Contemporary Seasonal Influenza A (H1N1) Virus Infection Primes for a More Robust Response To Split Inactivated Pandemic Influenza A (H1N1) Virus Vaccination in Ferrets

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Human influenza pandemics occur when influenza viruses to which the population has little or no immunity emerge and acquire the ability to achieve human-to-human transmission. In April 2009, cases of a novel H1N1 influenza virus in children in the southwestern United States were reported. It was retrospectively shown that these cases represented the spread of this virus from an ongoing outbreak in Mexico. The emergence of the pandemic led to a number of national vaccination programs. Surprisingly, early human clinical trial data have shown that a single dose of nonadjuvanted pandemic influenza A (H1N1) 2009 monovalent inactivated vaccine (pMIV) has led to a seroprotective response in a majority of individuals, despite earlier studies showing a lack of cross-reactivity between seasonal and pandemic H1N1 viruses. Here we show that previous exposure to a contemporary seasonal H1N1 influenza virus and to a lesser degree a seasonal influenza virus trivalent inactivated vaccine is able to prime for a higher antibody response after a subsequent dose of pMIV in ferrets. The more protective response was partially dependent on the presence of CD8+ cells. Two doses of pMIV were also able to induce a detectable antibody response that provided protection from subsequent challenge. These data show that previous infection with seasonal H1N1 influenza viruses likely explains the requirement for only a single dose of pMIV in adults and that vaccination campaigns with the current pandemic influenza vaccines should reduce viral burden and disease severity in humans.

Human influenza pandemics have occurred with some regularity throughout history (20). These pandemics occur when influenza viruses to which the population has little to no immunity emerge. In April 2009, the U.S. Centers for Disease Control and Prevention (CDC) reported two cases of a novel H1N1 influenza virus in children in the southern United States (2). It was retrospectively shown that these cases represented the continued spread of this virus, subsequently labeled pandemic influenza A (H1N1) 2009 (H1N1pdm) virus, from an ongoing outbreak in Mexico. The virus was quickly sequenced and shown to be a novel reassortant between lineages of influenza viruses known to circulate widely in swine (7). Within a matter of weeks, the virus had spread to a number of other continents, signaling the beginning of the first influenza pandemic of the 21st century.

The emergence of this pandemic came with a number of surprises, not the least of which was that the pandemic virus was of the H1N1 subtype. Much emphasis and pandemic planning had been based on the likelihood of the emergence of a novel avian strain into the human population (23). Although not explicitly stated, the immediate threat from viruses in the swine reservoir was, to some extent, minimized due to the fact that these viruses were of the same subtype as those already circulating in humans (1). In response to the emergent pandemic and in accordance with pandemic plans, many national and international public health authorities responded with the immediate manufacture of matching pandemic H1N1 vaccines. Although the H1N1pdm virus was of the same subtype as the circulating human H1N1 lineage, the viruses were shown to be antigenically distinct (11, 21), and early serologic studies implied that the seasonal influenza vaccines would be of limited utility (4). Based on these data and previous clinical trials with avian virus antigens (5, 22), there was reason to assume that two doses of a split unadjuvanted vaccine would be needed to induce a seroprotective response in humans (3). In contrast, the results of the first human clinical trials showed that a single 15-μg dose of H1N1pdm monovalent inactivated vaccine (pMIV) elicited a seroprotective response (hemagglutination inhibition [HI] titer of ≥40) in >90% of individuals (6, 15). Accordingly, Del Giudice et al. were able to show that a dose of 2008 to 2009 seasonal influenza virus trivalent inactivated vaccine (TIV) was able to prime ferrets for a more robust response to a subsequent dose of pMIV, despite a lack of reactivity of sera from TIV-immunized animals to the pandemic virus (9).

