Evaluation of the Humoral and Cellular Immune Responses Elicited by the Live Attenuated and Inactivated Influenza Vaccines and Their Roles in Heterologous Protection in Ferrets

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The humoral and cellular immune responses elicited by the trivalent live attenuated influenza vaccine (LAIV) and the trivalent inactivated influenza vaccine (TIV) were evaluated in the ferret model, using newly developed ferret immunological reagents and assays. In contrast to the TIV, which only induced immune responses in primed animals, LAIV induced strong influenza virus–specific serum antibody and T-cell responses in both naive and influenza-seropositive animals. The LAIV offered significant protection against a heterologous H1N1 virus challenge infection in the upper respiratory tract. Influenza virus–specific immunoglobulin A (IgA) and immunoglobulin G (IgG) antibody-secreting cells (ASCs) and influenza virus–specific CD4+ and CD8+ T cells were detected in the circulation and local paratracheal draining lymph nodes. The frequency of the influenza-specific ASCs in the local lymph nodes appeared to correlate with the degree of protection in the upper respiratory tract. The protection conferred by the LAIV could be attributed not only to the antibody response but also to the cell-mediated and local mucosal immune responses, particularly in naive ferrets. These findings may explain why the LAIV is immunologically superior and offers immediate protection after a single dose in children.

Keywords. influenza vaccines; LAIV; TIV; ferret; humoral and cellular immune responses.

Seasonal influenza epidemics continue to cause significant mortality and morbidity each year. Vaccination is the most effective means of protecting the public from influenza epidemics and occasional pandemics. Two types of influenza vaccines are currently licensed in the United States. The inactivated trivalent influenza vaccine (TIV), which is administered intramuscularly, consists mainly of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). The live attenuated influenza vaccine (LAIV), which was approved for use in the United States and Europe, is administered intranasally and contains three 6:2 reassortant viruses comprising the HA and NA from the wild-type influenza virus and the 6 internal protein gene segments from a cold-adapted and temperature-sensitive master donor influenza virus A or B strain. In addition to the induction of serum neutralizing antibodies, LAIV elicits local mucosal immunoglobulin A (IgA) antibody responses and stimulates cell-mediated immune (CMI) responses that are important for viral clearance and recovery [1, 2].

CMI responses to natural influenza virus infection are considered to play an important role in the protection of humans against drifted or shifted strains [3]. Although the CMI response does not confer sterilizing immunity, it provides cross-reactive T-cell responses that may reduce virus replication and improve clinical outcomes [4–7]. A number of studies conducted in mice have indicated that antigen-specific CD8+ T cells are important for virus clearance [8], while memory
CD4+ T cells are involved in maintaining CD8+ T-cell and B-cell memory responses [9, 10]. Furthermore, antigen-specific memory CD4+ T cells also have cytotoxic activity against virus-infected cells [11, 12].

Ferrets are susceptible to influenza virus infection and are considered to be an appropriate small animal model for influenza research. Ferrets have been extensively used to study influenza pathogenesis, transmission, and vaccine immunogenicity [13–15]. However, the lack of critical immunological reagents has hampered the study of influenza viruses and vaccines in the ferret model. To overcome this issue, we have developed several immunological reagents and methods that allow the detection of influenza virus–specific ferret interferon γ (IFN-γ)–secreting T cells by ELISpot and CD4+ or CD8+ T cells by intracellular cytokine staining assays. We characterized the protective immune responses induced by TIV and LAIV and evaluated the contributions of the humoral and CMI responses to the protection of influenza virus–seronegative and –seropositive ferrets against a heterologous virus infection.

**MATERIALS AND METHODS**

**Viruses and Vaccines**

Influenza viruses were obtained from the Centers for Disease Control and Prevention and expanded in 10–11-day-old embryonated hen eggs (Charles River). The 2010–2011 LAIV, swine-origin A/California/7/2009 (CA09, H1N1), A/Perth/19/2009 (Perth09, H3N2) and B/Brisbane/60/2008 (Bris08, Victoria lineage), was manufactured by MedImmune. The TIV containing the same vaccine components was manufactured by Sanofi Pasteur. A/Wisconsin/67/2005 (WI05, H3N2) and B/Malaysia/2506/2004 (ML04, Victoria lineage) were used in the seropositive-ferret study. A/Solomon Islands/3/2006 (SI06, H1N1) wild-type virus was used as the challenge virus.

