the absence of detectable neutralizing antibody.

Because the licensed seasonal and 2009 p-H1N1 LAIV share internal protein genes derived from the vaccine donor strain, A/Ann Arbor/6/60 ca (cold-adapted) (AA ca; H2N2), we first investigated whether the CD4+ T cells induced by p-LAIV would respond to s-WT and AA ca viruses (Fig. S4). Groups of three mice were immunized with two doses of p-LAIV and, on day 5 after secondary vaccination, lung cells were harvested and incubated with naïve splenocytes that had been pulsed with the s-WT virus, p-H1N1 virus, or AA ca virus and the numbers of CD4+ T lymphocytes that produced IFN-γ were determined by ICS assay. About 1.5% of the pulmonary CD4+ T cells produced IFN-γ after incubation with AA ca virus-pulsed splenocytes (Fig. S4). A substantial response (80% and 50% of the 1.5% detected with AA ca virus-pulsed cells) was also detected when cells were incubated with p-H1N1 virus or s-WT virus-pulsed splenocytes, respectively. These results suggest that CD4+ T-cell epitopes are shared between the ca and WT viruses. Our data are consistent with reports by others (18–20) including Greenbaum et al. that up to 41% of CD4+ T-cell epitopes are conserved between seasonal and 2009 p-H1N1 in humans and that these epitopes are mainly located in the internal protein genes.

To examine the secondary CD4+ T-cell responses, mice were primed and boosted as described in Fig. 1 and the number of IFN-γ+ CD4+ T cells in the lungs was determined 5 d after the mice received p-LAIV. Similar to naïve mice, mice that received s-TIV had few influenza-specific CD4+ T cells, whereas two out of three and three out of three mice that received primary vaccination with s-LAIV or p-LAIV, respectively, had influenza-specific CD4+ T-cell responses (Fig. 4). Mice primed with s-WT virus before p-LAIV vaccination had the highest number of influenza-specific T cells, and an average of 2.5% of the CD4+ T-cell population in the lungs were activated (Fig. 4). These findings parallel our observations of the CD8+ response and suggest a role for the pulmonary cellular immune response in mediating protection in the absence of detectable serum neutralizing antibody.

**Passive Transfer of Postvaccination Sera.** To determine the role of serum antibodies in the protection of mice that were primed with seasonal influenza vaccine or virus before vaccination with p-LAIV, postvaccination sera were transferred to naïve mice and the mice were challenged with the p-H1N1 virus. Mice that were primed with seasonal influenza vaccine did not develop a detectable neutralizing antibody response, and the recipient mice had no detectable neutralizing antibodies against the p-H1N1 virus (Table 1). Mice that received two doses of p-LAIV developed a significant neutralizing antibody response against the p-H1N1 virus [geometric mean titer (GMT) 160], transfer of which resulted in a GMT of 40 in the recipient mice. However, protection from p-H1N1 virus challenge was not observed in any of the groups that received postvaccination or nonimmune mouse sera (Table 1), suggesting that either a titer of 1:40 was not protective or that serum antibodies alone do not confer protection in this challenge system.

**Discussion**

Clinical trials evaluating the immunogenicity of the 2009 p-H1N1 vaccines suggest that there is evidence of priming in the population (7–10); however, the basis for this observation has not yet been elucidated. We designed this study in mice to examine the contribution of prior exposure to seasonal influenza vaccination or infection to the immune response to and efficacy of a 2009 p-H1N1 vaccine (6, 12–16). We focused on p-LAIV rather than the inactivated pandemic vaccine because the breadth of the immune responses induced by LAIV in naïve mice is greater than that induced by vaccine (Fig. S2), allowing us to evaluate the importance of recalling other arms of the adaptive immune system that might be primed by previous vaccination or infection.