Several recent reports have suggested that although the major neutralizing B-cell epitopes are not shared between the seasonal and pandemic H1N1 viruses, the CD4 and CD8 T-cell epitopes are relatively conserved between the two viruses (8, 14). For CD8, almost 70% of the epitopes recognized are invariant (14). That said, it is still not entirely clear how priming by previous infection with, or vaccination against, seasonal influenza viruses affects the response to pMIV or whether in vitro-predicted cross-reacting CD8+ cells could have a protective role. To address these questions, we performed a vaccina-
**TABLE 1. Prime/boost regimens**

| Group     | Priming stepa | Boosting agentb |
|-----------|----------------|-----------------|
| I/M       | Infection with seasonal virus | pMIV            |
|           | A/Brisbane/59/2007 (H1N1)     |                 |
| 1*/M      | Infection with seasonal virus | pMIV            |
|           | A/Brisbane/59/2007 (H1N1)     |                 |
| M/M       | Vaccination with pMIV (1 dose) | pMIV            |
| T/M       | Vaccination with TIV (1 dose) | pMIV            |
| P/M       | Mock vaccination with PBS      | pMIV            |
| P/P       | Mock vaccination with PBS      | PBS             |

a Ferrets were intranasally inoculated with 10^8 EID_{50} of live A/Brisbane/59/2007 (H1N1) in the presence or absence of CD8+ T cells, intramuscularly injected with 15 μg of either pMIV (Sanofi-Aventis, Bridgewater, NJ) or TIV (Fluzone; Sanofi-Aventis, Bridgewater, NJ), or mock vaccinated with PBS.

b Ferrets were boosted 3 weeks after receiving the priming infection or vaccination.

Immunization and challenge experiment in ferrets, in which we assessed a number of priming scenarios for impact on the antibody response to and protective efficacy of pMIV.

**MATERIALS AND METHODS**

**Viruses and cells.** The H1N1 viruses A/Brisbane/59/2007 (a contemporary seasonal virus vaccine strain, passed through three eggs in 10% MMEC cells before being used), A/Tennessee/1-560/2009 (a representative H1N1pdm virus vaccine strain, passed through three eggs before being used), and A/California/07/2009 (an H1N1pdm virus vaccine strain, rescued in eggs) were obtained from the World Health Organization influenza collaborating laboratories. MMEC cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in a humidified atmosphere of 5% CO2 at 37°C in 1% minimal essential medium (MEM) (Invitrogen, Carlsbad, CA). Supplements were 5% fetal calf serum (FCS), 200 mM l-glutamine, 40 mg/ml gentamicin, 1% MEM vitamin solution (Sigma, St. Louis, MO), and 1× antibiotic-antimycotic solution (Sigma).

**Immunization and challenge.** Young female ferrets 3 months of age and seronegative for currently circulating influenza A H1N1 and H3N2 and influenza B viruses were obtained from Triple F Farms (Sayre, PA) and were divided into 6 groups, with 5 ferrets per group (except for two groups with 4 ferrets each). The vaccines used in this study were the 2008 to 2009 seasonal influenza trivalent vaccine (Sanofi Pasteur, Inc. (Swiftwater, PA)), and were kindly provided to us by the DMID, NIH. As shown in Table 1, the first group (I/M) was intranasally inoculated with 10^5 50% egg infective doses (EID_{50}) of live A/Brisbane/59/2007 (H1N1) (the current H1N1 virus component of the TIV) (I) and vaccinated 3 weeks later with 15 μg of pMIV (M) to mimic the scenario of exposure followed by vaccination. The second group (1*/M) received the same treatment as the I/M group, but CD8+ cells were depleted prior to the priming infection as detailed below. The third group (M/M) received two 15-μg doses of pMIV 3 weeks apart. The fourth group (T/M) received a single human dose of TIV (T) and then a single 15-μg dose of pMIV. The fifth group (P/M) received a mock priming dose of phosphate-buffered saline (PBS) (P) followed 3 weeks later by a single dose of pMIV. The sixth group (P/P) was mock vaccinated with PBS. All animal experiments were performed in a biosafety level 2 laboratory, using biosafety level 3 practices. Three weeks after the second immunization, all animals were challenged with 10^6 EID_{50} of A/Tennessee/1-560/2009 (H1N1pdm), a representative pandemic virus strain. All animal experiments were approved by the Animal Care and Use Committee of St. Jude Children’s Research Hospital and performed in compliance with National Institutes of Health regulations and the USDA Animal Welfare Act. A subcutaneous implantable temperature transponder (Bio Medic Data Systems, Seaford, DE) was placed in each ferret for identification and to take temperature readings. For the priming infection and challenge, ferrets were anesthetized with isoflurane and then inoculated intranasally with a total volume of 1 ml.