**Ferret Studies**

The animal study protocol was approved by MedImmune’s Institutional Animal Care and Use Committee and was performed in an Association for Assessment and Accreditation of Laboratory Animal Care International–certified facility. Ferrets aged 9–10 weeks that were free of influenza virus infection were obtained from Simonsen Laboratory. On day 0, ferrets in groups of 4 were either vaccinated with phosphate-buffered saline (mock vaccine), with LAIV (10⁷ median tissue culture infective doses per strain) intranasally, or with TIV (7.5 µg per strain) intramuscularly. For the seropositive-ferret study, the ferrets were preinfected with 10⁵ plaque-forming units (PFU) each of wild-type WI05 (H3N2) and ML04 (Victoria lineage) viruses 29 days before vaccination. On day 34 (seronegative-ferret study) or 37 (seropositive-ferret study) after vaccination, the ferrets were challenged with 10⁶ median tissue culture infectious doses of SI06 (H1N1) wild-type virus (intranasally).

The study was terminated on day 4 or 5 after challenge to examine viral titers in the tissues and the CMI responses. Because of issues associated with scheduling, the ferrets were euthanized on day 4 (seronegative-ferret study) or day 5 (seropositive-ferret study) after challenge; serum, blood, paratracheal lymph nodes (TLNs), nasal turbinates, and lung tissues were collected. Virus titers in the nasal turbinates and lungs were measured in terms of the 50% egg infectious dose (EID₅₀) per gram.

**Measurement of Serum Antibody Titers**

Ferret sera were pretreated with a receptor-destroying enzyme (Denka Seiken), and the hemagglutination inhibition (HAI) antibody titers of the sera were determined using 0.5% turkey or chicken red blood cells. The H1 HA (CA09 or SI06)–specific serum immunoglobulin G (IgG) antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described [16], using a rHA from Protein Sciences.

**Preparation of Single-Cell Suspension of Peripheral Blood Mononuclear Cells (PBMCs) and TLN Cells**

A total of 5–10 mL of whole blood samples from ferrets was collected in ethylenediaminetetraacetic acid tubes and processed using Lympholyte-Mammal (Cedarlane) to isolate the PBMCs. The lymphocytes from the TLNs were isolated as previously described [16]. The same cell suspension preparation was used for the B-cell ELISpot, IFN-γ ELISpot, and flow cytometry analysis.

**B-Cell ELISpot Assay**

The B-cell ELISpot assay on PBMCs or lymphocytes isolated from the local TLNs was performed using 50 µL per well of antigen from either the 2010–2011 TIV (2000 HA/mL) or recombinant CA09 or SI06 HA protein (10 µg/mL) [16].

**IFN-γ ELISpot Assay**

Because influenza virus–specific CD4+ and CD8+ T-cell epitopes are not known for ferrets, whole influenza viruses or HA protein were used as stimulating antigens, using a cross-reactive mouse anti-canine IFN-γ monoclonal antibody as the capture antibody (MAB871, R&D Biosystems) with modifications [17]. The cells were stimulated with 100 µL of a 10 µg/mL preparation of the formalin-inactivated and sucrose-gradient-purified LAIV (2010–2011) or rHA. IFN-γ–secreting cells were detected with biotinylated goat anti-canine IFN-γ (BAF781, R&D Biosystems) and hors eradish peroxidase–conjugated streptavidin (BD Biosciences).

**Production of Flow Cytometry Antibody Reagents to Detect Ferret T Cells**

Ferret CD8- and CD5-specific monoclonal antibodies were produced by the immunization of BALB/c mice with ferret thymocytes, using standard procedures. A ferret CD4 antibody was produced from hybridoma cells generated from splenocytes of...
mice immunized with a recombinant ferret CD4 protein, which was expressed in CHO cells and purified using a HisTALON column (Clontech). Anti-bovine IFN-γ (MCA1738, AbDSerorec) is cross-reactive with ferret IFN-γ [18].