Several interesting findings were observed. First, prior exposure to s-H1N1 WT virus infection or two doses of p-LAIV provided comparable protective efficacy—both provided complete protection from viral replication in the upper and lower respiratory tracts following challenge with the p-H1N1 virus. Notably, however, the immunologic mechanisms that correlate with this protection differ (Table 2). Whereas mice immunized with two doses of p-LAIV developed a robust neutralizing antibody response against the p-H1N1 virus, exposure to an s-H1N1 WT virus before p-LAIV vaccination led to a robust serum ELISA antibody without a significant neutralizing antibody response against the p-H1N1 virus, suggesting that apart from neutralizing antibody there are other mechanisms capable of conferring complete protection from challenge. Second, we noted that priming with one dose of s-LAIV before p-LAIV also led to significant protection against viral replication, associated with a detectable ELISA antibody response without detectable neutralizing activity. Third, in contrast to priming with live virus, priming with a dose of s-TIV was not associated with a significant enhancement in ELISA or neutralizing antibody response or protection over that seen with one dose of p-LAIV alone. We do not expect that s-TIV administered at a higher dose will improve priming, because Laurie et al. reported similar observations in ferrets that received two doses of 15 μg (two full human doses) of the 2008–2009 s-TIV with Freund’s incomplete adjuvant. These ferrets developed robust hemagglutination inhibition (HI) antibodies against the s-H1N1 WT virus, but they manifested no significant protection against infection with the p-H1N1 WT virus. Most importantly, the HI titers that developed in these ferrets following p-H1N1 WT virus infection were not significantly different from postinfection titers in unprimed ferrets (30). On the other hand, ferrets that were preinfected with s-H1N1 WT virus showed accelerated clearance and reduced shedding following p-H1N1 infection (30). s-TIV-primed ferrets only showed a modest increase in HI and microneutralization titers over unprimed ferrets after receiving adjuvanted p-TIV, and this could be the result of induction of cross-reactive CD4+ T cells by s-TIV (21).

To better delineate the mechanisms underlying these observations, we evaluated other components of the immune response including mucosal and cellular immunity (Table 2). Overall, the mucosal response closely paralleled the serum antibody response. A serum and mucosal ELISA antibody response was...
detected in mice that were primed with a dose of s-LAIV, p-LAIV, or s-WT virus. However, with the exception of one mouse that received two doses of p-LAIV, a mucosal neutralizing antibody response was not detected. As the ELISA titer in the mucosal samples was ~200-fold lower than in the serum samples, the lack of neutralizing activity of the mucosal samples may be due to insufficiently high titer antibodies to neutralize 100 TCID<sub>50</sub> (50% tissue culture infectious dose) of input virus in the neutralizing antibody assay. Despite the lack of detectable neutralizing antibody in the serum and respiratory tract, mice that were primed with s-WT virus or s-LAIV required only a single dose of p-LAIV to be protected from challenge.

Consistent with previous studies (26, 31–33), we observed influenza-specific CD<sup>8+</sup> T cells in the lungs following primary infection with s-LAIV or s-WT virus and also showed that cellular immunity to the 2009 p-H1N1 virus can be primed by s-LAIV or s-WT virus infection. Following a heterologous infection, there was an expansion of these CD<sup>8+</sup> T cells in lungs with immediate effector functions such as the production of antiviral cytokines. The contribution of CD<sup>8+</sup> T cells to viral clearance from the lungs has been demonstrated in a number of studies, using adoptive transfer, depletion of CD<sup>8+</sup> T cells, or CTL-inducing vaccines (34–39). CD<sup>8+</sup> T cells can mediate viral clearance from the respiratory tract by lysing target cells following exocytosis of granules containing perforin and granzymes (40). We recently reported our observations of prominent perivascular and peribronchial lymphoid cuffs and general lymphoid hyperplasia in the lungs of mice that were vaccinated with an H5N1 LAIV (but not in mock-vaccinated mice) 2 d after challenge with the WT virus. The appearance of lymphocytes in the lungs coincided with viral clearance that was complete 4 d after challenge (41).

Table 2. Summary of immune responses and protection from challenge

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Serum antibody</th>
<th>Mucosal antibody</th>
<th>T-cell response</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>Secondary</td>
<td>ELISA</td>
<td>ELISA</td>
<td>CD8  CD4</td>
</tr>
<tr>
<td>p-LAIV</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>s-TIV</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>s-LAIV</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>s-WT</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>p-LAIV</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Responses were scored on a scale from – to +++, where – indicates no response, + indicates a weak response, ++ indicates a moderate response, and +++ indicates a robust response.