**Production of anti-CD8 monoclonal antibody and CD8+ cell depletion in ferrets.** The hybridoma-secreting anti-human CD8 (clone OKT 8, murine IgG2a) monoclonal antibody (MAb) was purchased from ATCC (CRL-8014). Hybridomas were grown in Fibercell cartridges (FiberCell Systems, Inc., Frederick, MD) with Dulbecco’s modified Eagle’s medium (DMEM) and adapted to 2.5% FCS (starting at 10%). The supernatant collected was passed over a 5-ml His Trap Protein G HP column (GE Healthcare Biosciences, Piscataway, NJ) by using an AKTA fast protein liquid chromatography (FPLC) instrument and eluted with 0.1 M glycine, pH 2.7. The purified MAb was desalted into PBS with a Zeba column (Pierce, Rockford, IL). Ferrets were injected intraperitoneally (i.p.) with 1 mg MAAb/kg of body weight 5, 3, and 1 day prior to and 7 days after infection with EID_{50} of the seasonal A/Brisbane/59/2007 (H1N1) influenza virus.

**Flow cytometry.** Peripheral blood mononuclear cells (PBMCs) were isolated from ferret blood by being spun with a Histopaque system (Sigma-Aldrich, Steinheim, Germany) at 400 x g for 30 min. The mononuclear cell liver was then washed in PBS. Next, cells were stained with either allophycocyanin (APC)- or fluorescein isothiocyanate (FITC)-conjugated goat anti-ferret IgG (H+L) antibody (Bethyl Labs, Montgomery, TX).

**Virus titration.** On days 1, 2, 4, 7, and 9 after virus inoculation, ferrets were anesthetized with ketamine (25 mg/kg), and 0.5 ml of PBS with antibiotics was slowly introduced into each nostril, recovered, measured, and brought to a volume of 1.0 ml with sterile PBS containing antibiotics. Bovine serum albumin (BSA) (7.5%) was added at a ratio of 1:20 (vol/vol) as a stabilizing agent. Virus was titrated in MMEC cells and expressed as log_{10} 50% tissue culture infective dose (TCID_{50}) per milliliter, as calculated by the method of Reed and Muench (18). The limit of virus detection was <0.75 log_{10} TCID_{50}/ml.

**Lung tissues** were collected 4 days after pandemic virus challenge. Two animals in each treatment group and the control group were euthanized by intracardiac injection of Euthanasia V solution, and lungs (total of 0.5 g each) were collected. Samples were homogenized in 1 ml sterile PBS with antibiotics, and the viral titer (log_{10} TCID_{50}/g) in MMEC cells was determined.

**Serologic tests.** Ferret sera were obtained before vaccination or infection, before vaccine boost, before challenge, and 10 days after challenge. Serum samples were treated with receptor-deactivating enzyme (Accurate Chemical and Scientific, NY) overnight at 37°C, heat inactivated at 56°C for 30 min, and diluted 1:10 in PBS. Virus neutralizing antibody titers in ferret sera were determined in MMEC cells. Briefly, the TCID_{50} was determined for each virus used, and 2-fold serial dilutions of serum were incubated with 100 TCID_{50}s of virus for 1 h at 37°C. The mixture was then added to MMEC cells and incubated for 72 h at 37°C in 5% CO2. After 72 h, the hemagglutination activity of the supernatant was assessed using 0.5% packed turkey red blood cells. Neutralizing titers were expressed as the reciprocal of the serum dilution that inhibited 50% of the hemagglutination activity of 100 TCID_{50}s of virus. Microneutralization (MN) assays were performed against both A/Brisbane/59/2007 and A/California/07/2009. For the antigen-specific enzyme-linked immunosorbent assay (ELISA), microtiter plates (Corning, Lowell, MA) were coated overnight at 4°C with purified whole A/Brisbane/59/2007 or A/Tennessee/1-560/2009 virus in PBS. After an overnight incubation with serial dilutions of ferret sera, influenza virus-specific antibodies were detected with a goat anti-ferret IgG–alkaline phosphatase conjugate (Biotrend, Cologne, Germany) diluted 1:1,000 in PBS with 1% BSA. The substrate p-nitrophenylphosphate (Sigma-Aldrich, Atlanta, GA) was added, plates were incubated for 30 min at room temperature for color development, and optical density (OD) values were determined at 405 nm in an ELISA reader (Bio-Rad, Los Angeles, CA).