For the flow cytometry analysis, 100 µL of the PBMC or TLN cell suspensions (5 × 10^5 cells/well) were added to microwell plates (BD Biosciences), mixed with 100 µL of LAIV (10 µg/mL) as stimulus or medium as a control. After overnight incubation at 37°C in a 5% CO2 incubator, the cells were treated with GolgiPlug (BD Biosciences) at 37°C for 2–3 hours. The cell surface staining was performed with the PE-Cy7-conjugated mouse anti-ferret CD5, A488-conjugated mouse anti-ferret CD4, and PE-conjugated mouse anti-ferret CD8 antibodies. For the intracellular IFN-γ staining, the cells were permeabilized using Cytofix/Cyoperm (BD Biosciences), followed by staining with the APC-conjugated mouse anti-bovine IFN-γ antibody. Approximately 250 000 events were captured using a FACS Canto II (BD Biosciences) and analyzed with FACS Diva software (BD Biosciences).

**Statistics**

Analysis of variance was used to compare the difference among the different groups using Prism software (GraphPad). A P value of ≤ 0.05 was considered to be significant.

**RESULTS**

Comparison of the Humoral and CMI Responses Induced by LAIV and TIV in Naïve Ferrets

The induction of vaccine-induced antibody and CMI responses to LAIV was compared to that of TIV in naïve ferrets (Figure 1). A single pediatric dose of the TIV was not immunogenic in seronegative ferrets, as evidenced by barely detectable HAI antibody titers to all 3 strains (Figure 1A). A single intranasal dose of the LAIV induced robust serum HAI antibody titers in naïve ferrets against each influenza vaccine strain component.

The LAIV induced strong effector T-cell responses in the PBMC samples stimulated with the respective recombinant HA proteins of the H1, H3, and B strain on day 7 after vaccination (Figure 1B). The number of IFN-γ⁺ T cells specific to the H3 HA was lower than that specific to the H1 HA and B HA. The number of IFN-γ⁺ T cells could be quantified following PBMC stimulation with live virus but not after their stimulation with other protein antigens [19]. Using this intracellular cytokine staining assay, we showed that LAIV recipients had significantly higher percentages of influenza virus-specific IFN-γ⁺ CD4⁺ or CD8⁺ T cells than the animals that were mock immunized or immunized with TIV on day 7 and day 34 after vaccination (Figure 1C), indicating that the LAIV elicited both effector and memory T-cell responses. In contrast, the TIV elicited very low levels of influenza virus-specific T-cell responses when evaluated by either assay. Low levels of influenza virus-specific antibody-secreting cells (ASCs), IFN-γ⁺CD4⁺ and IFN-γ⁺CD8⁺ T-cell responses were detected in the TLNs of the LAIV recipients at day 34 (data not shown).

Vaccine-Mediated Protection Against Heterologous Virus Challenge in Naïve Ferrets

CA/09 (H1N1) in the 2010–2011 vaccine is antigenically distinct from the previously circulating seasonal SI06 (H1N1)
used in the challenge infection [20]. On day 4 after challenge, the LAIV immunized ferrets had a significantly lower level of the SI06 challenge virus in the upper respiratory tract than the mock vaccine or TIV recipients (approximately 40-fold reduction; Figure 2A). The levels of the SI06 challenge virus in the ferret lungs were similar among all the groups (data not shown). On the basis of a pilot study result, the CMI recall responses peaked on day 7 for either vaccine, but the CMI responses after the challenge virus infection could be detected on day 4 after infection. Very low levels of IFN-γ+CD4+ or IFN-γ+CD8+ T-cell responses were detected in the PBMCs of ferrets that were either mock or TIV immunized (Figure 2B).

The percentage of influenza virus–specific IFN-γ+CD4+ and or IFN-γ+CD8+ T cells in the LAIV recipients was still significantly higher than in the other groups and slightly increased as compared to prechallenge values (Figure 1C), indicating a systemic recall response. In the local TLN, the LAIV recipients had the highest numbers of IFN-γ+CD4+ or IFN-γ+CD8+ T cells, although the differences in the level of IFN-γ+CD8+ T cells were not significant (Figure 2C). The influenza virus–specific IFN-γ+CD4+ and IFN-γ+CD8+ T cells detected in the TLNs of mock vaccine and TIV recipients mainly reflected the primary response to the challenge virus infection. The LAIV recipients had significantly higher numbers of influenza virus–specific IgG ASCs in