Table 1. Efficacy of passively transferred postvaccination serum in protection from challenge

<table>
<thead>
<tr>
<th>Passive transfer serum*</th>
<th>Neutralizing Ab titer in passive transfer serum pool†</th>
<th>Mean prechallenge neutralizing Ab titer in recipient mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>Nonimmune</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2 doses of p-LAIV</td>
<td>160</td>
<td>40</td>
</tr>
<tr>
<td>1 dose of p-LAIV</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2 doses of s-LAIV</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>s-LAIV ⇒ p-LAIV</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

*Five hundred microliters of serum pooled from immunized or uninfected mice was administered to recipient mice by i.p. injection.
†Neutralizing antibody titer against p-H1N1 virus; a titer of 10 indicates the absence of detectable neutralizing antibody in the sample.
generated by reverse genetics as previously described and was kindly provided by Dr. Hong Jin (MedImmune, Mountain View, CA) (23). The H1 NA and H1 N1 reassortants of the reasortant pandemic 2009 H1N1 CA-eg DNA virus were derived from the p-H1N1 virus, and the internal protein gene segments were derived from the CAa aa donor virus. The p-LAIV virus contains two additional amino acid changes (K119E and A186D) in the HA protein that enhanced vaccine virus yield in eggs without affecting vaccine antigenicity and immunogenicity in ferrets (23).

The WT viruses were propagated in the allantoic cavity of 9- to 11-d-old embryonated specific pathogen-free hen eggs. Infectious allantoic fluids were stored at −80 °C until use. The TCD50 for each virus was determined in Madin-Darby canine kidney (MDCK) cells and calculated by the method developed by Reed and Muench (49).

**Vaccines.** The unadjuvanted 2008–2009 s-TIV composed of A/Br/Brisbane/59/2007 (H1N1), A/Br/Brisbane/10/2007 (H3N2), and B/Fl/Florida/4/2006 was kindly provided by Dr. Derek O’Hagan (Novartis, Cambridge, MA). The 2008–2009 s-LAIV composed of A/South Dakota/6/2007 (H1N1), an A/Br/Brisbane/59/2007-like virus, A/Brisbane/10/2007 (H3N2), and B/Fl/Florida/4/2006 was kindly provided by Dr. Hong Jin and George Kemble at MedImmune.

Detailed descriptions of the experimental procedures are included in SI Materials and Methods.

**ACKNOWLEDGMENTS.** We thank Jadon Jackson for technical support. We also thank Drs. Ruben Donis and Alexander Klimov from the Centers for Disease Control and Prevention for providing the 2009 pandemic H1N1 viruses for the 2009 pandemic H1N1 live attenuated vaccine, and Dr. Derek O’Hagan and Kathryn Patton at Novartis for the 2008–2009 seasonal trivalent inactivated influenza vaccine used in this study. This work was supported in part by the Intramural Research Program of the National Institutes of Health and the National Institute of Allergy and Infectious Diseases.
Ten-week-old female BALB/c mice (Taconic Farms) were used as the recipients in the passive transfer studies; 6-wk-old female BALB/c mice (Taconic Farms) were used in all other experiments. The National Institutes of Health Animal Care and Use Committee approved all animal experiments.

Evaluation of Efficacy Against p-H1N1 Wild-Type Virus Challenge. We administered one dose of vaccine or seasonal (s)-WT (A/Brisbane/59/2007; H1N1) virus to groups of five BALB/c mice lightly anesthetized with isoflurane. The mice received either 50 μL of Leibovitz-15 (L-15) media, 10⁶ TCID₅₀ (50% tissue culture infectious dose) of s-WT, 10⁶ TCID₅₀ of seasonal live attenuated influenza vaccine (s-LAIV) intranasally (i.n.), or 100 μL of s-trivalent inactivated vaccine (s-TIV) (diluted to 0.1 μg HA of each strain for a total of 0.3 μg HA/100 μL) intramuscularly (i.m.) 1 mo before i.n. inoculation of 10⁶ TCID₅₀ of the pandemic live attenuated vaccine (p-LAIV). The dose of s-TIV was recommended by scientists at Novartis, and a number of publications have demonstrated that a single dose of inactivated influenza virus (0.1–0.15 μg) is sufficient to induce antibody production in mice (1, 2). For challenge infection, lightly anesthetized mice received 10⁶ TCID₅₀ of the p-H1N1 virus intranasally. Serum was collected before first and second inoculations and before the administration of the challenge virus.