**Histological analysis.** Lung specimens from each ferbe were collected at the time of necropsy, fixed in 10% neutral buffered formalin, and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. The pathologist remained blinded to the group/treatment to which the specimen belonged.

**Statistical analysis.** Viral titers in ferret nasal wash samples were compared by means of an unpaired 2-tailed t test. A probability value of 0.05 was prospectively chosen as the cutoff to indicate that findings were not the result of chance alone.

**RESULTS**

As shown in Table 1, ferrets were divided into 6 groups. The first group (I/M) was primed by intranasal inoculation with live A/Brisbane/59/2007 (H1N1) (the current H1N1 virus component of the TIV) (I) and vaccinated 3 weeks later with pMIV (M). The second group (1*/M) received the same treatment as the I/M group, but CD8+ cells were depleted prior to the priming infection as detailed below. The third group (M/M)
received two doses of pMIV 3 weeks apart. The fourth group (T/M) received a single dose of TIV (T) and then a single dose of pMIV. The fifth group (P/M) received a mock priming dose of PBS (P) followed 3 weeks later by a single dose of pMIV. The sixth group (P/P) was mock vaccinated twice with phosphate-buffered saline (PBS).

Depletion of CD8\textsuperscript{+} T cells in the ferret model. Cellular immunologic studies in ferrets have historically been hampered by a lack of validated reagents. Specific and cross-reactive reagents are now, however, becoming available. Rutigliano and colleagues were able to show that the anti-human CD8 (clone OKT 8, murine IgG2a) monoclonal antibody cross-reacts with ferret CD8\textsuperscript{+} cells (19). In order to determine the role of cross-reactive memory CD8\textsuperscript{+} cells in the ferret model, we used this monoclonal antibody to perform a CD8\textsuperscript{+} cell depletion experiment. Ferrets in the I*/M group were depleted of CD8\textsuperscript{+} cells prior to infection with A/Brisbane/59/2007 (H1N1) so that memory CD8\textsuperscript{+} cells would not be established. Ferrets were injected with 1 mg/kg of the MAb intraperitoneally 5, 3, and 1 day prior to and 7 days after virus inoculation. As shown in Fig. 1, a single i.p. injection of the purified anti-CD8 monoclonal antibody specifically depleted all circulating CD8\textsuperscript{+} cells without affecting the circulating B cells. This was shown by staining peripheral blood mononuclear cells (PBMCs) with antibodies specific for CD8\textsuperscript{+} cells or IgG-expressing cells as a control.

Antibody response to vaccination. In this study, we assessed the impact of a number of priming scenarios on subsequent pMIV administration. Ferrets in groups that were primed by infection with the seasonal virus A/Brisbane/59/2007 (H1N1) in the presence or absence of CD8\textsuperscript{+} cells (I/M and I*/M groups, respectively) showed positive neutralizing antibody titers against the pandemic virus only after being boosted with a dose of the pandemic virus vaccine (Table 2). The postboost geometric mean titers (GMTs) of antibodies against the pandemic virus in ferrets in the I/M group were 1,838 and 30 as measured by ELISA and microneutralization (MN) assays, respectively (Table 2). A similar observation was made for ferrets in the I*/M group, for which the GMTs were 2,785 and 40, respectively (Table 2). Although ferrets from both of these groups developed detectable ELISA antibody titers against the H1N1pdm virus after the priming infection with the seasonal H1N1 virus, the titers were greatly boosted (at least 10-fold increased) with a single dose of pMIV (Table 2).

Not surprisingly, ferrets that received two doses of the pandemic virus vaccine (M/M) also showed a detectable antibody response but only after receiving the second dose of the vaccine. The GMTs were 673 and 17 by ELISA and MN assay,
respectively (Table 2); these titers were lower than those observed in ferrets primed by infection with the seasonal H1N1 virus, despite the fact that they received only a single dose of the pandemic virus vaccine. Although these ferrets did not develop neutralizing antibody titers against the seasonal H1N1 virus, cross-reactive antibodies were detectable by ELISA (Table 2). The ELISA titers were enhanced after the second dose of vaccine (GMTs from 168 to 400 [Table 2]). Ferrets that were primed with TIV and then boosted with pMIV (the T/M group) displayed antibody titers against both seasonal and pandemic H1N1 viruses. Of note was the fact that ELISA titers to the seasonal strain were boosted (from 174 to 400) after administration of pMIV (Table 2).