Figure 2. Protection against heterologous virus challenge and immune responses after challenge in naive ferrets. Ferrets in groups of 4 received mock vaccine, trivalent live attenuated influenza vaccine (LAIV), or trivalent inactivated influenza vaccine (TIV) on day 0 and were challenged intranasally with 10⁶ plaque-forming units of SI06 (H1N1) wild-type virus on day 34 after vaccination. Blood, nasal turbinates, and paratracheal lymph nodes (TLNs) were harvested 4 days after challenge. A, SI06 (H1N1) virus titers in the nasal turbinates. B, Interferon γ (IFN-γ)+CD4+ or IFN-γ+CD8+ T cells in the PBMCs stimulated with the 2010–2011 LAIV. C, IFN-γ+CD4+ or IFN-γ+CD8+ T cells in TLNs stimulated with the 2010–2011 LAIV. D, Influenza virus–specific immunoglobulin A (IgA) or immunoglobulin G (IgG) antibody-secreting cells in TLNs tested with the 2010–2011 TIV. E and F, CA09 HA– and SI06 HA–specific serum IgG antibodies on day 34 after vaccination. *P < .05, by 1-way analysis of variance, for the difference between LAIV and mock vaccine or TIV. Abbreviation: EID₅₀, 50% egg infectious dose.
the TLNs than the others (Figure 2D), while the IgA ASC responses were low in all animals. Thus, the humoral and cellular immune responses from LAIV immunization may correlate with partial protection against a heterologous H1N1 virus infection in the upper respiratory tract of ferrets.

The cross-reactivity of CA09 H1N1–specific antibodies against the heterologous SI06 HA was examined by ELISA. The LAIV induced a much higher level of binding antibodies against the CA09 HA (Figure 2E), some of which also reacted with the SI06 HA (Figure 2F). A low level of binding antibodies was detected in the TIV recipients, but their reactivity to SI06 was not detected.

**Comparison of the Humoral and CMI Responses Induced by LAIV and TIV in Seropositive Ferrets**

TIV and other protein antigens require either priming or coadministration with an adjuvant to induce a robust immune response in naive animals [21]. The immune responses induced by the LAIV and TIV were therefore compared in influenza seropositive ferrets. Ferrets were intranasally infected with Wi05 (H3N2) and MAL04 (B) viruses and immunized with the 2010–2011 LAIV or TIV 29 days later. The serum antibodies induced by H3N2 and B wild-type virus infection cross-reacted with the H3N2 and B components of the vaccine, Perth09 H3N2 and Bris08 B viruses in mock-vaccinated ferrets (Figure 3A). CA09 HAI antibodies were not detected in the mock-immunized animals; the levels of the HAI antibodies induced by the LAIV and TIV were similar for the H3N2 and B strains. Although the animals were not previously infected with an H1N1 virus, the H3N2 and B strain infection primed the ferret immune system to respond not only to the antigens of H3N2 and B strains, but also to the H1N1 strain for the TIV.

The T-cell responses induced by the LAIV and TIV in the influenza seropositive ferrets were evaluated for HA-specific IFN-γ secreting T cells by an ELISpot assay (Figure 3B). On day 7, the T-cell response pattern is similar, albeit higher than that on day 37 (data not shown). CA09 H1N1 HA-specific IFN-γ secreting T cells were significantly higher in the LAIV immunized ferrets, which were not detected in the ferrets that received mock vaccine or TIV. In contrast, all ferrets showed H3 Perth09 and B Bris08 HA-specific T-cell responses; although the LAIV recipients still had significantly higher numbers of H3-HA specific IFN-γ secreting T cells than the other groups (Figure 3B). The LAIV and TIV induced comparable levels of circulating influenza-specific IFN-γCD4+ and IFN-γCD8+ T cells, which appeared to be higher than mock-immunized animals but the difference was not significant (Figure 3C).