On days 2 and 4 following challenge, lungs and nasal turbinates were harvested; organs were harvested from three mice on day 2 and two mice on day 4 in each group. Organs were homogenized in L-15 medium (Invitrogen-GIBCO) containing antibiotic-antimycotic (Invitrogen-GIBCO) to make 5% (wt/vol) (nasal turbinates) or 10% (wt/vol) (lungs, brains) tissue homogenates. Tissue homogenates were titrated on Madin–Darby canine kidney (MDCK) cell monolayers, as previously described (3). Titers are expressed as log₁₀ TCID₅₀/g of tissue.

Evaluation of the Serum Antibody Response. ELISA. ELISA was performed as previously described (4). Plates were coated with β-propiolactone-inactivated p-H1N1 and s-WT viruses at 1,000 hemagglutinating units (HAU)/mL (50 μL per well) or baculovirus-expressed recombinant HA proteins from p-H1N1 (NR15258) and s-WT (NR13411) viruses obtained through the National Institutes of Health Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases and used at 1 μg/mL (50 μL per well). Serum and mucosal samples were serially 10- and 5-fold-diluted, respectively, and incubated on the plates at 4 °C overnight. Bound antibodies were detected with HRP-conjugated goat anti-mouse Ig (Dako; 1:1,000 dilution). Wells with an OD of >0.2 at 405 nm were considered positive.

Neutralization assay. Neutralizing antibody titers in serum or mucosal samples were determined in a microneutralization (MN) assay as previously described (5) starting with a 1:10 or 1:20 dilution of serum and/or undiluted mucosal samples. Neutralizing antibody titers were defined as the reciprocal of the highest dilution of serum that completely neutralized the infectivity of 100 TCID₅₀ of the p-H1N1 virus indicated by the absence of viral cytopathic effect at day 4.

Supporting Information

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SI Materials and Methods

Animals. Ten-week-old female BALB/c mice (Taconic Farms) were used as the recipients in the passive transfer studies; 6-wk-old female BALB/c mice (Taconic Farms) were used in all other experiments. The National Institutes of Health Animal Care and Use Committee approved all animal experiments.

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Evaluation of the Mucosal Antibody Response. Mice were anesthetized with isoflurane and terminally exsanguinated by resection of the portal vein. An incision was made in the trachea and a catheter was inserted; nasal washes were collected at the nose by flushing 1 mL of 0.5% BSA in RPMI-1640 through the catheter. The mice were then perfused with PBS through the heart until all of the blood was removed, and the lungs and nasal turbinates were collected. The organs were homogenized in 1 mL of RPMI-1640 and clarified samples were stored at −20 °C. Influenza-specific antibodies in the samples were determined by ELISA and MN assay as described above.

Evaluation of the T-Cell Response. For primary immunization, groups of 11 mice received medium, s-TIV, s-LAIV, p-LAIV, or s-WT vaccine. All groups received p-LAIV for secondary immunization 28 d later. Three and six mice in each group were killed following primary and secondary immunization on days 8 and 36 and days 5 and 8, respectively, for cellular immunology studies. Single-cell suspensions were prepared from various tissues, and epitope-specific CD8⁺ T cells were enumerated using intracellular IFN-γ staining as previously described (6). In brief, after 5 h of antigenic stimulation (1 μM NP147:10147 SYQRTQLV) in the presence of GolgiPlug (BD Biosciences), cells were stained for CD8 (53-6.7) and IFN-γ (XMG1.2) using a Cytofix/Cytoperm Kit according to the manufacturer’s instructions (BD Biosciences). For enumeration of influenza-specific CD4⁺ T cells in various organs, syngeneic spleen cells from naïve mice were pulsed with various viruses for 60 min at 37 °C at a multiplicity of infection of 1, followed by washing in RPMI-1640 twice before receiving 2,200 rad of irradiation in a 660Co irradiator. The cells were resuspended in T cell medium (TCM; composed of RPMI 1640 medium supplemented with penicillin, streptomycin, glutamine, and 10% heat-inactivated fetal calf serum) and incubated at 37 °C overnight before single-cell suspensions from various organs were added to the wells for antigenic stimulation with GolgiPlug. Cells were stained for CD4 (RM4-5) and IFN-γ (XMG1.2) after 5 h to enumerate double-positive cells using the above-mentioned method. Cells were analyzed on a FACSCalibur (BD Biosciences) and further analysis was performed with FlowJo software (Tree Star).