Ferrets that were not primed before receiving a single dose of pMIV did not develop any neutralizing antibody titers against the pandemic or the seasonal H1N1 virus (Table 2). Antibodies against the pandemic strain in this group were detectable by ELISA, albeit at low levels (GMT of 43.5 [Table 2]). Taken together, these findings indicate that prior seasonal H1N1 influenza virus infection primed the highest pMIV response, with a

TABLE 2. Preboost and prechallenge ferret antibody titers against seasonal and pandemic influenza H1N1 viruses, as determined by ELISA and microneutralization (MN) assay

| Group | Ferret IDa | ELISA (IgG)b | MN assayc | Titer |
|-------|------------|--------------|-----------|-------|
|       |            | Brisbane 59d | TN 1-560e |       |
|       |            | Preboost     | Prechallenge | Preboost | Prechallenge | Preboost | Prechallenge |
| I/M   | 584        | 12,800       | 6,400      | 50      | 800       | 1,280     | 640       | <f       | 20       |
|       | 585        | 12,800       | 6,400      | 400     | 1,600     | 1,280     | 640       | <        | 40       |
|       | 586        | —           | 6,400      | —       | 1,600     | —         | 1,280     | —        | 20       |
|       | 587        | 12,800       | 6,400      | 200     | 3,200     | 1,280     | 640       | <        | 40       |
|       | 588        | 12,800       | 6,400      | 100     | 3,200     | 1,280     | 640       | <        | 40       |
|       | Allb       | 12,800.0     | 6,400      | 141.4   | 1,837.9   | 1,280.0   | 735.1     | <        | 30.3     |
| I*/M  | 591        | 12,800       | 6,400      | 400     | 1,600     | 1,280     | 640       | <        | 40       |
|       | 592        | 25,600       | 6,400      | 200     | 3,200     | 1,280     | 640       | <        | 40       |
|       | 593        | 12,800       | 12,800     | 200     | 6,400     | 1,280     | 640       | <        | 40       |
|       | 596        | 12,800       | 12,800     | 200     | 1,600     | 1,280     | 640       | <        | 40       |
|       | 597        | 25,600       | 12,800     | 200     | 3,200     | 1,280     | 640       | <        | 40       |
|       | All        | 16,889.7     | 9,700.5    | 229.7   | 2,785.7   | 1,280.0   | 640.0     | <        | 40.0     |
| M/M   | 600        | 100          | 400        | 200     | 800       | <         | <        | <        | 20       |
|       | 601        | 100          | 400        | 100     | 800       | <         | <        | <        | 20       |
|       | 602        | 200          | 200        | 100     | 400       | <         | <        | <        | 10       |
|       | 604        | 400          | 800        | 100     | 800       | <         | <        | <        | <        |<<        | 20       |
|       | All        | 168.1        | 400.0      | 118.9   | 672.7     | <         | <        | <        | 16.8     |
| T/M   | 610        | 100          | 200        | <       | 100       | <         | <        | <        | <        |
|       | 611        | 400          | 1,600      | 100     | 400       | 40        | 40        | <        | <        |
|       | 612        | 200          | 400        | <       | 400       | 20        | <        | <        | <        |
|       | 613        | 100          | 200        | <       | 100       | 10        | <        | <        | <        |
|       | 616        | 200          | 400        | <       | 400       | 20        | <        | <        | <        |
|       | All        | 174.1        | 400.0      | 57        | 229.7     | 15.1      | 7.5       | <        | <        |
| P/M   | 605        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | 606        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | 607        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | 608        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | 609        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | All        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
| P/P   | 617        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | 618        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | 620        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | 589        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |
|       | All        | <            | <          | <       | <         | <         | <         | <        |<<        | <        |