**Vaccine-Mediated Protection Against Heterologous Virus Challenge in Seropositive Ferrets**

The 2010–2011 LAIV- or TIV-mediated protection in seropositive ferrets against challenge infection with heterologous SI06 H1N1 wild-type virus was examined. Again, only the LAIV offered ferrets significant protection against upper respiratory tract infection, with approximately 1000-fold lower viral titers in nasal tissues as compared to those in mock vaccine or TIV recipients (Figure 4A).
The T-cell responses in PBMCs stimulated with the 2010–2011 LAIV were also examined by intracellular cytokine staining on day 5 after challenge (Figure 4B). The mock vaccine and TIV recipients had a higher percentage of INF-γ+CD4+ T cells than those immunized with the LAIV, and the numbers of CD8+ T cells in the PBMCs were low in all of the groups. In contrast, the percentage of INF-γ+CD4+ T cells was generally lower than that of INF-γ+CD8+ T cells in the TLNs stimulated with the 2010–2011 LAIV. D, Influenza virus–specific immunoglobulin A (IgA) or immunoglobulin G (IgG) antibody-secreting cells in TLNs tested with the 2010–2011 TIV. Dotted line indicates the detection limit. E and F, CA09 hemagglutinin (HA)–specific and SI06 HA–specific serum IgG antibodies. The circle in the LAIV group indicates the same animal in A and D. *P < .05, by 1-way analysis of variance, for the difference between LAIV and mock vaccine or TIV. Abbreviation: EID50, 50% egg infectious dose.

The T-cell responses in PBMCs stimulated with the 2010–2011 LAIV were also examined by intracellular cytokine staining on day 5 after challenge (Figure 4B). The mock vaccine and TIV recipients had a higher percentage of INF-γ+CD4+ T cells than those immunized with the LAIV, and the numbers of CD8+ T cells in the PBMCs were low in all of the groups. In contrast, the percentage of INF-γ+CD4+ T cells was generally lower than that of INF-γ+CD8+ T cells in the TLN, with no significant differences among the 3 groups (Figure 4C). Influenza virus–specific IgA and IgG ASCs in the TLNs on day 5 after challenge are shown in Figure 4D. IgA ASC levels in the TLNs of LAIV recipients were statistically significantly higher than those for mock vaccine and TIV recipients (P < .05). IgG ASCs in the TLNs of mock vaccine and TIV recipients were significantly higher than in the LAIV recipients, reflecting the primary response to the replication of the challenge virus. Interestingly, one animal, with the lowest influenza virus–specific IgA ASCs, had the highest challenge virus titer in the upper respiratory tract (Figure 4A). This seropositive-ferret study indicated that although the TIV and LAIV induced comparable antibody responses, only the LAIV offered protection against a challenge infection due to heterologous H1N1 virus in the upper respiratory tract. The H1-specific antibody response was examined by an ELISA (Figure 4E and 4F). No H1 HA–specific antibody
DISCUSSION

Both the LAIV and TIV have clinically proven efficacy against influenza via distinct immune mechanisms. We determined that the LAIV is superior to the TIV in inducing influenza virus–specific antibody and T-cell responses in seronegative ferrets. Although both the TIV and LAIV induced comparable humoral immune responses in the seropositive ferrets, only the LAIV conferred partial protection against a heterologous H1N1 virus challenge infection. The ferret data obtained in this study are consistent with clinical trial results in humans that demonstrate that the LAIV is more efficacious in children [22, 23] and more effective against drifted strains [24, 25] than the TIV.

The LAIV could activate Toll-like receptor 7 (TLR7) on plasmacytoid dendritic cells and other TLR7-independent pathways in other cells, thereby inducing a strong immune response in a naive host [21]. The de novo synthesis of the protein antigens from the LAIV replication in the nasal tissues provides a favorable environment for dendritic cells and other antigen-presenting cells to present viral antigens to major histocompatibility complex class I–restricted CD8+ or class II–restricted CD4+ effector T cells. Memory T cells elicited by conserved antigens shared between the priming and challenge viruses could be recruited to the site of infection to limit virus spread and reduce disease severity even in the absence of specific antibodies [26]. The number of specific memory CD4+ T cells in the periphery was found to be responsible for lower virus shedding and a shorter duration of illness in responses to a heterologous virus challenge [27]. In this study, we showed that a single dose of the LAIV induced strong systemic T-cell responses in naive ferrets (Figure 1C). The exposure of these ferrets to a heterologous virus challenge infection quickly recalled their influenza virus–specific T-cell responses. The level of systemic and local T-cell responses correlated inversely with the level of challenge virus replication in the upper respiratory tract (Figure 2A–C). The LAIV reduced the heterologous challenging virus replication in the nasal turbinates but not in the lungs of seronegative ferrets. The lung protection may require a more robust heterologous influenza virus–specific immune response since greater lung protection was observed in H3N2- and B-seropositive ferrets. The better protection in the nasal turbinates might also be attributed by the local mucosal immune responses. Thus, individuals who have never been exposed to influenza are recommended to receive 2 doses of influenza vaccine.