Passive Transfer Study. Postvaccination sera were generated in mice that received either one or two intranasal doses of p-LAIV or s-LAIV or a dose of s-LAIV followed by a dose of p-LAIV. The vaccines were administered in volumes of 50 μL to lightly anesthetized mice; the doses were separated by 28 d. Postvaccination sera were administered to groups of four mice; each mouse received 500 μL intraperitoneally. A pool of non-immune serum was collected from uninfected mice and 500 μL was administered intraperitoneally to the control group. Twenty-four hours later, the recipient mice were bled to determine the titer of antibodies achieved and all groups were challenged intranasally with 10⁵ TCID₅₀ p-H1N1 in 50 μL. At days 2 and 4 postchallenge, lungs and nasal turbinates were harvested and viral titers were determined as described above.

Statistical Analysis. The significance of difference between any two different groups was assessed by the unpaired t test. A two-sided P value <0.05 is considered significantly different.


**Fig. S1.** The impact of different priming strategies on the induction of humoral immunity by p-LAIV. Groups of three mice were primed and boosted as described in Fig. 2A. Serum samples were collected at the indicated time points. HA-specific serum antibody titers (log₁₀) were determined by ELISA using purified recombinant HA (rHA) proteins expressed in insect cells as coating antigen. Each dot represents the titer of an individual mouse and the bars represent the mean for each group. The lower limit of detection is represented by the dotted lines. The asterisk indicates that the difference between the two groups is statistically significant (*P* < 0.05). ND, not determined.

**Fig. S2.** The impact of humoral immunity induced by s-WT virus and s-TIV on the recall of pulmonary influenza-specific CD8⁺ cytotoxic T cell (CTL) responses. Homologous boosting with s-TIV or s-WT virus fails to induce a significant expansion of influenza-specific CD8⁺ T cells in lungs. Groups of three mice were vaccinated with 300 ng of s-TIV i.m. or 10⁵ TCID₅₀ of the seasonal H1N1 WT virus. To examine the primary responses induced by a single dose of each immunogen, mice were killed on day 8 or 36 after priming (1°). Memory responses were evaluated on day 5 or 8 after boosting with the same immunogen (2°). Lungs were collected and the numbers of epitope-specific CD8⁺ CTLs in the lungs were enumerated by intracellular cytokine staining and expressed as a percent of total CD8⁺ T cells in lungs. Each dot represents the result from one mouse and the bars represent the mean for each group.
Fig. S3. s-LAIV or s-WT virus, but not s-TIV, primed mice for robust recall of splenic CD8+ CTL responses to p-LAIV. Mice were vaccinated and primed as described in Fig. 3. The percent of NP147-specific CD8+ CTLs in the spleen was determined by intracellular cytokine staining. The dotted lines represent the magnitude of NP147-specific CD8+ T-cell response on day 8 during a primary response. A minimum of 50,000 lymphocytes were analyzed for each sample.

Fig. S4. Induction of cross-reactive influenza-specific CD4+ T cells induced by p-LAIV. Three mice were primed i.n. with p-LAIV and boosted 31 d later. To determine the level of cross-reactivity of these influenza-specific CD4+ T cells induced by p-LAIV, mice were killed on day 5 after boosting and single-cell suspensions from lungs were prepared and incubated with naive syngeneic splenocytes that were pulsed with the indicated viruses as antigen presenting cells (APC) at a multiplicity of 1:1. The numbers of IFN-γ+ CD4+ lymphocytes were determined. To determine the level of nonspecific IFN-γ production, uninfected splenocytes were used as APCs and the background was subtracted to enumerate the influenza-specific CD4+ T cells. A minimum of 30,000 lymphocytes were analyzed.