a ID, identifier.
b ELISA titers represent the highest dilution that yielded an optical density (OD) value three times higher than that for a 1:100 dilution of preimmune serum. To calculate GMTs, a titer of <50 was assigned a value of 25.
c MN assay titers are expressed as the reciprocal of the highest serum dilution that neutralized 100 TCID50s of each virus. To calculate GMTs, a titer of <10 was assigned a value of 5.
d Brisbane 59, A/Brisbane/59/2007 (H1N1).
e TN 1-560, A/Tennessee/1-560/2009 (H1N1).
f <, undetectable titer.
g —, serologic assay was not performed.
h Data in the rows labeled “All” represent the GMTs for that group.
substantially lower response induced by homologous pMIV vaccination, while TIV administration had a measurable but minimal impact on the response to subsequent pMIV immunization. Single pMIV immunization in a naive animal did not induce a titer measurable by MN assay in any animal.

**Effect of vaccination on H1N1pdm virus-induced morbidity.**
To assess the protective nature of the various vaccination strategies, all ferrets were challenged with A/Tennessee/1-560/2009 (H1N1pdm) 3 weeks after the last vaccine was received. As expected, no animals succumbed to infection. Nevertheless, after challenge with the pandemic strain, several clinical and virological features were observed. Animals from all groups developed typical signs of influenza, including fever and sneezing. All challenged ferrets had an increase in temperature, but there were no significant differences between groups (data not shown). However, as shown in Table 3, ferrets from the I/M and I*/M groups, which were primed by infection with the seasonal H1N1 influenza virus (in the presence or absence of CD8+ T cells) prior to vaccination with pMIV, lost significantly more virus than did animals from the control group (PBS) group at day 4 postchallenge ($P < 0.05$) (Fig. 2). At day 4 after infection, all ferrets from the 5 vaccinated groups shed significantly less virus than the PBS control group ($P < 0.05$) (Fig. 2). All vaccinated groups cleared virus by day 7 postchallenge, whereas virus could still be detected in PBS control animals. We did not detect any significant differences in virus shedding among the M/M, T/M, and P/M groups (Fig. 2). These results were consistent with the observed symptoms of disease progression as represented by weight loss.

On day 4 after challenge, we sacrificed 2 animals from each group to measure virus replication in the lungs. Viral titers in the lungs of ferrets belonging to the I/M and I*/M groups were below the assay detection limit (Table 3). Animals in the M/M group, which received 2 doses of pMIV, had substantially less virus in their lungs than did animals from either the PBS control group or the P/M group (Table 3). Surprisingly, the ferrets from the T/M group, which received single doses of TIV and pMIV, had 10-fold less virus than those from the PBS control group (Table 3). This was more than double the reduction in titer seen between animals from the P/P group and those from the P/M group, which received a single dose of pMIV alone, indicating a significant beneficial impact of prior TIV administration.

**Effect of vaccination on H1N1pdm virus-induced lung pathology.**
In addition to the determination of viral titers in the lungs at day 4 postchallenge, we fixed tissue for histopathologic examination. Pathological changes were present in the lungs of ferrets in the six groups. Bronchiolitis were commonly involved in all ferrets, whereas the levels of alveolar involvement were quite variable. Bronchiolar epithelial necrosis, sloughing, and regeneration were common to all six groups, but severity and composition of these three bronchiolar lesions varied. Pulmonary parenchymal changes consisting of alveolar pneumocyte hyperplasia and interstitial hypercellularity were common to some groups but not others. Inflammatory cell infiltrates of various proportions of macrophages and neutrophils were

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**TABLE 3. Weight loss and lung titers after H1N1pdm virus challenge**

| Group | Lung viral titers | Weight loss (%) | Day 4 | Day 7 |
|-------|------------------|----------------|-------|-------|
| I/M   | <c, <            | −0.8           | 1.8   |
| I*/M  | <c, <            | 1.8            | 2.6   |
| M/M   | 2.4, 4.2         | 4.1            | 8.3   |
| T/M   | 4.3, 5.1         | 7.8            | 11.5  |
| P/M   | 5.3, 5.5         | 5.7            | 10.2  |
| P/P   | 5.8, 6.1         | 7.5            | 13.2  |

*a* Lung viral titers are expressed as log_{10} TCID$_{50}$/g of lung tissue taken from 2 ferrets from each group 4 days after H1N1pdm virus challenge.

*b* Morbidity was assessed by monitoring weight loss after H1N1pdm virus challenge. Results are expressed as mean percentages of weight loss at 4 and 7 days postchallenge.