The TIV elicited serum antibodies as efficiently as the LAIV for H3N2 and B strains in H3N2- and B-positive ferrets, as well as to the H1N1 virus, to which they were not previously exposed (Figure 3A). In 2011, Hoft et al reported a study comparing the TIV and LAIV in children 6–35 months of age and found that even though both vaccines elicited comparable HAI antibody responses, only the LAIV induced broad influenza virus–specific T-cell responses [2]. Most of the children had detectable influenza virus–specific HAI antibody and T-cell responses before the vaccination and were therefore not immunologically naive. To our knowledge, this is the first report to show that animals previously infected with a different type or subtype of influenza virus could be primed to respond to the antigens of different subtypes.

Vaccination with the LAIV but not the TIV significantly increased the influenza virus–specific IFN-γ ’CD4+ and IFN-γ ’CD8+ T cells in children 5–9 years old. [19]. Despite the similar levels of antibody responses elicited by both the TIV and LAIV in the primed animals, the TIV induced a generally lower level of circulating influenza virus–specific T-cell responses than the LAIV (Figure 3B and 3C). Although the LAIV induced better total effector T-cell responses to CA09 H1N1 and Perth09 H3N2 than the TIV and had comparable responses to influenza B Bris08 virus in the primed ferrets (Figure 3B), the expansion of the CD4+ T cells after challenge infection was low in the LAIV recipients (Figure 4B). The magnitude of the acute T-cell expansion correlated with the levels of challenge virus replication in the upper respiratory tract in humans [27]. In addition to the HA and NA surface antigens present in the TIV, the LAIV offers viral internal proteins that are the major targets for the CD8+ T cells [28–30], which could contribute to the protection against heterologous strains. The circulating H1N1 cross-reactive CD4+ and CD8+ memory T-cell populations are capable of an immediate effector function against a heterologous H1N1 virus [31, 32]. The strong T-cell responses induced by LAIV in children [2, 33] and adults [34, 35] might partially explain why H1N1 CA09 LAIV had a clinical efficacy rate that was higher than predicted on the basis of the serum antibody response rate [36].

Local draining lymph nodes are the sites where B cells initially encounter the viral antigens presented by either dendritic cells from the infected airway epithelial tissues or directly captured and expressed by other B cells [37]. The local B cells can respond to the infection quickly [38, 39]. The low IgG ASCs elicited by the TIV in the TLNs of the naive ferrets was expected because the TLN is not the primary draining lymph node for intramuscularly delivered antigens. In the H3N2- and B-preinfected animals, the expansion of the IgG ASCs in the TLNs correlated with the level of challenge virus replication, but the expansion of the IgA ASCs had an inverse correlation with the level of challenge virus replication. Natural influenza virus infection and nasal vaccination with the LAIV have been known to induce local mucosal responses that serve as the first defense in local protection [40–42].
Heterologous protection can be attributed by T-cell and mucosal antibody responses, as well as by the cross-reactive binding antibodies targeting the conserved domains of influenza HA epitopes, such as the HA2 stalk region [43]. We showed that the antibodies to CA09 H1N1 from vaccinated ferrets indeed cross-reacted with the SI06 HA H1N1 antigen (Figures 2 and 4). The ability of the LAIV to elicit virus-specific antibodies, IFN-γ-secreting CD4+ and CD8+ T-cell subsets, and mucosal antibodies in the upper respiratory tract of naive ferrets could explain why the LAIV is immunologically superior in children, offers immediate protection after a single dose, and reduces influenza transmission in the community by the immunization of school children [44].

Notes

Acknowledgments. We thank Scott Jacobson, Stephanie Gee, Armando Navarro, Paulyrin Cha, Ernesto Madariaga, Janet Cetz, and Rosemary Broome, for performing the ferret studies; Dr Jennifer Woo and Loraine Gemmell, for ferret CD5 and CD8 hybridoma cells; Darren Heeke, for technical assistance; Yang He and Lily Yang, for tissue culture cells; Drs Xinyan Xu and Alexander Klimov, for wild-type influenza viruses; and Drs Kanta Subbarao, Chris Ambrose, Gary Van Nest, Zhongying Chen, Christopher Cotter, Anu Cherukuri, Kirsten Schneider, and April Spesock, for their critical review of the manuscript.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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