<, below assay detection limit.
FIG. 3. Priming by seasonal influenza virus infection protected ferrets from severe lung histopathology after H1N1pdm virus challenge. Illustrated are the histopathologic findings associated with the bronchioles and interbronchiolar alveoli and interstitium in the lungs of ferrets challenged with the H1N1pdm virus following various pretreatments (H&E staining, ×20 magnification). (A and B) Lung tissue from a ferret that belongs to the I/M group, which was primed by seasonal H1N1 virus infection prior to receiving a single dose of pMIV. (A) Neutrophils (arrows) and mucus are present in the lumens of two bronchioles. (B) The alveoli (a) are free of inflammatory cells, and the interstitial septa (arrows) are thin and narrow and have very few cells other than red blood cells. (C and D) Lung tissue from a ferret that belongs to the M/M group, which received two doses of pMIV 3 weeks apart prior to the H1N1pdm virus challenge. (C) Mononuclear inflammatory cells (m) fill the lumens of two bronchioles. Due to bronchiole epithelial necrosis and sloughing, one bronchiole is not lined with epithelium (thick arrows), whereas the other bronchiole is lined with some hypertrophied regenerating epithelial cells (thin arrows). There is also a mononuclear cell infiltrate surrounding the bronchioles. (D) Mononuclear inflammatory cells fill several of the alveoli (a) that make up the lung parenchyma between the bronchioles, and
The results of the presented studies show that a single 15-μg dose of pMIV is able to accelerate clearance of A/Tennessee/1-560/2009 (H1N1pdm) from infected ferrets compared to clearance from control animals, albeit in the absence of detectable neutralizing titers prior to challenge. Such results are not unique to the H1N1pdm virus, and protection in the absence of detectable neutralizing antibodies has also been seen in ferret models with the H5N1 virus (13, 16). Although showing reduced amounts of viral replication, ferrets vaccinated with a single dose of pMIV did not have either a significant difference in weight loss/gain pattern compared to that for mock-vaccinated controls or pathological involvement in the lung. A second dose of pMIV led to an increased antibody response as measured by ELISA and MN assay but only a marginal improvement in measures of vaccine efficacy. Contrary to the results of Del Giudice and colleagues (9), our MN assay results did not show a significant boosting of pMIV-induced antibodies by prior seasonal TIV administration. The reason for these differences is unknown but could be related to the vaccine formulations and/or schedules used or variations in antibody assays. Despite no priming of neutralizing titers, we did see a priming effect of TIV on pMIV-induced A/Tennessee/1-560/2009-specific antibodies detected by ELISA. Although it is possible that priming with TIV led to a preferential induction of nonneutralizing antibodies after pMIV vaccination, it is also possible that the ELISA was more sensitive than the neutralization-based assays. Whatever the nature of the antibody response from TIV priming, the pathological changes in the lungs of ferrets in this group were milder than those seen in the group receiving a single dose of pMIV, indicating that the antibodies were functional. It is also intriguing that the ELISA GMTs to A/Brisbane/59/2007 (H1N1)-specific antibodies in the group given TIV followed by pMIV were elevated after pMIV administration. The practical consequence of this phenomenon is that individuals given a vaccine containing H1N1pdm virus may also receive a boost to seasonal H1N1 influenza virus antibodies. This is of some importance as the recommended 2010 Southern Hemisphere and 2010 to 2011 Northern Hemisphere trivalent influenza vaccines contain only an influenza B, an influenza A (H3N2), and a pandemic influenza A (H1N1) virus antigen, due to the limited circulation of seasonal H1N1 virus since the emergence of H1N1pdm virus. A seasonal H1N1 virus boosting effect of vaccines containing H1N1pdm virus antigens would be an unexpected advantage in the unlikely instance that seasonal H1N1 viruses become dominant again in the coming seasons.

Perhaps not surprisingly, the most robust antibody response and subsequent protection were observed in animals primed with the seasonal virus strain A/Brisbane/59/2007 (H1N1), suggesting a priming and/or protective effect of cellular components of the immune response. Indeed, Greenbaum and colleagues have shown that PBMCs taken from normal individuals have CD4 and CD8 T cells that are reactive with the H1N1pdm virus strain (14). The results of this study are also consistent with pMIV clinical studies showing that most adult recipients achieve seroprotective antibody levels after only a single 15-μg dose, whereas young children do not (25). Our data suggest that the immunogenicity of pMIV is linked to prior exposure to seasonal H1N1 virus strains and that even contemporary viruses can provide this priming. Indeed, although protective, pMIV was not highly immunogenic in naive ferrets, a result not dissimilar to that seen with H5 vaccines, which have been shown to be poorly immunogenic in humans (22). Thus, the encouraging results seen with pMIV in humans are likely linked to preexisting cross-reactive immunity as opposed to a high intrinsic immunogenicity or prepriming due to administration of TIV. Both neutralizing and ELISA antibody titers were considerably higher in the seasonal virus infection
primed groups (I/M and I*/M) than in the seasonal virus vaccine primed group (T/M) (Table 2). That said, the difference in protection observed upon H1N1pdm virus challenge of these groups could also be attributed to other potential factors. First, unlike immunization with dead antigen, live virus infection generates cross-reactive mucosal antibody responses that can interfere with initial virus replication and is more likely to generate antibodies to other, more conserved, non-HA viral proteins.

A number of recent studies have shown that there are HA antibody epitopes that are conserved between historical seasonal H1N1 viruses and H1N1pdm viruses, and this has been used as an explanation for the relatively mild nature of the pandemic in the elderly (7, 10). Wei and colleagues have recently shown that an epitope located in the receptor binding region of HA is conserved between the 1918 and pandemic 2009 H1N1 viruses, although the more contemporary A/New Caledonia/20/99 virus had this site blocked by an additional glycosylation site (24). As such, although it is possible that the priming seen by A/Brisbane/59/2007 infection is due to cross-reactive B cells, the lack of neutralizing antibody priming by inactivated TIV suggests that other mechanisms are likely to explain the observation.

The major drawback to the more widespread use of the ferret as a model for influenza has been the lack of immunological reagents (19). A number of recent publications have, however, described the development of ferret-specific reagents as well as a number of cross-reactive reagents used in other systems (17, 19). In this study, we utilized an anti-human CD8 antibody to assess the role of memory CD8+ T cells in within-subtype heterologous protection. Our data showed that CD8+ T-cell memory derived from an A/Brisbane/59/2007 (H1N1) infection had a significant effect on subsequent A/Tennessee/1-560/2009 (H1N1pdm) replication after pMIV vaccination but not on the antibody response to the pMIV itself. The presence of the memory CD8+ T-cell response did not affect early virus replication at days 1 and 2 p.i., as measured by viral titers in nasal washes, but there was a significant increase in viral titers at day 4 in ferrets from the I/M group, which were previously depleted of CD8+ T cells, compared to those in ferrets from the I/M group, which were not depleted. It should be stressed that CD8+ T cells were depleted during A/Brisbane/59/2007 (H1N1) priming and not at the time of A/Tennessee/1-560/2009 (H1N1pdm) challenge and that both groups were able to respond with a functional primary CD8+ T-cell response to this challenge. These data clearly show, as predicted by analyses of human PBMCs (14), that some CD8+ T-cell epitopes are conserved between seasonal and pandemic H1N1 viruses and that such cross-reactive memory cells are able to contribute to the clearance of the pandemic virus. The ability to induce CD8+ T-cell memory was not, however, the sole correlate for improved clearance of the pandemic virus challenge, as the ferrets in the I/M group, which were depleted of CD8+ cells at the time of priming, still fared better than the animals receiving two doses of pMIV. Recent in vitro studies have shown that there are several CD4+ T-cell epitopes shared between the seasonal and the H1N1pdm viruses, and indeed, cross-reacting memory CD4+ T cells could be detected ex vivo by using peptides derived from the H1N1pdm viruses (12). Therefore, we think that the improved immunogenicity of pMIV following exposure to or vaccination with seasonal viruses is mediated partially by cross-reacting memory CD4+ T cells.

 Taken together, the results of this study show that the encouraging immunogenicity of pMIV in humans is likely linked to prior influenza virus exposure and that, as has been seen with other influenza vaccines, protection can be observed in the absence of detectable neutralizing antibody titers.

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We declare no conflicts of interest.

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